

**UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
CENTRO DE CIÊNCIAS DA SAÚDE
FACULDADE DE FARMÁCIA**



Daniel da Silva

**METFORMINA REVERTE A INIBIÇÃO DA
HEXOCINASE E DA FOSFOFRUTOCINASE DE
DIFERENTES TECIDOS DE CAMUNDONGOS
DIABÉTICOS**

Rio de Janeiro,

2011

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Ciências Farmacêuticas.

Orientador: Prof. Mauro Sola-Penna
Prof. Associado do Departamento de Fármacos, Faculdade de Farmácia, UFRJ.

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II. Metformina reverte a inibição da hexocinase e da fosfofrutocinase de diferentes tecidos de camundongos diabéticos

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Aprovada em 16 de novembro de 2011.



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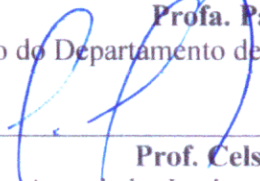
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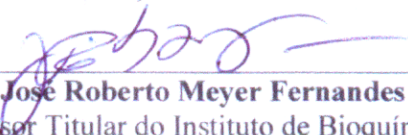
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RESUMO

O diabetes mellitus (DM) é caracterizado pela hiperglicemia e suas complicações associadas, incluindo cardiomiopatia. A metformina, além de reduzir a concentração sanguínea de glicose, pode causar efeitos cardioprotetores em pacientes diabéticos. No entanto, seu mecanismo de ação ainda não está completamente elucidado. A inibição da hexocinase (HK) e da fosfofrutocinase (PFK), enzimas limitantes da glicólise no músculo esquelético, fígado, tecido adiposo e coração, pode contribuir para o desenvolvimento de hiperglicemia e suas complicações, incluindo cardiomiopatia. O presente estudo descreve os efeitos da metformina sobre a regulação da HK e da PFK em diferentes tecidos de camundongos diabéticos induzidos com estreptozotocina. Camundongos diabéticos apresentam menor atividade da HK e da PFK no músculo esquelético, fígado, tecido adiposo e coração, em comparação com os controles. O tratamento com metformina 250 mg/kg reverte essa inibição enzimática, com reversão concomitante da hiperglicemia e da hipolactacidemia. A análise da expressão protéica indica que a metformina reverte a menor expressão de HK-1 no músculo esquelético, fígado e tecido adiposo de camundongos diabéticos; este efeito também ocorre para a HK-2, no músculo esquelético e tecido adiposo, e para a PFK, no fígado e tecido adiposo. Além disso, o tratamento aumenta a atividade da HK associada à mitocôndria em fígado, tecido adiposo e coração; e também aumenta a atividade da PFK associada ao citoesqueleto no músculo esquelético e coração. Esses efeitos têm sido descritos como um mecanismo importante para a ativação das enzimas. Ademais, esse efeito pode ser devido à reversão dos baixos níveis de fosforilação da PFK em resíduos de serina no músculo esquelético, uma modificação que é descrita como sendo capaz de aumentar a interação da PFK com f-actina. Por outro lado, a metformina reverte os níveis elevados de fosforilação da PFK em resíduos de serina dos corações dos diabéticos. Além disso, a ativação da HK e da PFK de células C2C12 por metformina parece ocorrer por vias dependentes e independentes da sinalização de insulina. Assim, este estudo suporta a hipótese de que os efeitos hipoglicemiantes da metformina envolvem a ativação da glicólise através de suas enzimas reguladoras, que podem ser alvos potenciais para o desenvolvimento de novos fármacos hipoglicemiantes.

Palavras-chave: Diabetes Mellitus; Fosfofrutocinase, Glicólise, Hexocinase, Metformina.

ABSTRACT

Diabetes mellitus (DM) is characterized by hyperglycemia and its associated complications, including cardiomyopathy. Metformin, in addition to reducing blood glucose levels, can cause cardioprotective effects in diabetic patients. However, its mechanism of action is still unclear. The inhibition of hexokinase (HK) and phosphofructokinase (PFK), limiting enzymes of glycolysis in skeletal muscle, liver, adipose tissue and heart, may contribute to the development of hyperglycemia and its complications, including cardiomyopathy. The present study describes the effects of metformin on hexokinase (HK) and phosphofructokinase (PFK) regulation in different tissues from streptozotocin-induced diabetic mice. Diabetic mice present lower HK and PFK activities in skeletal muscle, liver, adipose tissue and heart, when compared with controls. Treatment with 250 mg/kg metformin reverses this pattern of enzyme inhibition with concomitant reversal of hyperglycemia and hypolactacidemia. Analysis of protein expression indicates that metformin reverses the lower expression of HK-1 in skeletal muscle, liver and adipose tissue from diabetic mice; this effect also occurs for HK-2 in skeletal muscle and adipose tissue, and PFK in liver and adipose tissue. Furthermore, the treatment increases the mitochondria-associated HK and cytoskeleton-associated PFK activity in skeletal muscle and heart. This effect has been described as an important mechanism for the enzyme activation. This effect might be due to the reversed lower phosphorylation levels of serine residues in the enzyme from skeletal muscle, a modification which has been described to increase the interaction of PFK with f-actin. On the other hand, metformin reverses the higher levels of serine residues of PFK in the diabetic hearts. Furthermore, activation of HK and PFK in C2C12 cells by metformin treatment appears to occur by a mechanism independent and dependent of insulin signaling. The current study supports the hypothesis that metformin hypoglycemic effects involve the activation of glycolysis through its regulatory enzymes, which may be potential targets for the development of new hypoglycemic drugs.

Keywords: Diabetes Mellitus, Glycolysis, Hexokinase, Metformin; Phosphofructokinase.

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ABREVIACOES

ADP	Adenosina 5' difosfato
AMP	Adenosina 5' monofosfato
AMPC	Adenosina 3',5' monofosfato cclico
AMPK	Protena cinase ativada por adenosina 5' monofosfato
ATP	Adenosina 5' trifosfato
[γ-³²P]ATP	Adenosina 5' trifosfato marcado com ³² P na posio γ
CaM	Calmodulina
Con	Grupo controle
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DMG	Diabetes mellitus gestacional
DTT	Ditiotreitol
EDTA	cido etileno-diamino tetractico
F6P	Frutose 6-fosfato
F1,6BP	Frutose 1,6-bifosfato
[1-³²P]F1,6BP	Frutose 1,6-bifosfato marcado com ³² P no carbono 1
F2,6BP	Frutose 2,6-bifosfato
F- actina	Actina filamentosa
GAPDH	Gliceraldedo-3-fosfato desidrogenase
G6P	Glicose-6-fosfato
G6PDH	Glicose-6-fosfato desidrogenase
GLUT	Transportador de glicose por difuso facilitada
HK	Hexocinase
HT	Homogeneizado total
IRS-1	Substrato do receptor de insulina
LDH	Lactato desidrogenase
MCT	Transportador de monocarboxilato
Met	Metformina
MTT	Brometo de 3-(4,5-dimetil-2-tiazoli)-2,5-difenil-2H-tetrazlio
NAD⁺	Nicotinamida adenina dinucleotdeo oxidada
NADH	Nicotinamida adenina dinucleotdeo reduzida

P1	Fração celular rica em mitocôndrias
P2	Fração celular rica em proteínas do citoesqueleto
³²P_i	Fosfato inorgânico marcado com ³² P
PBS	Tampão fosfato salino
PDH	Piruvato desidrogenase
PGM	Fosfoglicerato mutase
PFK	Fosfofrutocinase
PFK-M	Isoforma muscular da PFK
PFK-L	Isoforma hepática da PFK
PFK-C	Isoforma de plaqueta da PFK
PFK-2	Fosfofrutocinase-2/frutose 2,6 bifosfatase
PK	Piruvato cinase
PKA	Proteína cinase A
PI3K	Fosfatidilinositol-3 cinase
PIP2	Fosfatidilinositol bifosfato
PIP3	Fosfatidilinositol trifosfato
RI	Resistência à insulina
S1	Fração celular rica em HK e PFK solúveis
S2	Fração celular rica em PFK solúvel
SDS	Dodecil sulfato de sódio
STZ	Estreptozotocina
Tris	Tris (hidroximetil) aminometano

1.0. INTRODUÇÃO

1.1. Panorama geral sobre o diabetes mellitus

Os primeiros relatos do diabetes mellitus (DM) datam do papiro de Ebers, aproximadamente 1550 a.C. No entanto, o grande marco foi a descrição de Arateus da Capadócia, no século II, que denominou esta enfermidade de diabetes com a clássica descrição de que “a carne do corpo e dos membros se derretia e se convertia em urina”. Somente em 1675, Thomas Willis chamou de DM a condição em que indivíduos apresentavam poliúria, com urina doce e espessa. Em 1921, Charles Best e Frederick Banting conseguiram isolar a insulina e demonstrar seu efeito hipoglicemiante. Esta descoberta significou uma das maiores conquistas médicas do século XX (OLIVEIRA e MILECH, 2004).

Na década de 1990, o DM afetou a saúde de pelo menos 110 milhões de indivíduos no mundo. A Organização Mundial de Saúde (OMS) considera que a incidência de casos de DM tenha atingido um nível tão elevado que estima uma pandemia até 2030, com um crescimento de 171 milhões de casos em 2000 para 366 milhões em 2030 (WILD et al., 2004). Lamentavelmente o Brasil ocupa uma posição de destaque neste cenário, sendo o oitavo país com maior número de casos de DM, esperando-se um aumento neste número de 4,6 milhões em 2000 para 11,3 milhões em 2030 (BÉLIVEAU e GINGRAS, 2010).

Atualmente o DM é responsável por 9% das mortes relatadas em todo o mundo, sendo que em 2005, 1,1 milhões de pessoas morreram em decorrência das complicações desta doença (SCHMIDT et al., 2011).

O DM é caracterizado principalmente pela incapacidade do hormônio insulina em exercer seus efeitos, seja pela ausência total ou parcial deste hormônio e/ou resistência celular ao mesmo, produzindo uma série de distúrbios no metabolismo de carboidratos, proteínas e lipídios (AMERICAN DIABETES ASSOCIATION, 2007).

Indivíduos diabéticos estão susceptíveis a complicações crônicas e agudas causadas principalmente pela hiperglicemia. As complicações agudas incluem: cetoacidose, sede intensa, poliúria e hipoglicemia, que pode estar relacionada ao tratamento do DM (OLIVEIRA e MILECH, 2004). Segundo Davidson (2001), as complicações crônicas podem ser agrupadas em três tipos principais: microvasculares (nefropatia e retinopatia), macrovasculares (isquemia cardíaca e doença vascular periférica) e neuropatia (periférica e autonômica). Desta maneira, esta enfermidade causa um impacto socioeconômico importante

devido à grande demanda pelos serviços ambulatoriais, hospitalizações prolongadas, invalidez e mortalidade geradas pelas complicações agudas e crônicas do DM (IDF, 2003; MINISTÉRIO DA SAÚDE, 2011).

A classificação etiológica do DM está representada na tabela 1. Esta classificação é feita de acordo com os fatores etiológicos peculiares envolvidos no aparecimento de cada uma dessas doenças. As duas principais formas de DM em incidência, prevalência e importância clínica são o diabetes mellitus tipo 1 (DM1) ou insulino-dependente e o diabetes mellitus tipo 2 (DM2). Ainda, existem outros tipos específicos de diabetes, além do diabetes mellitus gestacional (GROSS et al., 2002; AMERICAN DIABETES ASSOCIATION, 2007).

Tabela 1. Classificação etiológica do diabetes mellitus tipo 2 (American Diabetes Association, 2009)

-
- I. Diabetes tipo 1**
 - destruição das células beta, usualmente levando à deficiência completa de insulina
 - A. auto-imune
 - B. idiopático
 - II. Diabetes tipo 2**
 - graus variados de diminuição de secreção e resistência à insulina
 - III. Outros tipos específicos**
 - A. Defeitos genéticos da função da célula β
 - B. Defeitos genéticos da ação da insulina
 - C. Doenças do pâncreas exócrino
 - D. Endocrinopatias
 - E. Indução por drogas ou produtos químicos
 - F. Infecções
 - G. Formas incomuns de diabetes imuno-mediado
 - IV. Diabetes Gestacional**
-

Vale ressaltar que embora existam inúmeros subtipos de DM, os tipos 1 e 2 correspondem juntos a mais de 95% dos casos diagnosticados. Sendo a incidência do tipo 2 correspondente a 80-90% dos casos (AMERICAN DIABETES ASSOCIATION, 2009). Há ainda outro fator recentemente identificado, que é a hereditariedade epigenética, onde a prole pode adquirir diabetes a partir de pais obesos (SKINNER, 2010).

1.2. Panorama geral sobre o diabetes mellitus tipo 1

No DM1 ocorre destruição das células beta do pâncreas, usualmente por processo auto-imune (90% dos casos do tipo 1; tipo 1A) ou, menos frequentemente, por causa desconhecida (10% dos casos do tipo 1; forma idiopática; tipo 1B) (tabela 1). Suas características principais são: necessidade diária de insulina no tratamento, com controle metabólico lábil, grande oscilação na glicemia e tendência a desenvolver cetoacidose (OLIVEIRA e MILECH, 2004). O DM tipo 1A tornou-se uma das doenças autoimunes mais estudadas nos últimos anos (EISENBARTH, 2007).

A incidência do DM1 vem crescendo rapidamente no mundo inteiro e também está presente cada vez mais em idades mais tenras (DEVENDRA et al., 2004). Embora muita atenção tenha sido dada ao aumento da incidência do DM2, um aumento paralelo dos casos de DM1 tem ocorrido (ONKAMO et al., 1999). A taxa de incidência do DM1 varia entre 0,3 - 0,4% da população, de acordo com a área geográfica e com os grupos étnicos estudados, sugerindo diferenças na suscetibilidade genética, bem como o papel de fatores ambientais e sócio-econômicos. Esta doença atinge principalmente crianças e adolescentes, com pico de incidência entre 15 anos de idade, embora indivíduos de qualquer faixa etária possam ser acometidos (VOLTARELLI, 2004; GILLESPIE, 2006). Entre os americanos que são diagnosticados com DM, estima-se que 5 - 10% deles tenha DM1 (AMERICAN DIABETES ASSOCIATION, 2009).

Os sintomas mais comumente observados nesses pacientes são: sede intensa, poliúria, glicosúria, fadiga muscular, dificuldades de cicatrização periférica, entre outros. Com o passar dos anos, o paciente diabético que não consegue controlar a glicemia com o uso de insulina e/ou outros medicamentos hipoglicemiantes pode apresentar distúrbios graves, tais como cardiopatias, cegueira, entre outros. Mesmo com os diversos recursos terapêuticos atuais (SBD, 2011), o DM1 possui um risco de mortalidade aumentada quando comparado à população geral, em grande parte devido às doenças cardiovasculares (International Diabetes Federation, 2003; SKIRIVARHAUG et al., 2005).

1.3. Panorama geral sobre o diabetes mellitus tipo 2

O DM2, previamente conhecido como DM não dependente de insulina, é caracterizado por resistência à insulina em tecidos periféricos, principalmente músculo esquelético e tecido adiposo, incapacidade da insulina em inibir a produção hepática de

glicose e desregulação da secreção de insulina (DEFRONZO, 1997). O DM2 tem maior incidência do que o DM1, perfazendo cerca de 90% dos casos totais de DM (AMERICAN DIABETES ASSOCIATION, 2009). A idade de início do DM2 é variável, embora seja mais frequente após os 40 anos de idade e, muitas vezes, associada com a obesidade e o estilo de vida (GRILL e QVIGSTAD, 2000; SBD, 2011).

Os principais fatores de risco para o surgimento do DM2 estão resumidos na tabela 2.

Tabela 2. - Principais fatores de risco para o surgimento do diabetes mellitus (adaptado de MOTTA, 2009).

Idade \geq 45 anos
Histórico familiar de DM (pais, filhos e irmãos)
Excesso de peso (IMC \geq 27 kg/m ²) ou acúmulo de gordura visceral
Sedentarismo
HDL baixo (< 35mg/dL) e/ou triglicérides elevados (> 250mg/dL)
Hipertensão arterial (> 140/90 mmHg)
Doença coronariana
DM gestacional prévio
Macrossomia ou histórico de abortos de repetição ou mortalidade perinatal

O DM2 tem mecanismos fisiopatológicos complexos e não completamente elucidados. Neste tipo de DM, a intolerância à glicose devido a maior resistência à insulina de tecidos como músculo esquelético, tecido adiposo e fígado (principais órgãos responsáveis pela manutenção da normoglicemia) pode ser compensada pelas células beta com o aumento da secreção de insulina (HEATHER e CLARKE, 2011). Assim, os níveis plasmáticos de insulina estão elevados, ao menos no início, apesar de geralmente serem insuficientes para compensar a resistência presente nos tecidos periféricos (WILKIN, 2001). Devido ao aumento da produção e liberação de insulina em função da hiperglicemia, a resistência à insulina pode levar a morte destas células ao longo dos anos. As complicações crônicas mais comuns decorrentes desta doença são coronariopatias, angina pectoris, infarto do miocárdio, retinopatia diabética, neuropatia periférica, acidente vascular cerebral (AVC), depressão e nefropatia (SRINIVASAN et al., 2008; SBD, 2011). A glicação é um processo não enzimático e irreversível responsável pela ligação de açúcares redutores a proteínas, lipídeos e outras moléculas orgânicas, comprometendo as suas funções biológicas normais (SBD, 2011).

Pacientes com DM2 frequentemente apresentam um conjunto de fatores de risco cardiovascular, tais como obesidade, hipertensão, altos níveis de triglicérides e baixos níveis de lipoproteína de alta densidade (HDL), todos os quais também estão relacionados com a resistência à insulina (WISSE, 2004). O controle do peso, dieta equilibrada e a prática de atividades físicas são importantes componentes para o tratamento do DM. Entretanto, quando a dieta e o exercício falham em controlar a hiperglicemia, a intervenção farmacológica tem que ser associada (DEFRONZO, 1999; SBD, 2011).

Nos últimos anos, a síndrome metabólica (conjunto de fatores de risco cardiovasculares, relacionados com resistência à insulina e obesidade abdominal), que sempre foi correlacionada com o desenvolvimento de DM2, também passou a ser considerada no DM1 (DIB, 2006) por causa do aumento da prevalência de obesidade na infância e na adolescência durante as últimas décadas (JAMES et al., 2001; LIBMAN et al., 2003).

A obesidade e suas consequências têm se tornado um grande problema de saúde mundial. Mais de 2,5 milhões de mortes por ano são atribuídas à obesidade, um número que deverá dobrar até 2030 (IDF, 2006). No Brasil, de acordo com o Instituto Brasileiro de Geografia e Estatística (IBGE), mais da metade da população adulta está acima do peso (IBGE, 2009). Não há dúvidas de que a obesidade é o resultado de um desequilíbrio positivo entre a quantidade de energia ingerida e a quantidade de energia gasta pelo corpo. Diante deste quadro, o excesso de peso pode levar ao aparecimento de resistência à insulina que, por sua vez, pode promover o desenvolvimento de hipertensão, DM2, dislipidemias, câncer e/ou doenças cardiovasculares (CHANDALIA e ABATE, 2007).

Durante muitos anos, o tecido adiposo foi considerado simplesmente como o órgão responsável pelo estoque de energia corporal. No entanto, da década de 90 até os dias de hoje, este tecido tem ganhado muita importância como um órgão endócrino, que secreta vários fatores importantes para a regulação metabólica de outros tecidos, tais como músculo esquelético e fígado. Além disto, sabe-se que tanto o excesso de tecido adiposo, quanto a sua falta, pode levar ao desenvolvimento de resistência à insulina e a dislipidemias, enfatizando o importante papel deste tecido na regulação do metabolismo celular (WAKI e TONTONNOZ, 2007). Apesar dessas descobertas, ainda não está claro como o excesso de tecido adiposo pode levar ao desenvolvimento de resistência à insulina.

1.4. Outros tipos específicos de diabetes mellitus

Na medida em que os processos de patogênese do diabetes têm sido elucidados, tanto em relação a marcadores genéticos como aos mecanismos que podem levar ao desenvolvimento da doença, tem crescido o número de tipos distintos de DM, permitindo uma classificação mais específica e definitiva. Portanto, novas categorias têm sido acrescentadas à lista de tipos específicos de DM, incluindo defeitos genéticos da função das células beta, endocrinopatias, infecções, casos decorrentes do uso de medicamentos, entre outros (tabela1).

1.5. Panorama geral sobre o diabetes mellitus gestacional

O diabetes mellitus gestacional (DMG) é definido como a tolerância diminuída aos carboidratos, com graus variados de intensidade, diagnosticado pela primeira vez durante a gestação, podendo ou não persistir após o parto (SBD, 2011). Estima-se que o DMG ocorra em 14% das mulheres grávidas (JOVANOVIC e PETTITT, 2001). Os fatores de risco associados a esta doença são semelhantes aos descritos para o DM2, incluindo, ainda, idade superior a 25 anos, ganho excessivo de peso na gestação, deposição central excessiva de gordura corporal, baixa estatura, crescimento fetal excessivo, polidrâmia, hipertensão ou pré-eclâmpsia gestacional e antecedentes obstétricos de morte fetal ou neonatal (KING, 1998).

As causas do DMG ainda não estão bem esclarecidas. No entanto, hormônios placentários parecem ser os principais responsáveis por reprogramar o metabolismo da mãe, levando a resistência periférica à insulina, na tentativa de gerar maior aporte de glicose para o desenvolvimento do feto (SORENSEN e BRELJE, 2009).

Embora todos os tipos de diabetes sejam importantes do ponto de vista científico, este trabalho se limitará ao estudo do DM1.

1.6. Diabetes mellitus tipo 1

1.6.1. Fisiopatologia do diabetes mellitus tipo 1

No estado de jejum, a normoglicemia depende do balanço entre a produção de glicose hepática e a utilização de glicose pelos principais tecidos dependentes de insulina (por exemplo, tecido adiposo e muscular) e pelos tecidos não dependentes de insulina (cérebro e rins). Este balanço é principalmente regulado por dois hormônios pancreáticos: a insulina e o

glucagon. Assim, em indivíduos saudáveis, a resposta ao aumento na glicemia é um incremento na secreção de insulina pelas células β das ilhotas pancreáticas e uma inibição da secreção de glucagon (GERICH et al., 1974).

A elevação da insulinemia em resposta à glicose também afeta a captação desta pelos tecidos periféricos e suprime a degradação de triglicerídeos e a liberação dos ácidos graxos pelo tecido adiposo (GROOP et al., 1989; DEFRONZO, 1997; DEFRONZO, 1999). A ação da insulina, inibindo a produção hepática de glicose e estimulando a sua utilização nos tecidos periféricos, possibilita a diminuição da glicemia, permitindo a manutenção da glicemia dentro de níveis normais (BARG, 2003).

Numerosos defeitos têm sido postulados para delinear a patogênese do DM1 (HORNUM e MARKHOLST, 2004; PANAGIOTOPOULOS et al., 2004). Esta se caracteriza por ser uma doença multifatorial, dependente da complexa interação entre resposta imunológica, fatores genéticos e influência do meio-ambiente na destruição das células beta produtoras de insulina (SESTERHEIM et al., 2007). No entanto, independentemente dos mecanismos de patogênese, a destruição das células beta pancreáticas parece ser a última etapa de um processo coordenado pelos linfócitos do sistema imune (HORNUM e MARKHOLST, 2004).

Estudos que mediram a expressão de anticorpos relacionados ao DM1 em crianças sugerem que a aparição destes marcadores é o maior fator de risco para o futuro desenvolvimento da doença (YU e EISENBARTH, 2000; EISENBARTH, 2007). Geralmente, anticorpos que reagem contra a enzima descarboxilase do ácido glutâmico (GAD) e contra a insulina são detectados (EISENBARTH, 2007; MARASCHIN et al., 2010). Exames histopatológicos de ilhotas pancreáticas, obtidas a partir de biópsia de pacientes com DM1 recentemente diagnosticados, mostraram a presença de um infiltrado composto de linfócitos T (CD4 e CD8), linfócitos B e macrófagos, sugerindo, desta forma, que essas células possam ter um importante papel na destruição das células beta (IMAGAWA et al., 1999).

1.6.2. Tratamento do diabetes mellitus tipo 1

O tratamento do DM pode se dar pela dieta, prática de atividade física e pelo uso de insulina exógena e/ou de hipoglicemiantes orais (EURICH et al., 2007; SBD, 2011). Atualmente, o DM1 é tratado com injeções de insulina, mudanças no estilo de vida e um

cuidadoso monitoramento da glicemia. Em pacientes que apresentam um quadro de deficiência de insulina, como no DM1, a lista de formulações de insulina, que variam de acordo com sua origem e/ou tempo de ação, disponíveis no mercado é extensa (tabela 3), possibilitando um estilo de vida com menos privações (OLIVEIRA e MILECH, 2004).

Tabela 3 – Sequências de aminoácidos dos principais tipos de insulinas comercializadas (Adaptado de CROMMELIN, 2008).

Tipo de insulina	A⁸	A¹⁰	A²¹	B³	B²⁸	B³⁰	B³¹	B³²
Human (Humulin®, Novolin®)	Thr	Ile	Asn	Asn	Pro	Thr	-	-
Porcina	Thr	Ile	Asn	Asn	Pro	Ala	-	-
Bovina	Ala	Val	Asn	Asn	Pro	Ala	-	-
Lispro (Humalog®)	Thr	Ile	Asn	Asn	Lis	Thr	-	-
Aspart (NovoRapid®, NovoLog®)	Thr	Ile	Asn	Asn	Asp	Thr	-	-
Glulisina (Apidra™)	Thr	Ile	Asn	Lys	Pro	Thr	-	-
Glargina (Lantus®)	Thr	Ile	Gly	Asn	Pro	Thr	Arg	Arg
Detemir (Levemir®)	Thr	Ile	Asn	Asn	Lys-(N-tetradecanoil)			

A administração de insulina permanece o principal tratamento do DM1. No entanto, o risco de hipoglicemia ainda é o maior fator limitante no tratamento desta doença com este hormônio (DEVENDRA et al., 2004; UNGER e PARKIN, 2011a; UNGER e PARKIN, 2011b).

1.7. Síntese e sinalização de insulina

A síntese de insulina ocorre no retículo endoplasmático rugoso das células beta das Ilhotas de Langerhans, no pâncreas, a partir da pré-pro-insulina. Esta ao direcionar-se ao complexo de Golgi é convertida em pró-insulina. A partir da atuação de enzimas, endopeptidases e exopeptidases, ocorre à clivagem desta molécula em insulina e peptídeo C. A insulina na sua forma ativa tem 51 aminoácidos e é uma das menores proteínas conhecidas. Em humanos, a insulina tem um peso molecular de 5808 Da.

O peptídeo C é liberado no sangue em quantidades molares iguais à da insulina. Como insulinas exógenas não contêm peptídeo C, o nível plasmático desse peptídeo é um bom indicador da produção endógena de insulina. Os efeitos biológicos do peptídeo C ainda não

estão completamente elucidados, mas alguns trabalhos já demonstraram que ele é capaz de aumentar o consumo de glicose e o controle metabólico de pacientes com DM1 (FORST et al., 2008; HILLS e BRUNSKILL, 2009; HILLS e BRUNSKILL, 2010).

Geralmente a insulina é produzida e secretada para a corrente sanguínea em resposta a um estímulo glicêmico. Quando este hormônio alcança os tecidos-alvo, há a ligação da insulina no sítio das subunidades α do receptor de insulina (RI) e uma rápida mudança conformacional se segue, gerando a autofosforilação de múltiplos resíduos de tirosina na porção citosólica das subunidades β (VIRKAMÄKI et al., 1999). A autofosforilação resulta no aumento da atividade tirosina cinásica intrínseca do receptor de insulina, iniciando a propagação do sinal através de muitas outras proteínas. Entre essas proteínas estão os substratos do receptor de insulina (*Insulin receptor substrate* - IRS). O IRS fosforilado ativa a enzima fosfatidilinositol-3 cinase (*Phosphatidyl inositol – 3 kinase* - PI3K). Uma vez ativada, a PI3K fosforila o fosfolípídeo de membrana fosfatidilinositol bifosfato (PIP2) convertendo-o em fosfatidilinositol trifosfato (PIP3). O PIP3 recruta para a membrana a enzima proteína cinase B (*Protein kinase B* - PKB) (COHEN et al., 1997). O mecanismo de ativação da PKB pelo PIP3 ainda não está muito bem esclarecido, envolvendo provavelmente, a ação da enzima dependente de fosfoinositol (*Phosphoinositol dependent kinase* - PDK). Por fim, a PKB parece ser capaz de induzir o recrutamento de transportadores de glicose para a membrana celular (GLUT) (TANTI et al. 1997).

A ativação do RI pode ser desfeita quando a insulina desliga-se do seu receptor ou quando há a desfosforilação de resíduos específicos de tirosina do receptor, diminuindo a atividade cinásica intrínseca da subunidade β do RI. Já foi demonstrado que a desfosforilação do RI *in vivo* é um processo rápido, mediado por fosfatases específicas, levando a crer que a fosforilação nos resíduos de tirosina do RI seja um processo dinâmico e rapidamente reversível (DRAKE et al., 1998).

Além dos efeitos sobre a homeostase de glicose, a insulina também promove outros eventos celulares, incluindo a regulação do transporte de íons e aminoácidos, metabolismo de lipídios, síntese de glicogênio, transcrição gênica, síntese de proteínas, entre outros. Assim, os efeitos da insulina desempenham importantes funções no armazenamento normal de nutrientes ingeridos e no crescimento e diferenciação celular (ROSEN, 1987; DEFRONZO et al., 1992; SEINO et al., 2011).

1.8. Papel da insulina na homeostase da glicemia

Um dos primeiros papéis da insulina na regulação da homeostase de glicose é o estímulo à captação de glicose nos tecidos sensíveis à insulina. A captação de glicose ocorre por difusão facilitada através de proteínas transportadoras específicas, sendo denominadas de uma forma geral de GLUT (*glucose transporter* - transportadores de glicose) (MYERS e WHITE, 1996; HOLMAN e KASUGA, 1997).

Existem várias isoformas de GLUT, com diferentes afinidades e capacidades de transporte de glicose. Isoformas diferentes são expressas em tecidos diferentes. Várias isoformas de GLUT já foram identificadas e clonadas (MANOLESCU et al., 2007; AUGUSTIN, 2010). Estas proteínas transportadoras de hexoses são similares quanto à sequência e estrutura, mas únicas quanto à distribuição tecidual. O GLUT 1 foi o primeiro transportador facilitado identificado e está presente na placenta, cérebro, rins, eritrócitos e cólon; também está presente em baixa quantidade no tecido adiposo e muscular. O GLUT 2 está presente, predominantemente, no fígado e nas células beta pancreáticas, estando envolvido nos mecanismos que levam a secreção de insulina. O GLUT 3 é encontrado em múltiplos tecidos, incluindo cérebro, placenta e rins. GLUT 5 é encontrado, predominantemente, no intestino delgado. As isoformas de GLUT 6-14 ainda não têm um papel bem determinado para o transporte de glicose (ZHANG et al., 1999; MANOLESCU et al., 2007).

O GLUT 4 parece ser o único transportador de glicose que é regulado por insulina e é encontrado, exclusivamente, em tecidos sensíveis à insulina, os quais incluem os músculos esquelético e cardíaco e o tecido adiposo (BIRNBAUM, 1992; MYERS e WHITE, 1996; HOLMAN e KASUGA, 1997). Na ausência de insulina, quase a totalidade dos transportadores GLUT 4, e em menor extensão também os transportadores GLUT 1, é encontrada em vesículas intracelulares. Após a sinalização desencadeada pela insulina, estas vesículas são incorporadas à membrana plasmática, resultando num aumento de 10 a 20 vezes na captação de glicose (CUSHMAN e WARDZALA, 1980; JAMES et al., 1988; ZORZANO et al., 1989). O mecanismo exato de translocação de GLUT 4, induzido por insulina, é desconhecido. Entretanto, o pré-tratamento de células de adipócitos (3T3-L1) com inibidores de PI3K, como a wortmanina, inibe a captação de glicose basal e a captação estimulada por insulina (FINGAR et al., 1993; CHEATHAM et al., 1994). Deste modo, postula-se que a PI3K esteja envolvida neste processo.

Além disto, o maior translocamento de GLUT 4 para a membrana plasmática e o consequente aumento no transporte de glicose podem envolver uma sinalização intracelular que leva a ativação de proteínas cinases dependentes do complexo Ca^{2+} -calmodulina (CaM), proteína cinase C (PKC) e/ou proteína cinase ativada por AMP (AMPK) (ROSE e RICHTER, 2005; ZIERATH, 2002).

A ativação da AMPK está correlacionada com a fosforilação e modulação de várias proteínas envolvidas no metabolismo de lipídios, carboidratos e na transcrição gênica. Nesse sentido, uma maior oxidação de gorduras e carboidratos, bem como incrementos na expressão de GLUT4 e genes mitocondriais em músculo esquelético, já foram descritos como alvos da ação da AMPK (TOWLER e HARDIE, 2007). Vale ressaltar que a ativação da AMPK, levando ao aumento do consumo de glicose, independe da ação da insulina.

Independente do estímulo inicial, seja ele insulinêmico ou não, a glicose quando internalizada pode ser direcionada para várias vias metabólicas, como por exemplo: via das pentoses fosfato, síntese de glicogênio, via glicolítica, dentre outras.

1.9. Glicólise

A glicólise é a principal via de catabolismo de carboidratos em todos os tipos celulares, fornecendo energia e metabólitos para vias biossintéticas, sendo fortemente regulada por vários mecanismos (BEITNER, 1979; SOLA-PENNA et al., 2010). A oxidação da glicose é conhecida como glicólise ou via de Embden-Meyerhof em mamíferos (Fig. 1). Na maioria das células de mamíferos, a presença de oxigênio leva ao aumento do metabolismo mitocondrial, aumentando o estado energético da célula e, conseqüentemente, inibindo a glicólise. Esta inibição é chamada de 'Efeito Pasteur', em que o fluxo de glicose é reduzido pela presença de oxigênio (RACKER, 1974). Nas células capazes de metabolizar glicose independentemente do consumo de oxigênio (glicólise anaeróbia), tais como os eritrócitos, algumas fibras musculares e tumores, o produto final da glicólise (piruvato) é majoritariamente convertido em lactato (NELSON e COX, 2000; GATENBY e GILLIES, 2004).

A via glicolítica consiste em dez reações sequenciais que convertem uma molécula de glicose em duas moléculas de piruvato (Fig. 1), sendo essas etapas separadas em duas fases. A primeira etapa é a fase preparatória que requer energia na forma de ATP para ativar a molécula de glicose, através da sua fosforilação. A segunda fase é a fase de pagamento ou produtora de energia. Na primeira fase, dois equivalentes de ATP são utilizados para

converter a glicose em duas moléculas de gliceraldeído-3-fosfato. Na segunda fase, o gliceraldeído-3-fosfato é transformado em piruvato com a produção de 4 equivalentes de ATP e 2 equivalentes de NADH (Robergs, 2004).

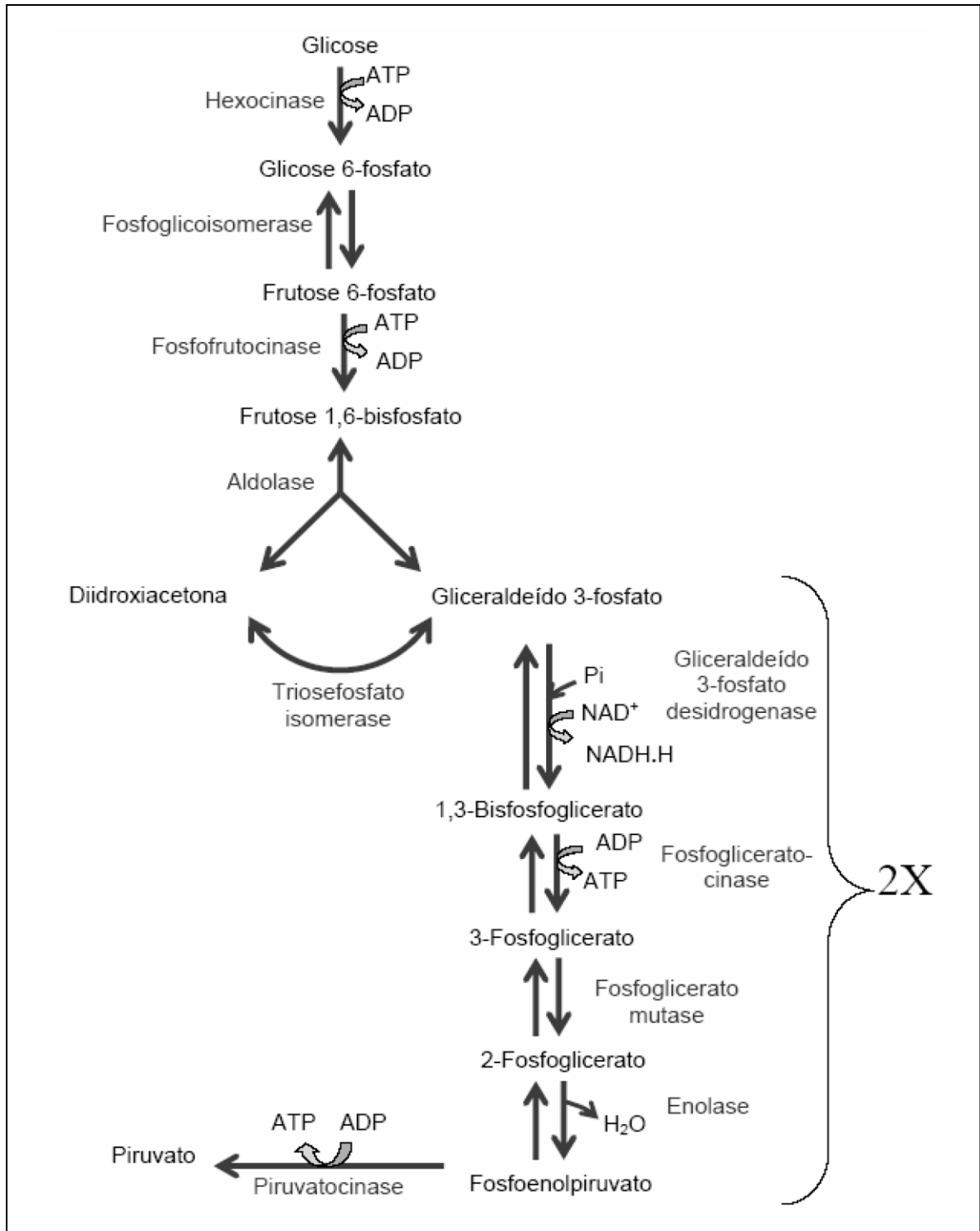
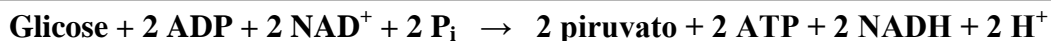


Figura 1. Via Glicolítica. Adaptado de NELSON e COX, 2000.

A oxidação parcial da glicose pela via glicolítica pode ser demonstrada pela seguinte equação:



Esquema 1. Equação geral da conversão de glicose em piruvato através da glicólise. Estas reações também produzem ATP e NADH a partir de ADP e NAD^+ , respectivamente.

O NADH gerado durante a glicólise pode ser utilizado pelas mitocôndrias para a síntese de ATP através da fosforilação oxidativa (NELSON e COX, 2000). A regulação da glicólise ocorre principalmente em três etapas limitantes, catalisadas pelas enzimas hexocinase (HK), fosfofrutocinase (PFK) e piruvato cinase (PK).

A fosforilação da glicose, utilizando ATP para formar glicose-6-fosfato (G6P), é a primeira reação da glicólise, sendo catalisada pela hexocinase (HK) (GROSSBARD e SCHIMKE, 1966). Esta reação é irreversível em condições fisiológicas, sendo um dos pontos de controle da via (Fig. 1). Com exceção do fígado e dos rins, que possuem a enzima glicose 6-fosfatase, capaz de converter a G6P em glicose livre, a G6P gerada pela HK não é capaz de sair da célula, assim sendo metabolizada. Isto ocorre porque a membrana plasmática e os transportadores de glicose são impermeáveis a G6P (WILSON, 2003). A segunda reação da glicólise é uma isomerização, na qual a G6P é convertida em frutose-6-fosfato (F6P), pela enzima fosfoglicoisomerase. Esta reação é livremente reversível em condições celulares normais.

A próxima reação da glicólise envolve a utilização de um segundo ATP para converter a F6P em frutose 1,6-bifosfato (F1,6BP). Esta reação é catalisada pela 6-fosfofruto-1-cinase (PFK) (Fig. 1). Esta é a segunda reação irreversível desta via, sendo descrita como o principal ponto de controle de todo o fluxo glicolítico (UYEDA, 1979; BEITNER, 1979; KEMP e FOE, 1983; SOLA-PENNA et al., 2010).

A aldolase catalisa a hidrólise da F1,6BP em dois produtos de 3 carbonos cada: a di-idroxiacetona fosfato e o gliceraldeído-3-fosfato. Estes dois produtos da reação da aldolase equilibram-se na reação catalisada pela triosefosfato isomerase. As reações subsequentes da glicólise utilizam o gliceraldeído-3-fosfato como substrato (Fig. 1). Assim, os produtos da reação da aldolase são direcionados no sentido da glicólise pelo princípio de ação das massas (NELSON e COX, 2000).

A segunda fase do catabolismo da glicose é caracterizada pelas reações que resultam na produção de ATP e NADH. Na primeira destas reações, a gliceraldeído-3-fosfato desidrogenase (GAPDH) catalisa a oxidação, dependente de NAD^+ , do gliceraldeído-3-fosfato em 1,3-bifosfoglicerato e NADH (ETO et al., 1999). O 1,3-bifosfoglicerato é utilizado para formar ATP e 3-fosfoglicerato pela enzima fosfoglicerato cinase. Em eritrócitos, a formação de 2,3-bifosfoglicerato, a partir de 1,3-bifosfoglicerato, pela ação da enzima bifosfoglicerato mutase é importante uma vez que ele modula a afinidade da hemoglobina por oxigênio (METIVIER et al., 2000).

Nas reações restantes da glicólise, o 3-fosfoglicerato é convertido em 2-fosfoglicerato pela fosfoglicerato mutase (PMG) e, em seguida, o produto desta reação é convertido em fosfoenolpiruvato pela enzima enolase.

A reação final da glicólise é catalisada pela piruvato cinase (PK). Nesta reação altamente exergônica, o ADP, juntamente com o fosfato de alta energia do fosfoenolpiruvato, é convertido em ATP. O piruvato formado pode seguir diferentes vias metabólicas dependendo da célula em questão e do perfil metabólico em que a mesma se encontra. Por exemplo, o piruvato pode ser translocado para a matriz mitocondrial através do transportador de monocarboxilatos (MCT - *monocarboxylate transporter*) (GLADDEN, 2004). No interior da mitocôndria o piruvato pode sofrer a ação do complexo multienzimático piruvato desidrogenase (PDH), resultando na formação de acetil-CoA. Na etapa seguinte, a condensação de quantidades equimolares de oxaloacetato e acetil-CoA, formando citrato, regulada pela enzima citrato sintase, controla diretamente a oxidação do acetil-CoA derivado tanto do piruvato como da oxidação dos ácidos graxos (NEWSHOLME e LEECH, 1988).

Em condições aeróbicas, o piruvato é metabolizado através do ciclo dos ácidos tricarbóxicos, na maioria das células. Por outro lado, em condições anaeróbicas, e em eritrócitos mesmo em condições aeróbicas, o piruvato pode ser reduzido a lactato pela lactato desidrogenase (LDH), reoxidando NADH, e depois transportado para fora das células, entrando na circulação sanguínea (Brooks, 2000).

A conversão de piruvato em lactato fornece à célula um mecanismo para a reoxidação do NADH (produzido durante a reação da GAPDH) em NAD^+ , garantindo a disponibilidade deste para a realização da glicólise. Normalmente, durante a glicólise aeróbica, os elétrons do NADH citosólico são transferidos para os carreadores mitocondriais da fosforilação oxidativa através das lançadeiras glicerol fosfato ou malato-aspartato, regenerando NAD^+ citosólico (NELSON e COX, 2000).

As concentrações sanguíneas e intracelulares de lactato podem variar significativamente em diferentes momentos metabólicos, dependendo da relação entre a sua taxa de produção e de remoção. Teoricamente, todas as células do organismo são capazes de sintetizar lactato, mas o músculo esquelético é o principal produtor deste metabólito, principalmente em exercícios físicos de alta intensidade (ROBERGS, 2004).

A glicólise em condições aeróbicas gera substancialmente mais ATP por mol de glicose oxidada do que em condições anaeróbicas, entretanto, neste último caso, a produção de ATP pela glicólise pode ser até aproximadamente 100 vezes mais rápida do que pela fosforilação oxidativa. Células com grande demanda energética como as fibras musculares esqueléticas durante exercícios físicos intensos, células cancerígenas com elevadas taxas de síntese de proteínas, entre outras, adaptam seu metabolismo para uma rápida e eficiente produção de energia (GATENBY e GILLIES, 2004). Esses fatores têm em comum a alteração na localização intracelular de enzimas glicolíticas, gerando um fenótipo glicolítico característico, o que potencializa a produção energética e a sobrevivência celular (EL-BACHA et al., 2003; SILVA et al., 2004; ZANCAN e SOLA-PENNA, 2005a, 2005b; MARINHO-CARVALHO et al., 2006; SPITZ et al., 2009; SOLA-PENNA et al., 2010). Por outro lado, a glicólise encontra-se comprometida em quadros de DM (BAZAES et al., 1982; CHEN-ZION et al., 1994; FULGENCIO et al., 2001). Assim, a ativação do metabolismo de carboidratos poderia ser importante para a promoção de ações hipoglicemiantes.

1.9.1. Regulação da glicólise

Para manter a sua homeostase, a célula tem que se adaptar a possíveis perturbações agudas ou crônicas que possam ocorrer no seu meio interno e/ou externo. Tais adaptações passam por modificações das suas vias metabólicas, ou seja, na atividade das enzimas que as constituem. A via glicolítica não é diferente, devendo se adaptar às necessidades celulares a cada momento. Dentre os grandes sinalizadores que modulam a atividade da glicólise podemos destacar os hormônios, como por exemplo, a insulina, a adrenalina e o glucagon.

Podemos considerar que a via glicolítica possui duas grandes funções: degradar a glicose para gerar ATP e fornecer substratos para a biossíntese de macromoléculas celulares. Nesse sentido, a velocidade da glicólise é regulada para atender a essas duas necessidades. Isso se dá através de regulações alostéricas, por modificações covalentes ou pelos níveis de substratos (NELSON e COX, 2000). Nesta via, algumas reações são limitadas por outras

reações que são altamente exergônicas e irreversíveis, sendo pontos onde o fluxo glicolítico é regulado. Essas etapas, já citadas anteriormente, são representadas pelas reações catalisadas pelas enzimas HK, PFK e PK (PHILIPS et al., 1981).

Com exceção da sua isoforma 4 (de fígado, HK-4), a HK é inibida pelo seu produto, a G6P. Outro modo de controlar a sua atividade é modulando a sua localização intracelular. Já foi demonstrado que, ao estar associada à mitocôndria, a sua atividade passa a estar incrementada (SOUTHARD e HULTIN, 1972).

Dentre as três enzimas regulatórias do fluxo glicolítico, a PFK é a que apresenta uma maior complexidade na sua regulação. Esta enzima é regulada por uma variedade de ligantes, incluindo substratos, produtos de reações e vários metabólitos celulares (PASSONNEAU e LOWRY 1962, UYEDA, 1979; KEMP e FOE, 1983, SOLA-PENNA et al., 2010). Além de substrato, o ATP também é um efetor alostérico negativo da enzima, diminuindo a afinidade da PFK pelo seu substrato (F6P). Altas concentrações de ATP (acima de 1 mM) são capazes de inibir fortemente a enzima (PASSONNEAU e LOWRY, 1962; LEITE et al., 2007; ZANCAN et al., 2008; MARINHO-CARVALHO et al., 2009). Esta inibição pode ser potencializada pela presença de altas concentrações de H^+ , citrato e de isocitrato, mas não por outros intermediários do Ciclo de Krebs (PARMEGGIANI e BOWMAN, 1963). Outros compostos fosforilados são capazes de inibir a PFK, como fosfocreatina, 3-fosfoglicerato, 2-fosfoglicerato, 2,3-bisfosfoglicerato e fosfoenolpiruvato. Mais recentemente, nosso grupo demonstrou que o lactato pode inibir a PFK purificada de músculo esquelético de coelho (LEITE et al., 2007) e de homogeneizados de músculo esquelético (LEITE et al., 2011), sugerindo que este metabólito tenha um importante papel no desenvolvimento de resistência à insulina.

Um dos efetores alostéricos positivos mais importantes da PFK é a frutose 2,6-bisfosfato (F2,6BP). Esta molécula é produto da fosforilação da F6P pela enzima bifuncional fosfofrutocinase-2/frutose 2,6 bifosfatase (PFK-2). Desta forma, a atividade cinásica desta enzima precisa estar ativa e a fosfatásica, que converte a F2,6BP em F6P e fosfato inorgânico, inibida. No músculo esquelético e cardíaco a atividade cinásica desta enzima bifuncional é ativada por fosforilação da enzima. Esta fosforilação ocorre após estímulos hormonais, como o promovido pela adrenalina (HUE e RIDER, 1987). Porém, no tecido hepático a fosforilação da enzima PFK-2 ativa a porção fosfatásica e inibe a cinásica, diminuindo a síntese de F2,6BP e o fluxo glicolítico. O resultado metabólico da fosforilação da enzima bifuncional no fígado é a interrupção da estimulação alostérica da PFK e a desinibição alostérica da frutose-1,6-

bifosfatase, que converte a F1,6BP em F6P, desta forma, contribuindo para a formação de glicose pelo fígado (PILKIS e CLAUS, 1991).

A F2,6BP atua de maneira sinérgica com o ADP e AMP, ambos efetores alostéricos positivos da PFK, sendo capaz de contrapor a inibição da PFK causada por altas concentrações de ATP (ZANCAN et al., 2007b, 2008; GRECHI et al., 2011), aumentando sua afinidade pelo substrato, F6P, porém sem efeitos sobre a atividade máxima da enzima (PILKIS et al., 1981; VAN SCHAFTINGEN et al., 1981; UYEDA et al., 1981). Outros moduladores positivos da PFK também já foram descritos: P_i , K^+ e NH_4^+ e CaM. Esses moduladores são capazes de reverter à inibição promovida por ATP e citrato (PASSONNEAU e LOWRY, 1962; LILLING e BEITNER, 1990; MARINHO-CARVALHO et al., 2006, 2009).

A insulina tem um importante papel na regulação da glicólise. Este hormônio é capaz de alterar rapidamente a distribuição celular das enzimas glicolíticas. A significância desta rápida ação é, provavelmente, fornecer ATP através da glicólise para este ser usado por outros processos, como fosforilação de proteínas, internalização de receptores e transporte de vesículas (CHEN-ZION et al., 1992a; SILVA et al., 2004; ZANCAN et al., 2005a, 2005b).

1.9.1.1 Hexocinase

A fosforilação da glicose, dependente de ATP, para formar glicose-6-fosfato (G6P) é a primeira reação da glicólise e é catalisada por isoenzimas tecido-específicas conhecidas como hexocinases (ATP: D-glicose 6-fosfo-transferase, EC 2.7.1.2), como mostrado na reação da figura 2 (GROSSBAR e SCHIMKE, 1966). De uma maneira geral, 4 isoformas de HK foram separadas por cromatografia de troca iônica ou eletroforese a partir de extratos de vários tecidos de mamíferos (GONZÁLEZ et al., 1964; KATZEN e SCHIMKE, 1965). Geralmente, essas são conhecidas como HK tipo 1, 2, 3 ou 4, sendo esta última também conhecida como glicocinase. Em mamíferos, a HK-4 tem 50 KDa, enquanto que as outras isoformas possuem 100 KDa cada uma (WILSON, 1995; CARDENAS et al., 1998). A G6P pode inibir as isoformas de HK-1, HK-2 e HK-3, porém não a HK-4 (WILSON, 1995).

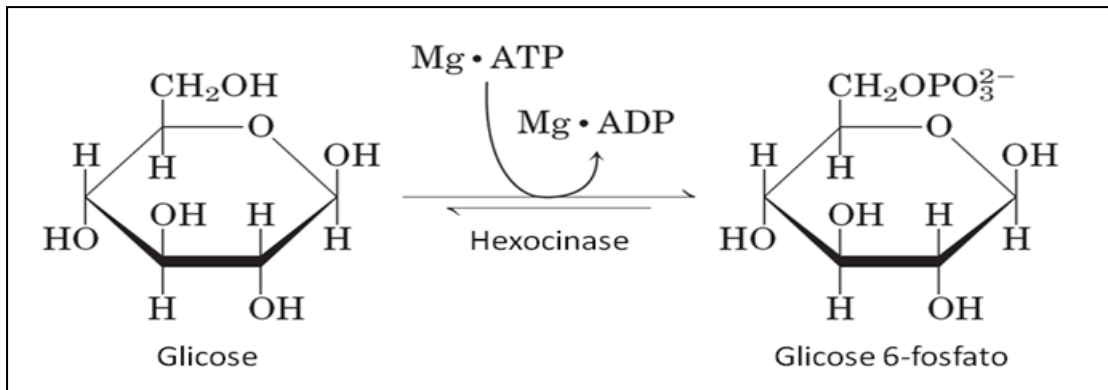


Figura 2. Reação da enzima hexocinase. Fosforilação da glicose gerando glicose-6-fosfato através da catálise enzimática da hexocinase. Adaptado de NELSON e COX, 2000.

A hexocinase, assim como outras enzimas glicolíticas, também está localizada em frações específicas das células. Esta enzima quando associada à mitocôndria possui maior atividade que a enzima na sua forma solúvel devido ao aumento de afinidade da enzima por glicose (VIITANEN et al., 1984). Este mecanismo vem sendo considerado como uma importante adaptação da enzima para a eficiente produção de energia. Walters e McLean (1968) demonstraram que o diabetes é capaz de reduzir a fração de HK ligada à mitocôndria e aumentar a fração solúvel da atividade da enzima em glândula mamária de rato. Além disso, sabe-se que ratos que foram induzidos ao diabetes com aloxano, também apresentam redução da atividade da HK ligada a frações específicas das células hepáticas (ANDERSON e ZAKIM, 1970). Por outro lado, o aumento fisiológico de cálcio intracelular parece aumentar a ligação da HK em músculo, com concomitante ligação de outras enzimas glicolíticas ao citoesqueleto muscular (CHEN-ZION et al., 1992b; LIVNAT et al., 1993). Esses estudos sugerem que o diabetes causa uma redistribuição celular da hexocinase e que este fato pode ser importante para o controle do metabolismo de glicose.

Já foi demonstrado que coração de camundongo que superexpressa uma isoforma de levedura de HK, resistente a inibição por G6P, mostrou elevado conteúdo de glicogênio e uma alta taxa de glicólise, indicando que a fosforilação da glicose é uma importante etapa que determina a utilização de glicose pelo coração (LIANG et al., 2002). A atividade da HK também é aumentada em mitocôndria de músculo cardíaco após estimulação insulinêmica (RUSSELL et al., 1992), indicando que a redistribuição da HK, do citosol para a mitocôndria, pode ser um importante mecanismo para o aumento da atividade desta enzima.

A hexocinase 1 (HK-1) está presente majoritariamente em tecidos não sensíveis à insulina, tais como cérebro, rins e eritrócitos. Esta isoforma da HK pode ser inibida por concentrações fisiológicas de G6P e tal inibição pode ser revertida por fosfato inorgânico (WILSON, 1995). Assim, o aumento da concentração de G6P pode inibir a HK, resultando na diminuição da captação e oxidação de glicose.

Tecidos sensíveis à insulina tais como músculo esquelético, coração e tecido adiposo apresentam predominantemente a HK-2. Nestes tecidos, a insulina leva ao translocamento da HK-2 do citosol para a mitocôndria (CHEN-ZION et al., 1992b), aproximando-a do fornecimento de ATP mitocondrial e, conseqüentemente, aumentando sua atividade (BESSMAN e GIEGER, 1980; RUSSELL et al., 1992).

Um dos mecanismos que podem levar a associação da HK-2 à mitocôndria é a sua fosforilação por Akt/PKB (proteína cinase B). Já foi demonstrado que a insulina estimula a fosforilação de HK-2, mediada por Akt, levando ao aumento da associação da enzima com a mitocôndria em tecido cardíaco, o que confere proteção a este tecido (ZUURBIER et al., 2005; MIYAMOTO et al., 2008). A HK-1 também pode se associar a membrana mitocondrial, no entanto a HK-2 parece se ligar com maior afinidade, o que sugere um diferente papel das isoformas em resposta a um estímulo insulinêmico. Vale ressaltar que ambas as isoformas possuem uma região consenso para a fosforilação por Akt (SOUTHWORTH et al., 2007).

A HK-4 é encontrada em hepatócitos e células beta pancreáticas e apresenta uma baixa afinidade para a glicose, sendo ativada principalmente com altas concentrações de glicose (WILSON, 2003). A HK-4 encontra-se em monômeros (CARDENAS et al., 2007) e apresenta somente um sítio para a ligação de glicose (CARDENAS et al., 1998), não apresentando cooperatividade com relação ao seu substrato, o que a permite se destacar como uma enzima muito sensível à variação na concentração de glicose intracelular.

Sabe-se também que a HK-4 pode sofrer regulação através da sua translocação entre diferentes compartimentos celulares. Apesar de não ser um consenso de que a HK-4 possa se ligar a mitocôndria (ARDEN et al., 2006; BUSTAMANTE et al., 2006), esta isoforma pode se translocar para o núcleo, quando ligada à proteína reguladora de glicocinase (GRP), sendo esta associação inibitória para a enzima. F6P aumenta a associação entre essas duas proteínas, enquanto que frutose 1-fosfato diminui, o que sugere um mecanismo indireto de inibição por *feedback* da HK-4, uma vez que a G6P está em equilíbrio com a F6P devido à ação da glicose-6-fosfato isomerase (VAN SCHAFTINGEN et al., 1994; DE LA IGLESIA et al., 1999).

Além da atividade gliconeogênica do fígado, a supressão da glicólise hepática também contribui para a elevação da glicemia (DAVIDSON, 1981). A menor captação de glicose em animais diabéticos está correlacionada com a inibição da síntese de HK-4, em resposta a queda dos níveis de insulina circulantes. Além disso, o diabetes está associado com menor atividade da glicocinase e das hexocinases de vários outros tecidos, sendo a terapia com insulina capaz de restaurar a atividade dessas enzimas (KATZEN, 1967; ANDERSON e ZAKIM, 1970).

A atividade da HK-4 está diminuída em ratos diabéticos induzidos com aloxano (SHARMA et al., 1964; PILKIS, 1970). Neste caso, as altas concentrações de glucagon parecem reduzir a síntese de HK-4 e, além disto, estimulam a sua degradação, contribuindo para a inibição da atividade dessa enzima (SIBROWSKI et al., 1982). Neste sentido, a regulação dos níveis da enzima parece envolver a ação de alguns hormônios, uma vez que baixas ou altas concentrações de glicose não parecem modular a síntese de HK-4 (SPENCE et al., 1979).

1.9.1.2. Fosfofrutocinase

A fosfofrutocinase (ATP: D-frutose-6-fosfato-1-fosfo-transferase, PFK, EC 2.7.1.11) catalisa a terceira reação da via glicolítica, sendo considerada como a principal enzima reguladora desta via metabólica (KEMP e FOE, 1983; RANDLE et al., 1966; DUNAWAY, 1988). A transferência do grupo fosfato gama terminal do $MgATP^{2+}$ para F6P, formando F1,6BP e ADP, é irreversível em condições metabólicas normais (Fig.3).

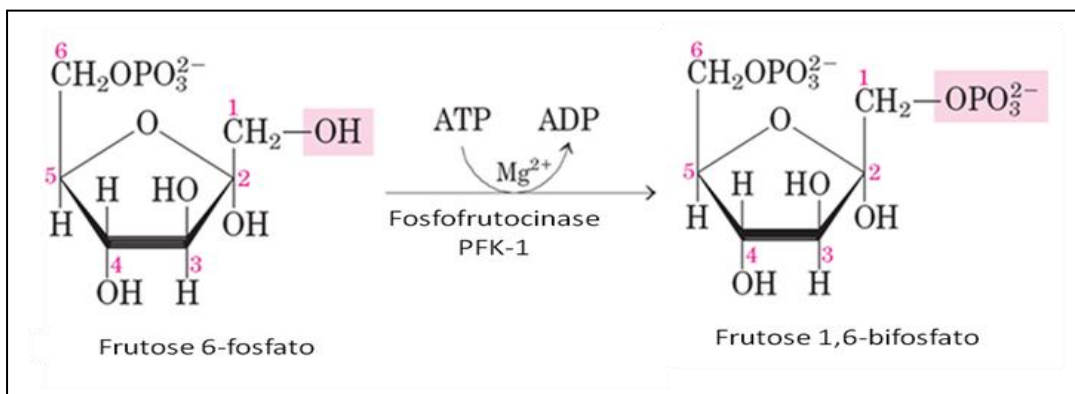


Figura 3. Reação da enzima fosfofrutocinase. Fosforilação da frutose-6-fosfato gerando frutose-1,6-bisfosfato através da catálise enzimática da fosfofrutocinase. Adaptado de NELSON e COX, 2000.

Dependendo do tecido, pode haver a expressão de um, dois, ou três genes que codificam a PFK. Os produtos destes genes podem associar-se randomicamente formando hetero-oligômeros ou homo-oligômeros. Três isoformas da PFK estão descritas e cada subunidade é codificada em cromossomos separados. Os genes para as subunidades PFK-L, PFK-M e PFK-C estão localizados nos cromossomos 21, 1 e 10, respectivamente (VORA e FRANCKE, 1981; VORA et al., 1982; SHARMA et al., 1989). A PFK-M (85,2 KDa) é a única isoforma expressa em músculo esquelético de adultos (KAHN et al., 1979; MEIENHOFER et al., 1979; VORA et al., 1980). Por outro lado, enquanto que a PFK-L (85,0 KDa) é a isoforma predominante no fígado, placenta, linfócitos, eritrócitos, plaquetas, rins e hepatomas (MEIERHOFER et al., 1979; KAHN et al., 1979), a PFK-C é expressa em cérebro, fígado, placenta, eritrócitos e coração (DUNAWAY et al., 1988).

Dunaway et al. (1988) mostraram que no fígado 62% e 29% das isoenzimas são PFK-L e PFK-M, respectivamente, e que 90% e 48% da atividade total da enzima foi precipitada com anti-L IgG e anti-M IgG, respectivamente. Consequentemente, quantidades significantes de híbridos (L-M) podem estar presentes no fígado humano. Conclusões similares já tinham sido postuladas para a PFK de fígado de rato (DUNAWAY e KASTEN, 1987). A homologia entre a PFK-M humana e a de rato é alta, tanto em nucleotídeos quanto em aminoácidos (89% para 2337 pares de base e 96% para os 779 resíduos de aminoácidos).

Recentemente os diversos fatores que podem regular a PFK foram revisados pelo nosso grupo, como mostrado na figura 4 (SOLA-PENNA et al., 2010). O grau de inibição da fosfofrutocinase provocado pelo ATP varia entre os diferentes tecidos e organismos. Em mamíferos, a isoforma de fígado é mais fortemente inibida que a de músculo esquelético, e esta, por sua vez, é mais sensível à inibição que a isoforma de cérebro (VORA e FRANCKE 1981). Da mesma maneira que a inibição por ATP, a sensibilidade à inibição por citrato também varia entre os tecidos (PASSONNEAU e LOWRY, 1962). Altos níveis de citrato potencializam a inibição promovida pelo ATP sobre a PFK (PASSONNEAU e LOWRY, 1962; TORNHEIM e LOWENSTEIN, 1976). A PFK-M é a menos sensível a inibição por ATP, enquanto que as isoformas de PFK-L e PFK-C são igualmente sensíveis a esta inibição (DUNAWAY e KASTEN, 1988).

A PFK pode ser modificada covalentemente em resíduos de serina e treonina pela proteína cinase A (PKA) (ALVES e SOLA-PENNA, 2003), PKC, (NETTELBLAD et al., 1986), e CaMK (MAHRENHOLZ et al., 1991). Além disso, foi demonstrado que a PFK, assim como outras enzimas glicolíticas, é substrato para a atividade tirosina cinásica do

receptor de insulina (SALE et al., 1987; ZANCAN e SOLA-PENNA, 2005b) e possivelmente outras cinases (COELHO et al., 2007; SOLA-PENNA et al., 2010).

Em um quadro de DM o efeito inibitório dos ácidos graxos e dos corpos cetônicos sobre a glicólise tem sido localizado na reação catalisada pela PFK (RANDLE et al., 1966). Propõe-se que o aumento da concentração intracelular de citrato, seria o responsável pela inibição da enzima. Recentemente, foi demonstrado que a PFK pode ser inibida por acil-CoA de cadeia longa (JENKINS et al., 2011). Além disto, Hosey et al., (1980) mostraram que a PFK isolada de fígado de camundongos geneticamente diabéticos (C57BL/KsJ-db) é mais suscetível à inibição por ATP que a enzima de um camundongo saudável. Bazaes et al. (1982) reportaram que a atividade da PFK é 30% menor em músculo esquelético de camundongos diabéticos que nos controles. Além disso, vários tecidos de animais com diabetes induzido com estreptozotocina (insulino-dependente) também apresentam reduzida atividade da PFK (RAJU et al., 2001). Apesar da atividade da PFK encontrar-se inibida em quadros de DM, a concentração desta enzima é a mesma em ratos diabéticos (induzido com aloxano) e controles: o valor médio para o músculo tibial anterior foi de 2,99 μM ; e do ventrículo esquerdo foi de 0.66 μM (HANSEN e VENEZIALE, 1980).

Tem sido postulado que a atividade reduzida da PFK, observada em alguns tecidos de animais diabéticos, foi devido a uma redução no metabólito F2,6BP (CHEN-ZION et al., 1994). Somando-se a esta ideia, em enterócitos isolados de ratos diabéticos, houve uma queda significativa nos níveis de F2,6BP e no fluxo glicolítico, sendo este efeito atribuído à diminuição da atividade da PFK (ROSSI et al., 1990).

Dentre os mais diversos fatores descritos como ativadores da PFK (SOLA-PENNA et al., 2010), a glicose-1,6-bisfosfato também já foi descrita como capaz de modular o fluxo glicolítico, estimulando alostericamente a PFK de fígado e de músculo esquelético, além de promover alterações em outras enzimas da via em resposta a estímulos hormonais (LILLING E BEITNER, 1990; CHEN-ZION et al., 1992b, 1994; MAGEN et al., 1995; ASHKENAZY-SHAZAR et al., 1997). A atividade reduzida da PFK do músculo gastrocnêmio e do tibial anterior já foi atribuída à redução nos níveis de glicose 1,6-bisfosfato (CHEN-ZION et al., 1994).

Dunaway et al. (1974) demonstraram que os níveis de PFK-L de fígado estão reduzidos no jejum e que este efeito está relacionado com a maior degradação da enzima. Neste mesmo trabalho, os autores demonstraram que o tratamento de ratos diabéticos com insulina reverte os baixos níveis da enzima, estando esse efeito correlacionado com a inibição da degradação enzimática. No entanto, além da insulina inibir da degradação da PFK, este

hormônio também pode aumentar a síntese de RNA da enzima, como demonstrado em fígado de camundongos (GEHNRICH et al., 1988).

Em tecido cardíaco, a atividade da PFK-M também parece ser inibida pelo DM (Dunaway et al., 1986), o que pode estar correlacionado com uma pequena alteração nos níveis da enzima neste tecido (NAKAJIMA et al., 1994).

Apesar de ainda não conhecermos a importância de cada isoforma da PFK no controle do metabolismo cardíaco, o trabalho de García et al. (2009) mostra que a deficiência na expressão de PFK-M em coração de camundongos, além de induzir alta letalidade dos animais perto da época do desmame, também reduz o seu tempo de vida, por causa das alterações metabólicas. A ausência de atividade da PFK inibe a glicólise, aumenta consideravelmente o estoque de glicogênio, baixa o conteúdo de ATP intracelular e causa hipertrofia cardíaca com o aumento da idade (García et al., 2009).

As interações da PFK com proteínas do citoesqueleto, como os filamentos de f-actina também são capazes de incrementar a sua atividade. Por outro lado, associações com microtúbulos, mais especificamente a tubulina, podem inibir a sua atividade enzimática (LIOU e ANDERSON, 1980; ANDRÉS et al., 1996; ALVES e SOLA-PENNA, 2003; SILVA et al., 2004). Outra forma importante de modular a atividade da PFK é alterando o seu estado oligomérico (SOLA-PENNA et al., 2010).

A PFK pode associar-se, formando complexos protéicos, com algumas enzimas citosólicas, como a aldolase e a GAPDH (RAIS et al., 2000; CAMPANELLA et al., 2005; MARCONDES et al., 2011), com elementos do citoesqueleto, como a actina filamentosa (F-actina) e microtúbulos (LIOU e ANDERSON, 1980; ANDRÉS et al., 1996; ALVES e SOLA-PENNA, 2003; EL-BACHA et al., 2003; SILVA et al., 2004), e também com proteínas de membrana plasmática, como o canal aniônico banda 3 presente na membrana plasmática de eritrócitos (CAMPANELLA et al., 2005).

A associação da PFK com estas proteínas ocorre de forma muito dinâmica e é capaz de alterar a atividade catalítica da enzima, provavelmente por alterar a sua afinidade por F6P (LIOU e ANDERSON, 1980; ANDRÉS et al., 1996; MARCONDES et al., 2011). Neste sentido, nosso laboratório demonstrou que esta associação está envolvida na regulação do fluxo glicolítico modulada por hormônios, como a adrenalina (ALVES e SOLA-PENNA, 2003) e a insulina (SILVA et al., 2004; ZANCAN e SOLA-PENNA, 2005a e 2005b) e a serotonina (COELHO et al., 2007) (FIG. 4). As enzimas glicolíticas, quando ligadas ao citoesqueleto, possuem maior atividade que as suas formas solúveis (CLARKE et al., 1985), o que poderia contribuir para um fluxo glicolítico mais eficiente.

Em quadros de hipermetabolismo, em que o fluxo glicolítico está aumentado, como no caso de células tumorais, ocorre aumento da associação da PFK com o citoesqueleto (EL-BACHA et al., 2003, COELHO et al., 2011). Esses efeitos são revertidos em presença do fármaco clotrimazol, deslocando a enzima da fração ligada a F-actina para a fração solúvel, com conseqüente inibição do fluxo glicolítico (COELHO et al., 2011), o que acarreta menor viabilidade de células de carcinoma mamário humano MCF-7 (MEIRA et al., 2005).

Alterações na associação da PFK com proteínas do citoesqueleto também foram vistas em quadros de DM e podem ser um possível alvo na redução dos efeitos danosos causados por esta disfunção metabólica (CHEN-ZION et al., 1994; CORRY et al., 2002).

Apesar dos vários estudos demonstrando a regulação da PFK por interação com outras proteínas celulares, pouco se conhece sobre a interação das diferentes isoformas de PFK sobre proteínas do citoesqueleto celular, o que sugere a necessidade de mais estudos neste assunto.

Os diferentes estados oligoméricos da PFK também regulam a atividade desta enzima, como mostrado na figura 4 (SOLA-PENNA et al., 2010). Os dímeros de PFK apresentam uma baixíssima atividade catalítica quando comparados aos tetrâmeros, considerados como a menor forma ativa da enzima (COLOMBO et al., 1975; DROZDOV-TIKHOMIROV et al., 1999). Os dímeros da PFK podem ser dissociados em monômeros, que se desnaturam rapidamente (FABER-BARATA e SOLA-PENNA, 2005) ou podem formar tetrâmeros a partir da sua associação. Por sua vez, os tetrâmeros são capazes de se associarem formando oligômeros mais complexos (UYEDA, 1979).

Bock e Frieden (1974) sugeriram um modelo de associação entre dímeros e tetrâmeros de PFK. Este equilíbrio entre diferentes formas oligoméricas é consideravelmente afetado pela concentração da enzima, pH e temperatura. Em valores baixos de pH (< 6,5), a inativação da enzima pode ser correlacionada com a mudança de uma forma ativa para uma forma inativa de menor peso molecular. Esta inativação também é mais pronunciada em baixas temperaturas e concentrações de PFK.

A ligação das formas diméricas e monoméricas da PFK com a tubulina e microtúbulos (Fig. 4), deslocam o equilíbrio entre oligômeros resultando em uma diminuição significativa da atividade enzimática (VÉRTESSY et al., 1997). De forma recíproca, a associação com a actina filamentosa estabiliza os tetrâmeros, ativando a enzima (CLARKE et al., 1983; ALVES e SOLA-PENNA, 2003; SILVA et al., 2004, ROBERTS e SOMERO, 1987). Curiosamente, a forma dimérica da PFK, originalmente inativa, quando associada com aldolase apresenta atividade similar à forma tetramérica, e é capaz de reverter a inibição promovida por clotrimazol, uma imidazole antifúngico capaz de inibir diretamente a enzima (MARCONDES

et al., 2011). Além disso, um trabalho do nosso laboratório mostrou que estes dímeros inativos de PFK, quando associados a cálcio-calmodulina (CaM), também possuem atividade similar aos tetrâmeros desta enzima (Fig. 4) (MARINHO-CARVALHO et al., 2006) e têm sua inibição por ATP e citrato atenuada (MARINHO-CARVALHO et al., 2009).

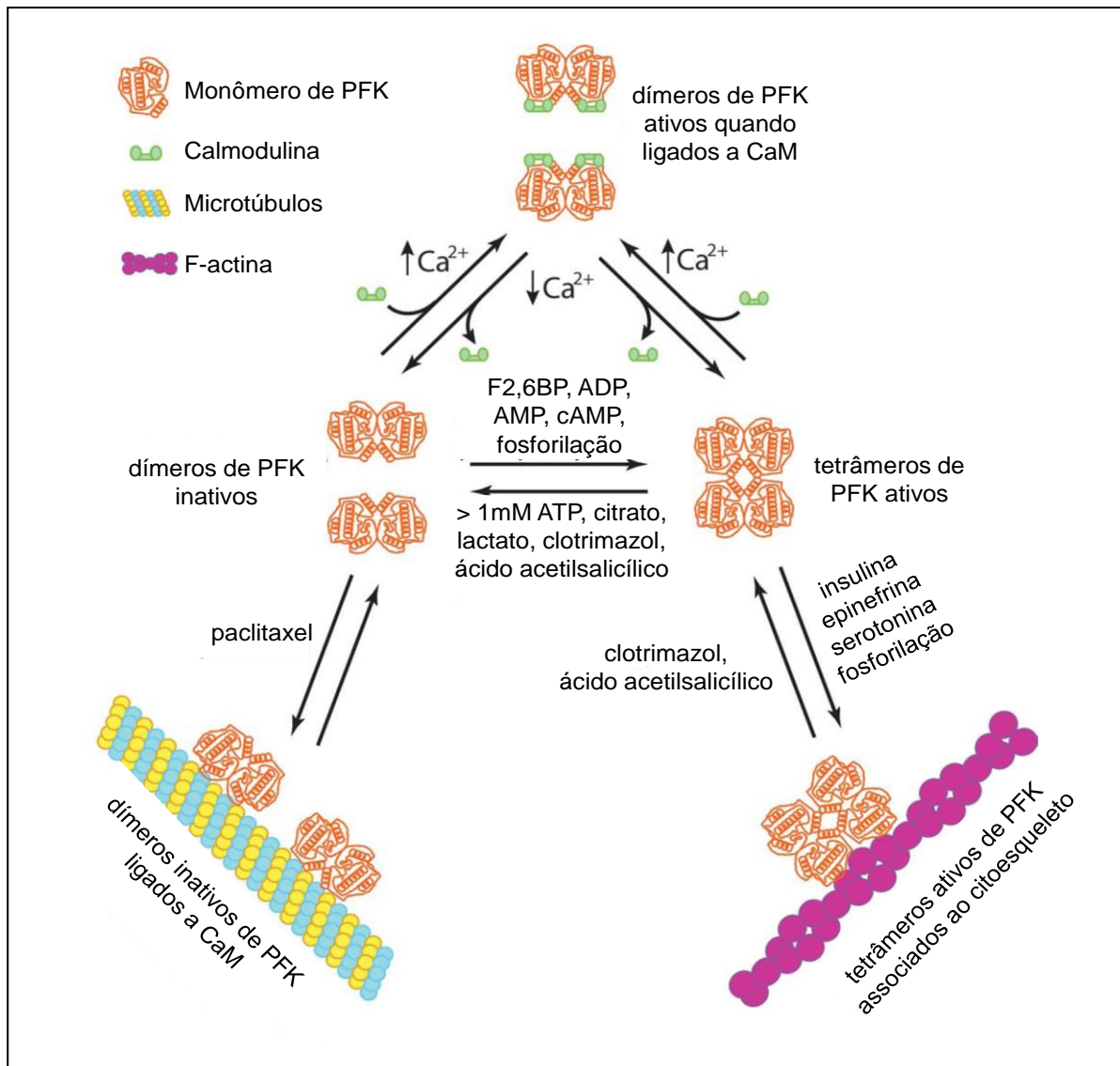


Figura 4: Modelo esquemático mostrando as diferentes formas de regulação da PFK (SOLA-PENNA et al., 2010).

A expressão de CaM é menor em animais diabéticos (MORLEY et al., 1984; SOLOMON et al., 1990; AYDIN et al., 1996), sugerindo que esta proteína possa ter um possível papel na estimulação do metabolismo glicolítico, uma vez que a CaM pode ativar

dímeros de PFK. No entanto, o envolvimento da CaM com dímeros de PFK ainda não foi demonstrado em modelos de DM.

Assim, o estudo da oligomerização da PFK e da associação desta enzima com elementos celulares tem aberto novas perspectivas no entendimento da regulação do metabolismo glicolítico.

1.10. Papel da insulina na regulação da glicólise

Os principais tecidos-alvo da ação da insulina e que são responsáveis pela homeostase da glicemia são músculo esquelético, fígado e tecido adiposo (BEITNER e KALANT, 1971; WEISBERG et al., 2003; XU et al., 2003). Entre os principais mecanismos que estão envolvidos na ação estimulatória da insulina sobre a glicólise em músculo estão: aumento da ligação das enzimas glicolíticas ao citoesqueleto muscular e aumento da produção de F2,6BP e glicose-1,6-bifosfato, potentes ativadores da PFK (BEITNER, 1990; CHEN-ZION et al., 1992a, 1992b; BEITNER, 1993; SILVA et al., 2004; SOLA-PENNA et al., 2010).

Diversos estudos sugerem que, além da regulação alostérica clássica, como por exemplo pela F2,6BP, a distribuição intracelular da PFK poderia desempenhar um importante papel na resposta celular rápida a estímulos externos, tais como sinalização hormonal pela epinefrina, serotonina ou insulina (LILLING e BEITNER, 1990; CHEN-ZION et al., 1992a; ALVES e SOLA-PENNA, 2003; EL-BACHA et al., 2003; ZANCAN e SOLA-PENNA, 2005a; COELHO et al., 2007).

Estes efeitos da insulina são inibidos pela presença de antagonistas de calmodulina (BEITNER, 1998), o que sugere fortemente que a calmodulina está envolvida no mecanismo de ação da insulina. Adicionalmente, a insulina promove um aumento na concentração de Ca^{2+} intracelular em adipócitos e a quelação do Ca^{2+} intracelular com quin-2 promove uma diminuição das ações da insulina, indicando um papel do Ca^{2+} na mediação dos sinais gerados por este hormônio nestas células (DRAZNIN, 1988). O aumento da concentração intracelular de Ca^{2+} promovido por insulina, aumentando o fluxo glicolítico, também foi observado em eritrócitos humanos (ZANCAN e SOLA-PENNA, 2005a). Além disso, quando as concentrações de Ca^{2+} estão muito altas ou muito baixas, a habilidade das células de responderem à insulina é significativamente diminuída (DRAZNIN, 1988). O aumento nas concentrações intracelulares de Ca^{2+} , induzido ou não por insulina, promove um rápido e temporário estímulo sobre a ligação das enzimas glicolíticas ao citoesqueleto e,

consequentemente, acelera a oxidação de glicose (LIVNAT et al., 1993), o que poderia ser importante para a promoção de ações hipoglicemiantes.

Além disso, Sale et al. (1987) demonstraram que as enzimas PFK, PGM, enolase, LDH e frutose-1,6-bifosfatase são fosforiladas quando incubadas com o receptor de insulina parcialmente purificado de células Fao de hepatoma de ratos e $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. As incorporações de ^{32}P na PFK e na PGM foram as mais evidentes, sendo estimuladas em cerca de 340 e 300%, respectivamente, por 10^{-7} M de insulina. Este estudo mostrou que, na ausência de insulina, a incorporação de ^{32}P nas proteínas PFK e PGM é baixa, e esta incorporação aumenta gradativamente em paralelo com o aumento da concentração de insulina até a concentração de 100 nM ser atingida. Concentrações maiores que esta não apresentam efeito adicional sobre a fosforilação das enzimas.

As demonstrações de que o receptor de insulina é uma proteína cinase estimulada por insulina (KASUGA et al., 1983; VAN OBBERGHEN et al., 1983) e o fato de que a insulina altera o estado de fosforilação de uma grande variedade de enzimas celulares (DENTON et al., 1981) sugerem uma cascata de fosforilação/desfosforilação de proteínas como um possível mecanismo pelo qual a insulina medeia suas ações. A fosforilação das enzimas glicolíticas, estimulada pela insulina, ocorre em resíduos de tirosina. Além disso, estas reações de fosforilação necessitam da presença de íons metálicos divalentes, tais como Mn^{2+} , Mg^{2+} e, em menor extensão, Ca^{2+} . Este resultado é similar àquele encontrado para a autofosforilação do receptor de insulina (SALE et al., 1987).

1.11. Medicamentos hipoglicemiantes

A indústria farmacêutica vem desenvolvendo, ao longo dos anos, diversos fármacos na tentativa de melhorar a qualidade de vida dos pacientes diabéticos. Antes do ano 2000, a escolha de agentes farmacológicos para o tratamento do DM, especialmente de medicamentos hipoglicemiantes orais, era limitada (SRINIVASAN et al., 2008). Hoje já existem diversos medicamentos hipoglicemiantes que atuam das mais diversas maneiras.

As sulfonilureias, usadas desde a década de 50, atuam estimulando a secreção pancreática de insulina. Devido ao seu mecanismo de ação, estes fármacos dependem de uma função residual mínima das células beta e, desta forma, podem tornar-se menos eficientes com a progressão do diabetes. Os efeitos colaterais promovidos por esta classe de fármacos são

raros, apesar de estarem associados com hipoglicemia e ganho de peso corporal (SRINIVASAN et al., 2008).

Os inibidores de glicosidases, como a acarbose, tornaram-se disponíveis desde a década de 90. Como atuam inibindo as enzimas necessárias à digestão de carboidratos no intestino delgado, estes fármacos devem ser ingeridos junto às refeições. Os efeitos colaterais mais comuns promovidos por esta classe de medicamentos são flatulência e diarreia (OLIVEIRA e MILECH, 2004).

As tiazolidinedionas (TZDs) atuam reduzindo a resistência periférica à insulina. Um dos principais efeitos colaterais é a retenção de água, levando ao ganho de peso. Além disto, o tratamento com TZDs é mais caro que o tratamento com sulfonilureias ou metformina (HERMANSEN et al., 2008).

A metformina é o único medicamento comercialmente disponível entre os fármacos do grupo das biguanidas, uma vez que a fenformina e a butformina foram retiradas do mercado. A metformina atua diminuindo a gliconeogênese e aumentando a utilização periférica de glicose (KIRPICHNIKOV et al., 2002).

A história do DM é repleta de terapias sendo que, quase todas, incluindo o tratamento com insulina, foram administradas pela primeira vez sem nenhum conhecimento do mecanismo de ação (WITTERS et al., 2001). No entanto, nos últimos anos, novas drogas hipoglicemiantes vêm sendo desenvolvidas, testadas e lançadas, como os análogos do peptídeo glucagon 1 (GLP1 - *glucagon-like peptide 1*), que reduzem a glicemia por estimular a liberação de insulina em resposta à ingestão de glicose e aumentar o consumo de glicose por vários tecidos, e os inibidores de dipeptidil peptidase, que atuam aumentando a sensibilidade das células beta à glicose, o que melhora a secreção de insulina dependente de glicose (KIM e EGAN, 2008).

Alguns medicamentos para o tratamento do DM já fazem parte do RENAME (Relação Nacional de Medicamentos Essenciais). Em 2008 foram adquiridas 960 mil ampolas de insulina regular e mais de 11 milhões de ampolas de insulina NPH, sendo o ministério da saúde brasileiro o maior comprador mundial de insulina. Apesar de a insulina ser muito utilizada, o medicamento hipoglicemiante fornecido pelo programa Farmácia Popular do governo federal mais consumido no Brasil é o cloridrato de metformina (VILA-NOVA, 2008).

1.11.1. Biguanidas

Três biguanidas foram disponibilizadas para tratamento do DM por volta de 1950. A metformina só foi aprovada para uso nos Estados Unidos da América em 1995 (WITTERS, 2001). No entanto, a butformina e a fenformina foram retiradas do mercado nos anos 70 devido à emergência de acidose láctica e aumento da mortalidade por problemas cardíacos (CUSI e DEFRONZO, 1998). Dentre os hipoglicemiantes orais, a metformina, fármaco da classe das biguanidas, é o único medicamento para o tratamento do DM que não está associado a danos diretos aos pacientes (FULGENCIO et al., 2001).

1.11.1.1. Metformina

1.11.1.1.1. História da metformina

O desenvolvimento da classe das biguanidas foi derivado do estudo dos efeitos da planta *Galega officinalis*. Galega é um gênero botânico pertencente à família *Fabaceae* (leguminosas), amplamente usada na Europa desde a idade média como um tratamento popular para a poliúria do DM. A galegina, substância ativa e derivada da guanidina presente nesta planta, era capaz de diminuir a glicemia de pacientes diabéticos. A guanidina era tóxica demais para ser usada como medicamento, mas o desenvolvimento de agentes derivados persistiu. Em 1957 foi publicada a primeira descrição científica da metformina (WITTERS, 2001).

A metformina é usada para o tratamento do DM2 há muitos anos (CUSI e DEFRONZO, 1998). Este medicamento entrou em uso clínico pela primeira vez na França, em 1979; nos Estados Unidos, foi aprovada somente em 1995, devido a preocupações de longa data a respeito da segurança das biguanidas.

1.11.1.1.2. Efeitos da metformina

O hidrocloreto de metformina (1,1-dimetilbiguanida) não está química ou farmacologicamente relacionado às sulfonilureias, tiazolidinedionas ou aos inibidores de

glicosidases. É um composto branco e cristalino com fórmula molecular de $C_4H_{12}ClN_5$ e peso molecular de 165,63. A metformina é altamente solúvel em água e seu pKa é 12,4. A estrutura molecular da metformina está abaixo.

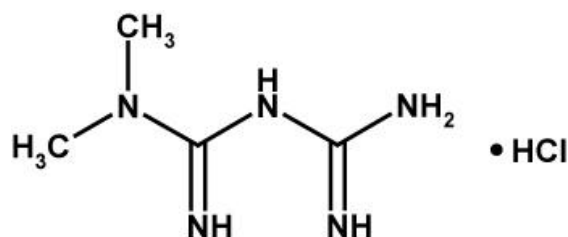


Figura 5. Estrutura molecular do hidrocloreto de metformina. Adaptado de STRACK (2008).

Usando-se doses clínicas (500 - 2000 mg) em humanos, a máxima concentração plasmática de metformina encontrada foi de 0,6 $\mu\text{g/mL}$ e 1,8 $\mu\text{g/mL}$, respectivamente. Durante experiências clínicas controladas, o nível máximo de metformina plasmática não excede 5 $\mu\text{g/mL}$, mesmo com doses máximas (STRACK, 2008). Em ratos que receberam metformina (320 mg/Kg) por via oral a concentração plasmática do fármaco variou entre 6 e 12 $\mu\text{g/mL}$ (WANJARI, 2008). A metformina é um agente hipoglicemiante com uma biodisponibilidade média de 50-60%. Este composto é eliminado primariamente por filtração e excreção renal, não é metabolizado e tem meia-vida de aproximadamente 6 horas em pacientes com DM2 (STRACK, 2008).

A terapia com metformina pode aumentar os níveis de lactato sanguíneo (HERMANN et al., 1994), o que fez com que alguns autores associassem este fato com o desenvolvimento de acidose láctica (DEFRONZO e GOODMAN, 1995; MISBIN et al., 1998). No entanto, a incidência estimada de acidose láctica associada ao tratamento com metformina é de 0,03 casos por 1000 pacientes por ano (BAILEY e TURNER, 1996). Além disto, um estudo recente demonstra que a acidose láctica ocorre em pacientes que possuem outras complicações, como problemas renais (LAAR et al., 2011).

Em um estudo publicado pela Associação Americana de Diabetes, a metformina foi capaz de diminuir a concentração de glicose plasmática de jejum em 60-70 mg/dL em pacientes com DM2. Interessantemente, a metformina não afeta a concentração de glicose sérica de animais saudáveis em jejum, mas a reduz significativamente em animais diabéticos (CHENG et al., 2001).

Em um estudo com pacientes diabéticos, feito no Reino Unido, a metformina foi o único medicamento que reduziu o número de mortes e ataques cardíacos relacionados com o

DM. O tratamento com metformina também reduziu o apetite, o peso e o conteúdo total de gordura corporal (CHARLES e ESCHWEGE, 1999).

De fato, estudos têm demonstrado redução na mortalidade relacionada a doenças cardiovasculares em usuários de metformina quando comparados com usuários de outros agentes antidiabéticos (JOHNSON et al, 2002; EURICH et al, 2005), sugerindo que a metformina possa apresentar efeitos cardiovasculares protetores adicionais, além de suas propriedades hipoglicemiantes. Entretanto, os mecanismos envolvidos nestes efeitos ainda não estão totalmente elucidados.

Em condições de isquemia, frequentemente encontradas em pacientes com DM, o coração depende mais do metabolismo glicolítico (NEELY e ROVETTO, 1975; STANLEY et al., 1997). Por outro lado, a glicólise está inibida em coração de pacientes com DM (STANLEY et al., 1997). Ficher et al. (1995) demonstraram que a metformina aumenta a captação de glicose em coração de ratos diabéticos, estando este efeito relacionado com um maior translocamento de transportadores de glicose para a membrana plasmática. Os mecanismos envolvidos neste efeito ainda não estão completamente elucidados, mas parecem ser independentes da ação da insulina (FISCHER et al., 1995).

O uso de metformina tem aumentado em pacientes com DM1 (MEYER e GUERCI, 2003), proporcionando um efeito coadjuvante ao tratamento com insulina em pacientes com este tipo de doença (GÓMEZ, et al. 2002; GUNTON e TWIGG, 2003). O tratamento apenas com metformina é insuficiente para controlar o DM1, mas permite uma queda de aproximadamente 30% nas doses de insulina utilizadas (BAILEY e TURNER, 1996; MATTHAEI et al., 2000) sem alterar a secreção pancreática deste hormônio (JOHNSON et al., 2002).

Detaille et al. (2005) demonstraram que a metformina pode aumentar o metabolismo anaeróbico independentemente da ação da insulina, atuando sobre a cadeia respiratória mitocondrial. Além da inibição do complexo 1 (DYKENS et al., 2008; PHIELIX et al., 2011), achados que demonstram que a metformina ativa a proteína cinase ativada por monofosfato de adenosina (AMPK) no músculo esquelético e no fígado (ZHOU et al., 2001), estimulando o transporte de glicose, reforçam um possível mecanismo de ação deste fármaco independente da ação insulinêmica.

Além disso, a AMPK responde a flutuações nos níveis energéticos celulares, promovendo a manutenção da homeostase energética, desativando vias consumidoras e ativando vias regeneradoras de ATP em situações de estresse, como por exemplo, durante o exercício físico, a isquemia cardíaca e a hipóxia (HUTCHINSON et al., 2008). No entanto,

Zhou et al. (2001) demonstraram que a AMPK pode ser ativada por outros mecanismos independentemente da mudança na relação AMP/ATP levando ao aumento da captação de glicose. No entanto, neste trabalho não foi determinado se o aumento da captação de glicose resulta em aumento da síntese de glicogênio ou na oxidação da mesma. Outras vias de sinalização celular, como as mediadas por Akt e HIF-1 α também podem estar diretamente envolvidas na ação celular das biguanidas (KOLA et al., 2006).

A metformina é capaz de diminuir a produção de glicose hepática (primariamente através da inibição da gliconeogênese e, em menor extensão, da glicogenólise) e aumentar a captação de glicose estimulada por insulina em músculo esquelético e adipócitos (BAILEY e TURNER, 1996; CUSI e DEFRONZO, 1998; WIERNSPERGER e BAILEY, 1999; HUNDAL et al., 2000).

Em muitos tecidos, incluindo músculo esquelético e adipócitos, a metformina facilita o tráfego de GLUT4 e GLUT1 para a membrana plasmática (BAILEY e TURNER, 1996; WIERNSPERGER e BAILEY, 1999). Em células musculares pré-expostas a metformina (2 mM, por 16 horas), a captação de 2-deoxiglicose foi estimulada em mais de 2 vezes (HUNDAL et al., 1992). Além deste estudo, Kumar e Dey (2002) mostraram que a metformina aumentava a captação basal de glicose em células C2C12 (uma linhagem de mioblasto) controles e resistentes à insulina. No entanto, quando a insulina era administrada juntamente com metformina não havia um aumento adicional na captação deste substrato, mostrando um efeito da metformina independente da presença de insulina.

Neste sentido, buscando entender como a metformina pode modular a glicemia, Silva et al. (2010) demonstraram que a metformina aumenta a glicólise e inibe o consumo de oxigênio em fígados perfundidos de ratos. Além disto, os níveis de ATP diminuiram, enquanto que os de AMP aumentaram, sugerindo um possível envolvimento da AMPK nesses efeitos (SILVA et al., 2010).

O tratamento com metformina de camundongos diabéticos induzidos com estreptozotocina não alterou a ligação da insulina com o seu receptor no músculo sóleo e em hepatócitos isolados, porém aumentou a síntese de glicogênio. Adipócitos isolados de ratos tratados *in vitro* com metformina tiveram um aumento da ligação de insulina aos seus receptores, mas este efeito (visto depois de 20 horas) foi secundário ao aumento da utilização de glicose (visto 2 horas depois) (FANTUS e BROSSEAU, 1986). Assim, apesar da metformina aumentar a ligação da insulina em seu receptor, sua ação direta parece ocorrer através de ações pós-receptor.

Outros trabalhos mostraram que a terapia com metformina diminui as concentrações de ATP em hepatócitos isolados de ratos (ARGAUD et al., 1993). Uma vez que o ATP é um inibidor alostérico da PFK, o aumento da glicólise neste tecido poderia ser o resultado do aumento da atividade desta enzima.

Embora as biguanidas estejam disponíveis para a terapia do DM2 desde a década de 50, os mecanismos envolvidos na ação hipoglicemiante da metformina ainda não foram completamente elucidados. Em vista disso, a compreensão dos mecanismos que levam ao aumento do catabolismo de glicose pelo músculo, fígado e tecido adiposo poderia representar uma importante ferramenta para o controle e/ou tratamento de patologias como o DM.

2.0.OBJETIVOS

2.1. Objetivo geral

O conhecimento acerca das questões envolvidas na regulação do fluxo glicolítico nos principais tecidos envolvidos no metabolismo de carboidratos pode representar uma importante ferramenta para o controle de patologias e colaborar para o desenvolvimento de novos alvos terapêuticos para o tratamento do DM.

Assim, o objetivo geral deste trabalho foi estudar o papel da metformina na regulação da atividade de enzimas glicolíticas do tecido muscular esquelético, do tecido hepático, do tecido adiposo epididimal e do tecido muscular cardíaco de camundongos com diabetes mellitus induzido por estreptozotocina (STZ).

2.2. Objetivos específicos

- Analisar o papel da metformina na glicemia, lactacidemia, insulinemia e outros parâmetros metabólicos de camundongos diabéticos;
- Avaliar a atividade da HK e da PFK de tecido muscular esquelético e cardíaco, adiposo epididimal e hepático desses camundongos tratados com diferentes doses de metformina;
- Analisar a atividade e a estrutura da HK e da PFK purificadas na presença de diferentes concentrações de metformina;
- Avaliar a atividade das duas enzimas nos tecidos pré-incubados com insulina;
- Estudar a sublocalização celular da atividade da HK e da PFK nos tecidos citados acima de camundongos diabéticos;
- Avaliar os efeitos do tratamento dos camundongos diabéticos com metformina sobre os níveis de proteína e RNAm das diferentes isoformas de HK e PFK dos tecidos;
- Estudar o efeito da metformina sobre os níveis de fosforilação em diferentes resíduos de aminoácidos da PFK dos diferentes tecidos;
- Investigar os efeitos da metformina sobre a atividade da HK e PFK e sobre a captação de glicose de células C2C12;
- Detectar a possível via de sinalização envolvida nos efeitos da metformina em células C2C12.

3.0. MATERIAL E MÉTODOS

3.1. Materiais

ATP, frutose-6-fosfato, estreptozotocina (STZ), metformina e hexocinase (HK) foram obtidas da Sigma Chemical (St. Louis, MO, USA) e ^{32}Pi do Instituto de Pesquisas Energéticas e Nucleares (SP). Insulina regular humana (Humulina) foi adquirida em farmácia. Os demais reagentes utilizados apresentavam grau analítico.

3.2. Métodos

3.2.1. Tratamento dos animais

O tratamento dos camundongos foi realizado de maneira similar à metodologia descrita por Fulgencio et al. (2001) e Shaw et al. (2005). Camundongos suíços machos (~8 semanas) foram divididos em quatro grupos: controle (Con), diabético (STZ), diabético tratado com metformina (STZ + Met) ou apenas metformina (Con + Met). Os camundongos foram mantidos em biotério com temperatura controlada e um ciclo de luz/escuro de 12:12 horas com água e ração *ad libitum*. O diabetes foi induzido com dose única de estreptozotocina (150 mg/Kg de peso corporal) injetada intraperitonealmente (ip.). Camundongos controles receberam apenas solução salina (100 mM de citrato de sódio, pH 4,5). O tratamento com metformina foi iniciado cinco dias após a indução do diabetes, caso os camundongos apresentassem glicemia ≥ 300 mg/dL. Uma vez ao dia, por três dias consecutivos, os camundongos do grupo STZ + Met e Con + Met receberam doses de metformina injetadas ip. nas concentrações indicadas nas figuras e/ou legendas. Os outros dois grupos (Con e STZ) receberam apenas o veículo de diluição da metformina (cloreto de sódio 0,9%). Após duas horas da última dose de metformina, gotículas de sangue foram retiradas da cauda dos camundongos para dosagem dos níveis de glicose (Glicosímetro Accu-Chek Active - Roche) e lactato (Lactímetro Accutrend lactate - Roche). Feito isto, os camundongos foram sacrificados através de deslocamento cervical e o sangue coletado para dosagem de insulina no soro, de acordo com o protocolo do fabricante (Rat/Mouse Insulin ELISA Kit, Millipore, MO, USA). Neste mesmo soro também foram analisados outros parâmetros metabólicos com kits, de acordo com as instruções do fabricante (DOLES, Brasil): colesterol total, lipoproteína de alta densidade (HDL), triglicerídeos (TG), hemoglobina glicada (HbA1c) e atividade das enzimas aspartato aminotransferase (AST) e alanina aminotransferase (ALT). Em seguida, os músculos esqueléticos das patas traseiras, fígado,

tecido adiposo epididimal e o coração foram removidos rapidamente e congelados em nitrogênio líquido até serem utilizados para os experimentos. O peso dos camundongos e o peso dos tecidos também foram analisados. O protocolo de tratamento dos animais foi aprovado pelo comitê de ética local (DFBCICB 038).

3.2.2. Incubação dos tecidos com insulina

Músculo esquelético, fígado e tecido adiposo foram removidos do nitrogênio líquido, divididos em dois pedaços de pesos iguais, diluídos em tampão de homogeneização (0,25 M de sacarose, 10 mM de EDTA, 46 mM de KCl, 20 mM de β -mercaptoetanol, 1 mM de pirofosfato de sódio e 100 mM de Tris-HCl, pH 7,4) e pré-incubados na ausência ou na presença de 100 nM de insulina por 30 minutos à temperatura ambiente. Depois deste período, os tecidos foram lavados três vezes com o tampão de homogeneização e homogeneizados por 30s com um Polytron (Brinkmann Instruments, Westbury, NY, USA) na proporção de volume do tampão (ml) por peso do tecido (g) de 6/1 músculo esquelético, 6/1 fígado e 15/1 para tecido adiposo epididimal. O homogeneizado foi centrifugado a 1.000g por 10 minutos (4°C) e a atividade da HK e da PFK medidas no sobrenadante (HT) de acordo com o método radiométrico, como descrito a seguir.

3.2.3. Fracionamento celular

Os fracionamentos foram feitos de acordo com o protocolo descrito por Lilling e Beitner (1990), com modificações feitas por Alves e Sola-Penna (Alves e Sola-Penna, 2003). Os tecidos de cada um dos quatro grupos foram descongelados, lavados com solução salina (0,9% de NaCl) e homogeneizados em tampão de homogeneização utilizando-se um Polytron. Os tecidos homogeneizados foram centrifugados por 10 minutos a 1.000g a 4°C para separar os restos celulares e tecidos não homogeneizados. O sobrenadante (HT) foi utilizado para medir a atividade total da HK e da PFK. Para separar a HK em fração solúvel e fração particulada, o HT foi submetido a uma centrifugação a 27.000g por 15 minutos (4°C), obtendo-se um precipitado rico em mitocôndria e núcleo (fração particulada, P1) e um sobrenadante com outros componentes celulares (fração solúvel, S1). A atividade da HK nestas frações foi avaliada para se analisar a distribuição celular da atividade desta enzima. Uma alíquota dessa última fração solúvel (S1) foi novamente centrifugada a 120.000g por 30 minutos (4°C), obtendo então um precipitado rico em componentes do citoesqueleto (fração particulada, P2) e um sobrenadante rico em componentes citoplasmáticos solúveis (fração solúvel, S2). Ambas as frações foram analisadas a fim de avaliarmos a distribuição celular da

atividade da PFK. Através destes processos de centrifugação diferencial, podemos estimar a proporção de HKs associadas a organelas, como mitocôndrias (fração particulada, P1), e estimar a proporção de PFKs associadas com componentes do citoesqueleto, como a f-actina (P2).

3.2.4. Atividade da hexocinase e da fosfofrutocinase

3.2.4.1. Método radiométrico: As atividades da HK e da PFK dos tecidos homogeneizados foram medidas pelo método radiométrico descrito por Sola-Penna et al., (2002) com as modificações introduzidas por Zancan e Sola-Penna (2005a, 2005b). A atividade enzimática foi analisada em um meio de reação contendo 50 mM Tris-HCl (pH 7,4), 5 mM MgCl₂, 1 mM ATP e 0,1 mM [γ -³²P]ATP (4 μ Ci/ μ mol). A atividade da HK foi avaliada neste meio de reação com mais 5 mM de glicose e a atividade da PFK com mais 1 mM de F6P e 5 mM de (NH₄)₂SO₄. A reação foi iniciada com a adição da proteína de cada fração e interrompida pela adição de 1 ml de carvão ativo suspenso em uma solução de HCl 0,1 N e manitol 0,5 M (25 g de carvão por litro de solução). A suspensão foi, então, centrifugada a 1.500g por 10 minutos e a radioatividade presente em 0,4 ml do sobrenadante foi mensurada em um contador de cintilação líquida (Modelo Tri-Carb - Perkin Elmer). Controles para cada tubo foram submetidos às mesmas condições experimentais na ausência de glicose (HK) ou F6P (PFK). A atividade da HK e da PFK foram avaliadas pela formação de [6 -³²P]glicose-6-fosfato ou [1 -³²P]frutose-1,6-bifosfato em função do tempo de reação, respectivamente. Todos os experimentos foram feitos pelo menos em duplicata.

As atividades da HK (Sigma Chemical, St. Louis, MO, USA) e da PFK purificadas também foram avaliadas de acordo com o método descrito acima. As demais informações estão descritas nas figuras e/ou legendas.

3.2.4.2. Método do sistema acoplado de enzimas: As atividades das enzimas HK, PFK, PK e G6PDH de culturas de células foram avaliadas em um espectrofotômetro (VICTOR 3, PerkinElmer) através da oxidação de NADH ou redução de NADP⁺ (340 nm) por um sistema acoplado de enzimas, como previamente descrito (SOLA-PENNA et al., 2002; KHUNDMIRI et al., 2004; ZANCAN et al., 2010). A atividade da PFK foi analisada em um meio de reação contendo 50 mM Tris-HCl, pH 7,4, 1 mM NADH, 10 mM MgCl₂, 1 mM ATP e 1 mM de F6P mais as enzimas aldolase (2 mU/mL), triose fosfato isomerase (2 mU/mL) e α -glicerofosfato desidrogenase (2 mU/mL). A atividade da HK foi analisada em

meio de reação contendo 100 mM Tris-HCl, pH 7,4, 1 mM NAD⁺, 2 mM MgCl₂, 1 mM ATP e 2 mU/mL glicose-6-fosfato desidrogenase (G6PDH). A atividade da PK foi medida em meio de reação contendo 100 mM Tris-HCl, pH 7,4, 1 mM NADH, 5 mM ADP, 120 mM KCl, 5 mM fosfoenolpiruvato (PEP), 5 mM MgCl₂ e 22 mU/mL lactato desidrogenase. A atividade da G6PDH foi avaliada em um meio de reação contendo 100 mM Tris-HCl, pH 7,4, 5 mM MgCl₂, 2 mM NADP⁺ e 1 mM G6P. Em todos os casos a reação foi iniciada pela adição do extrato celular. Brancos na ausência das enzimas G6PDH, aldolase ou lactato desidrogenase foram feitos para se analisar as reações inespecíficas das HK, PFK, PK, respectivamente. A atividade inespecífica da G6PDH foi analisada na ausência de G6P. Todos os experimentos foram feitos em triplicatas.

3.2.5. Espectro de fluorescência intrínseca da hexocinase e da fosfofrutocinase

Os espectros de fluorescência intrínseca da HK e da PFK purificadas foram analisados em um espectrofluorímetro JASCO 6300. A HK (5 µg/ml) ou a PFK (5 µg/ml) purificadas foram diluídas em meio de reação contendo 50 mM Tris-HCl, pH 7,4 e diferentes concentrações de metformina (0,1, 10, 100 e 1000 µM). As amostras foram colocadas em cubeta de quartzo com caminho óptico de 1,0 x 1,0 cm e excitadas em 280 nm. A emissão de fluorescência foi varrida entre 300 e 400 nm. O centro de massa espectral foi calculado pela equação:

$$\text{Centro de massa (nm)} = \frac{\sum (\lambda \cdot I_{\lambda})}{\sum I_{\lambda}}$$

Onde : λ = comprimento de onda

I_{λ} = intensidade de fluorescência em um dado comprimento de onda

3.2.6. Dosagem de glicose-6-fosfato (G6P)

Músculo esquelético, fígado e tecido adiposo e músculo cardíaco foram homogeneizados em 3% HClO e a concentração de G6P foi determinada em um meio de reação contendo 50 mM Tris-HCl, pH 8,0, 1 mM NADP⁺, 0,025 unidades/ml glicose 6-fosfato desidrogenase. A redução do NADP⁺ foi acompanhada através da medida da absorbância em 340 nm em um leitor de microplacas (Victor 3, PerkinElmer, USA).

3.2.7. Western blotting

O homogeneizado total (HT) dos tecidos de cada grupo (60-100 μ g), contendo um coquetel inibidor de proteases (Sigma Chemicals, St. Louis, MO, USA), foi misturado com tampão de amostra contendo 1% de SDS (dodecil sulfato de sódio), 0,5% de β -mercaptoetanol, fervidos por 3 minutos e, então, submetidos a SDS-PAGE (10%). As proteínas separadas foram transferidas para uma membrana de difluoreto de polivinilideno (PVDF Imobilon-P, Millipore), bloqueadas durante a noite com TBS-Tween (20 mM Tris-HCl, pH 7,5, 500 mM NaCl e 0,1% Tween 20) contendo 5% de leite em pó desnatado e posteriormente incubados, por 2h, com anticorpo monoclonal de camundongo anti-actina (1:1000, Santa Cruz), policlonal de coelho anti-PFK (1:1000, Santa Cruz), monoclonal de camundongo anti-HK-1 ou monoclonal de camundongo anti-HK-2 (1:1000, AbCam). Feito isto, as membranas foram lavadas três vezes com TBS-Tween e incubadas durante 1h com anticorpo secundário anti-coelho ou anti-camundongo conjugado a peroxidase (1:5000, GE). A mesma membrana utilizada para analisar a expressão de HK e PFK foi lavada e usada para marcação com anticorpo anti-actina para assegurar que a mesma quantidade de proteína foi adicionada em cada gel. As proteínas imunoreativas foram visualizadas por quimioluminescência utilizando-se um kit ECL (ECL - Amersham Pharmacia) e filme Kodac. As bandas imunoreativas foram quantificadas por densitometria usando o programa ImageJ (NIH, USA).

3.2.8. Imunoprecipitação

A imunoprecipitação, seguida de *Western blotting*, foi realizada como descrita anteriormente por Coelho et al. (2007). O homogeneizado total dos tecidos de cada grupo foi incubado durante a noite, a 8°C, com anticorpo anti-PFK (1:20)/proteína A-agarose. As esferas de agarose foram coletadas por centrifugação, lavadas três vezes com solução salina, uma vez com 10% SDS, ressuspendidas em 50 μ L de tampão de amostra e fervidas por 3 minutos. As amostras foram submetidas à SDS-PAGE (8%), transferidas para membrana de nitrocelulose, bloqueadas com TBS-Tween (20 mM Tris-HCl, pH 7,5, 0,15 M NaCl, 0,1% Tween 20) contendo 5% de leite em pó desnatado. A membrana foi subsequentemente incubada por 2h com anticorpo monoclonal anti-fosfotirosina, anti-fosfoserina ou anti-fosfotreonina (1:1000, Sigma Chemicals). Depois, a membrana foi lavada 5 vezes com TBS-Tween e, então, incubada por 1h com anticorpo secundário ligado a fosfatase alcalina (1:5000, Sigma Chemicals). Depois deste período, as membranas foram incubadas com o substrato da enzima e a marcação foi analisada usando-se o programa ImageJ (NIH, USA). Controles de carregamento para o total de PFK imunoprecipitada foram feitos em paralelo.

3.2.9. Reação em cadeia da polimerase - transcriptase reversa (RT-PCR)

O RNA total foi extraído a partir de 100 mg dos tecidos utilizando-se Trizol, de acordo com o protocolo fornecido pelo fabricante (Sigma Chemicals, St. Louis, MO, USA). A concentração de RNA total foi determinada utilizando um espectrofotômetro Nanodrop (Thermo Scientific, Waltham, MA, EUA) e sua integridade foi verificada em gel de agarose 1% (dados não mostrados). Quatro microgramas do RNA total foram transcritos reversamente com a transcriptase reversa (M-MLV, Uniscience) em 20 µL de uma mistura de reação usando *primers* randômicos (Invitrogen). A amplificação do cDNA de cada isoforma da PFK foi realizada com 0,2 µM de *forward primer* comum para todas as isoforma (5'-GGCTACTGTGGCTACCTGGC-3') e 0,2 µM de *reverse primer* específica para cada isoforma (PFK-M, NM_021514, 5'-GCATGGAGTACAGGGAAACC-3', 741 bp; PFK-L, NM_008826, 5'-CCAGCATCTTCAGCATCAGC, 533 bp; PFK-C, NM_019703, 5'-GGCAGACTTGATGAGATGGC-3', 606 bp). O tamanho do produto resultante da amplificação nos permitiu distinguir entre as diferentes isoformas de PFK. Os *primers* usados para amplificação da HK foram: HK-1 (NM_010438, *forward*: 5'-GATGGAGGTGAAGAAGAAGC-3' e *reverse*: 5'-GGAAACGAGAAGGTGAAGC-3') e HK-2 (NM_013820, *forward*: 5'-CGGTACACTCAATGACATCC-3' e *reverse*: 5'-GTAGACAGAGCCATCCACG-3'). A amplificação de GAPDH foi utilizada como um controle interno (NM_008084.2, *forward*: 5'-ATCACCATCTTCCAGGAGGCG3' e *reverse*: 5'-CCTGCTTCACCACCTTCTTG-3'). As condições do PCR foram: desnaturação inicial por 1 minuto a 98°C, 30 segundos a 96°C, 45 segundos a 58°C (PFKs), 57,5°C (GAPDH) ou 53°C (HK-1 e HK-2), 1 minuto a 72°C e, finalmente, 5 minutos a 73°C. A amplificação de cada cDNA foi quantificada em ciclos diferentes para garantir que a amplificação do PCR fosse em escala linear. Para PFKs, GAPDH e HKs foram realizados 26, 26 e 30 ciclos, respectivamente. Fragmentos de DNA foram separados por eletroforese em géis de agarose 1,0% contendo brometo de etídio e visualizados em transiluminador UV. Os géis foram analisados e quantificados usando o programa ImageJ (NIH, EUA).

3.2.10. Cultura de células e ensaios

Células C2C12 (linhagem de mioblasto) foram cultivadas em DMEM suplementado com soro fetal bovino (10%), a 37°C, em atmosfera humidificada contendo 5% de CO₂. Após as células tornarem-se confluentes, o meio foi substituído e as células foram subcultivadas em placas de 96 poços. Em um experimento, as células foram incubadas na ausência ou na

presença de 0,5 μM de metformina, 100 ou 1000 nM de insulina por 24 horas. Em outros experimentos, visando entender qual a possível via de sinalização envolvida nos efeitos da metformina, as células foram pré-incubadas por 1h na presença dos inibidores de PI3K (wortimanina, 0,1 μM) ou de AMPK (composto C, 20 μM). Após este período, foram adicionados ao meio 0,5 μM de metformina ou 100 nM de insulina por 24 h. Tanto em um, quanto no outro experimento, após este período de 24 h, o meio foi removido e utilizado para avaliar o consumo de glicose e a produção de lactato, enquanto as células remanescentes foram utilizadas para se avaliar: a viabilidade celular, através da redução de MTT; o conteúdo intracelular de ATP, através da reação luciferina-luciferase (Kit ATPLite, *Luminescence ATP Detection Assay System*, PerkinElmer); e as atividades das enzimas HK, PFK, PK e G6PDH que foram analisadas através do sistema acoplado de enzimas, como descrito anteriormente. O consumo de glicose foi analisado como descrito por (SPITZ et al., 2009), analisando-se a concentração de glicose remanescente no meio de cultura através de um ensaio enzimático contendo glicose oxidase/peroxidase (Glucox 500, Doles Ltda). A concentração de lactato também foi determinada neste meio, como previamente descrito por Meira et al. (2005), através da adição de 2 U/ml de lactato desidrogenase e 1 mM NAD^+ . A redução do NAD^+ foi acompanhada através da medida da absorvância em 340 nm em um leitor de microplacas (Victor 3, PerkinElmer, USA). Para se avaliar a viabilidade celular, as células foram lavadas com PBS e 5 mg/ml do reagente de MTT (brometo de 3-(4,5-dimetil-2-tiazoli)-2,5-difenil-2H-tetrazólio; Sigma-Aldrich Co., MA, EUA) foram adicionados. Depois de três horas, o reagente foi removido, os cristais de formazan formados foram dissolvidos em DMSO e a absorvância avaliada em leitor de microplacas nos comprimentos de onda de 570 e 660 nm.

3.2.11. Purificação da fosfofrutocinase de músculo esquelético de coelho

A PFK de músculo esquelético de coelho foi purificada segundo o protocolo proposto por Kemp (1975), e adaptado do original descrito por Parmeggiani et al. (1966). *Extração:* A eutanásia foi realizada por deslocamento cervical, sendo então os músculos da região dorsal e das patas traseiras removidos, limpos e mantidos em gelo. A massa muscular foi então processada em um moedor de carne, pesada e triturada em liquidificador com 3 volumes de tampão 1 (30 mM NaF, 4 mM EDTA e 15 mM β -mercaptoetanol, pH 7,5) por 30 segundos. A mistura foi então centrifugada a 14.000g por 10 minutos a 4°C (Hitachi-Himac). O sobrenadante foi filtrado através de gaze para a remoção dos resíduos lipídicos e o pH ajustado para 6,8 com adição de 1,5M Tris.

Precipitação com Isopropanol: A mistura foi mantida em constante agitação em gelo com sal grosso, com a temperatura entre -5°C e 0°C . Adicionou-se álcool isopropílico gelado (1/5 do volume total do extrato) gota a gota, sendo a mistura mantida por mais 20 minutos. O precipitado foi coletado por centrifugação a 14.000g por 30 minutos em centrífuga refrigerada a 4°C e dissolvido em 1/15 da fração inicial em tampão 2 (0,1 M Tris-fosfato, 0,2 mM EDTA, 0,2 mM F1,6BP, 1 mM β -mercaptoetanol, 5 mM pirofosfato de sódio, pH 8,0). Esta suspensão foi dialisada por 20h contra o mesmo tampão para a remoção do isopropanol remanescente.

Tratamento Quente: A suspensão dialisada foi transferida para um becker e colocada em banho maria a 70°C , sob agitação. A temperatura da suspensão foi mantida entre 57 e 59°C por 3 minutos quando, então, foi retirada e condicionada em banho de gelo até atingir a temperatura de 5°C . Depois disto, a suspensão foi centrifugada por 10 minutos (23.500g) a 4°C . O sobrenadante foi reservado e o precipitado ressuspense em tampão 2 e centrifugado novamente. O segundo sobrenadante obtido foi adicionado ao primeiro.

Fracionamento por Sulfato de Amônio: Ao sobrenadante, mantido em banho de gelo sob agitação, foi adicionado sulfato de amônio sólido até atingir 38% de saturação (21,3 g / 100 ml da solução inicial). Após 30 minutos, o precipitado foi removido por centrifugação a 14.000g por 15 minutos a 4°C , e ao sobrenadante foi adicionado sulfato de amônio até atingir 55% de saturação (30,8 g / 100 ml da solução inicial). O sedimento foi coletado por centrifugação a 14.000g por 20 minutos e dissolvido em tampão 3 (50 mM Tris-fosfato, 0,2 mM EDTA, 1 mM β -mercaptoetanol, 5 mM pirofosfato de sódio, pH 8,0).

Cristalização: A solução foi dialisada em banho de gelo contra o tampão 3, adicionada de sulfato de amônio até 38% de saturação (21,3 g / 100 ml). Após 24h o tampão de diálise foi trocado pelo mesmo volume de tampão 3 e, então, sulfato de amônio foi adicionado até atingir 40% de saturação (22,6 g / 100 ml). Depois da diálise, a suspensão foi centrifugada a 1.500g por 10 minutos em centrífuga clínica a 4°C . O precipitado foi dissolvido em tampão 3 e mantido em isopor com gelo dentro da geladeira.

3.2.12. Síntese de ATP radioativo

O $[\gamma\text{-}^{32}\text{P}]$ ATP foi preparado de acordo com Maia et al. (1983). A síntese do $[\gamma\text{-}^{32}\text{P}]$ ATP foi feita a partir da adição de 1 mL $[\text{}^{32}\text{P}]\text{Pi}$ e 40 μL de um coquetel enzimático de marcação em um meio contendo 0,115 mM Tris-HCl, pH 9,0, 0,0276 mM MgCl_2 , 0,0138 mM DTT, 0,276 μM L- α -glicerofosfato, 1,15 μM β -NAD, 0,115 μM ADP e 2,3 μM piruvato. Esse coquetel de enzimas contém glicerol-3-fosfato desidrogenase, triose-fosfato isomerase,

gliceraldeído-3-fosfato desidrogenase, 3-fosfoglicerato cinase e lactato desidrogenase. Durante 90 minutos esse meio de reação foi incubado em temperatura ambiente. Em seguida, o [γ - ^{32}P]ATP formado foi avaliado em um contador de cintilação líquida (Modelo Tri-Carb - Perkin Elmer) e aliqotado para ser estocado a -20°C até o seu uso.

3.2.13. Dosagem de proteína

A dosagem de proteína foi feita pelo método de Lowry et al. (1951). O padrão utilizado em todas as dosagens foi a albumina de soro bovino.

3.2.14. Análise estatística

Todos os resultados estão expressos como média \pm erro padrão. A análise estatística e as regressões lineares dos dados foram realizadas por meio do programa Sigma Plot (v. 10,0, Systat Inc., CA, USA) integrado com o *software* SigmaStat (v. 3.2, Systat Inc. CA, USA). Os valores para cada grupo foram comparados pelos testes *t*-Student pareados ou não pareados. As diferenças foram consideradas estatisticamente significativas quando $p \leq 0,05$.

4.0. RESULTADOS

4.1. PARTE 1

4.1.1. Caracterização dos animais diabéticos

De acordo com a literatura, camundongos que desenvolvem DM1 após indução com estreptozotocina apresentam perda de peso, alta concentração sanguínea de glicose e baixa concentração plasmática de insulina e lactato (KAKEMI et al., 1983; TANAKA et al., 1999). Com o objetivo de caracterizar os camundongos diabéticos utilizados neste estudo e investigar os efeitos da metformina sobre os parâmetros citados, os animais foram divididos em quatro grupos e tratados como descrito em Material e Métodos. A figura 6A mostra a variação do peso dos camundongos controles e diabéticos tratados por três dias com 250 mg/Kg de metformina com relação ao início da indução do DM. Os camundongos diabéticos apresentam menor peso que os controles e o tratamento com solução salina ou metformina não altera o peso desses animais. Os camundongos controles que receberam metformina também não apresentam alteração significativa do peso após o tratamento, quando comparados ao grupo controle não tratado.

A concentração de glicose sanguínea é aproximadamente quatro vezes maior no grupo diabético que no grupo controle, antes do tratamento com metformina (Fig. 6B). O tratamento com 50 e 100 mg/Kg de metformina não é capaz de alterar significativamente a glicemia tanto dos animais saudáveis quanto dos diabéticos. No entanto, a dose de 250 mg/Kg de metformina reduz a glicemia em 36% do grupo diabético, quando comparado com a glicemia antes do tratamento. Curiosamente, nenhuma das doses de metformina administradas é capaz de alterar a glicemia dos camundongos controles. Adicionalmente, camundongos controles morrem após a administração de apenas uma dose de 500 mg/Kg de metformina, enquanto que os diabéticos morrem somente após duas doses (uma por dia) (dados não mostrados). Apesar desse efeito inesperado, as alterações fisiológicas que levam a morte dos camundongos devido a dose de 500 mg/Kg não estão determinadas.

A diminuição da glicemia dos camundongos diabéticos tratados com metformina nos levou à hipótese de que este tratamento poderia estar aumentando o consumo de carboidratos pelos tecidos corporais. A figura 6C mostra que os camundongos diabéticos apresentam concentração plasmática de lactato cerca de 2 vezes menor que os animais controles ($p < 0,05$ teste t), o que poderia sugerir uma baixa taxa glicolítica nestes animais. No entanto, os

camundongos diabéticos tratados com a dose de 250 mg/Kg de metformina apresentam reversão da baixa lactacidemia, alcançando níveis similares aos dos animais controles não tratados com metformina. Esta dose do fármaco não é capaz de alterar a lactacidemia dos camundongos controles, corroborando com o fato de não haver alteração da glicemia deste grupo, como observado na figura 6B.

Para realmente confirmar o desenvolvimento do DM1 e investigar se a metformina poderia estar diminuindo a glicemia, por alterar as concentrações plasmáticas de insulina, as concentrações deste hormônio no soro dos quatros grupos de camundongos foram determinadas. A figura 6C mostra que os animais diabéticos apresentam concentração sérica de insulina significativamente menor que os animais controles ($p < 0,05$ teste t). Os camundongos diabéticos tratados com a dose de 250 mg/Kg de metformina não apresentam alteração da concentração de insulina, quando comparados aos camundongos diabéticos não tratados. Esta dose do fármaco não é capaz de alterar a insulinemia dos camundongos controles.

As concentrações de lactato e de insulina não foram analisadas nos camundongos que foram tratados com as doses de 50 e 100 mg/Kg de metformina, uma vez que a dose de 250 mg/Kg foi escolhida por ter melhor efeito sobre a glicemia. A análise do perfil lipídico dos animais mostra que a indução do DM aumenta a concentração sérica de triglicerídeos e colesterol total. No entanto, o tratamento com metformina é capaz de reduzir significativamente tais níveis nos camundongos diabéticos, sem alterar os valores dos controles. Não há alteração da concentração de HDL em nenhuma das condições estudadas (Fig. 6D).

Uma vez que a glicemia dos animais diabéticos diminui com o tratamento com metformina, procuramos analisar os possíveis efeitos crônicos deste tratamento medindo o percentual de hemoglobina glicada (HbA1c). A figura 6E mostra que o tratamento com metformina não tem efeito sobre os níveis aumentados de HbA1c nos camundongos diabéticos. A atividade da aspartato aminotransferase (AST) e da alanina aminotransferase (ALT) séricas estão aumentadas em aproximadamente quatro vezes nos camundongos diabéticos. Apesar da metformina diminuir significativamente a atividade tanto da AST quanto da ALT, com maior efeito sobre esta, suas atividades ainda permanecem maiores que as atividades encontradas nos animais controles (Fig. 6F). O efeito diabetogênico da estreptozotocina resulta em aumento da atividade sérica da AST e da ALT, podendo estar este efeito relacionado ao aumento da gliconeogênese e da cetogênese (BROWNLEE, 2001). Em conjunto, esses resultados sugerem que a metformina pode ter efeitos benéficos em

camundongos com DM1, sendo esses efeitos independentes da ação da insulina, como já proposto por outros autores (KLIP et al., 1992; FULGENCIO et al., 2001).

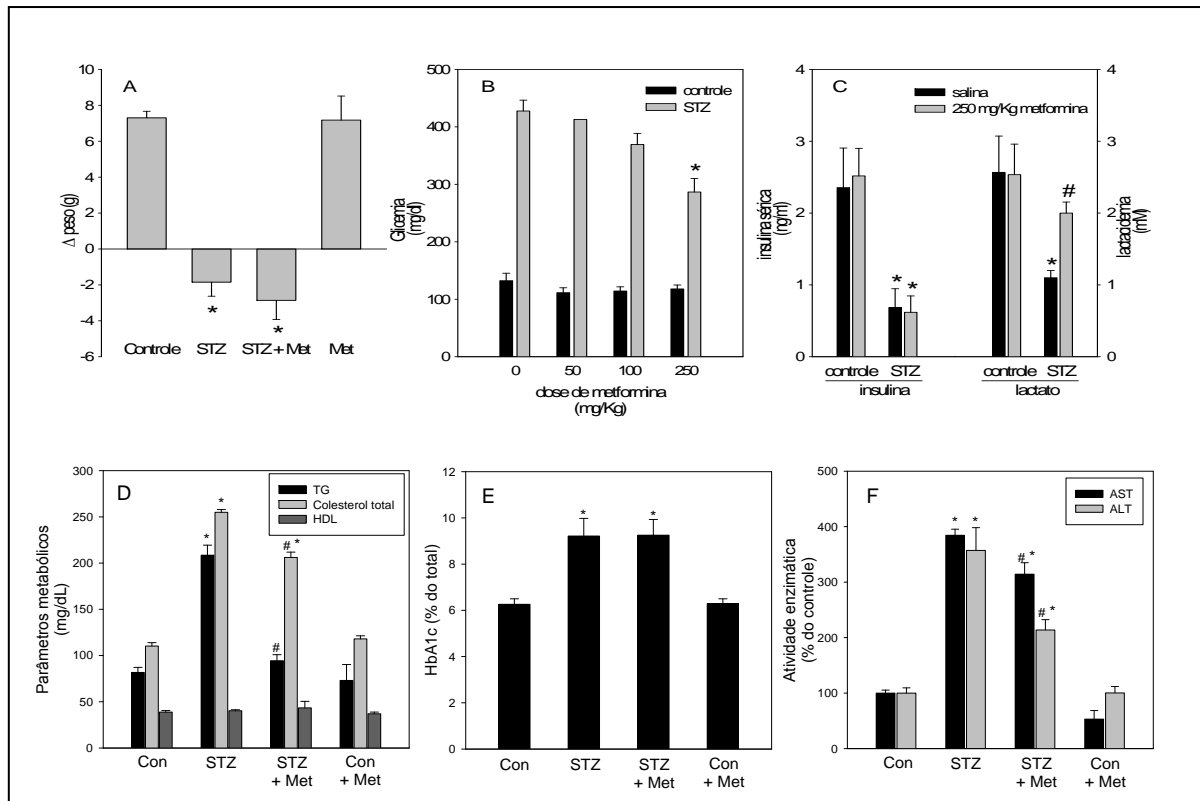


Figura 6. Características metabólicas de camundongos diabéticos tratados com metformina. Camundongos foram injetados intraperitonealmente com metformina (mg/Kg de peso corporal) diluída em solução salina, ou apenas solução salina, uma vez por dia, durante três dias consecutivos. **A:** variação do peso corporal (desde a indução do diabetes) de camundongos controles e diabéticos tratados ou não com 250 mg/Kg de metformina. $*p < 0,05$ comparado aos grupos controles. **B:** Concentração de glicose sanguínea de camundongos controles (barras pretas) e diabéticos (barras cinza) tratados com diferentes doses de metformina. Os valores representam a média \pm erro padrão ($n = 3$). $*p < 0,05$ comparado aos grupos controles. **C:** Concentração de insulina sérica (escala da esquerda) e concentração de lactato sanguíneo (escala da direita). Barras pretas representam camundongos tratados apenas com solução salina e barras cinza representam camundongos tratados com 250 mg/Kg de metformina. Os valores representam a média \pm erro padrão ($n = 3$). $*p < 0,05$ quando comparado aos controles dos seus respectivos grupos. $\#p < 0,05$ quando comparado ao grupo diabético tratado apenas com solução salina. **D:** Concentração de triglicerídeos (barras pretas), colesterol total (barras cinza claras) e DHL (barras cinza escuras) no soro de camundongos controles e diabéticos tratados ou não com 250 mg/Kg de metformina. $*p < 0,05$ comparado ao controle; $\#p < 0,05$ comparado ao diabético; $\#\#p < 0,05$ comparado ao controle e ao diabético. **E:** Percentual de hemoglobina glicada (HbA1C) no soro de camundongos controles e diabéticos tratados ou não com 250 mg/Kg de metformina. $*p < 0,05$ comparado ao controle. **F:** Atividade da AST e ALT do soro de camundongos controles e diabéticos tratados ou não com 250 mg/Kg de metformina. $*p < 0,05$ comparado ao controle; $\#p < 0,05$ comparado ao diabético; $\#\#p < 0,05$ comparado ao controle e ao diabético. Os demais procedimentos experimentais estão descritos em **Material e Métodos**. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.1.2. Atividade enzimática da hexocinase e da fosfofrutocinase em homogeneizado total (HT) de músculo esquelético, fígado e tecido adiposo epididimal de camundongos diabéticos tratados com diferentes doses de metformina.

Com o objetivo de caracterizar a atividade de enzimas-chave da glicólise em três dos principais tecidos responsáveis pela homeostase da glicemia, foram realizadas medidas de atividade enzimática em homogeneizado total (HT) de músculo esquelético (Fig. 7A, D), fígado (Fig. 7B, E) e tecido adiposo epididimal (Fig. 7C, F) de camundongos diabéticos tratados com diferentes doses de metformina, como descrito em Material e Métodos. As atividades da HK e da PFK são menores no músculo esquelético, fígado e tecido adiposo epididimal dos camundongos diabéticos, quando comparadas aos seus respectivos controles (HK ~ 46, 67 e 48% e PFK 55, 72 e 47%, respectivamente). A atividade dessas enzimas nos diferentes tecidos não é alterada pelo tratamento com 50 ou 100 mg/Kg de metformina, quando comparada aos animais diabéticos não tratados. Por outro lado, o tratamento dos camundongos diabéticos com 250 mg/Kg do fármaco é capaz de reverter completamente a baixa atividade da HK (painéis superiores) e da PFK (painéis inferiores) no HT de músculo esquelético e tecido adiposo. Entretanto, em HT de fígado a reversão da atividade de ambas as enzimas é parcial, sendo significativamente diferente do grupo controle e do grupo diabético não tratado. O tratamento com 50, 100 ou 250 mg/Kg de metformina não altera a atividade tanto da HK quanto da PFK de HT desses tecidos dos camundongos controles.

Interessantemente, as atividades da HK e da PFK são afetadas proporcionalmente no músculo e no tecido adiposo. Além disto, comparando entre os tecidos, a atividade dessas enzimas está mais inibida no fígado dos camundongos diabéticos do que nos outros tecidos. Neste sentido, nossos resultados sugerem que o fígado parece ser um dos tecidos mais afetados pelo desenvolvimento de um quadro diabético e, por isto, o tratamento com metformina por três dias poderia não ser suficiente para reverter completamente a baixa atividade das enzimas neste tecido. Em conjunto, esses resultados indicam que a baixa atividade enzimática da HK e da PFK, em decorrência do quadro diabético, em HT de músculo esquelético, fígado e tecido adiposo epididimal pode ser revertida pelo tratamento com metformina, podendo este efeito estar correlacionado com os efeitos hipoglicemiantes da metformina.

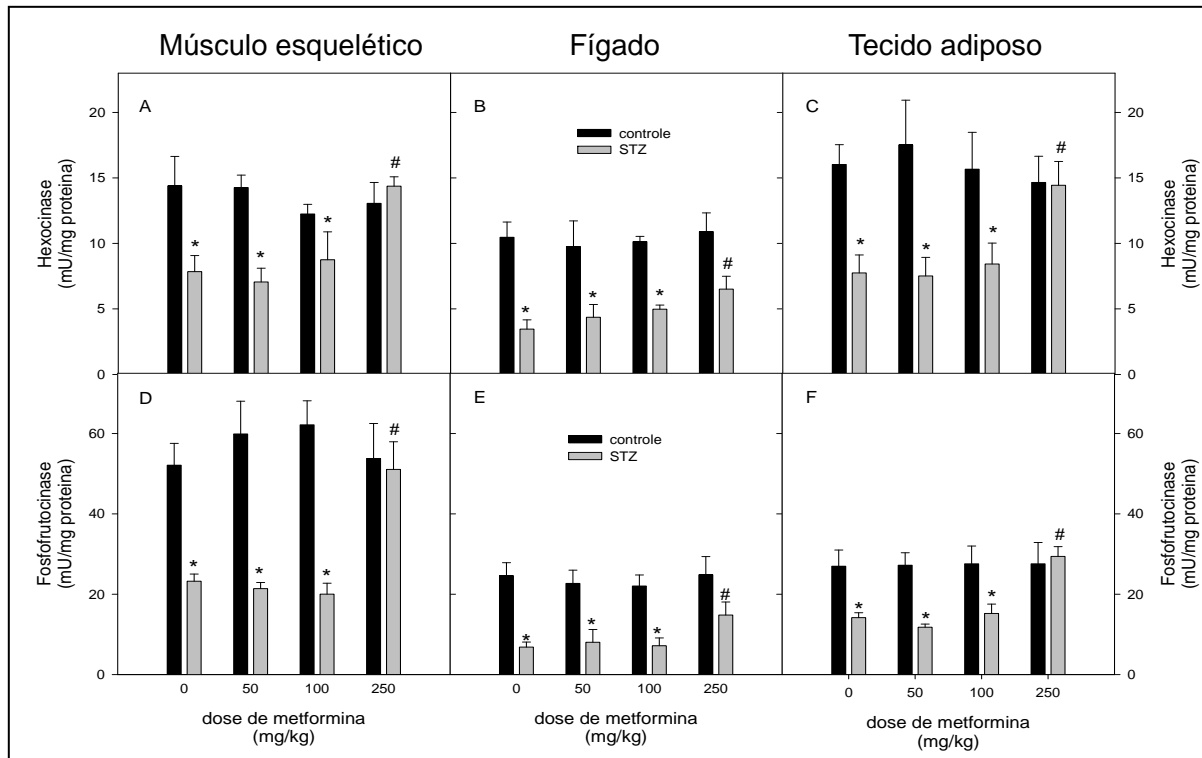


Figura 7. Atividade da hexocinase (A, B, C) e da fosfofrutocinase (D, E, F) de músculo esquelético (A, D), fígado (B, E) e tecido adiposo (C, F) de camundongos diabéticos tratados com diferentes doses de metformina. Camundongos controles (barras pretas) ou diabéticos (barras cinza) foram tratados com diferentes doses de metformina ou apenas solução salina. Os valores representam a média \pm erro padrão ($n=6$). * $p < 0,05$ quando comparado ao grupo controle não tratado. # $p < 0,05$ quando comparado ao grupo diabético não tratado. Os demais procedimentos experimentais estão descritos em material e métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.1.3. Atividade enzimática e análise estrutural da hexocinase e da fosfofrutocinase purificadas na presença de metformina

Resultados prévios do nosso laboratório demonstram que alguns fármacos, que alteram o consumo de glicose através da modulação de enzimas glicolíticas, tais como clotrimazol e ácido acetilsalicílico, interagem diretamente com a PFK alterando sua atividade e estrutura quaternária (ZANCAN et al., 2007a; SPITZ et al., 2009). Com o objetivo de investigar um possível efeito direto da metformina sobre a atividade enzimática da HK e da PFK, a atividade dessas enzimas purificadas foi medida na presença de diferentes concentrações do fármaco. Como mostrado na figura 8A, as atividades da HK (barras pretas) e da PFK (barras cinza) purificadas não são alteradas pela presença de diferentes concentrações de metformina (1, 10, 100 e 1000 μM). Na presença de concentrações superiores de metformina (2,0 e 3,0 mM), a atividade da PFK também não foi alterada (dados não mostrados).

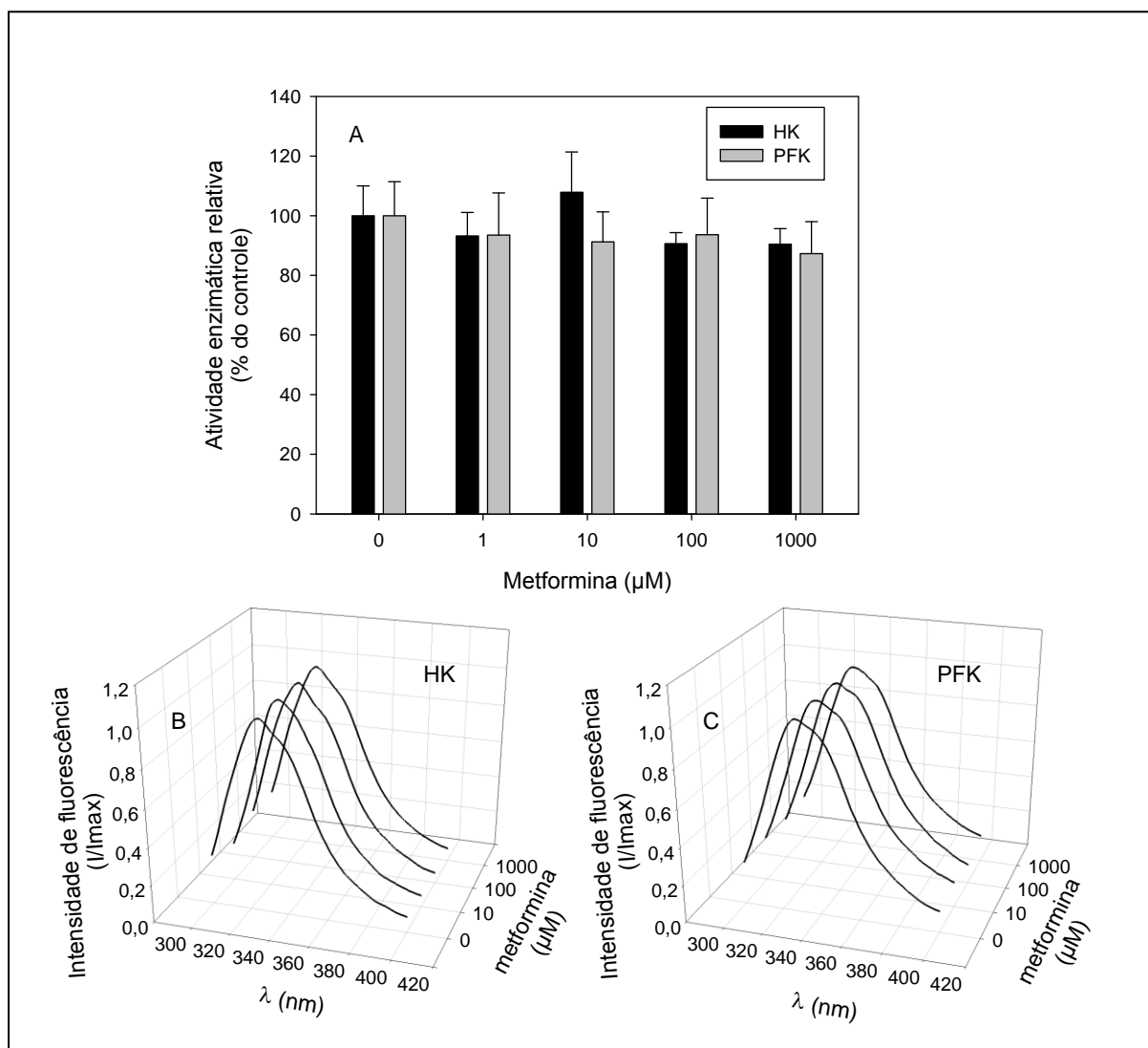


Figura 8. Atividade catalítica e espectro de fluorescência intrínseca da hexocinase (HK) e da fosfofrutocinase (PFK) purificadas na presença de diferentes concentrações de metformina. A atividade enzimática e o espectro de fluorescência intrínseca foram analisados como descrito em Material e Métodos. A: atividade da HK (barras pretas) e da PFK (barras cinza) na presença de diferentes concentrações de metformina. Os valores representam a média \pm erro padrão ($n=3$). B, C: espectro de fluorescência intrínseca da HK e da PFK, respectivamente, na presença de diferentes concentrações de metformina. Os valores representam a média \pm erro padrão ($n=3$).

Os espectros de fluorescência intrínseca da HK (Fig. 8B) e da PFK (Fig. 8C) não são alterados pela presença de 10, 100 ou 1000 μM de metformina. Com os dados obtidos a partir da leitura da amostra, foi possível calcular o centro de massa do espectro, como descrito em Material e Métodos. As concentrações de metformina citadas anteriormente não alteram o centro de massa espectral das enzimas (dados não mostrados). Desta forma, os resultados sugerem que a metformina não modifica a estrutura das enzimas e que a reversão das

atividades enzimáticas, tanto da HK como da PFK, encontradas nos tecidos, não ocorre devido a um mecanismo direto de ação da metformina sobre a atividade catalítica destas enzimas.

4.1.4. Efeitos da insulina sobre a atividade da HK da PFK de diferentes tecidos de camundongos diabéticos tratados com metformina

A insulina é capaz de aumentar a atividade da HK (Fig. 9A) e da PFK (Fig. 9D) de músculo esquelético de todos os grupos de camundongos estudados, sendo que o efeito do hormônio sobre a atividade da PFK de músculo esquelético de camundongos diabéticos não tratados é maior quando comparado com o seu efeito sobre a atividade da HK deste mesmo grupo. Além disto, o fato da atividade reduzida da PFK ser completamente revertida pelo tratamento com insulina *in vitro* sugere que os níveis de PFK não foram alterados pela indução do DM.

Interessantemente, a pré-incubação com insulina não aumenta significativamente a atividade da HK de fígado em nenhum dos grupos estudados (Fig. 9B). Por outro lado, esta incubação é capaz de aumentar significativamente a atividade da PFK em todos os tecidos (Fig. 9E), quando comparados com os tecidos incubados na ausência de insulina. Apesar de a insulina estimular a atividade da PFK de fígado do grupo diabético, esta atividade ainda permanece menor que a atividade do grupo controle estimulado com insulina. Este resultado pode ser um indicativo de que os níveis da enzima estão alterados nesta condição.

A pré-incubação com insulina não aumenta a atividade da HK de tecido adiposo epididimal do grupo controle, porém este hormônio aumenta significativamente a atividade da HK deste tecido nos outros três grupos (Fig. 9C). De forma similar, a incubação do tecido adiposo com insulina (barras cinza) é capaz de aumentar a atividade da PFK nos quatro grupos estudados, quando comparada com os seus respectivos grupos controles (barras pretas) (Fig. 9F). No entanto, a insulina não é capaz de reverter completamente a atividade da HK, nem a atividade da PFK, sugerindo novamente que o nível das enzimas possa estar diminuído.

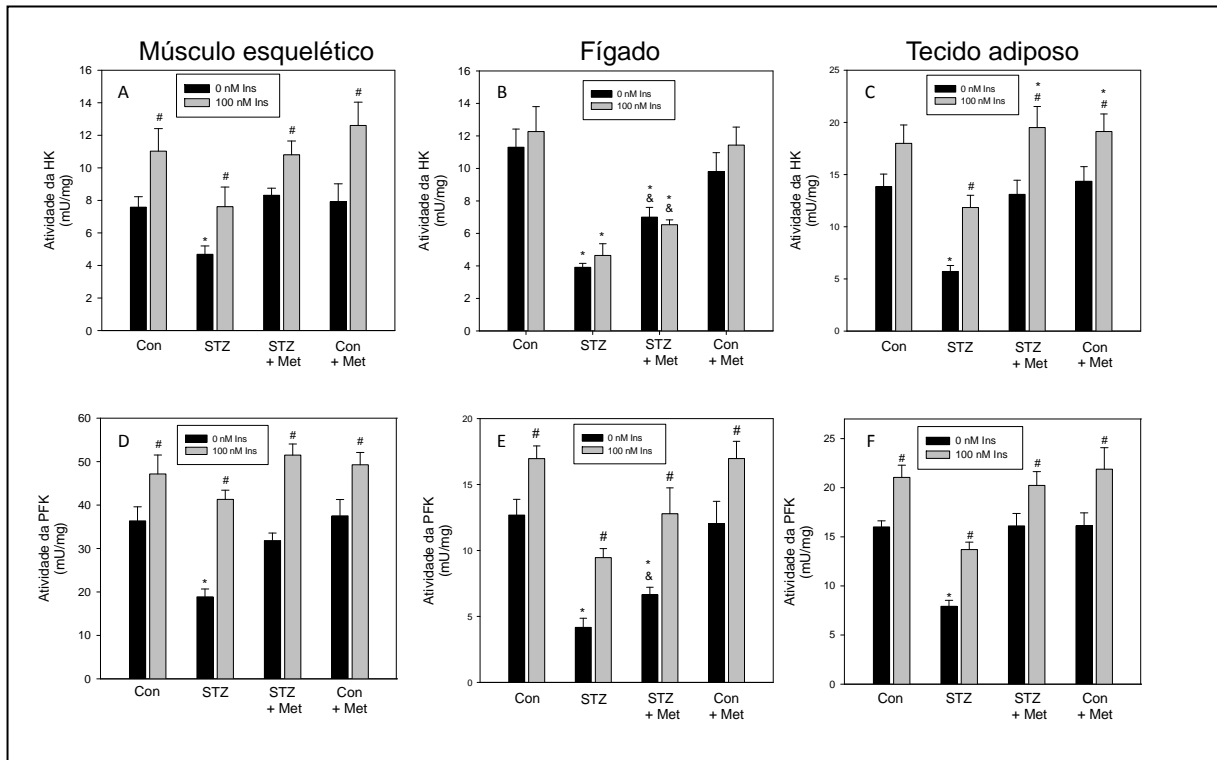


Figura 9. Atividade da hexocinase (painéis superiores) e da fosfofrutocinase (painéis inferiores) de músculo esquelético (A e D), fígado (B e E) e tecido adiposo epididimal (C e F) de camundongos diabéticos tratados com 250 mg/Kg de metformina e pré-incubados com insulina. Os tecidos foram pré-incubados por 30 minutos na ausência (barras pretas) ou na presença (barras cinza) de 100 nM de insulina. A atividade enzimática foi medida como descrito em Material e Métodos. Os valores representam a média \pm erro padrão ($n=5-6$). * $p < 0,05$ comparado ao grupo controle não tratado; # $p < 0,05$ comparado aos tecidos não incubados com insulina; & $p < 0,05$ comparado ao grupo diabético não tratado. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.1.5. Efeitos da metformina sobre o nível de proteína de HK e de PFK dos diferentes tecidos de camundongos diabéticos

A pré-incubação com insulina não é capaz de reverter completamente à baixa atividade das enzimas em todos os tecidos, desta forma sugerindo que os níveis enzimáticos possam estar reduzidos nos camundongos diabéticos. A análise dos níveis de HK-1 mostra que a indução do DM reduz os níveis da enzima, quando comparados com os controles não tratados, em músculo esquelético (Fig. 10A, D), fígado (Fig. 10B, E) e tecido adiposo (Fig. 10C, F). Por outro lado, o tratamento com metformina reverte esses baixos níveis de HK-1 dos animais diabéticos, sem apresentar efeito sobre os controles. Curiosamente, os níveis de HK-1 em tecido adiposo (Fig. 10C, F) dos controles tratados com metformina são maiores que os controles não tratados. Os níveis de PFK em músculo esquelético não são diferentes entre as condições estudadas.

No músculo esquelético (Fig. 10A, D) e tecido adiposo (Fig. 10C, F) os níveis de HK-2, principal isoforma de HK expressa nesses tecidos, também diminui com a indução do DM. De forma similar a HK-1, o tratamento com metformina também reverte os baixos níveis desta isoforma nesses tecidos, sem apresentar efeito sobre os controles.

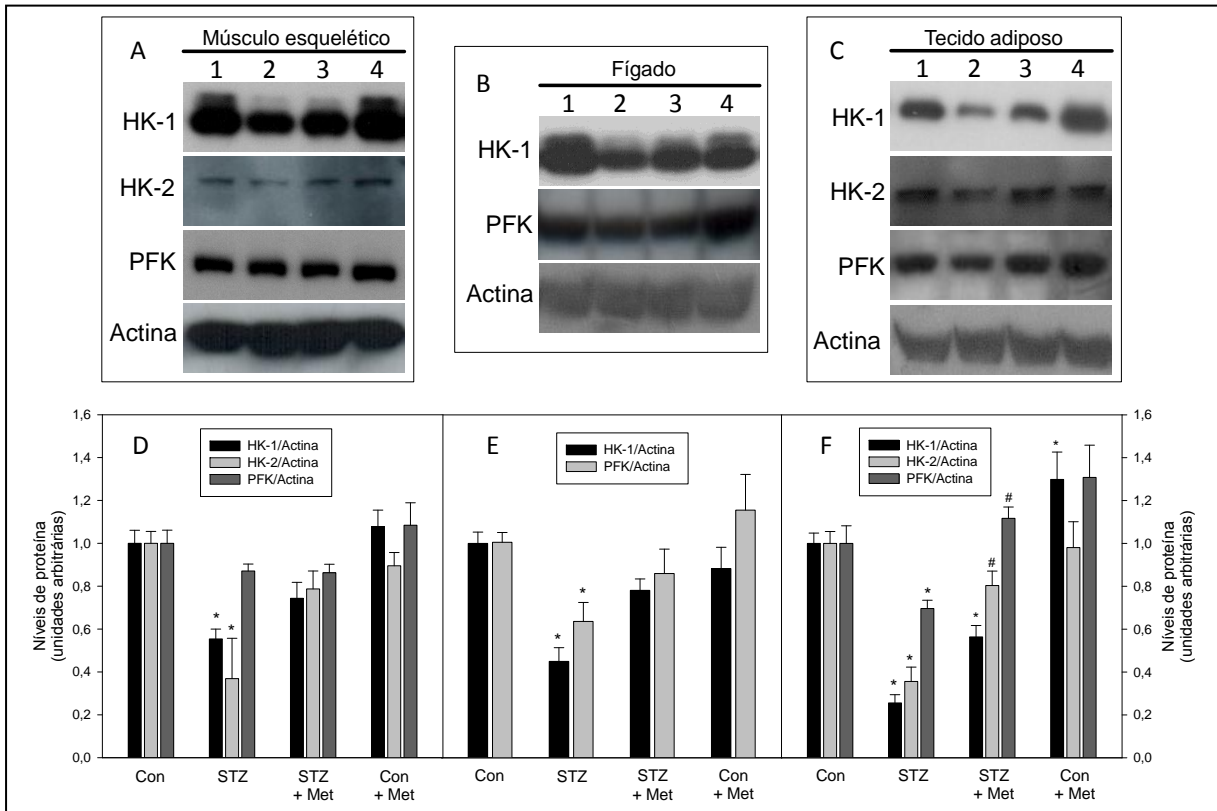


Figura 10. Níveis de hexocinase (HK-1 e HK-2) e fosfofrutocinase (PFK) em diferentes tecidos de camundongos diabéticos tratados com 250 mg/Kg de metformina analisadas através de *Western blotting*. Os painéis superiores mostram um experimento representativo e os painéis inferiores mostram a quantificação de pelo menos 3 diferentes experimentos para cada tecido. Músculo esquelético (A, D), fígado (B, E) e tecido adiposo epididimal (C, F) foram homogeneizados, entre 60 e 100 μ g de proteína foram submetidas a eletroforese (SDS-PAGE) e transferidas para uma membrana de PVDF. As membranas foram bloqueadas e depois incubadas com: anticorpo monoclonal anti-HK-1 ou anti-HK-2; anticorpo policlonal anti-PFK ou anti-actina. As bandas imunorreativas foram visualizadas através de reação quimioluminescente (Kit ECL), de acordo com as instruções do fabricante. Os níveis de actina foram utilizados como controle de carregamento. * $p < 0,05$ comparado ao grupo controle não tratado; # $p < 0,05$ comparado ao grupo diabético não tratado. Os demais procedimentos experimentais estão descritos em Material e Métodos. Con (1): controle; STZ (2): diabético; STZ + Met (3): diabético tratado com metformina; Con + Met (4): controle tratado com metformina.

Os níveis de PFK são menores em fígado (Fig. 10B, E) e tecido adiposo (Fig. 10C, F) dos camundongos diabéticos, quando comparados com os seus respectivos controles. Apesar da metformina apresentar um pequeno efeito sobre os níveis de PFK de fígado, em ambos os tecidos o tratamento com o fármaco reverte significativamente os baixos níveis da enzima.

Em geral, esses resultados sugerem que a metformina é capaz de reverter os baixos níveis tanto de HK quanto de PFK, podendo ser este efeito correlacionado com a reversão da baixa atividade das enzimas nos diferentes tecidos.

4.1.6. Efeitos da metformina sobre os níveis de RNAm das diferentes isoformas de HK e de PFK dos tecidos de camundongos diabéticos

Com o objetivo de analisar se as alterações nos níveis das enzimas se dão por modificações a nível transcricional, as concentrações de RNAm das diferentes isoformas de HK e PFK foram analisadas através de PCR, como descrito em Material e Métodos. Primeiramente, caracterizamos as principais isoformas de PFK transcritas nos diferentes tecidos. A figura 11A mostra, em um experimento representativo, que em músculo esquelético e fígado os níveis transcricionais de PFK-M e PFK-L, respectivamente, estão em maior concentração. Por outro lado, as diferentes isoformas de PFK parecem apresentar níveis similares em tecido adiposo epididimal.

Os resultados sugerem que tanto a indução do DM1 quanto o tratamento com metformina não alteram os níveis de RNAm de HK-1, HK-2 e PFK-M em músculo esquelético (Fig. 11B, E). No tecido hepático, os níveis de RNAm da HK-1 também são os mesmos entre as condições (Fig. 11C, F). Por outro lado, a indução do DM reduz significativamente os níveis de HK-4 e de PFK-L, sendo esta redução completamente revertida pelo tratamento com metformina. A redução dessas duas isoformas pode ser uma das causas da mais baixa atividade da HK e da PFK encontrada em fígado, quando comparada com os outros tecidos. Este tratamento também reverte os baixos níveis de RNAm da HK-2 e, interessante, não apresenta efeitos sobre os baixos níveis de PFK-C em tecido adiposo dos camundongos diabéticos (Fig. 11D, G). De forma similar ao músculo esquelético, no tecido adiposo também não há alterações nos níveis de HK-1, PFK-M e PFK-L. Em conjunto, esses resultados indicam que as modificações transcricionais induzidas pelo DM e/ou tratamento com metformina podem ser específicas para cada tecido e, até mesmo, para cada isoforma das enzimas.

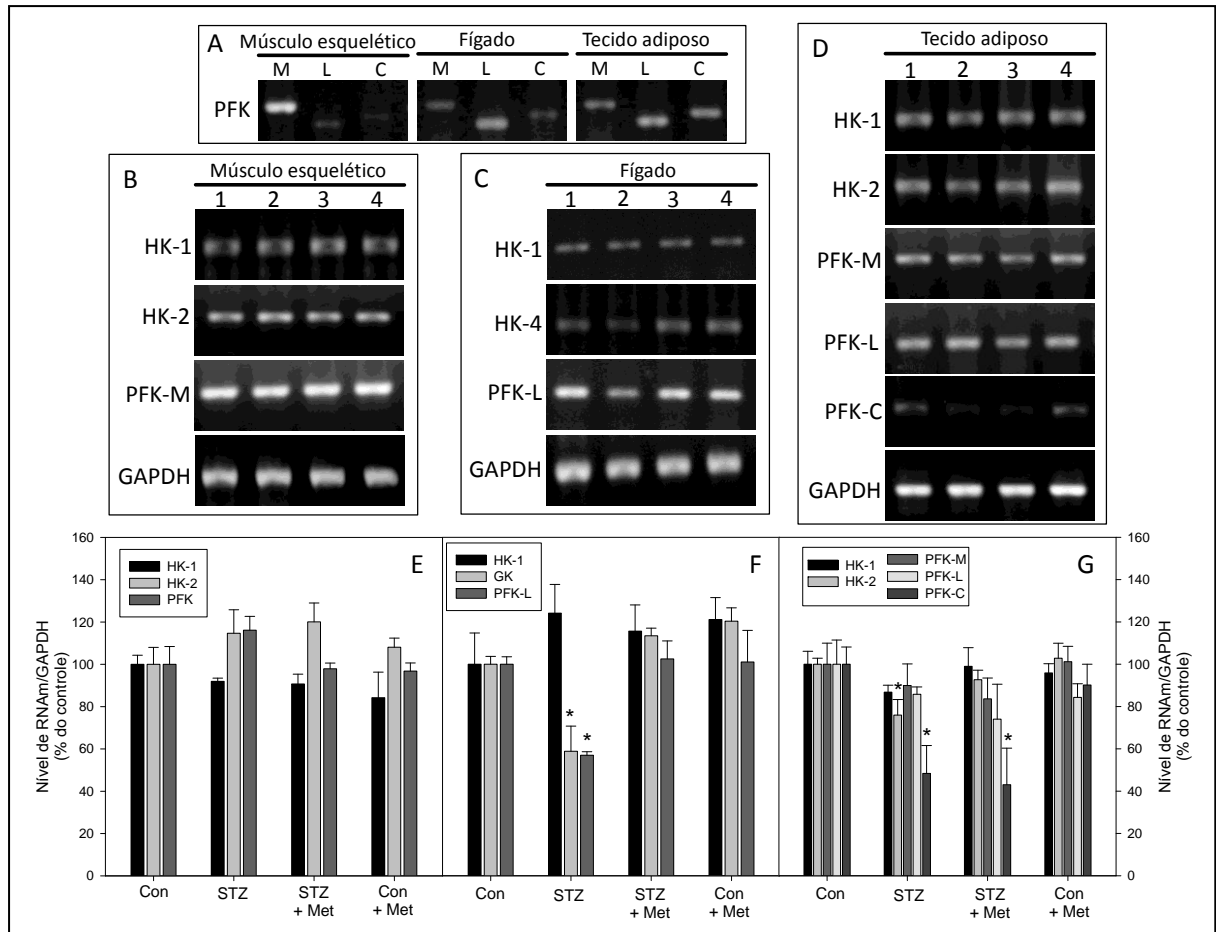


Figura 11. Identificação das diferentes isoformas de fosfofrutocinase (PFK) transcritas em diferentes tecidos de camundongos controles (painel A). Nível de RNAm de HK-1, HK-2, HK-4, PFK (isoformas M, L e P) transcritas em diferentes tecidos de camundongos diabéticos tratados com 250 mg/Kg de metformina. Os painéis A, B, C e D mostram um gel de agarose 1% representativo e os painéis E, F e G mostram a quantificação de pelo menos 3 diferentes experimentos para músculo esquelético, fígado e tecido adiposo epididimal, respectivamente. Os níveis de RNAm de GAPDH foram utilizados como controle de carregamento. As bandas correspondem a fragmentos de 361 pb (HK-1), 451 pb (HK-2), 574 pb (GAPDH), 741 pb (PFK-M), 606 pb (PFK-C) e 533 pb (PFK-L). * $p < 0,05$ quando comparado ao grupo controle não tratado. Os demais procedimentos experimentais estão descritos em Material e Métodos. Con (1): controle; STZ (2): diabético; STZ + Met (3): diabético tratado com metformina; Con + Met (4): controle tratado com metformina.

4.1.7. Efeitos da metformina sobre a localização celular da atividade da HK de diferentes tecidos de camundongos diabéticos

Com o objetivo de investigar o papel da metformina sobre a sublocalização celular da atividade da HK nos tecidos estudados, o HT destes foi submetido a um processo de centrifugação diferencial através do qual foram obtidas as diferentes frações celulares, ricas em mitocôndrias (P1) e com os demais componentes celulares (S1), como descrito em Material e Métodos. Como mencionado anteriormente, a atividade da HK de HT de músculo esquelético (Fig. 12A), fígado (Fig. 12C) e tecido adiposo (Fig. 12E) de camundongos

diabéticos é menor que o controle. No entanto, o tratamento com 250 mg/Kg é capaz de reverter esta baixa atividade enzimática.

A atividade da HK nos três tecidos controles é maior na fração P1 do que na fração S1. A indução do DM aumenta a atividade da HK na fração S1 e diminui na fração P1, levando ambas as frações a apresentarem atividades similares em músculo esquelético (Fig. 12B), fígado (Fig. 12D) e tecido adiposo (Fig. 12F). Por outro lado, o tratamento dos camundongos diabéticos com metformina aumenta a atividade da HK na fração P1, retornando esta ao mesmo nível dos controles em fígado (Fig. 12D) e tecido adiposo (Fig. 12F), porém sem ter efeito em músculo esquelético (Fig. 12B). O tratamento dos camundongos controles com metformina não altera o perfil de distribuição da atividade da HK em nenhum dos tecidos, quando comparada ao grupo controle não tratado. Assim, a alteração na sublocalização celular da atividade da HK pode ser uma das causas relacionadas com a menor atividade total da enzima encontrada nos tecidos. Apesar de a metformina modular esta distribuição em fígado e tecido adiposo, os mecanismos pelos quais este fármaco induz este efeito ainda não estão claros.

4.1.8. Efeitos da metformina sobre as concentrações de glicose-6-fosfato de diferentes tecidos de camundongos diabéticos

A diferente sublocalização da HK pode estar ligada as concentrações de G6P, uma vez que este produto da enzima é capaz de desligar a mesma da mitocôndria. Por isto, avaliamos as concentrações de G6P em músculo esquelético (Fig. 13A), fígado (Fig. 13B) e tecido adiposo epididimal (Fig. 13C). O resultado mostra que o tratamento dos camundongos diabéticos reverte parcialmente os baixos níveis de G6P encontrados em músculo esquelético e tecido adiposo. No entanto, entre as diferentes condições estudadas, não há diferença na concentração de G6P hepática. Desta forma, esses resultados sugerem que as diferentes concentrações de G6P não estão relacionadas com as diferentes distribuições celulares da HK. Por outro lado, a baixa concentração deste metabólito pode ser um indicativo da atividade total reduzida da HK.

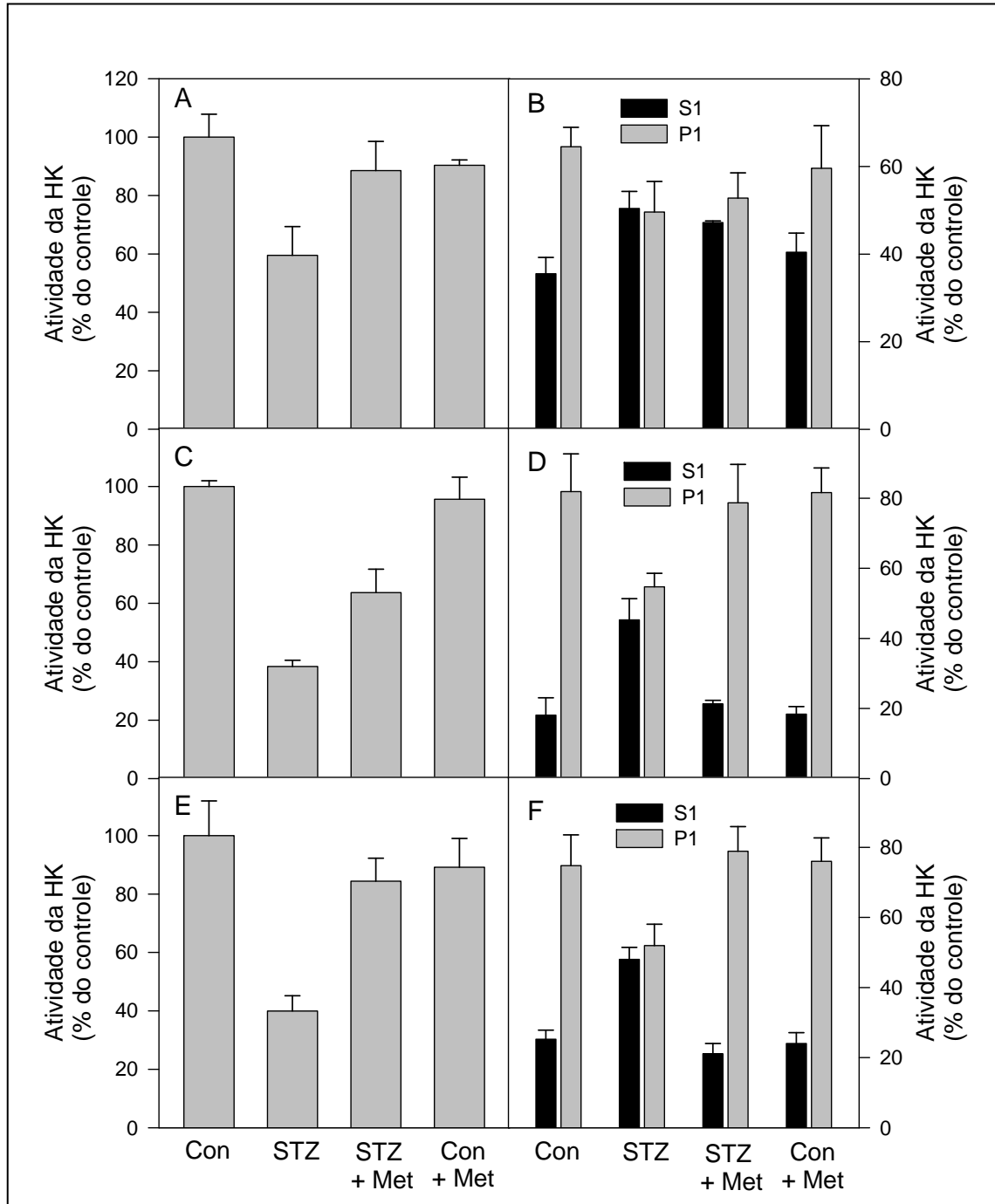


Figura 12. Distribuição celular da atividade da hexocinase (HK) de músculo esquelético (A e B), fígado (C e D) e tecido adiposo (E e F) de camundongos diabéticos tratados com 250 mg/Kg de metformina. Os gráficos A, C e E representam o percentual da atividade da HK em homogeneizado total (HT), relativos aos seus respectivos controles. Os gráficos B, D e F representam o percentual da atividade total da HK do sobrenadante (S1, barras pretas) e do precipitado (P1, barras cinza) após a centrifugação do HT a 27.000g. Os valores representam a média \pm erro padrão (n= 5-6). Os demais procedimentos experimentais estão descritos em **Material e Métodos**. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

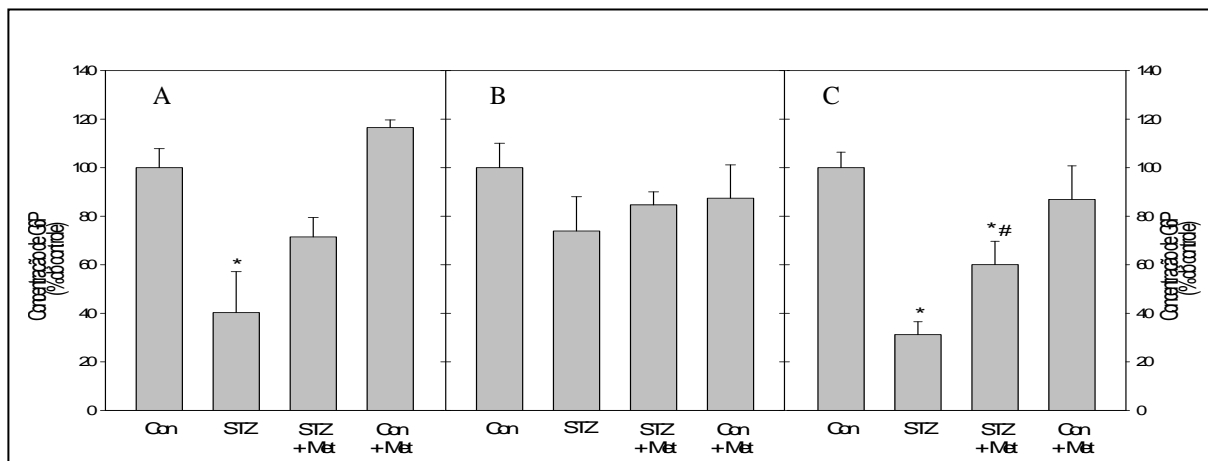


Figura 13. Concentração de glicose-6-fosfato (G6P) em músculo esquelético (A), fígado (B) e tecido adiposo (C) de camundongos diabéticos tratados com 250 mg/Kg de metformina. Os procedimentos para a determinação de G6P, bem como os demais procedimentos experimentais, estão descritos em Material e Métodos. Os valores representam a média \pm erro padrão (n= 3-4). * $p < 0,05$ comparado ao grupo controle. # $p < 0,05$ comparado ao grupo diabético. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.1.9. Efeitos da metformina sobre a localização celular da atividade da PFK de diferentes tecidos de camundongos diabéticos

Com o objetivo de investigar o papel da metformina na localização celular da PFK nos tecidos estudados, o HT destes foi submetido a um processo de centrifugação diferencial através do qual foram obtidas diferentes frações celulares (S1, P1, S2 e P2), como descrito em Material e Métodos. Como mencionado anteriormente, a atividade da PFK de HT de músculo esquelético (Fig. 14A), fígado (Fig. 14C) e tecido adiposo (Fig. 14E) de camundongos diabéticos é menor que o controle, e o tratamento com 250 mg/Kg é capaz de reverter esta baixa atividade enzimática. Praticamente toda a atividade da PFK permaneceu na fração S1 dos tecidos, sendo desta forma a fração P1 desprezível. O tratamento com metformina não altera a atividade da enzima nessas frações em nenhum dos grupos de animais (dados não mostrados). A partir da fração S1 foram isoladas outras duas frações: S2, na qual se encontra predominantemente a PFK na forma solúvel; e P2, enriquecida F-actina (a atividade da PFK nesta fração se deve a precipitação das proteínas do citoesqueleto juntamente com a PFK). Em músculo esquelético de camundongos controles, aproximadamente 60% da atividade da PFK encontra-se na fração P2, enquanto que nos animais diabéticos a atividade desta enzima é predominante na fração S2 (Fig. 14B). Em animais diabéticos tratados com 250 mg/Kg de metformina, a atividade da PFK é similar tanto na fração S2 quanto na P2. Curiosamente, a atividade da PFK de camundongos controles tratados com essa dose de metformina encontra-

se majoritariamente na fração P2. Assim, o tratamento com metformina parece ser capaz de alterar a localização celular da atividade da PFK de músculo esquelético para frações ricas em F-actina.

Os resultados da análise do perfil de distribuição da atividade da PFK no fígado de camundongos controles e diabéticos não tratados com metformina mostra o mesmo perfil de distribuição da atividade da PFK entre as frações S2 e P2. No entanto, os grupos tratados com metformina, controles e diabéticos, apresentam diminuição da atividade da PFK na fração S2, sem concomitante aumento significativo da atividade na fração P2 (Fig. 14D). Assim, o desenvolvimento do DM não altera a localização celular da atividade da PFK entre as frações S2 e P2 de fígado. Por outro lado, o tratamento dos animais diabéticos ou controles com metformina somente diminuiu a atividade da PFK na fração solúvel.

Ao contrário do músculo esquelético, em tecido adiposo epididimal de camundongos controles e controles tratados com metformina, aproximadamente 75-80% da atividade da PFK se encontra na fração S2 (Fig. 14F). Em animais diabéticos tratados e não tratados com metformina a atividade da PFK é similar tanto na fração S2 quanto na P2, porém apresentam diferença estatística quando comparados aos seus respectivos controles. Desta forma, os resultados sugerem que o desenvolvimento do DM altera a localização celular da atividade da PFK de frações solúveis para frações ligadas a F-actina, independentemente do tratamento com metformina.

Em conjunto, esses resultados apontam para mecanismos específicos de regulação da atividade da PFK quanto a sua sublocalização celular dependendo do tecido.

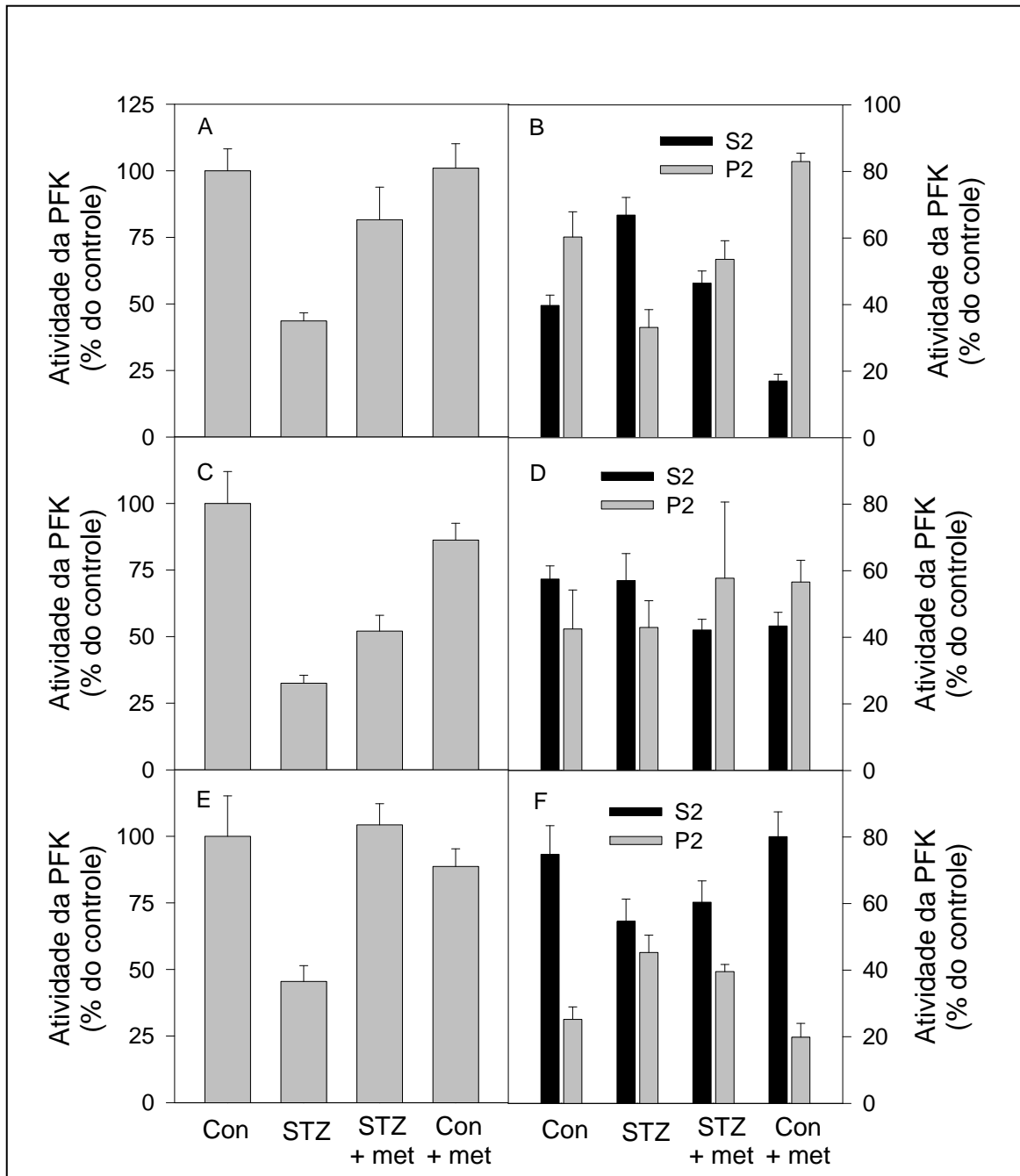


Figura 14. Distribuição celular da atividade da PFK de músculo esquelético (A e B), fígado (C e D) e tecido adiposo (E e F) de camundongos diabéticos tratados com 250 mg/Kg de metformina. Os gráficos A, C e E representam o percentual da atividade da PFK em homogeneizado total (HT), relativos aos seus respectivos controles. Os gráficos B, D e F representam o percentual da atividade total da PFK do sobrenadante (S2, barras pretas) e do precipitado (P2, barras cinza) após a centrifugação do S1 a 120.000g. Os valores representam a média \pm erro padrão (n= 5-6). Os demais procedimentos experimentais estão descritos em Material e Métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.1.10. Efeitos da metformina sobre o perfil de fosforilação da PFK de diferentes tecidos de camundongos diabéticos

Para entendermos melhor a sinalização que modula a distribuição intracelular da PFK no DM e os efeitos da metformina, os níveis de fosforilação da enzima foram analisados em músculo esquelético, fígado e tecido adiposo epididimal. A figura 15A mostra um resultado representativo de um *western blotting*. Não há mudança nos níveis de fosforilação dos resíduos de fosfotreonina e fosfotirosina em nenhum dos tecidos. A única mudança significativa é encontrada em relação aos níveis de fosfoserina da PFK de músculo esquelético, como mostra a quantificação de quatro experimentos independentes (Fig. 15B). Neste caso, o DM reduz os níveis e a metformina reverte estes baixos níveis de fosforilação. Em camundongos controles não há efeito da metformina. A baixa marcação mostrada na coluna quatro, correspondente a imunoprecipitação da PFK do tecido hepático (Fig. 15A), indica que menores quantidades da enzima estão imunoprecipitadas. Assim, a diferença no perfil de fosforilação da PFK de músculo esquelético, em relação ao fígado e ao tecido adiposo, sugere um possível padrão de regulação específico da enzima deste tecido que pode estar envolvido na redistribuição da enzima de frações citossólicas para frações ricas em F-actina.

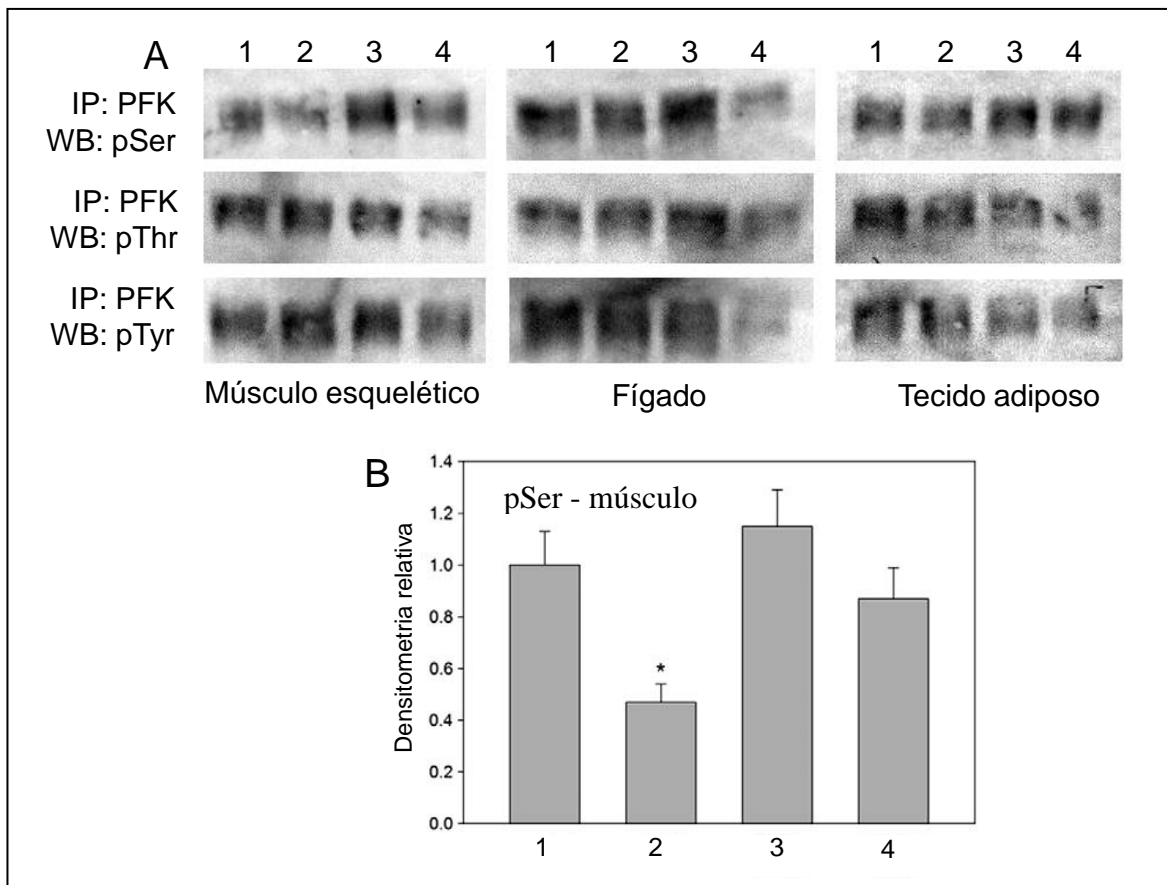


Figura 15. Perfil de fosforilação da PFK de diferentes tecidos de camundongos diabéticos tratados com 250 mg/Kg de metformina. (A) *Western blotting* representativo. A PFK de músculo esquelético, fígado, tecido adiposo epididimal dos camundongos foi imunoprecipitada com um anticorpo anti-PFK (IP:PFK) e marcada com um anticorpo antifososerina (pSer), antifosfotreonina (pThr) ou antifosfotirosina (pTyr). (B) Quantificação relativa dos níveis de pSer da PFK de músculo esquelético. Os valores representam a média \pm erro padrão (n= 4). * $p < 0,05$ comparado a todos os outros grupos. Os demais procedimentos experimentais estão descritos em Material e Métodos. 1: controle; 2: diabético; 3: diabético tratado com metformina; 4: controle tratado com metformina.

RESULTADOS

4.2. PARTE 2

4.2.1. Efeitos da metformina sobre a atividade total e sobre localização celular da atividade da HK e da PFK de coração de camundongos diabéticos

Como mostrado previamente, a atividade da HK e da PFK estão inibidas em músculo esquelético, fígado e tecido adiposo de camundongos diabéticos, sendo este efeito revertido pelo tratamento com metformina. Além desses tecidos, o coração também depende do

metabolismo glicolítico (STANLEY et al., 1997). Já foi demonstrado que camundongos deficientes em PFK-M têm alta mortalidade devido às alterações metabólicas causadas pelo bloqueio da glicólise, incluindo hipertrofia cardíaca (GARCÍA et al., 2009). Neste sentido, além da metformina diminuir a glicemia, também já foi mostrado que este fármaco confere cardioproteção a pacientes diabéticos (JOHNSON et al., 2002). Assim, com o objetivo de determinar se a metformina modula a HK e a PFK de coração de camundongos diabéticos, avaliamos a atividade total e a distribuição intracelular dessas enzimas em corações de camundongos com DM1 e os efeitos da metformina sobre esta distribuição (Fig. 16).

Os corações de cada um dos quatro grupos estudados foram homogeneizados e submetidos a fracionamento através de centrifugação diferencial. Através dessas centrifugações, podemos estimar a proporção das HKs associadas a organelas (P1), tais como mitocôndrias, e estimar a proporção das PFKs associadas aos componentes do citoesqueleto (P2), tais como F-actina.

As atividades da HK (Fig. 16A) e da PFK (Fig. 16C) no homogeneizado total, medidas pelo método radiométrico, são menores em coração de camundongos diabéticos, quando comparadas com as atividades dos controles, sendo a atividade da HK mais afetada do que a da PFK. Por outro lado, o tratamento dos camundongos diabéticos com metformina é capaz de reverter completamente à baixa atividade de ambas as enzimas, apesar deste fármaco não ter efeito em camundongos controles tratados com metformina.

O desenvolvimento do DM1 aumenta significativamente a atividade da HK na fração solúvel (S1), quando comparada com a fração particulada (P1) (Fig. 16B). O tratamento dos camundongos diabéticos com metformina aumenta bruscamente a atividade da HK na fração P1, sendo esta atividade maior que a atividade encontrada na fração particulada do controle. No entanto, não há alteração na distribuição da atividade da HK entre o grupo controle tratado e não tratado, sendo a atividade similarmente distribuída entre as frações S1 e P1 nessas duas condições.

O desenvolvimento do DM1 também aumenta a atividade da PFK na fração solúvel (S2), quando comparada com a fração particulada (P2). No entanto, o tratamento dos camundongos diabéticos com metformina restaura o perfil de distribuição da atividade da PFK, sendo este perfil similar entre o grupo controle tratado e não tratado com metformina (Fig. 16D).

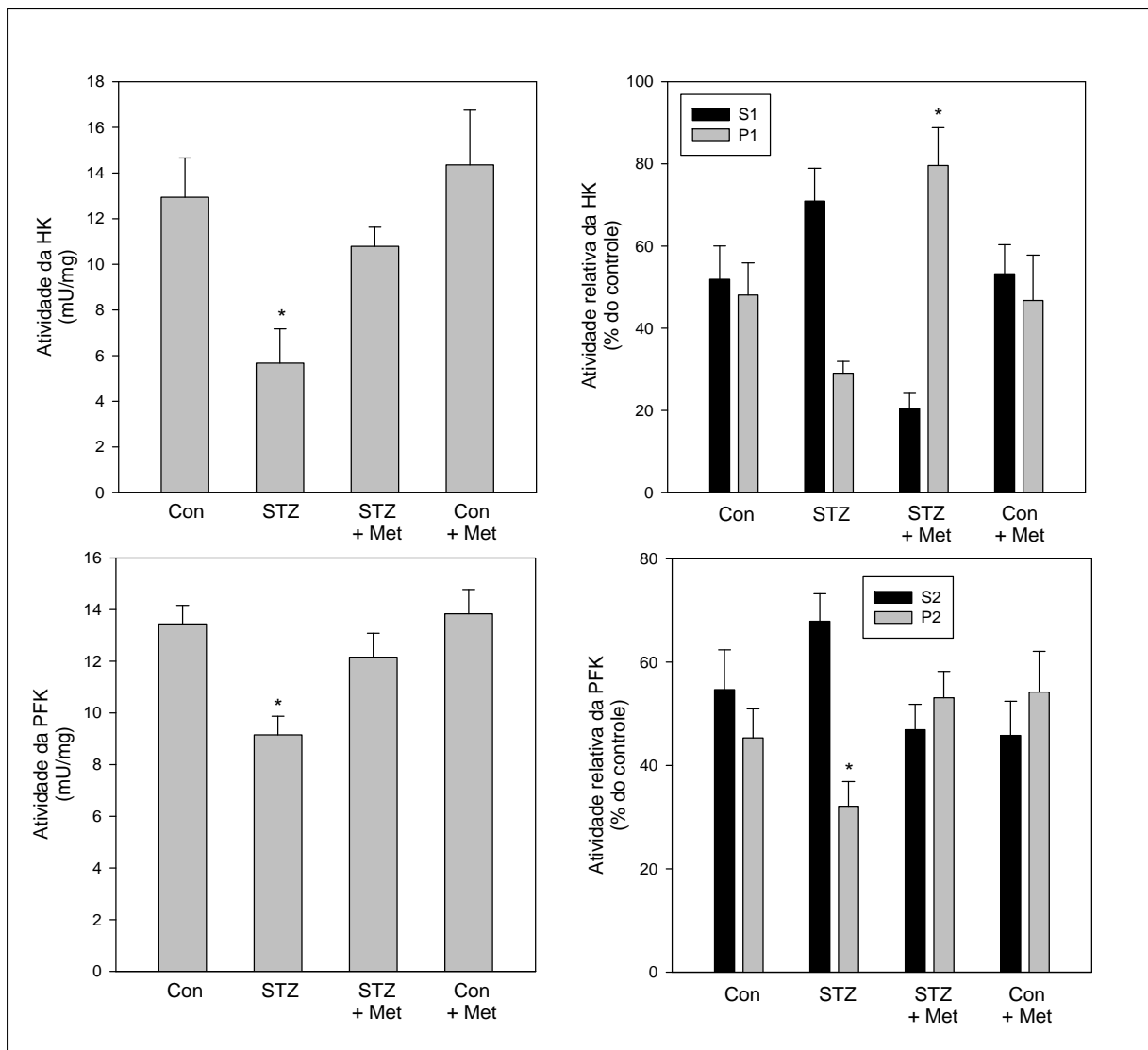


Figura 16. Distribuição celular da atividade da HK (A, B) e da PFK (C, D) de coração de camundongos diabéticos tratados com 250 mg/Kg de metformina. Os gráficos A e C representam a atividade total (mU/mg) da HK e da PFK de homogeneizado de coração. Os gráficos B e D representam o percentual da atividade total (solúvel mais particulada) da HK e PFK após a centrifugação do HT ou da fração S1, respectivamente. Os valores representam a média \pm erro padrão (n= 4-6). * $p < 0,05$ comparado aos seus respectivos grupos controles. Os demais procedimentos experimentais estão descritos em Material e Métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

A fim de investigar se os níveis de G6P estão possivelmente envolvidos na mudança da HK da mitocôndria para o citosol, uma vez que a elevação da concentração de G6P desliga a HK da mitocôndria, os níveis deste metabólito, produzido por catálise desta enzima, foi medido nos corações dos diferentes grupos. O resultado mostra que a concentração de G6P no coração dos camundongos diabéticos é menor do que o controle não tratado. No entanto, o tratamento com metformina reverte completamente esta diminuição do nível de G6P (dados não mostrados).

Em conjunto, esses resultados sugerem que a reversão da atividade das enzimas glicolíticas pode ocorrer devido à redistribuição da atividade da HK e da PFK para frações ricas em mitocôndrias ou F-actina, respectivamente. Além disto, a redistribuição da atividade da HK não parece estar relacionada com os níveis de G6P, sugerindo desta forma que outros fatores possam estar envolvidos na redistribuição celular da enzima.

4.2.2. Efeitos da metformina sobre os níveis de proteína da HK e da PFK de coração de camundongos diabéticos

A análise dos níveis de proteína, através de *Western blotting*, está demonstrada na figura 17A, como um experimento representativo. Na figura 17B está representada a análise densitométrica de pelo menos três experimentos independentes. Os resultados demonstram que os níveis de HK-2 e PFK são menores nos corações dos camundongos diabéticos, quando comparados com os seus respectivos controles. No entanto, o tratamento dos camundongos diabéticos com metformina é capaz de reverter os baixos níveis de PFK, sem alterar os baixos níveis de HK-2. Os níveis de HK-1 não são diferentes entre os corações de nenhum dos grupos estudados. Assim, esses resultados sugerem que a metformina poderia estar agindo especificamente sobre a inibição da degradação da PFK ou através do aumento da sua síntese.

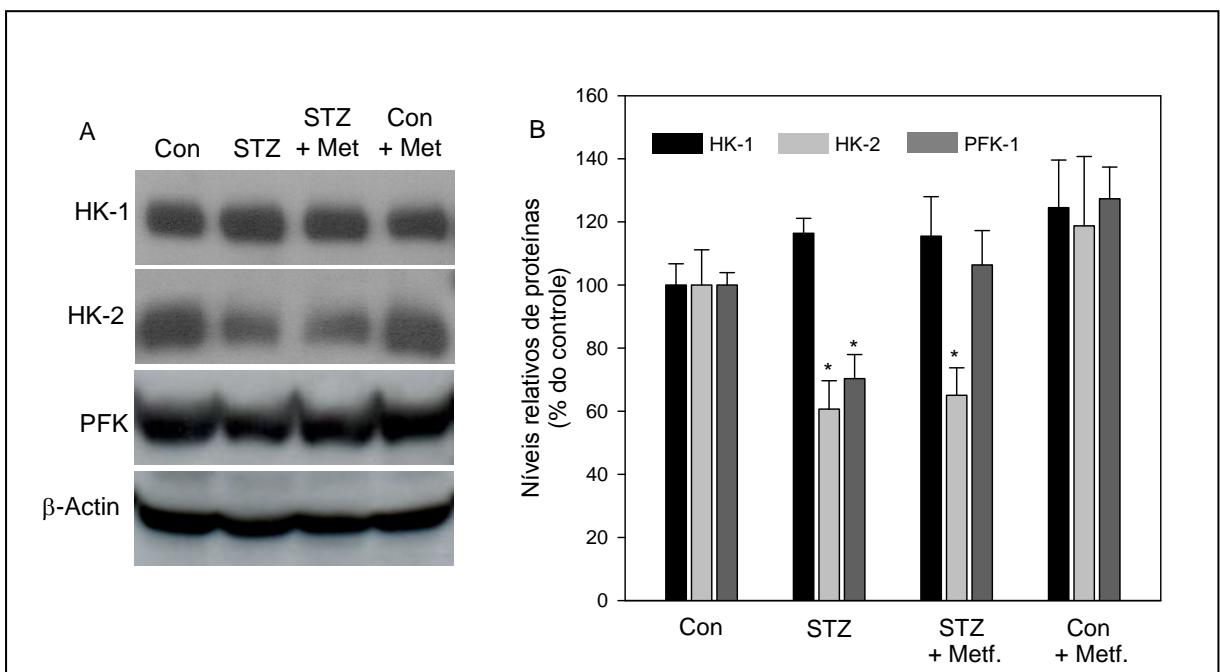


Figura 17. Níveis de hexocinase (HK-1 e HK-2) e fosfofrutocinase (PFK) em corações de camundongos diabéticos tratados com 250 mg/Kg de metformina analisadas através de *Western blotting*. O painel A mostra um experimento representativo e o painel B mostra a quantificação de pelo menos 3 diferentes experimentos. Os corações foram homogeneizados, entre 60 e 100 µg de proteína foram submetidas à eletroforese (SDS-PAGE) e transferidas para uma membrana de PVDF. As membranas foram bloqueadas e depois incubadas com: anticorpo monoclonal anti-HK-1 ou anti-HK-2; anticorpo policlonal anti-PFK ou anti-actina. As bandas imunorreativas foram visualizadas através de reação quimioluminescente (Kit ECL), de acordo com as instruções do fabricante. Os níveis de β-actina foram utilizados como controle de carregamento. * $p < 0,05$ comparado ao grupo controle não tratado. Os demais procedimentos experimentais estão descritos em Material e Métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.2.3. Efeitos da metformina sobre os níveis de RNAm da HK e da PFK de coração de camundongos diabéticos

Com o objetivo de compreender se as mudanças nos níveis das proteínas estão relacionadas com alterações transcricionais, os níveis de RNAm da HK-1, HK-2 e PFK foram analisados através de RT-PCR. Tanto a indução do DM1 quanto o tratamento com metformina não alteram os níveis de RNAm de HK-1, HK-2 e PFK (isoformas M, L e C) em coração (Fig. 18).

A figura 18A mostra que todas as três isoformas da PFK (M, L e C) são transcritas nos corações em quantidades muito similares, sendo a PFK-C apenas 12% menos expressa que a PFK-M. Neste sentido, ambas as isoformas são transcritas em níveis similares. Ainda, as principais isoformas de HK expressas em tecidos cardíacos são a HK-1 e HK-2 (QUINTENS et al., 2008). Assim, foram analisados os níveis de RNAm da PFK em um ensaio multiplex RT-PCR, em que as três isoformas são amplificadas na mesma reação, pois a temperatura de anelamento é muito semelhante entre os primers, como descrito em Material e Métodos. Os níveis de HK-1 e HK-2 foram analisados isoladamente. A figura 18B mostra um resultado representativo de um *western blotting* e a figura 18C mostra a quantificação de pelo menos três experimentos independentes. Os níveis de RNAm da PFK (isoformas M, L e C), HK-1 e HK-2 entre os grupos controles e diabéticos, tratados ou não com metformina, não são diferentes entre si. Os níveis de RNAm da PFK-M também são os mesmos quando analisados isoladamente (dados não mostrados). Assim, nossos resultados sugerem que a diminuição dos níveis de proteína de HK-2 e PFK pode ocorrer devido ao aumento da degradação proteica, uma vez que os níveis de RNAm não estão alterados.

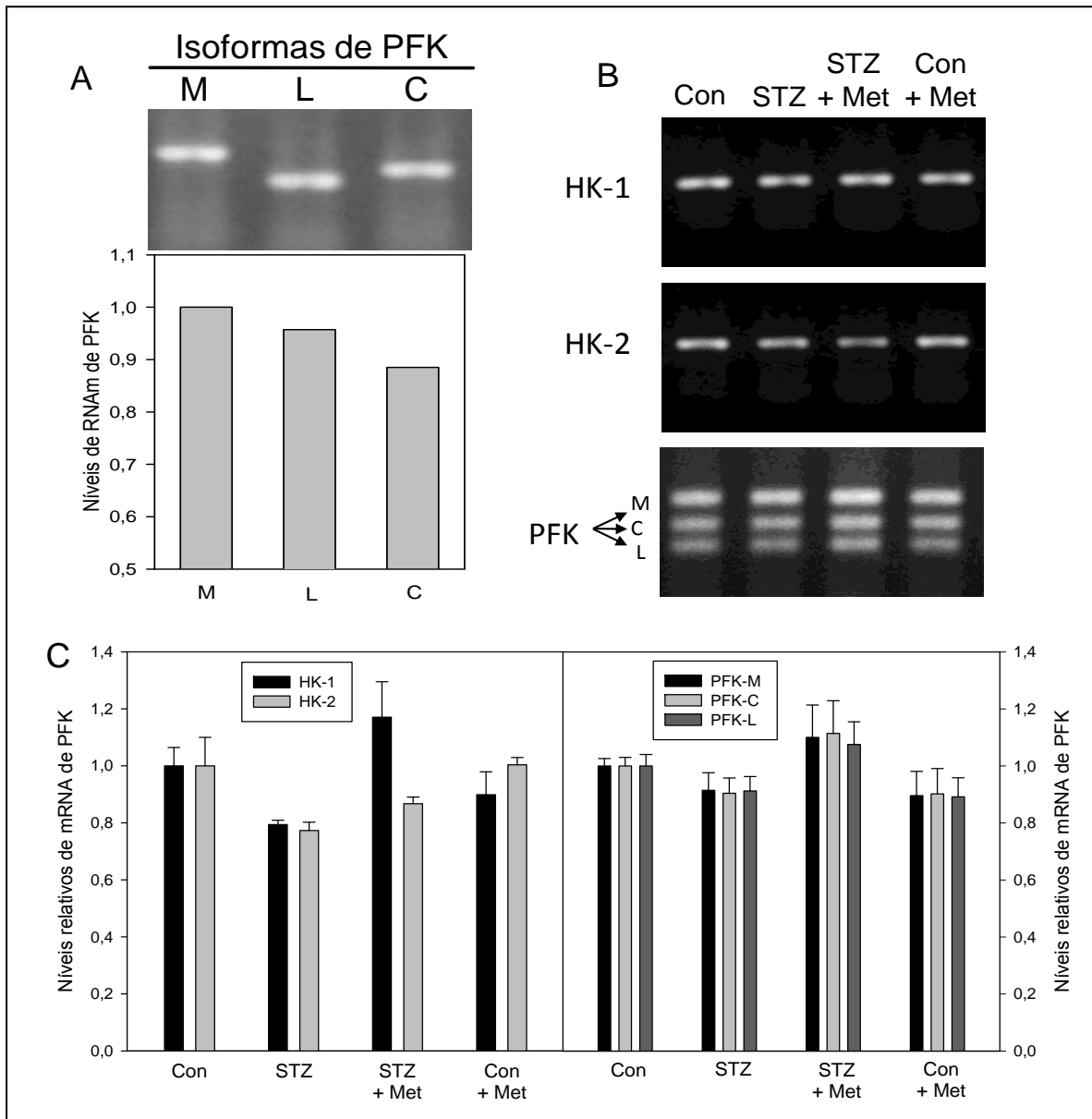


Figura 18. Identificação das diferentes isoformas de fosfofrutocinase (PFK) transcritas em coração de camundongos controles (painel A). Nível de RNAm de HK-1, HK-2 e PFK (isoformas M, L e C) transcritas em coração de camundongos diabéticos tratados com 250 mg/Kg de metformina. Os painéis A e B mostram um gel de agarose 1% representativo e o painel C mostra a quantificação de pelo menos 3 diferentes experimentos. Os níveis de RNAm de GAPDH foram utilizados como controle de carregamento. As bandas correspondem a fragmentos de 361 pb (HK-1), 451 pb (HK-2), 574 bp (GAPDH), 741 pb (PFK-M), 606 pb (PFK-C) e 533 pb (PFK-L). Os demais procedimentos experimentais estão descritos em Material e Métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.2.4. Efeitos da metformina sobre o perfil de fosforilação da PFK de coração de camundongos diabéticos

A fosforilação da PFK pode aumentar a afinidade de ligação da enzima com a F-actina e, conseqüentemente, aumentar a sua atividade (COELHO et al., 2007; LUTHER e LEE, 1986). Com o interesse em compreender como o tratamento com metformina afeta os níveis de fosforilação dos resíduos de aminoácidos da PFK, esta enzima foi imunoprecipitada e marcada com anticorpos primários anti-fosfoserina, anti-fosfotreonina ou anti-fosfotirosina. A figura 19A mostra um resultado representativo de um *western blotting* e a figura 19B mostra a quantificação de três experimentos independentes.

Os resultados mostram que há um aumento nos níveis de fosforilação em resíduos de serina da PFK em camundongos diabéticos, quando comparados aos controles (Fig. 19). Porém, o tratamento dos camundongos diabéticos com metformina reverte esses elevados níveis de fosforilação. Os níveis de fosforilação de treonina e tirosina não são modificados pela indução do DM ou pelo tratamento com metformina. Este aumento na fosforilação em resíduos de serina pode estar correlacionado com a redistribuição subcelular e com a diminuída atividade enzimática. Neste sentido, a diferença no perfil de fosforilação da PFK de coração em relação a músculo esquelético, fígado e tecido adiposo sugere um possível padrão de regulação específico da enzima de acordo com cada tecido. Porém, apesar do mecanismo de regulação da enzima ser diferente entre os tecidos, em ambas as situações a metformina promove a redistribuição da atividade enzimática no sentido de reverter os efeitos do DM1.

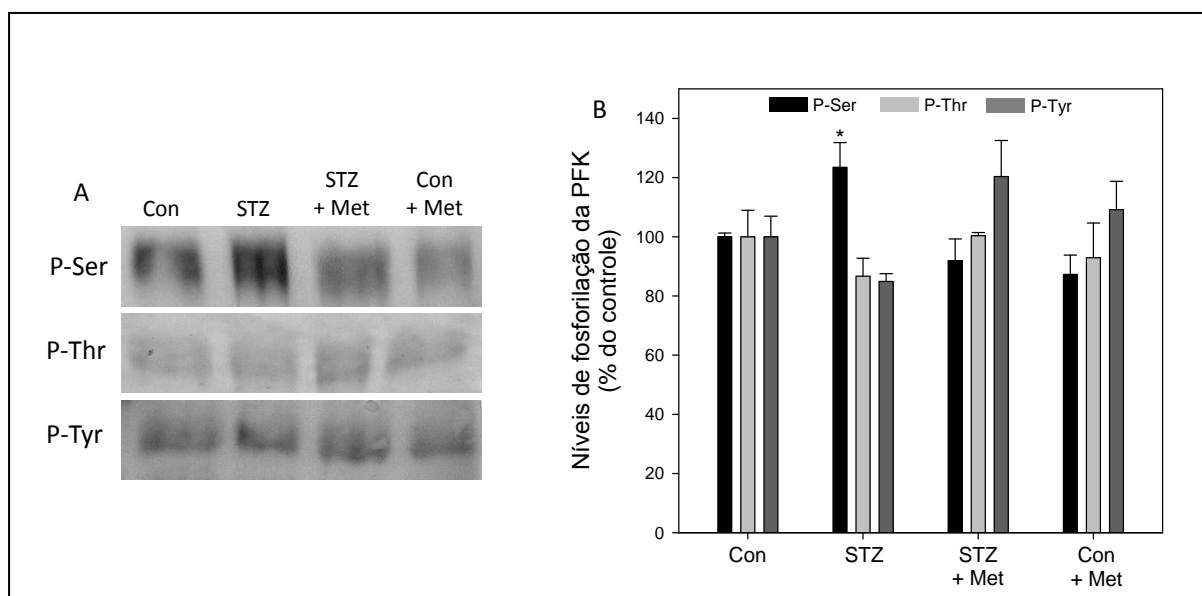


Figura 19. Perfil de fosforilação da PFK de coração de camundongos diabéticos tratados com 250 mg/Kg de metformina. O painel A mostra um *western blotting* representativo e o painel B mostra a quantificação de três diferentes experimentos (n= 3). A PFK dos corações dos camundongos foi imunoprecipitada com um anticorpo anti-PFK (IP:PFK) e marcada com um anticorpo antifosfoserina (P-Ser), antifosfotreonina (P-Thr) ou antifosfotirosina (P-Tyr). A quantidade de PFK total foi utilizada como controle de carregamento. Os demais procedimentos experimentais estão descritos em Material e Métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

4.2.5. Efeitos da metformina sobre os níveis de ATP de coração de camundongos diabéticos

Por último, buscamos investigar os efeitos da metformina sobre os níveis de ATP nos corações dos camundongos diabéticos. Surpreendentemente, a figura 20 mostra que a concentração de ATP está aumentada nos corações dos camundongos diabéticos, quando comparados ao grupo controle. Por outro lado, o tratamento com metformina é capaz de reverter parcialmente esse aumento da concentração, sem apresentar efeitos sobre os corações dos camundongos controles. Esse resultado da concentração de ATP pode estar relacionado ao aumento do metabolismo oxidativo, podendo contribuir para diminuição do fluxo glicolítico em corações de pacientes diabéticos, uma vez que ele é um potente inibidor da PFK (LEITE et al., 2007; ZANCAN et al., 2008).

Em conjunto, os resultados sugerem que a regulação positiva das enzimas glicolíticas no coração de camundongos diabéticos tratados com metformina pode estar correlacionada com a proteção cardíaca causada por este fármaco.

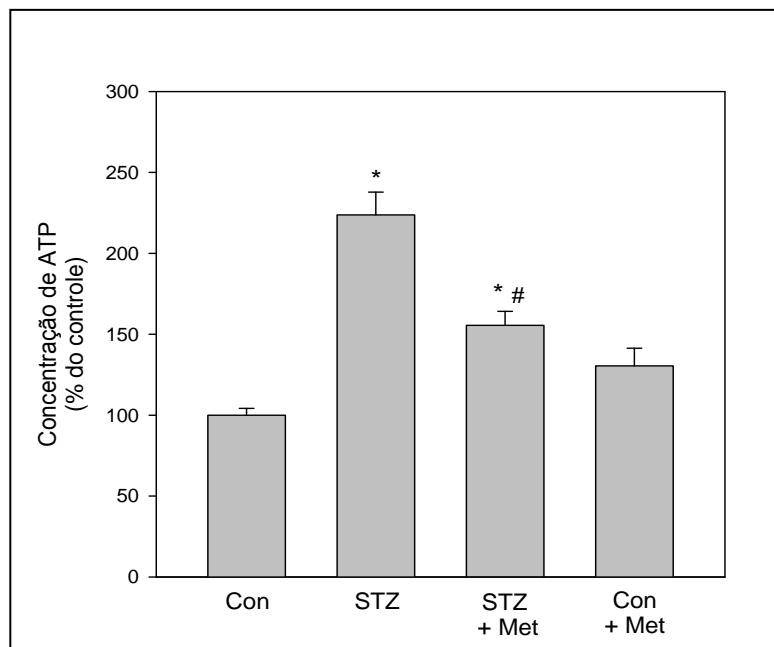


Figura 20. Concentração de ATP em coração de camundongos diabéticos tratados com 250 mg/Kg de metformina. A concentração de ATP foi determinada utilizando-se o kit *ATPlite* kit (PerkinElmer). Os valores representam a média \pm erro padrão (n= 3-4). * $p < 0,05$ comparado ao grupo controle; # $p < 0,05$ comparado ao grupo diabético. Os demais procedimentos experimentais estão descritos em Material e Métodos. Con: controle; STZ: diabético; STZ + Met: diabético tratado com metformina; Con + Met: controle tratado com metformina.

RESULTADOS

4.3. PARTE 3

4.3.1. Efeitos da metformina sobre diferentes parâmetros metabólicos de cultura de células C2C12

Procurando investigar se os efeitos da metformina ocorrem ao nível celular ou por meio de uma resposta hormonal, alguns parâmetros metabólicos foram analisados em cultura de células de mioblasto (C2C12) tratadas com metformina. Para a realização desses experimentos, as células foram pré-incubadas na ausência ou na presença de 100 ou 1000 nM de insulina por 24 horas. Estas concentrações de insulina foram usadas para alcançar a ação da insulina previamente estabelecidas como máxima (100 nM) ou para induzir resistência à insulina após a exposição a 1000 nM.

Os resultados mostram que a metformina induz a ativação da HK (Fig. 21A) e da PFK (Fig. 21B) de células C2C12 independentemente da concentração de insulina no meio de cultura. Ambas as enzimas são ativadas por 100 nM ou 1000 nM de insulina. Além disto, a presença de insulina apresenta um efeito adicional sobre a atividade da HK, mas não da PFK. A metformina também aumenta o consumo de glicose (Fig. 21C), mas não altera a produção de lactato (Fig. 21D). A captação de glicose é aumentada na presença de insulina (100 nM), porém na maior concentração não há diferença na captação, quando comparado ao controle sem o hormônio, sugerindo assim que as células tenham ficado resistentes a ação da insulina. Além disto, a insulina diminui a produção de lactato. Por outro lado, a metformina aumenta a redução de MTT sozinha ou na presença de 100 nM de insulina (Fig. 21E). Adicionalmente, o fármaco reverte à inibição causada neste parâmetro por 1000 nM de insulina. A figura 21F mostra que a metformina ou 100 nM de insulina aumentam a concentração intracelular de ATP. Além disto, a metformina se contrapõe a diminuição da concentração de ATP causada por 1000 nM de insulina.

Em conjunto, esses resultados demonstram que a metformina é capaz de estimular as enzimas HK e PFK e aumentar o consumo de glicose de maneira independente da sinalização insulinêmica. Ainda, apesar da metformina não alterar a produção de lactato, este fármaco aumenta a redução de MTT e a produção de ATP, sugerindo um aumento do metabolismo oxidativo nestas células.

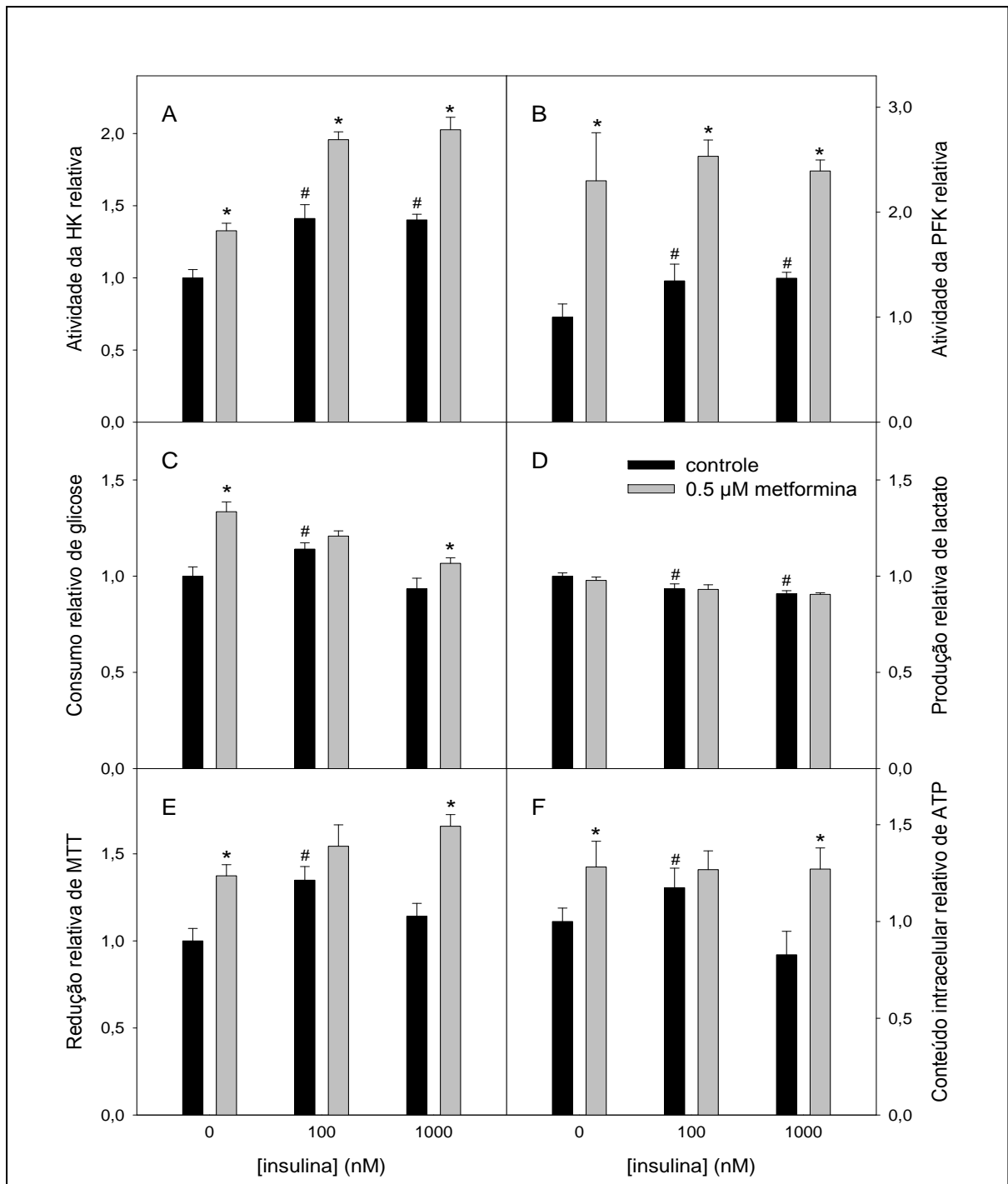


Figura 21. Efeitos metabólicos do tratamento de células C2C12 com metformina. As células foram tratadas como descrito em Material e Métodos. Barras pretas representam controles e barras cinza representam 0,5 μM de metformina. As células foram tratadas durante 24 horas na ausência ou na presença de metformina, 100 ou 1000 nM de insulina. A: atividade relativa da hexocinase. B: atividade relativa da fosfofrutocinase. C: consumo relativo de glicose. D: produção relativa de lactato. E: redução relativa de MTT. F: Concentração intracelular relativa de ATP. Os valores representam a média ± erro padrão (n= 8). * $p < 0,05$ comparado com os seus respectivos controles na ausência de insulina. # $p < 0,05$ comparado com os controles na ausência de metformina e insulina. Os demais procedimentos experimentais estão descritos em Material e Métodos.

4.3.2. Envolvimento das vias de sinalização mediadas por PI3K e por AMPK nos efeitos da metformina sobre a atividade da HK e da PFK

Procurando investigar quais são as possíveis vias de sinalização envolvidas nos efeitos da metformina em culturas de células C2C12, a wortimanina e o composto C foram utilizados, como descritos em Material e Métodos. Uma vez que a metformina mimetiza muitos dos efeitos da insulina, uma via dependente (wortimanina, Wm: inibidor de PI3K) e uma via independente (Composto C, CC: inibidor de AMPK) deste hormônio foram testadas sobre a atividade da HK e da PFK. Os resultados demonstram que tanto a metformina quanto a insulina aumentam a atividade da HK (Fig. 22A) e da PFK (Fig. 22B). Por outro lado, o composto C é capaz de antagonizar o efeito do fármaco, enquanto que a wortimanina é capaz de antagonizar o efeito do hormônio para ambas as enzimas. Assim, a ativação da HK e da PFK parece poder ocorrer por duas vias distintas, uma dependente e outra independente de insulina.

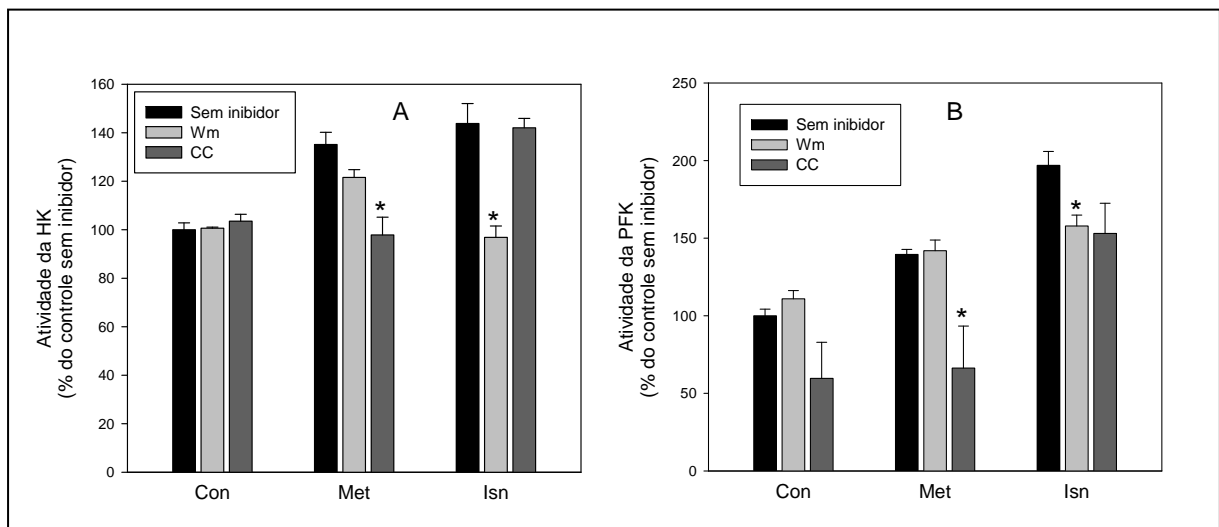


Figura 22. Atividade da hexocinase (A) e da fosfofrutocinase (B) de células C2C12 na presença de metformina (0,5 mM) ou insulina (100 nM). As Células foram tratadas com wortimanina (Wm) ou composto C (CC) antes do tratamento com metformina (Met) e insulina (Ins), como descrito em Material e Métodos A: atividade relativa ao controle (sem inibidor) da HK. B: atividade relativa ao controle (sem inibidor) da PFK. Os valores representam a média \pm erro padrão (n= 6). * $p < 0,05$ comparado aos seus respectivos controles na ausência de inibidor. Os demais procedimentos experimentais estão descritos em Material e Métodos.

4.3.3. Envolvimento das vias de sinalização mediadas por PI3K e por AMPK nos efeitos da metformina sobre a atividade da PK e da G6PDH

Uma vez que a metformina aumenta a atividade da HK e da PFK nas culturas de células, testamos se este fármaco também é capaz de modular outras enzimas importantes na regulação da etapa final da glicólise, piruvato cinase (PK), e da primeira reação da via das pentoses fosfato, glicose-6-fosfato desidrogenase (G6PDH). Como mostrado na figura 23, a metformina não estimula a atividade da PK (Fig. 23A) e nem da G6PDH (Fig. 23B). No entanto, a insulina aumenta a atividade da PK, sendo este efeito antagonizado apenas por wortimanina. Interessantemente, o composto C inibe a atividade da G6PDH tanto do controle quanto das condições estimuladas com metformina e insulina (Fig. 23B). Em geral, esses resultados sugerem que a metformina pode ter um efeito específico sobre as enzimas HK e PFK, uma vez que a PK e a G6PDH não são moduladas por este fármaco.

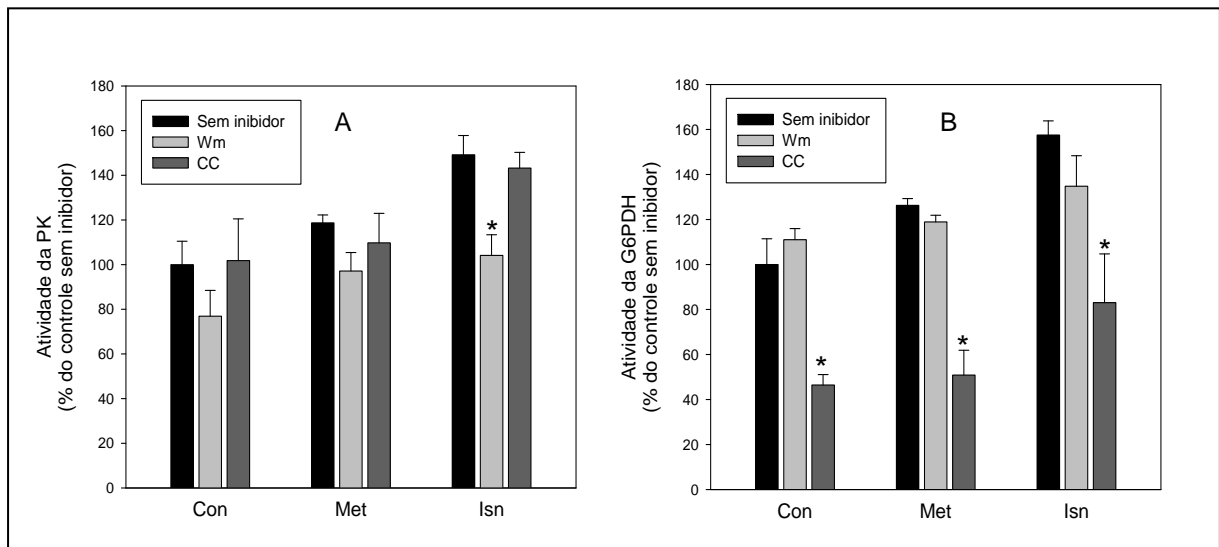


Figura 23. Atividade da piruvato cinase (A) e da glicose-6-fosfato desidrogenase (B) de células C2C12 na presença de metformina (0,5 μ M) ou insulina (100 nM). As Células foram tratadas com wortimanina (Wm) ou composto C (CC) antes do tratamento com metformina (Met) e insulina (Ins), como descrito em Material e Métodos. A: atividade relativa ao controle (sem inibidor) da PK. B: atividade relativa ao controle (sem inibidor) da G6PDH. Os valores representam a média \pm erro padrão (n= 3). * $p < 0,05$ comparado aos seus respectivos controles (na ausência de inibidor). Os demais procedimentos experimentais estão descritos em Material e Métodos.

4.3.4. Envolvimento das vias de sinalização mediadas por PI3K e por AMPK nos efeitos da metformina sobre a captação de glicose e produção de lactato em células C2C12

Por último, os efeitos dos antagonistas de PI3K e AMPK foram testados sobre importantes parâmetros metabólicos das células C2C12 tratadas com metformina ou insulina, como descrito em Material e Métodos. Como mostrado na figura 24A, a metformina e a insulina aumentam a captação de glicose das células. No entanto, curiosamente, tanto a wortimanina quanto o composto C impedem o efeito estimulatório do hormônio e do fármaco, respectivamente. Com o objetivo de saber se o aumento na captação de glicose leva a alterações na produção de lactato, este metabólito foi medido nas mesmas condições da figura A. A metformina não modula a produção de lactato pelas células (Fig. 24B). Por outro lado, a insulina diminui esta produção, tendo seu efeito antagonizado pela ação da wortimanina. Em conjunto, os resultados sugerem que o aumento na captação de glicose induzida por metformina pode ser devido ao estímulo das enzimas HK e PFK, sendo esses efeitos mediados por vias dependentes e independentes de insulina.

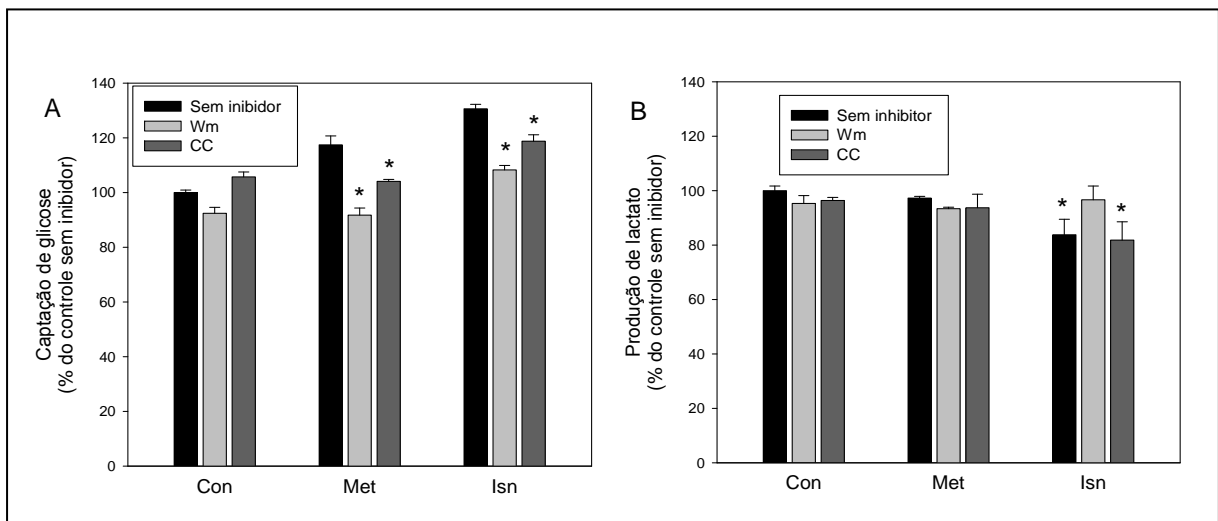


Figura 24. Parâmetros metabólicos de células C2C12 na presença de metformina (0,5 mM) ou insulina (100 nM). As Células foram tratadas com wortimanina (Wm) ou composto C (CC) antes do tratamento com metformina (Met) e insulina (Ins), como descrito em Material e Métodos. A: captação relativa ao controle (sem inibidor) de glicose. B: produção relativa ao controle (sem inibidor) de lactato. * $p < 0,05$ comparado aos seus respectivos controles (na ausência de inibidor).

5.0. DISCUSSÃO

A metformina é uma biguanida utilizada para o tratamento clínico do DM2 há muitos anos. Embora este fármaco esteja disponível para uso desde a década de 50, os mecanismos pelos quais a metformina diminui a glicemia em modelos animais de DM ainda permanecem controversos. Sabe-se que a metformina é capaz de diminuir a gliconeogênese e a glicogenólise, além de aumentar a captação de glicose pelos tecidos periféricos (CUSI e DEFRONZO, 1998; WIERNSPERGER e BAILEY, 1999; HUNDAL et al., 2000). Assim, o objetivo geral deste trabalho foi estudar o papel da metformina na regulação da atividade de enzimas glicolíticas dos tecidos muscular esquelético, hepático, adiposo epididimal e muscular cardíaco de camundongos com DM induzido por estreptozotocina (STZ).

Um dos modelos mais utilizados *in vivo* para o estudo do DM, com deficiência de insulina, é o de roedores tratados com STZ (MARLES e FARNSWORTH, 1995). A STZ estimula a produção de radicais livres, o que leva à destruição e disfunção das células β das ilhotas de Langerhans do pâncreas causando deficiência na produção de insulina e, conseqüentemente, o desenvolvimento de DM1 nestes animais (SZKUDELSKI, 2001; AKBARZADEH et al., 2007). De acordo com a literatura, camundongos que desenvolvem DM1 apresentam perda de peso, alta concentração sanguínea de glicose e baixa concentração plasmática de insulina e lactato (KAKEMI et al., 1983; TANAKA et al., 1999). Com o objetivo de caracterizar os camundongos diabéticos utilizados neste estudo e investigar os efeitos da metformina sobre os parâmetros citados, os animais foram divididos em quatro grupos (Con; STZ; STZ + Met; Con + Met) e tratados com diferentes doses de metformina. O tratamento dos camundongos diabéticos com metformina diminui a glicemia, reverte à baixa lactacidemia, diminui os níveis de triglicerídeos e de colesterol total, bem como a atividade de AST e ALT detectadas no plasma.

Apesar do tratamento com metformina já ter sido associado com a perda de peso de pacientes com DM2 (WONG e WONG, 2003), o tratamento por três dias com metformina não altera o baixo peso dos camundongos diabéticos e nem o peso do músculo esquelético, fígado, tecido adiposo epididimal e coração. Interessantemente, quando analisamos a relação do peso de cada tecido pelo peso corporal, somente o tecido adiposo epididimal apresenta queda significativa desta relação, sugerindo que este seja um dos tecidos mais afetados pelo quadro de DM (dados não mostrados) e que possa ser um dos mais importantes tecidos responsáveis pelo controle glicêmico. Além disto, este tratamento também não altera os níveis

elevados de hemoglobina glicada, sugerindo que o tempo de tratamento pode ter sido insuficiente para causar modificações crônicas.

De maneira similar a outros estudos (TANAKA et al., 1999; ASHOKKUMAR e PARI, 2005), a glicemia dos camundongos diabéticos induzidos com STZ é bastante elevada, sendo este efeito parcialmente revertido pelo tratamento com metformina. Contudo, este efeito parece ser independente da concentração de insulina, uma vez que a metformina não altera este parâmetro. Estudos em humanos também mostram que a metformina não é capaz de alterar as concentrações plasmáticas de insulina (DEFRONZO e GOODMAN, 1995). Os camundongos controles tratados com metformina não têm sua glicemia alterada, efeito já reportado por outros autores (EWIS e ABDEL-RAHMAN, 1995) e que pode estar de acordo com o fato da metformina não alterar a insulinemia. Desta forma, nossos resultados reforçam a hipótese de que a metformina possa ter um efeito hipoglicemiante por um mecanismo independente da insulina (FULGENCIO et al., 2001).

A queda da glicemia no grupo STZ + Metf está correlacionada com o aumento da sua lactacidemia. Há muitos anos já se sabe sobre a capacidade das biguanidas em elevar a concentração de lactato sanguíneo, o que levou muitos autores a associarem o uso da metformina com acidose láctica (DEFRONZO e GOODMAN, 1995; MISBIN et al., 1998). Este é um dos motivos que levou a fenformina e a butformina a serem retiradas do uso clínico na década de 70. Neste sentido, nosso trabalho mostra que não há hiperlactacidemia frente à administração de metformina. Estas observações nos levam a descartar uma possível ação tóxica desencadeada pelo tratamento dos camundongos com a dose de 250 mg/Kg de metformina. Além disto, a metformina diminui significativamente a atividade tanto da AST quanto da ALT, apesar das suas atividades ainda permanecerem maiores que as atividades encontradas nos animais controles. O efeito diabetogênico da estreptozotocina resulta em aumento da atividade sérica da AST e da ALT, podendo estar este efeito relacionado ao aumento da gliconeogênese e da cetogênese (BROWNLEE, 2001). Uma possível explicação para os efeitos relatados pode ser o fato da ação da metformina ser diretamente proporcional à elevação da glicemia, justificando sua eficácia no tratamento do DM.

Com as doses clínicas usuais de metformina (500 mg - 2500 mg), a concentração plasmática máxima do fármaco fica próxima de 5 µg/mL. Apesar das concentrações plasmáticas de metformina não terem sido analisadas neste estudo, a dose de 250 mg/Kg, já utilizada em outros estudos com camundongos (ZOU et al., 2004; SHAW et al., 2005), parece ser similar as doses orais recomendadas para humanos (ALEISA et al., 2007).

A glicose é a maior fonte de energia para muitas células de vários tecidos de mamíferos. Os níveis deste substrato no sangue são mantidos cuidadosamente por tecidos independentes e dependentes de insulina. A menor utilização de glicose pelo fígado, músculo esquelético e tecido adiposo no DM tem um importante papel na elevação da glicemia sistêmica (SOCHOR et al., 1987; VALVERDE et al., 2005). Além desses tecidos, a disfunção do metabolismo glicolítico de coração também pode ter importantes implicações no aparecimento de patologias cardíacas, como infarto agudo do miocárdio, angina severa e insuficiência cardíaca congestiva (STANLEY et al., 1997; LOPASCHUK, 2002; GARCÍA et al., 2009).

Os efeitos positivos do tratamento de animais diabéticos com metformina sobre a HK-4 já foram demonstrados tanto nos níveis de RNAm (FULGENCIO et al., 2001) quanto na atividade enzimática (ZHANG, 2008). Cabe ressaltar que a expressão de HK-4 é totalmente dependente de insulina, estando assim diminuída em animais diabéticos (IYNEDJIAN et al., 1998). Este resultado está de acordo com os mais baixos níveis de HK-4 encontrados em fígados dos camundongos diabéticos. O tratamento com metformina não foi capaz de alterar esta condição. No entanto, os efeitos deste fármaco sobre a regulação das outras isoformas de HK e de PFK em outros tecidos importantes para a manutenção da euglicemia ainda não foram elucidados. Neste sentido, o tratamento de camundongos diabéticos por três dias com 250 mg/Kg de metformina (uma dose por dia) é capaz de recuperar completamente a atividade da HK e da PFK de músculo esquelético, tecido adiposo e coração, apresentando um efeito parcial sobre essas enzimas do fígado.

Uma das possíveis explicações para os efeitos da metformina sobre a atividade da HK e da PFK seria a modulação dos níveis dessas enzimas. De fato, a indução do DM é capaz de diminuir os níveis de HK-1 e HK-2 no músculo esquelético e no tecido adiposo, sendo este efeito revertido pelo tratamento com metformina. Além disto, a análise dos níveis de RNAm dessas isoformas de HK mostra que somente os níveis de RNAm de HK-2 são acompanhados pelas mudanças nos níveis de proteína desta isoforma. Esses mesmos efeitos são observados para a PFK-L de fígado e de tecido adiposo. Interessantemente, os níveis de proteína da PFK e de RNAm da PFK-M, principal isoforma transcrita em músculo esquelético, não estão alterados neste tecido em nenhuma das condições. Em outro caso, o fato de haver diminuição nos níveis de proteína, mas não de RNAm para a HK-1 em músculo esquelético, fígado e tecido adiposo sugere um aumento na degradação da proteína. Por outro lado, o tratamento com metformina reverte os baixos níveis de proteína de PFK em fígado e tecido adiposo possivelmente por aumentar os níveis de RNAm desta enzima. Ademais, em coração o

tratamento não altera os níveis mais baixos de proteína da HK-2, apresentando efeitos positivos somente sobre os níveis de PFK. Apesar de ainda desconhecermos os diferentes papéis que cada isoforma das enzimas dos diferentes tecidos podem exercer sobre o controle glicêmico, em geral nossos resultados demonstram que o DM e/ou tratamento com metformina podem alterar de maneira tecido-específica os níveis de proteína e RNAm das enzimas. Interessantemente, o efeito da insulina sobre as diferentes isoformas de PFK de vários tecidos ainda é desconhecido.

A pré-incubação do músculo esquelético com insulina faz com que a atividade da PFK seja similar entre os diferentes grupos estudados. Desta forma, este resultado parece corroborar com o fato de não haver alterações nos níveis de expressão da enzima. A partir desses resultados, levantamos a hipótese de que a enzima poderia estar sofrendo outro tipo de regulação (que não fosse por expressão) que pudesse explicar sua baixa atividade neste tecido dos camundongos diabéticos. No entanto, a reversão da atividade enzimática da HK e da PFK encontrada em todos os tecidos estudados parece estar mais correlacionada às alterações a nível transcricional e, conseqüentemente, nos níveis dessas proteínas.

Em pacientes com DM1 e DM2 não são encontradas mudanças na expressão de RNAm da PFK de músculo esquelético (VESTERGAARD, 1993). Em outro trabalho, foi demonstrado que a expressão de PFK-M está reduzida em músculo cardíaco e esquelético de camundongos com DM1, indicando que a transcrição pode ser afetada pela doença (NAKAJIMA et al., 1994). O fato de não termos encontrado alterações nos níveis de PFK-M em músculo esquelético pode estar correlacionado com o tempo de indução do DM. Por outro lado, corroborando com o trabalho de Nakajima et al. (1994), nós também demonstramos menor expressão de PFK nos corações dos camundongos diabéticos, sendo este efeito revertido pelo tratamento com metformina. Uma vez que o tecido cardíaco possui dependência do metabolismo glicolítico (STANLEY et al., 1997), a deficiência de PFK-M deste tecido pode causar alta mortalidade devido às alterações metabólicas causadas pelo bloqueio da glicólise, incluindo hipertrofia cardíaca (GARCÍA et al., 2009).

O importante papel da PFK-M também está demonstrado em outros tecidos, como o tecido adiposo. Apesar das três isoformas serem transcritas neste tecido, Getty-Kaushik et al. (2010) demonstraram que camundongos nocautes para PFK-M deste tecido apresentam menor lipogênese e, conseqüentemente, menor massa adiposa e peso corporal. Por outro lado, sabe-se que em quadros patológicos, como o câncer e infecções virais (EL-BACHA et al., 2003; MEIRA et al., 2005; SPITZ et al., 2009), ou outras situações de alta demanda energética, como atividade física (VESTERGAARD, 1993), a atividade e a expressão de PFK podem ser

moduladas no sentido de suportar a maior demanda energética e gerar potencial redutor para os processos de síntese. Assim, a alteração de algumas isoformas específicas das enzimas causadas pelo tratamento com metformina pode estar sendo responsável por aumentar a glicólise, diminuir a glicemia e, possivelmente, conferir efeitos cardioprotetores aos diabéticos.

A glicólise é extremamente estruturada e suas enzimas podem interagir com diversos elementos celulares, como mitocôndrias e F-actina. A HK quando associada à mitocôndria possui maior atividade que a enzima na sua forma solúvel devido ao aumento de afinidade da enzima por glicose (WILSON, 2003). Vários trabalhos já demonstraram que o DM é capaz de reduzir a fração de HK ligada à mitocôndria e aumentar a atividade da enzima na fração solúvel em diversos tecidos (WALTERS e MCLEAN, 1968; ANDERSON e ZAKIM, 1970; CHEN-ZION et al., 1992b; LIVNAT et al., 1993). Em todos os tecidos estudados neste trabalho, a indução do DM1 leva a um desligamento da atividade da HK da fração mitocondrial. Por outro lado, a metformina reverte este efeito em coração, fígado e tecido adiposo, mas não em músculo esquelético. A menor atividade da HK total está correlacionada com os menores níveis de G6P em músculo esquelético, coração e tecido adiposo epididimal, no entanto não há diferença entre os grupos no tecido hepático. Neste tecido, o aumento da gliconeogênese pode sustentar os mesmos níveis de G6P entre animais saudáveis e diabéticos. Além disto, o aumento da concentração de G6P nos tecidos causada pelo tratamento com metformina, com exceção do fígado, pode não ser suficiente para desligar a HK da mitocôndria, uma vez que os níveis dele ainda não ultrapassam as concentrações dos grupos controles. Assim, outros mecanismos parecem estar envolvidos nas alterações subcelulares da HK.

Um dos mecanismos que pode levar a associação da HK-2 à mitocôndria é a sua fosforilação por Akt/PKB, através do estímulo insulínico, o que pode conferir proteção a este tecido (ZUURBIER et al., 2005; MIYAMOTO et al., 2008). A HK-1 também pode se associar a membrana mitocondrial, no entanto a HK-2 parece se ligar com maior afinidade, o que sugere um diferente papel das isoformas em resposta a um estímulo insulínico. Vale ressaltar que ambas as isoformas possuem uma região consenso para a fosforilação por Akt (SOUTHWORTH et al., 2007). No entanto, neste estudo, a possível fosforilação das diferentes isoformas de HK e as suas implicações para as alterações no catabolismo de glicose não foram investigadas.

Neste mesmo sentido, dentre todas as enzimas da via glicolítica que se associam a elementos do citoesqueleto, a PFK é a enzima que se liga com mais afinidade aos filamentos

de actina (ROBERTS e SOMERO, 1987; CLARKE e MORTON, 1982). Já foi demonstrado que a insulina é capaz de levar à fosforilação da PFK e que este fato aumenta a afinidade da enzima pela F-actina, o que poderia consequentemente aumentar o fluxo glicolítico (SALE, 1987; CHEN-ZION et al., 1992a; SILVA et al., 2004). Por outro lado, a menor atividade da PFK ligada ao citoesqueleto de músculo esquelético pode implicar na diminuição da glicólise desses tecidos (CHEN-ZION et al., 1994). Diante desses trabalhos, podemos pressupor que a distribuição celular da PFK está bem caracterizada em músculo esquelético. No entanto, os efeitos do tratamento de camundongos diabéticos com metformina sobre a distribuição celular da PFK nos diversos tecidos utilizados neste estudo ainda não foram descritas.

Nossos resultados mostram que o tratamento dos camundongos diabéticos com 250 mg/Kg de metformina altera a localização celular da atividade da HK e da PFK para frações ricas em mitocôndrias (P1) e para frações ricas em citoesqueleto (P2), respectivamente, em detrimento das frações solúveis (S1: HK e S2: PFK), em músculo esquelético e coração. Curiosamente, a atividade da PFK de músculo esquelético de camundongos controles tratados com metformina encontra-se majoritariamente na fração P2. Não sendo estes camundongos diabéticos, e a metformina tendo um efeito sensibilizador das ações da insulina (STRACK, 2008), o aumento da associação da PFK com F-actina pode estar ocorrendo devido à ação sinérgica entre a insulina e a metformina. Assim, o tratamento com metformina é capaz de alterar a localização celular da atividade da PFK para frações ricas em F-actina. Esta modulação em músculo esquelético de camundongos diabéticos pode ser de grande relevância, uma vez que este tecido desempenha um importante papel na captação de glicose no estado pós-prandial (NEWSHOLME e DIMITRIADIS, 2001), o que poderia ser importante para a promoção de ações hipoglicemiantes.

Ao contrário do músculo esquelético, a indução do DM é capaz de aumentar a atividade da PFK na fração P2 de tecido adiposo epididimal, sendo que o tratamento com metformina não altera a distribuição celular da enzima neste tecido. Este resultado pode estar correlacionado com os baixos níveis destes filamentos de actina no tecido adiposo quando comparado com o músculo esquelético. Os adipócitos possuem uma rede de microtúbulos formando o seu citoesqueleto de maneira similar ao da actina filamentosa em músculo esquelético (OATEY et al., 1997), porém, os microtúbulos são inibidores da atividade da PFK (VÉRTESSY et al., 1996). Adicionalmente, dímeros de tubulina não polimerizados podem permanecer no sobrenadante, inibindo a atividade da PFK na fração solúvel (VÉRTESSY et al., 1996).

Além disto, a actina no músculo esquelético adulto está presente na forma de alfa-actina, que é diferente das outras duas isoformas presentes em células não musculares, beta e gama-actina (GORDON et al., 1977). A actina em muitas células não musculares parece estar presente tanto na forma polimerizada quanto na forma não polimerizada (LINDBERG et al., 1979). Já foi sugerido que a actina filamentosa nesses tipos celulares poderia ter um importante papel no controle do metabolismo, devido ao controle de algumas enzimas (MASTERS, 1978). No entanto, as isoformas de PFK expressas em tecidos não musculares e a diferente organização do citoesqueleto dos adipócitos poderia ser responsável pelo diferente perfil de distribuição da atividade da PFK no tecido adiposo, quando comparado com músculo esquelético. Assim, em tecido adiposo a PFK pode estar se associando preferencialmente a microtúbulos (que precipitam juntamente com F-actina), sugerindo este fato como mais um possível fator responsável pela maior inibição da atividade da enzima.

No tecido hepático o perfil de distribuição celular da PFK foi diferente dos dois tecidos citados anteriormente, uma vez que a fração P1 teve sua atividade aumentada. Este resultado já foi reportado por outros trabalhos que trataram suas amostras com insulina (CHEN-ZION et al. 1992a; ASHKENAZY-SHAHAR et al., 1998). A capacidade da PFK em se ligar a mitocôndria ou outras frações celulares que precipitariam na fração P1 ainda não foi descrita. O desenvolvimento do DM não altera a localização celular da atividade da PFK entre as frações S2 e P2 de fígado. Por outro lado, o tratamento dos animais diabéticos ou controles com metformina somente diminuiu a atividade da PFK na fração solúvel (S2). Em geral, nossos resultados demonstram que tanto o DM1 quanto o tratamento com metformina podem alterar a sublocalização da atividade da HK e da PFK, podendo ser este um dos mecanismos pelos quais a metformina é capaz de estimular a atividade das enzimas.

Em modelo animal de DM1 e DM2 que superexpressa a porção fosfatásica inativada da PFK-2, o aumento dos níveis de F2,6BP estão correlacionados com a diminuição da glicemia (WU et al., 2006). Além disso, já foi demonstrado que a AMPK pode: (1) fosforilar a PFK-2 de coração, aumentando a formação de F2,6BP e, conseqüentemente, ativar a PFK (MARSIN et al., 2000); (2) aumentar o translocamento de GLUT 4 para a membrana celular, promovendo aumento da captação de glicose por uma via independente da sinalização por insulina (RUSSELL et al., 2006). Os efeitos benéficos do tratamento com metformina já foram correlacionados positivamente com a ativação da AMPK (ZHOU et al., 2001). Assim, a reversão da atividade enzimática da HK e da PFK de diferentes tecidos de camundongos diabéticos pode estar correlacionada com a alteração de efetores alostéricos das enzimas ou com as alterações covalentes das enzimas causadas por proteínas cinases.

Argaud et al. (1993) demonstraram que o tratamento de hepatócitos isolados de ratos com metformina é capaz de diminuir a concentração de ATP, um conhecido inibidor alostérico da PFK. Nossos resultados, apesar de surpreendentes, demonstram um aumento da concentração de ATP nos corações dos camundongos diabéticos, sendo este efeito parcialmente revertido pelo tratamento com metformina. Uma das possíveis causas da diminuição da concentração de ATP causada pelo tratamento com metformina pode ser devido à capacidade do fármaco em inibir o complexo I da cadeia de transporte de elétrons (EVANS et al., 1983). Este efeito poderia levar ao aumento da atividade da PFK, com consequente consumo de G6P. Além disto, Hosey et al., (1980) mostraram que a PFK isolada de fígado de camundongos geneticamente diabéticos (C57BL/KsJ-db) é mais suscetível à inibição por ATP que a enzima de um camundongo saudável. Por fim, a diminuição dos níveis deste composto em hepatócitos tratados com metformina (GUIGAS et al., 2006) poderia contribuir para o aumento da atividade da PFK, consumo de G6P e ativação da HK.

O aumento da oxidação de ácidos graxos visto em diabéticos pode inibir enzimas-chave da glicólise por acumular acetil-CoA e citrato (KELLEY e MANDARINO, 2000). Ácidos graxos livres inibem IRS associado à atividade da PI3K e, assim, podem atenuar o transporte de glicose através da membrana celular (SHULMAN, 1999). Além disto, recentemente foi demonstrado que a PFK pode ser inibida por acil-CoA de cadeia longa (JENKINS et al., 2011). No entanto, a metformina é capaz de reduzir a oxidação de ácidos graxos (WIERNSPERGER e BAILEY, 1999; HUNDAL et al., 2000), fato que também poderia contribuir para a reversão da atividade das enzimas HK e PFK.

Para entender melhor a sinalização que modula a distribuição intracelular da PFK no DM1 e a os efeitos da metformina, os níveis de fosforilação da enzima foram analisados nos diversos tecidos e condições. O tratamento dos camundongos diabéticos reverte os baixos níveis de fosforilação em resíduos de serina da PFK em músculo esquelético. Por outro lado, em coração este tratamento é capaz de reverter os elevados níveis de fosforilação em serina da enzima. Por outro lado, sabe-se que a fosforilação da PFK de músculo esquelético pode aumentar a afinidade da enzima para a f-actina e, conseqüentemente, aumentar a sua atividade (LUTHER e LEE, 1986; COELHO et al., 2007). Mas, apesar do mecanismo de regulação da enzima ser diferente entre os tecidos, em ambas as situações a metformina promove a redistribuição da atividade enzimática no sentido de reverter os efeitos do DM1.

Já foi demonstrado em coração de ovelha que a Ca^{2+} -calmodulina cinase (CaMK), bem como a PKA, pode fosforilar a PFK em resíduos de serina, o que resulta em um aumento da sensibilidade da enzima a inibição por ATP e diminui a afinidade por F6P

(MAHRENHOLZ et al., 1991). Além disto, ainda não está claro se o aumento da fosforilação da PFK em resíduos de serina poderia levar ao aumento da degradação da enzima, o que poderia explicar os menores níveis de PFK nos corações dos camundongos diabéticos. Por fim, as proteínas cinases envolvidas nestes efeitos ainda não foram estabelecidas.

Procurando investigar se os efeitos da metformina ocorrem ao nível celular ou por meio de uma resposta hormonal, alguns parâmetros metabólicos foram analisados em cultura de células de mioblasto (C2C12) tratadas com metformina. Nestas células tanto a metformina quanto a insulina aumentam a atividade das enzimas glicolíticas (HK e PFK), o consumo de glicose, a redução de MTT e a concentração intracelular de ATP. Porém, apesar da metformina não alterar a produção de lactato, a insulina reduz a produção deste metabólito. Assim, esses resultados sugerem que a metformina possa estimular mais o metabolismo oxidativo da glicose do que o fermentativo, uma vez que a redução de MTT é um indicativo de atividade mitocondrial (HUET et al., 1992).

O aumento da atividade tanto da HK quanto da PFK causada pelo estímulo com metformina parece ser dependente da via de sinalização envolvendo a AMPK, uma vez que o composto C é capaz de bloquear este estímulo. Além disto, esta via parece envolver especificamente a HK e a PFK, uma vez que a PK não foi modulada nem pela metformina nem pelo composto C. Corroborando com a diminuição da glicemia, este inibidor também é capaz de impedir o aumento na captação de glicose estimulado por metformina nas células, sem alterar a produção de lactato, sugerindo, desta forma, que a AMPK possa estar envolvida na ativação dessas enzimas nos diferentes tecidos dos animais.

Em conjunto, nossos resultados sugerem que o aumento da atividade de enzimas glicolíticas, como HK e PFK, estimuladas por metformina em um modelo de DM1, poderia contribuir para a cardioproteção e redução da glicemia sistêmica.

6.0. CONCLUSÕES

- ✓ O tratamento de camundongos diabéticos com 250 mg/Kg de metformina, uma dose por dia, durante três dias consecutivos, reduz a glicemia e reverte à baixa lactacidemia, sem alterar o peso corporal dos camundongos; esses efeitos da metformina parecem ser independentes da ação da insulina;
- ✓ As atividades da HK e da PFK são significativamente menores em músculo esquelético e cardíaco, fígado e tecido adiposo epididimal dos camundongos diabéticos. No entanto, o tratamento com 250 mg/kg de peso corporal de metformina é capaz de reverter completamente esta inibição no músculo esquelético, no coração e no tecido adiposo. No fígado, as atividades dessas enzimas são parcialmente revertidas com o tratamento descrito;
- ✓ A pré-incubação dos músculos esqueléticos das quatro condições com insulina aumenta a atividade da HK e da PFK. Este aumento também ocorre para a HK de tecido adiposo dos grupos STZ, STZ + Met e Con + Met. No entanto, a atividade da HK não é alterada pela presença do hormônio em fígado. Neste tecido, e em tecido adiposo epididimal, esta incubação é capaz de aumentar a atividade da PFK dos quatro grupos;
- ✓ Há menor nível de proteína de HK-1 em músculo esquelético, fígado e tecido adiposo de camundongos diabéticos. No entanto, o tratamento com metformina é capaz de aumentar estes níveis nos três tecidos. Este mesmo perfil ocorre para a HK-2 de músculo esquelético e tecido adiposo, sendo que o tratamento com metformina não aumenta os níveis desta isoforma em coração. Os níveis de PFK não são alterados em músculo esquelético, porém são menores em fígado, tecido adiposo e músculo cardíaco de camundongos diabéticos. Nesses três últimos tecidos, o tratamento com metformina é capaz de reverter os baixos níveis de PFK;
- ✓ No músculo esquelético não há alteração dos níveis de RNAm de HK-1, HK-2 e PFK-M. Os níveis de RNAm de HK-1 também são os mesmos entre os fígados dos quatro grupos estudados, enquanto que o tratamento com metformina reverte os baixos níveis de HK-4 e PFK-L neste tecido. Os níveis de RNAm de PFK-M, PFK-L e HK-1 não estão alterados em tecido adiposo epididimal. O tratamento com metformina não altera os baixos níveis de PFK-C encontrado neste tecido dos camundongos diabéticos, porém aumenta os níveis de HK-2. Nenhuma isoforma de PFK e HK testadas está alterada em músculo cardíaco de camundongos diabéticos e controles tratados ou não com metformina;
- ✓ A indução de DM aumenta a atividade da HK na fração solúvel em detrimento da fração mitocondrial. No entanto, a metformina reverte este efeito em fígado, tecido adiposo e músculo cardíaco, sem apresentar efeito em músculo esquelético;

- ✓ O tratamento com metformina reverte os baixos níveis de G6P em músculo esquelético e cardíaco e tecido adiposo. Por outro lado, a concentração de G6P é a mesma no fígado dos diferentes grupos;
- ✓ Além de estimular a reversão completa da atividade da PFK no músculo esquelético e cardíaco, nossos resultados sugerem que a metformina pode levar ao deslocamento da PFK da fração solúvel para a fração rica em f-actina, alterando a localização subcelular desta enzima nesses tecidos. O aumento da atividade da PFK ligada ao citoesqueleto celular ocorre apenas parcialmente em fígado e não ocorre em tecido adiposo epididimal. A atividade da HK de músculo cardíaco também aumenta na fração mitocondrial após o tratamento com metformina;
- ✓ O tratamento com metformina reverte os baixos níveis de fosforilação em resíduos de serina da PFK de músculo esquelético. Por outro lado, em músculo cardíaco, o tratamento reverte os elevados níveis de fosforilação da PFK em resíduos serina;
- ✓ A metformina não altera a atividade da HK nem da PFK purificadas em nenhuma das concentrações testadas. Este fármaco também não modifica o centro de massa do espectro de fluorescência intrínseca dessas enzimas;
- ✓ Os resultados mostram que a metformina aumenta a atividade da HK e da PFK, o conteúdo de ATP intracelular e a redução de MTT, sem alterar as concentrações de lactato, sugerindo aumento da glicólise e do metabolismo oxidativo em células C2C12.
- ✓ A ativação da HK e da PFK nesta cultura de células parece poder ocorrer por vias dependentes e independentes da sinalização de insulina.
- ✓ A metformina aumenta a captação de glicose nas células C2C12, sendo este efeito dependente da AMPK e de PI3K;
- ✓ Em conjunto, nossos resultados sugerem que o aumento da atividade de enzimas glicolíticas, como HK e PFK, estimuladas por metformina em um modelo de DM1, poderia contribuir para redução da glicemia sistêmica e, possivelmente, para a cardioproteção.

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8.0. APÊNDICES - Trabalhos publicados durante o doutorado

8.1. APÊNDICE A

Metformin reverses hexokinase and 6-phosphofructo-1-kinase inhibition in skeletal muscle, liver and adipose tissues from streptozotocin-induced diabetic mouse



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Metformin reverses hexokinase and 6-phosphofructo-1-kinase inhibition in skeletal muscle, liver and adipose tissues from streptozotocin-induced diabetic mouse

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ABSTRACT

The present work describes the effects of metformin on hexokinase (HK) and phosphofructokinase (PFK) activities and localization in different tissues from streptozotocin-induced diabetic mice. Diabetic mice present lower HK and PFK activities (50%) in skeletal muscle, liver and adipose tissue, as compared with control ($P < 0.05$). Treatment with 250 mg/kg metformin reverses this pattern of enzyme inhibition with concomitant reversal of hyperglycemia and hypolactacidemia. Furthermore, the treatment increases the cytoskeleton-associated PFK activity in skeletal muscle; this activity has been described as an important mechanism for the enzyme activation. This effect might be due to the increased phosphorylation of serine residues in the enzyme, a modification which has been described to increase the interaction of PFK with f-actin. The current work supports the hypothesis that metformin hypoglycemic effects involve the activation of glycolysis through its regulatory enzymes, which may be potential targets for the development of new hypoglycemic drugs.

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Introduction

Diabetes mellitus (DM)¹ is a life-threatening disease with a prevalence that is increasing worldwide [1]. Hyperglycemia is caused by abnormal regulation of glucose and impaired carbohydrate utilization, which results from a defective and/or deficient insulin secretory response. Hyperglycemia is the main cause of the development and progression of microvascular and macrovascular complications, suggesting that its control can prevent or reverse diabetic complications [1]. DM treatment mainly focuses on glycemia control and involves a controlled diet, regular exercise, insulin administration and/or hypoglycemic agents.

Metformin, a biguanide derivative (dimethylbiguanide), is the most common drug used for type II diabetes treatment [2]. Several studies have shown that metformin has insulin-sensitizing effects in the major tissues responsible for regulating the blood glucose concentration, including skeletal muscle, adipose tissue and liver

[3,4]. Metformin inhibits gluconeogenesis and stimulates glucose uptake by peripheral tissues, processes requiring the activation of AMPK [5]. In spite of stimulating basal glucose uptake, metformin does not augment insulin-stimulated glucose transport in C2C12 myoblasts [6,7]. In addition, it has been shown that metformin has hypoglycemic effects on streptozotocin (STZ)-induced diabetic rats, suggesting that these effects are not dependent on insulin action [8]. These findings support the increasing use of metformin for type 1 diabetic patients, providing an additional effect to insulin treatment [9].

Glycolysis is the main metabolic pathway that consumes glucose. Hexokinase (HK) and 6-phosphofructo-1-kinase (PFK) are key rate-limiting enzymes in glycolysis and are the most sensitive indicators of the glycolytic pathways in diabetic states [10]. HK plays an important role in maintenance of glucose homeostasis, while PFK represents a major control point in the metabolism of glucose. Regulation of these enzymes has been correlated with the regulation of complete glucose metabolism [11–13] and, thus, with glycemic control.

The aim of the present study is to determine whether metformin modulates key enzymes involved in glycolysis regulation in different tissues from STZ-induced diabetic mice. These findings will contribute to the understanding of the mechanisms of action of this drug and may shed light on novel targets for the development of new hypoglycemic drugs.

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¹ Abbreviations used: PFK, 6-phosphofructo-1-kinase, phosphofructokinase; DM, diabetes mellitus; HK, hexokinase; STZ, streptozotocin; F6P, fructose-6-phosphate; Met, metformin; i.p., intraperitoneal; TH, total homogenate.

Materials and methods

Materials

ATP, fructose-6-phosphate (F6P), purified HK, insulin, metformin and STZ were purchased from Sigma Chemical (St. Louis, MO, USA). Purified PFK was obtained from rabbit skeletal muscle according to [14] with modifications introduced by [15]. ^{32}P i was purchased from IPEN, Brazil. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared according to [16]. The C2C12 cell line was obtained from the Cell Bank of the Hospital Universitario Clementino Fraga Filho, UFRJ, Brazil, and maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, USA) supplemented with 10% (v/v) FBS (fetal bovine serum; Invitrogen, USA). Protein content measurements were performed according to [17]. All other chemicals were of the highest purity available.

Diabetes induction and treatment

Swiss male mice (8 weeks) were randomly divided in four groups: control (Control), diabetic (STZ), diabetic treated with metformin (STZ + Met) and control treated with metformin (Met). Mice were housed in a temperature-controlled room with a 12-h light-dark cycle and given free access to water and normal chows. Diabetes was induced by one intraperitoneal (i.p.) administration of STZ (200 mg/kg) dissolved in fresh 100 mM citrate buffer (pH 4.5). After 5 days, metformin treatment was started in mice whose glycemia was higher than 300 mg/dl. Other diabetic features, including polyuria, polydipsia and weight loss, were also observed. Mice were injected i.p. with metformin (50, 100 or 250 mg per kg body weight-mg/kg) in saline or only saline for 3 consecutive days, once a day. On the third day, 2 h after metformin administration, blood was drawn from the mice by tail snip, and glucose and lactate levels were measured using a glucometer (Accu-Chek[®] Active – Roche) and a lactometer (Accutrend[®] Lactate Analyzer – Roche), respectively. Then, mice were sacrificed by decapitation and the blood was used to measure serum insulin concentration (Rat/Mouse Insulin ELISA Kit, Millipore, MO, USA) in accordance with the manufacturer's instructions. Mouse skeletal muscle from back limbs, liver and epididymal adipose tissue were removed and immediately frozen in liquid nitrogen. The animal protocol was approved by the internal institutional animal care and use committee.

Preparation of soluble and bound PFK

Cytoskeleton-bound and soluble PFK were separated as described previously [18,19]. Tissues were homogenized in a potter-homogenizer in buffer containing 100 mM Tris-HCl, pH 7.4, 100 mM sucrose, 10 mM EDTA, 46 mM KCl, 20 mM β -mercaptoethanol and 1 mM sodium pyrophosphate. Homogenized tissues were centrifuged for 10 min at 100 g at 4 °C to separate cellular debris and non-digested tissues. The resultant supernatant, which we total homogenate (TH), was centrifuged for 30 min at 27,000g at 4 °C. The resulting low-speed pellet, P1, is a fraction enriched with mitochondria, lysosomes, and nuclear components. The low-speed supernatant, S1, containing most of cytoplasmic structures was centrifuged for 30 min at 120,000g at 4 °C. The resulting high-speed supernatant, S2, retained most of the soluble structures and the high-speed pellet, P2, contained a microsomal fraction and cytoskeleton components as described previously [18,19]. All fractions (TH, S1, P1, S2 and P2) were assayed for PFK activity and protein concentration. HK activity was also determined in HT from tissues.

Assay for HK and PFK activity

HK and PFK activities from tissue fractions and purified enzymes were measured as previously described [20] with modifications introduced by [12,13]. The enzymes' activities were determined in reaction media containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4 $\mu\text{Ci}/\mu\text{mol}$). For HK activity, 5 mM glucose was added to the basic medium while 1 mM F6P and 5 mM $(\text{NH}_4)_2\text{SO}_4$ were added to determine PFK activity. The reaction was started by the addition of tissue extracts or purified enzymes and the reaction was stopped by the addition of a suspension of activated charcoal in 0.1 M HCl and 0.5 M mannitol. After centrifugation (1500g at 4 °C for 10 min), the supernatant-containing labeled product was analyzed in a liquid-scintillation counter. Duplicates were performed for all assays and blanks were obtained in the absence of glucose or F6P, for HK and PFK activities, respectively and were subtracted from all measurements to discount the effect of ATP hydrolysis.

Intrinsic fluorescence measurements

Intrinsic fluorescence measurements were performed in medium containing 100 mM Tris-HCl (pH 7.4), 5 mM $(\text{NH}_4)_2\text{SO}_4$ and purified HK (5 $\mu\text{g}/\text{ml}$) or PFK (5 $\mu\text{g}/\text{ml}$) in the absence or presence of different concentrations of metformin (0, 1, 10, 100 or 1000 μM) using a Jasco Spectrofluorometer. Appropriate reference spectra were subtracted from the data to correct for background interference. The excitation wavelength was set at 280 nm, and fluorescence emission was recorded at 90° scanned from 300 to 400 nm according to [21].

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as previously described [22]. Briefly, mouse skeletal muscle, liver and epididymal adipose tissue homogenates from controls and diabetic mice treated with saline or 250 mg/kg of metformin were incubated for 4 h at 4 °C with anti-PFK (1:500)/protein A-agarose. The agarose beads were collected by centrifugation, washed three times with saline buffer and three times with 10% SDS, resuspended in 50 μl of sample buffer and boiled for 5 min. Samples were subjected to 8% SDS-PAGE, transferred to nitrocellulose membrane, blocked with Tween-TBS (20 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 0.1% Tween 20) containing 2% bovine serum albumin. The membrane was subsequently incubated with monoclonal mouse anti-phosphotyrosine (1:500; Sigma Chemicals Co.), monoclonal mouse anti-phosphoserine (1:500; Sigma Chemicals Co.) or anti-phosphothreonine (1:500; Sigma Chemicals Co.). The nitrocellulose membrane was washed five times with Tween-TBS, followed by a 1 h incubation with alkaline phosphatase-conjugated anti-mouse IgG (1:1000). Next, the membranes were stained with alkaline phosphatase staining substrate. Membranes were scanned and analyzed using ImageQuant software (Molecular Dynamics, USA). The same nitrocellulose membranes were probed with the antibody used for immunoprecipitation to ensure that equal amounts of protein were loaded in each lane.

C2C12 Cell culture

C2C12 myoblasts were cultured in DMEM supplemented with 10% FBS and antibiotics (Penicillin 100 IU/ml, Streptomycin 100 mg/ml) in 5% CO_2 at 37 °C until confluence. Then, the medium was removed and cells were seeded in 96 well plates in fresh medium in the absence or presence of 100 or 1000 nM insulin, with or without 0.5 mM metformin for 24 h. Controls without any treatment were performed. After this incubation, the media were re-

moved and used to evaluate glucose consumption and lactate production, while the remaining cells were used for cell viability evaluation through assays of the reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide to formazan (MTT assay) and ATP concentration. The MTT assay is also indicative of mitochondrial activity because the reduction occurs through the reductant potential of metabolizing mitochondria, as proposed previously [23]. Glucose consumption, lactate production and

MTT were assayed as described by [24]. ATP content was evaluated as described in [25].

Statistical analyses

Results are expressed as means \pm standard error of the mean (SEM). Analyses were performed using Sigma Plot software (v. 10.0, Systat Inc., CA, USA) integrated with SigmaStat (v. 3.2,

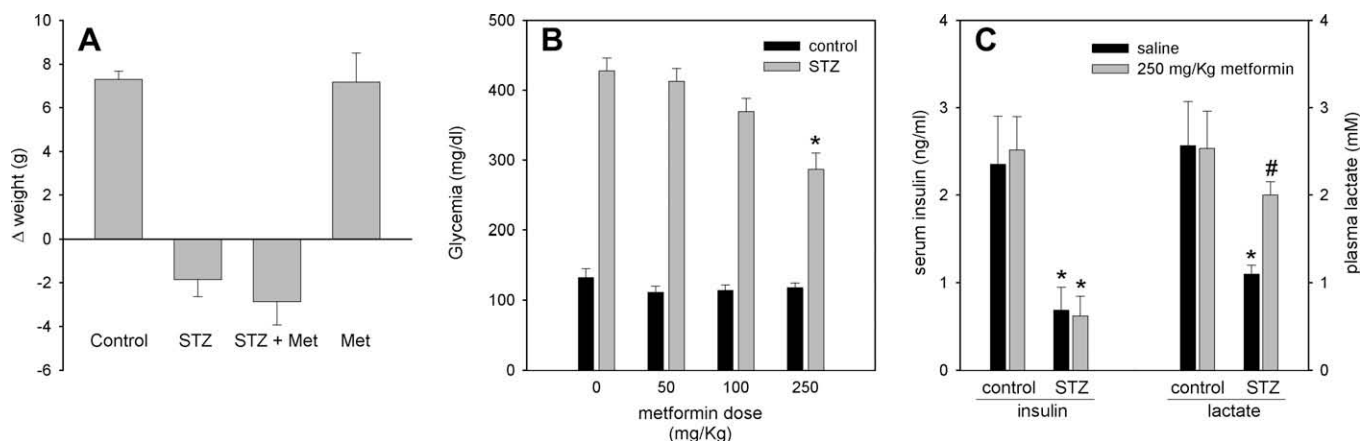


Fig. 1. Metabolic features of STZ-induced diabetic mice treated with metformin. Mice were injected intraperitoneally with either 250 mg metformin per Kg body weight (mg/kg) in saline or with saline alone once a day, for three consecutive days, as described in Materials and methods. (A) Body weight variation (since diabetes was induced) of control and STZ-induced diabetic mice treated with 250 mg/kg metformin. Data represent the mean \pm SEM for three mice from each group ($n = 3$). (B) Blood glucose concentration of control (black bars) and STZ-induced diabetic mice (light gray bars) treated with different metformin doses or saline. Data represent the mean \pm SEM for three mice from each group ($n = 3$). * $P < 0.05$ compared with control (Student's t -test). (C) Serum insulin concentration (left bars or scale) and blood lactate concentration (right bars or scale). Black bars represent mice treated just with saline and light gray bars represent mice treated with metformin 250 mg/Kg. Data represent the mean \pm SEM for three mice from each group ($n = 3$). * $P < 0.05$ compared with respectively control (Student's t -test). # $P < 0.05$ compared with untreated diabetic mice (Student's t -test).

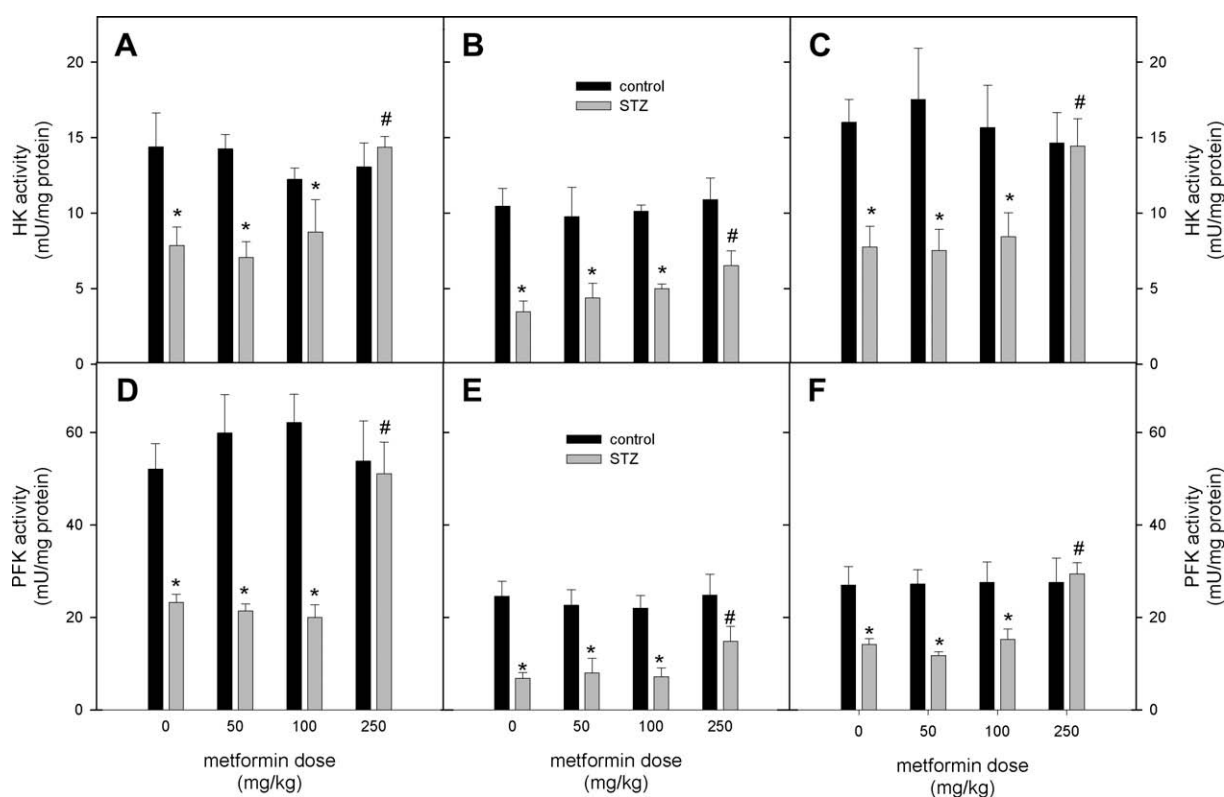


Fig. 2. Metabolic enzyme activities of skeletal muscle (A and D), liver (B and E) and epididymal adipose tissue (C and F). The hexokinase (A–C) and phosphofruktokinase (D–F) activities from control (black bars) and STZ-induced diabetic mice (light gray bars) treated with different metformin doses or saline were performed as described in Materials and methods. Data represent the mean \pm SEM of six independent experiments ($n = 6$). * $P < 0.05$ compared with untreated control mice (Student's t -test). # $P < 0.05$ compared with untreated diabetic mice (Student's t -test).

Systat Inc. CA, USA). Values for each group were compared by non-paired Student's *t*-test and the differences were considered significant if $P < 0.05$.

Results

Swiss mice were divided into two groups where one group received a single dose of 200 mg/kg STZ and the other group was treated with saline (control). The STZ-treated group developed diabetes within 5 days, presenting fasting plasma glucose levels 5.5 times higher than the control group's (349 ± 32 mg/dl glucose vs. 63 ± 1 mg/dl glucose for STZ-treated and control groups, respectively). After confirming that the STZ-treated group developed diabetes, further treatment with metformin or saline for 3 consecutive days was initiated; after this treatment some parameters were evaluated. STZ-treated mice lost body weight, in contrast with the gain of body weight observed for the control group (Fig. 1A). Treatment of these mice with 250 mg/kg metformin does not change this pattern for either STZ-treated or control groups (Fig. 1A). Metformin, however, promotes the reduction of glycemia measured in *ad libitum* fed STZ-treated mice in a dose-dependent manner, without affecting this parameter in control mice (Fig. 1B). Furthermore, metformin (250 mg/Kg) reverses the reduced plasma lactate observed in STZ-treated mice, but has no effect on lactacidemia in the control group (Fig. 1C). These effects are not due to changes in serum insulin levels of the mice because this parameter remains reduced in STZ-treated mice and unaffected in the control group (Fig. 1C). This last result confirms that the mice developed type I diabetes mellitus with low serum insulin levels

that does not respond to plasma glucose. Moreover, reduction of glycemia with a concomitant increase in lactacidemia suggests an augmented glycolytic flux, which might be a consequence of metformin action on target tissues of diabetic mice.

To test whether metformin can stimulate cellular glycolytic flux, we evaluated the activity of two key regulatory enzymes of glycolysis – HK and PFK – in major metabolic tissues (skeletal muscle, liver and adipose tissue) from diabetic mice. Our first observation is that both enzymes' activities are reduced in diabetic mice when compared to control samples of all tested tissues (Fig. 2). HK activity is approximately 45% lower in skeletal muscle (panel A), 67% lower in liver (panel B) and 52% lower in adipose tissue (panel C), while PFK is approximately 55% lower in skeletal muscle (panel D), 73% lower in liver (panel E) and 48% lower in adipose tissue (panel F). Treatment of diabetic mice for 3 days with 250 mg/kg metformin reverses the decreased glycolytic enzyme activities in all tested tissues. This reversion is complete in skeletal muscle and adipose tissue but only partial in the liver for both tested enzymes (Fig. 2). Lower doses of metformin (50 and 100 mg/kg) do not significantly alter the diminished enzyme activity patterns, such as those observed for the glycemia (Fig. 1B), revealing a correlation between plasma glucose removal and activation of glycolytic enzymes. The partial reversion of HK and PFK inhibition observed in the liver corroborates the hypothesis that this organ's contribution to lowering glucose levels is not related to augmented glucose utilization, but more likely to reduced glucose production via gluconeogenesis [4].

We have previously demonstrated that some drugs that alter cellular glucose consumption through modulation of glycolytic

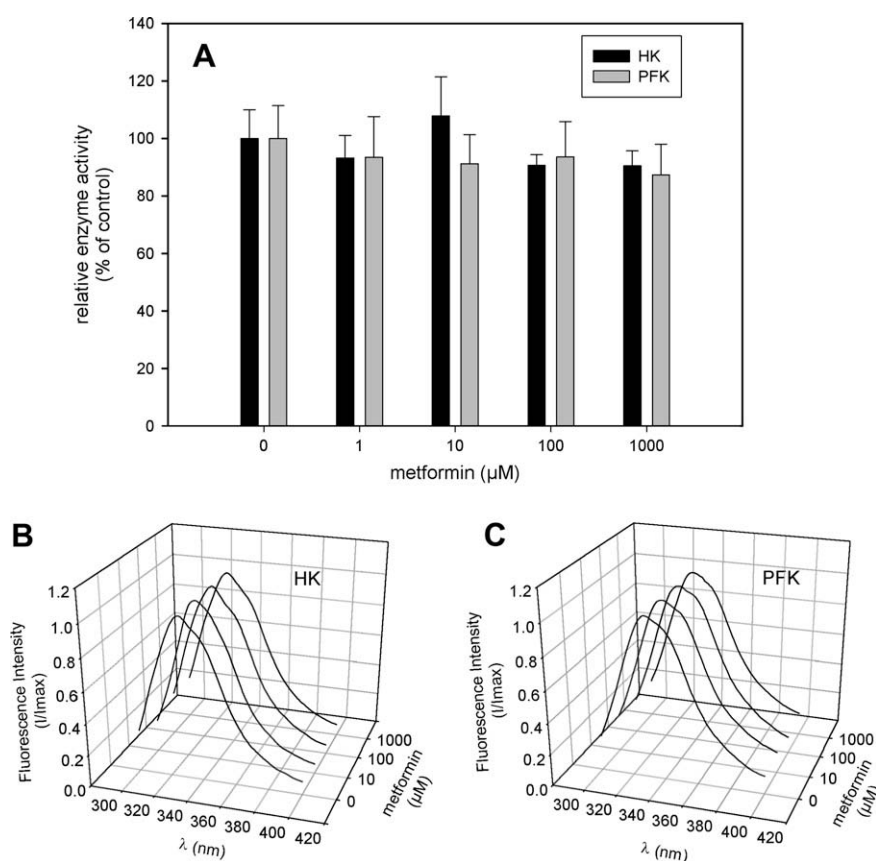


Fig. 3. Effects of metformin on the catalytic activities and intrinsic fluorescence of purified hexokinase (HK) and phosphofructokinase (PFK). Enzyme activities and intrinsic fluorescence spectra were assessed as described in Materials and methods. (A) Relative enzyme activities of purified HK (black bars) and PFK (light gray bars) in the presence of different metformin concentrations. Data represent the mean \pm SEM of three independent experiments ($n = 3$). (B) Intrinsic fluorescence spectra of purified HK (left panel) and PFK (right panel) in the presence of different metformin concentrations. Data represent the mean \pm SEM of three independent experiments ($n = 3$).

enzymes, such as clotrimazole and acetylsalicylic acid, directly interact with PFK, altering its quaternary structure and activity [24,26]. To evaluate whether metformin acts directly on HK or PFK we assessed the effects of the drugs on activities of purified enzymes and their tertiary/quaternary structures. No effects are observed on either enzyme's activity for up to 1 mM metformin (Fig. 3A) or structure, as shown through the intrinsic fluorescence emission spectra (Fig. 3B and C, for HK and PFK, respectively). These results are particularly interesting for PFK because several recent publications from our laboratory reported that many modulators of the enzyme, such as ATP, fructose-2,6-bisphosphate, citrate, calmodulin and phosphorylation, alter its quaternary structure [21,22,24,26–30]. The lack of direct effects of metformin on the enzymes indicates that the reversal of the enzymes' inhibition in diabetic tissues involves mechanisms other than modulation of quaternary structure.

One of the most intriguing properties of PFK is its ability to associate with cellular components, such as the cytoskeleton, altering its cellular location and regulating its catalytic activity [11,18,22,31–33]. It has been demonstrated that among other mechanisms, insulin modulates PFK activity, thereby altering its cellular distribution [12,13,34,35]. Based on these observations, we decided to evaluate the intracellular distribution of PFK in different tissues of diabetic mice and the effects of metformin on this distribution. For this purpose, skeletal muscle, liver and adipose tissue from the mice were homogenized and submitted to subcellular fractionation through differential centrifugation as previously described [18,19]. The first centrifugation consists of submitting the homogenized tissues to 27,000g for 30 min, obtaining the mitochondria- and nucleus-enriched pellet (P1) and supernatant containing the remaining cell components (S1). For the second centrifugation, S1 was submitted to 120,000g centrifugation for

30 min, obtaining the microsome- and cytoskeleton-enriched pellet (P2) and the soluble cytoplasmatic component (S2). Evaluating PFK activity in each fraction allowed us to estimate the proportion of the enzyme associated with organelles such as mitochondria and nucleus (P1) or the cytoskeleton (P2), as well as the free soluble enzyme (S2).

Fractionation of skeletal muscle revealed that diabetic mice possess an inverted distribution of PFK in the S2 and P2 fractions when compared to control (Fig. 4C). Control subjects present approximately 60% of PFK activity in P2 fraction, while in STZ-treated mice this distribution decreases to 33% (Fig. 4C). Treatment of diabetic mice with 250 mg/kg metformin promotes the redistribution of PFK, increasing the activity in P2 to levels similar to those of the control group (Fig. 4C). Control mice treated with metformin also presented an increased PFK activity in the P2 fraction when compared to control treated with saline (Fig. 4C). No alterations are observed in PFK activity distribution among S1 and P1 from skeletal muscle fractions in any of the groups (Fig. 4B). Reduction of P2-associated PFK activity, however, is accompanied by a reduction in the enzyme activity in the whole tissue homogenate (Fig. 4A). Moreover, metformin-induced redistribution of the enzyme occurs in parallel with the reversal of PFK activity reduction in the whole homogenate, although the augmented association with the P2 fraction in the control group treated with metformin is not followed by the activation of the enzyme in the whole homogenate (Fig. 4A). We have demonstrated that stimulation of skeletal muscle with epinephrine, which stimulates muscle glycolysis, alters the distribution of PFK, increasing the P2-associated enzyme and its catalytic activity [18]. Furthermore, insulin also augments the PFK content of the P2 fraction of rabbit skeletal muscle stimulated *in vitro* with the hormone [34]. In the present work, our results corroborate these findings because the diabetic mice

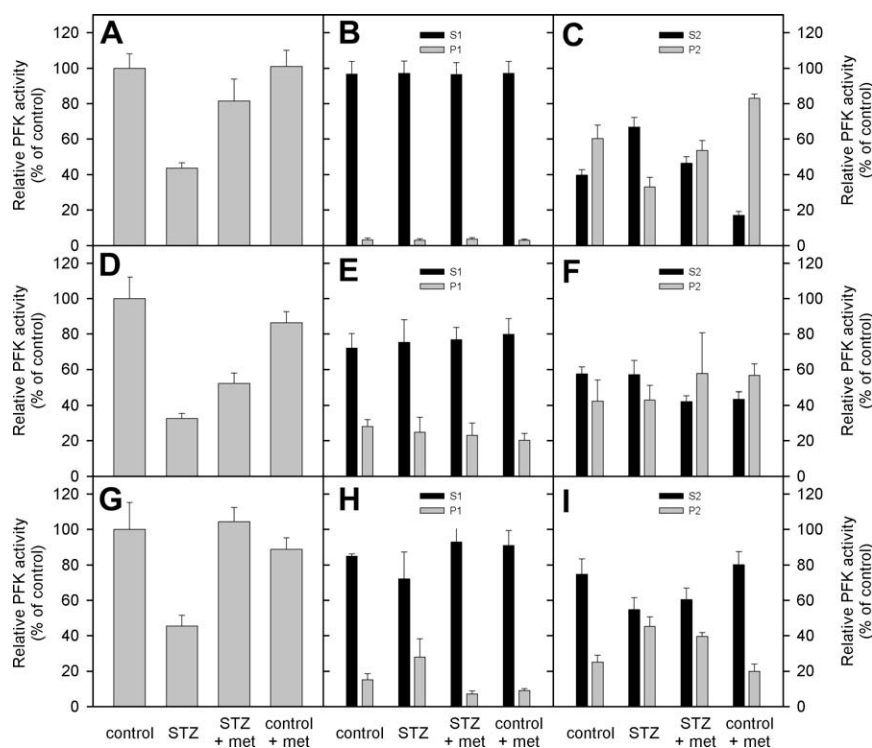


Fig. 4. Cellular distribution of phosphofructokinase activity in skeletal muscle (A–C), liver (D–F) and epididymal adipose tissue (G–I) from STZ-induced diabetic mice treated with 250 mg/kg metformin. Phosphofructokinase activity in control and STZ-induced diabetic mice was assessed in different cellular fractions (TH, S1, P1, S2 and P2) separated by differential centrifugation as described in Materials and methods. (A, D and G) Percentage of the total phosphofructokinase activity obtained in total homogenate (TH) from control mice. (B, E and H) Distribution of the PFK activity found in the fractions S1 and P1 represented as percentages of the total PFK activity (TH). (C, F and I) Distribution of the PFK activity found in the fractions S2 and P2 represented as percentages of the PFK activity found in S1 fraction. Data represent the mean \pm SEM of four to six independent experiments ($n = 4$ –6).

deficient in insulin production exhibited lower PFK content in the skeletal muscle P2 fraction. Moreover, metformin augments PFK content in this fraction, mimicking the reported effects of insulin. The stimulatory effects of cytoskeleton-associated PFK have been attributed to the interaction between the enzyme and the actin filaments present in this fraction [15,18,36–39]. Furthermore, the binding of PFK to actin filaments is recognized as an important mechanism of the enzyme's activation [15,37,40]. Because skeletal muscle possesses a surplus of actin filaments which sediment at 120,000g (P2), we may infer that PFK activity measured in this fraction is mainly due to the association of the enzyme and actin filaments. This statement is substantiated by the fact that, in skeletal muscle, the diminished PFK activity measured for the STZ-treated group and metformin-induced reversal of this inhibition are followed, respectively, by a decrease and an increase of the enzyme content in P2.

Despite partially reversing the lower enzyme activity in the liver (Fig. 4D), metformin does not affect the distribution of PFK activity among the fractions (Fig. 4E and F). In fact, no alterations of PFK distribution in the liver are observed when comparing control and STZ-treated groups (Figs. 4E and F). On the other hand, fractionation of adipose tissue revealed an unexpected pattern of PFK distribution. In these tissues, we found that control subjects present a higher content of PFK in the S2 fraction (~75%), compared with P2 (Fig. 4I), which is the inverse situation described above for skeletal muscle. In addition, the STZ-treated group presents equal PFK activity measured in the S2 and P2 fractions (Fig. 4I), showing a redistribution of the enzyme in diabetic subjects. Treatment with metformin, in spite of not affecting the control group, elevates the PFK content in the S2 fraction to $60 \pm 6\%$, which is different from the $40 \pm 2\%$ measured in the P2 fraction ($P < 0.05$, Student's *t*-test). No significant effects are observed for PFK distribution between P1 and S1 (Fig. 4H), as well as in the liver (Fig. 4E) and in skeletal muscle (Fig. 4B). As observed for skeletal muscle, the distribution of PFK in S2 and P2 derived from adipose tissue follows the activities of the enzyme measured in the total homogenate (Fig. 4G), but in a reciprocal way, i.e. in adipose tissue the decrease in PFK activity is followed by a diminished amount of the enzyme in P2. Although controversial when comparing to the data obtained for skeletal muscle fractionation, these results can be explained by the low levels of actin filaments in adipose tissue [41]. Nevertheless, adipocytes possess a microtubule network, forming their cytoskeleton in a manner similar to that of the actin filaments [41], a situation not found in skeletal muscle where actin-filaments are much more abundant than microtubules. PFK also binds to microtubules; as a result of this interaction the enzyme becomes inhibited [42–45]. Because PFK activity is lower in adipose tissue when the enzyme is found in higher proportion in P2, we suggest that PFK is associated with microtubules, and this association is augmented in STZ-treated subjects and diminished upon treatment with metformin.

To understand the signals that modulate the intracellular distribution of PFK in diabetes and upon its treatment with metformin, we evaluated the phosphorylation of the enzyme in the diverse conditions and tissues used in the present work. For this purpose, skeletal muscle, liver and adipose tissues of control, diabetic, and metformin treated diabetic and control mice were treated with anti-PFK antibodies to immunoprecipitate the enzyme and Western blot analyses were performed using anti-phosphoserine (anti-PS), anti-phosphothreonine (anti-PT) and anti-phosphotyrosine (anti-PY) antibodies. The results of a representative experiment are summarized in Fig. 5A. The only significant alteration is observed for the anti-PS analysis of skeletal muscle where a reduction in phosphorylation levels of PFK is observed for STZ-treated subjects, when compared to the control (Fig. 5B). Moreover, treatment of these diabetic mice with metformin reversed this reduc-

tion and elevated the phosphorylation of PFK serine residues to levels comparable to those of a control (Fig. 5B). No effect of metformin is observed in non-diabetic mice (Fig. 5B). All other conditions evaluated present no significant alterations, and the low staining levels of lane 8 are due to low amounts of immunoprecipitated PFK under this condition, as confirmed by phenol-red staining (data not shown).

Phosphorylation of PFK has been associated to the ability of the enzyme to interact with actin filaments [46,47]. The results obtained here in skeletal muscle for phosphorylation of PFK in serine residues are in accordance with the distribution of the enzyme in this tissue. We found that diabetic mice have less PFK associated to the actin-enriched fraction in skeletal muscle, when compared to control subjects (Fig. 4C) and in parallel, the enzyme presents a lower level of phosphorylation in serine residues (Fig. 5). Moreover, upon treatment with metformin the enzyme recovers its phosphorylation levels (Fig. 5) and its association to the actin-enriched fraction (Fig. 4C). However, the results for PFK distribution in adipose tissue (Fig. 4I) cannot be explained by amounts of enzyme phosphorylation because no differences are observed for these measurements (Fig. 5).

In an attempt to verify whether the effects of metformin occur at the cellular level and are not due to a hormonal response, we evaluated some metabolic parameters of C2C12 cells treated with metformin. For these experiments we assayed the cells after withdrawal of FBS in the absence and in the presence of either 100 nM or 1 μ M insulin. These concentrations of insulin were used to achieve the previously established maximal insulin action (at 100 nM) or insulin resistance after exposure to 1 μ M insulin

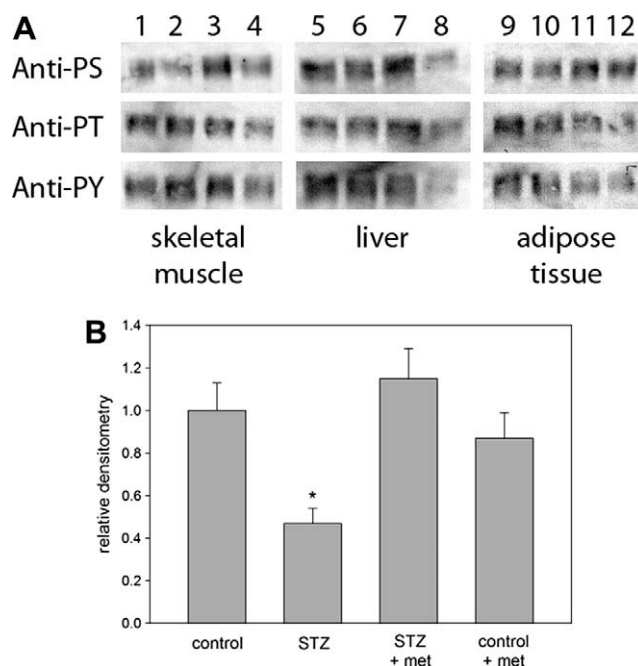


Fig. 5. Effects of metformin treatment on PFK phosphorylation from skeletal muscle (1–4), liver (5–8) and epididymal adipose tissue (9–12) from control and STZ-induced diabetic mice treated with metformin. (A) Representative Western blot of control (1, 5, 9), diabetic (2, 6, 10), diabetic treated with 250 mg/kg metformin (3, 7, 11) and control treated with 250 mg/kg metformin (4, 8, 12). The treatments are described in Materials and methods. Phosphofruktokinase from each tissue was immunoprecipitated using an anti-PFK antibody and probed with monoclonal antibodies against phosphoserine (Anti-PS), phosphothreonine (Anti-PT) and phosphotyrosine (Anti-PY). (B) Densitometric analysis Western blot experiments with skeletal muscle extracts immunoprecipitated using anti-PFK and probed with monoclonal antibody against phosphoserine. Bars are means \pm standard errors of 4 independent experiments ($n = 4$). * $P < 0.05$ compared to all other groups (Student's *t*-test).

[12,13]. Metformin induces activation of HK (Fig. 6A) and PFK (Fig. 6B) in C2C12 cells independently of the concentration of insulin in the culture media. Both enzymes are activated by the presence of 100 nM insulin in the culture media; this activation is not disrupted by the presence of 1 μ M insulin, in spite of the fact under this last condition the glucose consumption is similar to control and lower than that in the presence of 100 nM insulin (Fig. 6C), confirming the achievement of insulin-resistance. Despite increasing the activity of the major glycolytic enzymes and the rate of glucose utilization, metformin does not stimulate lactate production by C2C12 cells (Fig. 6D). This result corroborates our findings that treatment with metformin does not alter the lactacidemia of control mice (Fig. 1). Metformin is the only biguanide approved as hypoglycemic agent due to the fact that it does not augment lactacidemia, and, thus, is considered a safe drug [2]. On the other hand, metformin increased MTT reduction in the absence or presence of 1 μ M insulin to the same extent observed in the presence of 100 nM insulin in the absence of metformin (Fig. 6E). This result clearly indicates that metformin stimulates the oxidative metabolism of glucose, rather than the fermentative metabolism, because

the result of the MTT assay is indicative of mitochondrial activity [23]. Moreover, this last result is consistent with the ATP levels measured (Fig. 6F), reinforcing the importance of oxidative metabolism for the maintenance of the intracellular ATP pool.

Discussion

The results described in the present work demonstrate that metformin promotes oxidative metabolism in muscle cells. The pyruvate formed due to augmented glucose metabolism is likely oxidized in the mitochondria rather than fermented in the cytosol. As a result of the augmented glucose oxidation induced by metformin, the intracellular ATP levels are increased in C2C12 cell upon metformin treatment, regardless of insulin signaling in these cells. Moreover, when the cells are in the presence of 100 nM insulin, which induces the maximum effects induced by the hormone, some metabolic parameters, such as glucose consumption, lactate production, oxidative metabolism (MTT assay) and intracellular ATP levels, are similar to those observed upon treatment with met-

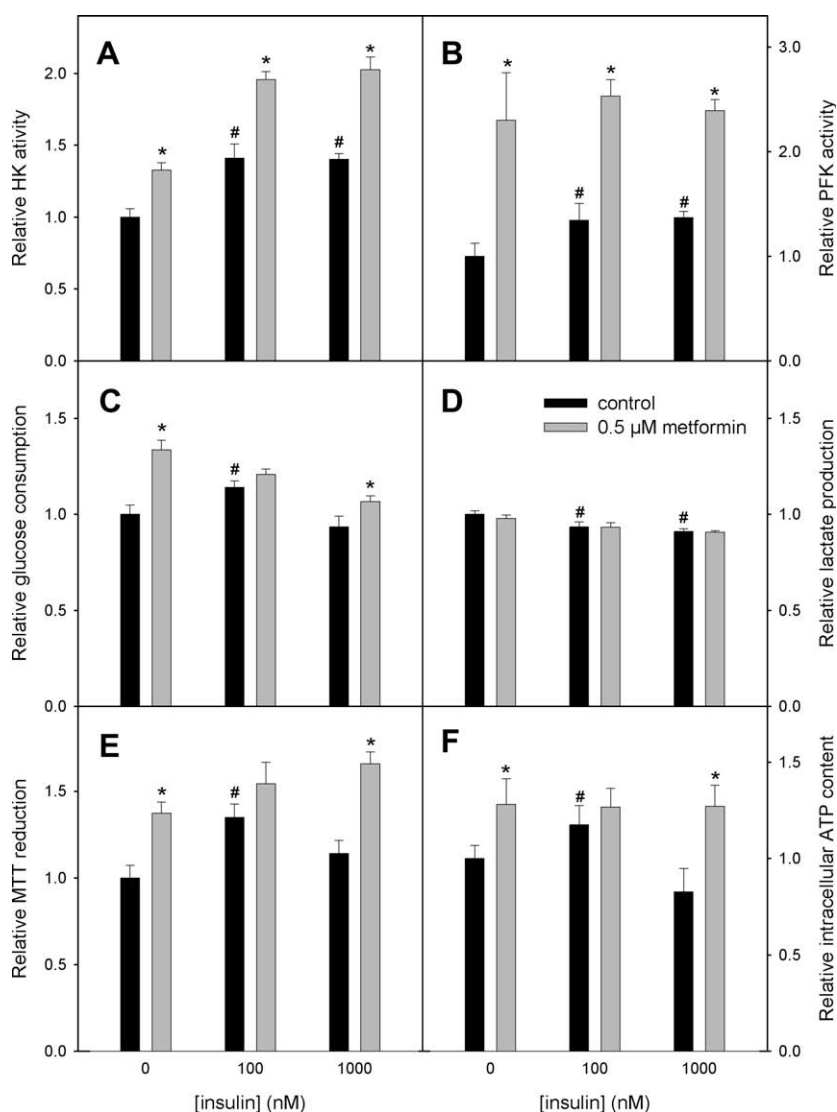


Fig. 6. Metabolic effects of metformin treatment on C2C12 myoblasts. Cells were treated as described in Materials and methods. Black bars represent controls and light gray bars represent C2C12 myoblasts treated with 0.5 mM metformin for 24 h in the presence of 0, 100 or 1000 nM insulin. (A) Relative hexokinase activity. (B) Relative phosphofructokinase activity. (C) Relative glucose consumption. (D) Relative lactate production. (E) Relative MTT reduction. (F) Relative intracellular ATP content. Data represent the mean \pm SEM of 8 independent experiments ($n = 8$). * $P < 0.05$ compared with respective control in the absence of metformin (Student's t -test). # $P < 0.05$ compared with control in the absence of metformin and insulin (Student's t -test).

formin. These data provide evidence that metformin mimics some of the effects of insulin on the cell, in a mechanism not demonstrated previously.

The described mechanism of glucose-lowering action of metformin is primarily to ameliorate insulin resistance [48]. This is mainly due to metformin effects on the liver and muscle and, to a lesser degree, on adipose tissue [48]. In the liver, metformin is shown to reduce gluconeogenesis, while in the muscle it increases glucose uptake and utilization. However, these effects have been directly associated with a higher insulin response by these tissues [48]. Our goal in this work is to demonstrate that metformin stimulates the major glycolytic enzymes in STZ-treated mice and on C2C12 cells in the absence of insulin, suggesting that the drug works at cellular level and is not dependent on the hormone's action. Direct stimulation of glycolytic enzymes is, per se, a novel mechanism of action contributing to the hypoglycemic effects of metformin, and should be included as one of its effects. Moreover, our results reveal that metformin has effects on HK, PFK, glucose consumption, lactate production, oxidative metabolism and ATP levels that are similar to those promoted by insulin alone. These data suggest that metformin may stimulate some of the intermediates in the insulin signaling cascade. However, further studies are needed to prove this hypothesis. In the end, our results corroborate the proposed usage of metformin for type 1 diabetes treatment [9] due to its insulin-dissociated effects.

Acknowledgments

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8.2. APÊNDICE B

Regulation of Mammalian Muscle Type 6-Phosphofructo-1-kinase and Its Implication for the Control of the Metabolism

Critical Review

Regulation of Mammalian Muscle Type 6-Phosphofructo-1-kinase and Its Implication for the Control of the Metabolism

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Summary

Phosphofructokinase (PFK) is a major regulatory glycolytic enzyme and is considered to be the pacemaker of glycolysis. This enzyme presents a puzzling regulatory mechanism that is modulated by a large variety of metabolites, drugs, and intracellular proteins. To date, the mammalian enzyme structure has not yet been resolved. However, it is known that PFK undergoes an intricate oligomerization process, shifting among monomers, dimers, tetramers, and more complex oligomeric structures. The equilibrium between PFK dimers and tetramers is directly correlated with the enzyme regulation, because the dimer exhibits very low catalytic activity, whereas the tetramer is fully active. Several PFK ligands modulate the enzyme, favoring the formation of its dimers or tetramers. The present review integrates recent findings regarding the regulatory aspects of muscle type PFK and discusses their relation to the control of metabolism. © 2010 IUBMB

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Keywords metabolism; glycolysis; regulation; cancer; drugs.

INTRODUCTION

The glycolytic enzyme 6-phosphofructo-1-kinase (PFK, ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) plays a central role in the regulation of glycolysis. This enzyme catalyzes the MgATP-dependent phosphorylation of fructose-6-phosphate (F6P), forming ADP and fructose-1,6-bisphosphate (F1,6BP). This reaction is virtually irreversible under intracellular conditions and is considered to be the pacemaker of the glycolytic flux (1). There has been evidence that the mammalian

PFK is derived from the duplication, tandem fusion, and divergence of an ancestral prokaryotic gene. Thus, the bacterial PFK is about half the size of the mammalian enzyme. As a result, mammalian PFK contains additional sites that have evolved into the known allosteric sites for ATP, which exhibits inhibitory properties, and for fructose-2,6-bisphosphate (F2,6BP), which is known as the major physiologic activator of the enzyme (2).

There are three different isoforms of PFK in mammals, named PFK-M, PFK-L, and PFK-P (also called PFK-C) because of their major expression in muscle, liver, and platelets (or cerebrum), respectively. In humans, the isoforms M, L, and P present 85.2, 85.0, and 85.6 kDa, and their genes are assigned to chromosomes 1, 21, and 10, respectively (3). Moreover, it has been shown that PFK-M and PFK-L share 68.6% amino acid sequence identity, PFK-M and PFK-P share 70.3%, and PFK-L and PFK-P share 66.6%. The level of expression of these isoforms is tissue-specific; skeletal muscle is the only tissue that expresses only one isoform (PFK-M), whereas all other tissues express distinct levels of the three isoforms (4). Therefore, PFK-M is considered as the only isoform expressed in all the mammalian cells evaluated. The distinct patterns of PFK isoforms expressed among tissues may contribute to their specific glycolytic rates. As an example, it has recently been shown that the glycolytic efficiency (lactate formed per glucose consumed) of some human mammary tumor cell lines is enhanced in more aggressive cells, which was attributed to a higher expression of PFK-L over the other two isoforms (5).

Once translated, PFK monomers rapidly associate forming dimers. In fact, PFK monomers are too unstable and easily unfold (6, 7); thus, the dimerization of monomers is essential for the maintenance of the enzyme tertiary structure. PFK dimers display minimal catalytic activity, but may associate to form PFK tetramers that are fully active (8, 9). These PFK tetramers are actually considered dimers of PFK dimers and can also associate into more complex structures without compromis-

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ing their catalytic activity (10). The equilibrium among the distinct PFK oligomeric structures is affected by many physical and chemical conditions (8) such as the enzyme concentration (a higher concentration of the enzyme leads to greater complexity of the oligomer), pH, temperature (an acidic medium and higher temperatures favor the dissociation of oligomers), and allosteric ligands, as will be discussed in the next section.

REGULATION OF MUSCLE TYPE PFK BY METABOLITES

PFK is tightly regulated and responds to diverse molecules and signals by changing its catalytic activity and behavior. This enzyme is one of the few examples in which inhibition by the substrate occurs. PFK is strongly inhibited by ATP, one of its substrates, showing two distinct sites for this molecule: the catalytic site, presenting a binding constant of ~ 0.15 mM, and the inhibitory allosteric site, presenting a binding constant of ~ 2.5 mM (11–13). The intracellular range of ATP concentration varies from 2 to 5 mM, depending on the tissue and the metabolic state. Hence, ATP constantly inhibits PFK at different levels, depending on the metabolic state. However, some metabolites, such as ADP, AMP, cAMP, and F2,6BP, counteract the inhibitory effects of ATP on PFK (13–15). On the other hand, other metabolites (e.g., citrate and lactate) potentiate the inhibitory effects of ATP on PFK (11, 13). Other strong activators of PFK are F1,6BP and glucose-1,6-bisphosphate (16). Therefore, the intracellular levels of all these metabolites are critical for the catalytic activity of PFK and normally are responsible for the low levels of ATP inhibition observed under cellular conditions.

Recently, it has been shown that these allosteric modulators of PFK exert stimulatory or inhibitory properties, interfering with the equilibrium between dimers and tetramers of the enzyme. Therefore, it seems plausible that ATP, citrate, and lactate inhibit PFK by stabilizing its dimeric conformation, whereas ADP, AMP, cAMP, and F2,6BP favor the formation of tetramers (7, 11, 13, 17). This characterization of the mechanism of PFK regulation simplifies much of the puzzle concerning the large number of molecules modulating the enzyme. A single mechanism driving the activity of the enzyme toward an active or inactive conformation explains the counterbalanced effects of PFK inhibitors and activators. Thus, it is not difficult to understand that F2,6BP reverses the inhibitory effects of ATP.

It is important to note that the metabolites that regulate the PFK activity that, in turn, modulates the enzyme oligomeric conformation are markers of cell metabolic status. High ATP levels clearly indicate that the glycolysis rate should be reduced, avoiding unnecessary ATP synthesis, which is achieved through inhibition of PFK. Cytosolic citrate is also indicative of a high glycolytic rate because it leaks from the mitochondria after a high input of pyruvate into the organelle, normally due to an elevated rate of oxidative glycolysis. The process of fermentative glycolysis is similar, in that lactate is produced and the

accumulation of this metabolite leads to the direct inhibition of PFK. On the other hand, the ADP and AMP that are elevated due to high ATP utilization by the cell counteract the inhibitory effects of the metabolites described previously, signaling the cellular need for ATP synthesis.

REGULATION OF MUSCLE TYPE PFK BY PHOSPHORYLATION

PFK is phosphorylated by diverse protein kinases (PKAs) at serine, threonine, or tyrosine residues (18–24). Very little is known regarding the sites in which these phosphorylations occur. Moreover, phosphorylation does not promote great changes in the enzyme activity. However, it modifies the pattern of activation by F6P, decreasing the cooperativity index for this substrate and making the enzyme less susceptible to inhibition by ATP. These effects are due to the stabilization of its tetrameric conformation by phosphorylation (18). Thus, PFK phosphorylation interferes with the regulation of the enzyme due to the modulators that affect the oligomerization of the enzyme. For example, on phosphorylation of muscle type PFK by cAMP-dependent PKA, lactate does not inhibit PFK (11). This effect can be particularly interesting, as PFK is phosphorylated by PKA under epinephrine stimulation in the skeletal muscle (23). Therefore, under certain situations such as during physical activity, the augmented epinephrine in circulation may attenuate the inhibition of PFK by the increased lactate produced due to elevated anaerobic glycolysis.

Phosphorylation of PFK results from stimulation by hormones such as epinephrine, insulin, or serotonin in the skeletal muscle (19, 23–27). These hormones all have the ability to activate glycolysis by stimulating glucose consumption in the skeletal muscles (19, 23, 24). There is a direct correlation between the phosphorylation of PFK and the activation of glycolysis triggered by these hormones, because the blockage of the former event impedes the latter (19, 24). However, PFK phosphorylation sites are not all the same, because epinephrine induces phosphorylation on serine residues, whereas insulin and serotonin promote phosphorylation on PFK tyrosine residues (19, 28). Interestingly, insulin may actually promote additional phosphorylations on serine residues of PFK, because in type 1 diabetes mellitus, there is a decrease in phosphorylated PFK serine residues (24). Regardless of which residue is targeted, phosphorylation seems to augment PFK activity in tissue extracts and glycolysis. The divergence between the effects of phosphorylation on the PFK activity evaluated using purified enzyme or tissue extracts is due to the intracellular redistribution of the enzyme on phosphorylation that occurs in the latter.

PFK ASSOCIATION WITH CYTOSKELETON ELEMENTS

Muscle type PFK reversibly associates with distinct cellular components such as actin filaments (f-actin), microtubules, and the integral membrane anion transporter, band 3 (16, 29–33).

The association with f-actin stabilizes the tetrameric conformation of PFK and, thus, stimulates the enzyme activity, whereas the association with microtubules or band 3 inhibits the enzyme due to stabilization of the dimers (16, 30, 34). The association/dissociation process is dynamic and regulated, playing a central role in the control of PFK activity and of the entire glycolytic flux. As an example, the level of oxygen controls the PFK association/dissociation with band 3 in erythrocytes because of the competition between PFK and deoxyhemoglobin that also binds to band 3 at the same site that PFK binds (34). Therefore, at low levels of O₂, deoxyhemoglobin displaces PFK from band 3, activating the enzyme and glycolysis. On the other hand, at high O₂ levels, the oxyhemoglobin formed dissociates from band 3 and, thus, PFK binds to band 3, resulting in the inhibition of the enzyme and the shift of glucose metabolism to the pentose phosphate pathway, which is critical in supplying NADPH to attenuate the highly oxidative environment (34).

Phosphorylation of PFK increases its affinity for f-actin and, thus, regulates the fraction of the enzymes associated with this cellular ultrastructure. Therefore, stimulation of skeletal muscle with epinephrine, insulin, or serotonin augments the f-actin-bound PFK fraction (19, 23, 25, 35). This association is described as part of the mechanism by which these hormones activate PFK and the entire glycolytic flux (19, 23–25, 36). In fact, this association is also described as being responsible for the increased PFK activity and glycolytic flux in cancer cells (5, 14, 37–42). Cancer cells exhibit an increased fraction of f-actin-associated PFK when compared with normal cells, which is confirmed by evaluating human breast cancer tissues (37). This pattern is directly correlated with the incidence of metastasis in human subjects (37). The association of PFK with f-actin is also shown to increase the glycolytic efficiency, that is, the rate of lactate produced per glucose consumed (5, 38). In other words, PFK association with f-actin is partially responsible for the so-called “Warburg effect,” a characteristic of tumors that accounts for ability to proliferate (43). Association of PFK to f-actin can prevent PFK inhibition by lactate (11). This fact is particularly important for tumor proliferation because these cells produce large amounts of lactate (5, 43), which would inhibit PFK if it were not associated with the cytoskeleton.

PFK REGULATION BY CALCIUM AND CaM

PFK is a calmodulin (CaM) binding protein, presenting two CaM-binding sites per protomer, each with distinct binding affinities (44). The high-affinity binding site presents a K_d of ~3 nM, whereas the low-affinity binding sites present a K_d of ~1 μM (44). CaM binding to PFK is dependent on Ca²⁺ concentrations and responds to the intracellular range of Ca²⁺ fluctuations (44, 45). Therefore, PFK binds to CaM in response to the elevation of the intracellular Ca²⁺ concentrations. The effects of CaM on PFK vary according to the number of CaM bound per PFK protomer. When both CaM sites are occupied, PFK is strongly inhibited, and this has been attributed to the

stabilization of the dimeric conformation of the enzyme by CaM (44). Recent studies have demonstrated that the exclusive binding of CaM to the high-affinity site on PFK is responsible for the dissociation of the tetramers of the enzyme into dimers. However, these PFK dimers with one molecule of CaM bound per monomer are not inhibited and exhibit catalytic activity similar to the tetramers (13, 45). As the intracellular levels of CaM are not compatible with the affinity of the second CaM-binding site, it may be that CaM can only bind to its high-affinity site on PFK (45).

The formation of active PFK dimers bound to CaM are of great regulatory relevance for the enzyme because this conformation is not affected by the allosteric modulators that inhibit PFK and stabilize its dimers (13). Therefore, once intracellular Ca²⁺ increases, PFK binds to CaM, which counteracts the inhibitory effects of ATP on the enzyme (13). This process is similar to the effects described for F2,6BP, which is considered to be the most relevant activator of PFK (15). Thus, CaM should be considered at least as relevant as F2,6BP with respect to the activation of PFK and glycolysis. Furthermore, CaM counteracts the inhibitory effects of ATP, citrate, lactate, and the combination of these metabolites more efficiently than F2,6BP (13). This higher efficiency is explained by the fact that F2,6BP counteracts the aforementioned inhibitory effects by shifting the oligomeric equilibrium of PFK in a direction opposite to that of the inhibitors. On the other hand, CaM binds to the dimers formed in the presence of the inhibitors, turning them into active forms (13). These effects are illustrated in Fig. 1, which also summarizes other structural and functional consequences of the PFK effectors. The relevance of the stimulatory effects of CaM on PFK is also related to hormonal signals such as insulin. It has been shown that insulin triggers a transient intracellular Ca²⁺ fluctuation because of the aperture of voltage-dependent calcium channels (26). Moreover, CaM-antagonists are able to attenuate the activation of PFK and glycolysis by insulin (36), supporting the claim that CaM participates in these insulin effects.

PFK INHIBITION BY PHARMACEUTICALS

PFK is a potential target for the control of cell growth because of the close relationship between its enzymatic activity and the rate of glycolysis as well as the relevance of glycolysis for tumorigenesis. Two main inhibitory strategies have been adopted: (a) the direct inhibition of the enzyme and (b) interference with the enzyme's ability to associate with the cytoskeleton (11, 12, 14, 19, 24, 38, 39, 46–49). Actually, these two strategies are interrelated because the pharmaceuticals that directly inhibit PFK act by dissociating the enzyme tetramers into dimers, which also affects the ability of PFK to associate with different components of the cytoskeleton. Clotrimazole and acetylsalicylic acid are examples of this category of pharmaceuticals. They directly inhibit PFK because of the dissociation of the enzyme tetramers (12, 14, 39). Moreover, these drugs

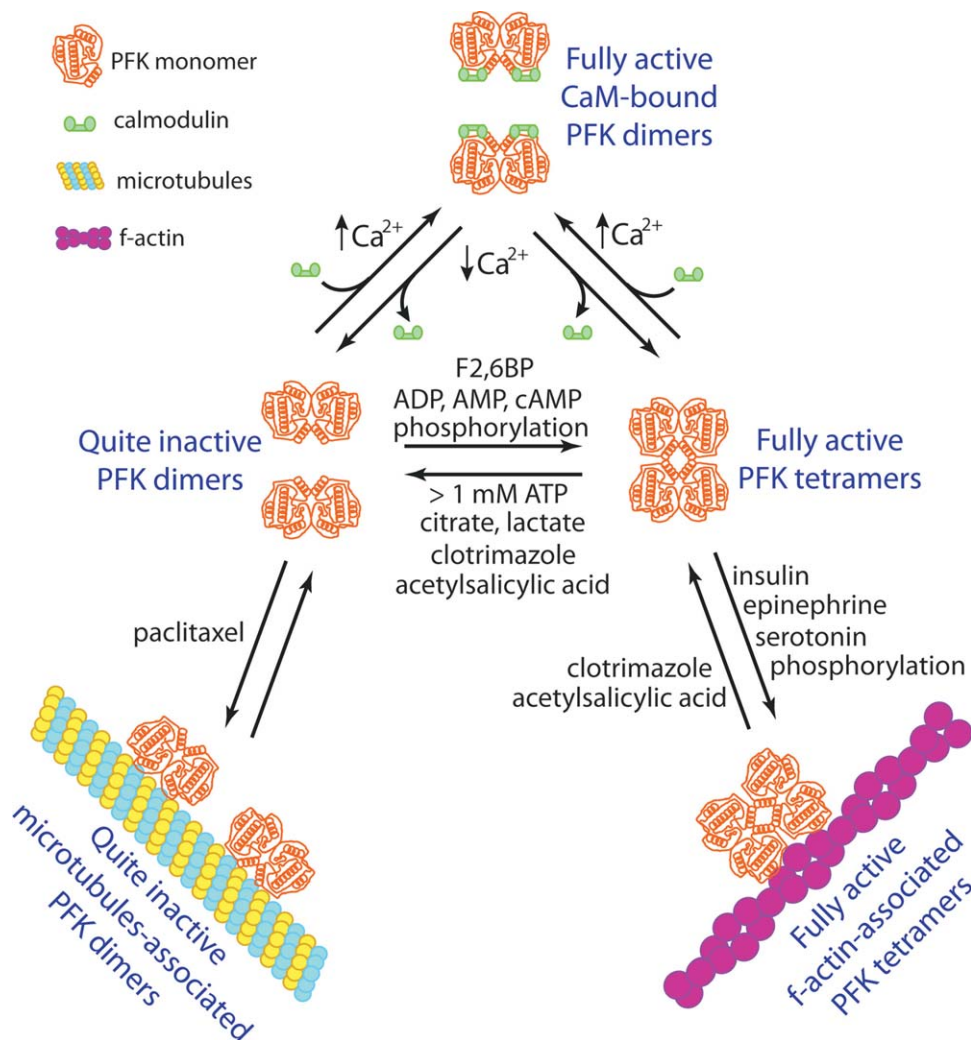


Figure 1. Schematic model of PFK activity and oligomeric structure modulation. PFK monomer illustration is a schematic model and does not represent the actual structure of the enzyme.

also promote the detachment of the enzyme from the cytoskeleton (14, 38, 40, 41). As a consequence, these drugs counteract the Warburg effect on tumor cells, that is, the increased glycolytic rate, even in the presence of oxygen, decreases their proliferation and viability (14, 38, 40, 41). Other drugs such as vinblastine, paclitaxel, lidocaine, and bupivacaine mainly affect the association of the enzyme with the cytoskeleton (46, 48, 49). Paclitaxel, because of its stabilizing properties on microtubules, decreases PFK activity and enhance the fraction of the enzyme bound to this ultrastructure (46, 49). On the other hand, lidocaine and bupivacaine detach PFK from f-actin, decreasing this fraction of the enzyme and, consequently, its activity and the entire glycolysis process (48). Regardless of whether they promote attachment to microtubules or the detachment from f-actin, all of these drugs inhibit PFK activity, glycolysis, and tumor cell viability (11, 12, 14, 19, 24, 38, 39, 46–49). Less is

known whether these mechanisms are involved on the clinical usage of these drugs. However, it is a possible explanation for some of their effects.

CONCLUSIONS

Muscle type PFK is a multiregulated enzyme and its activity drives the rate of glycolysis. Therefore, PFK plays a central role in metabolism and in various other cell functions, such as cell growth and viability. Despite the fact that several regulatory properties of this enzyme are well described in biochemistry textbooks, some of its physiologically important characteristics have been neglected. Among them, the inhibition of the enzyme by lactate and its activation by CaM in response to Ca^{2+} fluctuations should be highlighted. Lactate is the final product of anaerobic glycolysis and of aerobic glycolysis, that is, the War-

burg effect (important for tumor growth), and its effect on the inhibition of PFK is an important mechanism of feedback inhibition in glycolysis. The effects of lactate on PFK can be potentiated or counteracted by other inhibitors or activators of the enzyme, indicating the complexity of PFK regulation. Nevertheless, CaM appears to be a potent PFK activator that can override the inhibitory effects of several physiologically relevant negative modulators of the enzyme, such as ATP, citrate, and lactate. Moreover, CaM activates PFK in a calcium-dependent fashion, contributing to the glycolytic stimulation by several hormones, such as insulin and serotonin. Figure 1 summarizes the major regulatory aspects discussed here. In conclusion, it should be emphasized that PFK might be a prominent intervention target in cancer treatment because inhibiting its activity reduces tumor cell viability.

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8.3. APÊNDICE C

Differential expression of phosphofructokinase-1 isoforms correlates with the glycolytic efficiency of breast cancer cells



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Differential expression of phosphofructokinase-1 isoforms correlates with the glycolytic efficiency of breast cancer cells

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ABSTRACT

Cancer cells are characterized by increased aerobic glycolysis, which correlates with a negative prognosis. Although this correlation is well known, the mechanism of the elevated rate of glycolysis in cancer and the role of glycolytic enzymes have yet to be determined. The present work aims to evaluate the activity of the major enzymes that regulate glycolysis in breast cancer cell lines of varying aggressiveness. MCF10A, MCF-7 and MDA-mb-231 are human breast-derived cell lines with non-tumorigenic, tumorigenic and metastatic profiles, respectively. These cell lines have increasing degrees of glycolytic efficiency, i.e., lactate produced per glucose consumed, corresponding to their metastatic potential. Although, there are no differences in phosphofructokinase (PFK) or pyruvate kinase (PK) activities, the activity of hexokinase (HK) activity is higher in both tumorigenic cell lines compared to MCF10A cells. No difference in HK activity is observed between MCF-7 and MDA-mb-231 cells, suggesting that the difference in their glycolytic efficiency could not be attributed to this enzyme. However, we find that expression of the PFK-L isoform directly and strongly correlates with aggressiveness and glycolytic efficiency in these cell lines. Thus, we conclude that glycolytic efficiency, which is important for the survival of cancer cells, depends primarily on the preferential expression of PFK-L over the M and P isoforms.

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Introduction

A hallmark of malignant cancers is elevated glucose uptake and aerobic glycolysis (Warburg effect), and this is a negative prognostic indicator which may endow tumor cells with invasive potential as well as resistance to radiation and chemotherapies [1]. Despite wide acceptance of the functional importance of glycolysis in cancer, surprisingly little is known about the influence of glycolytic enzymes on cancer cell metabolism. Genes in the glycolytic pathway are over-expressed in the majority of clinically relevant cancers [2]. In addition to these genomic alterations, mammary gland tumors appear to use other survival strategies that also affect glycolysis.

Some authors have proposed that glycolytic activity correlates with the degree of tumor malignancy, so that the rate of glycolysis is higher in more de-differentiated and faster-growing tumors than in slow-growing tumors or normal cells [3–5]. In fact, a high level of lactate has been proposed as predictor of malignancy [6–9]. Furthermore, the glycolytic phenotype persists during metastasis due

to increased production of glucose-derived acid, potentiating invasion of tumor cells into the extracellular matrix of host tissue [10]. High glycolysis in tumor cells has also been related to resistance to chemo- and radio-therapy treatments [11–13]. Thus, blocking glycolysis might impede tumor progression and enhance efficacy of chemo- and radio-therapy.

In this study, we analyzed metabolic profiles and glycolytic enzyme activities in three breast cell lines with different metastatic potentials. We demonstrate that regardless glycolytic gene expression, aggressiveness of tumor cells was strongly associated with elevated glycolytic activity.

Materials and methods

Cells and reagents

The human breast cancer cell line MCF-7 was obtained from the Cell Bank of the Hospital Universitário Clementino Fraga Filho, UFRJ, Brazil. MDA-mb-231 and the non-tumorigenic epithelial MCF10A cell lines were kindly supplied by Prof. Dr. Mitzi Brentani from the Universidade de São Paulo, USP, Brazil.

The tumor cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% (v/v) FBS (fetal bovine serum; Invitrogen) and L-glutamine. The MCF10A cell

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line was maintained in DMEM/F12 medium (Invitrogen) supplemented with 5% FHS (fetal horse serum; Gibco) plus 0.02 µg/mL EGF, 5 µg/mL insulin, 1.25 µg/mL hydrocortisone and 0.1 µg/mL cholera toxin (Sigma Chemical, St. Louis, MO, USA). Cells were grown at 37 °C in 5% CO₂ atmosphere.

NAD⁺, NADH, ATP, glucose 6-phosphate dehydrogenase, fructose 6-phosphate, aldolase, lactate dehydrogenase, phosphoenolpyruvate, NADP⁺ and glucose 6-phosphate were obtained from Sigma. Other reagents were of the highest purity available.

Cell viability assay, glucose consumption, lactate production and ATP quantitative evaluation

To measure metabolic rates, cells were seeded in 96-well plates in the appropriate medium and grown to confluence. Then, the medium was removed and fresh medium was added, and the cells were returned to the incubator for 24 h. After this incubation, the medium was removed and used to evaluate glucose consumption, lactate production, and the amount of leaked lactate dehydrogenase (LDH), while the remaining cells were used to determine mitochondrial activity by MTT assay [14].

LDH activity was determined by spectrophotometrically monitoring by the conversion of NADH to NAD⁺ in a lactate-utilizing reaction [15]. Cellular ATP was assessed using a system based on firefly (*Photinus pyralis*) luciferase (PerkinElmer ATPLite) using a VICTOR3 multilabel reader (PerkinElmer).

Spectrophotometric assay for enzyme activity

Cells were seeded in 24-well plates and grown to confluence. Then, medium was removed and fresh medium was added, and cells were returned to the incubator for 24 h. After this incubation, cells were removed from the plates by trypsinization and counted in a hemocytometer. Protein concentrations of cell lysates were measured [16], and the glycolytic enzyme activities were evaluated. Phosphofructokinase-1 (PFK)¹ activity was assayed by an enzyme-coupled method, where the reduction of NAD⁺ was monitored in a spectrophotometer [17]. Hexokinase (HK), pyruvate kinase (PK) and glucose-6-phosphate dehydrogenase (G6PDH) activities were assayed as previously described [18,19]. NADH oxidation or NAD⁺ reduction was followed by measuring the absorbance at 340 nm in a microplate reader (VICTOR 3, PerkinElmer). Reactions were initiated by the addition of an aliquot of cellular homogenate. Blanks with none of the coupled enzymes were performed to control for non-specific oxidation/reduction. Each curve was performed in quadruplicate.

Western blotting

Western blotting was performed as previously described [17]. Briefly, samples of whole-cell lysates were subjected to 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF Imobilon-P, Millipore), which were blocked with Tween-TBS (20 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 0.1% Tween 20) containing 5% powdered milk, and subsequently incubated with polyclonal rabbit anti-PFK (1:5000; Santa Cruz). PVDF membranes were washed five times with Tween-TBS, followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibody (1:5000; GE). The same Western blots were reprobbed with anti-actin antibody (1:5000; Millipore) to ensure that equal amounts of protein were loaded in each lane. Immunoreactive proteins were

detected by chemiluminescence using Kodak film and quantified by densitometry using ImageJ 1.43r (NIH, USA).

Reverse transcriptase-PCR

To investigate PFK gene expression, total RNA from MCF10A, MCF-7 and MDA-mb-231 cells was extracted using Trizol (Sigma), according to the protocol provided by the manufacturer. The concentration of total RNA was determined using NanoDrop spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA) and its integrity was verified by 1% agarose gel electrophoresis (data not shown). Four micrograms of total RNA was reverse transcribed with reverse transcriptase (M-MLV, Uniscience) in a 20 µL reaction mixture using a random primer (Invitrogen). cDNA amplification was performed with a forward primer (5'-GCCTACTGTGGCTACCTGGC-3') and a reverse primer specific for each isoform (PFK-M: 5'-GCATGGAGTACAGGGAAACC-3', 741 bp; PFK-L: 5'-CCAGCATCTTCAGCATCAGC-3', 533 bp; PFK-P: 5'-GGCAGACTTGATGAGATGGC-3', 606 bp). The length of the resultant PCR products enabled us to distinguish between the different PFK isoforms. GAPDH amplification was used as an internal control (forward: 5'-ATCACCATCTTCAGGAGGCG-3' and reverse 5'-CCTGCTTCACCACCTTCTTG-3'). The PCR conditions were as follows: initial denaturation for 1 min at 98 °C, 28 cycles of 30 s at 96 °C, 45 s at 58 °C (PFK) or 57.5 °C (GAPDH) and 1 min at 72 °C, and finally 5 min at 73 °C. DNA fragments were separated by electrophoresis in 1.0% agarose gels containing ethidium bromide and visualized under UV transillumination. PFK isoforms were quantified by ImageJ 1.43r (NIH, USA).

Statistics

Statistical analysis and non-linear regression were performed using the software SigmaPlot 10.0 integrated with SigmaStat 3.1 packages (Systat, CA, USA). Student's *t*-test was used unless otherwise indicated. *P* values <0.05 were considered statistically significant.

Results

The metabolic profiles of three human breast cell lines were compared by measuring glucose consumption, lactate production, intracellular ATP and mitochondrial reduction activity. The cell lines used were the non-tumorigenic MCF10A and the tumorigenic MCF-7 and MDA-mb-231, representing noninvasive and invasive profiles, respectively. There was a negative correlation between glucose consumption and tumorigenic aggressiveness. The glucose consumption rate of MDA-mb-231 was 40% lower than that of MCF-7, which, in turn, was 10% lower than MCF10A (Fig. 1A). However, lactate production in both tumor cell lines was approximately 35% higher than that of MCF10A cells (Fig. 1A). Although lactate production was not different between MCF-7 and MDA-mb-231 cells, the ratio between lactate production and glucose consumption showed a strong positive correlation with the aggressiveness of the tumor cells (Fig. 1B). The more aggressive cell lines had a higher ratio of lactate production to glucose consumption, suggesting that the invasive tumor cells have increased rates of glycolysis and a higher efficiency of conversion of glucose into lactate (glycolytic efficiency). The mitochondrial reduction activity of the cell lines showed the opposite trend, where MCF10A cells had the highest activity, followed by MCF-7 and then MDA-mb-231 cells (Fig. 1C). These data indicate that cells with lower glycolytic efficiency compensate with higher mitochondrial metabolism. Moreover, this compensation appears to be very efficient, since higher intracellular ATP levels were observed in MCF10A compared to MCF-7 and MDA-mb-231 cells (Fig. 1D). Despite the lower glucose consumption, mitochondrial

¹ Abbreviations used: HK, hexokinase; PFK, phosphofructokinase; PK, pyruvate kinase; PFK-L, liver isoform of PFK; PFK-M, muscle isoform of PFK; PFK-P, platelet isoform of PFK; LDH, lactate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.

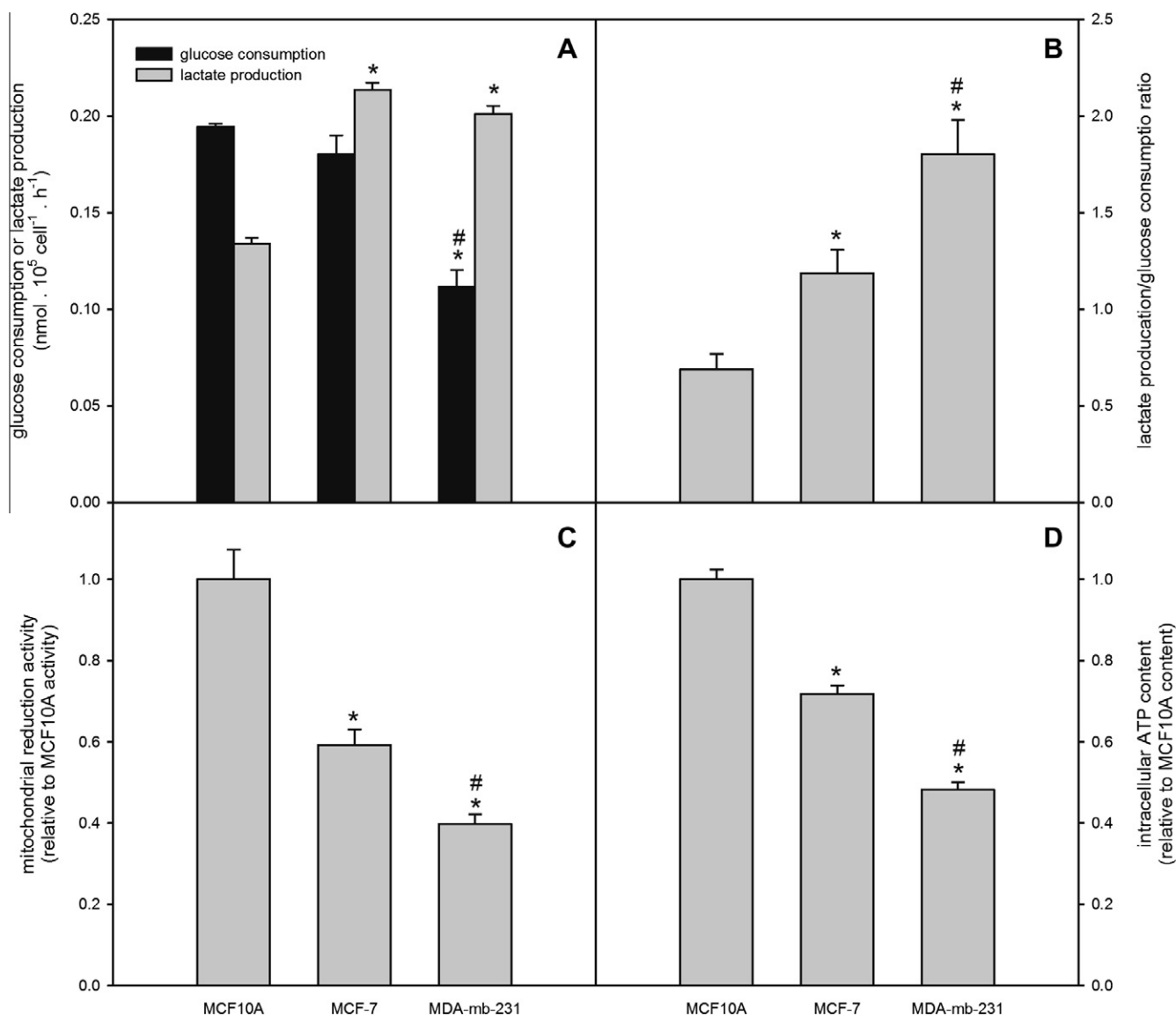


Fig. 1. Characterization of the metabolic profiles of human breast cancer cell lines. (A) Comparison of glucose consumption in MCF10A, MCF-7 and MDA-mb-231 cells. Cells were incubated for 24 h, and glucose consumption was determined as described in the Materials and methods. (B) Lactate production in the three cell lines. Cells in exponential growth phase were incubated with fresh culture medium for 24 h, and lactate production was measured as described. (C) Relative mitochondrial reduction activity, evaluated by MTT assay. All values were normalized to that of MCF10A cells. (D) Intracellular ATP content measured by relative firefly luciferase activity (PerkinElmer ATPLite Kit). Error bars represent standard errors from five independent experiments. * $P < 0.05$ vs. MCF10A; # $P < 0.05$ vs. MCF-7 (two-tailed t -test).

activity and ATP content, this elevated glycolytic efficiency might confer a proliferative advantage to MDA-mb-231 cells. However, we found a similar basal rate of cell proliferation among the three breast cell lines (data not shown).

To investigate the mechanism for the differences in tumor cell metabolism, we compared the activity of the rate-limiting enzymes of glycolysis in the three cell lines. Hexokinase, phosphofruktokinase and pyruvate kinase catalyze the three irreversible steps of glycolysis that determine the rate of the glycolytic flux [20]. We found that there is a positive correlation between HK activity and the tumorigenic aggressiveness of the cell lines, while no significant differences in activity were observed for PFK and PK (Fig. 2A). Since HK activity was positively correlated with the ratio between lactate production and glucose consumption, its activity might be the critical determinant for glycolytic efficiency in breast tumor cells. Although PFK and PK activities did not differ between the three cell lines, the activities of these two enzymes were 5- and 3-times higher than HK activity, respectively. These data suggest

that glucose, once phosphorylated by HK, can be rapidly converted into pyruvate due to the high activities of PFK and PK.

Moreover, the increased glycolytic efficiency observed in the tumor cell lines could be due, in part, to the activity of glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting catalyzing enzyme of pentose phosphate pathway (PPP). The PPP can divert glucose-6-phosphate from glycolysis, diminishing glycolytic efficiency. G6PDH activity was higher in MCF10A cells compared to MCF-7 and MDA-mb-231 cells (Fig. 2B). Thus, MCF10A might mobilize more glucose-6-phosphate for the PPP, resulting in lower rates of glycolysis.

The fact that PFK activity did differ among the three cell lines was intriguing because this enzyme is considered to be the pace-maker for glycolysis, determining its rate and, consequently, lactate production. To more closely investigate the discrepancy between PFK activity and lactate production, we evaluated total PFK content in the cell lines by Western blot analysis. Surprisingly, we observed that, despite the lack of difference in PFK activity, MCF10A cells had a higher PFK content compared to MCF-7 and

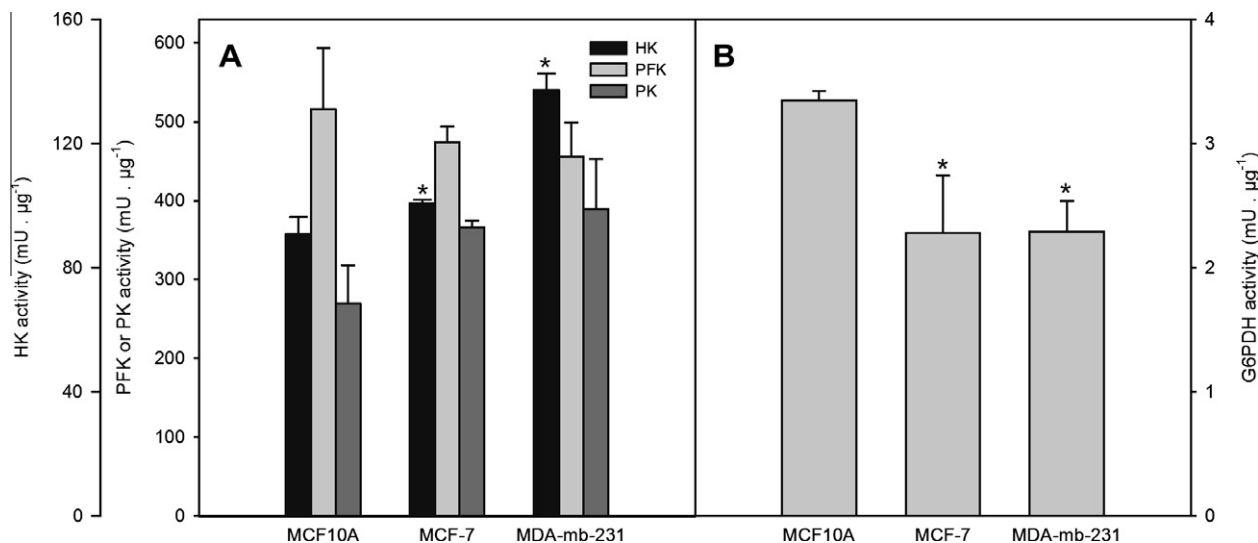


Fig. 2. Metabolic enzyme activity profiles in MCF10A, MCF-7 and MDA-mb-231 cell lines. Cell lines were grown to confluence in the indicated media as described in Materials and methods. Cell lysates were used to evaluate HK, PFK and PK enzyme activities (A) and G6PDH activity (B) as described in the Materials and methods. Plotted values are mean \pm standard errors ($n = 5$ separate experiments). * $P < 0.05$ compared to MCF10A cell line.

MDA-mb-231 cells (Fig. 3A). Densitometric analysis revealed that PFK content decreased with increasing aggressiveness of the cell line (Fig. 3C). These data seemed to conflict with the measured PFK activity and with lactate production, which was higher in the tumor cell lines than in non-tumorigenic MCF10A cells (Fig. 1). This fact led us to consider that the decrease in PFK content in aggressive tumor cell lines may be counterbalanced by the expression of a more active isoform. To determine the expression of specific PFK isoforms, we measured the mRNA for the three PFK isoforms (M, L and P) by RT-PCR (Fig. 3B). The total PFK mRNA content was comparable with the enzyme content quantified by Western blot

analysis, and decreased with the increasing aggressiveness of the cell line (Fig. 3C). However, we observed a drastic difference in the expression pattern of the distinct PFK isoforms. MCF10A cells showed equivalent expression of the three isoforms, with PFK-L slightly lower than the PFK-M and PFK-P (Fig. 3D). However, in the tumorigenic cell lines, PFK-L was the most highly expressed isoform (Fig. 3D). Indeed, PFK-L content increased with increasing aggressiveness of the cell line, whereas PFK-M and PFK-P expression decreased (Fig. 3D).

The changes observed in the expression of PFK isoforms among the cell lines correlate with the glycolytic efficiency plotted in

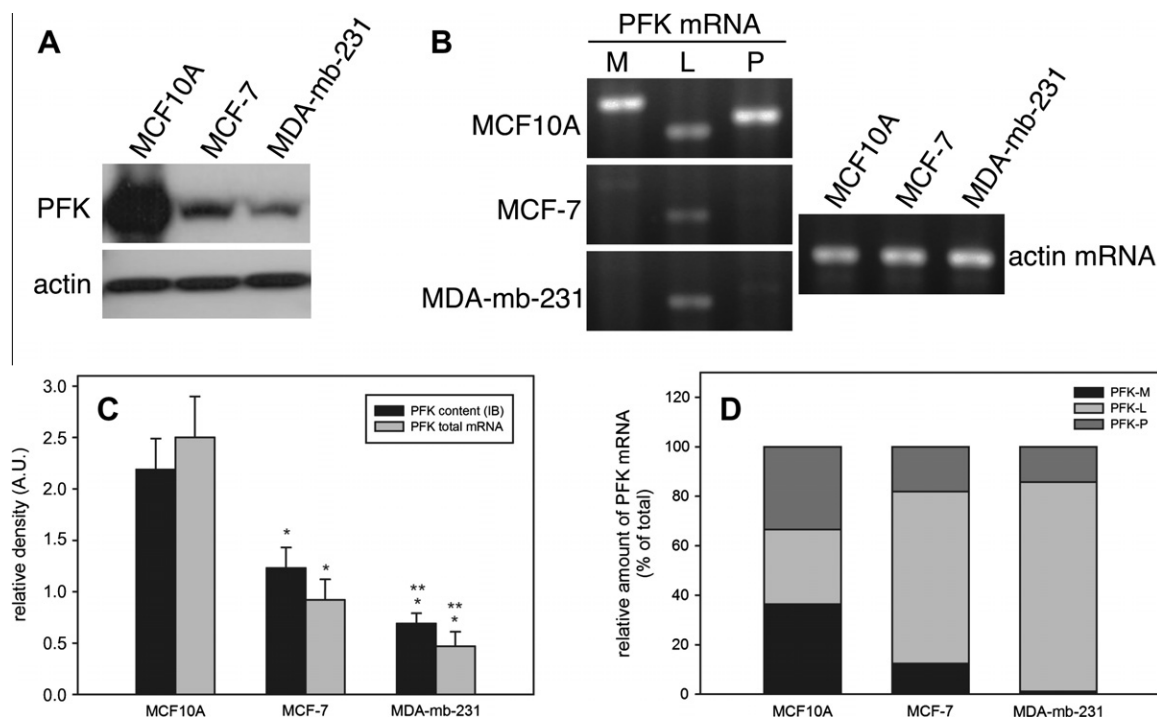


Fig. 3. Dysregulated expression of PFK and PFK mRNA expression in human breast cancer cell lines. (A) Western blot analysis was performed on whole-cell lysates, probed with rabbit polyclonal anti-PFK antibody, and re-probed with anti-actin as a loading control. (B) Agarose gel electrophoresis analysis of PFK RNA isoform expression in MCF10A, MCF-7 and MDA-mb-231 cell lines. RT-PCR was conducted as described in the Materials and methods. (C) Relative PFK content and PFK total mRNA levels. The PFK content was evaluated by immunoblotting as described. (D) Relative amounts of PFK mRNA isoforms as percentage of the control.

Fig. 1B. A strong negative correlation was found between glycolytic efficiency and PFK-M expression (Fig. 4A, correlation coefficient = -0.987 , $P < 0.05$), whereas a strong positive correlation was observed between glycolytic efficiency and PFK-L expression (Fig. 4B, correlation coefficient = 0.992 , $P < 0.05$). A small decrease in PFK-P expression with enhanced glycolytic efficiency was observed but did not reach a statistically significant level (Fig. 4C, correlation coefficient = -0.826 , $P > 0.05$).

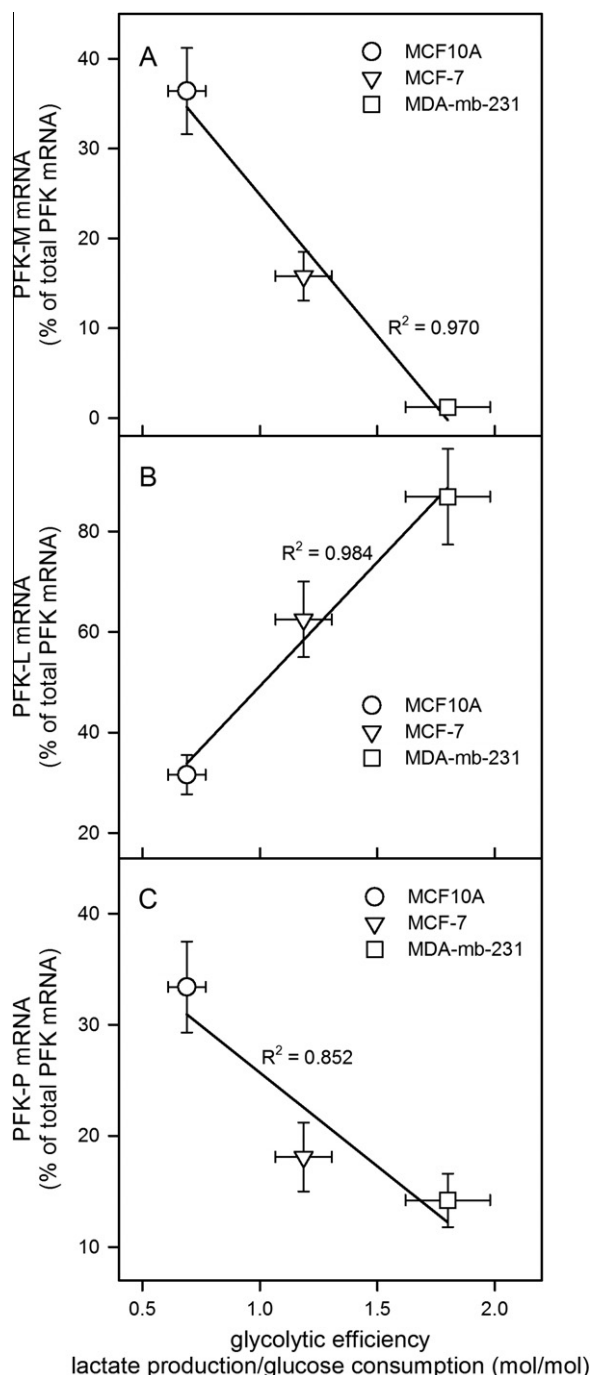


Fig. 4. Correlation plots between glycolytic efficiency and PFK isoforms expression. The glycolytic efficiency was calculated as the ratio of lactate production/glucose consumption (mol/mol). PFK isoforms mRNA levels were plotted as a percentage of total PFK mRNA. Values of PFK isoform expression and glycolytic efficiency are mean \pm standard errors of at least three and five independent experiments, respectively.

Discussion

It has long been known that tumors generally have higher glycolytic rates than normal cells [1,10]. In the present work, we compared glucose consumption among three cell lines and found a negative relationship between glucose utilization as well as tumor cell aggressiveness. The two tumorigenic cell lines showed higher rates of lactate production compared to the non-tumorigenic cell line, which supports the higher glycolytic rate described for tumors, mainly ascribed the Warburg effect [1,10,12,20–22]. In fact, the Warburg effect can be translated into glycolytic efficiency, as shown in Fig. 1B, since this parameter unveils the relationship between produced lactate and the glucose consumed. The highest possible glycolytic efficiency (2 mol/mol) would occur when all the glucose consumed by a cell is converted into lactate, bypassing other metabolic pathways that could utilize glucose or its metabolites. Our results support the hypothesis that the Warburg effect is positively correlated with cell aggressiveness, as has been previously proposed [1,10,21,23,24].

High glycolytic efficiency appears to be insufficient for maintaining ATP production, since the cell line showing the highest glycolytic efficiency had the lowest levels of intracellular ATP, and vice versa. Instead, intracellular ATP levels were higher in cell lines with higher mitochondrial activities, revealing the importance of mitochondria in the energy supply of the cell. It is well known that inhibition of cancer cell glycolysis leads to a decrease in ATP levels and to drug resistance [12]. This phenomenon is probably due to the deficiency of mitochondrial activity in aggressive tumors, making these cells highly dependent on glycolytic flux for energy. This dependence explains the high glycolytic efficiency found in more aggressive cells, which also have lower mitochondrial activity, since the maximal conversion of glucose into lactate represents the maximal production of ATP from glucose consumed. On the other hand, studying breast cancer brain metastases *in vivo*, Chen et al. demonstrated that these metastatic cells show enhanced mitochondrial respiratory pathways for energy production [25]. The fact that metastatic cells should adapt their metabolism to grow in the brain might explain the differences between our results and those published by Chen et al. [25]. In fact, the chances of finding different adaptations in metastatic cells grown in different tissues are high [26–31]. The present work shows the metabolic profiles of cancer cells grown to confluence and may reproduce only some aspects of the complex microenvironment milieu of a cancer tissue.

In the present work, we sought to correlate glycolytic efficiency with the activity of key enzymes that regulate glycolysis, HK, PFK and PK. However, we found differences only in HK activity, which was higher in tumorigenic cells than in the non-tumorigenic cell line. However, we found no difference in HK activity between MCF-7 and MDA-mb-231 cells, in spite of the latter having higher glycolysis. Because tumor cell aggressiveness did increase along with glycolytic efficiency we next considered the possibility that differences in PFK expression might be responsible for the observed metabolic variations between cell lines. This hypothesis was based on previous publications stating the importance of PFK for the regulation of glycolysis [14,17,32–36] and the survival of cancer cells [32,37–40]. Strikingly, the levels of PFK, as determined by Western blot, were highest in the non-tumorigenic cells, contrary to our expectations. However, upon measuring the expression of different PFK isoforms, we found a strong positive correlation between the expression of the PFK-L isoform and cell aggressiveness, with concomitant decrease of M and P isoform expression. Two major considerations should be presented here. First, despite the larger amount of PFK in MCF10A cells, compared to the other two cell lines, the enzyme activity is similar among

them (compare Fig. 3A with Fig. 2A). This indicates that the evaluation of an enzyme content is not always correlated to changes in the enzyme activity or a pathway rate. Several recent studies have used this approach (protein content) to predict metabolic fluxes (such as the case of the study of Chen et al. [25]) but their results should be interpreted with parsimony, or verified by an evaluation of enzymes activities or metabolic markers. Second, our results suggest that the changes of the expressed PFK isoform directly correlate with the glycolytic efficiency and the tumor aggressiveness. In addition, the expression of other glycolytic enzymes, such as phosphoglycerate mutase and glucosephosphate isomerase, has been correlated with increase in cell lifespan [41]. Actually, it has been stated that some glycolytic enzymes present additional non-glycolytic functions in transcriptional regulation, apoptosis control and cell motility [42]. Some of these functions only occur when the enzyme is active, highlighting the importance of these enzymes' activities to other cellular events other than energy production. A recent study has shown that PFK associates to the inactive phosphorylated form of the pro-apoptotic protein Bad [43]. This association stimulates the activity of the enzyme, increasing the glycolytic flux and inhibiting apoptosis [43]. Moreover, PFK also binds to the cytoskeleton stabilizing its structure and contributing to cell shape and motility [44,45]. Thus, the changes of the expressed PFK isoform observed here could have consequences not only to the rate of the reaction catalyzed but also to the putative additional function of the enzyme.

Alterations in PFK isoform expression in tumors have been reported in several types of tumors, including brain, kidney, lung and skin, which all preferentially express PFK-L [34]. Nevertheless, there has not yet been any information regarding alterations in PFK isoform expression in breast cancer. The data presented here clearly show that PFK-L is the major isoform expressed in the more aggressive breast tumor cell line (~90%). From these data, we may infer that during breast cancer tumorigenesis there is an increase in the expression of PFK-L over PFK-P and PFK-M. It has been proposed that tumors might preferentially over-express M or L isoforms due to the lower sensitivity of the former to ATP inhibition and the latter to citrate inhibition [46].

Conclusions

Our data also show that ATP content decreases with the increasing aggressiveness of the cell lines (Fig. 1), thus PFK inhibition by this metabolite might not be limiting in these cells. This could also explain the increased expression of L isoform, which has a high baseline activity and is less sensitive than the P isoform to ATP or citrate inhibition. Indeed, the preferential expression of PFK-L in MDA-mb-231 cells could be responsible for their high level of glycolytic efficiency. In summary, we conclude that glycolytic efficiency, which is critical for the survival of cancer cells, depends on the preferential expression of PFK-L over the M and P isoforms.

Acknowledgments

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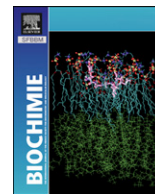
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8.4. APÊNDICE D

Filamentous actin and its associated binding proteins are the stimulatory site for 6-phosphofructo-1-kinase association within the membrane of human erythrocytes



Research paper

Filamentous actin and its associated binding proteins are the stimulatory site for 6-phosphofructo-1-kinase association within the membrane of human erythrocytes

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ABSTRACT

Glycolytic enzymes reversibly associate with the human erythrocyte membrane (EM) as part of their regulatory mechanism. The site for this association has been described as the amino terminus of band 3, a transmembrane anion transporter. Binding of glycolytic enzymes to this site is recognized to inhibit glycolysis, since binding inhibits the catalytic activity of these enzymes, including the rate-limiting enzyme 6-phosphofructo-1-kinase (PFK). However, the existence of a putative stimulatory site for glycolytic enzymes within the EM has been proposed. PFK has been described as able to reversibly associate with other proteins, such as microtubules, which inhibit the enzyme, and filamentous actin, which activates the enzyme. Here, it is demonstrated that PFK also binds to actin filaments and its associated binding proteins in the protein meshwork that forms the erythrocyte cytoskeleton. Through fluorescence resonance energy transfer experiments using either confocal microscopy or fluorescence spectroscopy, we show that, within the EM, PFK and actin filaments containing its associated binding proteins are located close enough to propose binding between them. Moreover, specifically blocking PFK binding to band 3 results in an association of the enzyme with the EM that increases the enzyme's catalytic activity. Conversely, disruption of the association between PFK and actin filaments containing its associated binding proteins potentiates the inhibitory action of the EM on the enzyme. Furthermore, it is shown that insulin signaling increases the association of PFK to actin filaments and its associated binding proteins, revealing that this event may play a role on the stimulatory effects of insulin on erythrocyte glycolysis. In summary, the present work presents evidence that filamentous actin and its associated binding proteins are the stimulatory site for PFK within the EM.

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1. Introduction

Most textbooks report that glycolytic enzymes are freely dispersed in the cytoplasm of cells [1]. However, experimental evidence has indicated that these enzymes associate with cytoskeletal components, forming a macromolecular structure that

becomes useful for regulation of the glycolytic flux [2–12]. Within the cytoskeleton, the major sites of binding for glycolytic enzymes are the following: (a) f-actin [2,8]; (b) microtubules [13,14]; and (c) the cytoplasmic domain of the anion exchanger band 3 [1,7,15]. The binding of glycolytic enzymes to f-actin normally presents a stimulatory effect [2,4,8], whereas binding to microtubules [10,13] or band 3 [15,16] inhibits glycolytic flux.

The binding of glycolytic enzymes to red blood cell (RBC) membranes under physiological conditions is correlated to the inhibition of the glycolytic flux, where band 3 is considered to be the only binding site for these enzymes [1,15,16]. Some of the binding sites for glycolytic enzymes have been identified and sequenced and have been shown to be located at the NH₂ terminus

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of band 3 [17]. In human erythrocytes, band 3 is part of the membrane cytoskeleton, which is composed of many other proteins such as protein 4.1, protein 4.2, spectrin, ankyrin and actin [18–20]. Actin forms filaments which bind to spectrin and stabilize the protein network of the membrane cytoskeleton [18,21]. These actin filaments are positioned perpendicularly to the membrane, forming an endoskeleton meshwork localized internal to the membrane cytoskeleton [22].

Actin filaments specifically bind many glycolytic enzymes, such as aldolase and 6-phosphofructo-1-kinase (PFK; phosphofructokinase), playing a stimulatory role in the activity of the enzymes and in the whole glycolytic flux [2,4,5,8,9]. Among glycolytic enzymes, PFK binds to actin filaments with the highest affinity and at two distinct sites [9], and plays a crucial role in activation of the glycolytic flux [2,4,5]. Nevertheless, it has not previously been demonstrated that actin filaments present in erythrocyte membrane meshwork bind and regulate the glycolytic enzymes within these cells. Therefore, the present study aimed to demonstrate that erythrocyte actin filaments and its associated binding proteins bind PFK, as occurs in other cell types, modulating its catalytic activity and thus the glycolytic flux.

2. Materials and methods

2.1. Materials

ATP, anti-actin antibody (whole antiserum), fructose-6-phosphate, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate-labeled phalloidin (TRITC-phalloidin) were purchased from Sigma Chemical (St. Louis, MO, USA). ^{32}P i was purchased from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared according to ref. [23]. Purified PFK was obtained from rabbit skeletal muscle according to the method developed by ref. [24], with modifications introduced by ref. [25]. Briefly, muscles from rabbits back were homogenized in three volumes (regarding the muscle mass weight) of a buffer containing 30 mM NaF, 4 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM 2-mercaptoethanol (pH 7.5). The homogenate was centrifuged ($1500 \times g$, 10 min, 4 °C) and the supernatant had its pH adjusted to 6.8 with 0.1 M H_3PO_4 . This supernatant was chilled to 4 °C in an ice/rock salt bath and 20% of the total volume of isopropanol was slowly added. After this procedure, the mixture was kept at –4 °C for 20 min followed by a centrifugation ($15,000 \times g$, 15 min, 4 °C). The pellet was resuspended in a 1/15 of the initial volume of a buffer containing 100 mM Tris- H_3PO_4 (pH 8.0), 0.2 mM EDTA, 1 mM 2-mercaptoethanol and 5 mM sodium pyrophosphate and dialyzed overnight against the 21 of the same buffer. Afterwards, the material was precipitated with 38% saturation ($(\text{NH}_4)_2\text{SO}_4$ (213 mg/ml) and centrifuged ($10,000 \times g$, 10 min, 4 °C), discarding the pellet, and further precipitated with 55% saturation of $(\text{NH}_4)_2\text{SO}_4$ (314 mg/ml). After centrifugation ($10,000 \times g$, 10 min, 4 °C), the resulting pellet contains 98% of PFK as confirmed through SDS-PAGE electrophoresis. Filamentous actin was prepared as described previously [26]. Briefly, the pellet from the initial centrifugation of the PFK preparation was washed twice with the same buffer and then extracted three times with 1 l acetone. The remaining material, named acetone powder, was dried overnight in a flux hood. This acetone powder (100 g) was resuspended in 1 l of a buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM 2-mercaptoethanol. After a centrifugation ($2000 \times g$, 20 min, 4 °C), 5 mM ATP and 2 mM CaCl_2 were added to the supernatant, which was incubated for 20 min at 4 °C to polymerize actin. This supernatant was centrifuged ($50,000 \times g$, 45 min, 4 °C) and the remaining pellet contains, at least, 98% actin, as confirmed by

SDS-PAGE. All protein content measurements were performed as described by ref. [27].

2.2. RBC separation

Blood samples were collected from volunteers in the morning after an overnight fast, and were drawn into blood collection tubes containing 15% EDTA. After centrifugation, plasma and buffy coat were removed by aspiration and the remaining erythrocytes were washed twice with a buffer containing 121 mM NaCl, 25.3 mM NaHCO_3 and 1.3 mM CaCl_2 (pH 7.8).

2.3. Erythrocyte membrane preparation

Erythrocyte membrane (EM) was prepared according to ref. [28] from outdated human blood obtained from the Universidade Federal do Rio de Janeiro Blood Bank Services. Briefly, the blood was centrifuged at $750 \times g$ for 15 min at 4 °C. Plasma and buffy coat were aspirated to remove polynuclear and mononuclear cells and erythrocytes were resuspended in ice-cold buffer containing 121 mM NaCl, 25.3 mM NaHCO_3 and 1.3 mM CaCl_2 (pH 7.8) to 50% hematocrit. The supernatant was removed after centrifugation at $750 \times g$ for 15 min at 4 °C. The pellet was washed four times with the same buffer to remove lymphocytes or granulocytes. Then, the cells were lysed in ice-cold distilled water containing 2 mM EDTA for 30 min with gentle shaking. Erythrocyte membranes were obtained by homogenization of the lysate in a Potter-Elvehjem glass homogenizer and were centrifuged at $30,000 \times g$ for 30 min. The pellet containing EM was washed five times with 2 mM EDTA and was stored at 4 °C.

2.4. TRITC-phalloidin-labeled EM

EM (10 mg/ml) was incubated for 30 min at room temperature in a medium containing 50 mM PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 , pH 7.4) and 3.3 μM TRITC-phalloidin, after which the reaction mixture was centrifuged at $100,000 \times g$ for 20 min. The pellet was resuspended in 50 mM PBS to a final concentration of 10 mg/ml and stored at –20 °C.

2.5. PFK activity

PFK activity was measured by the method described by ref. [29] with standard modifications [30,31]. Briefly, PFK activity was evaluated in a reaction medium containing 50 mM Tris-HCl (pH 8.2), 5 mM MgCl_2 , 100 μM CaCl_2 , 5 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4 $\mu\text{Ci/nmol}$), 1 mM fructose-6-phosphate, and 5 $\mu\text{g/ml}$ purified PFK. The reaction was stopped by addition of a suspension of activated charcoal in 0.1 M HCl and 0.5 M mannitol. After centrifugation, the supernatant containing $[\text{1-}^{32}\text{P}]\text{fructose-1,6-bisphosphate}$ was analyzed in a liquid scintillation counter. Appropriate blanks in the absence of fructose-6-phosphate were performed and subtracted from all measurements to discount ATP hydrolysis.

2.6. Fluorescence microscopy

Fluorescence confocal microscopy images were collected with an Axiovert 200M motorized inverted microscope and the detection setup consisted of a LSM 510 META NLO system (Carl Zeiss, Germany). Samples were excited with the 488 nm laser line of the 50 mW air-cooled argon ion laser under the control of an acousto-optical tunable filter (AOTF) set to 13% transmission. Excitation light was directed to the sample by a dichroic mirror (DC, HFT 488/543) and through a Zeiss Plan-Apochromat 63 \times /1.4 Oil DIC objective lens. The fluorescence light collected by the objective was sent

through the DC, split with a secondary DC NFT 545 and selected by band pass filters BP500–530 (FITC) and BP565–615 (TRITC) for two-channel acquisition. PFK-FITC (Donor) enzyme (2 μ l, 33 μ g/ml) was added to phalloidin-TRITC (Acceptor) labeled ghosts attached to poly-L-lysine coated coverslips and energy transfer was observed in real time. The final concentration of PFK was 1 μ g/ml (~12 nM PFK monomers), which presents PFK dimers and tetramers in equivalent proportion. Images were acquired and analyzed later using Zeiss LSM Image Browser software version 3.5.0.359 (Carl Zeiss, Germany). Energy transfer was interrupted by photobleaching of the Acceptor fluorescent probe bound to actin (phalloidin-TRITC). Photobleach was achieved by scanning the specified region of interest (ROI) at a scan speed of 5 (Zeiss control software units) with the 543 nm laser line under the control of an ATOF set to 50% transmission (100 iterations).

2.7. Fluorescence resonance energy transfer (FRET) assay

FRET experiments were carried on a JASCO 3500 spectrofluorometer (JASCO Inc. MD, USA). PFK or GAPDH at a concentration of 0.2 mM were labeled by incubation with 1 mM FITC in a medium containing 20 mM Hepes, pH 7.4 and 1 mM DTT, at 4 °C for 3 h. Free FITC was separated from labeled enzymes using Hitrap desalting column (GE Healthscience, WI, USA) equilibrated with 50 mM Tris-HCl pH 8.2. Enzyme labeling and efficiency (~0.7 mol FITC per mol PFK or GAPDH) were determined as described elsewhere [32]. TRITC-phalloidin-labeled EM (100 μ g/ml) and FITC-labeled PFK or FITC-labeled GAPDH (10 μ g/ml) were incubated in a medium containing 50 mM Tris-HCl pH 8.2, 5 mM MgCl₂ and 5 mM (NH₄)₂SO₄. Spectra for FITC-labeled proteins were obtained by exciting the samples at 494 nm and fluorescence emission was scanned from 505 to 555 nm. Spectra for TRITC-phalloidin-labeled EM were obtained by exciting the samples at 555 nm and fluorescence emission was scanned from 565 to 595 nm. FRET was obtained by exciting the samples at 494 nm and scanning the fluorescence emission from 505 to 595 nm. After correction of spectra, the TRITC emission fluorescence was observed from 565 to 595 nm. Controls were performed by exciting samples containing TRITC-phalloidin-labeled EM in the absence of the labeled enzymes at 494 nm and scanning from 505 to 595 nm, where no fluorescence emission was observed.

2.8. Statistical analyses

Statistical analyses (Student's *t*-test and two-tailed ANOVA) and non-linear regression were performed using the software SigmaPlot 10.0 integrated with SigmaStat 3.1 packages (Systat, CA, USA). Values $P < 0.05$ were considered statistically different mean values.

3. Results and discussion

It is well known that PFK, along with other glycolytic enzymes, associates to the inner face of EM resulting in the inhibition of the enzyme [1,17]. Here we mimicked this condition using purified PFK and EM and found that the enzyme activity is $20.2 \pm 1.9\%$ lower in the presence of EM when compared to control without EM (Fig. 1, $P < 0.05$, Student's *t*-test). The described site for this association is the anion transporter band 3, which also binds deoxy-hemoglobin, therefore playing a key role on the regulation of erythrocytes metabolism and oxygen transport [16]. However, the existence of a putative stimulatory binding site for glycolytic enzymes in EM has been proposed [17]. To test this hypothesis, we blocked the association of PFK to band 3 through addition of an excess of GAPDH, which competes with PFK binding to band 3 [17]. After this procedure, the resulting blocked EM exerts a stimulatory effect on

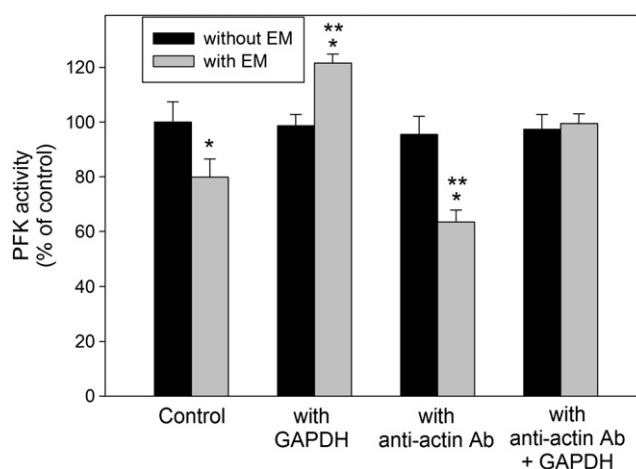


Fig. 1. A stimulatory site for PFK binding on EM. PFK activity was assessed as described in Section 2 in the absence and presence of EM. The binding of PFK to band 3 and f-actin was blocked by competition with GAPDH or anti-actin antibodies, respectively. Values are mean \pm standard error of four independent experiments ($n = 4$). * $P < 0.05$ compared to the same condition without EM. ** $P < 0.05$ compared to control in the presence of EM.

PFK activity, which becomes $21.6 \pm 2.0\%$ higher after blocking than in the absence of EM (Fig. 1, $P < 0.05$, Student's *t*-test). This result confirms the existence of the previously proposed stimulatory binding site for PFK within EM.

Association of PFK to ultrastructural elements followed by enzyme activation is not a novelty since the enzyme, along with other glycolytic enzymes, binds to the f-actin present in the cytoskeleton, becoming more active and less susceptible to inhibitors [2–6,8,25,26,33–35]. Although it is present within the membranous cytoskeleton of human erythrocytes [19,21], f-actin has never been considered as a possible binding partner for glycolytic enzymes in these cells. In order to evaluate whether f-actin and its associated proteins are the stimulatory binding site for PFK within EM, we designed an experiment obstructing binding to f-actin and its components through the pre-incubation of EM with anti-actin antibody (whole antiserum, Sigma Chemicals St. Louis, MO, USA) prior to assaying for PFK activity in the presence of these membranes. As a result, we observed that EM treated with anti-actin antibody inhibits PFK activity to a higher extent than non-treated EM (Fig. 1, $P < 0.05$ vs. control without EM or with non-treated EM, Student's *t*-test). Moreover, simultaneously blocking both sites, band 3 by GAPDH and f-actin and its associated proteins by anti-actin antibody, results in EM that are unable to affect PFK activity (Fig. 1). These results strongly suggest that filamentous actin containing its associated binding proteins are the stimulatory binding partner for PFK within the EM.

To demonstrate that PFK binds to actin filaments and its associated proteins in EM, we performed a set of FRET experiments labeling PFK with FITC and EM with TRITC-phalloidin, which specifically labels f-actin. FRET was assessed through two distinct techniques: fluorescence spectroscopy and fluorescence microscopy. For these experiments, TRITC-phalloidin-labeled EM was incubated for 1 h in the presence of FITC-labeled PFK. After incubation, the EM was washed twice to remove unbound enzyme and then used for fluorescence spectroscopy or attached to poly-L-lysine coated cover slips, which were analyzed by confocal fluorescence microscopy as described in Section 2.6. FITC-labeled PFK binds to the EM, which can be directly observed through fluorescence microscopy via the FITC signal of the EM subjected to the treatment described above (Fig. 2A). Moreover, energy transfer from FITC-labeled PFK to TRITC-labeled EM was observed, since the TRITC

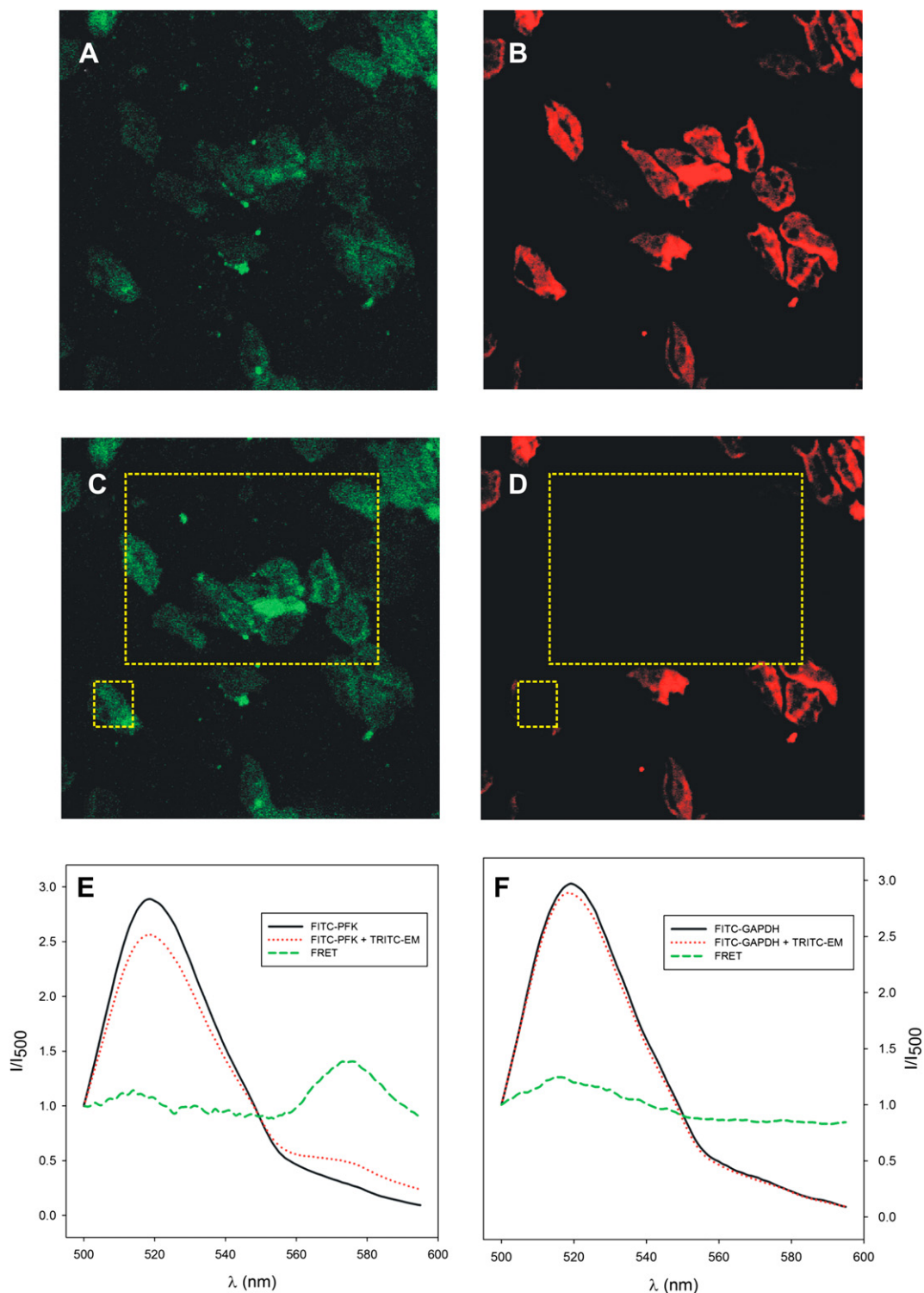


Fig. 2. PFK associates with f-actin within EM. Experiments were performed with FITC-labeled PFK and TRITC-phalloidin-labeled EM. FRET was analyzed through confocal microscopy and fluorescence spectroscopy as described in Section 2. Samples were excited at 488 nm, which only excites FITC. Panel A: confocal image of samples at the FITC channel. Panel B: confocal image of samples at the TRITC channel. Panel C: same as panel A after photobleaching of the region of interest highlighted by the dashed rectangles. Panel D: same as panel B after photobleaching of the region of interest highlighted by the dashed rectangles. Panel E: fluorescence spectrum of the samples. Panel F: fluorescence spectrum substituting FITC-labeled PFK by FITC-labeled GAPDH.

signal presented in Fig. 2B was obtained by exciting the sample at 488 nm, which excites FITC but not TRITC. In order to confirm that the TRITC signal observed in Fig. 2B is due to energy transfer and not to FITC emission at high wavelengths, we photobleached in

a specific region of the image (Figs. 2C,D, indicated by the dashed boxes). After this procedure, the TRITC signal was abolished in the specific region (Fig. 2D) with a concomitant increase of the FITC signal (Fig. 2C), which occurs since TRITC is not quenching FITC

fluorescence in this region. This result confirms that the TRITC signal observed is due to energy transfer from FITC-labeled PFK. Fluorescence spectroscopy experiments corroborate the findings described above. For these experiments, TRITC-phalloidin-labeled EM, FITC-labeled PFK and a mixture of both prepared as described above were excited with 488 nm (FITC excitation wavelength) and the fluorescence emission was recorded from 500 to 595 nm, covering the emission region for FITC (500–540 nm) and TRITC (560–595 nm). TRITC-phalloidin-labeled EM alone produced no fluorescence emission signal, as was expected since the TRITC dye is not excited at 488 nm (data not shown), while FITC-labeled PFK presented its expected emission spectrum 500–540 nm (Fig. 2E, solid black line). However, the fluorescence emission spectrum of TRITC-phalloidin-labeled EM pre-incubated with FITC-labeled PFK followed by removal of unbound PFK, as described above, presented the FITC signal, confirming the presence of FITC-labeled PFK, and an additional signal in TRITC emission region (Fig. 2E, dotted red line). Correcting this later spectrum with the one obtained with FITC-labeled PFK alone, the TRITC emission spectrum can be clearly observed, revealing energy transfer between the two dyes (Fig. 2E, dashed green line). To verify that this energy transfer is due to the binding of PFK to f-actin, we repeated the latter experiment substituting the enzyme with FITC-labeled GAPDH, which binds to band 3 but not to f-actin. The fluorescence emission spectra of FITC-labeled GAPDH alone or bound to TRITC-phalloidin-labeled EM are identical, revealing that no energy transfer occurred under this condition (Fig. 2F). Since FRET only occurs if the distance between the dyes is less than 10 nm, these results strongly suggest that PFK associates with f-actin and its associated binding proteins within the EM. However, since actin filaments can bind to a diversity of proteins including spectrin, troponin, tropomyosin among others, we cannot definitively state whether in RBCs PFK binds directly to f-actin or to one of the filamentous actin components. On the other hand, data obtained with purified PFK and f-actin reveals the direct association of these proteins, suggesting that even if other proteins participate in the PFK association with filamentous actin, it is possible that the enzyme also binds directly to f-actin in RBCs, in spite of this direct interaction is not demonstrated here.

Despite knowledge concerning the ability of actin filaments to bind PFK, it has neither been demonstrated nor proposed that this association occurs within erythrocytes. The unique site hitherto described for PFK binding at EM is band 3 [1,15–17,36], which also binds other glycolytic enzymes [7,15,17] and hemoglobin [15,16,37]. Binding of PFK and other glycolytic enzymes to band 3 inhibits enzymatic activity and thus the association of these enzymes with the membranous cytoskeleton of mammalian erythrocytes has been considered to decrease the glycolytic flux rate [1,7,15,17,36]. Moreover, the whole regulation of erythrocyte glycolysis has been reduced to the control of the equilibrium between band 3-bound and cytosolic-free glycolytic enzymes [38,39], which has been demonstrated to be modulated by tyrosine phosphorylation [1,15,31] and competition with hemoglobin, both of which detach the enzymes from band 3 [1,15,16]. Nevertheless, the results presented above demonstrate that PFK can also associate with f-actin and its associated binding proteins and that this association stimulates enzyme activity. However, what controls the binding of this enzyme to band 3 or filamentous actin and its associated binding proteins remains to be elucidated.

It has been demonstrated that phosphorylation of the glycolytic enzymes by tyrosine kinases detaches the enzymes from the inner face of the EM [1,15]. Furthermore, phosphorylation of PFK at tyrosine residues activates the enzyme and favors its association to f-actin in other cell types [3,26,31]. Moreover, PFK phosphorylation, similar to other stimulatory allosteric signals, stabilizes the tetrameric conformation of the enzyme [2,3,6,26,35,40–45], which has

high affinity for f-actin [2–6,35,42]. On the top of that, insulin signaling in muscle has been demonstrated to promote phosphorylation of PFK on tyrosine residues followed by association of the enzyme to f-actin [26], and appears to upregulate erythrocyte glycolysis in a similar manner [30,31]. All this information led us to hypothesize that insulin would be able to displace PFK from band 3 while promoting its association to f-actin and its associated binding proteins and thus stimulate enzyme activity. To test this hypothesis we evaluated the effects of insulin on PFK activity in the presence of EM under different conditions. Initially, we confirmed that 100 nM insulin stimulates PFK activity in the presence of EM (Fig. 3). However, this effect is partially prevented if EM was pre-incubated with anti-actin antibodies (Fig. 3). Moreover, in the presence of GAPDH, which stimulates PFK in the presence of EM (see Fig. 1), insulin is able to stimulate PFK activity to levels similar to those found in control (Fig. 3). According to these results, once PFK is prevented from associating to f-actin and its associated binding proteins, insulin is not able to fully activate the enzyme. The partial activation observed under this condition might be due to the displacement of the enzyme bound to band 3. This is corroborated by the experiments in the presence of GAPDH, which prevents PFK from associating to band 3, where insulin only partially activate PFK, probably due to promoting its association to f-actin and its associated binding proteins. Finally, in the simultaneous presence of GAPDH and anti-actin antibodies, where the enzyme is unable to associate to either f-actin (containing its associated binding proteins) or band 3, insulin does not affect the enzyme activity. From these results, we can infer that insulin-induced stimulation of PFK activity in human erythrocytes occurs through the modulation of the enzyme association to band 3 and f-actin and its associated binding proteins, confirming the hypothesis stated above.

It is known that PFK is a substrate for insulin receptor tyrosine kinase activity and is phosphorylated exclusively on tyrosine residues [46]. We have previously reported that stimulation of human erythrocytes by insulin leads to the phosphorylation of PFK tyrosine residues, which is essential for the stimulatory effects of the hormone on enzyme activity [31]. In order to verify whether phosphorylation of PFK is the only requirement for its binding to f-actin and its associated binding proteins and consequent activation the following set of experiments were designed. First EM was

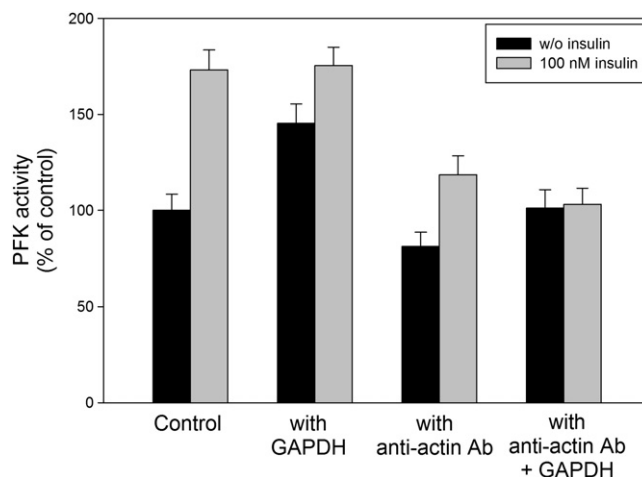


Fig. 3. Insulin promotes the binding of PFK to f-actin. PFK activity was assessed as described in Section 2 in the absence and in the presence of EM. The binding of PFK to band 3 and f-actin was blocked by competition with GAPDH or anti-actin antibodies, respectively. Values are mean \pm standard error of four independent experiments ($n = 4$). * $P < 0.05$ compared to the same condition without EM. ** $P < 0.05$ compared to control in the presence of EM.

incubated in the presence of PFK and was treated or not for 30 min with 100 nM insulin. After this treatment, the whole mixture was centrifuged at $40,000 \times g$ for 30 min to separate the EM and soluble PFK, so that four fractions were obtained: (1) non-treated PFK; (2) insulin-treated PFK; (3) non-treated EM; and (4) insulin-treated EM. PFK activity was assayed for each fraction and combination as represented in Fig. 4. There is no difference in the activity of the non-treated or insulin-stimulated PFK (Fig. 4, Bars 1 and 2). However, the presence of either EM fraction is able to inhibit non-treated PFK and to activate insulin-stimulated PFK, with no difference whether the EM was treated with insulin or not (Fig. 4). These results are indicative that no modifications of the EM are required for PFK binding to band 3 (presenting the inhibitory effect) or to f-actin and its associated binding proteins (presenting the stimulatory effect). Instead, once phosphorylated by the insulin receptor, PFK detaches from band 3 and attaches to f-actin and its associated binding proteins, and thus presents increased catalytic activity.

Altogether, the data presented in the present work indicate that the fraction of PFK within human erythrocyte membranes is associated to at least two distinct sites: band 3 and filamentous actin containing its associated binding proteins. The well-described association of PFK and band 3 inhibits for the enzyme [1,7,15,17]. Some factors have been described as modulating the association of PFK to band 3 and therefore regulating enzyme activity, e.g. deoxy-hemoglobin, which competes with PFK for binding to band 3 so that, at low-oxygen pressures, high levels of deoxy-hemoglobin displace PFK from band 3 and activate the enzyme [16]. Furthermore, phosphorylation of PFK by tyrosine kinases also displaces the enzyme from band 3 [1]. Our findings here reveal that activation of insulin receptor, which we have previously shown phosphorylates PFK tyrosine residues [30,31], not only displaces the enzyme from band 3 but also promotes its binding to filamentous actin containing its associated binding proteins, which stimulates the enzyme. However, the binding of PFK to filamentous actin containing its associated binding proteins is not dependent on the phosphorylation of the enzyme, since the simple displacement of the enzyme from band 3 by competition with GAPDH favors its binding to filamentous actin containing its associated binding proteins. Considering the above statements, we suggest that the EM-associated fraction of PFK is divided into two distinct populations: the band 3-associated and filamentous actin (containing other actin binding proteins)-associated. Nevertheless, under non-stimulated conditions the major population of PFK is associated to band 3 due to the higher amount of the transmembrane protein as

compared to f-actin [47]. However, any factor leading to the displacement of PFK from band 3 might induce its association to filamentous actin and its associated binding proteins. In conclusion, the regulation of PFK activity in human erythrocytes depends on the membranous site to which PFK is bound, where band 3 is inhibits and filamentous actin containing its associated binding proteins stimulates the enzyme

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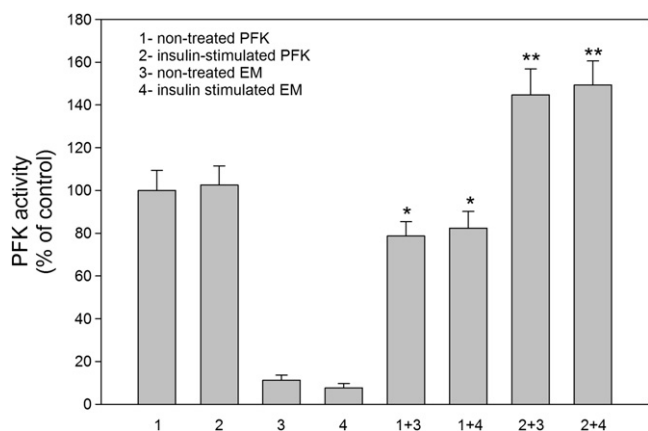


Fig. 4. The effects of insulin on PFK associated with EM are dependent on modification on the enzyme. PFK activity was assessed as described in Section 2. * $P < 0.05$ compared to bar 1. ** $P < 0.05$ compared to bar 2.

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8.5. APÊNDICE E

Microcapsules of alginate/chitosan containing magnetic nanoparticles for controlled release of insulin



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Microcapsules of alginate/chitosan containing magnetic nanoparticles for controlled release of insulin

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ABSTRACT

The challenge of this work was to investigate the potential of alginate/chitosan beads containing magnetite nanoparticles as a drug delivery system. The insulin beads were prepared by dripping a solution of sodium alginate containing insulin into a CaCl₂ solution. Magnetite nanoparticles of 5 nm mean size were synthesized inside the alginate egg-box structure by co-precipitation of Fe(III) and Fe(II) in the presence of NH₄OH. Quantitative analysis revealed that insulin encapsulation depends on the initial protein content and 35% of insulin was entrapped by alginate beads for a protein concentration of 10 wt%. It was verified that approximately 50% of the insulin was released to Milli-Q water in 800 h release experiments. The application of oscillating magnetic field increased three fold the insulin release. The results suggest that the alginate/chitosan system containing magnetite nanoparticles is a promising system for clinical applications of controlled release of insulin in the presence of an oscillating magnetic field in a subcutaneous implant approach.

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1. Introduction

Since its discovery in the decade of 20, the insulin has been managed through injections and other invasive methods. The discomfort associated with this type of administration has led diabetic patients of type I to neglect and even to give up the therapy. For these reason alternative procedures of insulin administration using controlled release systems such as protein [1], lipids [2] and polysaccharides [3,4] have been proposed. Insulin encapsulation in polymeric matrices as ethylene vinyl acetate, chitosan, polylactic acid, ethyl cellulose, has been tested [5].

Biopolymers are chosen predominantly to produce microcapsules because of the advantages of biocompatibility and biodegradability. Beyond that, the biopolymer can be associated to a specific device which could control the drug release. Alginate has been considered one of the most suitable biopolymer for microcapsules production; its composition and sequential structure has a great importance for its function as encapsulation material [6].

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It contains two uronic acids, β -(1-4)-linked D-mannuronic acid (M) and α -(1-4) linked L-guluronic acid (G), and is composed of homopolymeric blocks M–M or G–G, and blocks with an alternating sequence of M–G blocks [7–9]. In addition, sodium alginate has a unique property of cross-linking in the presence of multivalent cations, such as calcium ions in aqueous media, which rather complex with G–G sequences in the polymer chain to form the 'egg box junctions'. Alginate forms a reticulated structure in contact with calcium ions and this network can entrap proteins [10].

Chitosan is a linear copolymer polysaccharide consisting of β -(1-4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units [10]. In general, it is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans. The strong electrostatic interaction of the amino groups of the chitosan with the carboxylic groups of the alginate leads to formation of the complex chitosan/alginate that becomes the microcapsule more resistant to the release of molecules [11]. The polyelectrolyte complex between chitosan and alginate has been widely used in order to obtain microcapsules for cell encapsulation and devices for the controlled release of drugs or other substances. In these systems the efficiency of encapsulation or drug

release is directly dependent on pore dimension of the polymeric network. According to Pasparakis and Bouropoulos coating of the calcium–alginate beads with chitosan caused significant reduction of micro/macroscale pores and cracks observed on the surface and thus a decrease of its permeability [10].

Edelman and Langer defended that the release of biomolecules, from polymeric matrices, could be regulated by the movement of magnetic particles inside the matrix [12]. The magnitude of the release was directly connected to the intensity of the magnetic field, the magnetization of particles and the number of movements of particles in matrix for a specific period increases with frequency of the magnetic field. Saslawski et al. produced beads of insulin in alginate/polyllysine matrix recovered with polyethylenimine for a magnetic controlled release [13]. Particles of ferrite strontium in micrometer size were incorporated into alginate. The authors applied a magnetic field of 8900 G with the frequency of 4 Hz and verified that the release rate was 50 times bigger in the presence of the magnetic field, even though a fraction of insulin had been released also by passive diffusion. However, the mechanism responsible for the insulin release is not entirely clarified so far [13,14].

In order to control the release several nanostructured devices has been used as modulating agents in open-loop delivery systems. In this work nanoparticles of iron oxide were used to generate mechanical impulses to modulate the rate of protein delivery from alginate/chitosan matrix. The synthesis of biocompatible superparamagnetic materials has been used in biomedical applications including magnetic resonance imaging for clinical diagnosis, tissue engineering, magnetic drug targeting, hyperthermia anti-cancer strategy, drug delivery and enzyme immobilization [15,16]. Iron oxides such as magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) have been suggested for biotechnological applications because of their biocompatibility and magnetic properties. In our previous work we synthesized and characterized these nanoparticles, therefore the combination of magnetic properties of iron oxide nanoparticles with the biocompatibility of calcium–alginate suggests that these materials have a great potential to be used as drug delivery system [17]. In the current study, magnetite nanoparticles were synthesized inside alginate/chitosan matrix in order to obtain a system of controlled release of insulin through interaction with an applied oscillating magnetic field.

2. Materials and methods

2.1. Materials

Sodium alginate was purchased from Keltone LV. Sodium alginate solutions (2%, w/v) had viscosity at 25 °C and 60 rpm (No. 2 spindle) of 100–300 mPa s, as determined with LV model of the Brookfield viscosimeter. The ratio of mannuronic acid to guluronic acid residues (M/G) was between 0.4 and 1.9. The ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was obtained from Nuclear Inc. The solution of ferrous chloride (FeCl_2) was prepared by a reaction between $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Química Moderna) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Isofar) in a stoichiometric ratio. The methanol and ammonium hydroxide were obtained from Synth and Merck, respectively.

Insulin (Mw 6000 Da) was purchased from Lilly; this insulin is manufactured using genetic engineering techniques from human DNA. High molecular weight chitosan (Mw 474 kDa) was purchased from Sigma; calcium chloride and all other chemicals were obtained from Merck.

2.2. Preparation of alginate beads containing insulin

The beads containing insulin were prepared in triplicate by extrusion method (dripping method). Sodium alginate was dis-

solved in distilled water at a concentration of 3% (w/v) and a pre-calculated quantity of insulin was added (10%, w/v). The solution was stirred thoroughly to ensure complete mixing of drug. The alginate/insulin mixture was dropped into calcium chloride solution (2%, w/v) under constant stirring at 300 K. After gelation the beads were maintained for a period of 10 min in the CaCl_2 solution. The beads were then removed from the CaCl_2 solution and washed several times with Milli-Q water.

2.3. Preparation of alginate/chitosan beads containing insulin

Firstly the insulin was entrapped into alginate by extrusion as described above. Then for the recovering with chitosan the beads were placed in the 3 mg/mL chitosan solution under stirring for 60 min at 25 °C. In the literature, this method is generally called two step method. The chitosan was previously dissolved in acetate buffer pH 5.0 (0.02 mol/L of sodium acetate/acetic acid 1%). Lastly the beads were taken out and washed several times with Milli-Q water. Beads containing different contents of insulin were prepared by the same described procedure.

2.4. Efficiency of entrapment of insulin in beads

The insulin content of the beads was determined spectrophotometrically ($\lambda = 266$ nm; HACH DR/4000V). The alginate and alginate/chitosan beads loaded with insulin were completely dissolved in phosphate buffer pH 7.4 and measured. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at 266 nm. The efficiency (%) of entrapment was calculated using the following formula:

$$\begin{aligned} \text{efficiency (\% of entrapment)} \\ = \frac{\text{mass of insulin present in beads}}{\text{mass of insulin in the formulation}} \times 100 \end{aligned}$$

2.5. Synthesis of iron oxide nanoparticles into alginate/chitosan beads

The iron oxide nanoparticles were synthesized using the procedure described by Morales et al. [17]. A solution of Fe(III) and Fe(II) ions in an equal molar ratio was prepared from $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, FeCl_2 , and deionized water. Then, the iron solution was mixed with the alginate/chitosan beads containing insulin and kept at a constant temperature of 60 °C for 15 min. Next, ammonium hydroxide solution 25% (v/v) was added dropwise to the iron–polymer mixture to maintain the pH in the range of 11–12, while the mixture was kept at 60 °C for another 15 min, while being stirred vigorously. The beads containing the iron oxide nanoparticles were then washed several times in methanol solution (50%, v/v) and dried in the oven at 35 °C for about 24 h. The characterization of iron oxide nanoparticles were detailed in a previous work [17].

2.6. Beads microstructure and magnetic characterization

The microstructures of the magnetic alginate beads were studied by scanning electron microscopy (SEM). Randomly selected dried beads were deposited on double-coated carbon conductive tape previously adhered to SEM aluminum stubs. The beads samples were then sputter coated with a thin gold layer using a coating unit (Balzers Union model FL 9496), and analysed in a JEOL JSM 5310 operated at 15 or 20 kV. In order to assess the fine structure of the alginate/chitosan–magnetite composite, at the nanometer level, the beads were embedded in epoxy resin, ultrathin-sectioned using a RMC ultramicrotome, and analysed by transmission electron microscopy (TEM). Conventional TEM was performed in a JEOL

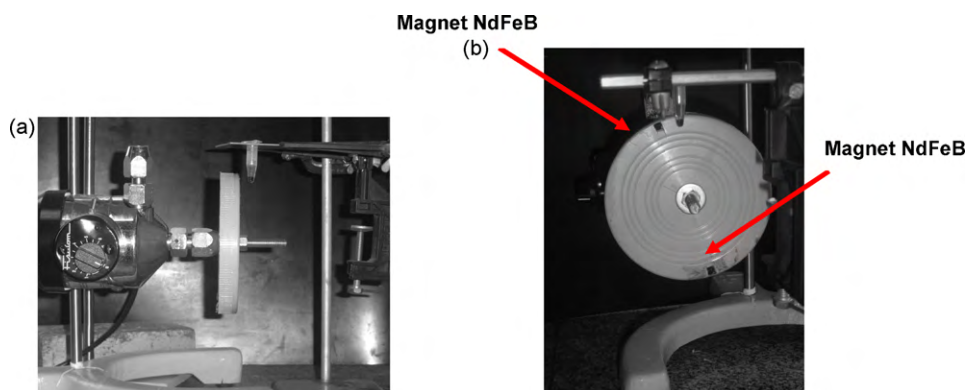


Fig. 1. Photo of the device for applying the magnetic field in the samples. (a) Side view and (b) frontal view.

1200 EX, while elemental mapping was done in a Zeiss CEM-902. Iron image (Fe-L_{2,3} edge) was obtained using inelastically scattered electrons (two window method with the following parameters: pos edge, 720 eV; pre-edge, 690 eV; energy-slit width, 20 eV; operating voltage, 80 kV). The iron map was obtained by subtracting the pre-edge image from the pos-edge one after normalizing the background intensity in both images (for details of the method, see [18]).

Magnetic characterization was performed using a Quantum Design MPMS-5S SQUID magnetometer at a magnetic field of 100 Oe.

2.7. *In vitro* insulin release studies

The *in vitro* insulin release profiles of beads were followed in 20 mL of Milli-Q water for 800 h under mechanical stirring rotation of 100 rpm at 37 °C. At predetermined time intervals agitation was stopped, 1.5 mL of the samples were withdrawn and replaced with fresh medium. The insulin content was determined spectrophotometrically at 266 nm.

In the experiment of controlled release, the magnetic field was generated from a prototype designed specifically for application in beads as shown in Fig. 1. It consists of a disc with two cylindrical neodymium permanent magnet (NdFeB, 10 × 10 mm) fixed radially on its ends. The disc with the magnets was fixed in a motor which could rotate at 33 Hz inducing an oscillating magnetic field. The magnetic field was of 1800 G for samples located at 0.5 cm from the rotating disk. The release tests were carried out using eppendorf containing 1.5 ml of Milli-Q water and the magnetic beads. At predetermined time intervals the oscillating magnetic field was stopped, all the volume of the samples were withdrawn and replaced with fresh medium. The insulin content was determined spectrophotometrically at 266 nm.

2.8. Preliminary *in vivo* studies

Swiss male mice (10 weeks) were divided into two groups: control microcapsules ($n = 3$) and insulin microcapsules ($n = 3$). For the subcutaneous microcapsules implantation, the animals in a fed state were sedated with ethyl ether but not placed under general anesthesia. The area of microcapsules implantations were shaved and surgically prepared under sterile conditions in laminar flow equipment. Subcutaneous implantations of microcapsules were performed in a longitudinal incision of approximately 1.5 cm on the dorsal region, followed by creation of a small cavity that received the material. Control group received 10 mg of microcapsules without insulin and the other group received 10 mg of insulin microcapsules. The material was inserted into the subcutaneous incision with a glass pasteur pipette and the incision closed

with sutures. Blood glucose concentration was determined using a glucometer (Accu-Chek Active – Roche) at time zero after the subcutaneous implantation of the control microcapsules or insulin microcapsules. After the surgical process mice were starved and glycemia was monitored at 2 and 6 h. Eight hours later, mice were refeed and glycemia monitored again at time of 22 h. During the experiments, mice were housed in a temperature-controlled room with a 12-h light and dark cycle and given free access to water. The animal protocol was approved by the internal institutional animal care and use committee [21].

3. Results and discussion

The dripping technique produced spherical droplets that, after falling down in the CaCl₂ solution, resulted in spherical thermostable gel particles due to the ionic interactions between guluronate blocks from alginate and Ca²⁺ ions. Insulin-loaded alginate/chitosan beads containing iron oxide nanoparticles showed a spherical geometry (1–1.5 cm diameter) and a compact structure as evidenced by the SEM analysis reported in Fig. 2a. The beads surface exhibited a non-homogeneous microstructure constituted by polyhedral particles of variable sizes (Fig. 2b). The particles arrangement originates a surface porosity with pores dimension of few micrometers. Fig. 2c shows a section just below the bead surface. The image reveals the existence of an external shell constituted by elongated particles radially oriented. This external shell may be associated to the first bead gelation layer. The bead interior in Fig. 2d presented a distinct morphology from the external bead layer. It is composed by smaller particles in a more compact arrangement than the external shell. The existence of two different morphologies is a consequence of the external gelation process. There was observed any influence of insulin on that morphology.

In order to assess the fine structure of the alginate/chitosan-magnetite composite, at the nanometer level, a transmission electron microscopy (TEM) was used. The small beads, produced as previously described (see Section 2), were embedded in epoxy resin and ultrathin-sectioned using a RMC ultramicrotome. The analysed area was the bulk region of the bead, near the surface. It was observed that the magnetite nanoparticles presented a narrow distribution of diameters and were homogeneously distributed in the whole area (Fig. 3 and inserts). This fact means that the organization pattern of the inner structure of the composite was reproducible allowing a correlation between structure and behavior of the whole ensemble of synthesized spheres.

At 300 K the M(H) loop showed negligible coercivity field (Fig. 4). The loop was fit to a Langevin function weighted with a lognormal distribution of particle size. The mean size and the standard deviation was 5.8 nm. Others results about characterization can be found in our previous work [17].

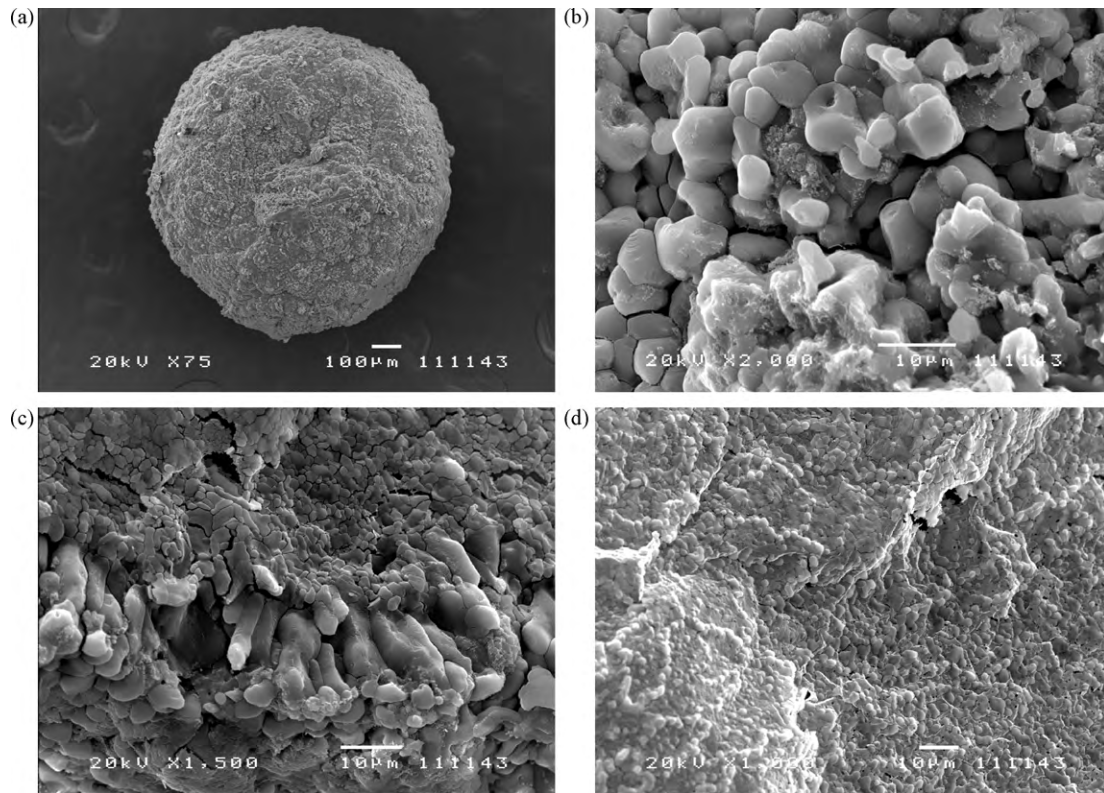


Fig. 2. SEM images of magnetite-containing alginate beads, (a) bead, (b) external surface, (c) fractured section near the surface of the bead, observed radially. Inner region (top half of the figure); external surface (bottom half of the figure) (d) fractured surface near the center of the bead.

The insulin encapsulation efficiency was $33.3 \pm 5.2\%$ and $34.0 \pm 5.0\%$ for alginate and alginate/chitosan beads, for insulin concentration of 10 wt%, respectively. This result indicated that insulin was not lost from beads during the procedure of alginate beads

coating with chitosan. The low insulin entrapment was due to insufficient cross-linking of alginate network which permitted the diffusion of insulin out from beads. The strength of the polymer cross-linking is related to the G/M ratio. Moreover, we must consider the insulin hydrophilic in nature and the small molecule size (6000 Da) which contribute to its diffusion out of the polymer structure. Gaumann et al. demonstrated the molecular weight cut-off in alginate capsules should be in the range of 60 000–200 000 Da [19].

The beads prepared with chitosan showed a tendency to agglomerate due to the adhesive properties of chitosan. Therefore constantly stirring was employed during the process to maintain the beads deagglomerated.

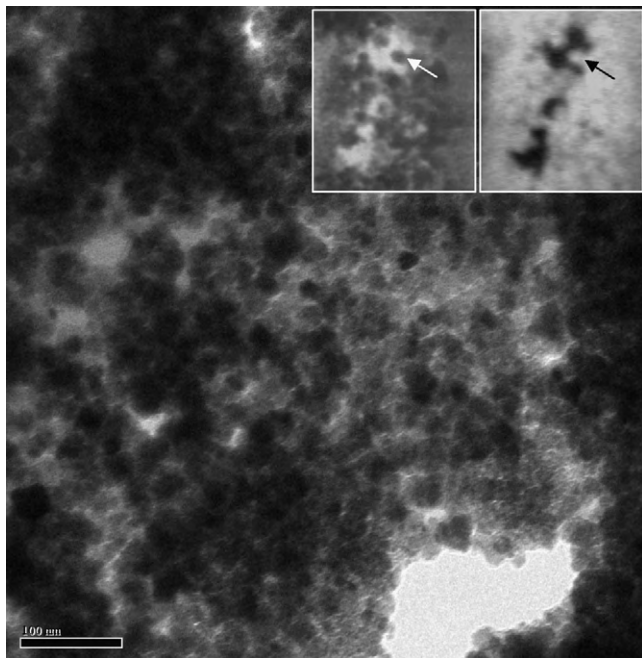


Fig. 3. Ultrathin sections of magnetite nanoparticles into alginate/chitosan beads, observed by TEM. The inserts show: bright field image of individual nanoparticles (left); iron mapping (Fe-L_{2,3} ionization edge) of the same region (right). Note that the nanoparticles, which appear dark in the bright field image, contain iron (they correspond to the white regions in the iron mapping image, e.g. arrows in the figure). Bar = 100 nm.

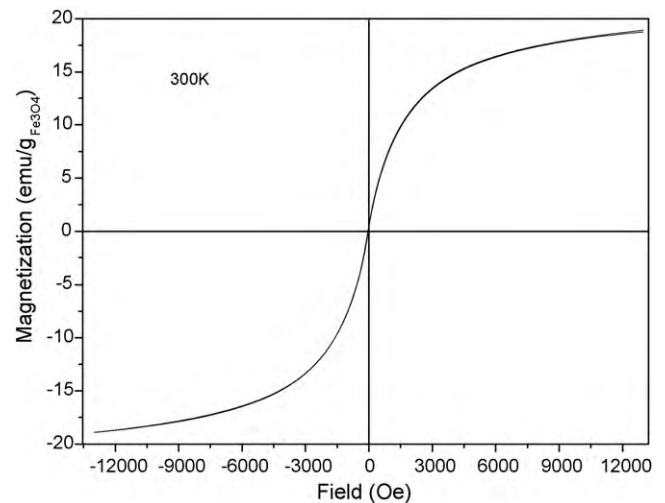


Fig. 4. M(H) loop for sample measured at 300 K. The solid line is the fit to a Langevin function weighted with a lognormal distribution of particle size.

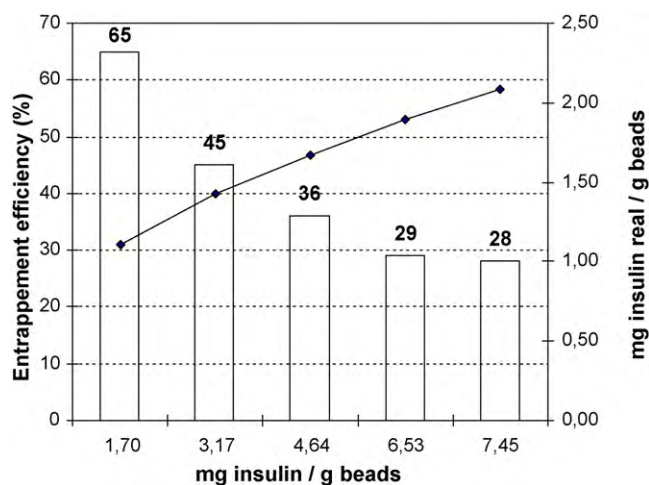


Fig. 5. The efficiency of entrapment of the insulin in alginate/chitosan matrix as a function of insulin content.

Beads containing different insulin contents had been prepared in order to understand the effect of protein concentration on encapsulation efficiency. Fig. 5 shows the relation between the entrapment efficiency and insulin initial concentration in beads. Increasing the insulin content from 1.7 to 7.5 mg/g of beads, the entrapment efficiency decreases from 65% to 28%. However, the total amount of entrapped insulin in the beads increased almost linearly with the protein concentration.

The purpose of these drug delivery systems is to implant the microcapsules subcutaneously, for that reason, the water release medium was chosen, instead of simulated gastric fluid or even a phosphate buffer, which present affinity to calcium and could influence the insulin release by alginate erosion. The release of insulin from alginate beads in Milli-Q water was monitored periodically until its concentration in the solution reached a constant value. It was verified (Fig. 6) that the insulin release occurs at least in three steps: a very fast release of about 18% in the first hour of the assay, which correspond to insulin physically entrapped to bead's external layer. Afterwards the insulin release keeps constant up to 48 h. In the third stage (48–140 h) released insulin reached its maximum value, which corresponds to 45% of the initial amount in the bead. This last stage can be attributed to the diffusion of the protein from the bead interior to the outside. After 140 h of assay, the insulin concentration was constant until the end of the experiment (~800 h).

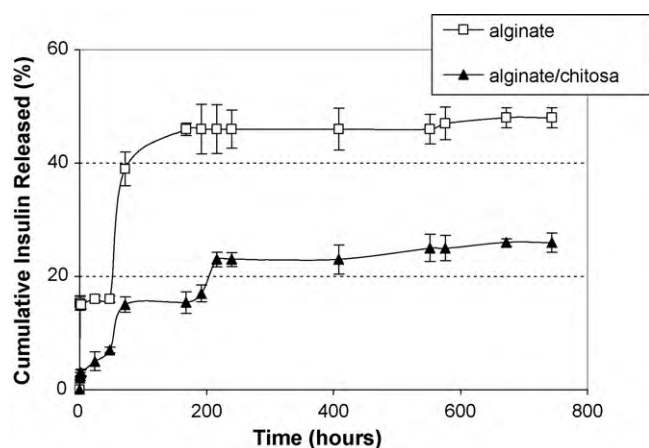


Fig. 6. Insulin release from alginate and alginate/chitosan beads in Milli-Q water under rotation of 100 rpm at 37 °C.

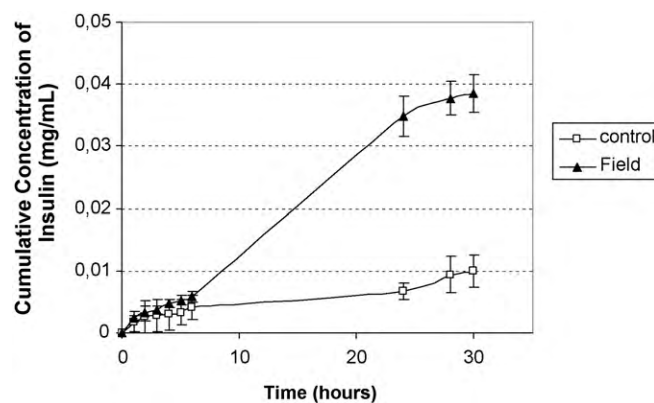


Fig. 7. Insulin controlled release from alginate/chitosan beads containing magnetic nanoparticles. Magnetic field strength of 1800 G and frequency of 33 Hz.

This many-stage release pattern is certainly due to the complexity of beads microstructure as presented before.

The kinetics of insulin release from alginate/chitosan bead showed a similar profile as the alginate beads without chitosan (Fig. 6). However, in this case, a significant reduction in the concentration of insulin released to solution was observed in all the steps of the process. The addition of chitosan provided a coating on the alginate beads surface increasing cross-linking density and decreasing pore size due to the polycationic property of chitosan. The chitosan is bound to alginate through strong ionic interactions between the carboxylate groups of alginate and the protonated amine groups in the chitosan. The consequence of this process was that less insulin was released from alginate/chitosan beads.

Electron paramagnetic resonance were performed previously [17] and indicated that an isolated paramagnetic Fe^{3+} ions may be in a structural site of polymer network probably substituting Ca^{2+} . A similar result was previously observed in alginate polymer cross-linked with Fe^{3+} [20]. The relative fraction of this specie is small because the method used to prepare the samples prioritizes the crosslink of alginate units by Ca^{2+} ions; therefore there is no significance influence in insulin release.

Fig. 7 shows the release of insulin from alginate/chitosan beads containing iron oxide nanoparticles, with and without the application of a magnetic field. As mentioned before the application of an oscillating magnetic field induces an oscillatory movement of magnetite nanoparticles causing a widening of the chains in the structure of the polymer and promoting the insulin release. In the first 6 h the insulin release occurred independently of the application of magnetic field. This behavior corresponds to the protein fraction released from bead external layer (first stage of kinetics). For longer periods a significant increase of insulin release was evidenced in beads subjected to oscillating magnetic field, in comparison to control samples. This result proves the efficiency of the proposed drug delivery system based on the magnetic nanoparticles agitation induced by oscillating magnetic field in an alginate/chitosan matrix.

In order to prove that the insulin was not affected by the process of encapsulation and magnetic nanoparticle synthesis, preliminary *in vivo* studies were carried out. The aim was investigate if the insulin maintained its *in vivo* activity after release, independently of oscillating magnetic field application. Thus, the Fig. 8 shows the glycemia control during 24 h after the microcapsules implant.

Swiss mice were divided into two groups where one group received an implant of microcapsules without insulin (control) and the other group received an implant of microcapsules containing insulin. During 6 h before the surgery, the animals were fed (*ad libitum*). At time zero, the animals were starved, thus the glycemia were reduced in both groups. After 8 h, an increase of glycemia

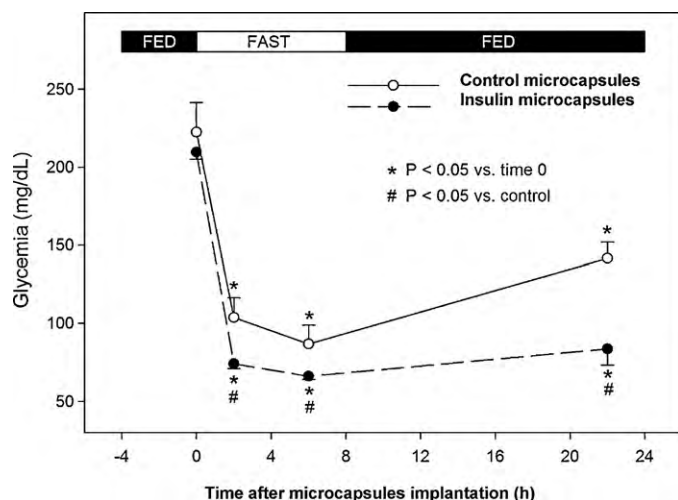


Fig. 8. Blood glucose concentration of control microcapsules (light beads) and insulin microcapsules (black beads) after implantation. Data represent the mean \pm SEM for three mice from each group ($n = 3$). * $P < 0.05$ compared with time zero and # $P < 0.05$ compared with control (Student's t -test).

can be observed because the animals were fed (*ad libitum*) once more, however, this occurred only in the control group. Insulin group maintained a high insulinemia, therefore the glycemia did not rise despite feeding at least for 22 days. Furthermore, this fact confirmed that the protein conformation were not affected by all process involved in the production of magnetic microcapsules.

4. Conclusion

The alginate showed a low efficiency of encapsulation of insulin and this is attributed to the size of pores on the surface, allowing the release of the protein during the gelation and the washing of microcapsules. After treatment with chitosan, there was no reduction in the efficiency of encapsulation.

The alginate beads allowed more release of insulin than alginate/chitosan beads. Thus, the association of these two polymers reinforced the structure of beads and improved their impermeability, decreasing the release of insulin. The association of magnetic nanoparticles to the beads promoted a more intense release profile,

when the oscillating magnetic field was applied. In addition, *in vivo* preliminaries tests established that the insulin released maintained its activity.

The results presented here encourage us to evaluate in deep the influence of the intensity and frequency of the applied field as well as the concentrations of the magnetic nanoparticles, in the release of insulin. This will be carried out by us in the near future.

Acknowledgements

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8.6. APÊNDICE F

Lactate downregulates the glycolytic enzymes hexokinase and phosphofructokinase in diverse tissues from mice



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1. Introduction

In the last century, lactate had been considered as the end product of glycolytic flux with no major metabolic functions other than inducing metabolic acidosis and tissue damage [1,2]. However, in recent years, lactate has been studied based on its ability to serve as an energy source and a cell-signaling and tissue-repairing molecule [1,3]. Chronic hyperlactatemia has been described as an independent risk factor for diabetes development, with lactate being an important factor for maintaining insulin resistance [4,5]. To date, only a few metabolic explanations have been provided for this effect of lactate. Depré et al. reported decreased tissue glucose consumption in the presence of lactate [6]. Lombardi et al. proposed that hyperlactatemia could decrease the GLUT-4 level and glucose uptake by skeletal muscle [7], a rate-limiting step of glucose metabolism in skeletal muscle. In addition, Choi et al. demonstrated that lactate could induce insulin resistance in skeletal muscle by inhibiting glycolytic flux through suppressing insulin signaling [8]. However, they did not specify which step(s) of the glycolytic flux was (were) inhibited. In a previous study, we

demonstrated that lactate could inhibit 6-phosphofructo-1-kinase (PFK, phosphofructokinase), a regulatory enzyme of glycolytic flux, by dissociating the active enzyme tetramers into the less active dimers [9].

This study aimed to contribute to, and to expand the knowledge regarding, the action mechanism of lactate in skeletal muscle, liver, kidney and heart. Our results demonstrate that lactate can inhibit both hexokinase (HK) and PFK, but not pyruvate kinase (PK) in a variety of tissues, supporting the hypothesis of negative regulation of glucose consumption by glycolytic flux downregulation.

2. Materials and methods

2.1. Materials

ATP, fructose-6-phosphate, fructose-2,6-biphosphate (F2,6BP), hexokinase, insulin and glucose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ³²Pi was obtained from the Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). [γ -³²P]ATP was prepared according to Maia et al. [10].

2.2. Mouse tissue homogenates

All mouse Experiments were performed according to the animal experimental protocols. Male Swiss mice (20–25 g) fed *ad libitum*

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were sacrificed by cervical dislocation. The heart, liver, kidney and quadriceps were promptly removed, stripped of fat and connective tissue and incubated in the homogenization buffer consisting of 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 20 mM KF, 0.2 mM β -mercaptoethanol and 0.5 mM PMSF (1:3). The tissues were then treated with or without 5 or 10 mM lactate, depending on the requirements of the experiments.

2.3. Tissue fractionation

Tissue fractionation was performed according to a modification of the Lilling and Beitner protocol [11] proposed by Alves and Sola-Penna [12]. The pH of all tissues homogenate and fractions were controlled before and after the addition of lactate to assure that the pH was controlled.

2.4. Enzymatic activity assays

HK and PFK enzymatic activities were assessed by the radiometric method described by Sola-Penna et al. [13] with the modifications proposed by Zancan and Sola-Penna [14,15]. This assay was performed at 37 °C in a 0.4-ml reaction system containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂ and [γ -³²P]ATP (4 μ Ci/ μ mol). PK was evaluated in a basic medium containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 5 mM phospho(enol)pyruvate (PEP), 5 mM ADP and 120 mM KCl. The reaction was initiated by the addition of enzyme preparation. Aliquots were withdrawn 2, 4, 6, 8 and 10 min after the reaction was initiated and the reaction was stopped by the addition of 0.1 N HCl. The medium was neutralized with NaOH and the ATP content was evaluated using the commercial kit ATPlite 1 step (PerkinElmer, MA, USA). Blanks were performed in parallel in the absence of PEP.

2.5. Intrinsic fluorescence spectroscopy

Intrinsic fluorescence analysis was performed on a spectrofluorimeter (Jasco[®]) in media consisting of 100 mM Tris-HCl (pH 7.4), 5 mM (NH₄)₂SO₄ and purified hexokinase (5 μ g/ml) in the presence or absence of 5 or 10 mM lactate. Respective spectra were subtracted for background and interference correction. The excitation wavelength used was 280 nm, and fluorescence emission was scanned from 300 to 400 nm (0.5 nm intervals at a rate of 100 nm/min). The center of mass of the intrinsic fluorescence spectrum was calculated according to Leite et al. [9] using the SigmaPlot 10.0 (Systat) software.

2.6. Immunoprecipitation and Western blotting

Immunoprecipitation and western blotting were performed as previously described [16], using a polyclonal anti-PFK antibody produced in our laboratory according to Meira et al. [17] and a monoclonal anti-phosphotyrosine antibody (clone pT-154, Sigma Chemical Co, MO, USA). Briefly, homogenates from tissues from mice were treated with or without 10 mM lactate in the presence or absence of 100 nM insulin for 3 h. Subsequently, the treated homogenates were subjected to analysis.

2.7. Statistical analysis

Results are expressed as the mean \pm standard error of the mean (S.E.M.). Statistical analysis was performed using the SigmaPlot software (v.10.0, Systat Inc., CA, USA) integrated with SigmaStat (v. 3.2, Systat Inc. CA, USA). We used the Student *t*-test to compare different groups, and *P* < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Effects of lactate on PFK activity

We assessed the ability of lactate to modulate PFK and found that lactate inhibited PFK activity in the analyzed tissues under several conditions (Fig. 1). Acute exposure to 10 mM lactate exerted no effect on PFK activity in skeletal and cardiac muscle. However, we observed a 20% and 33% reduction in PFK activity in the liver and kidney, respectively. Unlike the acute exposure to 10 mM lactate, lactate preincubation for three hours inhibited PFK activity in skeletal and cardiac muscle. However, in the liver and kidney, lactate preincubation did not cause a further decrease in PFK activity. To evaluate if the ability of lactate to inhibit PFK activity remains in the presence of hormones that stimulate glycolytic flux, isolated mouse tissues were incubated in a buffer containing 4.5 mM glucose and stimulated with 100 nM insulin (Fig. 1). We observed that after insulin exposure, the ability of lactate to inhibit PFK was lost, independent of the analyzed tissue type and time frame. The only exception was found in skeletal muscle, where we observed a 52% reduction in PFK activity in the tissue preincubated with 10 mM lactate, even in the presence of 100 nM insulin (Fig. 1). Insulin can promote an increase in intracellular synthesis of F2,6BP, a known positive allosteric factor of PFK [18,19]. Therefore, it is possible that the increased F2,6BP synthesis triggered by insulin could counterbalance the inhibitory effect of lactate on PFK activity.

To evaluate whether F2,6BP is capable of reversing the inhibitory effect of lactate on PFK activity, isolated mouse tissues were preincubated with or without 10 mM lactate for three hours and subsequently treated with 100 nM F2,6BP (Fig. 2). As expected, PFK was activated when tissues were treated with 100 nM F2,6BP. As shown in Fig. 2, PFK activity in skeletal muscle, heart, liver and kidney was increased by approximately 148%, 156%, 132% and 134%, respectively. However, the stimulatory effect of F2,6BP on PFK activity was decreased by preincubating the tissues with lactate for three hours. Under these conditions, PFK activity in skeletal muscle, heart, liver and kidney was approximately 70%, 121%, 104% and 96%, respectively, of the levels observed in control samples. Only in skeletal muscle was the PFK activity significantly lower than that in the control samples.

It has been shown that PFK is regulated by different protein kinases capable of phosphorylating its serine, threonine and/or tyrosine residues [20–22]. Therefore, we proceeded to examine if lactate is capable of regulating the phosphorylation levels of these residues in PFK. For this purpose, skeletal and cardiac muscle, as well as liver and kidney tissues, was incubated for three hours with 10 mM lactate or 100 nM insulin (positive control) for evaluating the PFK phosphorylation levels. As expected, insulin stimulation led to an increase in PFK phosphorylation in all the analyzed tissues (Fig. 3). The representative western blot in Fig. 3 showed that previous exposure of the tissues to 10 mM lactate for three hours caused a 40% decrease in PFK tyrosine phosphorylation level only in skeletal muscle. Phosphorylation of serine and threonine residues, however, was not altered under these conditions independent of the analyzed tissue type (data not shown). These data indicate that lactate can inhibit PFK activity through both a direct effect independent of the insulin signaling cascade (by inhibiting PFK phosphorylation) and an antagonistic effect to the insulin signaling cascade (by counteracting the effects of F2,6BP).

3.2. Effects of lactate on hexokinase/glucokinase activity

Another possible action mechanism of glycolytic flux inhibition by lactate is its ability to modulate hexokinase, another enzyme involved in controlling the flux. To evaluate the role of lactate in

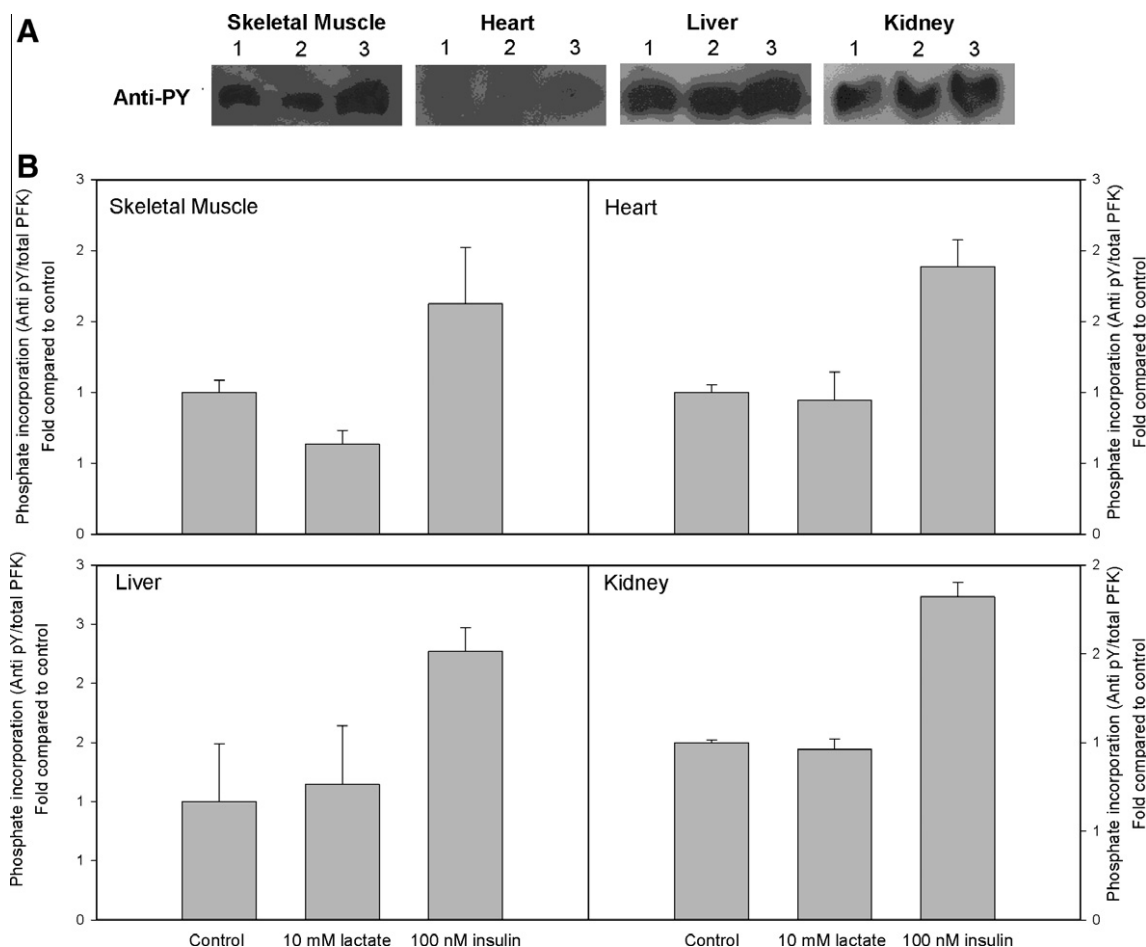


Fig. 3. Effect of lactate on phosphorylation level of PFK tyrosine residues. Tissues were isolated and preincubated for 3 h in the presence or absence of 10 mM lactate and/or 100 nM insulin for analyzing the phosphorylation levels of PFK tyrosine residues. (A) Western blot of different tissues blotted with an anti-phosphotyrosine antibody (1: control; 2: 10 mM lactate; 3: 100 nM insulin). (B) Phosphate incorporation in tyrosine residues normalized to total PFK. Exposure to 10 mM lactate for 3 h decreased the overall phosphorylation level of PFK tyrosine residues in skeletal muscle. Insulin treatment promotes an increase in the phosphorylation level of PFK tyrosine residues in all the analyzed tissues.

To examine the mechanism by which lactate inhibits hexokinase and glucokinase activity, we evaluated its ability to modulate the intracellular sublocalization of these enzymes. The binding of hexokinase I to mitochondria renders the enzyme less susceptible to the inhibition by its product [6]. In order to evaluate the cellular distribution of hexokinase in the presence of 10 mM of lactate, mouse tissues were isolated and subjected to differential centrifugation. We analyzed the enzymatic activity in two fractions, total homogenate (TH) and the mitochondria and nuclear compartment-enriched fraction (P1). As shown in Fig. 4C, lactate inhibited hexokinase activity in TH from skeletal muscle and liver, which was likely associated with cellular redistribution of hexokinase because we observed significant hexokinase inhibition in the P1 fraction from both tissues (Fig. 4C). Lactate did not modulate the activity or the cellular distribution of hexokinase in the heart and kidney (data not shown).

We next evaluated the ability of lactate to inhibit the hexokinase activity stimulated by increasing concentrations of the P1 fraction. For this purpose, the activity of purified hexokinase was determined in the presence or absence of 10 mM lactate in the P1 fraction from skeletal muscle, heart, liver and kidney. Fig. 5 showed the activity of purified hexokinase in the presence of the mitochondria-enriched P1 fraction from skeletal muscle. We noted that even the P1 fraction at the lowest analyzed concentration (1 μ g/ml) was sufficient to saturate the activity of the purified

hexokinase. The same activation pattern was observed in all the analyzed tissues (data not shown). We suggest that binding of the enzyme to mitochondria and the subsequent auto-activation caused the 4.5-fold increase in activity.

We further evaluated whether the hexokinase inhibition by lactate is direct, similar to that of PFK inhibition [9]. For this purpose, we performed an assay using purified hexokinase in the presence of different concentrations of glucose in the presence or absence of 10 mM lactate (Fig. 6A). The data showed that lactate did not modulate the activity of the purified hexokinase because it did not alter its affinity for glucose or ATP (data not shown). Together with the data shown in Figs. 4 and 5, these results suggest that lactate inhibits hexokinase activity by dissociating the enzyme from mitochondria or nuclei, rather than by a direct inhibition. Even though lactate does not directly inhibit hexokinase, it could indirectly inhibit hexokinase activity by mobilizing it to the soluble fraction. It is possible that lactate could cause enzyme dissociation through altering the structure of hexokinase. To test this hypothesis, we measured the center of mass of the intrinsic fluorescence spectrum of purified hexokinase in the presence or absence of 10 mM lactate (Fig. 6B). This technique is commonly used to evaluate the transitions between the intermediate states of proteins in response, for instance, to ligands [23] or different enzymatic concentrations [24,25]. As shown in Fig. 6B, lactate could indeed alter the structure of hexokinase. This structural alteration was not

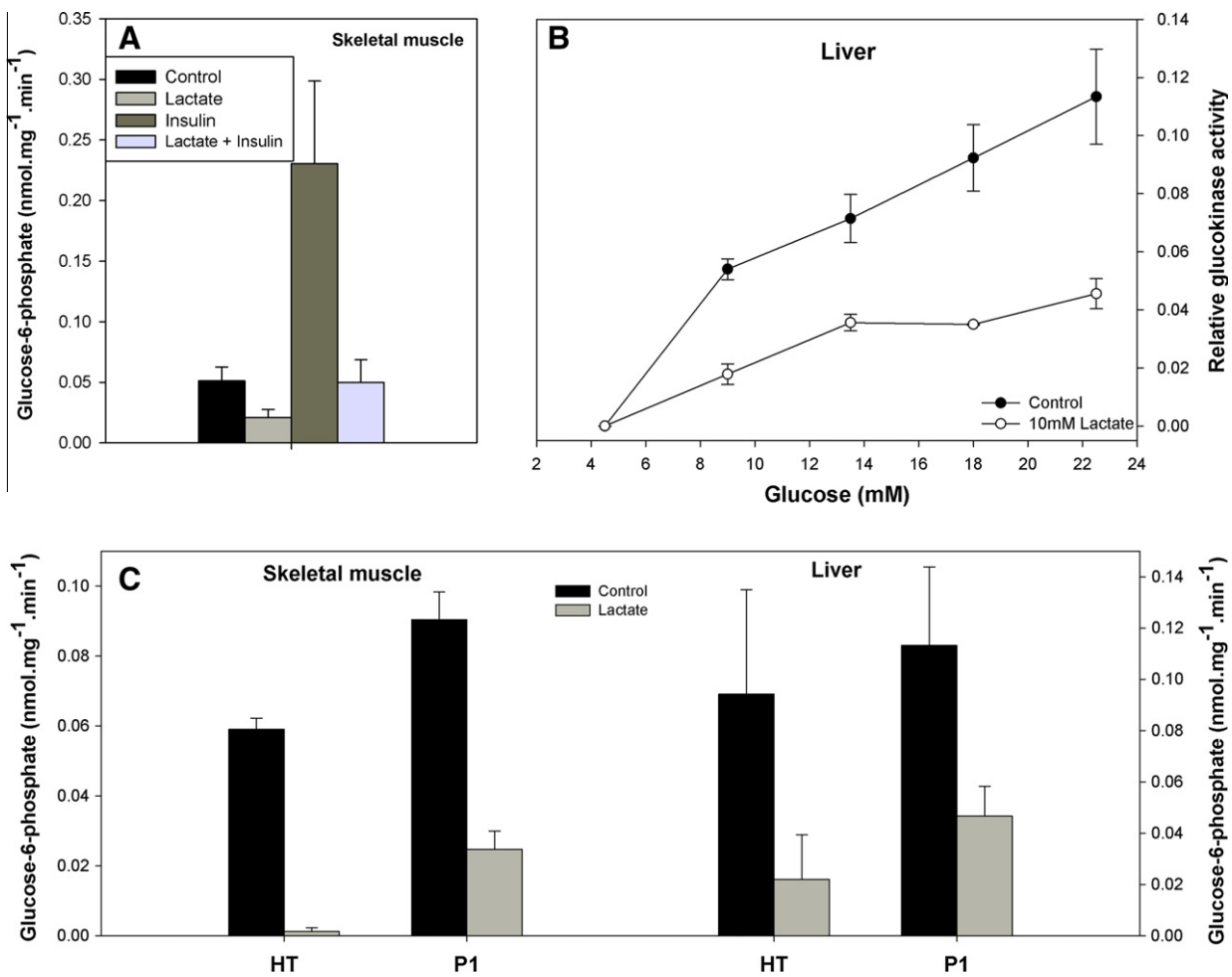


Fig. 4. Effect of lactate on the activity and cellular distribution of hexokinase and glucokinase. HK or glucokinase activity was assessed as described in Section 2 in skeletal muscle (A) and liver (B), respectively. Panel C shows the results after fractionation. Values are presented as the mean \pm S.E.M. of six independent assays.

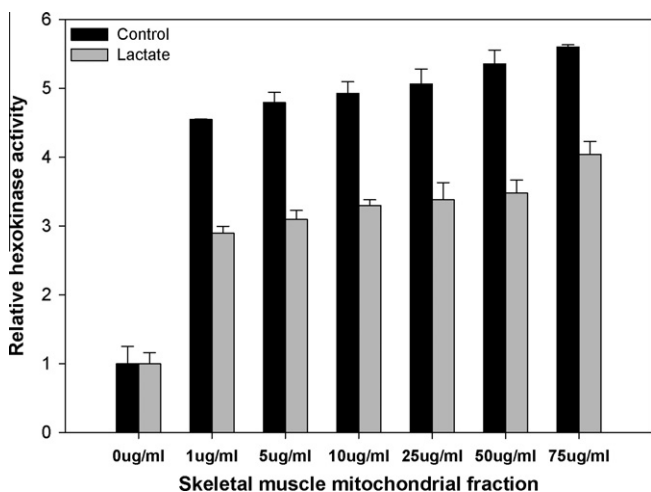


Fig. 5. Effect of lactate on the association of purified hexokinase to mitochondria in skeletal muscle. HK activity was assessed as described in Section 2. Values are presented as the mean \pm S.E.M. of three independent assays.

caused by protein denaturation because the enzymatic activity was preserved under these experimental conditions (Fig. 6A). Although these results do not provide a definitive explanation, they suggest that lactate can alter the structure of hexokinase and that this

modification may lead to the dissociation of the enzyme from mitochondria and nuclei, which, in turn, causes its inhibition.

The effects of lactate on PK activity was also evaluated. For this purpose, we used an alternative method for the assay of PF activity, instead of the classical coupled enzyme linked assay. This was necessary since the classical method uses lactate dehydrogenase to evaluate the pyruvate formed through its reduction measuring the coupled oxidation of NADH to NAD⁺. However, the presence of lactate in the reaction medium would displace the equilibrium of this reaction in the opposite direction, interfering with the measurements. To solve this issue, we analyzed the ATP formed using a commercial kit. The method was efficient but lactate did not promote any effect on PK activity under all tested conditions (data not shown).

Our results support the hypothesis that lactate is not a mere byproduct of glycolysis without relevant metabolic roles. In fact, several groups have shown that lactate metabolism is highly versatile and associated with several pathological conditions [26–31]. The inhibitory effects of lactate on the enzymes known to be important in regulating glycolytic flux, such as hexokinase and PFK, in tissues, such as skeletal muscle and liver, can have an inhibitory effect on carbohydrate metabolism [14,15,17]. Because skeletal muscle is one of the most important tissues in glucose metabolism, glycolytic flux inhibition caused by lactate in this tissue, even in the presence of insulin, can have a systemic impact on hexose metabolism in the whole body [32]. We suggest that the inhibitory effect of insulin is in part due to (I) antagonistic effects

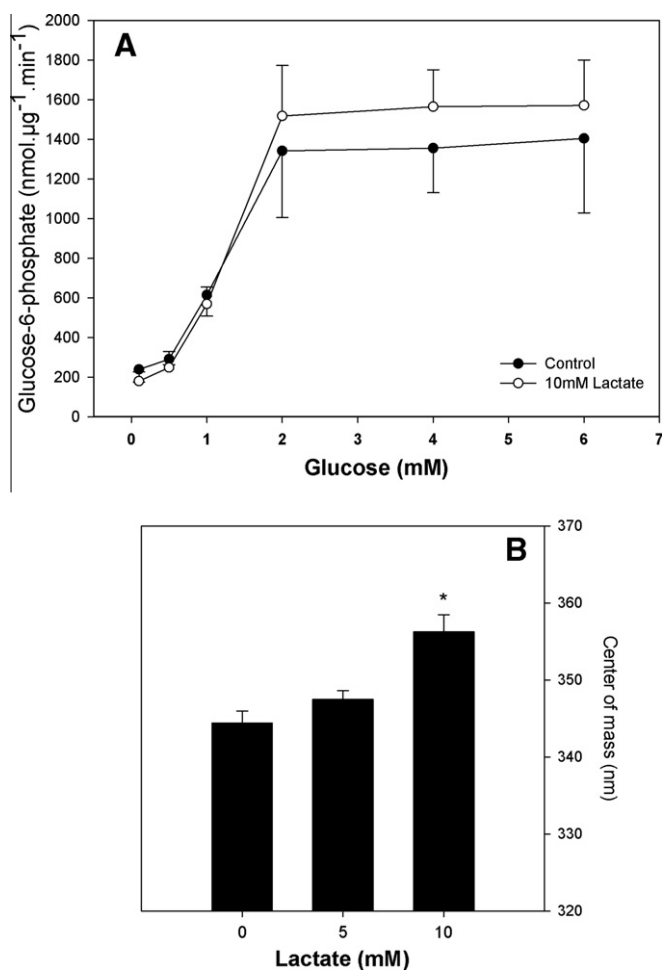


Fig. 6. Effects of lactate on the activity and structure of purified hexokinase. (A) HK activity (A) and intrinsic fluorescence spectra (B) were performed as described in Section 2. Values are presented as the mean \pm S.E.M. of three independent experiments. $P < 0.05$ indicates a statistically significant difference between the experimental group and the control group without lactate.

of lactate on PFK activity when stimulated with fructose-2,6-biphosphate; (II) decreased phosphorylation level of PFK tyrosine residues and (III) PFK dimerization that inhibits its activity [9]. Furthermore, hexokinase inhibition occurs mainly through regulating the association of the enzyme to cellular structures such as mitochondrial and nuclear membranes. There is no direct modulation of hexokinase I by lactate. The modulation of the association with mitochondrial and nuclear membranes is dependent on the alteration of the enzyme structure without protein denaturation, consistent with lactate-induced insulin resistance because the ability of insulin to increase glucose uptake depends on both GLUT4 externalization and increased mobilization of hexokinase to mitochondrial membrane [33], an effect antagonized by lactate. Therefore, we suggest that lactate acts as an antagonist of insulin regarding hexokinase activity and contributes to the decreased glucose consumption by skeletal muscle, a common characteristic under pathological conditions with insulin resistance. In the liver, lactate modulates the major enzymes of the gluconeogenesis pathway, thereby contributing to the hyperglycemia characteristic of diabetes. Finally, our results are in agreement with the theory supported by several groups that lactate should be recognized as an extremely versatile molecule, not simply the end product of fermentation glycolysis.

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8.7. APÊNDICE G

(Aceito) Serotonin modulates hepatic 6-phosphofructo-1-kinase in an insulin synergistic manner



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The International Journal of Biochemistry
& Cell Biologyjournal homepage: www.elsevier.com/locate/biocel1 Serotonin modulates hepatic 6-phosphofructo-1-kinase in an insulin synergistic
2 manner3 **Wagner Santos Coelho^{a,b}, Daniel Da Silva^a, Mônica Mesquita Marinho-Carvalho^a, Mauro Sola-Penna^{a,*}**4 ^a Laboratório de Enzimologia e Controle do Metabolismo (LabECoM), Departamento de Fármacos, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil5 ^b Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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Human and rat hepatic tissue express many serotonin (5-HT) receptor subtypes, such as 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT₇ receptors, which mediate diverse effects. 5-HT is known to regulate several key aspects of liver biology including hepatic blood flow, innervations and wound healing. 5-HT is also known to enhance net glucose uptake during glucose infusion in fasted dogs, but little is known about the ability of 5-HT to control hepatic glucose metabolism, especially glycolysis. This study addresses the potential of 5-HT to regulate PFK activity and the mechanisms related to the enzyme activity. Based on our results, we are the first to provide evidence that 5-HT up-regulates PFK in mouse hepatic tissue. Activation of the enzyme occurs through the 5-HT_{2A} receptor and phospholipase C (PLC), resulting in PFK intracellular redistribution and favoring PFK association to the cytoskeletal f-actin-enriched fractions. Interestingly, 5-HT and insulin act in a synergistic manner, likely because of the ability of insulin to increase fructose-2,6-bisphosphate because the presence of this PFK allosteric regulator enhances the 5-HT effect on the enzyme activity. Together, these data demonstrate the ability of 5-HT to control hepatic glycolysis and present clues about the mechanisms involved in these processes, which may be important in understanding the action of 5-HT during the hepatic wound healing process.

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21 **1. Introduction**

22 Serotonin (5-hydroxytryptamine; 5-HT¹) is a biogenic amine
23 that functions as a ligand for a large family of 5-HT receptors (Hoyer
24 et al., 1994). Enterochromaffin cells of the gastrointestinal tract are
25 responsible for the synthesis of 90% of the total 5-HT in the body
26 (Ruddell et al., 2008). Serotonin is an important neurotransmitter
27 within the central nervous system (CNS) and the autonomic
28 nervous system (ANS), where the systems controlled by 5-HT are
29 determined by the variable expression pattern of 5-HT receptors
30 within the CNS and ANS (Hoyer et al., 1994). In the CNS, serotonin
31 is known to control mood, behavior, learning, sleep and anxiety

(Leysen, 2004; Harvey et al., 2004). Peripherally, serotonin is able
to mediate vascular contraction and relaxation, gastrointestinal
motility, cell proliferation, apoptosis and platelet aggregation (Roth
et al., 1998).

Human hepatic metabolic functions are known to be regulated
by both sympathetic (Cruise et al., 1987) and parasympathetic
(Kato and Shimazu, 1983) branches of the ANS. Serotonergic nerve
fibers are part of the peptidergic family of the ANS, localized in
the tunica media on branches of the hepatic artery, portal vein,
bile ducts and the connective tissue of the interlobular septa from
humans (el-Salhy et al., 1993) and rats (Stoyanova, 2004). Serotonin
presents the ability to elicit portal resistance and thereby
regulate portal vein pressure (Richardson and Withrington, 1977;
Cummings et al., 1986). The effects of serotonin on isolated mesenteric
veins are blocked by ketanserin, a known 5-HT_{2A} antagonist,
suggesting the involvement of the 5-HT_{2A} receptor (Cummings
et al., 1986). The hepatic stellate cell (HSC) system, which is pro-
posed to regulate hepatic blood flow, undergoes activation and
acquires a smooth muscle cell-like phenotype with enhanced
contractile capabilities in response to liver injury, has been demon-
strated to express functional 5-HT_{2A} and 5-HT_{2B} receptors (Ruddell
et al., 2006). Sinusoidal endothelial cells (SECs) also respond to a

Abbreviations: 5-HT, serotonin; 5-HT_{2A}, subtype 2A of the serotonin receptor; PI3K, phosphatidylinositol 3 kinase; PFK, 6-phosphofructo-1-kinase; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; F2,6BP, fructose-2,6-bisphosphate; PKC, protein kinase C; PLC, phospholipase C.

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54 5-HT-mediated stimulus (Brauneis et al., 1992, Gatmaitan et al.,
55 1996), which induces the activation of the myosin light chain
56 through rapid influx of extracellular Ca^{2+} (Gatmaitan et al., 1996).
57 5-HT also inhibits cAMP synthesis and activates phospholipase A2
58 in these cells (Gatmaitan et al., 1996). In fact, many studies have
59 demonstrated the responsiveness of the human hepatic vascula-
60 ture to serotonin and ketanserin (Hadengue et al., 1987, 1989; Islam
61 et al., 2000; Vorobioff et al., 1989), where serotonin plays a role pro-
62 moting the contraction of the human hepatic artery (Islam et al.,
63 2000).

64 The liver demonstrates a remarkable capacity for regeneration.
65 Following a 70% partial hepatectomy (PHx), rodents are able to
66 almost completely restore the lost mass within 14 days (Mabuchi
67 et al., 2004). The process of wound healing is tightly regulated
68 and is known to involve many hepatic and extra-hepatic tis-
69 sues. In addition, recent studies highlighting the importance of
70 serotonin in rodent hepatic wound healing after injury (Kulinskii
71 et al., 1983a,b,c) led to increased interest in understanding the
72 role of 5-HT in hepatic regeneration. It has been shown that sero-
73 tonin elevates cAMP and cGMP within rat hepatocytes (Aslamova
74 et al., 1985) and enhances hepatic poly-(A) RNA synthesis in nor-
75 mal healthy Wistar rats (Roy et al., 1985). Ketanserin has been
76 demonstrated to be a potent inhibitor of liver regeneration and
77 hepatocyte proliferation following PHx (Papadimas et al., 2006).
78 Platelets have been identified as the major source of serotonin,
79 which drives liver regeneration in a process that is mimicked
80 by the 5-HT₂ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI)
81 and antagonized by ketanserin (Lesurtel et al., 2006). Again, these
82 observations suggest a key role for the 5-HT₂ family of receptors
83 in mediating hepatic regeneration, likely through 5-HT mitoge-
84 nic effects (Lesurtel et al., 2006; Hsu, 1995). However, the
85 mechanisms underlying these observations are yet to be fully
86 explored.

87 Serotonin acts within the liver, on the vessels supplying the
88 liver with blood, and within the serotonergic nerve connections
89 that regulate liver function (Ruddell et al., 2008). However, little
90 is known about the actions of serotonin on carbohydrate metabolism
91 in the liver. Moore and colleagues (Moore et al., 2004a,b, 2005b)
92 conducted a series of studies and demonstrated enhanced hepatic
93 glucose uptake after portal infusion of 5-HT or inhibitors of
94 serotonin uptake. They further reported that fluvoxamine, a selec-
95 tive serotonin reuptake inhibitor, increases net hepatic glucose
96 uptake, enhancing hepatic glycogen storage under condition of
97 hyperglycemia and hyperinsulinemia, but not under euinsulinemic
98 conditions (Moore et al., 2005a). Nevertheless, there have no stud-
99 ies that investigated the effects of serotonin upon hepatic glycolysis
100 control.

101 In this study, we demonstrate that 5-HT up-regulates
102 6-phosphofructo-1-kinase (PFK; phosphofructokinase; ATP:D-
103 fructose-6-phosphate-1-phosphotransferase; EC 2.7. 1.11), the
104 rate-limiting enzyme of glycolysis, through the 5-HT_{2A} receptor by
105 altering the enzyme cellular localization in an insulin synergistic
106 manner.

107 2. Materials and methods

108 2.1. Materials

109 ATP, fructose-6-phosphate, ketanserin tartrate salt, fructose 2,6
110 bisphosphate (F-2,6-BP), insulin, serotonin, genistein, and wort-
111 mannin were purchased from Sigma Chemical St. Louis, MO, USA.
112 U-73122, phorbol-12-myristate-13-acetate (PMA) was purchased
113 from Calbiochem. ³²Pi was obtained from the Instituto de Pesquisas
114 Energeticas e Nucleares (SP). [γ -³²P]ATP was prepared according
115 to (32a).

116 2.2. Mouse liver homogenate

117 Animal experimentation was conducted according to the Ani-
118 mal Care Procedures. Male Swiss mice (20–25 g) were fed *ad libitum*
119 and killed by cervical dislocation with prompt removal of the liver.

120 Liver samples were weighed and homogenized for 30s in a
121 Polytron (Brinkmann Instruments, Westbury, NY, USA) in the pres-
122 ence of 6 vol of buffer containing 100 mM Tris-HCl, pH 7.4, 30 mM
123 KF (potassium fluoride), 4 mM EDTA, 1.5 mM 2-mercaptoethanol
124 and 250 mM sucrose, 1 mM sodium pyrophosphate (homogeniz-
125 ing buffer). Homogenized tissues were centrifuged for 10 min at
126 $100 \times g$ at 4 °C to separate cellular debris and non-digested tissues.
127 The resulting supernatant was used for all measured parameters.

128 The protein content measurements were performed according
129 to (Bradford, 1976), and the total protein concentration used in each
130 experiment was 50 $\mu\text{g/ml}$.

131 2.3. Preparation of soluble and bound PFK

132 Cytoskeleton-bound and soluble PFK were separated as
133 described previously (El-Bacha et al., 2003; Gomes Alves and Sola-
134 Penna, 2003). Tissues were homogenized as described above, and
135 they were centrifuged for 10 min at $100 \times g$ at 4 °C to separate the
136 cellular debris and the non-digested tissues. The resulting super-
137 natant, which we named total homogenate (TH), was centrifuged
138 for 30 min at $27,000 \times g$ at 4 °C. The resulting low-speed pellet,
139 P1, is a fraction that is enriched with mitochondria, lysosomes,
140 and nuclear components. The low-speed supernatant, S1, contain-
141 ing most of cytoplasmic structures was centrifuged for 30 min
142 at $120,000 \times g$ at 4 °C. The resulting high-speed supernatant, S2,
143 retains most of the soluble structures and the high-speed pellet,
144 P2, contains the microsomal fraction and the cytoskeleton compo-
145 nents as described previously (El-Bacha et al., 2003; Gomes Alves
146 and Sola-Penna, 2003). All fractions (TH, S1, P1, S2 and P2) were
147 assayed for PFK activity and protein concentration.

148 2.4. Radioassay for PFK activity

149 PFK activity was measured using the method described by
150 (Sola-Penna et al., 2002), with the modifications introduced by
151 (Zancan and Sola-Penna, 2005a) in medium containing 50 mM
152 Tris-HCl (pH 7.4), 5 mM (NH₄)₂SO₄, 5 mM MgCl₂, and 1 mM [γ -
153 ³²P]ATP (4 $\mu\text{Ci}/\mu\text{mol}$), 1 mM F6P was added after incubation in
154 the appropriate conditions and length of time as indicated in the
155 figures. The reaction was started with the addition of enough
156 muscle homogenate to reach a final concentration of 100 μg pro-
157 tein/ml. The reaction was ended by the addition of a suspension
158 of activated charcoal in 0.1 M HCl and 0.5 M mannitol. In addi-
159 tion, after centrifugation (10 min, $27,000 \times g$), the supernatant
160 containing [1 -³²P]fructose-1,6-bisphosphate was analyzed in a liq-
161 uid scintillation counter. Appropriate blanks (samples without
162 fructose-6-phosphate) were measured, and their values were sub-
163 tracted from all measurements to account for any ATP hydrolysis.
164 One unit of PFK was attributed to the formation rate of 1 nmol of
165 fructose-1,6-bisphosphate per minute.

166 3. Results

167 Incubation of mouse liver samples with 5-HT results in a signif-
168 icant upregulation of enzyme activity on TH in a dose-dependent
169 manner (Fig. 1b). The maximum effect is observed with a dose of
170 10 μM 5-HT. At this dose, PFK activity was increased $50.13 \pm 8.91\%$
171 compared to control ($p < 0.05$, Student's *t*-test). We next incubated
172 the liver homogenate with 10 μM 5-HT for 60 min, and aliquots
173 were retrieved from the samples throughout the incubation at the

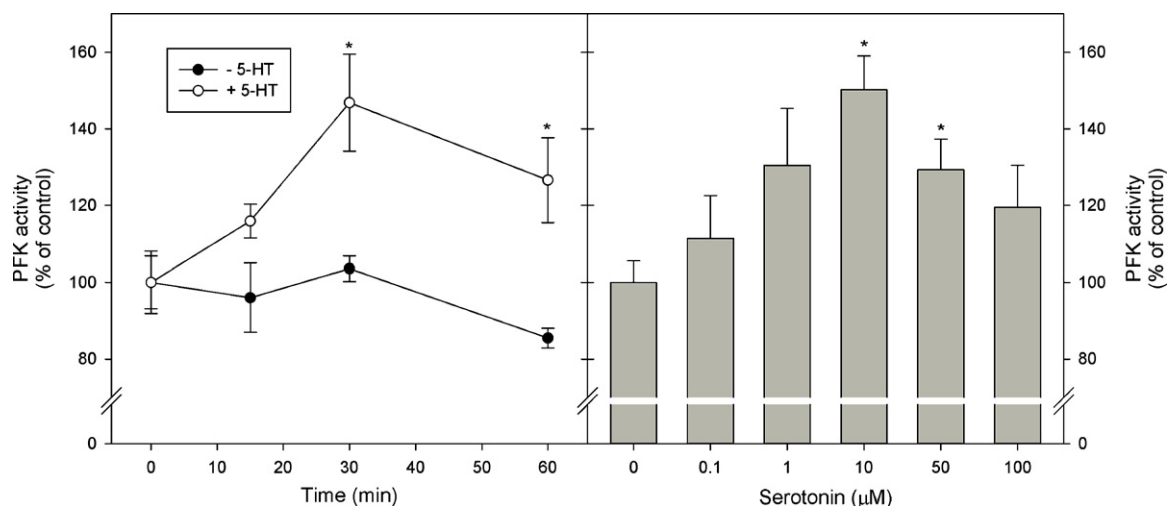


Fig. 1. The effects of 5-HT on PFK activity in mouse hepatic tissue is time and dose dependent. PFK activity was measured as described in the Section 2. The assay was initiated by the addition of hepatic homogenates, which were preincubated with the conditions indicated in the panels, to a final concentration of 50 $\mu\text{g}/\text{ml}$. The reaction was ended after 6 min when the formation of the product was linear (first order kinetics). The data are plotted as mean \pm standard error of 4 independent experiments ($n=4$). (a) The presence of 10 μM 5-HT (open circles) up-regulates PFK activity after a 30 min incubation ($*p < 0.05$ vs. control). (b) The effects of 5-HT are dose-dependent and promote PFK activation at concentrations between 10 μM and 50 μM 5-HT ($*p < 0.05$ vs. control).

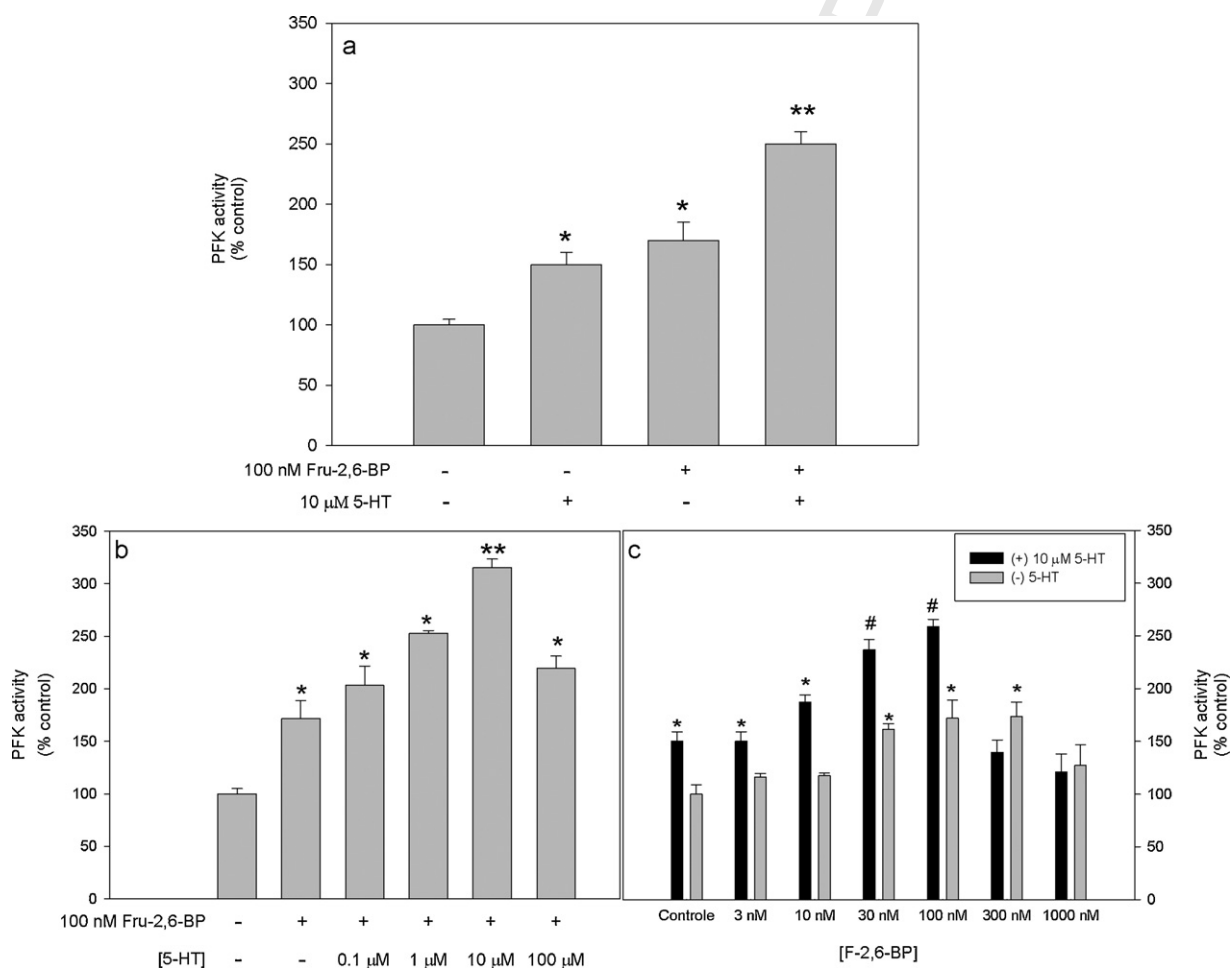


Fig. 2. The synergistic effects of 5-HT and F-2,6-BP on PFK activity are shown. Hepatic mouse samples were preincubated for 30 min in the conditions indicated in the figures. PFK activity was measured as described in the Section 2. The data are plotted as mean \pm standard error of 6 independent experiments ($n=6$). (a) Simultaneous stimulation with 10 μM 5-HT and 100 nM F-2,6-BP upregulates PFK activity compared to control or the action of either alone. (b) Dose dependent response of 5-HT upon PFK activity in the presence of 100 nM F-2,6-BP ($*p < 0.05$ vs. control; $**p < 0.05$ vs. 5-HT or F-2,6-BP alone). (c) The activity of PFK in response to various doses of F-2,6-BP activity is plotted (black bars: with, or gray bars: without) ($\#p < 0.05$ vs. respective gray bar).

times indicated in Fig. 1a to measure PFK activity. The effects of 5-HT on PFK activity are time dependent until 30 min of incubation, and the increased enzyme activity remains statistically significant compared to control until 60 min ($p < 0.05$, Student's *t*-test). These results indicate that the effects of 5-HT on liver PFK activity require at least 30 min of hormonal exposure, with the maximum effect observed in the presence of $10 \mu\text{M}$ 5-HT. Therefore, subsequent experiments were conducted under these conditions. When the liver samples were exposed to 100 nM F-2,6-BP, the PFK activity increased significantly when compared to the control ($p < 0.05$, Student's *t*-test), as expected. The order of magnitude of this increase is similar to the increase observed after a 5-HT stimulus. Curiously, when both conditions were simultaneously added to the liver preparations, they acted synergistically and significantly increase ($p < 0.05$, Student's *t*-test) the PFK activity beyond the activation observed with either stimulus alone (Fig. 2a). To gain further insight on the synergistic action of 5-HT and F-2,6-BP on hepatic PFK activity, we preincubated increasing doses of 5-HT in the absence or presence of 100 nM F-2,6-BP for 30 min and assayed enzymatic activity (Fig. 2b). We observed an incremental increase in the positive effect of F-2,6-BP over PFK activity that was dependent on the dose of 5-HT. The effect of $10 \mu\text{M}$ 5-HT in the presence of 100 nM F-2,6-BP increased PFK activity approximately $215 \pm 8\%$ compared to control, and $143 \pm 8\%$ compared to the action of F-2,6-BP alone. We next assayed PFK activity in the presence of increasing concentrations of F-2,6-BP in the absence or presence of $10 \mu\text{M}$ 5-HT. We demonstrated that F-2,6-BP significantly up-regulates PFK activity when the concentration is between 30 nM and 300 nM . When $10 \mu\text{M}$ 5-HT was added to the samples, we observe a synergistic effect on PFK activity in the presence of 30 nM and 100 nM of F-2,6-BP. Interestingly, at high concentrations of F-2,6-BP, 5-HT does not increase PFK activity when compared to negative control (gray bar Fig. 2c).

F-2,6-BP is recognized as the most potent activator of PFK (Hers and Van Schaftingen, 1982; Uyeda et al., 1981), and its effects are not restricted to PFK control because this modulator also plays a role in regulating gluconeogenesis enzymes (Pilkis et al., 1981; Van Schaftingen and Hers, 1981). Hepatic levels of F-2,6-BP vary according to the metabolic state of the organism, such as during fasting or post prandial periods, which reflects insulinemia and glucagonemia. Insulin has the ability to control hepatic glycolytic flux through the control of the bi-functional enzyme PFK-2, leading to F-2,6-BP production and PFK activation (Okar et al., 2001, 2004). Therefore, we investigated PFK activity after exposure to 5-HT in the presence of 100 nM insulin. The results obtained from these experiments were compared to the dose-dependent effect described previously for the serotonergic stimulus and were also compared to the action of 100 nM insulin alone, which acted as the positive control. These results reveal that when both hormones are preincubated in the hepatic tissue, the enzyme activity rises significantly above that observed for the action of either hormone alone (Fig. 3) suggesting a synergistic action between 5-HT and insulin in the control of PFK activity.

To understand whether the PFK activation mediated by 5-HT involves the cellular redistribution of the enzyme, we fractionated liver TH and measured the effects of $10 \mu\text{M}$ 5-HT and F-2,6-BP on PFK activity in the soluble fraction (S2) and the microsomal fraction, which contains most of the cytoskeleton components (El-Bacha et al., 2003; Gomes Alves and Sola-Penna, 2003). As shown in Fig. 4, treatment with $10 \mu\text{M}$ 5-HT favors the association of PFK to the cytoskeleton resulting in a significant increase in PFK activity in the particulate fraction when compared to the soluble fraction and to the control ($p < 0.05$, Student's *t*-test). F-2,6BP alone did not promote PFK redistribution, and both stimuli together did not induce the effect observed with 5-HT alone, indicating that the redistribution of the enzyme is not an important event to explain the

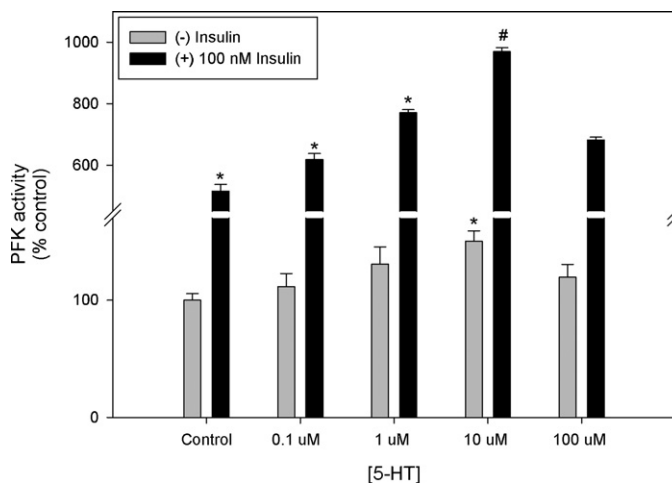


Fig. 3. The synergistic effects of 5-HT and insulin on PFK activity are shown. Homogenates of mouse muscles were assayed for PFK activity after incubation with 5-HT alone, at the concentration indicated in the figure (black bars), or with the addition of 100 nM insulin (gray bars). The data are plotted as mean \pm standard error of 6 independent experiments ($n = 6$). Insulin upregulates hepatic PFK activity, and acts synergistically with 5-HT to activate the enzyme (* $p < 0.05$ vs. control; # $p < 0.05$ vs. respective black bar).

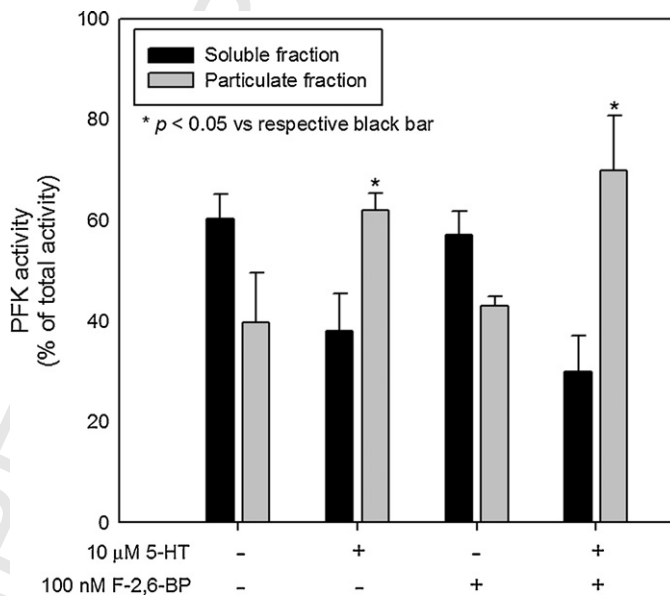


Fig. 4. The effects of 5-HT on PFK cellular localization are described. Mouse hepatic homogenates were incubated in the absence (control) or in the presence of $10 \mu\text{M}$ 5-HT, 100 nM F-2,6BP, or both for 30 min, subjected to ultracentrifugation, and the soluble and particulate fractions were assayed for PFK activity as described in the Section 2. The data are plotted as mean \pm standard error of 4 independent experiments ($n = 4$), and they are represented as total PFK activity relative to control (total PFK activity was $235 \pm 31 \text{ mU/mg}$). Black bars: PFK activity in the soluble fractions. Gray bars: PFK activity in the particulate fractions. 5-HT promotes an increase in the total enzyme activity in the particulate fraction, an effect not observed for the F-2,6-BP stimulus alone or in the presence of 5-HT (* $p < 0.05$ vs. corresponding black bar).

synergistic effect between 5-HT and F-2,6-BP in the regulation of PFK activity.

We next stimulated the hepatic tissue with $10 \mu\text{M}$ 5-HT and challenged it with ketanserin, a 5-HT_{2A} receptor antagonist. The presence of $1 \mu\text{M}$ of ketanserin reverts the action of 5-HT upon PFK activity, either alone or in the presence of F-2,6-BP. However, ketanserin could not abolish the activation of F-2,6-BP during the activation of the enzyme (Fig. 5). Interestingly, the effect of ketanserin in the presence of 5-HT significantly reduced the

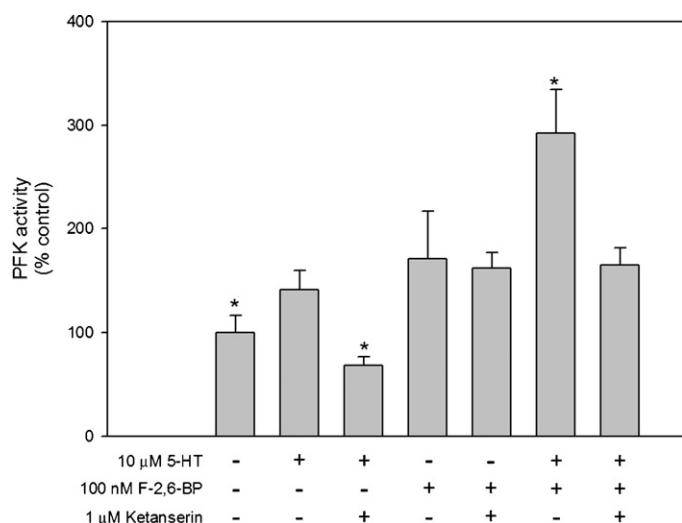


Fig. 5. The effects of 5-HT on PFK of mouse hepatic tissues is mediated through the 5-HT_{2A} receptor. Mouse hepatic tissue homogenates were assayed for PFK activity after incubation with 5-HT or F-2,6-BP, in the absence and presence of ketanserin (a 5-HT_{2A} receptor antagonist). The data are plotted as mean ± standard error of 5 independent experiments (n=5). PFK activity is expressed as fold change over activity of the control in the absence of additives. *Statistically significant difference compared to the control (p < 0.05, Student's t-test).

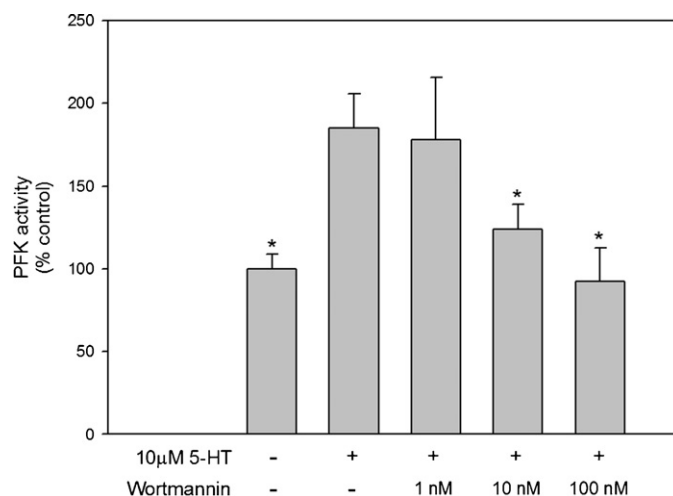


Fig. 7. 5-HT induced PFK activation is dependent on PI3K. Mouse hepatic tissue homogenates were assayed for PFK activity after incubation with 5-HT, in the absence and presence of wortmannin (1 nM, 10 nM and 50 nM), a PI3K inhibitor. The data are plotted as the mean ± standard error of 4 independent experiments (n=4). PFK activity is expressed as fold change relative to the activity of the control in the absence of additives. *Statistically significant difference compared to stimulation by 5-HT (p < 0.05, Student's t-test).

enzymatic activity below control levels, likely due to the intrinsic action of the 5-HT receptor subtype. When the skeletal muscle isoform, 5-HT_{2A}, is activated by 5-HT, it leads to PFK activation by a pathway that is dependent on tyrosine kinases (Coelho et al., 2007). Therefore, we treated hepatic homogenates for 30 min with 5-HT and genistein, a tyrosine kinase inhibitor, and then measured PFK activity. Genistein treatments between 10 μM and 50 μM were able to limit the action of 5-HT on PFK activity to control the level of activity (Fig. 6). We next investigated whether PI3K plays a role as a signaling component of the 5-HT and PFK-induced activation. To test this hypothesis, we preincubated the samples with wortmannin, a PI3K inhibitor. The presence of wortmannin abolished the action of 5-HT in a dose-dependent manner, where 10 nM of

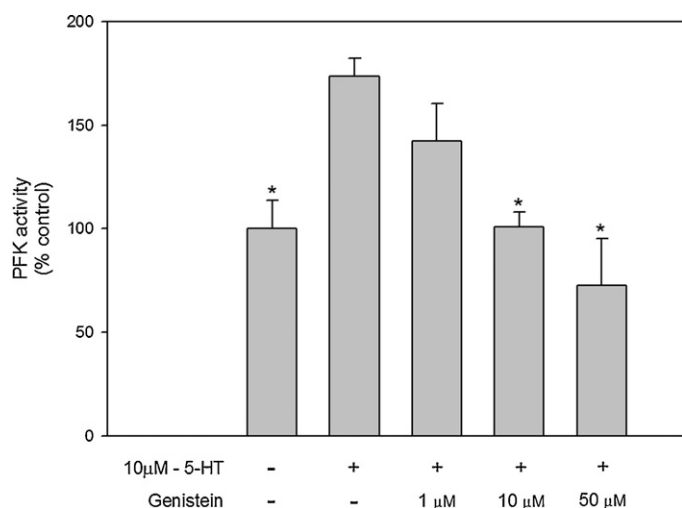


Fig. 6. Inhibition of tyrosine kinases with genistein abrogates the effects of 5-HT on enzyme control. Mouse hepatic tissue homogenates were assayed for PFK activity after incubation with 5-HT, in the absence and presence of genistein (1 μM, 10 μM and 50 μM), a tyrosine kinase inhibitor. The data are plotted as mean ± standard error of 4 independent experiments (n=4). PFK activity is expressed as the fold change relative to the activity of the control in the absence of additives. *Statistically significant difference compared to stimulation with 5-HT (p < 0.05, Student's t-test).

wortmannin partially prevented PFK activation, and concentrations up to 100 nM brought the enzymatic activity to levels seen in the control (Fig. 7). These results suggest that the positive control exerted by 5-HT over PFK is shared with an insulin pathway. However, the synergic effect observed by the stimulus of both hormones together suggests an additive pathway or an alternative mechanism for the activation of the enzyme.

The 5-HT_{2A} receptor subtype is able to induce the activation a PLC. Therefore, we challenged the hepatic tissue in the presence of 10 μM U-73122, a PLC inhibitor, and assayed for PFK activity. These experiments revealed that the action of 5-HT is dependent on PLC activation because the inhibition of this phospholipid reduced the ability of 5-HT to promote enzyme activity (Fig. 8). To further characterize this pathway, we pre treated the tissue preparations with the phorbol ester, PMA, for 30 min, which mimics the action of diacylglycerol (DAG), to activate protein kinase C (PKC). PMA was

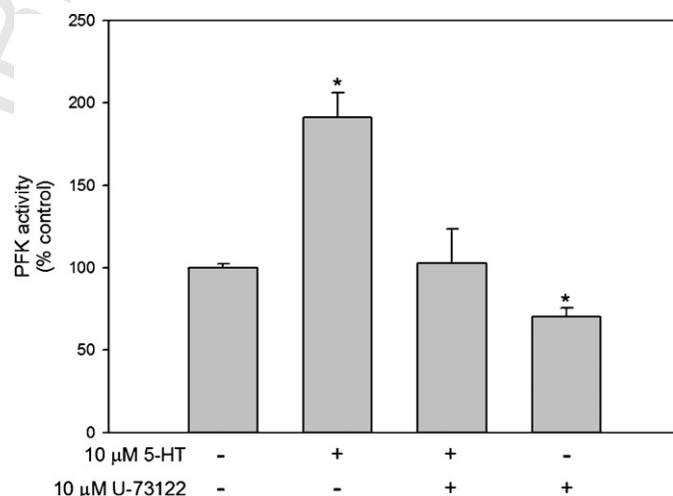


Fig. 8. Inhibition of PLC prevents 5-HT-mediated upregulation of PFK activity. Mouse hepatic tissue samples were incubated with 5-HT in the absence or presence of 10 μM U-73122, a PLC inhibitor, and the activity of PFK was measured as described in the Section 2. The data are plotted as the mean ± standard error of 4 independent experiments (n=4). *Statistically significant difference compared to stimulation with 5-HT (p < 0.05, Student's t-test).

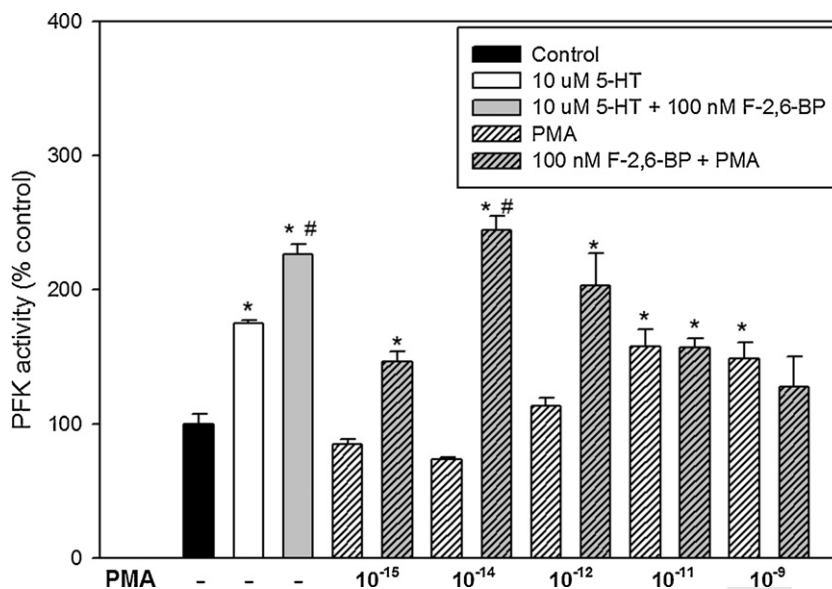


Fig. 9. The phorbol ester mimics, serotonin and serotonin Fru-2,6-BP, show synergistic effects upon the control of PFK activity. Mouse liver samples were pre-incubated for 30 min with PMA alone (white dashed bars), or in the presence of 100 nM F-2,6-BP (gray dashed bars), at the concentrations indicated in the figure. PFK activity was measured as described in the Section 2. The data are plotted as the mean \pm standard error of 6 independent experiments ($n = 6$). *Statistically significant differences compared to control (black bar) ($p < 0.05$, Student's t -test). #Statistically significant differences compared to control (black bar) and to 10 μ M 5-HT (white bar) ($p < 0.05$, Student's t -test).

added to the samples in the concentrations indicated in Fig. 9, in the presence or absence of 100 nM F-2,6-BP. We demonstrated that PMA alone is able to upregulate PFK activity in a dose-dependent manner that is similar to the effect observed with the treatment of 5-HT. Additionally, the presence of PMA acts synergistically with F-2,6-BP to activate PFK and depends on lower concentrations of PMA compared to the action of PMA alone to promote PFK activity.

Together, these results indicate that 5-HT is able to activate hepatic PFK through at least two independent signaling pathways. 5-HT_{2A} is coupled to the activation of PI3K and PLC, which leads to PKC recruitment. Cellular redistribution of PFK is an important mechanism that regulates the enzyme after a 5-HT stimulus and acts in a synergistic manner with insulin.

4. Discussion

Serotonergic nerve fibers have previously been shown to be localized to the tunica media on branches of hepatic artery and portal vein, as well as in bile ducts and the connective tissue of the interlobular septa (el-Salhy et al., 1993). Some authors have provided evidence for 5-HT-mediated liver regeneration (Hsu, 1995; Choi et al., 2002) where it acts as a neurotransmitter and an endocrine hormone (Ruddell et al., 2006). Despite these observations, the importance of serotonergic innervations to the human liver is not completely understood, and very few studies have investigated the effects of serotonin in the control of glucose metabolism. Physiological studies conducted by Moore and colleagues (Moore et al., 2004a,b, 2005a,b) revealed an augmented glucose uptake after 5-HT infusion in the portal vein. Here, we describe for the first time, the role of 5-HT in regulating the glycolytic enzyme PFK in mice hepatic tissue.

Preincubation of liver samples with 10 μ M 5-HT after 30 min of hormone exposure promotes a dose and time-dependent activation of PFK that is significantly increased compared to control. The activation promoted by 5-HT is potentiated by the presence of F-2,6-BP or insulin. Insulin is known to increase liver F-2,6-BP production through its ability to upregulate the bi-functional enzyme PFK-2 (Hamer and Dickson, 1990). These observations suggest that the synergistic effect between 5-HT and insulin is due to increased

levels of F-2,6-BP in hepatic tissue. F-2,6-BP is the most potent allosteric modulator of PFK and plays a crucial role in the control of hepatic glucose metabolism (Okar et al., 2004; Choi et al., 2002; Wu et al., 2006). Additionally, α -metil-5-HT, a 5-HT₁/5-HT₂ receptor agonist, stimulates glycogen synthesis and acts synergistically with the most effective insulin concentration (Hampson et al., 2007). In fact, an increase in hepatic glycolysis promoted by PFK-2 or over expression of glucokinase reduces obesity (Wu et al., 2005), glycemia in diabetes (Choi et al., 2002), and the hepatic production of glucose, which suppresses hepatic insulin resistance in type 2 diabetes (Wu et al., 2002). Similarly, treatment with 5-HT selective reuptake inhibitors improves glucose tolerance in type 2 diabetes (Pestell et al., 1989).

The interaction between PFK and f-actin is promoted by 5-HT and is an important mechanism in the upregulation of the enzyme activity, and this observation is consistent with previous work (Coelho et al., 2007). In addition, many investigators have shown that the ability of PFK to associate with f-actin after a variety of stimuli is a crucial event that precedes enzyme activation (Zancan and Sola-Penna, 2005a,b; Gomes Alves and Sola-Penna, 2003; Assouline-Cohen et al., 1998; Silva et al., 2004; Kuo et al., 1986; Leite et al., 2007; Marinho-Carvalho et al., 2006, 2009; Meira et al., 2005; Real-Hohn et al., 2010; Sola-Penna et al., 2010; Spitz et al., 2009). However, F-2,6-BP did not increase PFK activity in the f-actin enriched fraction, nor did it potentiate 5-HT effect, suggesting that the synergic action of 5-HT and F-2,6-BP likely involves the enzyme redistribution stimulated by 5-HT and the allosteric regulation promoted by F-2,6-BP.

The reported effects of 5-HT on PFK control in hepatic tissue occur after recruitment of the 5-HT_{2A} receptor. Ketanserin abrogates the 5-HT-induced PFK activation in the skeletal muscle (Coelho et al., 2007). The participation of this receptor isotype has been previously described as important for 5-HT to promote liver wound healing after injury (Ruddell et al., 2008). Furthermore, ketanserin is a potent inhibitor of this process (Papadimas et al., 2006). Moreover, the ability of 5-HT to control hepatic blood flow and vascular tonus is reduced after the administration of various 5-HT_{2A} antagonists (Ruddell et al., 2008). Treatment of liver samples with genistein and wortmannin, a tyrosine kinase and a PI3K

inhibitor, respectively, was able to prevent the 5-HT effect, suggesting a role for an insulin mutual pathway that is distinct from the effects of 5-HT in PFK control in skeletal muscle (Coelho et al., 2007). Furthermore, the inhibition of PLC and PKC also abolished the 5-HT effect, and the presence of PMA was able to mimic the 5-HT effect alone; the inhibition also resulted in a synergic activation of PFK when the samples were preincubated simultaneously with PMA and F-2,6-BP. Together, these results suggest that 5-HT-mediated regulation of PFK activity is dependent on a PLC-PKC and a PI3K tyrosine kinase pathway. Furthermore, this role is consistent with the findings of many studies that have correlated the role of these pathway components with the activation of 5-HT_{2A} receptor (Coelho et al., 2007; McKune and Watts, 2001; Ogden et al., 2006; Lu et al., 2008; Honda et al., 1998; Tang and Tan, 2003; Guillet-Deniau et al., 1997; Quinn et al., 2002; Banes et al., 2005; Liu and Fanburg, 2006).

Additional experiments are required to characterize the other pathway elements that may participate in the 5-HT-mediated control of glycolysis. CaMK may be an interesting candidate for future investigation because previous studies have presented evidence of glycolytic enzyme control being modulated by this signaling protein (Mieskes et al., 1987). The liver presents the extraordinary ability to recover from injury (Mabuchi et al., 2004), and many questions related to this topic are yet to be investigated. Although there is a consensus in the literature that 5-HT plays an important role in wound healing processes, growth factor expression, cell proliferation and blood flow regulation (Ruddell et al., 2008), the correlation between the control of hepatic glycolysis and these processes requires more investigation.

In conclusion, we are the first to provide evidence that hepatic PFK control is mediated by 5-HT in an insulin synergic manner requiring the activation of the PI3K and PLC-PKC pathways and that changes in PFK cellular localization is a mechanism of PFK activation.

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8.8. APÊNDICE H

Temperature dependence of cardiac sarcoplasmic reticulum Ca^{2+} -ATPase from rainbow trout
Oncorhynchus mykiss

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Temperature dependence of cardiac sarcoplasmic reticulum Ca^{2+} -ATPase from rainbow trout *Oncorhynchus mykiss*

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In this work, the temperature dependence of the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA2) activity from rainbow trout *Oncorhynchus mykiss* cardiac ventricles was measured and compared with the mammalian SERCA2 isoform. The rate of ATP-dependent Ca^{2+} transport catalysed by *O. mykiss* vesicles was totally abolished by thapsigargin and the Ca^{2+} ionophore A_{23187} . At warm temperatures (25 and 30° C), the SERCA2 from *O. mykiss* ventricles displayed the same rate of Ca^{2+} uptake. At 35° C, the activity of the *O. mykiss* enzyme decreased after 20 min of reaction time. The rate of Ca^{2+} uptake catalysed by the mammalian SERCA2 was temperature dependent exhibiting its maximal activity at 35° C. In contrast to the rate of Ca^{2+} uptake, the rate of ATP hydrolysis catalysed by *O. mykiss* SERCA2 was not significantly different at 25 and 35° C, but the rate of ATP hydrolysis catalysed by the rat *Rattus norvegicus* SERCA2 isoform at 35° C was two-fold higher than at 25° C. At low temperatures (5 to 20° C), the rate of Ca^{2+} uptake from *O. mykiss* SR was less temperature dependent than the *R. norvegicus* isoform, being able to sustain a high activity even at 5° C. The mean \pm s.e. Q_{10} values calculated from 25 to 35° C for ATP hydrolysis were 1.112 ± 0.026 ($n = 3$) and 2.759 ± 0.240 ($n = 5$) for *O. mykiss* and *R. norvegicus*, respectively. Taken together, the results show that the *O. mykiss* SERCA2 was not temperature dependent over the 10 to 25° C temperature interval commonly experienced by the animal *in vivo*. The Q_{10} value of SERCA2 was significantly lower in *O. mykiss* than *R. norvegicus* which may be key for cardiac function over the wide environmental temperatures experienced in this eurythermal fish.

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Key words: ATPase activity; cardiac SERCA2; cold active fish; *R. norvegicus*; ventricles.

INTRODUCTION

During cardiac excitation–contraction (E–C) coupling, Ca^{2+} enters the cell through depolarization-activated L-type Ca^{2+} channels as an inward Ca^{2+} current (I_{Ca}). Sarcolemmal Ca^{2+} entry induces Ca^{2+} release from the sarcoplasmic reticulum (SR)

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through activation of the ryanodine receptor or SR Ca^{2+} channel (RyR 2). Subsequent release of Ca^{2+} raises the free intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ and triggers contraction. For relaxation to occur $[\text{Ca}^{2+}]_i$ must be removed from the cytoplasm, which requires Ca^{2+} transport out of the cytosol by the sarco-endoplasmic reticulum Ca^{2+} -ATPase cardiac isoform (SERCA2), sarcolemmal Na^+ - Ca^{2+} exchange or sarcolemmal Ca^{2+} -ATPase. In mammalian cardiac myocytes, the SERCA2 isoform plays the major role during cardiac muscle relaxation by transporting the Ca^{2+} from the cytosol back into the SR (Carafoli, 1987; Bers, 2002).

In fish hearts, extracellular Ca^{2+} entry through the L-type sarcolemma (SL) channel is generally the major source of Ca^{2+} used during cardiac contraction (Tibbitts *et al.*, 1992; Tiitu & Vornanen, 2001). The SR, however, can play an important role during fish cardiac E-C coupling in some more active tunas, *e.g.* bluefin tuna *Thunnus thynnus* (L. 1758) (Landeira-Fernandez *et al.*, 2004; Castilho *et al.*, 2007; A. M. Landeira-Fernandez, C. Castilho & B. A. Block, unpubl. data) and cold-tolerant species active at low temperatures, *e.g.* rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) and burbot *Lota lota* (L. 1758). Electron microscopy studies have demonstrated the presence of SR in the heart of *T. thynnus* (Di Maio & Block, 2008), *L. lota* (Tiitu & Vornanen, 2002) and *O. mykiss* (Santer, 1985). In these species, blockage of SR Ca^{2+} release channels with ryanodine results in a significant decrease in the force of contraction (Hove-Madsen & Gesser, 1989; Hove-Madsen, 1992; Keen *et al.*, 1992, 1994; Shiels & Farrell, 1997; Hove-Madsen *et al.*, 1998; Shiels *et al.*, 1999; Brill & Bushnell, 2001). Moreover, [^3H]Ryanodine binding experiments and Western blotting analysis have also demonstrated the presence of RyRs in *T. thynnus* (Castilho *et al.*, 2007), lamnid sharks *Lamna ditropis* Hubbs & Follett 1947 (Weng *et al.*, 2005), *O. mykiss*, *L. lota* and in the crucian carp *Carassius carassius* (L. 1758) (Vornanen, 1989; Tiitu & Vornanen, 2003).

Other studies by Shiels & White (2005), using confocal microscopy, demonstrated the temporal and spatial properties of cellular Ca^{2+} flux in *O. mykiss* ventricles and recorded significant changes in the Ca^{2+} transient after SR inhibition by ryanodine and thapsigargin. In addition, caffeine was shown to induce large contractions and Ca^{2+} transients that were 4.5 times greater than those stimulated by field electrodes. These results indicate that *O. mykiss* myocytes not only have Ca^{2+} in the SR, but that the Ca^{2+} is both released from and resequenced by the SR during the cardiac cycle. Recently, a similar functional role for the SR has now been shown in *T. thynnus* by Shiels *et al.* (2011).

The cardiac SERCA2 pump has also been examined in SR vesicles isolated from several fishes of the family Scombridae. In *T. thynnus*, the SERCA2 enzyme present either in ventricle and atrium was four to six-fold higher than in the ectothermic mackerel *Scomber japonicus* Houuttuyn 1782 (Landeira-Fernandez *et al.*, 2004; Castilho *et al.*, 2007). Furthermore, it was also found that the endothermic *L. ditropis* also had high SERCA2 activity in cardiac tissues, similar to mammals at temperatures from 10 up to 25°C (Weng *et al.*, 2005). The results indicate a crucial role of cardiac SERCA2 activity in fishes that have expanded their ecological niche into cooler waters.

The *O. mykiss* is a temperate teleost that is able to maintain a high swimming performance over broad ambient temperatures ranging between 5 and 25°C. The cardiac system has to function across these large ambient temperature ranges maintaining oxygen delivery to the tissues (Threader & Houston, 1983; Matthews & Berg, 1997;

Randall & Brauner, 1998). Aho & Vornanen (1998) first revealed a thapsigargin-sensitive Ca^{2+} -ATPase activity in crude homogenates of fish hearts conducted on *O. mykiss* and *C. carassius*. Experiments performed at 25° C with cold-acclimated *O. mykiss* indicated a two to three times higher Ca^{2+} -ATPase activity than *C. carassius* and almost the same activity as rat *Rattus norvegicus* ventricles. This study first indicated the potential role for a SERCA2 activity in the *O. mykiss* heart.

In this work, the effect of acute temperature changes on the activity of the SERCA2 in vesicles derived from *O. mykiss* ventricles is examined in comparison to mammalian tissues. Although several different physiological and molecular studies have demonstrated a role of SR during the *O. mykiss* cardiac E–C coupling process, direct measurements of the activity of the SR Ca^{2+} -ATPase in relationship to temperature are limited. Thus, the primary objective of this study was to investigate the temperature dependence of the SERCA2 from *O. mykiss* ventricles to verify the physiological role of this enzyme in SR Ca^{2+} cycling at a broad range of temperatures. In addition, experiments with the SERCA2 isoform from *R. norvegicus* ventricles were conducted in order to compare the temperature dependence of cardiac SERCA2 of these animals at the same experimental conditions.

MATERIALS AND METHODS

MATERIALS

All reagents were of analytical grade. [^{32}P]Pi was from Instituto de Pesquisas Energéticas e Nucleares (www.ipen.br). [γ - ^{32}P]ATP was synthesized as described by Walseth & Johnson (1979) and adapted by Maia *et al.* (1983).

ANIMALS AND TISSUE COLLECTION

The animals used in this study were 40 *O. mykiss* (250–280 g) collected during 2005 and 2006 in a trout farm, Teresópolis, Rio de Janeiro, Brazil and 20 Wistar female *R. norvegicus* (250–280 g) housed in a temperature-controlled room (*c.* 21° C) with a 12L:12D light cycle and given free access to water and food. All animals were euthanized, the hearts were removed by dissection and the ventricles were freeze-clamped in liquid nitrogen immediately. Most cardiac tissues still retained conductive activity and were beating post-mortem prior to freezing. At the laboratory, the tissues were stored in the freezer at –70° C until used for preparations. The rate of Ca^{2+} uptake catalysed by the *O. mykiss* SR vesicles did not change when *O. mykiss* samples were collected during the summer or winter months, where the temperature of the water varied from 18 to 22° C and 16 to 20° C. All procedures were in accordance with the university's animal handling protocols licence numbers: IBQM 034A and IBQM 034 B.

SR VESICLES ISOLATION

Microsomes enriched in SR vesicles were prepared as previously described (Landeira-Fernandez *et al.*, 2004). Approximately eight *O. mykiss* ventricles and four *R. norvegicus* ventricles were used to isolate SR vesicles and at least three different preparations were tested at the same temperature. Briefly, frozen cardiac muscle ventricle (5–10 g) was homogenized in 10 volumes of buffer containing 20 mM Mops-Tris (pH 7.0), 100 mM KCl, 100 mM NaCl, 1 mM MgCl_2 and 1 mM phenylmethylsulphonyl fluoride using a Teflon pestle. The homogenate was centrifuged at 10 000 g twice for 20 min in a Sorvall SS34 rotor (www2.dupont.com) at 4° C. The supernatant was filtered through two layers of cheesecloth

and then centrifuged at 37 000 *g* for 30 min in a Beckman 70Ti rotor (www.beckmancoulter.com) at 4° C. The pellet was resuspended in a high ionic strength medium containing 20 mM 3-(N-morpholino)propanesulfonic acid–tris(hydroxymethyl)aminomethane (Mops–Tris; pH 7.0) and 0.6 M KCl using a Teflon pestle and then centrifuged at 100 000 *g* for 30 min at 4° C. The pellet of SR microsomes was resuspended in a small volume of cold buffer containing 50 mM Mops-Tris (pH 7.0), 50 mM KCl and 0.32 M sucrose, and stored in liquid nitrogen until use. Protein concentrations were determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard. Prior to use, vesicles were diluted in a medium containing 50 mM Mops-Tris (pH 7.0), 0.1 mM MgCl₂, 1 mM CaCl₂ and 100 mM KCl. It was not possible to make direct comparisons on the absolute values of the activity found in *O. mykiss* and *R. norvegicus* SR vesicles because the total protein yield found in these preparations were different. The percentage of protein was calculated in relation to the total mass of ventricles used during vesicles isolation. The mean ± s.e. values found were 0.087 ± 0.002 (*n* = 3) for *O. mykiss* and 0.231 ± 0.020 (*n* = 3) for *R. norvegicus*. Therefore, the focus of this work was comparing the temperature dependence of each preparation.

WESTERN BLOTTING ANALYSIS

Briefly, 10, 20 and 100 µg of SR vesicles from *R. norvegicus* and *O. mykiss* ventricles were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (7.5%) according to Laemmli (1970) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked overnight, at 4° C, in a Tris-buffer saline containing 1% Tween 20 (TBS-T) and 5% non-fat milk. After that, the membrane was incubated overnight with a polyclonal antibody number MA3-919 (www.pierce-antibodies.com) raised against mammalian SERCA2 protein. The membrane was washed with TBS-T and then, incubated with a peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were visualized by incubation with luminol according to the manufacturer's instructions (ECL Western blotting detection system; www.gelifesciences.com). No significant result with the previous antibody was detected, thus a different polyclonal antibody raised against a conserved cardiac SERCA2 protein sequence was tested at a 1:750 dilution. Blots were developed using an alkaline phosphatase goat anti-rabbit secondary antibody kit (www.bio-rad.com) according to Landeira-Fernandez *et al.* (2004).

Ca²⁺ UPTAKE

Ca²⁺ uptake was determined using Millipore filtration technique (Chiesi & Inesi, 1979). The reaction medium composition was 50 mM Mops-Tris (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 5 mM sodium azide, 100 mM KCl, 5 mM oxalate and 10 µM CaCl₂. The reaction was started by the addition of 0.040 mg ml⁻¹ vesicles and aliquots were taken at the time and temperature as shown in the figures or legends. For Ca²⁺ uptake, trace amounts of ⁴⁵Ca²⁺ were included in the assay medium. The reaction was arrested by filtering samples of the assay medium through Millipore filters. After that, the filters were washed six times with 5 ml of 2 mM LaCl₃ and the radioactivity remaining on the filters was counted using a liquid scintillation counter. The temperature of the reaction medium was controlled by a temperature-controlled water bath.

ATPase ACTIVITY

The ATP hydrolysis was assayed by measuring the release of ³²Pi from [γ -³²P]ATP (Fabiato, 1983). The Mg²⁺-dependent activity was measured in a medium with no added Ca²⁺ and in the presence of 5 mM of ethylene glycol tetraacetic acid (EGTA). Ca²⁺-ATPase activity was determined by subtracting Mg²⁺-dependent activity from the total activity measured in the presence of both Mg²⁺ and Ca²⁺. The reaction medium was composed of 50 mM Mops-Tris (pH 7.0), 100 mM KCl, 2 mM MgCl₂, 5 mM sodium azide, 1 mM of ATP and 10 µM CaCl₂ or 5 mM EGTA. At the desired temperature, the reaction was started by the addition of 0.040 mg ml⁻¹ SR vesicles and stopped by the addition of 50% trichloroacetic acid (w/v). The suspension was then mixed with charcoal to remove [γ -³²P]ATP, centrifuged

and the remaining radioactivity was counted using a liquid scintillation counter. The Q_{10} values for ATP hydrolysis were calculated from the degree of activation that occurred on increasing the temperature by 10° C. The temperature of the reaction medium was controlled by a temperature-controlled water bath.

STATISTICS AND CALCULATIONS

All experiments were performed with at least three different tissues preparations and the results were expressed as means \pm s.e. The number of experiments (n) is shown in parentheses. Statistical significance of difference was assessed by a t -test or one-way ANOVA followed by Student–Newman–Keul *post hoc* test using the Sigma Plot–SigmaStat integrated software packages (www.systat.com). Differences were considered significant if $P < 0.05$.

Kinetic coefficients for ATP hydrolysis were calculated through linear regression. Maximal Ca^{2+} uptake activity was calculated through non-linear regression using the experimental data to fit the coefficients of the equation: $C_a = C_{am} (1 - e^{-kt})$; where C_a is the calcium uptake for a given time (t) of reaction and C_{am} is the maximum calcium uptake and k is a time constant.

RESULTS

SR vesicles derived from the *O. mykiss* ventricle retained a membrane-bound protein that was able to transport Ca^{2+} at the expense of ATP hydrolysis (Fig. 1). Activity was measured in the presence of oxalate, as a Ca^{2+} precipitating agent, ATP and MgCl_2 (Fig. 1). The rate of Ca^{2+} transport was prevented when thapsigargin, a specific inhibitor of the SERCA enzyme, was added to the reaction medium (Fig. 1). After the steady state was reached, the addition of $5 \mu\text{M}$ of the Ca^{2+} ionophore A_{23187} completely dissipated the Ca^{2+} gradient formed across the vesicles (Fig. 1). Western blotting analyses of the SR vesicles preparations were performed and it was found that in *R. norvegicus*, a protein band with a molecular mass of *c.* 110 kDa cross reacted with the mammalian SERCA2 antibody but not with *O. mykiss* SR vesicles, even at high concentration of *O. mykiss* protein (100 μg). The same antibody previously used to successfully detect SERCA2 in *T. thynnus* was tested

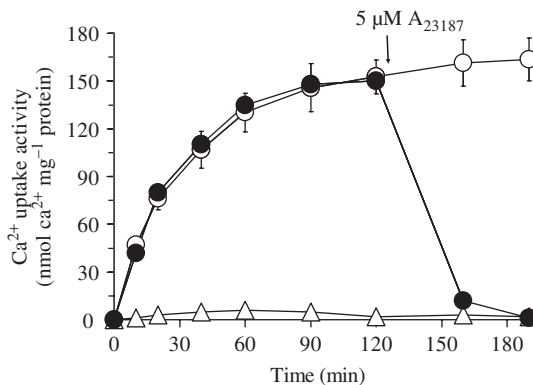


FIG. 1. The effect of different SERCA inhibitors (O, control; ●, addition of $5 \mu\text{M}$ A_{23187} after 120 min of reaction; Δ , $1 \mu\text{M}$ of thapsigargin) on the rate of Ca^{2+} uptake catalysed by sarcoplasmic reticulum vesicles derived from *Oncorhynchus mykiss* ventricles. The temperature of the medium was 20° C and values are means \pm s.e. ($n = 12$: control and A_{23187} ; $n = 4$: thapsigargin).

(Landeira-Fernandez *et al.*, 2004), but again, the cross reactivity was low. This is similar to the result found by Birkedal *et al.* (2009), working with the L-type Ca^{2+} channels and the Na^{+} - Ca^{2+} exchange (NCX) *O. mykiss* cardiac E-C coupling proteins.

In order to investigate the physiological contribution of the SR as an intracellular Ca^{2+} store in ventricular myocytes, the activity of the *O. mykiss* SERCA2 was measured at temperatures from 25 to 35° C, the approximate physiological temperature for *O. mykiss* (a relevant temperature for this population of *O. mykiss* living in the mountains of Rio de Janeiro, Brazil) and *R. norvegicus* (Figs 2 and 3).

Vesicles derived from *O. mykiss* SR ventricles were able to sustain a similar rate of Ca^{2+} transport at 25 and 30° C, but not at 35° C [Fig. 2(a)]. At this temperature, the rate of Ca^{2+} transport was lower and decreased after 20 min of reaction, possibly due to thermal inactivation [Fig. 2(a)]. In contrast, the rate of Ca^{2+} uptake in SR vesicles derived from *R. norvegicus* ventricles increases as the temperature of the medium increased, reaching its maximal activity at 35° C [Fig. 2(b)]. Measurements of the rate of ATP hydrolysis showed that, distinct from the Ca^{2+} uptake results, the *O. mykiss* SERCA2 enzyme had a similar activity either at 25 or 35° C [Fig. 3(a)], while the *R. norvegicus* isoform increased the enzyme activity two-fold from 25 to 35° C, similar to Ca^{2+} uptake [Fig. 3(b)].

Temperature-dependent regulation of cardiac contractility is crucial for cold active ectothermic fishes like *O. mykiss*, as they experience a wide range of low temperatures in their natural environment. To explore how the cardiac SR functions over a range of temperatures <20° C, the rate of Ca^{2+} uptake from 5 up to 15° C was measured.

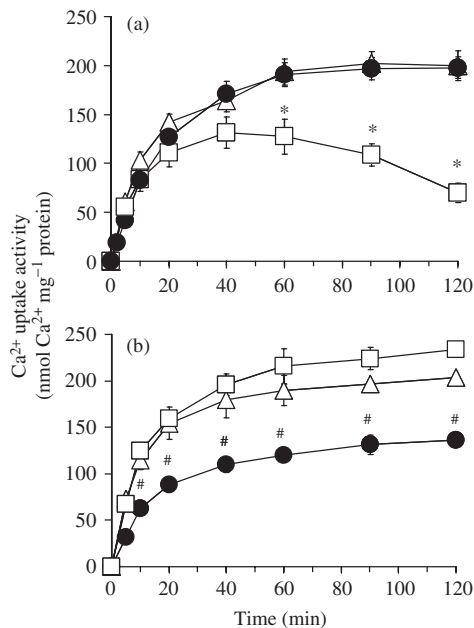


FIG. 2. Rate of Ca^{2+} uptake catalysed by sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA2) from (a) *Oncorhynchus mykiss* and (b) *Rattus norvegicus* at: 25° C (●), 30° C (Δ) and 35° C (□). Data are means \pm S.E. ($n = 12$: *O. mykiss*; $n = 4$: *R. norvegicus*). *, Statistically different from 30 and 25° C; #, statistically different from 30 and 35° C ($P < 0.05$).

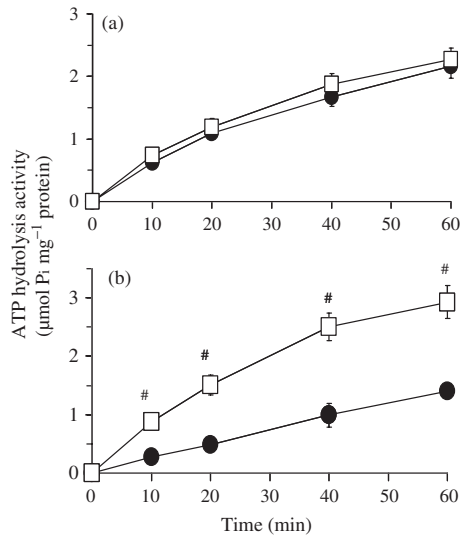


FIG. 3. Rate of ATP hydrolysis catalysed by sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA2) ventricles from (a) *Oncorhynchus mykiss* and (b) *Rattus norvegicus* at: 35° C (□) and 25° C (●). Data are means \pm S.E. ($n = 5$: *O. mykiss*; $n = 3$: *R. norvegicus*). #, Statistically different from 25° C ($P < 0.05$).

Oncorhynchus mykiss SR vesicles were able to sustain a high activity at all these temperatures [Fig. 4(a)]. Even at very cold temperature (5° C), the *O. mykiss* SERCA2 isoform was able to sustain a significantly high activity, *c.* 50% of the activity measured at 10° C [Fig. 4(a)]. In contrast, at 5° C, the mammalian SERCA2 isoform did not show a significant activity. Also, in contrast to the *O. mykiss* SERCA2, the *R. norvegicus* isoform displayed higher temperature dependence. The increase of the temperature from 10 to 20° C increased the activity three-fold in *R. norvegicus* [Fig. 4(b)], while the *O. mykiss* enzyme increased only 1.5 fold [Fig. 4(a)].

The maximal Ca^{2+} uptake activity reached by the *R. norvegicus* SERCA2 was at 35° C, a temperature at which the *O. mykiss* loses its enzymatic activity. At 30° C, *O. mykiss* and *R. norvegicus* SERCA2 isoforms have similar SERCA2 activity (Fig. 5). As the temperature of the medium decreases, the SERCA2 from *O. mykiss* ventricles was still able to maintain a high activity, but the mammalian SERCA2 decreased its activity and no significant Ca^{2+} accumulation was measured at 5° C (Fig. 5). These results indicate that the activity from the *O. mykiss* ventricles SERCA2 isoform is less temperature sensitive maintaining high activity throughout the temperature range of 5 to 30° C.

The effects of the temperature on the enzyme activity can be quantified by calculating the temperature coefficient or Q_{10} of the process. For ATP hydrolysis, the Q_{10} value was calculated using the results from Fig. 3, in the temperature interval of 25 to 35° C and the values found were 1.112 ± 0.026 ($n = 3$) and 2.759 ± 0.240 ($n = 5$) for *O. mykiss* and *R. norvegicus*, respectively.

The effect a 10° C change in temperature has on the rate of Ca^{2+} uptake catalysed by *O. mykiss* and *R. norvegicus* SERCA2 isoforms is shown in Table I. Similar to the rate of ATP hydrolysis, the SERCA2 from *O. mykiss* ventricles displayed very low Q_{10} *c.* 1.3 in all temperature ranges, which was different from the values found with

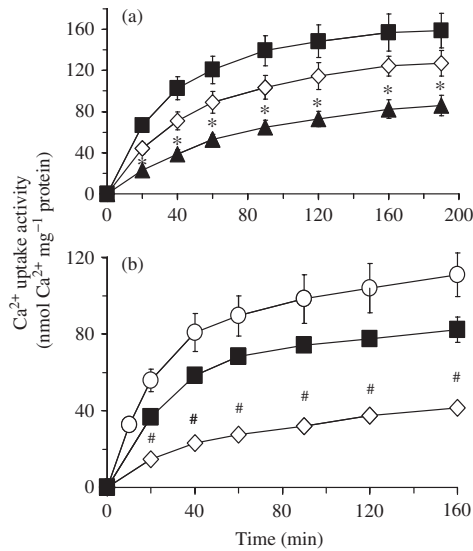


FIG. 4. Rate of Ca²⁺ uptake catalysed by sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA2) ventricles from (a) *Oncorhynchus mykiss* and (b) *Rattus norvegicus* at: 10° C (◇) and 15° C (■), and (a): 5° C (▲) and (b): 20° C (○). Data are means ± s.e. ($n = 10$: *O. mykiss*; $n = 3$: *R. norvegicus*). *, Statistically different from 10° and 15° C; #, statistically different from 15 and 20° C ($P < 0.05$).

the *R. norvegicus* isoform that was *c.* 2.0 (Table I) and similar to the other SERCA2 isoforms from different tunas species, *L. ditropis* and *R. norvegicus*, described in the literature (Landeira-Fernandez *et al.*, 2004; Weng *et al.*, 2005; Castilho *et al.*, 2007).

DISCUSSION

In this work, the first measurements of the acute temperature sensitivity of SERCA function in *O. mykiss* ventricles were performed and a comparison with the mammalian isoform from *R. norvegicus* was made.

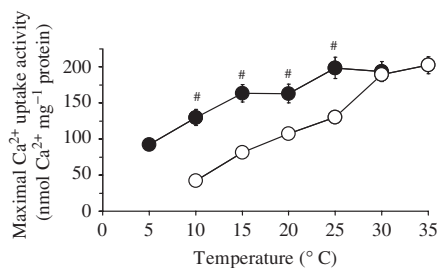


FIG. 5. Temperature dependence on the maximal Ca²⁺ uptake activity catalysed by *Oncorhynchus mykiss* (●) and *Rattus norvegicus* (○) sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA2) ventricles. Data were taken from Figs 1, 2 and 4. #, Statistically different from *R. norvegicus* at the temperature indicated ($P < 0.05$).

TABLE I. Comparison of the Q_{10} values (mean \pm S.E.) for Ca^{2+} uptake found for *Oncorhynchus mykiss* and *Rattus norvegicus* at different temperature intervals

Species	Temperature intervals ($^{\circ}$ C)			
	10–20	15–25	20–30	25–35
<i>O. mykiss</i>	1.25 \pm 0.06 (n = 3)	1.22 \pm 0.04 (n = 3)	1.19 \pm 0.08 (n = 3)	—
<i>R. norvegicus</i>	2.55 \pm 0.20 [#] (n = 3)	1.60 \pm 0.08 [#] (n = 3)	1.76 \pm 0.09 [#] (n = 3)	1.56 \pm 0.10 (n = 3)

The values were calculated from data in Fig. 5. #, Statistically different from other Q_{10} values found in *O. mykiss* ($P < 0.05$).

The physiological role of the SR during *O. mykiss* heart E–C coupling at different temperatures, especially at the cold end of the thermal range remains enigmatic. In comparison with mammals, the *O. mykiss* SR is less well developed morphologically and lacks T-tubules (Santer, 1985; Shiels & White, 2005). Recent studies, however, indicate that SR from *O. mykiss* cardiac myocytes can play an important role in regulating E–C coupling at physiological temperatures. Cellular studies with permeabilized ventricular myocytes demonstrated that the thapsigargin-sensitive SR Ca^{2+} uptake was only reduced by *c.* 50%, when the temperature of the medium was lowered from 20 to 5 $^{\circ}$ C, with an average Q_{10} value of *c.* 1.6 (Hove-Madsen *et al.*, 1998). More recently, using the perforated patch-clamp technique, combined with rapid caffeine applications, different groups found that *O. mykiss* cardiac cells were able to accumulate and liberate SR Ca^{2+} at 21 and 7 $^{\circ}$ C to the same extent (Hove-Madsen *et al.*, 2001; Shiels & Vornanen, 2002). These results are distinct from those reported in the mammalian heart, where low temperatures have been shown to cause prolonged opening of the RyR, with resulting arrhythmia and cold-induced contractile dysfunction (Bridge, 1986; Bers, 1991). The heart of these *O. mykiss* and other active fishes (*e.g.* *T. thynnus*) is potentially utilizing internal calcium stores, and calcium cycling, to maintain contraction at lower temperatures than mammals (Shiels *et al.*, 2011).

In contrast to mammals, as the temperature of the medium decreases, the activity of the *O. mykiss* SERCA2 isoform did not decrease and instead maintained a high activity, even at temperatures as low as 10 $^{\circ}$ C [Figs 4(a) and 5]. As a result, either for ATP hydrolysis and Ca^{2+} uptake, the Q_{10} value of *c.* 1.3 was found for *O. mykiss* ventricles, which was significantly lower than the value of *c.* 2.0 found for mammals (Table I). This result provides functional evidence for the capacity to maintain relaxation at cold temperatures in cardiac myocytes as the SERCA2 is functional at cold temperatures.

When the temperature was raised to the mammalian physiological temperature (*c.* 35 $^{\circ}$ C), the rate of ATP hydrolysis catalysed by the *O. mykiss* ventricle SERCA2 isoform was linear [Fig. 3(a)] but the Ca^{2+} uptake activity decreased [Fig. 2(a)], leading to an uncoupling state. This is distinct from the mammalian SERCA2 isoform that reached its maximal activity at 35 $^{\circ}$ C [Figs 2(b) and 3(b)].

The *in vitro* results indicate that the different SERCA2 isoforms from the ectothermic and endothermic animals were specialized to have maximal enzymatic activity at their physiological temperatures experienced at the organismal level. These data

demonstrate that *O. mykiss* and *R. norvegicus* SERCA2 isoforms, share distinct kinetic properties. A similar difference in the kinetic properties of the SERCA1 isoform were previously observed with vesicles derived from the SR skeletal muscle from *O. mykiss* and mammals (Chini *et al.*, 1993). Distinct from the mammalian SERCA1 isoform, the SERCA1 activity from *O. mykiss* skeletal muscle is temperature independent, being able to sustain a high activity even at temperatures as low as 5° C (Chini *et al.*, 1993). Taken together, the results suggest that the functional capacity of the skeletal and cardiac muscle cells in this eurythermal fish is maintained in part by the broad range of activity exhibited by the SERCA P-type ATPases.

At temperatures <20° C, the SERCA2 activity of the ventricular and atrial tissues of the endothermic *T. thynnus* and *L. ditropis* differ from *O. mykiss* and seem to be more similar to mammals (Landeira-Fernandez *et al.*, 2004; Weng *et al.*, 2005; Castilho *et al.*, 2007). The SERCA2 isoform from endothermic fishes is highly temperature dependent indicating that the SERCA2 kinetic properties are more similar to mammals than to the *O. mykiss* isoform.

A possible explanation for the kinetic differences between *R. norvegicus* and *O. mykiss* microsomes observed here is that there may be significant differences in the SR bilayer lipid composition, as this study was conducted with native microsomal membrane preparations. In order to address this hypothesis, it is necessary to purify and reconstitute the SERCA2 from *O. mykiss* ventricles and further work needs to be done.

The comparative analyses conducted in this study indicate that *O. mykiss* and *R. norvegicus* SERCA2 isoforms have distinct kinetic properties which enable the *O. mykiss* heart to have a high enzyme activity over a broad range of temperatures. Furthermore, cardiac SERCA2 from *O. mykiss* were not recognized by antibodies against the mammalian, endothermic fish (*T. thynnus* and *L. ditropis*) and *S. japonicus* SERCA2 isoform, indicating that *O. mykiss* SERCA2 isoform bears unique protein structure and conformational features, at least at the antibody domains. Importantly, the capacity of the SERCA2 to function over a large eurythermal range explains in part the capacity of the *O. mykiss* to successfully exploit habitats across a wide range of cold temperatures. Future efforts to discern population-specific differences in the enzyme function with temperature may help to explain the regional variation in thermal tolerances of *O. mykiss* and other salmonids across their ranges.

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