MARCO AURÉLIO SALOMÃO FORTES

**HIPERTROFIA DOS MÚSCULOS SÓLEO E EDL DE RATOS NO
INÍCIO DO DIABETES INDUZIDO POR ESTREPTOZOTOCINA**

Tese apresentada ao Programa de Pós-Graduação em Fisiologia Humana do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

São Paulo
2018

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **23** nas fls. **16** do livro **03** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a) **Rui Curi**, Coordenador (a) da Linha de pesquisa "*Hipertrofia do músculo esquelético no início do diabetes induzido por estreptozotocina*" do qual participam o(s) aluno(s) **Marco Aurélio Salomão Fortes**, Pesquisador **Carlos Hermano da Justa Pinheiro**, está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) em **20.03.2014**, com validade de 4 anos.

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Prof. Dr. WOTHAN TAVARES DE LIMA
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Secretária- CEUA - ICB/USP

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Confia no SENHOR de todo o teu coração e não se apoie somente em
sua inteligência – Provérbios 3.5

RESUMO

FORTES, M. A. S **Hipertrofia dos músculos sóleo e EDL de ratos no início do diabetes induzido por estreptozotocina. 2018. 151 f.** Tese (Doutorado em Fisiologia Humana) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

Pacientes com diabetes *mellitus* apresentam perda de massa e força muscular esquelética. O treinamento de força é prescrito aos pacientes diabéticos como parte do tratamento, pois melhora o controle glicêmico além de promover aumento da massa muscular. Foram investigados os mecanismos envolvidos na hipertrofia muscular induzida por sobrecarga mecânica durante o estabelecimento do estado diabético do tipo I induzido por estreptozotocina em ratos. Os experimentos foram realizados nos músculos com predominância de fibras oxidativas (sóleo) ou glicolíticas (extensor digital longo - EDL). Avaliou-se a modulação da via de síntese de proteínas PI3K-AKT-mTOR sete dias após indução de hipertrofia dos músculos sóleo por tenotomia do músculo gastrocnêmio e do EDL pela ablação do músculo tibial. Determinou-se também a expressão de mRNA de outras vias de sinalização que controlam a hipertrofia muscular: mecanotransdução (FAK), Wnt/ β -catenina e miostatina e folistatina. Os músculos sóleo e EDL quando submetidos à sobrecarga funcional sofreram hipertrofia semelhante em animais controles e diabéticos. O aumento das forças tetânica e isotônica, absolutas e específicas, ocorreu na mesma magnitude que a hipertrofia muscular. A hipertrofia do músculo EDL nos animais diabéticos envolveu principalmente a via PI3K-AKT-mTOR além da redução no conteúdo de AMPK e diminuição da expressão de miostatina. No músculo sóleo, a hipertrofia foi mais pronunciada nos animais diabéticos por ativação mais intensa da via pelas proteínas rpS6 e aumento na expressão de mRNA de IGF-1, MGF e folistatina além de diminuição nos conteúdos de miostatina, MuRF-1 e atrogina-1. As modificações relacionadas à sinalização permitiram ao músculo sóleo alcançar valores de força e massa muscular similares ao grupo controle.

Palavras-chave: Músculo esquelético, Síntese de Proteínas, Força muscular, Hiperglicemia, Eletroestimulação.

ABSTRACT

FORTES, M. A. S. **Hypertrophy of soleus and EDL muscles in the early diabetes induced by streptozotocin in rats.** 2018. 151 pgs. Ph. D. these (Human Physiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

Patients with diabetes mellitus have reduction in skeletal muscle mass and strength. Strength training is prescribed to diabetic patients as part of the treatment since it improves glycemic control and promotes an increase of skeletal muscle mass. The mechanisms involved in the overload-induced muscle hypertrophy during the establishment of the type I diabetic state, induced by streptozotocin, were investigated in rats. The experiments were performed in muscles with predominance of oxidative (soleus) or glycolytic (EDL) fibers. PI3K/AKT/mTOR protein synthesis pathway was evaluated seven days after the overload-induced hypertrophy of the soleus muscle by tenotomy of the gastrocnemius muscle and of the EDL muscle by tibialis anterior muscle ablation. The mRNA expression of genes associated with different signaling pathways that control muscle hypertrophy was also evaluated: mechanotransduction (FAK) signaling, Wnt/ β -catenin, myostatin and follistatin. The soleus and EDL muscles when submitted to overload had similar hypertrophic responses in control and diabetic animals. The increase of twitch and tetanic, absolute and specific, forces had the same magnitude as the muscle hypertrophic response. Hypertrophy of the EDL muscle from diabetic animals mostly involved mechanical loading-stimulated PI3K/AKT/mTOR pathway in addition to the reduced activation of AMPK and decrease of myostatin expression. Hypertrophy was more pronounced in the soleus muscle of diabetic animals due to a more potent activation of rpS6 and increased mRNA expression of IGF-1, MGF and follistatin, and decrease of the myostatin, MuRF-1 and atrogin-1 contents. The activated signaling pathways enabled the soleus muscle mass and force of the diabetic rats to reach the values of the control group.

Keywords: Skeletal muscle, protein synthesis, muscle strength, hyperglycemia, electrostimulation.

LISTA DE ABREVIATURAS E SIGLAS

4E-BP1	<i>4E (eIF4E)-binding protein 1</i>
AGE	Produtos avançados de glicação <i>Advanced glycation end products</i>
ActRIIA	<i>Activin A receptor, type IIA</i>
ActRIIB	<i>Activin A receptor, type IIB</i>
ALK 4	<i>Activin receptor-like kinase 4</i>
ALK5	<i>Activin receptor-like kinase 5</i>
AKT ou PKB	Proteína quinase B
AMPK	Proteína quinase ativada por AMP <i>AMP-activated protein kinase</i>
ANOVA	<i>Analysis of variance</i>
CEUA	Comissão de Ética no Uso de Animal
COBEA	Colégio Brasileiro de Experimentação Animal
CL	Pata contralateral
CT	<i>Cycle threshold</i>
CTRL	Grupo controle
Daam1	<i>Dishevelled associated activator of morphogenesis 1</i>
DM	Grupo diabético
Dsh	<i>Dishevelled</i>
EDL	Extensor digital longo
EDTA	Ácido diaminoetanotetraacético
eIF2 α	Fator eucariótico de iniciação da tradução 2alfa <i>Eukaryotic translation initiation factor 2alpha</i>
eIF4E	Fator eucariótico de iniciação da tradução <i>Eukaryotic translation initiation factor 4E</i>
EPM	Erro padrão da média
EROs	Espécies reativas de oxigênio
FAK	Quinase de adesão focal <i>Focal adhesion kinase</i>
FoxO	<i>AKT/Forkhead O</i>
GLUT4	Transportador de glicose 4 <i>Glucose transporter 4</i>
H	Pata hipertrofiada

HPRT1	<i>Hypoxanthine Phosphoribosyltransferase 1</i>
HRT	Tempo de relaxamento <i>Half relaxation time</i>
IGF-1	Fator de crescimento semelhante à insulina-1 <i>Insulin-like growth factor-1</i>
IP3	<i>Inositol trifosfato</i>
FZD	<i>Frizzled</i>
JNK	<i>Jun N-terminal kinase</i>
LEF	<i>Lymphoid enhancer factor</i>
LRT	Tempo de relaxamento tardio <i>Late relaxation time</i>
LRP	<i>Lipoprotein receptor-related protein</i>
MAPK	Proteína quinase ativada por mitógeno <i>Mitogen activated protein kinases</i>
MARP	<i>Muscle ankryin repeat proteins</i>
MLP	<i>muscle LIM protein</i>
MG53	<i>Mitsugumin 53</i>
MGF	Fator de crescimento mecânico <i>Mechano growth factor</i>
mTOR	<i>Mammalian target of rapamycin</i>
mTORC1	<i>Mammalian target of rapamycin complex 1</i>
MuRF1	<i>Muscle RING-finger protein 1</i>
MyoD	<i>Myogenic differentiation factor</i>
Myf5	<i>Myogenic factor 5</i>
rpS6	<i>Ribossomal protein S6</i>
PBS	Tampão salina fosfato
PDK1	Proteína quinase fosfoinositídeo-dependente 1 <i>Phosphoinositide-dependent kinase-1</i>
PIP2	<i>Phosphatidylinositol-2 phosphate;</i>
PIP3	<i>Phosphatidylinositol-3 phosphate</i>
PLC	Fosfolipase C
PMSF	Fenilmetilsulfonilfluoreto
PRAS40	<i>Proline-rich AKT substrate 40 kDa</i>
Rheb	<i>Ras homolog enriched in brain</i>
ROCK	<i>Rho-associated kinase</i>

RT-PCR	Reação em cadeia da polimerase quantitativa em tempo real <i>Quantitative real-time polymerase chain reaction</i>
S6K1	<i>p70 ribosomal S6 kinase 1</i>
SBE	<i>Smad-binding element</i>
SDS-PAGE	Eletroforese em gel de poliacrilamida-dodecil sulfato de sódio <i>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</i>
SERCA1 e 2	ATPase de cálcio do retículo sarcoplasmático 1 e 2 <i>Sarcoplasmic reticulum calcium ATPase 1 e 2</i>
SMAD	<i>Mothers against decapentaplegic (MAD) e Caenorhabditis elegans SMA</i>
TCF	<i>T-cell factor</i>
TGF- β	<i>Transforming Growth Factor-β</i>
TSC1	<i>Tuberous sclerosis complex 1 - Hamartin</i>
TSC2	<i>Tuberous sclerosis complex 2 - Tuberin</i>
TTP	Tempo de contração <i>Time to peak</i>
Ub	Ubiquitina
YWHAZ	<i>tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide</i>
Wnt	<i>Wingless-related integration site</i>
Wnt7a	<i>Wingless-type MMTV integration site family, member 7A</i>

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1. INTRODUÇÃO

A insulina estimula a síntese e inibe a degradação de proteínas no músculo esquelético (SANDRI, 2008; SCHIAFFINO *et al.*, 2013). Estados de deficiência de insulina, como diabetes *mellitus* tipo 1, apresentam redução na síntese e aumento na degradação de proteínas, levando à perda de massa muscular esquelética (BARAZZONI *et al.*, 2004). A baixa quantidade de massa muscular corporal associa-se com a resistência à insulina, condições de pré-diabetes e síndrome metabólica, independente da adiposidade central e visceral (ATLANTIS *et al.*, 2009; LARSEN *et al.*, 2015; MIZGIER *et al.*, 2014; SRIKANTHAN; KARLAMANGLA, 2011).

O IGF estimula a via de síntese de proteínas quando há indução por sobrecarga funcional promovendo hipertrofia (COLEMAN *et al.*, 1995; ROMMEL *et al.*, 2001; SCHIAFFINO; MAMMUCARI, 2011). Animais com diabetes *mellitus* induzido por administração de estreptozotocina apresentam aumento da atividade proteolítica no músculo esquelético em curto prazo (1-3 dias após indução do diabetes) que retorna a valores semelhantes aos controles após 5 a 10 dias (PEPATO *et al.*, 1996). O aumento na expressão de miostatina em curto-prazo está associado à severidade da perda de massa muscular, sendo esta revertida pelo tratamento com insulina (CHEN *et al.*, 2009). Contudo, oito semanas após a indução do diabetes, a expressão do mRNA de miostatina retorna ao valor basal o que reduz sua influência na diminuição da massa muscular (BARAZZONI *et al.*, 2004; CHEN *et al.*, 2009).

Em ratos Zucker obesos e resistentes à insulina, a hipertrofia por sobrecarga funcional é menor em relação aos controles. Há menor ativação da via de sinalização da *mammalian target of rapamycin* (mTOR) caracterizada por menor fosforilação de p70S6-quinase e aumento de tuberina/TSC2 no músculo sóleo de animais obesos submetidos à hipertrofia (PATURI *et al.*, 2010). Menor ativação da via da mTOR foi também demonstrada por Katta e colaboradores (2010) no músculo sóleo de ratos Zucker durante hipertrofia induzida por sobrecarga funcional (PATURI *et al.*, 2010). Animais diabéticos induzidos por estreptozotocina apresentam aumento da fosforilação de AMP-quinase (AMPK) e efeito supressor sobre a hipertrofia muscular, reduzindo a ativação das seguintes proteínas da via da mTOR: AKT em Ser473, mTOR em Ser2448, *ribosomal protein S6 kinase* em Thr389 e *eukaryotic initiation factor (eIF4E) binding protein* em Thr37 (BOLSTER *et al.*, 2002; THOMSON; GORDON, 2005).

De modo geral, os trabalhos sobre diabetes mellitus e músculo esquelético envolvem abordagem indistintamente diabetes tipos 1 e 2 (LARSEN et al., 2016; SALA; ZORZANO, 2015). Contudo, há muitas diferenças entre os tipos 1 e 2. Em particular, há diminuição na resposta hipertrófica do músculo esquelético quando submetido à sobrecarga funcional no diabetes tipo 2 (KATTA et al., 2010; PATURI et al., 2010) enquanto que no tipo 1 o mesmo efeito não é observado (FORTES et al., 2015). Desta forma, identificar e avaliar possíveis alvos moleculares associados à sarcopenia em doenças metabólicas é importante para atuar na prevenção (RODEN, 2015).

O exercício físico é prescrito para pacientes diabéticos com o objetivo de melhorar o controle glicêmico. Contudo, pouco se sabe sobre as respostas do ganho de massa muscular esquelética induzido pelo exercício físico resistido na condição de diabetes *mellitus*. A sobrecarga funcional por ablação da musculatura sinergista é a abordagem experimental mais utilizada para o estudo dos mecanismos envolvidos na hipertrofia do músculo esquelético (BODINE et al., 2001a; ISHIDO et al., 2006; KATTA et al., 2010; PATURI et al., 2010; PHELAN; GONYEA, 1997; ROSENBLATT; PARRY, 1992). A hipertrofia dos músculos extensor digital longo (EDL) e sóleo é induzida por ablação do tibial anterior e tenotomia do gastrocnêmio, respectivamente (BODINE; BAAR, 2012; BOOTH; THOMASON, 1991). Há evidências de que, sete dias após a cirurgia de ablação sinergista, há ativação máxima da via de síntese proteica e após 30 dias é observada resposta trófica máxima nos músculos esqueléticos (ARMSTRONG; IANUZZO, 1977; FARRELL et al., 1999; KATTA et al., 2010). A miopatia diabética causa várias alterações no músculo esquelético (D'SOUZA; AL-SAJEE; HAWKE, 2013) e promove redução na força e massa muscular (ANDERSEN et al., 1996, 1997, 2004). Contudo, apesar de haver hipotrofia muscular na condição crônica de DM, a capacidade dos músculos sóleo e EDL de responderem a um estímulo hipertrófico não se altera em ratos induzidos ao diabetes por estreptozotocina (FORTES et al., 2015). Por outro lado, após 3 dias de indução do diabetes por injeção de estreptozotocina em ratos, já há redução significativa da massa dos músculos sóleo, EDL e gastrocnêmio (PEPATO et al., 1996; PRICE et al., 1996). Em pacientes adolescentes, a redução da massa muscular é evidenciada em menos de quatro semanas após o diagnóstico de diabetes (KRAUSE; RIDDELL; HAWKE, 2011; RESKE-NIELSEN; HARMSEN; VORRE, 1977).

1.1. Vias de sinalização que controlam a hipertrofia muscular

1.1.1 PI3K-AKT-mTOR

A sobrecarga funcional está associada à ativação da via canônica de sinalização da mTOR (BODINE et al., 2001a). Na Figura 1 estão ilustradas as vias de sinalização que contribuem para a resposta hipertrófica frente à sobrecarga funcional.

Figura 1. Vias de sinalização que participam no controle da resposta hipertrófica do músculo esquelético.

Abreviaturas: MEC – matrix extracellular; PIP2 – *phosphatidylinositol-2 phosphate*; PIP3 – *phosphatidylinositol-3 phosphate*; PI3K – *phosphatidylinositol 3-kinase*; IRS1 – *Insulin Receptor Substrate 1*; AKT – *Protein Kinase B*; TSC – *Tuberous Sclerosis Complex*; TSC1- *hamartin*; TSC2 – *tuberin*; mTORC1 – *mammal target of rapamycin complex 1*; eIF4E – *eukaryotic initiation factor 4E*; 4E-BP1 – (*eIF4E*)-binding protein 1; S6K – *p70 ribosomal S6 kinase 1*; FAK – *Focal adhesion kinase*; Wnt 7a – *Wingless-Type MMTV Integration Site Family, Member 7A*; MuRF-1 – *Muscle RING-finger protein-1*; IGF-1 Eb – *Insulin-like growth factor 1 Eb-peptide*; Ankrd2 – *Ankyrin repeat domain 2*; ActRIIB – *Activin type 2 B receptor*. Os artigos que subsidiaram a elaboração da figura foram: (BARAZZONI et al., 2012; BODINE, 2006; HAY; SONENBERG, 2004; LAPLANTE; SABATINI, 2009, 2012; MIYAZAKI et al., 2011; SAXTON; SABATINI, 2017).

A via canônica mais comum que culmina em síntese de proteínas no músculo esquelético é conhecida como PI3K-AKT-mTOR e é ativada quando a insulina ou o IGF-1 se liga ao receptor correspondente (BODINE et al., 2001a). Uma etapa chave

é a ativação da proteína mTOR por meio de fosforilação (LAPLANTE; SABATINI, 2009; SCHIAFFINO et al., 2013). Após a ligação ao receptor, o substrato do receptor de insulina 1 (IRS1) é recrutado e fosforilado, iniciando a transdução de sinal na via, recrutando as subunidades p85 e p110 que formam a PI3K. Esta adiciona um fosfato ao fosfatidil inositol difosfato (PIP2) transformando-o em fosfatidil inositol trifosfato (PIP3). A PIP3 é um sítio de ligação para a proteína quinase fosfoinositídeo-dependente 1 (PDK1) e também a AKT, que está localizada na membrana sarcoplasmática. PDK1 ativa AKT por fosforilação em resposta ao aumento de PIP3. A ligação de PIP3 ao domínio da AKT promove translocação desta para a membrana plasmática (SAXTON; SABATINI, 2017; ZONCU; EFEYAN; SABATINI, 2011). Esses eventos de fosforilação são necessários para a ativação completa da AKT (HAY; SONENBERG, 2004). Três isoformas de AKT foram descritas, AKT1/PKB α , AKT2/PKB β e AKT3/PKB γ , mas somente AKT1 e 2 estão presentes no músculo esquelético (PERSAD et al., 2001; SAKAMOTO et al., 2002; SCHIAFFINO; MAMMUCARI, 2011), sendo que a AKT1 está mais relacionada ao trofismo celular e AKT2 com o metabolismo de glicose (SCHIAFFINO; MAMMUCARI, 2011). Para se tornar ativa, a AKT necessita ser fosforilada em 2 sítios, treonina 308 (^{thr308}) e serina 473 (^{Ser473}), sendo que a PI3K fosforila o sítio Thr308 e a PDK1 a Ser473 (COFFER; JIN; WOODGETT, 1998; PERSAD et al., 2001; VANHAESEBROECK; ALESSI, 2000). A AKT ativada fosforila 2 alvos subsequentes: o complexo TSC1/TSC2 e a *proline-rich AKT substrate 40 kDa* (PRAS40) (LAPLANTE; SABATINI, 2009). PRAS40 é uma proteína inibitória do complexo mTORC1 que quando fosforilada se dissocia do complexo permitindo que este seja ativado. O complexo TSC1/TSC2 ativa a proteína *Ras homolog enriched in brain* (Rheb) que subsequentemente inibe mTORC1 (LAPLANTE; SABATINI, 2009). TSC1/TSC2 atua como ativador de Rheb inibindo a sinalização de mTOR *in vivo* (LONG et al., 2005; MANNING; CANTLEY, 2003). Então, quando AKT fosforila o complexo TSC1/TSC2, este não ativa Rheb, o que permite a ativação de mTORC1. mTORC1 então fosforila o fator de iniciação eucariótico 4E (eIF4E)-*binding protein 1* (4E-BP1) e a *p70 ribosomal S6 kinase 1* (S6K1). No final da sequência de nucleotídeos do mRNA há uma estrutura especificamente reconhecida por *eukaryotic translation initiation factor 4E* (eIF4E) que se une a esta e à proteína eIF4A para formar o complexo eIF4F. Este juntamente com uma helicase iniciam a tradução ribossomal por facilitar a ligação da subunidade 40S do ribossomo ao mRNA (GINGRAS; RAUGHT; SONENBERG, 1999). O 4E-BP1 é um peptídeo inibitório que se liga à eIF4E e eIF4A não

permitindo a sua interação com a proteína eIF4G e eIF4A, anulando a ligação destes ao ribossomo (RICHTER; SONENBERG, 2005). A fosforilação de 4E-BP1 não permite a sua ligação à proteína eIF4E e a deixa livre para promover síntese proteica (EGERMAN; GLASS, 2014; GINGRAS et al., 1998; MAYER et al., 2004).

1.1.2 Mecanotransdução

A mecanotransdução ocorre quando o músculo esquelético converte sinais mecânicos em bioquímicos, os quais regulam a síntese de proteínas no músculo esquelético (HORNBERGER; ESSER, 2004). Essas alterações mecânicas estão associadas a proteínas de adesão, passando pela matriz extracelular, costâmero, citoesqueleto, filamentos intermediários e ao sarcômero (fibra muscular). O trabalho mecânico gera deformação da lamina basal e das proteínas constituintes do costâmero e essas são importantes sensores mecânicos (BAUM et al., 2000; BURKHOLDER, 2007; DANOWSKI et al., 1992; FISCHER et al., 2016; PATEL; LIEBER, 1997). O costâmero, que também é chamado de placas de adesão focal, foi primeiramente descrito por Pardo e colaboradores em 1983 (PARDO; SILICIANO; CRAIG, 1983). São moléculas sinalizadoras e de adesão sendo a distroglicana e a integrina as principais proteínas constituintes, que também são receptores da laminina (BURKHOLDER, 2007; COHN et al., 1999; COLOGNATO et al., 1999). A laminina é uma glicoproteína e um dos principais componentes da matriz extracelular e da membrana basal (HOLMBERG; DURBEEJ, 2013).

A deformação localizada do sarcolema, permite a possibilidade de que mesmo pequenas modificações na célula possam induzir alterações situadas no sarcolema e no citoesqueleto (DULHUNTY; FRANZINI-ARMSTRONG, 1975). No músculo isolado, a hipertrofia estimulada por estiramento/alongamento mecânico pode conduzir à fosforilação de efetores da via PI3K, sem qualquer fosforilação de AKT ou mTOR (HORNBERGER; CHIEN, 2006).

A ligação da integrina e distroglicana à quinase de adesão focal (FAK) na matriz extracelular (Figura 2) leva sinais mecânicos do costâmero e da rede da matriz extracelular para o núcleo (COLOGNATO et al., 1999; HILENSKI; TERRACIO; BORG, 1991). Uma extensa rede de sinalização envolvendo proteínas quinases ativadas por mitógeno (MAPKs) e PI3K está associada à integrina (CARSON; WEI, 2000; INGBER, 1997). O sistema de mecanotransdução é modulado pela sinalização e grau de deformação e quantidade de carga aplicada (IQBAL; ZAIDI, 2005; SAWADA; SHEETZ, 2002).

Uma das primeiras moléculas de sinalização identificada foi a FAK, que regula a renovação de moléculas de adesão, ativação de GTPases, migração celular e *cross-talk* entre a sinalização de fatores de crescimento e integrinas (MITRA; HANSON; SCHLAEPFER, 2005). Constatou-se aumento do conteúdo de FAK após estímulo hipertrófico por sobrecarga funcional no músculo sóleo de ratos e fusão de mioblastos demonstrando seu potencial em estimular o aumento da massa muscular esquelética (FLÜCK et al., 1999). FAK é de extrema importância para a hipertrofia do músculo esquelético quando estimulada por fatores mecânicos, e, quando autofosforilada no resíduo 397 em tirosina, ativa a p70S6K e consequentemente promove hipertrofia independente da AKT, que não é ativada neste processo (FLÜCK et al., 1999; KLOSSNER et al., 2009). Em células C2C12, a ablação da FAK causa diminuição da resposta hipertrófica dos miotubos reduzindo o aumento no diâmetro destes e da síntese total de proteínas na célula (CROSSLAND et al., 2013).

Figura 2. Costâmero do músculo esquelético e filamentos intermediários de suporte e sinalização que controlam a resposta hipertrófica no músculo esquelético.

Diagrama esquemático das várias proteínas do costâmero e citoesqueleto envolvidos na transferência de força lateral e longitudinal. Os complexos de glicoproteínas, integrinas e distrofina são componentes-chave na transmissão de força lateral. Titina, nebulina, as *muscle ankyrin repeat proteins* (MARP) e *muscle LIM protein* (MLP) (LIM é uma sigla resultante das iniciais dos três primeiros membros da família de proteínas: lin-11, islet-1, mec-3), estão envolvidos na transferência

de força longitudinal e são importantes para o desenvolvimento de força no músculo. O artigo que subsidiou e elaboração da figura foi: Hughes, Wallace e Baar, 2015.

1.1.3 Miostatina e folistatina

A miostatina inibe e a folistatina induz hipertrofia muscular esquelética (GILSON et al., 2009). A ausência de miostatina no organismo por eliminação, mutação ou perda da função induz crescimento muscular excessivo, enquanto que a expressão aumentada ou administração provoca hipotrofia muscular (MCPHERRON; LEE, 1997; MCPHERRON; LAWLER; LEE, 1997; RODRIGUEZ et al., 2014; SCHUELKE et al., 2004; WURTZEL et al., 2017). A miostatina é uma proteína polimérica da família TGF- β (*Transforming Growth Factor- β*). É secretada por células musculares e atua de formas parácrina e autócrina quando se liga ao seu receptor de superfície. Como consequência, desencadeia uma cascata de sinalização que leva à translocação da proteína Smad2/3 para o núcleo alterando a expressão de genes alvo (HAN et al., 2013). A atividade da miostatina é controlada não só por transcrição, mas também por eventos pós-traducionais. Após a clivagem do seu precursor, a promiostatina, a maior parte da miostatina é armazenada em um complexo no espaço extracelular (ANDERSON; GOLDBERG; WHITMAN, 2008; LEE; MCPHERRON, 2001; THIES et al., 2001).

As activinas pertencem à subfamília activinas-inibinas da família TGF- β . Nos músculos, a activina A e a miostatina ligam-se ao mesmo receptor de superfície e ativam a mesma cascata de sinalização que promove a translocação de Smad2/3 para o núcleo. Há e classes de proteínas SMAD (Figura 3) que são homólogas de ambas as proteínas de *Drosophila*, *mothers against decapentaplegic* (MAD) e da *Caenorhabditis elegans* SMA (SAVAGE et al., 1996; SEKELSKY et al., 1995) e as Smads 2/3 pertencem à classe 1 (WU et al., 2001).

A miostatina e a activina exercem atividades biológicas ligando-se ao mesmo complexo heterodimérico da membrana que abrange os receptores de activina dos tipos I e II (BILEZIKJIAN et al., 2006). Este complexo é composto por dois receptores de tipo-II, ActRIIA e ActRIIB, e dois receptores de tipo-I, *activin receptor-like kinase 4* (ALK 4) e *activin receptor-like kinase 5* (ALK5). ActRIIB é abundante no músculo esquelético comparado à ActRIIA e apresenta maior afinidade à miostatina (HAN et al., 2013; LEE; MCPHERRON, 2001).

Figura 3. Via de sinalização da miostatina e activina no músculo esquelético.

A miostatina ou activina se liga ao receptor ActRIIB na membrana muscular, o que leva ao recrutamento e ativação de ALK4 ou ALK5 causando fosforilação de Smad2 e Smad3 e o recrutamento de Smad4 formando o complexo Smad. O complexo de Smad se transloca para o núcleo e promove alterações de transcrição de genes, o que por fim resulta na perda de massa

muscular. A ligação de miostatina/activina ao receptor reduz a atividade de AKT e *AKT/Forkhead O* (FoxO). A FoxO inativada entra no núcleo para ativar a transcrição de genes ligados à hipotrofia muscular, às E3 ligases, MuRF1 e atrogina-1, que promovem a ubiquitinação e degradação de proteínas musculares pelo sistema proteassoma. Abreviaturas: SBE, *Smad-binding element*; Ub, ubiquitina. O artigo que subsidiou e elaboração da figura foi: Han et al., 2013.

A ligação da miostatina ou a activina A causa dimerização nos seus receptores de membrana, o que leva ao recrutamento e à ativação dos receptores do tipo I transmembranares ALK4 e ALK5. Essa ação no receptor causa fosforilação de Smad2 e Smad3 no citosol e subsequente recrutamento de Smad4 em um complexo Smad2/3/4. Este complexo entra no núcleo e induz transcrição gênica, levando à perda de massa muscular (HAN et al., 2013). Ocorre também supressão da atividade da AKT levando à redução e acúmulo de FoxOs (FoxO1, FoxO3 e FoxO4). FoxOs não fosforiladas são as formas ativas que regulam a transcrição do gene. Quando a atividade de AKT é diminuída, os FoxOs são desfosforilados e podem entrar no núcleo e ativar a transcrição de genes específicos que levam à hipotrofia muscular, as E3 ligases atrogina-1 e MuRF1. Estas causam ubiquitinação e subsequente degradação pelo proteassoma de proteínas musculares, incluindo as contráteis (HAN et al., 2013).

A miostatina também pode regular a hipertrofia muscular atuando diretamente nas células satélites. As células satélites quiescentes nas fibras musculares são ativadas em resposta à lesão do músculo para dar origem a mioblastos (FRY et al., 2013; MCCROSKERY et al., 2003). Quando há um estímulo hipertrófico, essas células se proliferam e se fundem com uma fibra pré-existente ou também podem se diferenciar em miotubos nascentes (FRY et al., 2013). A miostatina é um regulador negativo da progressão do ciclo celular. Concentrações elevadas de miostatina em células satélites bloqueiam a ativação e as mantêm quiescentes (MCCROSKERY et al., 2003). Aumento da miostatina é detectado em estados de hipotrofia muscular. Aumento transitório do mRNA de miostatina no músculo de roedores na hipotrofia induzida pelo desuso de fibras de contração rápida foi observado (CARLSON; BOOTH; GORDON, 1999). O breve aumento na expressão de miostatina após quatro dias da indução do diabetes por estreptozotocina está associado à severidade da perda de massa muscular, sendo revertido pelo tratamento com insulina, retornando aos valores basais após 4 semanas (CHEN et al., 2009). Já após 8 semanas, a expressão de miostatina não está elevada em animais diabéticos induzidos por estreptozotocina (BARAZZONI et al., 2004). A expressão de mRNA da

miostatina não está elevada no estado diabético crônico, indicando que outros mecanismos estão envolvidos na sinalização que modula o trofismo muscular além desta proteína (FORTES et al., 2015, 2017).

A folistatina é uma glicoproteína monomérica que foi isolada, inicialmente, do fluido folicular ovariano. Esta tem a habilidade de suprimir a secreção do hormônio folículo estimulante (FSH) *in vitro* (TORTORIELLO et al., 2001; UENO et al., 1987). Sua principal ação é antagonizar a atividade de vários membros da família TGF- β , incluindo a activina e a miostatina (GILSON et al., 2009; MINETTI et al., 2006; SARTORELLI; FULCO, 2004) e possui grande capacidade de induzir hipertrofia do músculo esquelético por diferentes vias de sinalização (GILSON et al., 2009; HAIDET et al., 2008). A folistatina também interage diretamente com a miostatina, bloqueando sua capacidade de inibir a hipertrofia muscular esquelética (AMTHOR et al., 2004; BARBÉ et al., 2015). Camundongos *knockout* para miostatina foram cruzados com portadores de um transgene de folistatina e, como resultante, houve aumento de 4 vezes na massa muscular em comparação ao observado somente com a falta da primeira (HAIDET et al., 2008; LEE, 2007). Outro mecanismo de indução da hipertrofia por folistatina requer ativação do IGF-1 pela via da insulina (PI3K/AKT/mTOR). É necessário a presença da insulina ou de IGF-I para que a folistatina seja capaz de exercer efeito anabólico sobre o músculo esquelético (BARBÉ et al., 2015). Em animais deficientes de insulina, como naqueles tratados com estreptozotocina, a folistatina, não é efetiva em estimular o crescimento muscular (BARBÉ et al., 2015; KALISTA et al., 2012). A hipertrofia induzida pela folistatina envolve ativação das proteínas Smad1/5 nos resíduos S463/465 e *cross-talk* desta com a via PI3K/AKT/mTOR (AMTHOR et al., 2002; WINBANKS et al., 2013).

A hipertrofia induzida pela folistatina é caracterizada por aumento do diâmetro das fibras e do conteúdo de DNA e proteínas miofibrilares contráteis (BARBÉ et al., 2017). A quantidade de DNA do músculo esquelético é equivalente ao número de mionúcleos, incluindo aqueles das células satélites que depende da replicação das células quiescentes. Este fenômeno se confirma também pela observação de aumento da expressão do *proliferating cell nuclear antigen* (PCNA), essencial para a replicação celular, após superexpressão de folistatina (GILSON et al., 2009). A ativação da via de sinalização Wnt canônica induz expressão de folistatina em mioblastos e promove a fusão de mioblastos de maneira dependente da folistatina (JONES et al., 2015). A via canônica de Wnt/ β -catenina leva os

mioblastos a se diferenciarem ativando seu programa miogênico e estimulando a expressão de folistatina e miogenina (JONES et al., 2015). Esses mecanismos podem estar envolvidos na hipertrofia muscular a longo prazo envolvendo ativação e fusão de células satélites a fibras pré-existentes.

1.1.4 Wnt/ β -Catenina

A via de sinalização Wnt é antiga evolutivamente e conservada. Regula principalmente aspectos cruciais da especificação do destino, migração e polaridade celulares e padronização neural e organogênese durante o desenvolvimento embrionário (GIRARDI; LE GRAND, 2018; KOMIYA; HABAS, 2008).

A família de proteínas Wnt compreende um grupo de glicoproteínas secretadas em vários processos biológicos. Cada Wnt se liga ao seu receptor específico *frizzled* (Fzd), ativando a via de sinalização. Estes peptídeos ricos em cisteína sofrem modificações químicas por reações de óxido redução ou por ligação a ácidos graxos como por exemplo palmitoilação (HUELSKEN; BEHRENS, 2002; KATOH; KATOH, 2007; VAN AMERONGEN; NUSSE, 2009; WODARZ; NUSSE, 1998). O primeiro gene da sinalização Wnt descoberto foi o *Int-1* em 1982 (NUSSE; VARMUS, 1982) e compreende pelo menos três vias diferentes. A primeira, referida como canônica, contém a β -catenina, que ativa a transcrição de genes alvo no núcleo da célula. A via não canônica envolve a proteína *jun N-terminal kinase* (JNK) e rearranjos no citoesqueleto celular. E, a última via, também não canônica, é a Wnt/ Ca^{2+} , que culmina na liberação do íon cálcio no citoplasma celular (HUELSKEN; BEHRENS, 2002; HULSKEN; BEHRENS, 2000).

A via canônica promove ativação do receptor Fzd e seu co-receptor *lipoprotein receptor-related protein* (LRP) pela ligação do Wnt levando ao recrutamento de *Dishevelled* (Dsh) até a membrana celular para interagir com Fzd (HUELSKEN; BEHRENS, 2002; KLAUS; BIRCHMEIER, 2008). A ligação do Wnt ao seu receptor promove a estabilização da proteína β -catenina no citoplasma celular. Assim, a β -catenina se estabiliza, migra para o núcleo da célula e forma um complexo transcricional com os fatores de transcrição *lymphoid enhancer factor* (LEF) e *T-cell factor* (TCF). Estes levam à ativação de genes alvos do Wnt (Figura 4) (HUELSKEN; BEHRENS, 2002; KLAUS; BIRCHMEIER, 2008). Dentre esses genes, estão o fator de transcrição *Myogenic differentiation factor* (MyoD). A expressão aumentada deste caracteriza o comprometimento das células para o programa miogênico e o fator de transcrição *Myogenic fator 5* (Myf5), que define o início e

regula a miogênese no músculo esquelético do adulto (BENTZINGER; WANG; RUDNICKI, 2012; COSSU; BORELLO, 1999; LACOUR et al., 2017; RIDGEWAY et al., 2000). Caso não haja ligação de Wnt com o receptor, ocorre uma interação da axina com LRP fosforilado e Dsh formando um polímero agregado ao receptor que inativa o complexo, assim, a β -catenina é ubiquitinada e destinada a ser destruída pelo sistema ubiquitina proteassoma.

A via não canônica promove organização assimétrica do citoesqueleto e polarização coordenada de células epiteliais que ocorre com a ligação do Wnt ao receptor Fzd independente do co-receptor LRP (GORDON; NUSSE, 2006; HUELSKEN; BEHRENS, 2002; KOMIYA; HABAS, 2008). A via é então ativada por meio de Dsh que recruta *Dishevelled associated activator of morphogenesis 1* (Daam1) formando um dímero que ativa duas vias paralelas por meio de duas GTPases Rho e *Rho-associated kinase* (ROCK). Estas, por fim, levam a modificações e rearranjos do citoesqueleto (HUELSKEN; BEHRENS, 2002; KOMIYA; HABAS, 2008).

A via não canônica Wnt/ Ca^{2+} leva à liberação de Cálcio do retículo endoplasmático dependente de uma proteína G trimérica. Após ligação do Wnt em Fzd, Dsh é recrutado e junto com a proteína G ativam a fosfolipase C (PLC) e subsequentemente o inositol trifosfato (IP_3) culminando na liberação de Cálcio. Com o aumento deste íon, a calcineurina é ativada desfosforilando o fator de transcrição *nuclear factor of activated T-cells* (NFAT) promovendo destinos de células ventrais (no tubo neural) e como um modulador crítico das vias de polaridade celular planar e perpendicular (a divisão planar é a divisão em paralelo das células e a divisão perpendicular acontece na orientação apicobasal, normalmente uma divisão assimétrica) (HUELSKEN; BEHRENS, 2002; KOMIYA; HABAS, 2008).

Figura 4. Representação esquemática das vias do Wnt canônica, não canônica e Wnt/Ca²⁺.

Abreviaturas: MEC – matrix extracelular; Myc – *myelocytomatosis oncogene*; Nkd – *naked cuticle*; Ubx – *Ultrabithorax*; En-2 – *Engrailed 2*; PPAR δ – peroxisome proliferator activated receptor gamma; Xbra – *Xenopus homologue of Brachyury*; ID2 – *inhibitor of DNA binding 2*; Xnr3 – *Xenopus nodal-related 3*; MMP7 – *matrix metalloproteinase 7*; TCF-1 – *T-Cell-Specific Transcription Factor 1*; CK1 α – *Casein kinase 1 alpha*; GSK3 β – *Glycogen synthase kinase-3 β* ; APC – *Adenomatous polyposis coli*; CBP – *cAMP response element-binding protein*; HDAC – *Histone deacetylases*. O artigo que subsidiou e elaboração da figura foi: Huelsken e Behrens, 2002.

A β -catenina é ativada durante a sobrecarga do músculo esquelético, pois aumenta as frações citoplasmática e nuclear desta quando o músculo é submetido à sobrecarga funcional por meio de sinalização Wnt. A ativação de β -catenina induzida por Wnt foi associada com aumento da expressão de muitos componentes de sinalização da via do Wnt, incluindo Fzd1, Dsh (ARMSTRONG; ESSER, 2005; ROCHAT et al., 2004). Além da hipertrofia, a sinalização do Wnt no músculo esquelético regula a miogênese no reparo tecidual, fibrose e a sinapse neuromuscular (CISTERNAS et al., 2013; RUDNICKI; WILLIAMS, 2015). A ligação de Wnt7a ao seu receptor Fzd7 ativa diretamente a via PI3K-AKT-mTOR induzindo hipertrofia miofibrilar. O complexo receptor Fzd7 associa-se a proteína G α_s e PI3K e estes componentes são necessários para Wnt7a ativar a via PI3K-AKT-mTOR em

miotubos, o que levou a descoberta de uma via alternativa do Wnt, a Fzd7/PI3K/AKT/mTOR (VON MALTZAHN; BENTZINGER; RUDNICKI, 2012). Além disso, o Wnt7a regula a proliferação de células satélites musculares (GRAND et al., 2009). Wnt7a estimula a expansão simétrica de células satélites, mas não afeta o crescimento nem a diferenciação de mioblastos (GRAND et al., 2009).

2. HIPÓTESE

A hipótese do presente estudo é de que vias de sinalização específicas são ativadas durante a sobrecarga funcional sobre o músculo esquelético na condição de diabetes *mellitus* tipo 1 para garantir que ocorra hipertrofia.

3. OBJETIVO

O objetivo do estudo foi o de avaliar o efeito da ablação do músculo tibial anterior e tenotomia do músculo gastrocnêmio e consequente sobrecarga sobre os músculos sóleo e EDL concomitante ao estabelecimento do estado diabético em ratos. As seguintes vias de sinalização foram estudadas: PI3K-AKT-mTOR, mecanotransdução, miostatina e folistatina e Wnt/ β -catenina.

4. JUSTIFICATIVA

O estado diabético compromete a massa e a função do músculo esquelético e causa miopatia atrofica (D'SOUZA et al., 2013). A regulação da massa muscular é fator importante na regulação do metabolismo da glicose e manutenção da glicemia (DEFRONZO et al., 1981a, 1981b). A fraqueza muscular e dificuldade de locomoção de pacientes diabéticos incapacitam-nos para as atividades físicas rotineiras, como caminhar e subir escadas (ANDERSEN, 2012). A diminuição da mobilidade também está associada com a alta incidência de comorbidades e mortalidade de pacientes diabéticos (PAHOR et al., 2014). Portanto, a melhora do trofismo do músculo esquelético (D'SOUZA et al., 2013) preserva a capacidade dos pacientes diabéticos para atividades físicas, melhorando sua condição clínica.

Há evidências de que a ausência de insulina não prejudica a resposta hipertrófica muscular esquelética induzida por sobrecarga funcional ou exercício resistido em animais diabéticos do tipo 1 (FARRELL et al., 1999b; FORTES et al., 2015; GOLDBERG, 1968a). O treinamento físico pode diminuir a degradação de

proteínas induzida pelo quadro de diabetes. Farrell e colaboradores (1999) reportaram indução de hipertrofia muscular esquelética em animais diabéticos pela prática de exercícios resistidos (FARRELL et al., 1999). Conforme os pesquisadores, a resposta hipertrófica no diabético é desencadeada por ativação compensatória da via de sinalização do IGF (*Insulin-like growth factor*). No entanto, os mecanismos envolvidos ainda não estão elucidados. A compreensão dos mecanismos que asseguram a resposta hipertrófica no estado de hiperglicemia e hipoinsulinemia contribuirá para o desenvolvimento de estratégias de preservação da massa muscular no paciente diabético.

Foi verificado previamente que a hipertrofia do músculo esquelético induzida por sobrecarga ocorre na mesma magnitude que em controles nos ratos diabéticos induzidos por estreptozotocina (FORTES et al., 2015). Este estudo foi desenvolvido em animais com a hiperglicemia do estado diabético já estabelecida, portanto, crônico, por um período de 30 dias. No presente estudo foi avaliado o efeito da hipertrofia por sobrecarga concomitante ao início do estado diabético em ratos.

5. PROTOCOLO EXPERIMENTAL

Figura 5 – Desenho experimental

Os animais controles foram injetados com tampão citrato. STZ = estreptozotocina

A: Indução de diabetes por injeção i.v. de estreptozotocina (65 mg/kg de massa corporal). Após a cirurgia, os animais foram submetidos à sobrecarga funcional por 30 dias e então foram coletados os músculos sóleo e EDL para análise da massa muscular e diâmetro das fibras. Outro grupo de animais foi utilizado para avaliação da função contrátil (força máxima e resistência à fadiga).

B: Indução do diabetes semelhante ao do tipo I por injeção i.v. de estreptozotocina (65 mg/kg de massa corporal). Após a cirurgia, os animais foram submetidos à sobrecarga funcional por 7 dias e então foram coletados os músculos sóleo e EDL para análise dos conteúdos de AKT, rpS6, 4EBP-1 e AMPK totais e fosforiladas e MuRF-1 e atrogina-1 e análise dos conteúdos de mRNA dos genes FAK (*Focal Adhesion Kinase*), Wnt7a, Axina, miostatina, follistatina, IGF-1.

6. MATERIAIS E MÉTODOS

6.1 Animais

Ratos Wistar de 3 semanas de idade foram obtidos do Biotério Central do Instituto de Ciências Biomédicas da Universidade de São Paulo. Os procedimentos experimentais nos animais foram iniciados quando os animais atingiram um peso mínimo de 200±50 g. Os procedimentos realizados neste estudo foram aprovados pela Comissão de Ética em Experimentação Animal (CEEA) do Instituto de Ciências Biomédicas da Universidade de São Paulo (nº 23 na fl. 16 do livro 03).

6.2 Indução ao diabetes mellitus semelhante ao do tipo I

Para indução do diabetes *mellitus*, os animais receberam uma única injeção intravenosa de estreptozotocina (65 mg/kg de massa corpórea i.v. caudal) dissolvida em tampão citrato, pH 4,2. Animais controle receberam volume equivalente de tampão citrato pela mesma via. A glicemia foi determinada em um monitor de glicose (Roche Diagnostics Corporation, IN, EUA), utilizando-se amostras de sangue obtidas da extremidade da cauda dos animais. Foram utilizados somente animais com glicemia superior a 400 mg/dL (22,2 mM).

6.3 Cirurgia de ablação sinérgica do músculo tibial e tenotomia do músculo gastrocnêmio

Na tenotomia do gastrocnêmio, uma incisão longitudinal foi realizada na pata traseira esquerda estando o animal sob anestesia (quetamina e xilazina, 200mg/kg e 100 mg/kg de massa corpórea, por via i.p., respectivamente), expondo o músculo a ser removido. Os tendões proximal e distal foram então isolados para a excisão do distal sem comprometimento do nervo, suprimento de sangue e outros músculos. O

músculo gastrocnêmio não foi removido para não afetar a volemia, massa corporal total e função da pata a ser analisada. A pele foi então suturada e o corte coberto com solução contendo iodo (povidine) para prevenir contaminação. Na pata direita foi realizada simulação (*sham*) do procedimento cirúrgico (BODINE; BAAR, 2012; FORTES et al., 2015; GUTH; YELLIN, 1971; JAMES, 1976; ROY et al., 1982).

Na ablação do tibial anterior, os mesmos procedimentos operacionais foram aplicados, exceto que a incisão foi realizada na porção anterior da pata traseira esquerda expondo o músculo tibial anterior. Simulação (*sham*) do procedimento cirúrgico foi realizada na pata direita. Imediatamente após a cirurgia, os animais foram injetados com gentamicina (40 mg/kg i.p) (ARMSTRONG et al., 1979; BALDWIN et al., 1982; BODINE; BAAR, 2012; DEGENS; TUREK; BINKHORST, 1993; FORTES et al., 2016).

O modelo de sobrecarga funcional é utilizado há bastante tempo (BODINE; BAAR, 2012; GOLDBERG, 1968b). Este modelo experimental utilizado para induzir a hipertrofia muscular esquelética proporciona resposta satisfatória para análise em um curto período de tempo após intervenção cirúrgica (ALWAY et al., 2005). A sobrecarga compensatória é alcançada por inativação da musculatura sinergista, enquanto o músculo que permanece no animal é sobrecarregado durante a locomoção (SAKUMA et al., 1998). Os músculos sinergistas podem ser inativados ou por tenotomia, ablação ou desnervação. Na tenotomia, não se faz remoção da musculatura, somente o tendão distal dos músculos sinergistas é seccionado, por consequência ocorre sobrecarga no músculo remanescente, o que não afeta a locomoção nem a volemia do animal. A tenotomia é utilizada principalmente para o estudo dos músculos plantar e sóleo (GOLDBERG, 1968b; PEHME et al., 2004).

A ablação sinergista é caracterizada pela remoção do músculo sinergista, mantendo no animal somente o músculo alvo de estudo (ARMSTRONG et al., 1979; GUTH; YELLIN, 1971; JAMES, 1976). A ablação dos músculos gastrocnêmio e sóleo sobrecarrega o músculo plantar (GUTH; YELLIN, 1971; JACKSON et al., 2012; ROY et al., 1982) e a ablação do músculo tibial anterior promove sobrecarga sobre o músculo EDL (HAMILTON et al., 2010; JAMES, 1976). Nesses modelos, a pata contralateral serve como controle interno (ALWAY et al., 2005).

6.4 Western blotting de AKT, rpS6, AMPK e 4EBP-1 totais e fosforiladas, MuRF-1 e atrogina-1

Os músculos EDL e sóleo foram retirados após 7 dias da indução de hipertrofia. O tecido foi homogeneizado em solução tampão para extração de proteínas contendo ácido diaminoetanotetraacético (EDTA) 10 mM, Tris-Base 100 mM, pirofosfato de sódio 10 mM, fluoreto de sódio 100 mM, ortovanadato de sódio 10 mM, PMSF (fenilmetilsulfonilfluoreto) 2 mM e aprotinina 0,01 mg/mL. O conteúdo total de proteínas do sobrenadante de cada amostra foi estimado conforme descrito anteriormente (BRADFORD, 1976). Quantidades iguais (30 g) de proteína das amostras foram separadas por eletroforese em gel de poliacrilamida-dodecil sulfato de sódio (SDS-PAGE - *sodium dodecyl sulfate polyacrylamide gel electrophoresis*) como previamente descrito (SHAPIRO et al., 1967). Em seguida foram transferidas para membrana de nitrocelulose (TOWBIN et al., 1979). As membranas foram expostas durante períodos variados de tempo utilizando o fotodocumentador de quimioluminescência Amersham Imager 600UV (Amersham Biosciences, Little Chalfont, RU). As bandas contendo as proteínas estudadas foram quantificadas por análise de densitometria óptica utilizando o *software* de domínio público *Image J* (<http://rsbweb.nih.gov/ij/>) e normalizadas pelo controle interno de proteína da amostra (utilizando o corante Ponceau) (FERGUSON et al., 2005; FORTES et al., 2016). As quantidades de proteínas das membranas foram normalizadas utilizando um pool de 12 amostras contendo volumes iguais de 3 amostras dos quatro grupos. Os resultados também foram normalizados pela média de unidade arbitrária do grupo controle em sua pata contralateral que não sofreu hipertrofia, o que foi considerado como 1 para cada gel. Procedimento similar foi utilizado em estudos anteriores do grupo (FORTES et al., 2015, 2016, PINHEIRO et al., 2010, 2011). Os anticorpos primários utilizados foram: p-AKT em Ser 473 (9271), AKT (9272), p-rpS6 em Ser 240/244 (5364), rpS6 (2217), p-4E-BP1 em Thr 37/46 (2855), 4E-BP1 (9644), p-AMPK-alpha em Thr 172 (2535), AMPK-alpha (2532) da *Cell Signaling Technology* (Danvers, MA, EUA) e atrogina-1 (AP2041) e MuRF-1 (MP3401) da *ECM Biosciences* (Versailles, KY, EUA). O anticorpo secundário utilizado foi HRP-conjugated antibody anti-rabbit (Millipore, Temecula, CA, EUA).

As proteínas normalmente utilizadas como controle constitutivo sofrem variação de expressão neste modelo experimental (FERGUSON et al., 2005; FORTES et al., 2016). Foi realizado um estudo com cinco diferentes proteínas constitutivas (GAPDH, β -actina, α -tubulina, γ -tubulina e α -actinina) para elucidar se

no modelo de hipertrofia induzida por sobrecarga funcional e no diabetes ocorre modulação e encontrou-se variação significativa em todas as proteínas constitutivas avaliadas (FORTES et al., 2016).

É comum avaliar pelo menos uma proteína constitutiva na análise por *western blotting*, porque se acredita que algumas enzimas metabólicas ou estruturais não alteram sua concentração em tratamentos diferentes (EATON et al., 2013; FERGUSON et al., 2005; WELINDER; EKBLAD, 2011). As proteínas mais comumente utilizadas são actina, tubulina e gliceraldeído 3-fosfato desidrogenase (GAPDH) (EATON et al., 2013; GARCÍA-PRAT et al., 2016; HAMILTON et al., 2014; RIVERO-GUTIÉRREZ et al., 2014; WELINDER; EKBLAD, 2011). Sabe-se que várias proteínas constitutivas variam significativamente entre as células, tecidos e diferentes condições experimentais (FERGUSON et al., 2005; FORTES et al., 2016). A utilização de proteínas constitutivas deve ser cuidadosamente escolhida em relação aos tipos de células ou tecidos, o tratamento ou estado da doença, e em nenhuma circunstância deverão ter o sinal saturado (FERGUSON et al., 2005; GHOSH; GILDA; GOMES, 2014). Para resolver este problema, o desenvolvimento de alternativas recentemente foi criada, por exemplo Ponceau S e Stain-Free Tecnologia (GILDA; GOMES, 2013, 2015; ROMERO-CALVO et al., 2010; ZENG et al., 2013). Ponceau S é uma técnica de coloração que envolve a preparação de uma solução corante de vermelho Ponceau e ácido acético e incubar a membrana de modo que as faixas de proteína e as bandas ficam claramente visíveis após uma breve lavagem com água destilada, a membrana se torna incolor (NAKAMURA et al., 1985; ROMERO-CALVO et al., 2010). Postula-se que a utilização de uma única proteína constitutiva como controle de carregamento é inadequada para normalizar os resultados das análises por *western blot* (GILDA; GOMES, 2015). Isso não significa que as proteínas constitutivas não podem ser usadas como controles de carregamento, mas que a sua utilização deve ser abordada com cautela (EATON et al., 2013). Nos modelos utilizando o diabetes induzido por STZ e a hipertrofia do músculo esquelético, concluímos que o Ponceau S é um método mais preciso para a quantificação de proteína constitutivas quando comparadas com as proteínas constitutivas testadas (FORTES et al., 2016). A coloração por Ponceau S é rápida, de baixo custo e não tóxica e a ligação é totalmente reversível em poucos minutos (ROMERO-CALVO et al., 2010).

6.5 Extração e quantificação de RNA total, síntese de cDNA e reação em cadeia da polimerase em tempo real (RT-PCR) de FAK, AKT, mTOR, β -catenina, MuRF-1 e atrogina-1, MG53, IGF-1, MGF, miostatina, Folistatina, Wnt7a, axina2

Os músculos sóleo e EDL foram isolados e o RNA total foi extraído utilizando-se o kit RNeasy (Qiagen, Hilden, Alemanha), conforme instruções do fabricante. A concentração de RNA foi estimada por densidade óptica em 260 nm (cada unidade de leitura corresponde a 40 μ g/mL de RNA), utilizando-se o espectrofotômetro NanoDrop (Thermo Scientific, Wilmington, Delaware, EUA). O grau de pureza do RNA foi determinado pela razão de absorbância nos comprimentos de onda de 260/280 nm (A_{260}/A_{280}) onde uma razão entre 1,8 e 2 indica grau aceitável de pureza. Foram utilizados 2 μ g de RNA para transcrição reversa e síntese do cDNA, utilizando *primers* oligo-dT e o kit de transcriptase reversa M-MLV (Invitrogen/Life Technologies, Carlsbad, Califórnia, EUA) de acordo com as especificações do fabricante. A reação de transcrição reversa foi realizada na ordem: 70 °C por 10 minutos, 37 °C por 60 minutos e 95 °C por 10 minutos. Para amplificação das sequências de interesse do cDNA foram usados 2 μ L de cDNA, 5 μ L de SYBR Green (Invitrogen/Life Technologies), 1 μ L (10 μ M) de cada *primer sense* e *anti-sense* do gene-alvo e 1 μ L de água milli-Q autoclavada. A RT-PCR foi realizada com as seguintes etapas: 50 °C por 2 minutos (ativação da enzima), 95 °C por 10 minutos (desnaturação) e 45 ciclos de três fases – a primeira a 95 °C por 20 segundos (desnaturação), a segunda a 58 °C por 30 segundos (anelamento) e a terceira a 72 °C por 30 segundos (extensão). A análise da fluorescência proveniente do SYBR Green, conforme amplificação específica da sequência de interesse, foi realizada no detector Rotor-Gene Q (Qiagen, Hilden, Alemanha). O *cycle threshold* (CT), ciclo no qual a sequência de interesse foi amplificada o suficiente para gerar fluorescência distinguível daquela de fundo, foi avaliado em duplicata para cada amostra. A subtração do CT do gene de interesse do CT do gene de referência ou *housekeeping* apresenta como resultado a variação de CT (Δ CT). As amostras avaliadas serão normalizadas pela média de Δ CT dos animais controle (Δ CT amostra – média Δ CT controle), obtendo-se o valor de $\Delta\Delta$ CT. A expressão relativa dos genes de interesse, em unidades arbitrárias, foi calculada pela fórmula $2^{-\Delta\Delta CT}$. O gene de referência inicialmente escolhido foi o *Hypoxanthine Phosphoribosyltransferase 1* (HPRT1). Os *primers* utilizados para a avaliação da expressão de mRNA estão listados na tabela 1 e foram desenhados com a ferramenta PRIMER BLAST em <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>.

Tabela 1 – Primers utilizados para avaliação da expressão de mRNA por RT-PCR

	Primer Sense	Primer Antisense
FAK	5'-AAGGAGCACCTCTCAAACCG-3'	5'-CATCGCTCCGACAGCATTTG-3'
AKT1	5'-GCCCAAGCACCGTGTGACCA-3'	5'-GCGACCTGTGGCCTTCTCCT-3'
mTOR	5'-AGGGACCACTGTGCCAGAATCCA-3'	5'-TGAGAGAAATCCCGACCAGTGAGC-3'
B-Catenina	5'-AGCGCTGGTGAAATGCTTG-3'	5'-CGCACTGCCATTTAGCTCC-3'
MuRF-1	5'-GGACCGGCATGGGGGTACG-3'	5'-TTTCTGCAGGGGCCGACTGG-3'
Atrogina-1	5'-CGGCACCTTCGTGAGCGACC-3'	5'-GTGCAGATATCCATGGCGCTCCT-3'
MG53	5'-CAGGCGCTAAGCACTAACCT-3'	5'-GGTCCTGCTCGCAGTAGATG-3'
IGF-1	5'-TGAGCGCACCTCCAATAAAGA-3'	5'-GAACTGAAGAGCGTCCACCA-3'
MGF	5'-TGGTGGACGCTCTTCAGTTC-3'	5'-TCCGGAAGCAACACTCATCC-3'
Miostatina	5'-TACCACGGAAACAATCATTACCAT-3'	5'-TGCCATCCGCTTGCATT-3'
Folistatina	5'-AGCGAGTGTGCCATGAAG-3'	5'-GAGTGAAGAGATAGGGAAGC-3'
Wnt 7a	5'-GCGCTCTAGGACAGTCTCCA-3'	5'-GGGGCAATCCACATAGCCTG-3'
Axin 2	5'-CTCAGCAAAAAGGGAAATTACAGGTAT-3'	5'-ACTGTCTCGTCGTCGCCAGATCTC-3'
HPRT1	5'-GCGAAAGTGGAAAAGCCAAGT-3'	5'-GCCACATCAACAGGACTCTTGTA-3'

Abreviaturas: FAK – Focal Adhesion Kinase; AKT1 – *Protein Kinase B*; mTOR – *Mammalian target of rapamycin*; MuRF-1 – *Muscle RING-Finger protein-1*; MG53 – *Mitsugumin 53*; IGF-1 – *Insulin-like growth factor 1*; MGF – *Mechano growth factor*; Wnt 7a – *Wingless-Type MMTV Integration Site Family, Member 7A*; HPRT1 – *Hypoxanthine Phosphoribosyltransferase 1*.

6.6 Análise gravimétrica dos músculos sóleo e EDL

Os músculos sóleo e EDL retirados em 7 e 30 dias após a indução da hipertrofia tiveram as massas úmidas determinadas em balança de precisão. Em seguida, foram colocados em estufa aquecida, durante 72 horas, a 37 °C, para evaporação do conteúdo de água. Após esse período, a massa seca foi determinada.

6.7 Teste das forças tetânica e isotônica, absoluta e específica

O protocolo de estimulação elétrica foi adaptado de Wojtaszewski et al. (1996) e Silveira et al. (2007) e realizado conforme Fortes et al. (2015). Após 30 dias de sobrecarga, os animais foram submetidos ao teste avaliação da função muscular. Os ratos foram anestesiados com quetamina e xilazina (quetamina e xilazina, 200 mg/kg e 100 mg/kg de massa corpórea, por via i.p.) e a pele da pata foi removida. Uma incisão foi realizada na região proximal e lateral da coxa. Em seguida, o nervo ciático foi localizado e um eletrodo de platina foi acoplado neste conforme descrito

anteriormente (FORTES et al., 2015; PINHEIRO; VITZEL; CURI, 2012). A aquisição dos dados foi realizada com o auxílio do *software* AqDados® (LYNX - Tecnologia Eletrônica Ltda., São Paulo, Brasil). O estímulo para produção de contrações tetânicas (máximas) consistiu de um pulso de 500 μ s de duração e frequência de 100 Hz. A voltagem foi ajustada para induzir produção de força máxima. Foi realizada uma série de 10 contrações tetânicas. A força isotônica máxima desenvolvida foi determinada por amplitude dos abalos musculares obtidos com frequência de estimulação de 1Hz e pulso de 500 μ s de duração. A resistência à fadiga foi avaliada pela queda da força ao longo de 10 contrações tetânicas sucessivas. Os dados foram analisados com o auxílio do programa *AqAnalysis*® (LYNX - Tecnologia Eletrônica Ltda., São Paulo, Brasil). Após o teste, os animais foram imediatamente eutanasiados por decapitação.

6.8 *Análise das propriedades contráteis dos músculos EDL e sóleo*

Uma série de dez contrações foi realizada e usada para determinar a força isotônica absoluta máxima, o tempo de contração (TTP – *time to peak* – tempo entre o início do desenvolvimento de força até a tensão máxima durante a contração) e o tempo de relaxamento (HRT – *half relaxation time* – tempo entre a tensão máxima e a redução de 50% da força durante o relaxamento e LRT - *late relaxation time* – tempo entre a tensão a 50% da força até 25% durante o relaxamento muscular) e a taxa de desenvolvimento de força (RFD – *rate of force development* - força gerada por unidade de tempo durante a contração muscular).

Foi realizada uma série de dez contrações tetânicas. A primeira contração teve duração de dez segundos e foi utilizada para avaliação da força tetânica absoluta máxima. As nove contrações subsequentes tiveram duração de 1 segundo cada e foram realizadas em intervalos de dez segundos entre elas. Estas contrações foram utilizadas para estimar a queda relativa de força em comparação à força tetânica máxima inicial. As forças isotônica e tetânica específicas foram obtidas por normalização da força absoluta pela massa seca do músculo.

6.9 *Análise histológica*

A preparação das lâminas foi realizada a partir de cortes seriados na porção central dos músculos sóleo e EDL dos animais após 30 dias de sobrecarga funcional. Após a coleta, os músculos foram emblocados em OCT (*Optimal Cutting Temperature* – OCT, 4583, Sakura Finetek, Inc., Torrance, CA, EUA) e mantidos em

nitrogênio líquido. Os cortes foram realizados transversalmente ao músculo (10 μ m), utilizando criostato (Leica CM 3050, Wetzlar, Alemanha). As lâminas foram coradas com hematoxilina e eosina (HE) para análise da área transversa das fibras musculares. Para a determinação das áreas das fibras musculares, imagens foram obtidas em aumento de dez vezes, utilizando microscópio óptico (Nikon Eclipse E1000, Fukuoka, Japão) e uma câmera acoplada a este (Nikon DXM 1200, Melville, EUA). Foram obtidas cinco imagens por músculo e estas foram analisadas utilizando o programa AxionVision® (version 4.8.1.0, Carl Zeiss Imaging Solutions). Foram mensuradas as áreas de 30 fibras musculares em cada imagem, totalizando 150 fibras por músculo (BODINE; BAAR, 2012; MARZUCA-NASSR et al., 2016).

6.10 Análise estatística

Os dados estão apresentados como média $\pm$ erro padrão da média (EPM) e foram analisados pelo teste *t* de *Student* (para comparação entre dois grupos) ou por análise de variância (ANOVA *two-way*) somente ou seguida pelo pós-teste de *Bonferroni* (para comparação entre três ou mais grupos). As diferenças entre os resultados foram consideradas significativas para valores de $p < 0,05$. Para a avaliação da área de secção transversa (CSA) dos músculos, utilizou-se o teste de normalidade de Anderson–Darling. As diferenças foram consideradas significativas quando não foi encontrada sobreposição entre o intervalo de confiança de 95% da média, pois os dados apresentaram distribuição normal (FORTES et al., 2015; PINHEIRO et al., 2011).

7. RESULTADOS

7.1 Caracterização do estado diabético

A massa corporal dos animais controles aumentou gradativamente (63%) enquanto nos animais diabéticos permaneceu quase inalterada (aumento de 11%) em 30 dias (Figura 6A). A glicemia permaneceu inalterada nos controles enquanto nos diabéticos aumentou em 17% ao longo de 30 dias (Figura 6B), sendo 3,9 vezes mais elevada do que no grupo controle, 72h após a indução de diabetes, confirmando o estabelecimento do estado hiperglicêmico.

Figura 6 – Massa corporal e glicemia dos animais durante 4 semanas.**A** **B**

(A) Evolução ponderal e (B) glicêmica dos animais controles e diabéticos ao longo de 4 semanas. Os valores estão apresentados como média $\pm$ EPM de no mínimo 13 animais. *** $p < 0,001$, pelo teste de ANOVA *Two-way* de medidas repetidas seguido do pós-teste de *Bonferroni*.

7.2 Resultados obtidos após 30 dias de sobrecarga funcional

Há evidências experimentais de que após 30 dias de sobrecarga é observada resposta trófica máxima nos músculos esqueléticos (ARMSTRONG; IANUZZO, 1977; FARRELL et al., 1999; KATTA et al., 2010). A análise realizada 30 dias após

indução de sobrecarga possibilitou investigar a correlação da ativação da sinalização celular com a hipertrofia muscular e a recuperação da atividade contrátil e função dos músculos sóleo e EDL.

7.3 Análise gravimétrica dos músculos EDL e sóleo

Após 30 dias de sobrecarga funcional por ablação do músculo tibial anterior, observou-se resposta hipertrófica significativa no músculo EDL. Nos animais controles, houve aumento de cerca de 25% da massa úmida absoluta e da massa úmida normalizada pelo comprimento da tíbia. Nos animais diabéticos, houve aumento de cerca de 38% e 33% da massa úmida absoluta e da massa úmida normalizada pelo comprimento da tíbia, respectivamente (Figura 7). Nos animais controles, houve aumento de 25% e 26% da massa seca absoluta e da massa seca normalizada pelo comprimento da tíbia. Nos animais diabéticos, houve aumento de 31% e 37% da massa seca absoluta e da massa seca normalizada pelo comprimento da tíbia, respectivamente (Figura 7). A hipertrofia foi semelhante nos animais diabéticos e controles conforme percentuais de aumento que não foram diferentes entre si. A responsividade à hipertrofia, representada pela média da razão de aumento, também não foi diferente entre os grupos.

Figura 7 – Massas úmida e seca, absoluta e normalizada pelo comprimento da tíbia, e razão de aumento na hipertrofia após 30 dias de sobrecarga do músculo EDL.

(A) Massa úmida normalizada pelo comprimento da tíbia do músculo EDL após 30 dias de sobrecarga funcional. (B) Massa seca normalizada pelo comprimento da tíbia do músculo EDL após 30 dias de sobrecarga funcional. (C) Razão de aumento da massa seca. (D) Massa úmida absoluta do músculo EDL após 30 dias de sobrecarga funcional. (E) Massa seca absoluta do músculo EDL após 30 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de no mínimo 6 animais. ** $p < 0,01$; *** $p < 0,001$, pelo teste ANOVA *Two-way* seguido do pós-teste de Bonferroni. CL = pata contralateral, H = pata hipertrofiada, CTRL = grupo controle, DM = grupo diabético.

Após 30 dias de sobrecarga funcional, o músculo sóleo apresentou resposta hipertrófica significativa. A hipertrofia do músculo sóleo foi semelhante nos animais diabéticos e controles. Observou-se aumento de 38% da massa úmida absoluta e da massa úmida normalizada pelo comprimento da tíbia no grupo controle. No grupo diabético, houve incremento de 42% e 48% da massa úmida absoluta e da massa úmida normalizada pelo comprimento da tíbia, respectivamente (Figura 8). Nos animais controles, 28% da massa seca absoluta e da massa seca normalizada pelo comprimento da tíbia aumentaram com a hipertrofia. Nos animais diabéticos, houve aumento de cerca de 45% da massa seca absoluta e da massa seca normalizada pelo comprimento da tíbia (Figura 8). A responsividade à

hipertrofia, representada pela razão de aumento, mostra ganho similar de massa muscular no músculo sóleo (Figura 8).

Figura 8 – Massa úmida e seca absoluta e normalizada pelo comprimento da tíbia e razão de aumento na hipertrofia após 30 dias de sobrecarga do músculo sóleo.

(A) Massa úmida normalizada pelo comprimento da tíbia do músculo sóleo após 30 dias de sobrecarga funcional. (B) Massa seca normalizada pelo comprimento da tíbia do músculo sóleo após 30 dias de sobrecarga funcional. (C) Razão de aumento da massa seca. (D) Massa úmida absoluta do músculo EDL após 30 dias de sobrecarga funcional. (E) Massa seca absoluta do músculo EDL após 30 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de no mínimo 8 animais. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$, pelo teste ANOVA *Two-way* seguido do pós-teste de Bonferroni. CL = pata contralateral, H = pata hipertrofiada.

7.4 Análise da força muscular e propriedades contráteis

Após 30 dias de sobrecarga, as forças tetânica absoluta e relativa do músculo EDL hipertrofiado foram diferentes entre os animais diabéticos e controles. A força tetânica absoluta aumentou em 49% nos animais controle e em 102% nos diabéticos (Figura 9A). A hipertrofia causou efeito significativo sobre a força específica (aumento de 91%), normalizada pela massa seca do músculo, nos animais diabéticos, mas não houve diferença nos animais controles. O aumento da força específica do músculo hipertrofiado em relação ao contralateral não foi diferente entre os grupos. A força isotônica absoluta no músculo EDL aumentou nos grupos controle e diabético em 79% e 114%, respectivamente (Figura 9C). O grupo

diabético apresentou força isotônica específica do músculo EDL mais elevada (67%) quando comparada ao controle que não teve aumento significativo (Figuras 6D).

Figura 9 – Análise das forças tetânicas e isotônicas, absolutas e específicas, do músculo EDL após 30 dias de sobrecarga nos grupos controle e diabético.

(A) Força tetânica absoluta do músculo EDL após sofrer sobrecarga funcional por 30 dias. (B) Força tetânica relativa do músculo EDL após sofrer sobrecarga funcional por 30 dias. (C) Força isotônica absoluta do músculo EDL após sofrer sobrecarga funcional por 30 dias. (D) Força isotônica relativa do músculo EDL após sofrer sobrecarga funcional por 30 dias. Os valores estão apresentados como média $\pm$ EPM de pelo menos 5 animais. * $p < 0,05$; *** $p < 0,001$, por ANOVA *Two-way* seguido do pós-teste de Bonferroni. CL = pata contralateral, H = pata hipertrofiada.

No músculo EDL, os grupos controle e diabético não apresentaram alteração no tempo de contração, indicado por TTP (Tabela 2). Também não apresentaram alteração no tempo de relaxamento muscular, indicado por HRT. Entretanto, o músculo EDL dos ratos diabéticos apresentou diminuição no LRT em 82% no músculo contralateral e de 28% no hipertrofiado, quando comparado ao controle (Tabela 2). A taxa de desenvolvimento de força, caracterizada por RFD, foi maior nos músculos hipertrofiados. Nos controles, houve aumento de 43% em relação ao

contralateral enquanto que no grupo diabético esse aumento foi de 59% no músculo EDL hipertrofiado.

Tabela 2 – Parâmetros contráteis do músculo EDL após 30 dias de sobrecarga nos animais controles e diabéticos.

Parâmetros Contráteis	Controle		Diabético	
	Contralateral	Hipertrofiado	Contralateral	Hipertrofiado
EDL				
TTP (ms)	22,85 ± 1,8	20 ± 0,0	24,0 ± 2,4	21,6 ± 1,6
HRT (ms)	20,3 ± 2,1	21,47 ± 1,7	17,3 ± 3,0	16,1 ± 1,6
LRT (ms)	13,9 ± 2,3	10,9 ± 0,7	7,6 ± 0,8*	8,5 ± 0,8
RFD (mN/ms)	41,1 ± 10,5	72,4 ± 10,9 ^{##}	22,9 ± 8,3	56,5 ± 11,1

Os valores estão apresentados como média ± EPM de pelo menos 5 animais. Os resultados foram significantes para LRT entre diabéticos vs controles considerando os músculos hipertrofiado e contralateral. Os resultados foram significantes para RFD entre os músculos dos grupos diabético e controle hipertrofiados vs os músculos dos grupos diabético e controle contralaterais. * $p < 0,05$ para CTRL-CL vs DM-CL, indicados somente por ANOVA *Two-way*; ^{##} $p < 0,01$ para CTRL-CL / DM-CL vs CTRL-H / DM-H, indicados somente por ANOVA *Two-way*. TTP – *time to peak*; HRT – *half relaxation time*; LRT – *late relaxation time*; RFD – *rate of force development*.

Após 30 dias de sobrecarga, o aumento da força tetânica absoluta do músculo sóleo hipertrofiado foi diferente do contralaterais nos dois grupos. A força tetânica absoluta aumentou em 77% nos animais controle e em 43% nos diabéticos (Figura 10A). O aumento da força tetânica específica do músculo sóleo hipertrofiado em relação ao contralateral não foi diferente entre os grupos. A força isotônica absoluta do músculo sóleo aumentou nos grupos controle e diabético em 31% e 35%, respectivamente (Figura 10C). O aumento da força isotônica específica do músculo sóleo hipertrofiado em relação ao contralateral não foi diferente entre os grupos.

Figura 10 – Análise das forças tetânica e isotônica, absoluta e específica, do músculo sóleo após 30 dias de sobrecarga nos grupos controle e diabético.

(A) Força tetânica absoluta do músculo sóleo após sofrer sobrecarga funcional por 30 dias. (B) Força tetânica relativa do músculo sóleo após sofrer sobrecarga funcional por 30 dias. (C) Força isotônica absoluta do músculo EDL após sofrer sobrecarga funcional por 30 dias. (D) Força isotônica relativa do músculo EDL após sofrer sobrecarga funcional por 30 dias. Os valores estão apresentados como média $\pm$ EPM de pelo menos 5 animais. * $p < 0,05$; analisado por ANOVA *Two-way* seguido do pós-teste de Bonferroni em A. Em C, $##p < 0,01$ para CTRL vs DM analisado por ANOVA *Two-way* e * $p < 0,05$ para CTRL-CL / DM-CL vs CTRL-H / DM-H analisado por ANOVA *Two-way*. CL = pata contralateral, H = pata hipertrofiada. # $p < 0,05$ = Grupo controle VS Grupo diabético analisado por ANOVA *Two-way*.

No músculo sóleo, os grupos controle e diabético não apresentaram alteração no TTP (Tabela 3). Também não apresentaram alteração no HRT. Entretanto, o músculo sóleo de ratos controles apresentaram diminuição de 56% no LRT quando sofreu hipertrofia (Tabela 3). A taxa de desenvolvimento de força foi menor no grupo diabético, tanto nos músculos controle quanto nos hipertrofiados.

Tabela 3 – Parâmetros contráteis do músculo sóleo após 30 dias de sobrecarga nos animais controles e diabéticos.

Parâmetros Contráteis	Controle		Diabético	
	Contralateral	Hipertrofiado	Contralateral	Hipertrofiado
Sóleo				
TTP (ms)	20 ± 0,0	25 ± 2,2	21,7 ± 1,7	21,7 ± 1,7
HRT (ms)	5,8 ± 1,1	5,0 ± 1,1	6,3 ± 1,5	4,5 ± 1,0
LRT (ms)	7,9 ± 0,5	3,5 ± 0,6*	6,0 ± 1,4	4,9 ± 1,4
RFD (mN/ms)	63,1 ± 9,2	74,4 ± 7,3	45,2 ± 5,9	54,6 ± 7,7§

Os valores estão apresentados como média ± EPM de pelo menos 5 animais. Os resultados foram significativos para LRT entre diabéticos vs controles considerando os músculos hipertrofiado e contralateral. Os resultados foram significativos para RFD entre os músculos dos grupos diabético e controle hipertrofiados vs os músculos dos grupos diabético e controle contralaterais. * $p < 0,05$ para CTRL-CL vs CTRL-H, analisado por ANOVA *Two-way* e pós teste de Bonferroni; § $p < 0,05$ para CTRL vs DM, analisado por ANOVA *Two-way*. TTP – *time to peak*; HRT – *half relaxation time*; LRT – *late relaxation time*; RFD – *rate of force development*.

7.5 Análise da área de secção transversa (CSA) das fibras dos músculos EDL e sóleo

A área da secção transversa (CSA – *cross sectional area*) das fibras dos músculos sóleo e EDL nos animais diabéticos e controles aumentou significativamente após 30 dias de sobrecarga funcional. No músculo EDL, no grupo controle, houve aumento de 97% e nos animais diabéticos de 69% (Figura 11). No músculo sóleo, houve aumento de 103% no grupo controle e de 70% nos animais diabéticos (Figura 12).

Figura 11 – Cortes transversais do músculo EDL dos grupos controle e diabético submetidos a 30 dias de sobrecarga funcional.

(A) Imagens histológicas dos cortes transversais do músculo EDL submetido a 30 dias de sobrecarga funcional com aumento de 20 vezes. (B) Área da seção transversa das fibras do músculo EDL. Os valores estão apresentados como intervalo de confiança de 95% da média. (C) Distribuição das fibras musculares de acordo com o intervalo no tamanho das fibras: 0–7000 μm^2 . CTRL-CL = Grupo controle, músculo contralateral, CTRL-H = Grupo controle, músculo hipertrofiado, DM-CL = Grupo Diabético, músculo contralateral, DM-H = Grupo diabético, músculo hipertrofiado, CSA = *cross sectional area*.

Figura 12 – Cortes transversais do músculo sóleo dos grupos controle e diabético submetidos a 30 dias de sobrecarga funcional.

(A) Imagens histológicas dos cortes transversais do músculo sóleo submetido a 30 dias de sobrecarga funcional com aumento de 20 vezes. (B) Área da seção transversa do músculo sóleo. Os valores estão apresentados como intervalo de confiança de 95% da média. (C) Distribuição das fibras musculares de acordo com o intervalo no tamanho das fibras: 0–7000 μm^2 . CTRL-CL = Grupo controle, músculo contralateral, CTRL-H = Grupo controle, músculo hipertrofiado, DM-CL = Grupo Diabético, músculo contralateral, DM-H = Grupo diabético, músculo hipertrofiado, CSA = *cross sectional area*.

Tabela 4. Resumo dos resultados no músculo EDL após 30 dias de sobrecarga.

	Efeito do diabetes	Resposta à hipertrofia induzida por sobrecarga funcional	
		Diabéticos	Controles
Massa úmida	↓	↑	↑
Massa seca	↓	↑	↑
Força tetânica absoluta	↔	↑	↑
Força tetânica relativa	↔	↑	↔
Força isotônica absoluta	↔	↑	↑
Força isotônica relativa	↔	↑	↔
TTP	↔	↔	↔
HRT	↔	↔	↔
LRT	↓	↓	↔
RFD	↔	↑	↑
CSA	↓	↑	↑

Tabela 5. Resumo dos resultados no músculo sóleo após 30 dias de sobrecarga.

	Efeito do diabetes	Resposta à hipertrofia induzida por sobrecarga funcional	
		Diabéticos	Controles
Massa úmida	↓	↑	↑
Massa seca	↓	↑	↑
Força tetânica absoluta	↔	↑	↑
Força tetânica relativa	↔	↔	↔
Força isotônica absoluta	↔	↑	↑
Força isotônica relativa	↔	↔	↔
TTP	↔	↔	↔
HRT	↔	↔	↔
LRT	↔	↔	↓
RFD	↔	↑	↑
CSA	↓	↑	↑

7.6 Resultados obtidos após 7 dias de sobrecarga funcional

A análise realizada 7 dias após indução de sobrecarga possibilitou avaliar a sinalização celular referente à hipertrofia muscular esquelética quando esta encontra-se em seu pico de ativação (ARMSTRONG; IANUZZO, 1977; FARRELL et al., 1999a; FORTES et al., 2015; HAMILTON et al., 2014; KATTA et al., 2010; MIYAZAKI et al., 2011; PETERSON; BRYNER; ALWAY, 2008; THOMSON; GORDON, 2006).

7.7 Conteúdos de rpS6, AKT, 4EBP-1 e AMPK totais e fosforiladas e MuRF-1 e atrogina-1 do músculo EDL

O efeito de 7 dias de sobrecarga funcional sobre os conteúdos das proteínas rpS6, AKT, 4EBP-1 e AMPK totais e fosforiladas, MuRF-1 e atrogina-1 foram avaliados no músculo EDL em animais submetidos a hipertrofia e hiperglicemia por 7 dias. Os resultados foram normalizados pelo conteúdo total de proteínas coradas com Ponceau conforme descrito em materiais e métodos.

O conteúdo de AKT total aumentou em 2,7 vezes no músculo EDL em 7 dias de sobrecarga no grupo controle (Figura 13). O conteúdo da p-AKT^{Ser473} aumentou em 3,2 vezes no grupo controle enquanto no diabético houve aumento de 2,6 vezes após 7 dias de sobrecarga (Figura 13). Após este mesmo período de sobrecarga no músculo EDL, o conteúdo de S6 total aumentou em 4,2 vezes (Figura 14) no grupo controle mas não se alterou no diabético. Os conteúdos de p-rpS6^{Ser244/240} aumentaram em 3,2 vezes no grupo controle e em 2,6 vezes no diabético em função da hipertrofia (Figura 14). O conteúdo de 4EBP-1 total, após 7 dias de sobrecarga, aumentou em 88% (Figura 14) no grupo controle, mas não se alterou no diabético. Os conteúdos de p-4EBP-1^{Thr37/46} não foram diferentes no músculo EDL entre os grupos (Figura 15). Já o conteúdo de AMPK total no grupo controle submetido à sobrecarga funcional foi reduzido em 38% (Figura 16). O conteúdo de p-AMPK^{Thr172} reduziu em 34% no grupo controle e em 54% no diabético submetidos à sobrecarga funcional (Figura 16). O conteúdo de MuRF-1 só se modificou no grupo diabético com diminuição de 51% (Figura 17) devido à hipertrofia. A atrogina-1 não apresentou mudança significativa decorrente da hipertrofia no músculo EDL (Figura 17).

Figura 13 – Análise das proteínas AKT total e fosforilada por *western blotting* no músculo EDL após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* no músculo EDL após 7 dias de sobrecarga funcional com a respectiva imagem representativa. (B) Análise quantitativa de *western blotting* da proteína AKT fosforilada no músculo EDL após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de no mínimo 6 animais. * $p<0,05$; *** $p<0,001$. A análise estatística foi realizada por ANOVA *Two-way* e pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A1.

Figura 14 – Análise das proteínas rpS6 total e fosforilada por *western blotting* do músculo EDL após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* no músculo EDL após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína rpS6 fosforilada no músculo EDL após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de no mínimo 6 animais. * $p<0,05$; *** $p<0,001$. A análise estatística foi realizada por ANOVA *Two-way* e pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A2.

Figura 15 – Análise das proteínas 4EBP-1 total e fosforilada por *western blotting* do músculo EDL após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína 4EBP-1 total no músculo EDL após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína 4EBP-1 fosforilada no músculo EDL após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 6 animais. Em A, $*p < 0,05$ para CTRL vs DM, analisado por ANOVA *Two-way*. Em B, $*p < 0,05$ analisado por ANOVA *Two-way* seguido do pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. A proteína 4EBP-1 total e fosforilada é representada por duas bandas. Os géis utilizados para a montagem da figura estão no quadro A3.

Figura 16 – Análise das proteínas AMPK total e fosforilada por *western blotting* no músculo EDL após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína AMPK Total no músculo EDL após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína p-AMPK no músculo EDL após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 6 animais. $*p < 0,05$; $**p < 0,01$ em A por ANOVA *Two-way* seguido de pós-teste de Bonferroni. Em B, $*p < 0,05$ para CTRL vs DM, analisado por ANOVA *Two-way*. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A4.

Figura 17 – Análise das proteínas MuRF-1 e atrogina-1 por *western blotting* no músculo EDL após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína MuRF-1 no músculo EDL após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína atrogina-1 no músculo EDL após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 7 a 8 animais. * $p < 0,05$. A análise estatística foi realizada por ANOVA *Two-way* e pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A5.

7.8 Conteúdos de rpS6, AKT, 4EBP-1 e AMPK totais e fosforiladas e MuRF-1 e atrogina-1 do músculo sóleo

Os conteúdos das proteínas rpS6, AKT, 4EBP-1, AMPK totais e fosforiladas, MuRF-1 e atrogina-1 foram avaliados no músculo sóleo em animais submetidos a hipertrofia e ao estado diabético por 7 dias. Os resultados foram normalizados pelo conteúdo total de proteínas corado com Ponceau.

O conteúdo de AKT total no músculo sóleo em 7 dias de sobrecarga no grupo controle aumentou em 83% enquanto que no diabético houve aumento de 1,6 vezes (Figura 18). O conteúdo da p-AKT^{Ser473} aumentou em 2,4 vezes no grupo controle enquanto que no diabético houve aumento de 3 vezes após 7 dias de sobrecarga (Figura 18). Após este mesmo período de sobrecarga no músculo sóleo, o conteúdo de rpS6 total aumentou em 3,7 vezes (Figura 19) no grupo controle e em 11,3 vezes no grupo diabético. Os conteúdos de p-rpS6^{Ser244/240} aumentaram em 2 vezes no grupo controle e em 8,6 vezes no diabético em função da hipertrofia (Figura 19). O conteúdo de 4EBP-1 total, após 7 dias de sobrecarga, aumentou em 66% (Figura 20) no grupo controle, no diabético houve aumento de 69%. Os conteúdos de p-4EBP-1^{Thr37/46} no músculo sóleo aumentou em 69% no grupo controle e em 65% no diabético (Figura 20). Já o conteúdo de AMPK total no grupo controle submetido à sobrecarga funcional reduziu em 40% e no grupo diabético em 27% (Figura 21). O

conteúdo de p-AMPK^{Thr172} não foi significativamente diferente entre os grupos (Figura 21). O conteúdo de MuRF-1 diminuiu em 26% (Figura 22) devido à hipertrofia no grupo controle e em 78% no grupo diabético. A atrogina-1 apresentou 36% de diminuição no grupo controle e de 45% no grupo diabético (Figura 22).

Figura 18 – Análise das proteínas AKT total e fosforilada por *western blotting* no músculo sóleo após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína AKT total no músculo sóleo após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína AKT fosforilada no músculo sóleo após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 6 animais. * $p < 0,05$; *** $p < 0,001$. A análise estatística foi realizada por ANOVA *Two-way* e pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A6.

Figura 19 – Análise das proteínas rpS6 total e fosforilada por *western blotting* no músculo sóleo após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína rpS6 total no músculo sóleo após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína rpS6 fosforilada no músculo sóleo após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 6 animais. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$. A análise estatística foi realizada por ANOVA *Two-*

way e pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A7.

Figura 20 – Análise das proteínas 4EBP-1 total e fosforilada por *western blotting* no músculo sóleo após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína 4EBP-1 total no músculo sóleo após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína 4EBP-1 fosforilada no músculo sóleo após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 6 animais. Em A, * $p < 0,05$ para CTRL vs DM analisado por ANOVA *Two-way* e # $p < 0,05$ para CTRL-CL / DM-CL vs CTRL-H / DM-H analisado por ANOVA *Two-way*. Em B, * $p < 0,05$ análise realizada por ANOVA *Two-way* seguido de pós teste de Bonferroni CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético, # = Grupo controle VS Grupo diabético. A proteína 4EBP-1 total e fosforilada é representada por duas bandas e os géis utilizados para a montagem da figura estão no quadro A8.

Figura 21 – Análise das proteínas AMPK total e fosforilada por *western blotting* no músculo sóleo após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* da proteína AMPK Total no músculo sóleo após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da p-AMPK no músculo sóleo após 7 dias de sobrecarga funcional. Os valores estão apresentados como média $\pm$ EPM de 6 animais. Em

A, * $p < 0,05$ para CTRL-CL / DM-CL vs CTRL-H / DM-H analisado por ANOVA *Two-way*. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A9.

Figura 22 – Análise das proteínas MuRF-1 e atrogina-1 por *western blotting* no músculo sóleo após 7 dias de sobrecarga funcional nos grupos controle e diabético.

(A) Análise quantitativa de *western blotting* com imagem representativa correspondente da proteína MuRF-1 no músculo sóleo após 7 dias de sobrecarga funcional. (B) Análise quantitativa de *western blotting* da proteína atrogina-1 no músculo sóleo após 7 dias de sobrecarga funcional com respectiva imagem representativa. Os valores estão apresentados como média $\pm$ EPM de 6 animais. Em **A**, * $p < 0,05$ para CTRL-CL/DM-CL vs CTRL-H/DM-H analisado por ANOVA *Two-way*. Em **B**, * $p < 0,05$ analisado por ANOVA *Two-way* e pós-teste de Bonferroni. CL = contralateral, H = hipertrofiada, CTRL = Grupo controle, DM = Grupo diabético. Os géis utilizados para a montagem da figura estão no quadro A10.

7.9 Expressão do mRNA dos genes de FAK, AKT, mTOR, β -catenina, MuRF-1, atrogina-1, MG53, IGF-1, MGF, miostatina, folistatina, Wnt7a, axina2 no músculo EDL

A quantidade de mRNA foi avaliada após 7 dias de sobrecarga funcional no músculo EDL (Figura 23). Em relação à resposta hipertrófica, a quantidade de mRNA no grupo controle foi diferente para os genes: FAK, redução na expressão de 46%; AKT1, aumento de 2,5 vezes; β -catenina, redução de 64%; folistatina, aumento de 4,6 vezes. Em relação à resposta hipertrófica, a quantidade de mRNA no grupo diabético foi diferente nos genes: AKT1, aumento de 3,6 vezes; β -catenina, redução de 68%; miostatina, redução de 68%; Wnt7a, redução de 62%. A quantidade de mRNA foi diferente entre a pata contralateral do grupo controle em relação á diabética para os seguintes genes: FAK, 49% menor no grupo diabético; IGF-1, 74%

menor no grupo diabético; miostatina, 2,6 vezes maior no grupo diabético; folistatina, 3,6 vezes maior no grupo diabético.

Figura 23 – Expressão do mRNA avaliada por RT-PCR no músculo EDL.

A expressão relativa dos genes de interesse, em unidades arbitrárias, foi calculada através da fórmula $2^{-\Delta\Delta CT}$. O gene de referência utilizado foi o HPRT1. Os valores estão apresentados como média $\pm$ EPM de pelo menos 6 animais. * $p < 0,05$, ** $p < 0,01$, como indicado por ANOVA *Two-way* e pós-teste de Bonferroni. Em mTOR, # $p < 0,05$ para CTRL-CL/DM-CL vs CTRL-H/DM-H analisado por ANOVA *Two-way*. Abreviaturas: AKT - *Protein Kinase B*; mTOR - *mammal target of rapamycin*; FAK - *Focal adhesion kinase*; Wnt 7a - *Wingless-type MMTV integration site family, member 7A*; MuRF-1 -

Muscle RING-finger protein-1; IGF-1 – *Insulin-like growth factor 1*; MGF – *Mechano growth factor*; MG53 – *Mitsugumin 53*.

7.10 Expressão do mRNA dos genes de FAK, AKT, mTOR, β -catenina, MuRF-1, atrogina-1, MG53, IGF-1, MGF, miostatina, folistatina, Wnt7a, axina2 no músculo sóleo

A quantidade de mRNA foi avaliada após 7 dias de sobrecarga funcional no músculo sóleo (Figura 24). Em relação à resposta hipertrófica, a quantidade de mRNA no grupo controle foi diferente para os genes: FAK, redução na expressão de 43%; mTOR, redução de 51%; atrogina-1, redução de 58%; IGF-1, aumento de 98%; MGF, aumento de 57%; miostatina, redução de 79%; Wnt7a, redução de 33%, axina2, redução de 42%.

Em relação à resposta hipertrófica, a quantidade de mRNA no grupo diabético foi diferente em relação aos seguintes genes: FAK, redução na expressão de 27%; mTOR, redução de 33%; MuRF-1, redução de 60%; atrogina-1, redução de 45%; IGF-1, aumento de 4,5 vezes; miostatina, redução de 65%; folistatina, aumento de 52%; Wnt7a, redução de 30%.

A quantidade de mRNA foi diferente entre a pata hipertrofiada do grupo controle em relação à diabética para os seguintes genes: β -catenina, 23% menor no grupo diabético. Houve diferença na quantidade de mRNA entre a pata contralateral do grupo controle em relação á diabética para os seguintes genes: atrogina-1, 50% maior no grupo diabético; MGF, 51% menor no grupo diabético.

As modificações na sinalização celular que ocorrem no músculo sóleo e EDL mediante hipertrofia muscular com um estímulo de sobrecarga funcional por 7 dias são mostradas nas Figuras 25 e 26.

Figura 24 – Expressão do mRNA avaliada por RT-PCR no músculo sóleo.

A expressão relativa dos genes de interesse, em unidades arbitrárias, foi calculada através da fórmula $2^{-\Delta\Delta CT}$. O gene de referência utilizado foi o HPRT1. Os valores estão apresentados como média $\pm$ EPM de pelo menos 9 animais. * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ como indicado por ANOVA *Two-way* e pós-teste de Bonferroni. Em MG53, * $p < 0,05$ CTRL vs DM e # $p < 0,05$ para CTRL-CL / DM-CL vs CTRL-H / DM-H analisado por ANOVA *Two-way*. Em Wnt7a para * $p < 0,05$ para CTRL-CL / DM-CL vs CTRL-H / DM-H analisado por ANOVA *Two-way*. Abreviaturas: AKT - *Protein Kinase B*; mTOR - *mammal target of rapamycin*; FAK - *Focal adhesion kinase*; Wnt 7a - *Wingless-type MMTV integration site family, member 7A*; MuRF-1 - *Muscle RING-finger protein-1*; IGF-1 - *Insulin-like growth factor 1*; MGF - *Mechano growth factor*; MG53 - *Mitsugumin 53*.

Figura 25. Resumo dos resultados no músculo EDL após 7 dias de sobrecarga.

Modificações na sinalização celular que ocorrem no músculo EDL mediante hipertrofia muscular com um estímulo de sobrecarga funcional por 7 dias.

Figura 26. Resumo dos resultados no músculo sóleo após 7 dias de sobrecarga.

Modificações na sinalização celular que ocorrem no músculo EDL mediante hipertrofia muscular com um estímulo de sobrecarga funcional por 7 dias.

8. DISCUSSÃO

A sobrecarga compensatória por 30 dias causou hipertrofia semelhante nos músculos sóleo e EDL nos animais controles e diabéticos. Houve aumento significativo das forças tetânica e isotônica, absolutas e específicas, no músculo EDL em relação a pata que não sofreu sobrecarga funcional.

Após sofrer sobrecarga funcional por 30 dias, a força específica do músculo EDL se elevou mais nos animais diabéticos. A concentração intrassarcoplasmática de cálcio é mais elevada em animais diabéticos e como consequência as respostas contráteis se tornam mais intensas no músculo esquelético nessa condição (NAKAGAWA et al., 1989). O excesso de cálcio no sarcoplasma pode sobrecarregar as bombas (SERCA - sarco/endoplasmic reticulum Ca^{2+} -ATPase) de recaptção deste íon, causando acúmulo no sarcômero, comprometendo a contração muscular e podendo provocar também fadiga precoce. Em contrapartida, animais diabéticos induzidos por estreptozotocina apresentam maior conteúdo de calsequestrina, o que parece agir como tamponante ao excesso de Cálcio presente no sarcoplasma dos músculos desses animais (HOWARTH et al., 2002). O músculo sóleo de ratos e camundongos apresenta menor densidade de SERCA quando comparado ao EDL (SCHIAFFINO; REGGIANI, 2011). Há inibição da SERCA por exposição a altas concentrações de espécies reativas de oxigênio, as quais estão mais elevadas no músculo de animais diabéticos (ANDRADE et al., 2001; ANDRADE et al., 1998). Assim, ocorre menor recaptção de Cálcio, aumentando a sensibilidade miofibrilar a este íon e alterando a cinética da contração muscular (ANDRADE et al., 2001; ANDRADE et al., 1998). Em nosso estudo, houve aumento da força específica no músculo EDL hipertrofiado dos animais diabéticos, mas não foi observada a mesma resposta no músculo sóleo. O transiente de Cálcio é mais prejudicado no músculo EDL do que no sóleo, mantendo a concentração deste íon mais elevada e sua cinética de recaptção é prejudicada no músculo esquelético de animais diabéticos induzidos por STZ (ESHIMA; POOLE; KANO, 2015). O músculo sóleo foi menos afetado pelo diabetes e respondeu com mais intensidade à sobrecarga funcional.

Várias vias de sinalização que regulam a hipertrofia muscular mediante estímulo mecânico foram estudadas. Contudo, o fenômeno hipertrófico em sua totalidade se dá por um balanço entre síntese e degradação de proteínas (TOIGO; BOUTELLIER, 2006). No presente trabalho analisamos as vias: PI3K-AKT-mTOR, mecanotransdução, miostatina e folistatina e Wnt/ β -catenina. É necessário mencionar que outras vias estão também envolvidas na regulação da hipertrofia

muscular merecem ser investigadas em estudos futuros tais como: as calpaínas (μ e mili), sistema ubiquitina-proteassoma (contemplados em parte neste estudo por meio de MuRF-1 e atrogina-1), caspases, autofagia, estresse de retículo endoplasmático por meio da ativação da *unfold protein response* (UPR) (BAEHR et al., 2016; DELDICQUE, 2013; DELDICQUE; HESPEL; FRANCAUX, 2012; FOLETTA et al., 2011; SANDRI, 2008, 2010).

8.1 Via PI3K/AKT/mTOR

No músculo EDL, a ativação da via de síntese de proteínas (AKT e rpS6 fosforiladas) ocorreu de forma semelhante nos animais controles e diabéticos. Pode-se induzir hipertrofia muscular esquelética em animais diabéticos por meio de exercícios resistidos (FARRELL et al., 1999). No entanto, a hipótese apresentada para que isso ocorra foi de que, na resposta hipertrófica, a via de sinalização do IGF substitui a da insulina. O IGF estimula a via de síntese proteica quando, por exemplo, esta é induzida por sobrecarga funcional, promovendo hipertrofia (BODINE et al., 2001; COLEMAN et al., 1995; SCHIAFFINO; MAMMUCARI, 2011). Contudo, a hipertrofia por sobrecarga funcional não depende exclusivamente de estímulo proveniente da via do IGF-I no músculo esquelético, pois ocorre mesmo quando os receptores desse fator de crescimento estão inativados (KLOSSNER et al., 2009; SPANGENBURG et al., 2008). O IGF-I, quando ligado ao seu receptor, ativa a via PI3K/AKT/mTOR e seus principais efetores (p70S6K, S6, 4EBP-1) que atuam também na síntese de proteínas miofibrilares. A fosforilação subsequente da p70S6K leva à fosforilação da 4EBP-1, permitindo a ligação em eIF4E e assim promove síntese proteica ligando-se ao ribossomo (RICHTER; SONENBERG, 2005; BODINE et al., 2001; BODINE, 2006). Animais diabéticos induzidos por estreptozotocina apresentam concentração residual de insulina no plasma, que é inferior à observada no jejum de 24 horas (MOORE; MUNFORD; PILLSWORTH, 1983).

Os conteúdos das proteínas AKT e rpS6 fosforiladas aumentaram no músculo EDL quando submetido à sobrecarga funcional por 7 dias. Essas observações corroboram resultados encontrados por outros autores (MIYAZAKI et al., 2011; THOMSON; GORDON, 2006) quando induziram sobrecarga funcional por 7 dias no músculo plantar em ratos por ablação sinergista. A proteína 4E-BP1 é um peptídeo inibitório que ao ser fosforilado não permite a sua ligação na proteína *eukaryotic translation initiation factor 4E* (eIF4E) e a deixa livre para promover a síntese

proteica (MAYER et al., 2004; RICHTER; SONENBERG, 2005). Houve aumento no conteúdo total e fosforilado desta proteína corroborando estudos anteriores (BODINE, 2006; POTTER et al., 2013). AKT e mTOR, importantes componentes da via canônica, mostraram expressão de mRNA diferente entre os músculos sóleo e EDL. AKT teve expressão aumentada no músculo EDL hipertrofiado enquanto que no músculo sóleo não houve mudança. No músculo sóleo, a expressão de AKT foi diminuída na pata contralateral dos animais diabéticos comparados aos controles. A expressão de mTOR aumentou no músculo EDL hipertrofiado mas diminuiu no sóleo. Apesar da fosforilação e quantidade total de AKT estarem mais elevadas nos grupos controle e diabético e em outros estudos (FORTES et al., 2015; MIYAZAKI et al., 2011), a expressão do mRNA não foi similar às alterações na proteína no músculo sóleo, o que indica modificações pós traducionais (LOURENÇO DOS SANTOS et al., 2015; OHLENDIECK, 2011, 2013; WENDE, 2016).

A proteína quinase ativada por AMP (AMPK) atua como sensor de ATP e modula a captação de glicose e a oxidação de ácidos graxos no músculo esquelético (BOLSTER et al., 2002). No presente estudo, o estímulo hipertrófico reduziu os conteúdos da proteína AMPK total no músculo EDL do grupo controle e no músculo sóleo dos dois grupos, enquanto que o conteúdo de AMPK fosforilada diminuiu no músculo EDL em ambos os grupos. A proteína AMPK pode diminuir a atividade de mTORC1 por fosforilação de TSC2 em Thr1345 (INOKI; ZHU; GUAN, 2003) ou raptor em Ser792 (GWINN et al., 2008). Mounier e colaboradores, após realizarem ablação do gene da AMPK α 1, observaram hipertrofia mais acentuada induzida por sobrecarga (MOUNIER et al., 2009). Há também aumento na ativação de AMPK no músculo plantar (HAMILTON et al., 2014; MCGEE et al., 2008) relacionado à diminuição na hipertrofia do músculo esquelético (BOLSTER et al., 2002; THOMSON; GORDON, 2005). Houve redução significativa no conteúdo de MuRF-1 no músculo sóleo em animais diabéticos após 7 dias de sobrecarga. Com a ativação da via PI3K/AKT/mTOR, MuRF-1 e atrogina-1 podem ser inativadas pela inibição de uma proteína sinalizadora *upstream*, a FOXO (STITT et al., 2004).

MuRF-1 e atrogina-1 afetam o processo de remodelamento que ocorre durante o crescimento do músculo esquelético (BAEHR; TUNZI; BODINE, 2014), além de apresentarem conteúdos elevados nos estados de hipotrofia muscular (BODINE et al., 2001b). No músculo sóleo, a expressão de atrogina-1 no grupo diabético na condição basal se apresentou mais elevada do que no controle além de

apresentar redução mediante hipertrofia induzida por sobrecarga nos dois grupos. A expressão de MuRF-1 foi reduzida pela hipertrofia do sóleo no grupo diabético.

MG53 está envolvida na regulação de *feedback* negativo de miogênese e cliva o substrato do receptor de insulina-1 (IRS-1) (JUNG; KO, 2010; LEE et al., 2010). Atua de forma sinérgica com outros dois fatores de regulação miogênicos (MyoD – *myogenic differentiation factor* e MEF2 – *muscle enhancer factor 2*) para ser expresso (JUNG; KO, 2010; LEE et al., 2010). Por ubiquitinar e promover degradação do IRS-1, MG53 também pode agir sobre a cascata de sinalização PI3K-AKT-mTOR culminando em síntese de proteínas miofibrilares contráteis deficiente (YI et al., 2013).

8.2 Mecanotransdução

O perfil de expressão de mRNA das proteínas da via de mecanotransdução após 7 dias de hipertrofia foi diferente entre os músculos com predominância de fibras oxidativas e glicolíticas. O padrão de expressão dessas proteínas depende do tempo em que o animal permanece diabético (FORTES et al., 2015). A expressão de FAK, muito importante para a hipertrofia do músculo esquelético (FLÜCK et al., 1999; KLOSSNER et al., 2009), aumentou nos músculos sóleo e EDL hipertrofiados em 7 dias quando os ratos foram submetidos a um período de 37 dias de diabetes (FORTES et al., 2015). Contudo, com 7 dias de sobrecarga funcional e diabetes, a expressão de mRNA de FAK se comportou de modo contrário, diminuindo tanto no músculo sóleo quanto EDL, indicando que a expressão de proteínas relacionada às vias de hipertrofia sofre mudanças expressivas quando submetidas a diferentes ambientes, ou seja, início do diabetes ou estado crônica da doença.

O IGF-1 é um fator de crescimento estruturalmente semelhante à insulina que regula a diferenciação, crescimento, regeneração e manutenção do tecido muscular no adulto (DUAN; REN; GAO, 2010a, 2010b; SARTORELLI; FULCO, 2004). O MGF está amplamente envolvido no processo hipertrófico, pois é uma variante do gene do IGF-1, sendo produzido localmente no músculo esquelético quando este é submetido a estímulo mecânico (HILL; GOLDSPINK, 2003). Também sinaliza às células satélites, induzindo seu recrutamento para o local da lesão e permitindo a regeneração do músculo esquelético (GOLDSPINK, 2005a, 2005b, PHILIPPOU et al., 2007, 2009). No músculo sóleo, houve aumento de expressão do mRNA de IGF-1 e MGF nos grupos diabético e controle. Em animais submetidos a 37 dias de diabetes a expressão de mRNA desses genes no músculo sóleo hipertrofiado é

marcadamente mais elevada (FORTES et al., 2015). No músculo EDL, a expressão de IGF-1 foi menor no grupo diabético na pata contralateral do que nos controles. Em animais submetidos a 37 dias de diabetes, a expressão de mRNA desses genes no músculo EDL hipertrofiado é marcadamente maior (por volta de dez vezes maior nos controles e cinco vezes maior nos diabéticos) nos dois grupos (FORTES et al., 2015). Esta diferença é indicativa de que não é somente o período de tempo que o músculo permanece sob estímulo mecânico, mas também o microambiente em que este está que regula a expressão gênica de MGF. O período em que os animais permanecem diabéticos/hiperglicêmicos modifica a expressão dos genes relacionados às vias alternativas de hipertrofia muscular esquelética.

8.3 *Miostatina e follistatina*

O balanço entre miostatina e follistatina também regula a hipertrofia muscular esquelética, sendo que a miostatina inibe e a follistatina estimula (GILSON et al., 2009). A ausência de miostatina induz crescimento muscular excessivo enquanto que a elevação da sua expressão e administração exógena provoca hipotrofia muscular (MCPHERRON; LEE, 1997; MCPHERRON; LAWLER; LEE, 1997; RODRIGUEZ et al., 2014; SCHUELKE et al., 2004). No músculo EDL, o grupo diabético apresentou maior expressão basal de miostatina que reduziu mediante hipertrofia. A follistatina aumentou devido à hipertrofia no músculo EDL do grupo controle e foi marcadamente mais elevada no músculo EDL do grupo diabético sem hipertrofia. No músculo sóleo, ocorreu redução na expressão de miostatina nos dois grupos e aumentou de follistatina somente no grupo diabético.

8.4 *Via Wnt/ β -catenina*

A β -catenina é ativada durante a sobrecarga do músculo esquelético (ARMSTRONG; ESSER, 2005; ARMSTRONG; WONG; ESSER, 2006). A expressão de β -catenina no músculo EDL sofreu redução, o que não aconteceu no músculo sóleo. A axina2 está presente na via não canônica que também compreende a β -catenina e forma um complexo que impede a destruição da β -catenina (HUELSKEN; BEHRENS, 2002; KLAUS; BIRCHMEIER, 2008). No músculo sóleo, houve redução na expressão de axina2 em função da hipertrofia no grupo controle.

O Wnt7a, quando ligado a seu receptor Fzd7, ativa a via Fzd7/PI3K/AKT/mTOR induzindo hipertrofia por mecanismo alternativo à ativação dos receptores de IGF e insulina (VON MALTZAHN; BENTZINGER; RUDNICKI,

2012). Houve redução na expressão de Wnt7a no músculo EDL no grupo diabético em função da hipertrofia. No músculo sóleo, houve redução na expressão de Wnt7a com a hipertrofia no grupo diabético e controle.

CONSIDERAÇÕES FINAIS

Os músculos sóleo e EDL submetidos a sobrecarga apresentaram resposta hipertrófica semelhante em animais controle e diabéticos. O aumento nas forças musculares ocorreu na mesma magnitude que a hipertrofia muscular.

No músculo EDL de animais diabéticos, a hipertrofia promovida por sobrecarga mecânica, foi estimulada pela via PI3K-Akt-mTOR, redução da ativação de AMPK e diminuição da expressão de miostatina.

A hipertrofia foi mais pronunciada no sóleo de animais diabéticos, em comparação com a EDL, mantendo a massa muscular e força em valores semelhantes ao do músculo controle contralateral. Isso pode ser devido a uma sinalização hipertrófica mais preservada, em relação ao grupo controle, incluindo um maior conteúdo e ativação de rpS6 do que no músculo EDL, aumento da expressão de mRNA de IGF-1, MGF e folistatina e diminuição da expressão de mRNA da miostatina, MuRF-1 e atrogina-1 (Figura 27).

Conclui-se que o exercício de resistência é um aliado para a prevenção da miopatia diabética (hipotrofia) e, quando iniciado no início da progressão diabética, sua eficácia pode ser maior.

Figura 27 – Vias de sinalização que sofrem alteração mediante sobrecarga funcional por sete dias nos músculos sóleo e EDL.

(A) Efeito do estado diabético no seu início sobre o conteúdo de mRNA e (B) modulação das principais vias de sinalização envolvidas na hipertrofia dos músculos sóleo e EDL nos animais controles e diabéticos e nas duas condições.

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APÊNDICE A – Quadros de Resultados

Figuras dos géis das proteínas rpS6, AKT, 4EBP-1 e AMPK totais e fosforiladas e MuRF-1 e atrogina-1 do músculo EDL

A1) Resultados utilizados para calcular as médias apresentadas na Figura 13

A2) Resultados utilizados para calcular as médias apresentadas na Figura 14

A3) Resultados utilizados para calcular as médias apresentadas na Figura 15

Ponceau e géis obtidos por *western blotting* da proteína 4EBP-1 total e sua forma fosforilada no músculo EDL após 7 dias de sobrecarga funcional. Para a montagem do gráfico foi utilizado um n de 6 animais. DMCL – Pata contralateral grupo diabético; DMH – Pata hipertrofiada grupo diabético; CL – Pata contralateral; H – Pata hipertrofiada.

A4) Resultados utilizados para calcular as médias apresentadas na Figura 16

Ponceau e géis obtidos por *western blotting* da proteína AMPK total e sua forma fosforilada no músculo EDL após 7 dias de sobrecarga funcional. Para a montagem do gráfico foi utilizado um n de 5 a 6 animais. DMCL – Pata contralateral grupo diabético; DMH – Pata hipertrofiada grupo diabético; CL – Pata contralateral; H – Pata hipertrofiada.

A5) Resultados utilizados para calcular as médias apresentadas na Figura 17

Figuras dos géis das proteínas rpS6, AKT, 4EBP-1 e AMPK totais e fosforiladas e MuRF-1 e atrogina-1 do músculo sóleo

A6) Resultados utilizados para calcular as médias apresentadas na Figura 18

A7) Resultados utilizados para calcular as médias apresentadas na Figura 19

A8) Resultados utilizados para calcular as médias apresentadas na Figura 20

A9) Resultados utilizados para calcular as médias apresentadas na Figura 21

Ponceau e géis obtidos por *western blotting* da proteína AMPK total e sua forma fosforilada no músculo sóleo após 7 dias de sobrecarga funcional. Para a montagem do gráfico foi utilizado um n de 6 animais. DMCL – Pata contralateral grupo diabético; DMH – Pata hipertrofiada grupo diabético; CL – Pata contralateral; H – Pata hipertrofiada.

A10) Resultados utilizados para calcular as médias apresentadas na Figura 22

Ponceau e géis obtidos por *western blotting* da proteína atrogina-1 e MuRF-1 no músculo sóleo após 7 dias de sobrecarga funcional. Para a montagem do gráfico foi utilizado um n de 6 animais. DMCL – Pata contralateral grupo diabético; DMH – Pata hipertrofiada grupo diabético; CL – Pata contralateral; H – Pata hipertrofiada.

ANEXO: TRABALHOS PUBLICADOS NO PERÍODO

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ORIGINAL RESEARCH

Overload-induced skeletal muscle hypertrophy is not impaired in STZ-diabetic rats

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Keywords

Diabetes mellitus, electrical stimulation, hyperglycemia, muscle mass regulation, streptozotocin.

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Abstract

The aim of this study was to evaluate the effect of overload-induced hypertrophy on extensor digitorum longus (EDL) and soleus muscles of streptozotocin-induced diabetic rats. The overload-induced hypertrophy and absolute tetanic and twitch forces increases in EDL and soleus muscles were not different between diabetic and control rats. Phospho-Akt and rpS6 contents were increased in EDL muscle after 7 days of overload and returned to the pre-overload values after 30 days. In the soleus muscle, the contents of total and phospho-Akt and total rpS6 were increased in both groups after 7 days. The contents of total Akt in controls and total rpS6 and phospho-Akt in the diabetic rats remained increased after 30 days. mRNA expression after 7 days of overload in the EDL muscle of control and diabetic animals showed an increase in MGF and follistatin and a decrease in myostatin and Axin2. The expression of FAK was increased and of MuRF-1 and atrogin-1 decreased only in the control group, whereas Ankrd2 expression was enhanced only in diabetic rats. In the soleus muscle caused similar changes in both groups: increase in FAK and MGF and decrease in Wnt7a, MuRF-1, atrogin-1, and myostatin. Differences between groups were observed only in the increased expression of follistatin in diabetic animals and decreased Ankrd2 expression in the control group. So, insulin deficiency does not impair the overload-induced hypertrophic response in soleus and EDL muscles. However, different mechanisms seem to be involved in the comparable hypertrophic responses of skeletal muscle in control and diabetic animals.

Introduction

Type 1 diabetes (T1DM) is associated with marked changes in skeletal muscle morphology, electrical, and contractile properties and metabolism leading to impaired muscle function (Cotter et al. 1989, 1993; Cameron et al. 1990; McGuire et al. 2001). Recovery from injury and skeletal muscle regeneration are impaired in diabetes (Vignaud et al. 2007; Krause et al. 2011, 2013). Under these conditions, skeletal muscle displays a shift in the fiber types toward more oxidative or slow-twitch pheno-

types (Armstrong et al. 1975). Growth and development of skeletal muscle are also impaired in T1DM (Krause et al. 2009; D'Souza et al. 2013), resulting in reduced myofiber diameter (Andersen et al. 1997, 2004) along with lowered capillary density and deficient angiogenesis (Leinonen et al. 1982; Krause et al. 2009; Rennert et al. 2014). These alterations together are diagnosed as diabetic myopathy (Krause et al. 2009; D'Souza et al. 2013).

The decrease in protein synthesis and increase in protein degradation lead to skeletal muscle mass loss and atrophy, a common feature of T1DM (Barazzoni et al.

2004; Krause et al. 2009). PI3K-Akt-mTOR is the major signaling pathway involved in maintaining skeletal muscle trophism and growth in response to hormones (e.g., testosterone, insulin and IGF-1) (Sukho 2003; Bolster et al. 2004; Basualto-Alarcón et al. 2013; Egner et al. 2013; Schiaffino et al. 2013) and nervous stimuli (Kern et al. 2014). Activation of this pathway increases protein synthesis and induces muscle hypertrophy (Taha and Klip 1999; Bodine et al. 2001; Miyazaki and Esser 2009; Yu and Baylies 2013) but its activity is attenuated in streptozotocin-induced diabetic animals due to reduced plasma levels of insulin and IGF-1 (Like and Rossini 1976; Yang et al. 1990). These animals also display a short-term increase in the proteolytic activity in the soleus and extensor digitorum longus (EDL) muscles 1–3 days after entering into the diabetic state returning to control levels after 5 days (Pepato et al. 1996). Hyperglycemia has been associated to reduced skeletal muscle mass (Oku et al. 2001; Russell et al. 2009) and in long-term leads to increased production of reactive oxygen species (ROS) and of advanced glycation end products (AGEs) that exacerbate the loss of skeletal muscle mass (Baynes 1991; Riboulet-chavey et al. 2006; Grzelkowska-Kowalczyk et al. 2013).

Several diabetes complications are caused by atrophic myopathy (D'Souza et al. 2013) that leads to reduced glucose homeostasis (DeFronzo et al. 1981a,b). Muscle weakness and limited mobility in long-term diabetes have been associated with incapacitation for routine physical activities such as walking, climbing stairs, and running (Andersen 2012). Decreasing mobility is also associated with high incidence of other morbidities, disabilities and mortality (Pahor et al. 2014). Therefore, improvements in skeletal muscle health and trophism are beneficial for glucose homeostasis (D'Souza et al. 2013) and preservation of the capacity of diabetic patients for physical activities and so to reach a better clinical condition.

Experimental models of muscle overload have been widely used to study skeletal muscle hypertrophy (Goldberg 1968; Armstrong et al. 1979). These models provide a large hypertrophic response in a short period of time, allowing the researcher to investigate hypertrophy in a way that is not possible in a low/moderate muscle hypertrophic condition (Lowe and Alway 2002).

The information above led us to examine the effects of overload-induced hypertrophy in EDL (fast-twitch) and soleus (slow-twitch) muscles from streptozotocin-induced diabetic rats. The rats were rendered diabetic for 30 days and then submitted to muscle overload protocol for further 7 or 30 days. Hypertrophy of EDL and soleus muscles was evaluated by measuring fiber cross-sectional areas (CSA) and dry and wet muscle weight. Strength and contractile properties such as maximum isotonic contractions (muscle twitch) and tetanic forces were also measured.

Contents of total and phosphorylated Akt and rpS6 proteins, after 7 and 30 days of overload, and mRNA levels, after 7 days, of several genes associated to signaling pathways regarding skeletal muscle hypertrophy were determined.

Methods

Animals

Male Wistar rats (200 ± 50 g, at least six animals per group) were obtained from the Institute of Biomedical Sciences, University of São Paulo, and maintained in groups of three with water and food ad libitum in a room with 12/12 h light–dark cycle at 22°C. All experimental procedures were approved by the Animal Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo (CEUA – USP) and were carried out in agreement with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC) and the principles of the Brazilian College of Animal Experimentation (COBEA).

Induction of diabetes mellitus

Diabetes *mellitus* was induced by injection of 65 mg/kg b.w. streptozotocin dissolved in citrate buffer (pH 4.5) into the caudal vein (Ungvari et al. 1999). Control animals received the same volume of citrate buffer. Blood glucose levels above 400 mg/dL (22.2 mmol/L) after 24-h of the streptozotocin injection confirmed the diabetic state. Glucose levels were weekly measured to ensure that the animals remained diabetic up to the experiment being performed. Similar procedure was used in our previous studies (Vitzel et al. 2013). Animals in diabetic state for 30 days were submitted to surgery for skeletal muscle overload induction.

Overload protocol of EDL and soleus muscles

To induce soleus muscle hypertrophy, unilateral synergistic tenotomy of the gastrocnemius muscles was performed as previously described (Goldberg 1972; Armstrong et al. 1979; Owino et al. 2001). Before surgery, animals were anesthetized by a single intraperitoneal injection of xylazine (10 mg/kg b.w.) and ketamine (90 mg/kg b.w.). An incision was made in the lower limb, exposing muscles, and tendons. The Achilles tendon was carefully separated in portions of gastrocnemius and soleus muscles, and then severed ensuring that no damage to the vasculature, nerves, and surrounding muscles occurred, leaving the

soleus muscle insertion intact. All procedures were performed under aseptic conditions. The contralateral limb was used for sham surgery, in which soleus and gastrocnemius tendons were identified but not severed.

To induce EDL muscle overload, in a different set of animals, unilateral synergistic ablation of the tibialis anterior muscle was carried out as previously described (Rosenblatt and Parry 1992; Hamilton et al. 2010). The contralateral limb was submitted to sham surgery. Immediately after surgery, animals were intraperitoneally injected with gentamicin (40 mg/kg b.w.).

Animals remained under muscle overloading during 7 or 30 days. The unilateral ablation and tenotomy are advantageous to allow paired comparison between sham and overloaded muscles avoiding inaccuracies of the use of different animals (Thomson and Gordon 2006).

Analysis of skeletal muscle contractile function

Evaluations of skeletal muscle contractile function were performed as previously described (Bassit et al. 2010; Pinheiro et al. 2010, 2011, 2012a). The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (75 mg/kg b.w.) and both hindlimbs were fixed on an acrylic platform. The stimulated limb was placed on the platform with the hip joint at 120°–140° angle and the knee joint at a 120°–140° angle. A hook was placed under the Achilles tendon for soleus muscle measurements, or under the distal tendon of EDL muscle and connected to a force transducer (Grass Technologies, West Warwick, RI). Skin was removed and the sciatic nerve accessed. A platinum electrode was placed at the nerve. The fascia was separated from the muscles and all the synergistic and antagonist muscles were tenotomized to avoid any interference in force measurements. Muscle contractions were induced by electrical stimulation using symmetrical monophasic square waves with the MultiStim System D330 (Digitimer Ltd, Welwyn Garden City, Hertfordshire, UK). The resting length (L_0) of the target muscle was adjusted to obtain maximum tension (the ankle joint approximately at a 90° angle) upon stimulation by regulating the traction of the hook coupled to the tendon and force transducer. Electrical stimulation-induced muscle contraction pulls the hook placed in the muscle tendon. For determination of the muscle twitch force, the stimulus consisted of 500 μ s pulse duration at 1 Hz with adjusted voltage to produce maximum force. To determine the tetanic force, electrical stimulus frequency was increased to 100 Hz. Muscle twitch force (maximum tension generated during twitch) and tetanic force (maximum tension generated) were recorded using the AqDados[®] software (version 4.16, Lynx Tecnologia Eletrônica Ltda, São Paulo,

Brazil). The muscle strength and contractile properties were analyzed using the AqAnalysis[®] software (version 4.16, Lynx Tecnologia Eletrônica Ltda, São Paulo, Brazil). The same measurements were carried out in our previous studies (Pinheiro et al. 2011, 2012b).

Histological analysis

Evaluation of fiber CSA was performed as previously described (Bodine and Baar 2012). Ten μ m thick sections from EDL and soleus muscles of the midportion region were obtained using a cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany) and stained with hematoxylin and eosin (HE) for fiber CSA examination. Sections were imaged using an upright microscope with camera (Nikon DXM 1200; Nikon Instruments, Melville, NY). Digitized images were analyzed using the Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD) in a blinded manner. Similar analysis was performed in our previous studies (Pinheiro et al. 2011).

Western blotting

Proteins were extracted in buffer containing Tris–HCl (100 mmol/L), pH 7.5; EDTA (10 mmol/L); sodium pyrophosphate (10 mmol/L); sodium fluoride (0.1 mmol/L); sodium orthovanadate (10 mmol/L); phenylmethylsulfonyl fluoride (PMSF) (2 mmol/L); and aprotinin (10 μ g/mL). The muscle extracts were sonicated for 30 s at 4°C. The homogenates were centrifuged at 12,000 g for 20 min at 4°C. The supernatants were treated with Triton X-100 (1%) and immediately transferred to a –80°C freezer for storage until western blotting analysis being performed. Bradford method was used to determine the total protein content in an aliquot from the supernatant. Laemmli buffer was added to the samples, followed by a 95°C heating for 5 min and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After protein transfer to a nitrocellulose blotting membrane and nonspecific sites blocking with free-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against total Akt and ribosomal protein S6 (rpS6), phospho-Akt (Thr³⁰⁸) and phospho-rpS6 (Ser^{244/240}) (Cell Signaling Technology, Beverly, MA) followed by treatment with a secondary HRP-conjugated antibody (Millipore, Temecula, CA) for 60 min at room temperature. Similar analysis was performed in our previous studies (Vitzel et al. 2013).

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from skeletal muscles using RNeasy RNA isolation kit (Qiagen Inc, Valencia, CA)

according to the manufacturer's protocol. The expression of the genes was determined by RT-PCR. Total RNA quality and quantity were determined using a Nanodrop spectrophotometer (Thermo Scientific Nanodrop; Nanodrop Technologies, Wilmington, DE) and it was reverse transcribed with oligo(dT) using cDNA Synthesis Kit containing RevertAid M-MuLV reverse transcriptase (Invitrogen/Life Technologies, Carlsbad, CA). The cDNA levels of the genes were quantified using the SYBR green qPCR kit. RT-PCR was performed in a Rotor-Gene 3000 (Corbett Robotics, Australia). The mRNA content of the genes was normalized to Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) mRNA levels. The following genes were evaluated: FAK – Focal Adhesion Kinase; Wnt 7a – Wingless-Type MMTV Integration Site Family, Member 7A; MuRF-1 – Muscle RING-Finger protein-1; IGF-1 Eb (MGF) – Insulin-like growth factor 1 Eb-peptide (mechanogrowth factor); Ankrd2 – Ankyrin repeat domain 2. The primers sequences used in the experiments are displayed in the Table 1.

Statistical analysis

The results are presented as mean $\pm$ SEM. Student's *t* test was used for the comparisons of the responses in muscle dry weight and twitch and tetanic forces between the diabetic and the control groups. Two-way analysis of variance (ANOVA) followed by Bonferroni post test was used for all analysis. The Bonferroni post test was used for comparisons between contralateral versus hypertrophied muscles of the same group, and contralateral versus contralateral muscles and hypertrophied versus hypertrophied muscles of different groups. As indicated in the text and the Figure legends, two-way ANOVA only was also used for comparison between diabetic versus control rats considering both contralateral and hypertrophied muscles

and for comparison between diabetic and control hypertrophied muscles versus diabetic and control contralateral muscles. Grubb's test was used to exclude outliers. Differences between values were considered statistically significant for $P < 0.05$. All results were analyzed using the GraphPad Prism 5.0 statistical software (GraphPad Software, San Diego, CA). Analysis of fibers CSA was performed as previously described using the 95% confidence interval of the mean (Pinheiro et al. 2011).

Results

Muscle weight

After 30 days of overload, the increase in EDL muscle wet weight per tibia length was of 10.6 mg/cm (39%) in diabetic animals and of 24.7 mg/cm (52%) in controls (Table 2). Soleus muscle presented an increase in absolute wet weight per tibia length of 18 mg/cm (59%) in diabetic animals and 21.6 mg/cm (55%) in controls. The wet weight of the contralateral muscles (on the tibia length basis) of the control group was higher than that of the muscles from diabetic animals. Although the absolute wet weight of the muscles is different, being the muscles from diabetic rats smaller, the hypertrophic response in percentage was comparable. In fact, hypertrophic response (as indicated by percentage increase in dry weight) of the EDL and soleus muscles was not different between diabetic and control rats.

Muscle forces

Absolute tetanic force was increased by 35% in the overloaded EDL muscle of the diabetic and the control groups. Specific tetanic force expressed on a muscle dry weight basis was elevated by 33% in diabetic and by 48%

Table 1. Primers sequences of the genes which expressions were measured by RT-PCR.

	Primer sequence (forward)	Primers sequence (reverse)
FAK	5'-AAGGAGCACCTCTCAAACCG-3'	5'-CATCGCTCCGACAGCATTTG-3'
Wnt 7a	5'-GCGCTCTAGGACAGTCTCCA-3'	5'-GGGGCAATCCACATAGCCTG-3'
MuRF-1	5'-GGACCGGCATGGGGTGTACG-3'	5'-TTTCTGCAGGGGCCGACTGG-3'
Atrogin-1	5'-CGGCACCTTCGTGAGCGACC-3'	5'-GTGCAGATATCCATGGCGCTCCT-3'
IGF-1 Eb (MGF)	5'-GCTTGCTCACCTTTACCAGC-3'	5'-AAGTGTACTTCTTCTCTCTC-3'
Myostatin	5'-TACCACGGAAACAATCATTACCAT-3'	5'-TGCCATCCGCTTGCAAT-3'
Follistatin	5'-AGCGAGTGTGCCATGAAG-3'	5'-GAGTGGAGAGATAGGGAAGC-3'
Ankrd2	5'-TGATGCCGTGAGACTCAACC-3'	5'-TTAGCCATCATGTCTGCCCC-3'
Axin 2	5'-CTCAGCAAAAAGGGAAATTACAGGTAT-3'	5'-ACTGTCTCGTCGCCAGATCTC-3'
HPRT1	5'-GCGAAAGTGGAAAAGCCAAGT-3'	5'-GCCACATCAACAGGACTCTTGATAG-3'

FAK – Focal Adhesion Kinase; Wnt 7a, Wingless-Type MMTV Integration Site Family, Member 7A; MuRF-1, Muscle RING-Finger protein-1; IGF-1Eb (MGF), Insulin-like growth factor 1Eb-peptide (mechanogrowth factor); Ankrd2, Ankyrin repeat domain 2; HPRT1, Hypoxanthine Phosphoribosyltransferase 1.

Figure 1. Cross-sectional areas of EDL and soleus muscles after 30 days of overload. Panel (A) Cross-sectional areas of the EDL muscle fibers. Panel (B) Cross-sectional areas of the soleus muscle fibers. Results are expressed as mean $\pm$ SEM of six animals (120 fibers per muscle). CTRL-CL = Control group, contralateral muscle, CTRL-H = Control group, hypertrophied muscle, DM-CL = Diabetic group, contralateral muscle, DM-H = Diabetic group, hypertrophied muscle. * $P < 0.05$ using the 95% confidence interval of the mean.

fold in the diabetic groups. An increase in the total content of rpS6 was observed in the control and in the diabetic groups (by 3.6- and fourfold, respectively). The phospho-rpS6 content was increased by 4.8-fold in the control and by 2.1-fold in the diabetic groups (Fig. 2). After 30 days of overload, total rpS6 and Akt and phosphorylated rpS6 and Akt returned to basal levels in the EDL muscle (Fig. 2).

In the soleus muscle ($n = 6$), after 7 days of overload, total Akt content was increased by 2.7-fold in both

groups. Phospho-Akt content was increased by 2.2-fold in control and by 2.5-fold in diabetic animals. Total content of rpS6 was not different between the contralateral muscles of the control and the diabetic groups. The overload caused a similar increase in the levels of this protein in both groups. An increase in the content of phospho-rpS6 was observed in the diabetic and the control groups (by 2.9- and 4.7-fold, respectively) (Fig. 2). After 30 days of overload, total Akt was increased by 2.5-fold in both groups, whereas phospho-Akt was increased by 1.7-fold in the diabetic group only (Fig. 2). The muscle overload raised total rpS6 content by 3.1-fold in diabetic rats but it was not significantly changed in the control group. Phospho-rpS6 content was not altered by the overload. However, there was a reduction of 47% in the content of this protein in the contralateral muscle of the diabetic animals when compared to controls.

mRNA expression of FAK, Wnt7a, MuRF-1, atrogen-1, IGF-1 Eb (MGF), myostatin, follistatin, Ankrd2, and Axin2

mRNA content of the genes was evaluated after 7 days of functional overload in the EDL and soleus muscles. The hypertrophied EDL muscle of diabetic rats presented increased expression of FAK (24%) when compared to the contralateral muscle whereas in the control group the expression of this gene was 133% higher. The mRNA content of MGF was enhanced by sixfold in the diabetic and by 10-fold in the control groups in EDL muscle due to overload stimulus. mRNA content of MuRF-1 was reduced by 59% in the control group as observed by Bonferroni post test. Using two-way ANOVA only, the diabetic state also reduced MuRF-1 expression. Atrogen-1 mRNA expression was reduced by 68% in the control but it was not changed in the diabetic groups. However, as indicated by two-way ANOVA only, diabetes reduced atrogen-1 expression in EDL muscle. Expression of myostatin was reduced by 67% in the control group but no significance was founded by the post test in the diabetic group. Analysis by two-way ANOVA showed a significant reduction in the expression of most genes by hypertrophy. Bonferroni post test did not show any difference in mRNA expression of follistatin between groups. However, muscle hypertrophy presented a significant effect on increasing follistatin mRNA levels as pointed out by two-way ANOVA. Ankrd2 mRNA content was increased by 165% in diabetic but showed no significant change in control rats. Hypertrophic stimulus decreased mRNA expression of Axin2 in the overloaded EDL muscle of both groups as indicated by two-way ANOVA only (Fig. 3).

Figure 2. Western Blots of EDL and soleus muscles after 7 and 30 days of overload. Panel (A) Quantitative analysis of western blotting and representative images of total rpS6 and Akt and phosphorylated rpS6^{240/244} and Akt³⁰⁸ of the EDL muscle after 7 and 30 days of overload. Panel (B) Quantitative analysis of western blotting and representative images of total rpS6 and Akt and phosphorylated rpS6^{240/244} and Akt³⁰⁸ of the soleus muscle after 7 and 30 days of overload. Data are expressed as mean $\pm$ SEM of at least six animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Also using two-way ANOVA only, total rpS6 protein was different between diabetic and control hypertrophied muscles versus diabetic and control contralateral muscles ($P < 0.05$). CTRL, Control group; DM, Diabetic group; CL, Contralateral muscle; H, Hypertrophied muscle.

Hypertrophied soleus muscle presented higher mRNA levels of FAK as compared to the contralateral muscle of the diabetic (88%) and the control (83%) groups. Wnt7a expression was significantly reduced by hypertrophy, as indicated by two-way ANOVA only. The increase in MGF mRNA content induced by overload stimulus in soleus muscle was remarkable even compared with that observed in the EDL: by 52- and 24-fold in diabetic and control rats, respectively. There was a reduction in the expression of MuRF-1 and atrogin-1 in the hypertrophied muscle: MuRF-1 by 58% in the diabetic group and by 62% in controls; atrogin-1 by 66% in the diabetic group and by

68% in controls. Myostatin expression was significantly lower (68%) in the hypertrophied soleus muscle of the control group. Two-way ANOVA indicated a decreasing effect of hypertrophy on mRNA content of myostatin in soleus muscle. Follistatin mRNA levels presented a 79% increase due to the overload in soleus muscle from diabetic animals only. Ankr2 mRNA content was decreased by 51% in controls due to hypertrophy and by 47% in the contralateral muscle of diabetic animals when compared to contralateral soleus muscle of the control group. Increased mRNA expression of Axin2 due to the diabetic state was also found by two-way ANOVA only (Fig. 4).

Figure 3. mRNA expression of several genes as measured by RT-PCR in the EDL muscle. Results are expressed in arbitrary units and presented as mean $\pm$ SEM of 6 animals. Data were analyzed using two-way ANOVA followed by Bonferroni post test. * $P < 0.05$, *** $P < 0.001$. As indicated by the two-way ANOVA only ($P < 0.05$), the expressions of the following genes were different between the diabetic versus the control groups considering both the contralateral and hypertrophied muscles: MuRF-1 and atrogin-1. Also using two-way ANOVA only ($P < 0.05$), the expressions of the following genes were different between diabetic and control hypertrophied muscles versus diabetic and control contralateral muscles: myostatin, follistatin and Axin2. EDL, extensor digitorum longus; CL, Contralateral muscle; H, Hypertrophied muscle; DM, Diabetic group; CTRL, Control group.

Discussion

Advanced glycation end products are generated under hyperglycemic conditions and can impair several signaling pathways such as protein kinase C (PKC), nuclear factor κ B (NF- κ B) and transforming growth factor beta (TGF- β) (Brownlee 2001; Riboulet-chavey et al. 2006), leading to a decrease in the hypertrophic signal. Impaired hypertrophy of the skeletal muscle has been associated with low-mitochondrial biogenesis and an increase in AMPK activity that attenuate S6K1, 4E-BP1, and eEF2 activities (Thomson and Gordon 2005; Thomson et al. 2008). Impaired regeneration of the skeletal muscle and low-force production are also observed in diabetic mice (Vignaud et al. 2007). Hypertrophy is lowered in insulin resistance state due to less activation of mTOR as observed in Zucker rats (Paturi et al. 2010). Mice fed a hyperlipidic diet show less

activation of Akt and p70S6K1 after functional overload in the plantaris muscle (Sitnick et al. 2009). It is also important to mention that diabetic condition is associated with low angiogenesis and capillary density in mice skeletal muscle (Emanuelli et al. 2002; Kivelä et al. 2006, 2008). However, despite the mentioned vascular impairment, we observed similar percentage increase in skeletal muscle hypertrophic response in both diabetic and control rats.

Specific force was more elevated in EDL and soleus muscle of diabetic rats. Sarcoplasmic concentration of calcium has been reported to be increased in diabetic animals (Nakagawa et al. 1989; Eshima et al. 2013). Sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibition occurs by high contents of reactive oxygen species (Andrade et al. 1998, 2001), which are significantly increased in the skeletal muscles from diabetic animals (Gumieniczek et al. 2001; Aragno et al. 2004; Bravard et al.

Figure 4. mRNA expression of several genes as measured by RT-PCR in the soleus muscle. Results are expressed as arbitrary units and presented as mean $\pm$ SEM of six animals. Data were analyzed using two-way ANOVA followed by Bonferroni post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. As indicated by two-way ANOVA only ($P < 0.05$), the expression of the following genes was different between diabetic versus control groups considering both contralateral and hypertrophied muscles: Axin2. Also using two-way ANOVA only ($P < 0.05$), the expressions of the following genes were different between diabetic and control hypertrophied muscles versus diabetic and control contralateral muscles: Wnt7a and myostatin. CL, Contralateral muscle; H, Hypertrophied muscle; DM, Diabetic group; CTRL, Control group.

2011). Excess of calcium in the sarcoplasm is likely to overwhelm calcium pumps reuptake activity and accumulation of this ion in the sarcoplasmic reticulum then occurs (Eshima et al. 2013). Under this condition, the intrinsic response of the skeletal muscle contraction may become more intense (Nakagawa et al. 1989).

Phospho-Akt and rpS6 contents were increased in the EDL muscle after 7 days of overload. These results corroborate previous studies (Thomson and Gordon 2006; Miyazaki et al. 2011) performed in plantaris muscles of rats also after 7 days of a similar muscle overload protocol used herein. When the EDL muscle was submitted to 30 days of overload, Akt and rpS6 did not present any significant difference in the total or phosphorylated proteins levels, indicating that this protein synthesis pathway activity returned to pre-overload values. Peterson and coworkers (Peterson et al. 2008) showed that the overload-induced response on the plantaris muscle also returns to pre-overload values after 21 days.

After 7 days of overload in the soleus muscle, the contents of total and phospho-Akt and rpS6 were increased in both groups. After 30 days of overload in soleus muscle, the contents of total Akt in controls and of total rpS6 and phospho-Akt in the diabetic group remained increased. Thus, protein synthesis pathway may have sustained an elevated activity under these conditions, even after 30 days of overload, particularly in the soleus muscle of diabetic animals. Gastrocnemius muscle presents, after 20 days of overload, elevated contents of phosphorylated mTOR, p70S6K1, and Akt confirming a persistent activation of this signaling pathway during a prolonged period of time (Ochi et al. 2010). The persistent activation of the protein synthesis pathway that lasted until to 30 days probably occurred due to higher susceptibility of the soleus muscle to a mechanical load-induced injury in diabetic rats (Coprav et al. 2000). In spite of the observed difference between soleus and EDL in the persistence of protein synthesis pathway activation, both muscles from

control and diabetic animals presented corresponding hypertrophic responses after 30 days of overload.

The occurrence of hypertrophy in soleus, plantaris and gastrocnemius muscles in response to 8 weeks of resistance exercise has previously been shown in diabetic rats (Farrell et al. 1999). The authors postulated that hypertrophy occurred because the IGF-1 increase was able to compensate for the low-plasma insulin levels. In fact, IGF-1 has been shown to play a central role in the stimulation of protein synthesis pathway induced by overload (Coleman et al. 1995; Bodine et al. 2001; Rommel et al. 2001; Schiaffino and Mammucari 2011). IGF-1 administration stimulates cell proliferation and enhances myogenic differentiation and hypertrophy in cultured muscle cells (Engert et al. 1996) and transgenic mice (Coleman et al. 1995). These effects of IGF-1 could produce a satisfactory hypertrophic response in skeletal muscle from diabetic rats. We observed a large increase in IGF-1 Eb (MGF) expression by overload both in control and diabetic animals, but it may not account alone for their similar hypertrophic response. For instance, we showed herein that MGF gene expression is almost twice as high in the control as compared to the diabetic groups in the EDL muscle, however, hypertrophic response was similar between groups. In fact, muscle hypertrophy due to overload does not depend exclusively on IGF-1 stimulus (Spangenburg et al. 2008; Klossner et al. 2009) and it can also occur without Akt activation (Miyazaki et al. 2011). Therefore, MGF may not be the major player in the similar hypertrophic responses observed in the diabetic and control groups. For instance, the MGF that is locally produced in skeletal muscle submitted to mechanical stress causes hypertrophy by activating the IGF receptors (Hill and Goldspink 2003) and, as discussed below, FAK stimulates p70S6K1 without Akt activation (Klossner et al. 2009). We showed herein that 7 days of overload caused noticeable variation in the mRNA contents of several genes associated to the control of muscle mass, and each experimental group presented a specific response.

Fluck and coworkers stated that FAK plays an important role in skeletal muscle hypertrophy (Flück et al. 1999). FAK has the ability to phosphorylate p70S6K1 in Tyr 397, independently of Akt and mTOR (Klossner et al. 2009). Hypertrophy mediated by IGF-1 requires the participation of FAK in C2C12 cells (Crossland et al. 2013). A decrease in the hypertrophic process after removal of FAK that affects mTOR-rpS6-eIF4F (eIF4F – eukaryotic initiation factor 4F) signaling pathway has been reported in C2C12 cells (Crossland et al. 2013). In the EDL muscle, FAK expression was increased by the overload in control but it did not change in diabetic animals. However, in soleus muscle, FAK was increased in both groups, being more pronounced in diabetic rats, suggesting a pos-

sible compensatory hypertrophic mechanism. This difference in FAK expression response to hypertrophy was also shown in other muscles such as the plantaris muscle (Gordon et al. 2001). Also, FAK activity is increased in young men after resistive training and was associated with muscle remodeling when its total content was diminished after bed rest (Li et al. 2013). Therefore, FAK signaling pathway may play a key role for the response of skeletal muscle hypertrophy in diabetic states.

Ankrd2 has been associated to muscle hypertrophy being triggered by mechanical signals (Tsukamoto et al. 2002; Kojic et al. 2004). Herein, we showed different responses of Ankrd2 expression in the overloaded EDL and soleus muscles: in the EDL, an increase was observed in the diabetic group only, whereas a decrease was found in the soleus muscle from the control group only.

FAK and Ankrd2 play an important role for muscle mechanotransduction. In the soleus muscle, when FAK expression was increased, Ankrd2 expression was unchanged or even lowered. Apparently, overload-induced hypertrophy of the soleus muscle relies more on FAK signaling rather than Ankrd2, regardless the diabetic condition. EDL muscle from control animals showed the same feature observed for soleus muscle. On the other hand, in the EDL muscle of diabetic animals, FAK expression was unchanged and Ankrd2 increased upon overload. Thus, mRNA expression in the EDL muscle suggests that under diabetic condition, FAK hypertrophic signaling was suppressed, being replaced by Ankrd2. A compensatory mechanism of FAK and Ankrd2 may ensure the muscle hypertrophic responses even in catabolic conditions such as diabetes.

Wnt7a binds to Frizzled7 (Fzd7) receptor and activates the Fzd7-PI3K-Akt-mTOR pathway, leading to skeletal muscle hypertrophy (Von Maltzahn et al. 2012a,b). Wnt signaling pathway also promotes hypertrophy through the canonical pathway, involving β -catenin and Axin2, that are activated during overload of the skeletal muscle (Armstrong and Esser 2005). We showed no change in gene expression of Wnt7a and Axin2 in the EDL muscle. However, in the overloaded soleus muscle of both groups, there was a decrease in Wnt7a expression that was not followed by changes in Axin2 mRNA content. The diabetic animals presented higher mRNA levels of Axin2 than controls, without any further alteration by the overload, indicating a possible modulation through β -catenin signaling pathway. Wnt7a nor Axin2 does not seem to be involved on overload-induced hypertrophy in this present study.

MuRF-1 and atrogin-1 are two muscle-specific E3 ubiquitin ligases that are increased in atrophic conditions and are involved in the ubiquitin binding and the consequent protein degradation by the 26S proteasome (Bodine

and Baehr 2014). When the PI3K-Akt-mTOR pathway is activated, these proteins have their expression reduced due to inhibition by the upstream Forkhead box O3 (FOXO3) (Stitt et al. 2004). The decrease in the mRNA expression of MuRF-1 and atrogin-1 was observed after 7 days of overload in the soleus muscle of both groups. In contrast, the EDL muscle showed a reduction in

mRNA levels of MuRF-1 and atrogin-1 only in the control group as indicated by two-way ANOVA. The diabetic group presented lower levels of MuRF-1 and atrogin-1 before muscle overload. Expression of MuRF-1 and atrogin-1 was increased after 1 day of overload, however, there was a reduction in the expression of the E3 ligases after 7 days corroborating our results (Baehr et al. 2014).

Figure 5. mRNA features of EDL and soleus muscles after 7 days of overload. Panel (A) Diagram of differences and similarities between mRNA expression of the genes of contralateral versus contralateral and hypertrophied versus hypertrophied EDL and soleus muscles from diabetic and control rats after 7 days of overload. Panel (B) Venn diagram of differences in mRNA expression of genes between contralateral versus hypertrophied EDL and soleus muscles from diabetic and control rats after 7 days of overload. FAK, Focal Adhesion Kinase; Wnt 7a, Wingless-Type MMTV Integration Site Family, Member 7A; MuRF-1, Muscle RING-Finger protein-1; MGF, Mechano growth factor; Ankrd2, Ankyrin repeat domain 2.

Also, hypertrophy seems to occur when both synthesis and degradation of proteins are more elevated (Baehr *et al.* 2014). In control animals, reduction in the expression of MuRF-1 and atrogin-1, and a possible decrease in proteasome-dependent protein degradation, may be involved in the hypertrophic response to overload in soleus and EDL muscles. In diabetic animals, reduction in the expression of MuRF-1 and atrogin-1 was present in the soleus muscle, but it was lost in the EDL muscle, that had already a lower basal expression of these genes. Therefore, reduced expression of E3 ubiquitin ligases cannot be accounted as a mechanism associated to overload-induced hypertrophy in EDL muscle from diabetic rats.

Follistatin promotes muscle hypertrophy by inhibiting the repressive effects of myostatin on differentiation and growth of myogenic precursor cells and by increasing protein synthesis through mTOR-p70S6K1 pathway via Smad3-dependent mechanism (Winbanks *et al.* 2012). Myostatin is a negative regulator of skeletal muscle growth being associated with down-regulation of the Akt-mTOR signaling pathway and decreased phosphorylation of Akt, rpS6, p70S6K1, and 4E binding protein 1 (4E-BP1) (Amirouche *et al.* 2009; Rodriguez *et al.* 2014). Taking as a whole, follistatin increases muscle hypertrophic response and myostatin decreases it (Gilson *et al.* 2009). Myostatin expression was decreased due to overload in both muscles whereas follistatin expression was increased in the overloaded EDL and soleus muscles of the diabetic group only. These changes in follistatin and myostatin balance in diabetic animals may assist in the hypertrophic response herein observed.

Differences between EDL and soleus muscles regarding signaling pathways in the hypertrophic response remain to be elucidated. Ablation and tenotomy protocols lead remaining muscles to hypertrophy in a not fully comparable way. The load that tibialis anterior muscle presents on EDL muscle upon ablation is different from that which gastrocnemius muscle presents on soleus muscle upon tenotomy. Also, soleus muscle is an oxidative and postural muscle, and it is more recruited than the glycolytic EDL muscle. The variability between EDL and soleus muscles in response to overloading could also be associated to differences in blood perfusion, and this possibility still needs clarification.

As comparable hypertrophic responses were found, different patterns of mRNA expression were involved leading to similar outcome. Regarding contralateral versus contralateral soleus muscles, there was a decrease in the *Ankrd2* and an increase in *Axin2* expression in the diabetic group, however, there was no alteration in the EDL muscle. When comparing hypertrophied versus hypertrophied muscles, EDL muscle showed a decreased expression of FAK and MGF in the diabetic group as compared to the

control. In contrast, soleus muscle presented an increase in FAK and *Axin2* expression in overloaded EDL muscle (Fig. 5A).

The overloaded EDL muscle of control and diabetic animals showed an increase in MGF and follistatin expression and a decrease in myostatin and *Axin2* mRNA levels. The expression of FAK was increased and of MuRF-1 and of atrogin-1 decreased only in the control group, whereas *Ankrd2* expression was enhanced only in the diabetic group. The overloaded soleus muscle caused similar changes in control and diabetic rats: increased FAK and MGF and decreased in *Wnt7a*, MuRF-1, atrogin-1, and myostatin. Differences were observed only in the increased expression of follistatin in diabetic animals and the decreased *Ankrd2* expression in the control group (Fig. 5B). Both soleus and EDL muscles of control animals shared common features in expression of genes associated to hypertrophic mechanisms (e.g., increased FAK and IGF-1 *Eb*, and reduced Myostatin, MuRF-1, atrogin-1) upon overload. However, under diabetic state, this response was different for each muscle, nevertheless, achieving a similar hypertrophic response (in percentage increase) to control. Further experiments are necessary to fully elucidate, which specific pathways are involved in the hypertrophic response of skeletal muscle in diabetic state.

Conclusion

Soleus and EDL muscles of diabetic rats submitted to functional overload for 30 days presented similar hypertrophic response when compared to controls. Muscle hypertrophy was accompanied by an increase in absolute tetanic and twitch forces. Regarding the protein synthesis pathway, phospho-Akt and phospho-rpS6 levels were elevated after 7 days of overload in both muscles, remaining increased for as long as 30 days in the soleus muscle only. Different expression patterns of several genes related to skeletal muscle hypertrophy were observed in both muscles of diabetic and control animals, indicating that different mechanisms seem to be involved. Our results indicate that insulin deficiency does not impair the overload-induced hypertrophic response of soleus and EDL muscles.

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Conflict of Interests

The authors declare that there is no conflict of interest that could jeopardize the impartiality of this study.

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Notes & Tips

Housekeeping proteins: How useful are they in skeletal muscle diabetes studies and muscle hypertrophy models?

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ABSTRACT

The use of Western blot analysis is of great importance in research, and the measurement of housekeeping proteins is commonly used for loading controls. However, Ponceau S staining has been shown to be an alternative to analysis of housekeeping protein levels as loading controls in some conditions. In the current study, housekeeping protein levels were measured in skeletal muscle hypertrophy and streptozotocin-induced diabetes experimental models. The following housekeeping proteins were investigated: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, α-tubulin, γ-tubulin, and α-actinin. Evidence is presented that Ponceau S is more reliable than housekeeping protein levels for specific protein quantifications in Western blot analysis.

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The use of gel electrophoresis and protein immunoblots (Western blot analysis) is of great importance in biomedical science research. These methods allow us to assess quantitative changes in the protein of interest in various experimental conditions. For consistent comparisons between protein blots, the amount of sample protein loaded and the efficiency of its transfer to an analytical nitrocellulose or PVDF (polyvinylidene difluoride) membrane using an electrophoretic procedure needs to be precisely determined [1,2]. It is standard to measure at least one housekeeping protein in Western blot analysis because it is thought that some structural or metabolic enzymes do not alter in concentration between various treatments [1,3,4]. Examples of the most frequently used proteins are actin, tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [1,4–7].

In spite of the information above, it is now known that commonly used housekeeping proteins vary significantly in levels among cells, tissues, and experimental conditions [3]. Therefore,

the use of housekeeping proteins should be carefully chosen in relation to the cell or tissue types and the treatment or disease state, and under no circumstance should the signal be saturated [3,8]. To solve this particular problem, development of alternatives has recently occurred, for example, Ponceau S and Stain-Free technology [2,9–11]. Stain-Free technology refers to a protein staining method where a distinctive compound is mixed within the gel solution and then regularly distributed in the whole gel [12]. After this, the gel is exposed to ultraviolet (UV) light after electrophoresis has been terminated, and the stain compound is then activated and the proteins become excitable, emitting a strong fluorescent signal that can be quantified [12]. Ponceau S is a staining technique that involves preparation of a dye solution of Ponceau Red and acetic acid to incubate the membrane so that the protein lanes and bands are clearly visible, followed by a brief wash in distilled water so that the membrane becomes colorless [2,13].

The main purpose of this article was to report the reliability of five commonly used housekeeping proteins with respect to protein loading control in Western blot analysis in extensor digitorum longus (EDL) muscle studies in experimental models of hypertrophy and/or streptozotocin-induced diabetes. The following commonly used housekeeping proteins were investigated: GAPDH,

Abbreviations used: PVDF, polyvinylidene difluoride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EDL, extensor digitorum longus; CEUA–USP, Institute of Biomedical Sciences at the University of São Paulo; BW, body weight; STZ, streptozotocin; ANOVA, analysis of variance.

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β -actin, α -tubulin, γ -tubulin, and α -actinin. Analysis of protein loading using Ponceau S was performed for comparison.

Male Wistar rats weighing 200 ± 50 g (initial weight), 6 animals per group, were used in the studies reported here. The rats were obtained from the Institute of Biomedical Sciences at the University of São Paulo (CEUA–USP) and were maintained in groups of 3 with water and food provided ad libitum in a room with a 12/12-h light/dark cycle (at 22 °C environment temperature). Protocols for all experimental procedures were approved by the animal ethics committee of the CEUA–USP and were carried out in agreement with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC, USA) and the principles of the Brazilian College of Animal Experimentation (COBEA).

Hyperglycemia induced by pancreatic beta cell destruction was performed by injection of 65 mg/kg BW (body weight) streptozotocin (STZ) (S0130, Sigma–Aldrich, St. Louis, MO, USA) dissolved in citrate buffer (pH 4.5) into the caudal vein [14]. Animals from the control group received the same volume of citrate buffer only. Blood glucose levels above 400 mg/dl after 24 h of the streptozotocin injection confirmed the hyperglycemic state. Blood glucose levels were measured before euthanasia to ensure that the animals remained diabetic. A similar procedure was used in our previous study [15]. Animals in a hyperglycemic/diabetic state for 3 days were submitted to surgery for skeletal muscle overload induction.

EDL muscle overload was induced by unilateral synergistic ablation of the tibialis anterior muscle as described previously [15–17] in both control and diabetic rats. Before surgery, animals were anesthetized by a single intraperitoneal injection of xylazine (10 mg/kg BW) and ketamine (90 mg/kg BW). An incision was made in the lower limb, exposing muscles and tendons. Tibialis anterior muscle was carefully removed, ensuring that no damage to the vasculature, nerves, and surrounding muscles occurred. All procedures were performed under aseptic conditions. The contralateral limb was used for sham surgery in which tibialis anterior tendons were identified but not severed. Immediately after surgery, animals were injected

with gentamicin (40 mg/kg BW, i.p.). Animals were killed after 7 days of muscle overload. The unilateral ablation is advantageous to allow paired comparison between sham and overloaded muscles avoiding inaccuracies of the use of different animals [18]. Similar procedures were used in our previous study [15].

Proteins were extracted in buffer containing Tris–HCl (100 mM, pH 7.5), ethylenediaminetetraacetic acid (EDTA, 10 mM), sodium pyrophosphate (10 mM), sodium fluoride (0.1 mM), sodium orthovanadate (10 mM), phenylmethylsulfonyl fluoride (PMSF, 2 mM), and aprotinin (10 μ g/ml). The muscle extracts were sonicated for 30 s at 4 °C. The homogenates were centrifuged at 12,000g for 20 min at 4 °C. The supernatants were treated with Triton X-100 (1%) and immediately transferred to a –80 °C freezer for storage until Western blotting analysis was performed. The Bradford method was used to determine the total protein content in an aliquot from the supernatant. Laemmli buffer was added to the samples, followed by 95 °C heating for 5 min and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). After protein transfer to a nitrocellulose blotting membrane and nonspecific sites blocking with fat-free milk, the membranes were incubated overnight at 4 °C with primary antibodies anti- α -tubulin (cat. no. 32-2500, Invitrogen, Carlsbad, CA, USA), anti- γ -tubulin (cat. no. T6557, Sigma–Aldrich), anti- α -actinin (cat. no. 3134S, Cell Signaling Technology, Danvers, MA, USA), anti- β -actin antibody (cat. no. 4967, Cell Signaling Technology), and anti-GAPDH (cat. no. sc-32233, Santa Cruz Biotechnology, Dallas, TX, USA), followed by treatment with a secondary horseradish peroxidase (HRP)-conjugated antibody anti-rabbit or anti-mouse (Millipore, Temecula, CA, USA) for 60 min at room temperature. Ponceau S staining was used to calculate the relative protein content. The loading protein amounts of the membranes were normalized by using a pool of 12 samples containing equal volumes of 3 samples from the four groups. The arbitrary optical density unit was acquired using ImageJ software (version 1.46, Wayne Rasband, National Institutes of Health [NIH], Bethesda, MD, USA). The results were also normalized by the average of contralateral control (C-CL) arbitrary

Fig. 1. Quantitative analysis of Western blotting and representative images of GAPDH, β -actin, α -tubulin, γ -tubulin, α -actinin, and Ponceau S of the EDL muscle after 7 days of overload (H) and under control conditions. Results are expressed as means $\pm$ standard errors of at least 5 animals. * $P < 0.05$; ** $P < 0.01$ (Bonferroni post-test). In addition, using two-way ANOVA only, GAPDH and β -actin were different between the diabetic and control groups (# $P < 0.05$). CTRL, control group; DM, diabetic group; CL, contralateral muscle; H, hypertrophied muscle.

units, which was considered as 1 for each gel [15]. A similar analysis was performed in our previous study [19].

The results are presented as means $\pm$ standard errors. Two-way analysis of variance (ANOVA) followed by Bonferroni post-test was used for comparisons. The Bonferroni post-test was used for comparisons of contralateral versus hypertrophied muscles of the same group and comparisons of contralateral versus contralateral muscles and hypertrophied versus hypertrophied muscles of the control and diabetes groups. As indicated in the text and the figure legends, two-way ANOVA was used only for comparison of diabetic versus control rats considering both contralateral and hypertrophied muscles. Grubb's test was used to exclude outliers. Differences between values were considered statistically significant for $P < 0.05$. All results were analyzed using GraphPad Prism 5.0 statistical software (GraphPad Software, San Diego, CA, USA). A similar analysis was used in our previous studies [15,19].

Significant variations in the levels of the five housekeeping proteins (GAPDH, β -actin, α -tubulin, γ -tubulin, and α -actinin) associated with STZ-induced hyperglycemia/diabetes and skeletal muscle hypertrophy models were found (Fig. 1). In contrast, the levels of the loading sample protein determined by Ponceau S staining were not significantly different between samples obtained from the various experimental conditions.

The EDL muscle GAPDH and β -actin levels were significantly changed by hyperglycemia/diabetes. Overload-induced hypertrophy of the EDL muscle resulted in more pronounced changes in α -tubulin, γ -tubulin, and α -actinin levels. Concerning EDL muscle γ -tubulin levels, the hyperglycemic state attenuated the increase of γ -tubulin levels induced by EDL muscle hypertrophy. Hyperglycemia promoted an increase in the GAPDH levels in contralateral muscle (by 41%) and in the hypertrophied muscle (by 33%) as compared with controls. Regarding β -actin levels, hyperglycemia resulted in an increase of 48% in the contralateral muscle and of 30% in the hypertrophied muscle. The α -tubulin, γ -tubulin, and α -actinin levels in hypertrophied EDL muscle were markedly increased as compared with the contralateral muscle of control rats by 3.2-, 2.6-, and 2.0-fold, respectively.

There has been considerable concern and debate over the use of housekeeping proteins for loading analysis and determination of changes in specific protein concentration [3]. It is postulated that there is a probability that using only a single housekeeping protein as loading control is inappropriate for normalizing results from Western blot analysis [9]. In fact, other methods have been postulated as more efficient for quantification of total protein, for example, Coomassie blue, Ponceau S, and Stain-Free method [1,2,10].

This does not mean that housekeeping proteins cannot be used as loading controls; rather, it means that their use should be approached with caution [4]. Our results demonstrate that in the particular models described, using STZ-induced diabetes and skeletal muscle hypertrophy, Ponceau S was a more accurate method for protein loading quantitation when compared with the tested housekeeping proteins. Ponceau S staining is fast, inexpensive, and nontoxic, and the binding is fully reversible in a few minutes [2]. Western blot housekeeping protein analysis is, of course, suitable for comparison between experimental samples analyzed on the same gel following protein extraction under similar conditions. Other methods may be considered as alternative approaches to improve measurement sensitivity and accuracy of protein loading for quantitative Western blotting analysis [4]. Although this test was performed using only overload-induced hypertrophy and STZ-induced diabetes, it is quite important to test housekeeping proteins and Ponceau staining in order to choose the most appropriate loading control for any experimental protocol under investigation.

Our results indicate that in STZ-induced diabetes and skeletal muscle hypertrophy experimental models, Ponceau S is a better choice for total protein quantification in Western blot analysis and

for determination of changes in protein levels when comparing with common housekeeping proteins in skeletal muscle.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2016.03.023>.

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Hypertrophy Stimulation at the Onset of Type I Diabetes Maintains the Soleus but Not the EDL Muscle Mass in Wistar Rats

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Diabetes mellitus induces a reduction in skeletal muscle mass and strength. Strength training is prescribed as part of treatment since it improves glycemic control and promotes increase of skeletal muscle mass. The mechanisms involved in overload-induced muscle hypertrophy elicited at the establishment of the type I diabetic state was investigated in Wistar rats. The purpose was to examine whether the overload-induced hypertrophy can counteract the hypotrophy associated to the diabetic state. The experiments were performed in oxidative (soleus) or glycolytic (EDL) muscles. PI3K/Akt/mTOR protein synthesis pathway was evaluated 7 days after overload-induced hypertrophy of soleus and of EDL muscles. The mRNA expression of genes associated with different signaling pathways that control muscle hypertrophy was also evaluated: mechanotransduction (FAK), Wnt/ β -catenin, myostatin, and follistatin. The soleus and EDL muscles when submitted to overload had similar hypertrophic responses in control and diabetic animals. The increase of absolute and specific twitch and tetanic forces had the same magnitude as muscle hypertrophic response. Hypertrophy of the EDL muscle from diabetic animals mostly involved mechanical loading-stimulated PI3K/Akt/mTOR pathway besides the reduced activation of AMP-activated protein kinase (AMPK) and decrease of myostatin expression. Hypertrophy was more pronounced in the soleus muscle of diabetic animals due to a more potent activation of rpS6 and increased mRNA expression of insulin-like growth factor-1 (IGF-1), mechano-growth factor (MGF) and follistatin, and decrease of myostatin, MuRF-1 and atrogin-1 contents. The signaling changes enabled the soleus muscle mass and force of the diabetic rats to reach the values of the control group.

Keywords: skeletal muscle, protein synthesis, muscle strength, hyperglycemia, electrostimulation, muscle mass regulation

INTRODUCTION

Reduced protein synthesis stimulation and increased protein degradation (Sandri, 2008; Schiaffino et al., 2013) are associated with the loss of skeletal muscle mass in type 1 diabetes (Barazzoni et al., 2004). Animals with diabetes mellitus induced by streptozotocin administration have increased short-term proteolytic activity in the skeletal muscle (1–3 days after diabetes induction) that returns to control values after 5–10 days (Pepato et al., 1996). The increase in short-term myostatin expression is associated with the severity of skeletal muscle loss, which is abolished by insulin treatment (Chen et al., 2009).

The mechanisms associated with sarcopenia and consequently on its prevention have been investigated (Roden, 2015). Streptozotocin-induced diabetic animals exhibit increased AMP kinase (AMPK) phosphorylation (Vitzel et al., 2013b) and impairs muscle hypertrophy through a reduced activation of protein synthesis signaling such as protein kinase B (Akt) in Ser⁴⁷³, mammalian target of rapamycin (mTOR) in Ser²⁴⁴⁸, ribosomal protein S6 (rpS6) in Thr³⁸⁹, and 4E binding protein-1 (4EBP-1) in Thr³⁷ (Bolster et al., 2002; Thomson and Gordon, 2005).

Physical exercise is prescribed for diabetic patients in order to improve glycemic control. However, little is known about the association of diabetes mellitus and the skeletal muscle mass hypertrophy induced by resistive physical exercise. The overload due to ablation of the synergistic muscles is the most used experimental approach for the study of the mechanisms involved in the skeletal muscle hypertrophy (Rosenblatt and Parry, 1992; Phelan and Gonyea, 1997; Bodine et al., 2001; Ishido et al., 2006; Katta et al., 2010; Paturi et al., 2010). Peak activation of protein synthesis pathway is described after 7 days of the synergistic ablation surgery. After 30 days of hypertrophic stimulus, the skeletal muscle mass increase reaches a plateau (Armstrong and Ianuzzo, 1977; Farrell et al., 1999; Katta et al., 2010).

Diabetic myopathy has several consequences for skeletal muscle (for review see Krause et al., 2011; D'Souza et al., 2013) such as reduction in physical abilities, muscle mass, and strength (Andersen et al., 1996, 1997, 2004). In fact, only 3 days after diabetes induction by streptozotocin injection in rats, a significant reduction in soleus, EDL, and gastrocnemius muscle mass is described (Pepato et al., 1996; Price et al., 1996). However, despite the exacerbated muscular hypotrophy in the diabetic state, the ability of the soleus and extensor digitorum longus (EDL) muscles to respond to a hypertrophic stimulus, even under uncontrolled hyperglycemic state, is not impaired in streptozotocin-induced diabetic rats (Fortes et al., 2015).

Taking into account the rapid onset of muscle loss upon diabetes induction and the prompt hypertrophic response caused by overload, we investigated if a hypertrophic stimulation could prevent or attenuate the muscle mass loss induced by the onset of the type 1 diabetes. For this purpose, the effects of tibialis anterior muscle ablation and gastrocnemius muscle tenotomy and the consequent overload-induced hypertrophy of the EDL and soleus muscles, respectively, concomitantly with the establishment of the diabetic state, were investigated in rats. We examined and compared the effects of short-term diabetic condition

associated with a state of intense skeletal muscle stimulus for hypertrophy induced by overload on signaling pathways associated with protein synthesis and degradation controlled by phosphoinositide 3-kinase (PI3K)-Akt-mTOR, including the E3 ubiquitin ligase that are increased in atrophic conditions, MuRF-1 and atrogin-1; mechanotransduction by rpS6 phosphorylation and mRNA expression of Focal Adhesion Kinase (FAK) and MGF; mRNA expressions of myostatin and follistatin, Wnt/ β -catenin and MG53 that are involved in myogenesis and may affect insulin signaling (Jung and Ko, 2010; Lee et al., 2010). The purpose of our study is to investigate the pathways associated to overload-induced muscle hypertrophy and to examine whether the hypertrophy would counteract the hypotrophy occurring soon after the onset of type I diabetes.

The hypothesis of the present study is that different signaling pathways are activated during skeletal muscle overload in type 1 diabetes mellitus in order to overcome the consequences of the low plasma insulin levels and so prevent/attenuate the marked loss of skeletal muscle mass observed after the onset of the diabetic state.

METHODS

Animals and Diabetes Induction Protocol

Type 1 diabetes was induced in male Wistar rats weighing 200 ± 50 g obtained from the Institute of Biomedical Sciences at the University of São Paulo. The animals were housed in groups of three with water and food provided *ad libitum* in a room with a 12/12-h light/dark cycle at 22°C. Protocols for all experimental procedures were approved by the animal ethics committee (CEUA-ICB-USP, 26/16/03) and performed according with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC, USA) and the principles of the Brazilian College of Animal Experimentation (COBEA).

Hyperglycemic condition similar to type 1 diabetes was achieved by injection of 65 mg/kg b.w. streptozotocin dissolved in citrate buffer (pH 4.5) into the caudal vein (Ungvari et al., 1999; Fortes et al., 2015) whereas only the control group received the same volume of citrate buffer. After a 24-h period of the streptozotocin injection, blood glucose levels confirmed the diabetic state and only animals with or above 400 mg/dL (22.2 mmol/L) were used in the experiments. Animals displaying sustained hyperglycemia for 3 consecutive days (confirmation of the diabetic state) were submitted to surgery for skeletal muscle overload induction. Also, glucose levels were measured weekly and before tissue collection to ensure that the animals remained diabetic up to the end of the experiments as previously described (Fortes et al., 2015, 2016).

EDL and Soleus Muscles Overload Protocol

Soleus muscle hypertrophy was induced by unilateral synergistic tenotomy of the gastrocnemius muscle as previously described (Goldberg, 1972; Armstrong et al., 1979; Owino et al., 2001; Fortes et al., 2015). EDL muscle overload was induced by unilateral synergistic ablation of the tibialis anterior muscle, and

it was performed in a different set of animals, as previously described (Rosenblatt and Parry, 1992; Hamilton et al., 2010; Fortes et al., 2015).

Muscle overloading was kept during 7 days for evaluation of protein content and mRNA expression because muscle protein synthesis is at its peak under this condition (Baillie and Garlick, 1991). In another set of animals, 30 days of overload was employed for evaluation of fiber cross-sectional area (CSA), twitch and tetanic forces, muscle mass and twitch contractile properties. After 30 days of overload, hypertrophied muscle no longer has increased protein synthesis activity and trophic response has reached its peak, therefore, being an appropriate time point for measurements of contractile activity (Armstrong and Ianuzzo, 1977; Farrell et al., 1999; Katta et al., 2010). The unilateral ablation and tenotomy allows paired comparison between sham and overloaded muscles and avoid inaccuracies due to the use of different animals (Thomson and Gordon, 2006). By the end of the experimental protocol, the control (CTRL), and diabetic (DM) groups were obtained, each one with a contralateral limb that does not undergo surgical intervention, the sham (CL), and the other limb that was ablated or tenotomized for hypertrophy (H).

Analysis of Skeletal Muscle Contractile Function and *In Situ* Electrical Stimulation

Evaluations of skeletal muscle contractile function were performed as previously described (Pinheiro et al., 2012; Fortes et al., 2015). After being anesthetized by using intraperitoneal injection of ketamine and xylazine (90 and 10 mg/kg b.w., ip., respectively), the rats were fixed on an acrylic platform and the hindlimb skin was excised for the assessment of the sciatic nerve and a platinum electrode was placed at the nerve. The synergistic and antagonist muscles were tenotomized to avoid interference in the measurements. The resting length of soleus or EDL muscles was adjusted to obtain maximum tension (the ankle joint approximately at a 90° angle) upon stimulation using the MultiStim System D330 electrical stimulation device (Digitimer Ltd, Welwyn Garden City, Hertfordshire, UK) by traction regulation of the hook coupled to the distal tendon and the force transducer (Grass Technologies, West Warwick, RI). Five twitch contractions were employed for determination of the muscle twitch force, time to peak (TTP—time between the onset of force development until peak tension), half relaxation time (HRT—time of muscle relaxation half-way from peak tension), late relaxation time (LRT—time of muscle relaxation between 50 and 25% of peak tension), and rate of force development (RFD—amount of force generated per time unit during skeletal muscle contraction). The twitch stimulus consisted of 500 μ s pulse duration at 1 Hz with adjusted voltage to produce maximum force. For determination of the tetanic force, 10 successive contractions were elicited at 100 Hz, with 2 s duration each and 10 s of recovery period between them, being the first tetanic contraction used to assess the maximum tetanic force. Data were recorded using the AqDados® software (version 4.16, Lynx Tecnologia Eletrônica Ltda, São Paulo, Brazil) and analyzed using AqAnalysis®

software (version 4.16, Lynx Tecnologia Eletrônica Ltda, São Paulo, Brazil).

Histological Analysis

Evaluation of fiber CSA was performed as previously described (Bodine and Baar, 2012; Marzuca-Nassr et al., 2016). Images were taken using an optical microscope (Nikon Eclipse E1000, Fukuoka, Japan) attached to a digital camera (NixonDXM1200). The images were analyzed using the AxioVision® program (version 4.8.1.0, Carl Zeiss Imaging Solutions, Jena, Germany).

Western Blot

Proteins were extracted and assayed as previously described (Vitzel et al., 2013a; Fortes et al., 2015; Marzuca-Nassr et al., 2016). The primary antibodies used were: p-Akt at Ser 473 (9271), Akt (9272), p-rpS6 at Ser 240/244 (5364), rpS6 (2217), p-4EBP-1 at Thr 37/46 (2855), 4EBP-1 (9644), p-AMPK- α at Thr 172 (2535), AMPK- α (2532) from Cell Signaling Technology (Beverly, MA, USA), and atrogen-1 (AP2041) and muscle RING-finger protein-1 (MuRF-1) (MP3401) from ECM Biosciences (Versailles, KY, USA). Cell Signaling Technology (Beverly, MA, USA) provided the secondary antibody conjugated to peroxidase. The images were captured using an Amersham Imager 600 (Amersham/GE Healthcare, Little Chalfont, UK). The densitometry of the specific bands was measured using the ImageJ software (NIH, Bethesda, MD, USA). Results were normalized to the total loading of protein in each sample, quantified by Ponceau S staining (Romero-Calvo et al., 2010; Fortes et al., 2016). The contralateral muscle was considered as 1. Western blotting gels and ponceau staining are presented as Supplementary Material.

Real-Time PCR

Total RNA was extracted according to the manufacturer's specification using the RNeasy kit (QIAGEN, Hilden, Germany). RNA concentration was estimated based on the optical density of the samples at 260 nm obtained in a DS-11 FX spectrophotometer (DeNovix, Wilmington, DE, USA). One microgram of RNA was used for reverse transcription and cDNA synthesis, using oligo-dT primers and the RevertAid M-MuLV reverse transcriptase kit (Invitrogen/Life Technologies, Carlsbad, CA, USA), following the manufacturer's instruction. The amplification of target genes sequences in the cDNA was performed through utilization of a SYBR Green qPCR kit (Invitrogen/Life Technologies, Carlsbad, CA, USA) together with specific sense and antisense primers (Exxtend, São Paulo, Brazil) (Table 1) performed in a Rotor Gene 6000 equipment (Corbett Research, Mortlake, Australia). Relative gene expression calculation was performed by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Results were normalized to the expression of hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism® software (version 4.01; El Camino Real, CA, USA). Results are presented as mean $\pm$ standard error of the mean (SEM) and were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni post-test (for comparison between

TABLE 1 | Primer sequences used for the Real-time PCR assays.

	Primer sense	Primer antisense
FAK	5'-AAGGAGCACCTCTCAAACCG-3'	5'-CATCGCTCCGACAGCATTTG-3'
Akt1	5'-GCCCAAGCACCGTGTGACCA-3'	5'-GCGACCTGTGGCCTTCTCCT-3'
mTOR	5'-AGGGACCACTGTGCCAGAATCCA-3'	5'-TGAGAGAAATCCCGACAGTGAGC-3'
β-Catenin	5'-AGCGCTGGTAAAAATGCTTG-3'	5'-CGCACTGCCATTTTAGCTCC-3'
MuRF-1	5'-GGACCGGCATGGGGTGTACG-3'	5'-TTTCTGCAGGGGCCGACTGG-3'
Atrogin-1	5'-CGGCACCTTCGTGAGCGACC-3'	5'-GTGCAGATATCCATGGCGCTCCT-3'
MG53	5'-CAGGCGCTAAGCACTAACCT-3'	5'-GGTCTGCTCGCAGTAGATG-3'
IGF-1	5'-TGAGCGCACCTCCAATAAGA-3'	5'-GAAGTGAAGAGCGTCCACCA-3'
MGF	5'-TGGTGGACGCTCTTCAGTTC-3'	5'-TCCGGAAGCAACACTCATCC-3'
Myostatin	5'-TACCACGGAACAATCATTACCAT-3'	5'-TGCCATCCGCTTGCATT-3'
Follistatin	5'-AGCGAGTGTGCCATGAAG-3'	5'-GAGTGGAAGAGATAGGGAAGC-3'
Wnt 7a	5'-GCGCTCTAGGACAGTCTCCA-3'	5'-GGGGCAATCCACATAGCCTG-3'
Axin 2	5'-CTCAGCAAAAAGGGAATTACAGGTAT-3'	5'-ACTGTCTCGTCGTCCAGATCTC-3'
HPRT1	5'-GCGAAAGTGAAAAAGCCAAGT-3'	5'-GCCACATCAACAGGACTCTTGTAG-3'

FAK, Focal Adhesion Kinase; Akt1, Protein Kinase B; mTOR, Mammalian target of rapamycin; MuRF-1, Muscle RING-Finger protein-1; MG53, Mitsugumin 53; IGF-1, Insulin-like growth factor 1; MGF, Mechano growth factor; Wnt 7a, Wingless-Type MMTV Integration Site Family, Member 7A; HPRT1, Hypoxanthine Phosphoribosyltransferase 1.

three or more groups). Differences were considered statistically significant for $p < 0.05$. CSA of the EDL and soleus muscles fibers were not normally distributed, therefore, difference was considered significant when there was no overlap between the 95% confidence interval of the median calculated for each experimental group (Gehrig et al., 2008; Fortes et al., 2015; Marzuca-Nassr et al., 2016).

RESULTS

Body Weight and Glycaemia

The body mass of the control animals increased regularly whereas in diabetic animals remained almost unchanged for 30 days (Figure 1). Glycaemia remained unchanged in controls whereas in diabetic rats it was 3.9-fold higher than in the control group 72 h after induction of diabetes and further increased by 17% over the 30-day period.

Muscle Mass, Strength, and Contractile Properties

After 30 days of overload, the absolute and relative tetanic forces of the hypertrophied EDL muscle were different between diabetic and control animals. Absolute tetanic force increased by 40% in control and by 102% in diabetic rats (Table 2). EDL absolute twitch force was increased in the control and diabetic groups by 79 and 114%, respectively (Table 2).

In the EDL muscle of control animals, there was an increase of 25% in both absolute and normalized muscle wet weight due to the overload. In diabetic animals, there was an increase of 38 and 33% in the wet weight and the wet weight normalized by the tibia length, respectively (Table 2). In the control animals, there was an increase of 25% in the dry weight and the dry weight normalized by the tibia length. In diabetic animals, there was a 31 and 37%

increase in dry weight and dry weight normalized by tibia length, respectively (Table 2).

The EDL muscle of diabetic rats had a decrease (by 45%) in LRT in the contralateral and in the hypertrophied (by 22%) EDL muscle when compared with control (Table 2). The RFD was higher in all hypertrophied muscles. In controls, there was an increase of 76% in relation to the contralateral muscle, whereas in the diabetic group it was raised by 2.5-fold.

After 30 days of overload, the absolute tetanic force of the hypertrophied soleus was different between the hypertrophied and contralateral muscles in both groups. Absolute tetanic force was increased by 77% in control animals and by 43% in diabetic rats (Table 3). The absolute twitch force of the soleus muscle was increased in the control and diabetic groups by 31 and 35%, respectively (Table 3).

Soleus muscle also presented an increase of 38% in the wet weight and the wet weight normalized by tibia length in the control group. In the diabetic group, there was an increase of 42 and 48% of the wet weight and the wet weight normalized by the tibia length, respectively (Table 3). In the control animals, the dry weight and the dry weight normalized by the tibia length were increased by 28% upon hypertrophy. In diabetic animals, there was an increase of about 45% in the dry weight and the dry weight normalized by the tibia length (Table 3).

The soleus muscle of control animals had a decrease in the LRT of 56% when they underwent hypertrophy (Table 3). The RFD was lower in the diabetic group both in the control and hypertrophied muscles.

CSA of the EDL and Soleus Muscles Fibers

CSA of the EDL and soleus muscles fibers were markedly increased due to overload in all groups as compared to non-hypertrophied muscles (Figures 2A,B, 3A,B). This effect, quantitatively assessed by the 95% confidence interval of the

TABLE 2 | EDL mass, strength, and contractile properties after 30 days of overload.

Measurements	Control group		Diabetic group	
	Hypertrophy	Contralateral	Hypertrophy	Contralateral
EDL MUSCLE				
Wet weight (mg)	214.23 $\pm$ 11.16 ^{aa}	171 $\pm$ 5.06	105.7 $\pm$ 9.09 ^{a bbb}	76.17 $\pm$ 4.5 ^{bbb}
Dry weight (mg)	57.03 $\pm$ 2.92 ^{aa}	45.41 $\pm$ 1.2	28.8 $\pm$ 2.38 ^{a bbb}	22 $\pm$ 1.75 ^{bbb}
Wet weight per tibia length (mg/mm)	4.96 $\pm$ 0.26 ^{aa}	3.94 $\pm$ 0.12	2.63 $\pm$ 0.22 ^{a bbb}	1.97 $\pm$ 0.11 ^{bbb}
Dry weight per tibia length (mg/mm)	1.32 $\pm$ 0.07 ^{aa}	1.05 $\pm$ 0.03	0.71 $\pm$ 0.05 ^{a bbb}	0.52 $\pm$ 0.05 ^{bbb}
Absolute Tetanic Force (mN)	5132.29 $\pm$ 669.18 ^a	3667.16 $\pm$ 410.77	3970.99 $\pm$ 496.61 ^a	1962.24 $\pm$ 369.08
Specific Tetanic Force (mN/mg)	118.9 $\pm$ 35.94	103.03 $\pm$ 26.02	79.27 $\pm$ 25.91	49.7 $\pm$ 16.35
Absolute Twitch Force (mN)	1379.29 $\pm$ 196.61 ^a	769.77 $\pm$ 97.51	1214.26 $\pm$ 165.12 ^a	567.16 $\pm$ 124.38
Specific Twitch Force (mN/mg)	37.81 $\pm$ 9.56	22.25 $\pm$ 6.09	26.32 $\pm$ 7.43	15.24 $\pm$ 6.34
TTP (ms)	20 $\pm$ 0.02	22.85 $\pm$ 1.8	21.6 $\pm$ 1.6	24.0 $\pm$ 2.4
HRT (ms)	21.47 $\pm$ 1.7	20.3 $\pm$ 2.1	16.1 $\pm$ 1.6	17.3 $\pm$ 3.0
LRT (ms) ^c	10.9 $\pm$ 0.7	13.9 $\pm$ 2.3	8.5 $\pm$ 0.8	7.6 $\pm$ 0.8
RFD (mN/ms) ^{dd}	72.4 $\pm$ 10.9	41.1 $\pm$ 10.5	56.5 $\pm$ 11.1	22.9 $\pm$ 8.3

Results are expressed as mean $\pm$ SEM of at least six animals. Data were analyzed using two-way ANOVA followed by Bonferroni post-test. ^a $p < 0.05$, ^{aa} $p < 0.01$ CTRL-CL vs. CTRL-H and DM-CL vs. DM-H; ^{bbb} $p < 0.001$ CTRL-CL vs. DM-CL and CTRL-H vs. DM-H; ^c $p < 0.05$ CTRL vs. DM using two-way ANOVA only; ^{dd} $p < 0.01$ CTRL-CL/DM-CL vs. CTRL-H/DM-H using two-way ANOVA only. CTRL, Control group; DM, Diabetic group; CL, Contralateral; H, Hypertrophy; TTP, time to peak; HRT, half relaxation time; LRT, late relaxation time; RFD, rate of force development.

median, was supported by qualitative analysis of the proportion of fibers in different ranges of CSA (Figures 2C, 3C). Frequency distribution (Figures 2C, 3C) was calculated and expressed as previously performed by others (Baehr et al., 2011; Ge et al., 2011; Kulakowski et al., 2011; Pistilli et al., 2011; Watson et al., 2012; Callahan et al., 2014; Marzucca-Nassar et al., 2016). In the EDL muscle of the control group there was a 69% increase and in the diabetic animals of 97% (Figure 2). In the soleus muscle, there was an increase of 103% in the control group and of 70% in the diabetic group (Figure 3).

Modulation of the Signaling Protein Contents after 7 Days of Muscle Overload

There was an increase in the content of total Akt by 2.2-fold in the EDL muscle of the control group after 7 days of overload. The content of p-Akt^{Ser473} was increased by 2.5-fold in the control and in the diabetic groups. Total rpS6 content was increased by

4.2-fold in the control group but did not change in the diabetic rats. The content of p-rpS6^{Ser244/240} was increased by 3.2-fold in the control group and by 2.6-fold in the diabetic due to overload stimulus. The total 4EBP-1 content, after 7 days of overload, was increased by 37% in the control group and by 23% in the diabetic. The contents of p-4EBP-1^{Thr37/46} was increased by 2.5-fold in the control group and by 2-fold in diabetic rats. The total AMPK content in the control group submitted to overload was reduced by 38%. The p-AMPK^{Thr172} content was decreased by 34% in the control and by 54% in the diabetic group submitted to overload. The content of MuRF-1 changed only in the diabetic group submitted to overload with a 51% decrease. Quantitative analysis of western blots after 7 days of overload in the EDL muscle is in Figure 4.

The total Akt content in the soleus muscle after 7 days of overload in the control group was increased by 83% whereas in the diabetic rats there was a 2.6-fold increase (Figure 5A). The

TABLE 3 | Soleus mass, strength, and contractile properties after 30 days of overload.

Measurements	Control group		Diabetic group	
	Hypertrophy	Contralateral	Hypertrophy	Contralateral
SOLEUS MUSCLE				
Wet weight (mg)	211.51 ± 10.34 ^{aaa}	152.37 ± 7.74	156.12 ± 9.67 ^{aaa bbb}	109.89 ± 7.72 ^{bb}
Dry weight (mg)	51.58 ± 2.64 ^{aaa}	40.05 ± 1.86	40.43 ± 1.6 ^{aaa bbb}	27.75 ± 1.53 ^{bbb}
Wet weight per tibia length (mg/mm)	4.96 ± 0.21 ^{aaa}	3.58 ± 0.17	3.9 ± 0.22 ^{aaa bb}	2.74 ± 0.18 ^b
Dry weight per tibia length (mg/mm)	1.21 ± 0.05 ^{aaa}	0.94 ± 0.04	1.01 ± 0.03 ^{aaa bb}	0.69 ± 0.05 ^{bbb}
Absolute Tetanic Force (mN)	4838.04 ± 379.19 ^{aaa}	2729.97 ± 177.59	4571.73 ± 503.6 ^a	3193.56 ± 341.29
Specific Tetanic Force (mN/mg)	93.41 ± 6.92	70.36 ± 7.08	97.57 ± 16.7	103.43 ± 8.57
Absolute Twitch Force (mN) ^{cc d}	1825.7 ± 217.96	1397.92 ± 166.21	1293.44 ± 52.34	956.23 ± 102.21
Specific Twitch Force (mN/mg)	35.17 ± 3.94	35.53 ± 4.3	30.24 ± 2.38	31.07 ± 2.61
TTP (ms)	25 ± 2.22	20 ± 0.0	21.7 ± 1.7	21.7 ± 1.7
HRT (ms)	5.0 ± 1.1	5.8 ± 1.1	4.5 ± 1.0	6.3 ± 1.5
LRT (ms)	3.5 ± 0.6 ^a	7.9 ± 0.5	4.9 ± 1.4	6.0 ± 1.4
RFD (mN/ms) ^d	74.4 ± 7.3	63.1 ± 9.2	54.6 ± 7.7	45.2 ± 5.9

Results are expressed as mean ± SEM of at least six animals. Data were analyzed using two-way ANOVA followed by Bonferroni post-test. ^a*p* < 0.05, ^{aaa}*p* < 0.001 CTRL-CL vs. CTRL-H and DM-CL vs. DM-H; ^b*p* < 0.05, ^{bbb}*p* < 0.01, ^{bbb}*p* < 0.001 CTRL-CL vs. DM-CL and CTRL-H vs. DM-H; ^{cc}*p* < 0.01 CTRL vs. DM using two-way ANOVA only; ^d*p* < 0.05 CTRL-CL/DM-CL vs. CTRL-H/DM-H using two-way ANOVA only. CTRL, Control group; DM, Diabetic group; CL, Contralateral; H, Hypertrophy; TTP, time to peak; HRT, half relaxation time; LRT, late relaxation time; RFD, rate of force development.

content of p-AKT^{Ser473} increased by 2.4-fold in the control group whereas in diabetic rats there was a 3-fold increase (**Figure 5B**). The total rpS6 content increased by 3.7-fold (**Figure 5C**) in the control group and by 11-fold in the diabetic group. The contents of p-rpS6^{Ser244/240} increased by 2-fold in the control group and by 8.6-fold in the diabetic rats due to hypertrophy (**Figure 5D**). The total 4EBP-1 content, after 7 days of overload, increased by 68% (**Figure 5E**) in the control and in the diabetic groups. The contents of p-4EBP-1^{Thr37/46} in the soleus muscle increased (by 67%) in the control and in the diabetic groups (**Figure 5F**). The content of MuRF-1 decreased by 26% (**Figure 5G**) due to soleus muscle hypertrophy in the control and by 47% in the diabetic groups. Atrogin-1 had 36% decrease in the control and 45% reduction in the diabetic group (**Figure 5H**). The total AMPK content in the soleus muscle of the control group submitted to overload reduced by 40% and in the diabetic group by 27% (**Figure 5I**).

Modulation of mRNA Content after Overload

In the control group, regarding the hypertrophic response, the amount of mRNA was different for the genes: FAK, 46% reduction in expression; Akt1, 2.5-fold increase; mTOR, 3.3-fold increase; β-catenin, 64% reduction; follistatin, 4.6-fold increase (**Figure 6**). In the diabetic group submitted to overload, mRNA response was different for the following genes: Akt1, increase of 3.6-fold; mTOR, 92% increase; β-catenin and myostatin, both 68% reduction; Wnt7a, reduction of 62%.

mRNA expression in the contralateral EDL muscle was different between the control and the diabetic groups for the following genes: FAK, 49% lower in the diabetic group; IGF-1, 74% lower in the diabetic group; myostatin, 2.6-fold higher in the diabetic group; follistatin, 3.6-fold higher in the diabetic group.

mTOR increased upon hypertrophy only in both groups (by 2.3-fold in CTRL and by 92% in DM) and MuRF-1 decreased due to diabetes (by 29% in the contralateral muscle and by 57% in the hypertrophied muscle).

The mRNA expression was also assessed after 7 days of overload in the soleus muscle (**Figure 7**). Regarding the hypertrophic response, the amount of mRNA in the control group was different for the genes: FAK, 43% reduction; mTOR, 51% reduction; Atrogin-1, 58% reduction; MG53, 11% reduction; IGF-1, 98% increase; MGF, 57% increase; myostatin, 79% reduction; Wnt7a, 33% reduction, axin2, 42% reduction.

In the diabetic group upon hypertrophy, the amount of mRNA was different for the following genes: FAK, 27% reduction; mTOR, 33% reduction; MuRF-1, reduction by 60%; Atrogin-1, 45% reduction; MG53, reduction of 25%; IGF-1, 4.5-fold increase; MGF, 2.9-fold increase; myostatin, reduction by 65%; follistatin, 52% increase; Wnt7a, reduction of 30%. MG53 presented a decrease due to diabetes only (by 14% in the contralateral muscle and by 26% in the hypertrophied muscle).

The mRNA expression in the hypertrophied soleus muscle was different between the control and diabetic groups for the following genes: β-catenin, 23% lower in the diabetic group. mRNA content in the contralateral soleus was different between the control and diabetic groups for the following genes: Akt 1, 19% lower in diabetic group; Atrogin-1, 50% higher in the diabetic group; MGF, 51% lower in the diabetic group.

DISCUSSION

In our previous study, EDL and soleus muscles (exhibiting a myopathy condition) of 30-days streptozotocin-induced diabetic rats had similar response to hypertrophic stimulus as the control animals (Fortes et al., 2015). Our previous

FIGURE 2 | Cross-sectional areas (CSA) of the EDL muscle fibers. **(A)** Representative histological hematoxylin and eosin staining images of EDL muscle CSA. Reference bar represents 50 μ m. **(B)** Dispersion graph of EDL muscle fibers CSA. **(C)** Frequency distribution of the EDL muscle fibers according to CSA ranges: 0–5,500 μ m². The results were analyzed as previously described using the 95% confidence interval of the median. *Significant different considering the 95% confidence interval of the median. CTRL-CL, control group, contralateral muscle; CTRL-H, control group, hypertrophied muscle; DM-CL, diabetic group, contralateral muscle; DM-H, diabetic group, hypertrophied muscle.

work was focused on a skeletal muscle hypertrophic response after an already established chronic diabetic condition (in diabetic myopathy) using two different protocols. First, animals were rendered diabetic and remained hyperglycemic for 30 days before being submitted to overload-induced hypertrophy for 7 days. Second, in a different set of animals, diabetes was induced for 30 days before overload-induced hypertrophy for another 30 days period. In the present study, we investigated the mechanisms associated to overload-induced muscle hypertrophy and tested if the hypertrophy would be able to counteract the hypotrophy observed at the onset of type I diabetes (before diabetic myopathy manifestation).

Several signaling pathways control protein synthesis and degradation act synergistically during hypertrophic stimulation. We reported in the previous study that the contribution of each pathway in the control of muscle mass varies between the control and diabetic groups. The diabetic rats had to overcome diabetic myopathy and the consequences of low plasma insulin levels to ensure skeletal muscle mass gain. The differences between groups may be also associated with the muscle recruitment trend and the susceptibility of each muscle to the metabolic alterations caused by the disease, and the distinct composition of fiber types such as in the soleus muscle (predominantly type I) and EDL (predominantly type II) muscles (Cotter et al., 1989). Although the response to overload was comparable to

FIGURE 3 | Cross-sectional areas (CSA) of the soleus muscle fibers. **(A)** Representative histological hematoxylin and eosin staining images of the soleus muscle CSA. Reference bar represents 50 μm . **(B)** Dispersion graph of soleus muscle fibers CSA. **(C)** Frequency distribution of the soleus muscle fibers according to the CSA ranges: 0–7,000 μm^2 . The results were analyzed as previously described using the 95% confidence interval of the median. *Significant difference considering the 95% confidence interval of the median. CTRL-CL, control group, contralateral muscle; CTRL-H, control group, hypertrophied muscle; DM-CL, diabetic group, contralateral muscle; DM-H, diabetic group, hypertrophied muscle.

control, the hypertrophic response in diabetic animals was not able to restore muscle mass to control values (of the contralateral muscle), which could be related to the onset of hypertrophic stimulation that occurs after a severe hypotrophy caused by chronic diabetes. In the present study, the hypertrophic stimulus was initiated concomitantly with the diabetes induction. The purpose was to promote hypertrophic stimulation in the early stages of diabetes, when protein degradation signaling is more intense, as an attempt to prevent diabetes-induced muscle hypotrophy.

The overload maintained soleus muscle mass, CSA of the muscle fibers and force production in diabetic rats to similar values or even higher than in the contralateral muscle of

control animals. The EDL muscles of diabetic animals were not able to reach the absolute contralateral values of the control group even after 30 days of hypertrophic stimulus. The greater response of the soleus muscle may be related to activation of different signaling pathways of protein synthesis and degradation as compared to EDL (**Figure 8**). EDL muscle is more susceptible to force and muscle mass loss than soleus muscle (Paulus and Grossie, 1983). The EDL muscle of diabetic rats suffered greater hypotrophy than the soleus muscle. A more severe hypotrophy in the glycolytic/fast-twitch fibers may be a result of the effects of glucocorticoids; the plasma levels of corticosterone are elevated in streptozotocin-induced diabetic rats (Rhees et al., 1983) and these are the most susceptible fibers

to the catabolic action of this hormone (Goldberg and Goodman, 1969). Reactive oxygen species (ROS) have been reported to, in long-term along with advanced glycation end-products (AGEs), promote skeletal muscle loss especially in the diabetic condition through a tightly coupling with PI3K/AKT signaling pathway (Grzelkowska-Kowalczyk et al., 2013; Zuo and Pannell, 2015; Zuo et al., 2015). Diabetic oxidative stress also impairs protein turnover and apoptotic process leading to muscle loss (Powers et al., 2012). Signaling differences (Figure 8A) might play an important role in the more pronounced atrophy of the EDL muscle reported.

The signaling pathway involved in the soleus muscle hypertrophy in diabetes was comparable to that of the control rats. The same was not observed for EDL muscle hypertrophy that may have contributed to the lower hypertrophic response in the diabetic group. mRNA expression of IGF-1 and MGF in the EDL muscle did not respond to hypertrophic stimulus in diabetic animals as observed in the soleus muscle. This may have contributed for the low increase in the phosphorylated and total rpS6 contents and for the lack of responsiveness of atrogin-1 expression, which was not reduced as in the soleus due to hypertrophic stimulation. In the EDL muscle, there was a reduction of myostatin, whereas in the soleus a reduction

of myostatin concomitantly with an increase in follistatin was described. Therefore, the restoration of muscle mass for values similar to the contralateral control was far more effective in the soleus.

IGF stimulates protein synthesis during overload and promotes hypertrophy (Coleman et al., 1995; Bodine et al., 2001; Schiaffino and Mammucari, 2011). However, overload-induced hypertrophy does not depend exclusively on the IGF-1 pathway stimulus since it occurs even when IGF-1 receptors are inactivated (Spangenburg et al., 2008; Klossner et al., 2009). IGF-1 activates the PI3K/Akt/mTOR pathway and its main effectors (p70S6K, rpS6, 4EBP-1) that induce synthesis of myofibrillar proteins (Bodine et al., 2001; Richter and Sonenberg, 2005; Bodine, 2006). Seven days of soleus muscle overload promoted an increase of IGF-1 and MGF mRNA expression in the diabetic and the control groups. In animals submitted to 37 days of diabetes, the MGF mRNA expression in the hypertrophied soleus muscle was also markedly elevated (Fortes et al., 2015). IGF-1 expression was lowered in the EDL muscle of the diabetic group even after 7 days of overload. As mentioned above, it may have contributed to the reduced hypertrophic response of the EDL muscle in diabetic animals when compared to the soleus. The lack of IGF-1/MGF response might be associated with the severe limitation for

the muscle mass maintenance in diabetic condition. In animals submitted to 37 days of diabetes, IGF-1 mRNA expression in the hypertrophied EDL muscle was markedly elevated (about 10-fold in the control and 5-fold in the diabetic groups) (Fortes et al., 2015). This difference between soleus and EDL muscles responses in our previous study indicates that both the period that the muscle remains under mechanical stimulation and the long-term diabetic myopathy regulate the gene expression of growth factors when compared to the initial phase diabetes onset. The diabetic condition affects the skeletal muscle, hypertrophic response, the susceptibility to contraction-induced injury, the quantity and activation of satellite cells, and the amount and composition of extracellular matrix (Brannon et al., 1989; Umpierrez et al., 1989; Gulati and Swamy, 1991; Law et al., 2012; D'Souza et al., 2016). Protein synthesis signaling reaches the activation peak within 7 days of muscle overload regardless hormonal stimulation does not follow the same time course (Armstrong and Ianuzzo, 1977; Farrell et al., 1999; Katta et al., 2010), indicating p-Akt and p-rpS6 protein levels were increased in the EDL and soleus muscles when submitted to 7 days of overload as also observed by others (Thomson and Gordon, 2006; Hamilton et al., 2010; Miyazaki et al., 2011). There was an increase in the total and phosphorylated content of 4EBP-1 protein upon

hypertrophic stimulus corroborating previous studies (Bodine, 2006; Potter et al., 2013). Akt and mTOR, important components of the canonical pathway of protein synthesis, had different mRNA expression in the soleus and EDL muscles. Despite phosphorylation and total amount of Akt being higher in the hypertrophied control and diabetic groups, as also reported by others (Miyazaki et al., 2011; Fortes et al., 2015), mRNA expression changes did not closely follow the changes of protein content in the soleus muscle, which indicates posttranslational modifications (Ohlndieck, 2011, 2013; Lourenço dos Santos et al., 2015; Wende, 2016).

AMPK may decrease mTORC1 activity by phosphorylation of Tuberin (TSC2) in Thr1345 (Inoki et al., 2003) or raptor in Ser792 (Gwinn et al., 2008). Mounier et al. (2009), after AMPK α 1 gene ablation, reported more pronounced overload-induced hypertrophy in plantar muscle (Mounier et al., 2009). Increased AMPK activation in the plantar muscle (McGee et al., 2008; Hamilton et al., 2014) associated with a decrease in skeletal muscle hypertrophy (Bolster et al., 2002; Thomson and Gordon, 2005) have also been described. We reported a more pronounced response of the PI3K/Akt/mTOR pathway in the soleus muscle in control and diabetic animals, despite not observing a reduction on the activation of AMPK. The AMPK activation may not be a

FIGURE 6 | (A,B,D, F-M) mRNA expression of several genes in the EDL muscle after 7 days of overload. Results are presented as mean $\pm$ SEM of at least six animals. Results were analyzed using two-way ANOVA followed by Bonferroni post-test. * $p < 0.05$, ** $p < 0.01$. In **(C)**, # $p < 0.05$ for CTRL-CL/DM-CL vs. CTRL-H/DM-H, in **(E)**, # $p < 0.05$ for CTRL vs. DM using two-way ANOVA only. CL, Contralateral muscle; H, Hypertrophied muscle; DM, Diabetic group; CTRL, Control group.

determinant inhibitor of protein synthesis under the conditions of this study.

As a result of the PI3K/Akt/mTOR pathway activation, the MuRF-1, and atrogin-1 mRNA expression was inhibited by their upstream signaling protein, FOXO (Stitt et al., 2004), reducing proteasome-associated protein degradation. MuRF-1 and atrogin-1 might also affect skeletal muscle remodeling that occurs during growth (Baehr et al., 2014). MuRF-1 and atrogin-1 expression was decreased in the soleus muscle of the control

and diabetic rats upon overload, and the same did not occur in the EDL muscle. This may be due to the lower activation of PI3K/Akt/mTOR pathway that contributed for the lower hypertrophic response in the EDL muscle.

MG53 expression was reduced by the diabetic condition as well by hypertrophic stimulus in the soleus muscle. This might be involved in the increased hypertrophic response of the soleus in diabetic animals as compared with the EDL muscle. MG53 is involved in the regulation of myogenic negative feedback and

FIGURE 7 | (A–F, H–K, M) mRNA expression of several genes in the soleus muscle after 7 days of overload. Results are presented as mean $\pm$ SEM of at least nine animals. Data were analyzed using two-way ANOVA followed by Bonferroni post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. In (G), * $p < 0.05$ CTRL vs. DM and # $p < 0.05$ for CTRL-CL/DM-CL vs. CTRL-H/DM-H, in (L), # $p < 0.05$ for CTRL-CL/DM-CL vs. CTRL-H/DM-H using two-way ANOVA only. CL, Contralateral muscle; H, Hypertrophied muscle; DM, Diabetic group; CTRL, Control group.

cleaves the insulin receptor substrate 1 (IRS-1) (Jung and Ko, 2010; Lee et al., 2010). MG53 acts synergistically with other two myogenic regulatory factors (MyoD—myogenic differentiation factor and MEF2—muscle enhancer factor 2) (Jung and Ko, 2010; Lee et al., 2010). MG53 promotes ubiquitination followed by degradation of IRS-1 and acts on the PI3K/Akt/mTOR signaling causing deficient synthesis of contractile myofibrillar proteins (Yi et al., 2013).

The expression of FAK, an enzyme involved in the mechanical signaling associated with skeletal muscle hypertrophy (Flück et al., 1999; Klossner et al., 2009), was increased in hypertrophied

soleus and EDL muscles after 7 days of overload when animals were previously submitted to 30 days of diabetes (Fortes et al., 2015). However, when animals were submitted to a period of 7 days of overload initiated at the onset of diabetes, FAK mRNA expression had a decrease in both soleus and EDL muscles. These findings indicate that the expressions of proteins related to muscle hypertrophy changes with the period of the diabetic state.

The balance between myostatin and follistatin expressions also regulate skeletal muscle hypertrophy, with myostatin inhibiting and follistatin stimulating this process (Gilson et al.,

2009). The absence of myostatin induces muscle growth whereas the elevation of its mRNA expression and exogenous administration causes muscle hypotrophy (McPherron and Lee, 1997; McPherron et al., 1997; Schuelke et al., 2004; Rodriguez et al., 2014). We reported a reduction in the myostatin mRNA in the EDL muscle. However, in the soleus, in addition to the reduction in myostatin, there was also an increase in follistatin mRNA. This combined response may contribute to a greater hypertrophic response in the soleus muscle in diabetes.

Wnt, β -catenin and axin influence skeletal muscle myogenesis, being necessary for skeletal muscle repair in response to injury (Newmire and Willoughby, 2015; Huraskin et al., 2016; Rudolf et al., 2016). There was no similar response of these three genes in the present study. The expression of β -catenin in the EDL muscle was reduced upon hypertrophy, which did not occur in the soleus muscle. Increased expression of β -catenin also participated in the soleus muscle hypertrophy process (Armstrong and Esser, 2005; Armstrong et al., 2006). β -Catenin expression was increased in the soleus muscle and at the same time Wnt remained almost unaltered. Since PI3K/Akt/mTOR pathway is more active in the soleus, there may be a greater inhibition of GSK3 β in this muscle, corroborating for the increase (or maintenance) in β -catenin levels and therefore contributing for the hypertrophic response (Jones et al., 2015).

CONCLUDING REMARKS

The soleus and EDL muscles from diabetic animals submitted to overload at the onset of the disease (before diabetic myopathy manifestation) exhibited similar hypertrophic response. The increase of muscle forces occurred at the same magnitude as the muscle hypertrophy.

In the EDL muscle of diabetic animals, overload promoted hypertrophy via mechanical action, which involved the PI3K/Akt/mTOR pathway, reduced AMPK activation and decreased myostatin expression.

Hypertrophy was more pronounced in the soleus muscle of diabetic animals, as compared with the EDL, maintaining the muscle mass and force to similar values of the contralateral control muscle. This may be due to a more preserved hypertrophic signaling, in relation to the control group, including a higher content and activation of pS6 than in the EDL muscle, increased mRNA expression of IGF-1, MGF, and follistatin and decreased mRNA expression of myostatin, MuRF-1 and atrogen-1.

We concluded that resistance exercise is an ally for the prevention of diabetic myopathy (hypotrophy) and when initiated early in the diabetes progression its effectiveness might be greater.

AUTHOR CONTRIBUTIONS

MF conceived of the study, carried out the molecular experiments, statistical analysis, conducted the animals' surgeries, interpreted the results, and wrote the manuscript. MS assisted in the molecular experiments. GM participated in the molecular studies, interpreted the results and assisted in the draft of the manuscript. KV assisted its design and coordination, interpreted the results and revised the manuscript for important intellectual content. CdJP participated in the interpretation of the results and revised for important intellectual content. RC revised for important intellectual content, assisted in the statistical analysis and draft the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2017.00830/full#supplementary-material>

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Research Paper

Contractile function recovery in severely injured gastrocnemius muscle of rats treated with either oleic or linoleic acid

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New Findings

- What is the central question of this study?

Oleic and linoleic acids modulate fibroblast proliferation and myogenic differentiation *in vitro*. However, their *in vivo* effects on muscle regeneration have not yet been examined. We investigated the effects of either oleic or linoleic acid on a well-established model of muscle regeneration after severe laceration.

- What is the main finding and its importance?

We found that linoleic acid increases fibrous tissue deposition and impairs muscle regeneration and recovery of contractile function, whereas oleic acid has the opposite effects in severely injured gastrocnemius muscle, suggesting that linoleic acid has a harmful effect and oleic acid a potential therapeutic effect on muscle regeneration.

Oleic and linoleic acids control fibroblast proliferation and myogenic differentiation *in vitro*; however, there was no study in skeletal muscle *in vivo*. The aim of this study was to evaluate the effects of either oleic or linoleic acid on the fibrous tissue content (collagen deposition) of muscle and recovery of contractile function in rat gastrocnemius muscle after being severely injured by laceration. Rats were supplemented with either oleic or linoleic acid for 4 weeks after laceration [$0.44 \text{ g (kg body weight)}^{-1} \text{ day}^{-1}$]. Muscle injury led to an increase in oleic-to-stearic acid and palmitoleic-to-palmitic acid ratios, suggesting an increase in Δ^9 desaturase activity. Increased fibrous tissue deposition and reduced isotonic and tetanic specific forces and resistance to fatigue were observed in the injured muscle. Supplementation with linoleic acid increased the content of eicosadienoic (20:2, $n-6$) and arachidonic (20:4, $n-6$) acids, reduced muscle mass and fibre cross-sectional areas, increased fibrous tissue deposition and further reduced the isotonic and tetanic specific forces and resistance to fatigue induced by laceration. Supplementation with oleic acid increased the content of docosahexaenoic acid (22:6, $n-3$) and abolished the increase in fibrous tissue area and the decrease in isotonic and tetanic specific forces and resistance to fatigue induced by muscle injury. We concluded that supplementation with linoleic acid impairs muscle regeneration and increases fibrous tissue deposition, resulting in impaired recovery of contractile function. Oleic acid supplementation reduced fibrous tissue deposition and improved recovery of contractile function, attenuating the tissue damage caused by muscle injury.

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Introduction

Skeletal muscle regeneration from injuries requires various overlapping phases, such as the following: (i) migration of inflammatory cells to the injured area; (ii) phagocytosis of dead cells and necrotic debris by macrophages and neutrophils; and (iii) stimulation of the process of myogenesis (Millay *et al.* 2013; Grasman *et al.* 2015; Domingues-Faria *et al.* 2016). Along with these events, re-innervation, angiogenesis and extracellular matrix remodelling take place in order to promote full recovery of the injured muscle tissue and muscle function (Fukushima *et al.* 2001; Charge & Rudnicki, 2004; Ciciliot & Schiaffino, 2010; Mann *et al.* 2011). Although the underlying mechanisms involved in the regeneration are not completely understood, several key proteins have been described to participate in this process, including striated muscle activator of Rho signalling, serum- and glucocorticoid-inducible kinase-1, phosphatase and tensin homologue, the Notch pathway, latent transforming growth factor- β binding protein-4/transforming growth factor- β signalling, and transient receptor potential channel subfamily A1 (Hu *et al.* 2010; Church *et al.* 2014; Lamon *et al.* 2014; Swaggart & McNally, 2014; Steinberger *et al.* 2015; Osterloh *et al.* 2016; Wallace *et al.* 2016). Severe injuries (e.g. laceration) cause rupture of large myofibres and extensive basal lamina and extracellular matrix damage, and so muscle regeneration and function recovery are eventually not fully achieved (Mann *et al.* 2011; Murphy *et al.* 2011; Sakuma *et al.* 2015). Extracellular matrix overgrowth and extensive fibrosis are closely associated with impaired regeneration and incomplete recovery of contractile function of skeletal muscle after severe injury (Chan *et al.* 2005; Ten Broek *et al.* 2010; Murphy *et al.* 2011).

Therapeutic interventions in injured skeletal muscle include local application of ice and/or heat, compression or elevation of the injured limb and immobilization (Jackson & Feagin, 1973; Caplan *et al.* 1988). These treatments, however, are often not effective for full recovery of the injured muscle (Huard *et al.* 2002; Sato *et al.* 2003). New strategies for improving muscle regeneration are required. For instance, nutritional strategies have been investigated in order to improve muscle regeneration in animals submitted to muscle injury (Baptista *et al.* 2013; Pereira *et al.* 2014,b).

Effects of fatty acids on *in vitro* fibroblast proliferation (Cardoso *et al.* 2004; Menendez & Lupu, 2006; Magdalon *et al.* 2011) and myogenic differentiation have been reported (Hurley *et al.* 2006; Lee *et al.* 2009; Briolay *et al.* 2013; Markworth & Cameron-Smith, 2013). However, *in vivo* effects of specific fatty acids (such as oleic or linoleic acid) on muscle regeneration have not been addressed. Oleic and linoleic acids are the major fatty acids in western human diets (British Nutrition

Foundation, 1992; US Department of Agriculture, 2008). Our group has investigated the effects of either oleic or linoleic acid on cultured neutrophils (Rodrigues *et al.* 2010) and macrophages (Magdalon *et al.* 2012) and on wound healing of skin in a rat model (Rodrigues *et al.* 2012). Supplementation with either oleic or linoleic acid accelerated the process of wound healing. In spite of these findings, there are no studies on *in vivo* effects of either oleic or linoleic acid on skeletal muscle regeneration. The aim of this study was to assess and compare the effects of supplementation with either oleic or linoleic acid on fibrous tissue area (collagen deposition) and recovery of contractile function in rat gastrocnemius muscle after severe injury by laceration. Our hypothesis was that oleic or linoleic acid or both could accelerate regeneration of injured muscle as observed in the wound-healing process (Rodrigues *et al.* 2012).

Methods

Ethical approval

All experimental procedures of this study were performed in accordance with the *Ethical Principles in Animal Research* followed by the Brazilian College of Animal Experimentation and in strict accordance with the *Guide for Care and Use of Laboratory Animals* of the US National Institutes of Health (NIH publication no. 85-23, eighth edition, revised 2011) and with the principles and regulations as previously described (Grundy, 2015). The Ethics Committee in Animal Research of the Institute of Biomedical Sciences, University of São Paulo, approved this study (protocol number 2011/78).

Animals

Male Wistar rats weighing 182–206 g (initial body weight) were used in all experiments. Animals were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo (São Paulo, Brazil) and maintained at 23°C with a light–dark cycle of 12 h–12 h. Animals received standard chow and water *ad libitum*. Standard chow for rodents (Nuvital, Curitiba, Brazil) contained 22% protein, 4.5% fat, 40.8% carbohydrate and 8% fibres. The main fatty acids of the dietary fat were linoleic acid (42%), oleic acid (17%) and palmitic acid (17%).

Diet and supplementation with either linoleic or oleic acid

Non-esterified linoleic acid (18:2 ω -6; $\geq 99\%$) and oleic acid (18:1 ω -9; $\geq 99\%$), both obtained from Sigma (L1376 and L1381, respectively; St Louis, MO, USA), were administered daily by gavage {0.44 g [kg body

weight (b.w.)⁻¹] for 4 weeks (<0.2 ml per animal). Control animals received mineral oil (100%; Tayuyana Ltda, São Paulo, Brazil) at the same dose and by the same route. Animals were killed 1 h after the last fatty acid supplementation. Our group previously observed that this treatment does not alter any nutritional parameter (such as food ingestion, caloric intake or body weight gain) or the general health condition of the animals as indicated by no alteration in plasma activities of alanine transaminase, aspartate transaminase and lactate dehydrogenase (Rodrigues *et al.* 2010).

Muscle laceration protocol

Gastrocnemius muscle laceration was induced as previously described by Garrett *et al.* (1984) and Menetrey *et al.* (1999), with slight modifications. Briefly, 24 h before surgery, animals received a single dose [80 mg (kg b.w.)⁻¹, i.p.] of gentamicin (antibiotic for preventing bacterial infections; Hipolabor Farmacêutica Ltda, Belo Horizonte, MG, Brazil). Rats were anaesthetized by i.p. injection of ketamine [90 mg (kg b.w.)⁻¹] and xylazine [10 mg (kg b.w.)⁻¹], both obtained from Vetbrands Brasil Ltda (Jacareí, SP, Brazil). Reflexes (tail pinch and palpebral) and respiratory rate were monitored before and during the surgery to verify anaesthetic depth. After shaving hair from the right hindlimb, dissection of the skin and subcutaneous tissue was performed to expose the gastrocnemius muscle. Sectioning of the gastrocnemius muscle (laceration) was performed as follows: at 60% of the length from the distal insertion (2.5 cm from the calcaneus flexed at 90 deg), 75% of the width (1.0 cm) and 50% of the thickness. All measurements were performed using a digital calliper rule (Digimed, São Paulo, Brazil). The skin was then sutured with nylon thread (3–0). The contralateral (left) hindlimb was submitted to a sham procedure. After surgery, a local antiseptic procedure was performed using povidone iodine (Laboratory Tayuyana Ltda, Nova Odessa, SP, Brazil), and animals were kept in individual cages in controlled environmental conditions (24°C) until fully recovered from anaesthesia in order to avoid hypothermia (Pereira *et al.* 2014a,b).

Rats were randomly divided into equal numbers and submitted to the following treatments for 4 weeks: (i) mineral oil (Mo); (ii) linoleic acid (Lin); or (iii) oleic acid (Ole). The following six groups were then studied: contralateral/uninjured muscles (Control) and lacerated/injured muscles (Injury) from Mo, Lin and Ole rats, i.e. Mo Control, Mo Injury, Lin Control, Lin Injury, Ole Control and Ole Injury groups. One hour after the last dose of linoleic or oleic acid or mineral oil, rats were again deeply anaesthetized by i.p. injection of ketamine [90 mg (kg b.w.)⁻¹] and xylazine [10 mg (kg b.w.)⁻¹], and gastrocnemius muscles (from left and right hind limbs) were removed and weighed. Muscles were stored

at –80°C until analysis was performed. Rats were then killed by exposure to CO₂ while still under deep general anaesthesia.

Determination of gastrocnemius muscle fatty acid composition

The composition of fatty acids in gastrocnemius muscle (100 mg) was determined by gas chromatography, according methods previously described (Feldsine *et al.* 2002; Ratnayake *et al.* 2002). A similar procedure has been used in our studies (Afonso *et al.* 2013; Hirabara *et al.* 2013; Fariña *et al.* 2014; de Santana *et al.* 2015). The results are presented as the mean ± SD of four animals per group.

Histological analysis

Immediately after removal, gastrocnemius muscles were embedded into Tissue-Tek OCT solution and frozen in liquid N₂, using a cold-resistant recipient containing isopentane solution. Sections (10 µm thick) from the mid-belly region of the injured muscle were obtained using a cryostat (Leica CM 3050, Wetzlar, Germany). Some sections were stained using the Haematoxylin and Eosin method for evaluation of muscle fibre cross-sectional area (CSA) and others processed according to the Picrosirius-polarization method for analysis of fibrous tissue areas (collagen deposition; Sweat *et al.* 1964).

For determination of the CSA (Kost & Kost, 1982), sections were photographed under a microscope (Nikon Eclipse E1000, Fukuoka, Japan) coupled to a photograph camera (Nikon DXM 1200). Five non-overlapping images from the central region of each section were analysed using Image Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA). Mean values of CSA were determined by measuring the circumferences of 25 adjacent fibres, giving a total of 500 fibres per group, using a scale bar of 100 µm. We used a similar procedure in previous studies (Pinheiro *et al.* 2012; Fortes *et al.* 2015).

Tissue collagen deposition was evaluated by Picrosirius Red histochemical staining. Briefly, sections were photographed using a polarized light microscope and images captured on a JVC TK-1280E digital colour video camera coupled to the microscope. Image analysis was performed as described above. Collagen distribution was determined in a Picrosirius-stained section on a bundle of muscle fibres surrounded by perimysium, containing 10–100 fibres, with ×375 magnification, as previously reported (Junqueira *et al.* 1979). Non-blinded analysis was performed, and the results were confirmed by two independent investigators. Results are reported as the mean ± SD from seven animals per group for analysis of the gastrocnemius muscle mass and from four animals for evaluation of the muscle fibre CSA.

Muscle strength and resistance to fatigue by electrical stimulation *in situ*

Gastrocnemius muscle contractile function was evaluated *in vivo* by electrical stimulation as previously described (Wojtaszewski *et al.* 1996) and performed by our group (Silveira *et al.* 2007; Bassit *et al.* 2010; Pinheiro *et al.* 2012). All procedures since initial surgery to the terminal anaesthesia for harvesting of tissues were performed in the same session. Briefly, rats were anaesthetized [$80 \text{ mg (kg b.w.)}^{-1}$ of pentobarbital by i.p. injection], and the Achilles tendon of the hindlimb muscle was attached to a hook connected to a force transducer (Grass Technologies, West Warwick, RI, USA). A 2 mm incision was made in the skin for sciatic nerve assessment. Tenotomy of all other synergistic and antagonist muscles was performed to allow assessment of the gastrocnemius muscle only. Resting length (L_0) was determined to obtain maximal tension upon stimulation (ankle joint at a 90 deg angle), using the MultiStim System D330 (Digitimer Ltd, Welwyn Garden City, UK). For determination of twitch force, muscles were stimulated using 500 μs pulse duration, at 1 Hz and an appropriate voltage to generate maximal force. For analysis of tetanic force, the frequency of electrical stimulation was increased to 100 Hz. Muscle twitch force (maximal tension during twitch), tetanic force (maximal tension during tetany), muscle strength and resistance to fatigue were determined using AqDados and AqAnalysis Software, version 4.16, from Lynx Tecnologia Eletronica Ltda (São Paulo, Brazil). Specific muscle strength was calculated by dividing the muscle twitch force and tetanic force by muscle mass dry weight (in millinewtons per gram). Muscle resistance to fatigue was determined through curve decline from the initial tetanic force during 10 successive contractions. We followed the protocol reported in our previous studies (Silveira *et al.* 2007; Bassit *et al.* 2010; Pinheiro *et al.* 2012) on resistance to fatigue in *in situ* muscle electrical stimulation. The results are presented as the mean $\pm$ SD of at least five animals per group.

Statistical analysis

Results are presented as means $\pm$ SD and were analysed using GraphPad Prism Software (4.0; GraphPad Inc., San Diego, CA, USA). Two-way ANOVA (factors: fatty acid supplementation and muscle injury) for repeated measures (contralateral *versus* injured muscles from the same animal), followed by the Bonferroni *post hoc* test, was used for multiple comparisons. Grubb's test was used to exclude outliers. Analysis of fibre CSA was performed as previously described using the 95% confidence interval of the mean (Pinheiro *et al.* 2012). Statistical differences were considered for $P \leq 0.05$. However, in addition to the P value, other factors were considered for analysis of the results, as follows: quality/distribution measure, result

relevance and size effect, as recently proposed by the American Statistical Association (Wassersteina & Lazara, 2016).

Results

Linoleic acid reduced and oleic acid did not change injured gastrocnemius muscle mass and muscle fibre CSA

Body weight was not changed by administration of either fatty acid (data not shown); however, significant differences were observed in gastrocnemius muscle mass and fibre CSA. Linoleic acid supplementation caused a minor reduction in the contralateral/uninjured muscle mass (by 7%; Mo Control *versus* Lin Control) and in the injured muscle mass (by 15%; Mo Injury *versus* Lin Injury; Fig. 1A). A significant decrease in injured muscle mass (14%), compared with contralateral/injured muscle, was observed in animals supplemented with linoleic acid (by 14%; Lin Control *versus* Lin Injury; Fig. 1A). There was no interaction between fatty acid supplementation and muscle lesion, but a more pronounced effect was observed in injured muscle from animals supplemented with linoleic acid (Fig. 1A). Oleic acid supplementation had no significant effect on gastrocnemius muscle mass; results are presented as the mean $\pm$ SD from seven animals per group (Fig. 1A).

Similar changes were observed in the CSA of gastrocnemius muscle fibres. Supplementation with linoleic acid decreased CSA in the contralateral leg (by 20%; Mo Control *versus* Lin Control) and in the injured leg (by 25%; Mo Injury *versus* Lin Injury; Fig. 1B and C). A minor reduction of the CSA in the injured muscle, compared with contralateral/uninjured muscle, was observed in animals supplemented with linoleic acid (by 10%; Lin Control *versus* Lin Injury; Fig. 1B and C). Oleic acid supplementation did not modify gastrocnemius muscle fibre CSA in either contralateral or injured muscle (Mo Control *versus* Ole Control and Mo Injury *versus* Ole Injury; Fig. 1B and C). A significant statistical interaction was observed between the linoleic acid supplementation and muscle injury ($P < 0.01$). Results of muscle fibre CSA are the mean $\pm$ SD of four animals.

Linoleic acid exacerbated and oleic acid abolished the increase of fibrous tissue area in injured gastrocnemius muscle

Laceration itself increased the area of fibrous tissue, as demonstrated by increased collagen deposition, in gastrocnemius muscle from the Mo group (by 61%; Mo Control *versus* Mo Injury). This increase was exacerbated by linoleic acid supplementation (by 53%; Mo Injury *versus* Lin Injury; P value interaction <0.01 ; Fig. 2).

Figure 1. Effect of either linoleic or oleic acid on muscle mass (A), fibre cross-sectional area (B) and representative histological images of Haematoxylin and Eosin staining (C) of the gastrocnemius muscle, 28 days after injury induced by laceration

Histological images (scale bars represent 100 μm) were obtained from the following groups: Mo Control, contralateral muscles from animals supplemented with mineral oil (Mo); Mo Injury, injured muscles from animals supplemented with Mo; Lin Control, contralateral muscles from animals supplemented

Compared with contralateral/uninjured muscle, linoleic acid increased collagen deposition in the injured muscle (by 1.2-fold; Lin Control *versus* Lin Injury). Oleic acid supplementation abolished the increase in collagen deposition induced by laceration (Fig. 2A). Results of fibrous tissue area are presented as the mean $\pm$ SD from four rats.

Linoleic acid impaired and oleic acid did not modify twitch and tetanic specific forces and resistance to fatigue in injured gastrocnemius muscle

Laceration itself (Mo Control *versus* Mo Injury) promoted a reduction in specific twitch force by 28% (Fig. 3A) and specific tetanic force by 23% (Fig. 3B). Laceration also decreased the resistance to fatigue during tetanic contraction test during the last three contractions (the eighth, ninth and 10th; by $\sim$ 37%; Fig. 3C). These findings suggest that, even 28 days after the laceration injury, the recovery of contractile function of gastrocnemius muscle was incomplete. Linoleic acid supplementation affected specific twitch force in the contralateral muscle (by 24%; Mo Control *versus* Lin Control; Fig. 3A) and in injured muscle (by 36%; Mo Injury *versus* Lin Injury) compared with mineral oil (Fig. 3A). Linoleic acid also reduced the specific twitch force in the injured muscle compared with the contralateral muscle (by 40%; Lin Control *versus* Lin Injury; Fig. 3A), the specific tetanic force in the contralateral muscle (by 25%; Mo Control *versus* Lin Control; Fig. 3B) and the resistance to fatigue at the eighth, ninth and 10th tetanic contractions, compared with mineral oil (by $\sim$ 29%; Mo Control *versus* Lin Control; *P* value interaction <0.001). Linoleic acid decreased the resistance to fatigue in injured muscle at the eighth, ninth and 10th tetanic contractions compared with mineral oil (by 35%; Mo Injury *versus* Lin Injury; Fig. 3C). Although the statistical analysis did not indicate any interaction between fatty acid supplementation and muscle lesion, a more pronounced effect was observed in injured muscle from animals treated with linoleic acid (Fig. 3A).

Oleic acid supplementation prevented the reduction in specific twitch (Fig. 3A) and tetanic (Fig. 3B) forces observed in injured muscle compared with mineral oil. Oleic acid also attenuated the decrease in the resistance to fatigue at the eighth, ninth and 10th tetanic contractions in the contralateral (by 26%; Mo Control *versus* Ole Control) and injured (by 14%; Mo Injury *versus* Ole Injury) muscles compared with mineral oil (Fig. 3C).

Supplementation with either linoleic or oleic acid did not modify the other contractile properties measured, i.e. the time from the onset of contraction to maximal tension and the relaxation capacity (data not shown). Results are the mean $\pm$ SD of at least five animals.

Fatty acid composition of gastrocnemius muscle after laceration and treatment with either linoleic or oleic acid

Laceration itself (Mo Control *versus* Mo Injury) increased palmitoleic acid by 1.9-fold (1.01 ± 0.68 *versus* 2.02 ± 0.81) and decreased stearic acid by 38% (11.3 ± 2.32 *versus* 7.09 ± 1.47) in gastrocnemius muscle of rats supplemented with mineral oil. In the contralateral/uninjured muscle, linoleic acid supplementation (Mo Control *versus* Lin Control) diminished palmitoleic acid by 88.1% (1.01 ± 0.68 *versus* 0.12 ± 0.07) and linoleic acid by 17.1% (28.6 ± 1.60 *versus* 23.7 ± 2.83), and increased eicosatrienoic acid fourfold (0.60 ± 0.51 *versus* 2.48 ± 2.18) and arachidonic acid by 40% (11.6 ± 2.28 *versus* 16.3 ± 0.95). In injured muscles, linoleic acid supplementation (Mo Injury *versus* Lin Injury) decreased palmitoleic acid by 45% (2.02 ± 0.81 *versus* 1.10 ± 0.21) and increased stearic acid by 58% (7.09 ± 1.47 *versus* 11.3 ± 2.33) compared with mineral oil. Oleic acid supplementation (Mo Injury *versus* Ole Injury) diminished palmitoleic acid by 48% (2.01 ± 0.81 *versus* 1.04 ± 0.65) and increased stearic acid by 64% (7.09 ± 1.47 *versus* 11.6 ± 3.26) and docosahexaenoic acid (DHA, $n-3$) by 2.4-fold (1.89 ± 1.04 *versus* 4.63 ± 2.40) in injured muscle. Interestingly, the oleic acid content was not modified by the supplementation with this fatty acid, suggesting that oleic acid is less efficiently incorporated into skeletal muscle (e.g. as triacylglycerol or membrane phospholipids), being more easily oxidized for energy production.

No interaction between fatty acid supplementation and muscle condition (control or injured) was found. Values are presented as the means $\pm$ SD of four animals, and differences were assessed by two-way ANOVA with Bonferroni *post hoc* test for $P < 0.05$ (Table 1).

Oleic-to-stearic acid and palmitoleic-to-palmitic acid ratios were calculated (Fig. 4A and B, respectively). Laceration itself (Mo Control *versus* Mo Injury) increased the oleic-to-stearic acid ratio by 2.4-fold and the palmitoleic-to-palmitic acid ratio by twofold. Linoleic acid supplementation (Mo Injury *versus* Lin Injury) decreased the oleic-to-stearic acid ratio by 51% and

with linoleic acid (Lin); Lin Injury, injured muscles from animals supplemented with Lin; Ole Control, contralateral muscles from animals supplemented with oleic acid (Ole); and Ole Injury, injured muscles from animals supplemented with Ole. Data are presented as means $\pm$ SD from seven animals per group for analysis of the gastrocnemius muscle mass and from four animals for evaluation of the muscle fibre cross-sectional area.

Figure 2. Effect of either linoleic or oleic acid on the fibrous tissue area, as indicated by collagen deposition, in gastrocnemius muscle 28 days after injury induced by laceration (**A**) and representative images from each group (**B**)

Gastrocnemius muscle sections were stained with Picrosirius Red, and collagen deposition was analysed in a polarized light microscope. Images (scale bars represent 100 μm) were obtained from the groups defined in Fig. 1. Results are presented as the mean $\pm$ SD from four animals per group.

the palmitoleic-to-palmitic acid ratio by 40% in injured muscle. Oleic acid supplementation (Mo Injury *versus* Ole Injury) reduced the oleic-to-stearic acid ratio by 48% and the palmitoleic-to-palmitic acid ratio by 52% in injured muscle. No significant interaction was observed at the two-way ANOVA. Results are presented as the mean $\pm$ SD from four animals per group.

Discussion

This is the first study to evaluate the effects of treatment with either oleic or linoleic acid on recovery of muscle contractile function and fibrous tissue area (collagen deposition) after severe muscle injury induced by laceration. We evaluated muscle mass, muscle fibre CSA, fibrous tissue area, contractile function and fatty

Figure 3. Effect of either linoleic or oleic acid on specific twitch (A) and tetanic forces (B) and resistance to fatigue (C) in gastrocnemius muscle, 28 days after injury induced by laceration

C shows differences among the eighth, ninth and 10th contractions during the resistance to fatigue test. The groups were as described in Fig. 1. Results are expressed as the mean $\pm$ SD of at least five animals and were analysed by two-way ANOVA and Bonferroni *post hoc* test. Mo Control versus Mo Injury, Lin Control or Lin Injury at the 8th (* $p < 0.01$), 9th (+ $p < 0.05$), and 10th (# $p < 0.01$) tetanic contractions.

Table 1. Effect of mineral oil, linoleic or oleic acid supplementation on fatty acid profile of the gastrocnemius muscle 28 days after muscle injury induced by laceration

Fatty acid	Mineral oil		Linoleic acid		Oleic acid	
	Contralateral	Injured	Contralateral	Injured	Contralateral	Injured
Palmitic (16:0)	17.8 ± 3.26	18.9 ± 0.75	18.5 ± 2.97	18.1 ± 0.95	19.1 ± 0.57	20.1 ± 0.71
Palmitoleic (16:1, <i>n</i> –7)	1.01 ± 0.68	2.02 ± 0.81 [†]	0.12 ± 0.07 [†]	1.10 ± 0.21*	0.54 ± 0.26	1.04 ± 0.65*
Stearic (18:0)	11.3 ± 2.32	7.09 ± 1.47 [†]	14.4 ± 0.42	11.3 ± 2.33*	14.0 ± 2.08	11.6 ± 3.26*
Oleic (18:1, <i>n</i> –9)	11.3 ± 3.21	17.8 ± 2.22	8.41 ± 2.09	13.8 ± 4.37	11.8 ± 4.48	14.6 ± 4.11
Vaccenic (18:1, <i>n</i> –7)	2.26 ± 0.40	2.27 ± 0.12	2.62 ± 0.19	2.47 ± 0.14	2.66 ± 0.13	2.54 ± 0.12
Linoleic (18:2, <i>n</i> –6)	28.6 ± 1.60	35.3 ± 2.09	23.7 ± 2.83 [†]	29.3 ± 3.17	26.1 ± 1.83	30.1 ± 6.24
Eicosatrienoic (20:3, <i>n</i> –6)	0.60 ± 0.51	0.49 ± 0.23	2.48 ± 2.18 [†]	1.35 ± 0.72	n.d.	n.d.
Arachidonic (20:4, <i>n</i> –6)	11.6 ± 2.28	8.58 ± 2.29	16.3 ± 0.95 [†]	11.9 ± 2.39	13.8 ± 1.10	10.8 ± 1.73
Docosahexaenoic (22:6, <i>n</i> –3)	4.48 ± 1.63	1.89 ± 1.04	5.39 ± 1.04	3.90 ± 1.16	6.32 ± 1.75	4.63 ± 2.40*

Results are presented as means ± SD from three to four animals per group and compared by two-way ANOVA and Bonferroni *post hoc* test. **P* < 0.05 when compared with the injured muscle of the mineral oil group. [†]*P* < 0.05 when compared with contralateral muscle of the mineral oil group. n.d., not detected.

acid composition in gastrocnemius muscle 28 days after laceration. According to previous studies, this time period (28 days) is not sufficient for complete recovery of muscle function after severe injury (Menetrey *et al.* 1999; Kääriäinen *et al.* 2000; Grounds *et al.* 2005; Ten Broek *et al.* 2010; Murphy *et al.* 2011), which allowed us to evaluate whether the administration of either oleic or linoleic acid could modulate, negatively or positively, muscle regeneration and recovery of contractile function.

Muscle laceration promoted an increase in the area of fibrous tissue, resulting in decreased specific muscle twitch and tetanic forces and resistance to fatigue in injured gastrocnemius muscle. As expected, these findings suggest partial recovery of skeletal muscle contractile function and

an increase in fibrous tissue area (collagen deposition) after 28 days of severe injury, similar to what was reported by others (Wynn, 2008; Gillies & Lieber, 2011; Mann *et al.* 2011; Meyer & Lieber, 2012).

Laceration itself increased the oleic-to-stearic acid and palmitoleic-to-palmitic acid ratios, probably as a result of increased Δ^9 desaturase activity. These increases were attenuated by either oleic or linoleic acid, suggesting that these fatty acids reduce the activity of Δ^9 desaturases. Further studies are required to confirm this assumption. Treatment with linoleic acid increased the content of eicosadienoic acid (20:2, *n*–6) and arachidonic acid (20:4, *n*–6) in gastrocnemius muscle, suggesting an increase in Δ^6 desaturase activity (Jung *et al.* 2010; Shin *et al.* 2012; Ohminami *et al.* 2014; Reinders *et al.* 2015). This

Figure 4. Effect of either linoleic or oleic acid on the oleic-to-stearic acid ratio (A) and the palmitoleic-to-palmitic acid ratio (B) in gastrocnemius muscles 28 days after injury induced by laceration The groups were as described in Fig. 1. Results are presented as the mean + SD from four animals per group and compared by two-way ANOVA and Bonferroni *post hoc* test.

enzyme catalyses the first and rate-limiting step of the metabolism of linoleic acid, converting it into γ -linolenic acid, for the synthesis of arachidonic acid (Horrobin, 1993). Accordingly, dietary supplementation with linoleic acid leads to an increase of arachidonic acid content in the tissues (Soriguer *et al.* 2000). Arachidonic acid is the precursor of pro-inflammatory mediators, including 4-series leukotrienes and 2-series prostaglandins and thromboxanes, resulting in an increased inflammatory response through activation of signalling pathways, inflammatory cell recruitment and production of inflammatory cytokines (Calder, 2006, 2008). Metabolic dysfunctions associated with alterations in the expression of endoplasmic reticulum stress-inducible genes (e.g. binding immunoglobulin protein, C/EBP homologous protein and X-box binding protein 1) are aggravated by high consumption of linoleic acid and, consequently, increased content of arachidonic acid in soleus muscle (Ohminami *et al.* 2014). These authors also reported that dietary linoleic acid itself promotes inflammation through c-Jun NH₂-terminal kinase activation in soleus muscle. Arachidonic acid generates 4-series prostaglandins that, in association with macrophage recruitment and transforming growth factor- β 1 production, control inflammation and so skeletal muscle healing after injury (Leask & Abraham, 2004; Shen *et al.* 2008). Linoleic acid supplementation impaired skeletal muscle regeneration and recovery of muscle contractile function probably because of increased arachidonic acid content and synthesis of pro-inflammatory mediators, persistence of the inflammatory state and enhanced profibrotic factor production and fibrotic tissue deposition.

Treatment with linoleic acid reduced muscle mass and the CSA of muscle fibres and increased fibrous tissue area in injured muscle and, as a consequence, decreased muscle contractile function as indicated by lowered specific muscle twitch force and muscle resistance to fatigue. The effects of linoleic acid on muscle mass and CSA of muscle fibres were also observed in the contralateral, uninjured muscle and led to impaired muscle contractile function (as indicated by the results of specific forces and muscle resistance to fatigue). The oleic acid treatment reduced collagen deposition and attenuated the decrease in specific muscle twitch and tetanic forces and resistance to fatigue induced by laceration in gastrocnemius muscle. Contrary to linoleic acid, oleic acid did not induce significant alterations in linoleic acid and arachidonic acid contents, probably resulting in a reduced inflammatory response. Moreover, this fatty acid increased docosahexaenoic acid concentrations in injured gastrocnemius muscle. This $n-3$ fatty acid has been associated with the synthesis of several molecules with anti-inflammatory properties, including resolvins, protectins and maresins (Serhan *et al.* 2002, 2009; Bannenberg *et al.* 2005), which might have contributed to the beneficial effects of oleic acid in

attenuating the tissue damage and improving muscle contractile function recovery.

Similar to our study, increased availability of fatty acids resulting from the consumption of a high-fat diet leads to decreased myofibre size and elevated collagen deposition in cardiotoxin-induced injured anterior tibialis muscle (Hu *et al.* 2010). These authors did not investigate whether a specific fatty acid could promote the effects on regenerating skeletal muscle. Reinders *et al.* (2015) reported that a reduction in muscle size and strength (knee-extension strength and grip strength) is associated with an increase in arachidonic acid content in skeletal muscle. Rodemann & Goldberg (1982) described a slight increase in inflammation and marked elevation of protein degradation in rat soleus and extensor digitorum longus muscles incubated with arachidonic acid. Therefore, an increase in arachidonic acid content attributable to linoleic acid treatment might play a role in the reduced muscle mass and muscle fibre CSA and, consequently, impairment of muscle contractile function observed in the present study.

An inverse relationship between concentrations of oleic acid and linoleic acid in skeletal muscles has been reported in experimental animal and human studies (Høstmark & Haug, 2013, 2014). Supplementation with oleic acid had no effect on $n-6$ polyunsaturated fatty acids but increased DHA in gastrocnemius muscle. An increased oleic acid level has been associated with elevation of $n-3$ polyunsaturated fatty acids [eicosapentaenoic acid (EPA) and DHA], reduced levels of oxidative stress biomarkers and improved muscle regeneration (Sanjurjo *et al.* 2006; Finlin *et al.* 2012; Liu *et al.* 2013). Increased intake of either $n-3$ polyunsaturated fatty acids or oleic acid has been associated with positive effects on muscle regeneration and recovery of muscle function. Peng *et al.* (2011) and Salvadó *et al.* (2013) described how oleic acid abolishes palmitic acid-induced metabolic dysfunction in skeletal muscle. The increase of DHA content in gastrocnemius muscle, after supplementation with fish oil for 21 days, attenuated lipopolysaccharide-induced muscle atrophy (Liu *et al.* 2013). Decreased DHA content impairs calcium homeostasis (Ye *et al.* 2010) and, thus, it can contribute to the impairment of contractile function in skeletal muscle observed in the present study (Vandebrouck *et al.* 2002). Tuazon & Henderson (2012) showed that high linoleic acid and low DHA in muscle phospholipids are positively correlated with serum creatine kinase activity and reduced grip strength in animals lacking dystrophin.

Evidence is presented in the present study that treatment with linoleic acid impairs skeletal muscle regeneration, as indicated by reduced muscle mass and muscle fibre CSA and by increased fibrous tissue area formation (collagen deposition), resulting in impaired recovery of contractile function. Oleic acid optimized muscle regenerative capacity by reducing the fibrous tissue area,

resulting in improved recovery of contractile function. The increase in Δ^9 desaturase activity induced by laceration itself and the positive effects of oleic acid administration indicate that monounsaturated fatty acids might play an important role in skeletal muscle regeneration.

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Additional information

Competing interests

None declared.

Author contributions

P.A., C.H.J.P., S.M.H. and R.C. participated in the conception and design of the study. P.A., K.F.V., D.A.A.V., R.P.T., M.S.F.

and G.N.M.-N. performed the methodological procedures used in the work and helped with the draft of the manuscript. P.A., K.F.V., C.H.J.P., J.M.-F., S.M.H. and R.C. conducted analysis and interpretation of results. P.A., C.H.J.P., J.M.-F., S.M.H. and R.C. critically revised the work. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Research

J A B PEDROSO and others

SOCS3 effects on resistance exercise

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SOCS3 expression in SF1 cells regulates adrenal differentiation and exercise performance

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Abstract

Many hormones/cytokines are secreted in response to exercise and cytokine signaling may play a pivotal role in the training adaptations. To investigate the importance of cytokine signaling during vertical ladder climbing, a resistance exercise model, we produced mice lacking SOCS3 protein exclusively in steroidogenic factor-1 (SF1) cells (SF1 Socs3 KO mice). SF1 expression is found in steroidogenic cells of the adrenal cortex and gonads, as well as in neurons of the ventromedial nucleus of the hypothalamus. Histological markers of the fetal adrenal zone (or X-zone in rodents) were still present in adult males and postpartum SF1 Socs3 KO females, suggesting a previously unrecognized effect of SOCS3 on the terminal differentiation of the adrenal gland. This change led to a distinct distribution of lipid droplets along the adrenal cortex. Under basal conditions, adult SF1 Socs3 KO mice exhibited similar adrenal weight, and plasma ACTH and corticosterone concentrations. Nonetheless, SF1 Socs3 KO mice exhibited a blunted ACTH-induced corticosterone secretion. The overall metabolic responses induced by resistance training remained unaffected in SF1 Socs3 KO mice, including changes in body adiposity, glucose tolerance and energy expenditure. However, training performance and glucose control during intense resistance exercise were impaired in SF1 Socs3 KO mice. Furthermore, a reduced counter-regulatory response to 2-deoxy-D-glucose was observed in mutant mice. These findings revealed a novel participation of SOCS3 regulating several endocrine and metabolic aspects. Therefore, cytokine signaling in SF1 cells exerts an important role to sustain training performance possibly by promoting the necessary metabolic adjustments during exercise.

Key Words

- ▶ adrenal cortex
- ▶ counter-regulatory response
- ▶ cytokine signaling
- ▶ steroidogenic factor-1
- ▶ ventromedial nucleus.

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Introduction

Cytokines normally regulate cellular behavior via the activation of plasma membrane receptors (Krebs & Hilton 2001). Many cytokine receptors recruit the Janus kinase/signal transducer and activator of transcription

(JAK/STAT) pathway, which controls the transcription of numerous genes and thereby regulates cell proliferation, differentiation and apoptosis (Krebs & Hilton 2001). Cytokine signaling is modulated by proteins from the

family of suppressors of cytokine signaling (SOCS). SOCS proteins comprise 8 proteins that can bind tyrosine-phosphorylated residues of cytokine receptors or JAK, blocking their capacity to activate intracellular signaling pathways (e.g., activation of STAT proteins) or leading them to proteosomal degradation (Krebs & Hilton 2001, Howard & Flier 2006). Interestingly, cytokine signaling can robustly increase the SOCS expression, indicating that SOCS proteins regulate cytokine signaling via a negative feedback loop (Krebs & Hilton 2001, Howard & Flier 2006).

Among the different SOCS proteins, SOCS3 has received considerable attention because several studies described its effects controlling important metabolic aspects. Obese animals exhibit increased SOCS3 expression in different tissues (Bjorbaek *et al.* 1998, Jorgensen *et al.* 2013). Notably, brain-specific or muscle-specific SOCS3 ablation improves insulin sensitivity and may partially prevent diet-induced obesity (Mori *et al.* 2004, Jorgensen *et al.* 2013, Pedroso *et al.* 2014). These effects occur because SOCS3 inhibits several cytokines/hormones that regulate the metabolism, such as leptin, insulin, growth hormone, prolactin, interleukin-6 and insulin-like growth factor-1 signaling (Krebs & Hilton 2001, Howard & Flier 2006).

Physical exercise produces robust metabolic consequences (Laing *et al.* 2016). Importantly, the levels of many hormones/cytokines are markedly altered by acute or chronic exercise, including those inhibited by SOCS3 (Strasser *et al.* 2012, Golbidi & Laher 2014). Exercise increases SOCS3 expression in different tissues (Spangenburg *et al.* 2006, Trenerry *et al.* 2007, Pereira *et al.* 2015), suggesting that SOCS3 may have a profound impact on cytokine signaling during exercise, which highlights a possible participation of this protein in the regulation of training adaptations. In the present study, we hypothesize that exercise-induced cytokine signaling plays a pivotal role in the training adaptations, which may depend on the activation of signaling pathways either in specific brain areas or peripheral tissues. For example, earlier studies observed that physical exercise activates cytokine signaling pathways in the hypothalamus (Pereira *et al.* 2015) or skeletal muscle (Spangenburg *et al.* 2006, Trenerry *et al.* 2007, Begue *et al.* 2013).

Since whole-body SOCS3-knockout mice exhibit embryonic lethality (Roberts *et al.* 2001), we employed the Cre-loxP system to produce tissue-specific SOCS3 ablation, as previously described (Mori *et al.* 2004, Jorgensen *et al.* 2013, Pedroso *et al.* 2014, Zampieri *et al.* 2015, Bohlen *et al.* 2016). To investigate the potential role of SOCS3

in exercise-induced metabolic adaptations, we generated mice lacking SOCS3 only in cells that express the nuclear receptor steroidogenic factor-1 (SF1). In the central nervous system, SF1-positive cells are specifically found in the ventromedial nucleus of the hypothalamus (VMH) (Ikeda *et al.* 1995, Segal *et al.* 2005, Kim *et al.* 2011), which comprises an important neuronal population involved in the regulation of the energy balance, glucose homeostasis and autonomic nervous system (Brobeck 1946, Powley 1977, Ikeda *et al.* 1995, Majdic *et al.* 2002, Seoane-Collazo *et al.* 2015). Additionally, VMH neurons express receptors for several hormones whose levels change in response to physical exercise, including insulin (Klockener *et al.* 2011), leptin (Nagaishi *et al.* 2014), growth hormone (Furigo *et al.* 2017), prolactin (Furigo *et al.* 2014, 2017) and interleukin-6 (Schobitz *et al.* 1993). Therefore, cytokine signaling in the SF1/VMH cells may promote metabolic adjustments during or after exercise. In fact, a recent study showed that a hypothalamic SF1 expression is required for the beneficial metabolic effects of exercise (Fujikawa *et al.* 2016). Pituitary gonadotropic cells as well as steroidogenic cells of the adrenal cortex and gonads also express SF1, which controls the transcription of key enzymes that synthesize steroid hormones (Ikeda *et al.* 1995, Zhao *et al.* 2001, Parker *et al.* 2002). Since exercise performance is also affected by glucocorticoids and sex hormone levels, the SOCS3 expression in these endocrine cells possibly modulates training adaptations as well. Thus, the objective of the present study was to characterize the endocrine and metabolic consequences of SOCS3 ablation in SF1 cells. Subsequently, these conditional knockout mice were subjected to a resistance exercise protocol in order to investigate whether the SF1-specific SOCS3 expression regulates training performance and the metabolic effects of exercise.

Material and methods

Animals and genotyping

Mice expressing the Cre recombinase under SF1 promoters (Tg(Nr5a1-cre)7Lowl/J, The Jackson Laboratory, Bar Harbor, ME, USA) were bred with animals carrying a loxP-flanked *Socs3* allele (B6.129S4-Socs3^{tm1Ayo}/J, The Jackson Laboratory). Mice carrying an SF1-specific SOCS3 inactivation (SF1 *Socs3* KO group) were homozygous for the loxP-flanked *Socs3* allele and carried the Cre transgene, whereas the control group was composed of littermate mice carrying only the loxP-flanked *Socs3* allele

in homozygosity. SF1-Cre animals were also bred with the Cre-inducible tdTomato-reporter mouse (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J, The Jackson Laboratory). The purpose of this breeding was to allow the visualization of SF1-positive cells through the red fluorescent tdTomato protein. Mice were weaned at 3–4 weeks of age and genotyped through PCR using DNA extracted from the tail tip (REDExtract-N-Amp Tissue PCR Kit, Sigma-Aldrich). Mice were bred and maintained under standard conditions of light (12-h light/darkness cycle; lights on at 8:00h) and temperature ($22 \pm 2^\circ\text{C}$). In all experiments, animals received a regular rodent chow diet (2.99 kcal/g; 9.4% calories from fat; Quimtia, Colombo, Brazil). The animal procedures were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo (protocol number 71/2016).

Characterization of SF1 Socs3 KO mice

To identify Cre-mediated DNA recombination, the hypothalamus, cerebellum, adrenal gland and liver of SF1 Socs3 KO mice were collected. After the same DNA extraction protocol employed for genotyping, we performed a PCR reaction to identify a 250-bp DNA fragment that would indicate Cre-mediated SOCS3 ablation (Mori *et al.* 2004). Control primers were also used to confirm that DNA extraction and PCR reaction did not fail in negative controls (cerebellum and liver). To analyze the effects of SF1-specific SOCS3 ablation in the hypothalamus, SF1-Cre/tdTomato-reporter or SF1 Socs3 KO mice ($n=3$) were transcardially perfused with formalin and their brains were prepared for histological analysis, as previously described (Furigo *et al.* 2014, Nagaishi *et al.* 2014). The distribution of tdTomato-expressing cells in the brain was assessed. Additionally, the VMH cytoarchitecture was analyzed through DAPI staining using Dapi-Fluoromount-G mounting medium (EMS, Hatfield, PA, USA), standard thionin staining and after a peroxidase reaction to label estrogen receptor- α immunoreactivity (ER α -ir) in the VMH of control and SF1 SOCS3 KO mice, as previously described (da Silva *et al.* 2014). To analyze the consequences of SF1-specific SOCS3 ablation in the gonadal function, the testicles were weighed and plasma samples were collected to assess testosterone levels by enzyme-linked immunosorbent assay (Calbiotech; TE187S-100; Spring Valley, CA, USA). Additionally, fertility was evaluated in males and females by measuring the time required for control and SF1 Socs3

KO mice to produce litters. The potential effects in the adrenal gland were evaluated by measuring adrenal weight, and basal plasma corticosterone (DetectX Enzyme Immunoassay Kit, Arbor Assays, Ann Arbor, MI, USA) and adrenocorticotrophic hormone levels (ACTH; Milliplex MAP Mouse Bone Magnetic Bead Panel, Millipore Corporation). An ACTH stimulation test was also performed in 3-month-old male mice as previously described (Scheys *et al.* 2011). Briefly, mice received i.p. injections of 5 mg/kg dexamethasone at the lights off 1 day before and at 10:00h on the test day to suppress the hypothalamic–pituitary–adrenal (HPA) axis. Two hours later, a blood sample was collected (time 0), followed by an i.p. injection of 1 mg/kg ACTH (Bachem, Torrance, CA, USA). Mice were killed after 60 min to assess serum corticosterone levels. The histological analysis of the adrenal gland was evaluated in another group of adult males (5 months) and in virgin or postpartum adult females. In the latter case, females were allowed to nurse the pups for 5 days. Then, the pups were removed and the adrenals were collected after approximately 5 days. The counter-regulatory response to 2-deoxy-D-glucose (2DG) was evaluated by assessing changes in glycemia after an i.p. infusion of 0.5 mg/kg 2DG (Sigma) for 3 h (Flak *et al.* 2014). Serum ACTH and corticosterone levels were also determined 60 min after an i.p. injection of 0.5 mg/kg 2DG.

Experimental design

Experimental design is summarized in Fig. 1. Four-month-old male mice were distributed in 4 groups according to genotype and training condition: sedentary control ($n=20$), exercise control ($n=20$), sedentary SF1 Socs3 KO ($n=16$) and exercise SF1 Socs3 KO ($n=16$). The exercise groups were subjected to an 8-week resistance exercise protocol, as described in detail later, whereas sedentary groups were exposed to the same experimental conditions, except for the training protocol. Exercise training was performed in the light period from 14:00h to 16:00h (lights off at 20:00h) and researchers were blinded to the experimental groups. Along the experiment, mice were weighted weekly. Performance was measured through the increments of the weight lifted during the 8-week training period and by one repetition maximum (1RM) tests performed at the beginning of training protocol and after 4 and 8 weeks of training. Glycemia was assessed just before the beginning of the last 1RM test and immediately after exhaustion using a portable glucometer (OneTouch, Johnson & Johnson).

Figure 1

Scheme that summarizes the experimental design. 1RM, one repetition maximum; CLAMS, Comprehensive Lab Animal Monitoring System; GTT, glucose tolerance test; ITT, insulin tolerance test.

After 4 and 5 weeks of training, mice were subjected to a glucose tolerance test (GTT; 2 g glucose/kg; i.p.) and an insulin tolerance test (0.8 IU insulin/kg; i.p.), respectively. During the last 2 weeks of training, mice were placed in the OxyMax/Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) to analyze O_2 consumption (VO_2), CO_2 production (VCO_2), respiratory exchange ratio (RER; VCO_2/VO_2), food intake, water intake and locomotor activity (through infrared beam sensors). After 3 days of adaptation inside the CLAMS, the metabolic parameters of each mouse were evaluated for 4 consecutive days. Therefore, the results presented here were the average of this period. Both sedentary and exercise groups were evaluated in the CLAMS, and the exercise groups were maintained in their regular training protocol. The data produced by the CLAMS were presented as pre-training (the light cycle before the training session from 8:00h to 14:00h), post-training (the light cycle immediately after the training session from 16:00h to 20:00h) and dark cycle (from 20:00h to 8:00h). In the following day after the last training session, food was removed from cages for 4h and mice were killed by decapitation (approximately from 14:00h to 16:00h). Body adiposity was calculated by summing the weight of the perigonadal, subcutaneous and retroperitoneal fat pads. The soleus muscle was removed in order to assess cross-sectional area (CSA) of individual fibers after a standard hematoxylin and eosin (HE) staining. Quantification and evaluation of fiber CSA (150 fibers per muscle) were performed as previously described (Fortes *et al.* 2015). After removing the soleus muscle, sections from the midportion region were obtained. A cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany) was used to acquire a 10 μ m thick section from each muscle.

Sections were imaged using an optical microscope with camera (Nikon DXM 1200; Nikon Instruments). Digitized images were analyzed using the AxioVision software (version 4.8.1.0, Carl Zeiss).

Resistance exercise protocol

A 110 cm high and 18 cm wide ladder angled at 80° and with a 2 cm spacing between the steps (Hornberger & Farrar 2004) was used for the training sessions. A 20 × 20 × 20 cm chamber is located at the top of the ladder and serves as a shelter during the rest periods between the series of climbs. Initially, the animals from the exercise groups were familiarized to the apparatus for 3 days, which consisted of three climbing attempts. Before the first attempt, mice were kept in the shelter for 90 s to feel safe. On the first attempt, the mouse was placed at the ladder top portion at an approximate distance of 35 cm from the door of the shelter. On the second attempt, the animal was positioned at the middle of the ladder at a distance of approximately 55 cm from the door of the shelter. On the third attempt, the animal was placed at the bottom of the ladder at a distance of approximately 110 cm from the door of the shelter. Afterward, animals were adapted to the exercise protocol. The resistance training started after the adaptation period, which consisted of 4 series of 8 climb repetitions during 1 week without any load. The protocol was adapted from Kim and coworkers and consisted of climbing a vertical ladder with progressive loads attached to the mouse tail base (Kim *et al.* 2015). To determine the initial training workload, all mice were subjected to a 1RM test, which consisted of climbing repetitions carrying a progressively heavier load. In the initial climbing, mice had to carry 75% of the animal's own body mass load. When mice reached the top of the ladder, a rest of 2 min was given before the next attempt. During subsequent attempts, an additional 5 g load was added until the animal was unable to climb the ladder completely. Thus, the load carried in the last successful climbing was considered as the 1RM load. The training protocol consisted of 4 series of climbing repetitions with progressive overload. Each series contained an average of 8 repetitions. In the first series, animals had to carry 75% of 1RM load. An additional 5–10% 1RM load was added in the following series. The load was kept until the animal could climb the whole ladder. If the animal could perform all series, the load of the last series would be the initial load on the following day. Resistance ladder climbing exercise was performed 5 days per week for 8 weeks.

Adrenal and pituitary analysis

For the HE staining, left adrenal glands from 5-month-old males and 3-month-old virgin or postpartum females of control and SF1 Socs3 KO groups (5 per group) were removed, fixed in formalin and embedded in paraffin. Serial histological sections (10 µm thickness) were used for a standard HE staining procedure. The cell morphology and the cortical areas images were captured under a light microscope (Nikon Eclipse 80i) and analyzed by Neurolucida and Neurolucida Explorer software (MBF Bioscience). For the quantification of the adrenal X-zone, images were captured with 10× magnification. The X-zone area was manually delimited based on the strong eosin staining. The area occupied by eosinophilic cells in the corticomedullary junction was determined using ImageJ software (ImageJ 1.51n, Wayne Rasband, National Institute of Health, Bethesda, MA, USA). For the oil red O (ORO) staining and immunofluorescence (IF) against anti-cytochrome P450 family 17 subfamily A member1 (Cyp17a1), right adrenal glands were collected and the excess fat was removed. The tissue was rapidly frozen in dry ice and embedded in optimal cutting media (OCT, Sakura) for cryosection. For the ORO staining and quantification, we performed a standard protocol, as previously described (Mehlem *et al.* 2013). The relative quantification of ORO staining was performed in 15 different images of the adrenal cortex per animal and analyzed using the ImageJ software, following the protocol previously described (Mehlem *et al.* 2013). For the IF, sections (3 animals from each experimental group) were fixed in ice-cold acetone (10 min) and washed in PBS. Blocking solution (10% donkey serum) was applied in each section and incubated for 1 h. After washing, primary anti-Cyp17a1 (ab134910; Abcam) at 1:250 dilution (PBS+0.3% Triton X+1% donkey serum) was applied and sections were incubated overnight at 4°C. Sections were washed and incubated with 1:1000 secondary donkey anti-rabbit IgG Alexa Fluor 594 (Jackson ImmunoResearch Laboratories). Sections were mounted on antifade reagent (ProLong Gold, Life Technologies) and analyzed under a fluorescent microscope (Nikon Eclipse 80i). Fresh pituitary glands from adult male mice were collected to analyze the mRNA levels of pituitary hormones. The protocol to extract the total RNA, produce the complementary DNA and perform the RT-PCR has been described in previous studies from our group (Pedroso *et al.* 2014, 2016, Zampieri *et al.* 2015, Bohlen *et al.* 2016). The expression of the constitutive gene cyclophilin A was used to normalize the data. The following primers were used: cyclophilin A (forward: tatctgactgccaagactgag;

reverse: cttcttgctggtcttgcattcc), GH (forward: gcttctcagagaccatcccg; reverse: agcgagaagcgaagcaattc), POMC (forward: tagatgtgtggagctggtgc; reverse: ccagcgagaggtcgagtttg), LHβ (forward: agttctgccagtctgcatc; reverse: actcggaccatgctaggaca) and FSHβ (forward: aataccacttggtgtgcggg; reverse: gccaggcaatcttacgtct).

Statistical analysis

When data from control and SF1 Socs3 KO groups were compared, independently of training protocol, the unpaired two-tailed Student's *t*-test was used. To analyze the effects of exercise training and/or SF1-specific SOCS3 ablation, a two-way ANOVA test followed by Bonferroni posttests was employed. GraphPad Prism software was used for the statistical analyses and the results were expressed as mean ± S.E.M. Only *P* values <0.05 were considered to be statistically significant.

Results

Characterization of SF1 Socs3 KO mice

It is well established that the hypothalamus and adrenal cortex contain SF1-expressing cells, whereas no SF1 expression is normally found in cerebellum and liver (Ikeda *et al.* 1995, Kim *et al.* 2011). Using primers that amplify a 250-bp DNA fragment, which is only expressed after Cre-mediated recombination in Socs3 gene (Mori *et al.* 2004), we confirmed that SF1 Socs3 KO mice had SOCS3 ablation in the hypothalamus and adrenal gland, but not in the cerebellum or liver (Fig. 2A). To identify the distribution of SF1-expressing cells in the brain, we used a Cre-inducible tdTomato-reporter mouse. So, SF1-positive cells could be visualized by the red fluorescent protein tdTomato. In accordance with previous studies that mapped the distribution of SF1 expression in the brain (Ikeda *et al.* 1995, Segal *et al.* 2005, Kim *et al.* 2011), the tdTomato expression was abundantly found in the VMH and only few scattered cells were present in other brain nuclei (Fig. 2B). Therefore, central SOCS3 ablation essentially affected VMH neurons. SF1 is critically involved in the VMH formation (Ikeda *et al.* 1995, Majdic *et al.* 2002) and ablation of SF1 in the central nervous system drastically reduces the expression of steroid receptors in the VMH (Kim *et al.* 2010). We assessed VMH cytoarchitecture and integrality through thionin staining (Fig. 2C and D) and by the presence of ERα in the ventrolateral part of the VMH (VMHv; Fig. 2E and F), but no changes were observed in SF1 Socs3 KO mice.

Figure 2

Characterization of SF1 Socs3 KO mice. (A) Agarose gel showing the products of a PCR reaction from different tissues of SF1 Socs3 KO mice. A 250-bp DNA fragment indicates Cre-mediated recombination in the Socs3 gene, whereas a ~800-bp fragment is a positive control band. (B) Epifluorescence photomicrograph illustrating the distribution of SF1 neurons in the ventromedial nucleus of the hypothalamus (VMH), using a reporter mouse that expresses the red fluorescent tdTomato protein under Cre recombination. DAPI staining was used as an anatomical reference. (C and D) Thionin staining illustrating a normal VMH cytoarchitecture in SF1 SOCS3 KO mice. (E and F) Peroxidase reaction to label estrogen receptor- α immunoreactivity (ER α -ir) in the VMH of control and SF1 SOCS3 KO mice. 3V, third ventricle; ARH, arcuate nucleus of the hypothalamus; VMHc, central part of the VMH; VMHdm, dorsomedial part of the VMH; VMHvl, ventrolateral part of the VMH. Scale bar = 200 μ m.

SOCS3 expression in SF1 cells is required for the terminal differentiation of the adrenal gland and maximal adrenal steroidogenic capacity

Previous studies have indicated that prolactin signaling induces the SOCS3 expression in the adrenal cortex of rats (Tam *et al.* 2001), and maternal dietary restriction can induce adrenocortical hypertrophy by upregulating cytokine signaling (Zhang *et al.* 2013). Whether SOCS3 plays a role in the adrenal cortex physiology remains unclear. Thus, here we investigated morphological and functional aspects of the adrenal cortex of SF1 Socs3 KO mice. HE staining revealed a different pattern of the cortex morphology in the medullary boundary (Fig. 3), with some clusters of eosinophilic cells matching the description and localization of the fetal adrenal zone or X-zone in rodents (Jones 1952). Indeed, these cells were positive for the steroidogenic enzyme Cyp17A1 (Fig. 4A and B), which is a marker for X-zone in rodents (Keeney *et al.* 1995).

ORO staining showed that the presence of this zone disturbed the morphology of the adjacent zones, especially the distribution of lipid droplets along the cortex (Fig. 4C, D, E and F). Due to this change, the total amount of lipids in adrenal cortex was significantly decreased ($P=0.0011$) in the SF1 Socs3 KO animals compared to control mice (Fig. 5A). We then evaluated possible functional consequences in the adrenal of SF1 Socs3 KO mice. Under basal conditions, control and SF1 Socs3 KO mice exhibited similar adrenal weight (Fig. 5B) as well as plasma ACTH (Fig. 5C) and corticosterone (Fig. 5D) levels. However, when the adrenal capacity to secrete corticosterone was evaluated during an ACTH stimulation test, SF1 Socs3 KO mice exhibited a blunted response in comparison with control animals (Fig. 5E). These results indicate a lower

Figure 3

SF1-specific SOCS3 ablation induces histological alterations in the adrenal cortex. (A, B, C, D, E, F, G and H) Photomicrographs of the adrenal gland of control (A, C, E and G) and SF1 Socs3 KO (B, D, F and H) mice after hematoxylin/eosin staining. The arrows indicate the localization of the fetal adrenal zone (or X-zone in rodents) in the medullary boundary, which is visible only in SF1 Socs3 KO mice. m, adrenal medulla; x, X-zone; zf, zona fasciculata; zg, zona glomerulosa. Scale bars=0.05 mm.

maximal adrenal steroidogenic capacity in SF1 Socs3 KO mice, even though their adrenal function seems to be normal during basal conditions.

SF1-specific SOCS3 ablation leads to a lower testis weight without affecting testosterone levels, fertility or pituitary function

SF1 expression is also found in the gonads (Ikeda *et al.* 1995, Kim *et al.* 2011), indicating that SOCS3 ablation may have occurred in the testis of SF1 Socs3 KO mice.

In fact, SOCS3 can modulate leptin and interleukin-6 signaling and affect testicular functions (Yuan *et al.* 2014, Huang *et al.* 2016). To assess the consequences of SF1-specific SOCS3 ablation in testicular functions, we initially measured testis weight. SF1 Socs3 KO mice exhibited significantly lighter testes compared to control animals (Fig. 6A). However, no changes in plasma testosterone levels were observed between groups (Fig. 6B). In addition, fertility was not impaired in male SF1 Socs3 KO mice (Fig. 6C), indicating that despite the lower testis weight, SF1 Socs3 KO mice

Figure 4

Presence of X-zone in adrenal glands of SF1 Socs3 KO mice. (A and B) Photomicrographs of the adrenal gland of control (A) and SF1 Socs3 KO (B) mice after immunofluorescence against Cyp17a1 ($n=3$ /group). Insets show a high-powered field of the adrenal cortex in the medullary boundary. (C, D, E and F) Photomicrographs of the adrenal gland of control (C and D) and SF1 Socs3 KO (E and F) mice after oil red O staining. m, adrenal medulla; x, X-zone; zf, zona fasciculata; zg, zona glomerulosa. Scale bars: (A, B and C) E=0.1 mm; D and F=0.05 mm; insets=0.01 mm.

showed no apparent reproductive dysfunction. Pituitary-specific SF1 inactivation causes infertility due to a robust reduction in LH and FSH expressions, but does not affect other pituitary cell types, such as somatotropes, corticotropes and thyrotropes

(Zhao *et al.* 2001). To investigate whether SF1-specific SOCS3 ablation affects the pituitary gland, we analyzed the mRNA levels of several pituitary hormones. SF1 Socs3 KO male mice showed similar mRNA levels of GH, POMC, LH β and FSH β in the pituitary gland, compared

Figure 5

Effects of SF1-specific SOCS3 ablation in the adrenal gland function. (A) Quantification of oil red O (ORO) staining, which represents the total amount of lipids in adrenal cortex ($n=18$ /group). (B, C and D) Basal adrenal weight (B) and plasma ACTH (C) and corticosterone (D) levels in control ($n=3-5$) and SF1 Socs3 KO ($n=3-6$) mice. (E) Serum corticosterone levels during an ACTH stimulation test ($n=7$ /group). *significant ($P<0.05$) different than control group (unpaired two-tailed Student's *t*-test).

Figure 6

Effect of SF1-specific SOCS3 ablation in gonadal and pituitary functions. (A) Testis weight in control ($n=8$) and SF1 Socs3 KO ($n=9$) mice. (B) Plasma testosterone levels in control ($n=5$) and SF1 Socs3 KO ($n=4$) mice. (C) Fertility test evaluated by measuring the number of days required for control ($n=20$) and SF1 Socs3 KO ($n=30$) males to produce litters when breeding with female mice. (D) Gene expression analysis in the pituitary gland of control ($n=4$) and SF1 Socs3 KO ($n=4$) mice. (E) Quantification of the area occupied by eosinophilic cells in the corticomedullary junction, representing the X-zone. Unpaired two-tailed Student's t -test was used in the statistical analyses. *, significant ($P<0.05$) different than virgin control and postpartum SF1 Socs3 KO groups.

to control animals (Fig. 6D). Therefore, SF1-specific SOCS3 inactivation does not induce apparent defects in the pituitary function.

SOCS3 expression in SF1 cells is required for X-zone regression in postpartum adult females

In female mice, the X-zone atrophy disappears after the first pregnancy (Tanaka & Matsuzawa 1995). As expected,

a histological analysis in the adrenal gland of virgin control females confirmed the presence of the X-zone in the medullary boundary (Figs 6E and 7A, D and G), which disappeared in postpartum control females (Figs 6E and 7B, E and H). Remarkably, SF1 Socs3 KO postpartum females retained the X-zone in the adrenal cortex (Figs 6E and 7C, F and I), indicating that the SOCS3 expression in SF1 cells is required for X-zone regression in postpartum adult females. The fertility of female mice was also

Figure 7

Presence of X-zone in adrenal gland of SF1 Socs3 KO postpartum female mice. (A, D and G) Photomicrographs of the adrenal gland of a virgin control female. (B, E and H) Photomicrographs of the adrenal gland of a postpartum control female. (C, F and I) Photomicrographs of the adrenal gland of postpartum SF1 Socs3 KO female. Black arrows indicate the X-zone. m, adrenal medulla. Scale bar = 0.02 mm.

analyzed. However, the number of days breeding until giving birth (control: 27.8 ± 2.5 days; SF1 Socs3 KO: 27.3 ± 1.7 days) and the number of pups per litter (control: 5.9 ± 0.4 ; SF1 Socs3 KO: 6.7 ± 0.6) were similar between the groups. Thus, SF1-specific SOCS3 ablation did not affect the fertility.

Resistance exercise reduces body adiposity and improves glucose tolerance

We assessed the metabolic consequences of 8-week resistance training. Compared to sedentary animals, exercise training reduced the body weight (Fig. 8A), weight gain (Fig. 8B) and body adiposity (Fig. 8C). Furthermore, glucose tolerance was improved by exercise training (Fig. 8D). These effects were equally observed in control and SF1 Socs3 KO mice (Fig. 8A, B, C and D). SOCS3 deletion or resistance exercise had no significant effect on insulin sensitivity (Fig. 8E). Therefore, SF1-specific SOCS3 deletion did not change the beneficial effects of exercise on energy balance and glucose homeostasis.

Metabolic changes induced by exercise training or SF1-specific SOCS3 ablation

Additional metabolic parameters were obtained in the last 2 weeks of training when mice were evaluated by the OxyMax/CLAMS (Fig. 9). During the post-training period (4h after the exercise session), exercise groups exhibited increased VO_2 (Fig. 9B), as well as reduced RER (Fig. 9E) and locomotor activity (Fig. 9H) compared to sedentary animals, independently of the genotype. Exercise also increased water intake during the dark cycle (Fig. 9O). We observed a significant effect of SF1-specific SOCS3 ablation by reducing the RER in the dark cycle (Fig. 9F),

the food and water intake during the pre-training period (Fig. 9J and M) and the water intake in the post-training period (Fig. 9N).

Impaired training performance, glucose control during intense exercise and counter-regulatory response to 2DG in SF1 Socs3 KO mice

Training performance was initially assessed through the weight lifted by the animals during the training sessions. During the first weeks of training, SF1 Socs3 KO and control mice carried similar loads during the climbing series (Fig. 10A). However, while control animals progressively increased the weight lifted, SF1 Socs3 KO mice were not able to lift similar loads from the fifth week of training, compared to control animals (Fig. 10A). The 1RM tests also confirmed the impaired exercise performance of SF1 Socs3 KO mice along the training protocol (Fig. 10B). Regarding the 1RM tests performed in the first and fourth weeks of training, SF1 Socs3 KO mice exhibited a similar performance compared to control animals (Fig. 10B). However, at the eighth week of training, SF1 Socs3 KO mice did not present improvements in their performance to the same extent as control animals (Fig. 10B). The analysis of the CSA of individual fibers of the soleus muscle revealed an interaction effect between the training status and the genotype ($P=0.0494$). While exercise training increased in 22% soleus CSA of individual fibers in control animals, the exercise SF1 Socs3 KO group exhibited no increment compared to sedentary mutants (data not shown).

An adequate blood glucose level is necessary to maintain performance during exercise (Coyle 1999). Since both the adrenal cortex and the VMH are related to the control of blood glucose (Verberne *et al.* 2014, Meek *et al.* 2016), we investigated whether SOCS3 ablation in SF1 cells affects the glycemia regulation during exercise. Thus,

Figure 8

Resistance exercise reduces body adiposity and improves glucose tolerance. (A, B, and C) Body weight (A), weight change (B) and body adiposity (C) at the end of experiment in sedentary control ($n=20$), exercise control ($n=20$), sedentary SF1 Socs3 KO ($n=16$) and exercise SF1 Socs3 KO ($n=16$). (D and E) Glucose tolerance test (D) and insulin tolerance test (E) after 4 and 5 weeks of training, respectively ($n=13$ /group). *, significant ($P < 0.05$) effect of exercise (two-way ANOVA test).

Figure 9

Metabolic changes induced by exercise training or SF1-specific SOCS3 ablation. (A, B, C, D, E, F, G, H, I, J, K, L, M, N and O) VO_2 (A, B and C) respiratory exchange ratio (RER, D, E, and F), locomotor activity (G, H, and I), food intake (J, K and L) and water intake (M, N and O) in sedentary control ($n=14$), exercise control ($n=15$), sedentary SF1 Socs3 KO ($n=13$) and exercise SF1 Socs3 KO ($n=14$). These data represent the average analysis of 4 consecutive days and were presented as pre-training (the light cycle before the training session from 8:00h to 14:00h), post-training (the light cycle immediately after the training session from 16:00h to 20:00h) and dark cycle (from 20:00h to 8:00h). *significant ($P<0.05$) effect of exercise (two-way ANOVA test). #significant ($P<0.05$) effect of SF1-specific SOCS3 ablation.

we assessed glycemia just before the beginning of the last 1RM test and immediately after exhaustion. Despite the high intensity of the 1RM test, in which mice carried approximately 4 times their body weight during the ladder climbing, control animals were able to sustain the same pre-exercise glycemia values at the fatigue moment (Fig. 10C). On the other hand, SF1 Socs3 KO mice exhibited a significantly lower glycemia at the exhaustion compared to pre-exercise glycemia (Fig. 10C). Therefore, the SOCS3 expression in SF1 cells is required to sustain training performance and glycemia during exercise. SF1-positive neurons in the VMH are critically involved in the glucoregulatory responses required to reestablish glycemia under metabolic challenges like hypoglycemia or 2DG administration (Flak *et al.* 2014, Verberne *et al.* 2014, Meek *et al.* 2016). Thus, we assessed whether SF1

Socs3 KO mice exhibit changes to the counter-regulatory response evoked by 2DG administration. Notably, the conditional knockout mice showed an attenuated glucoregulatory response compared to control animals (Fig. 10D and E). This defect occurred despite a similar activation of the HPA axis, since serum ACTH (Fig. 10F) and corticosterone (Fig. 10G) levels showed no differences between the groups after 2DG infusion.

Discussion

In the present study, we investigated whether the SOCS3 expression in SF1 cells regulates adrenal, gonadal and pituitary functions, resistance training performance as well as the metabolic effects of exercise. Our hypothesis was that exercise-induced cytokine signaling exerts an

Figure 10

Impaired training performance, glucose control during intense exercise and counter-regulatory response to 2-deoxy-D-glucose (2DG) in SF1 Socs3 KO mice. (A) Changes in the weight lifted by control ($n=9$) and SF1 Socs3 KO ($n=8$) mice along the 8-week training period. (B) Maximal weight lifted during 1RM tests performed in the first, fourth and eighth weeks of training ($n=9$ /group). (C) Glycemia measured just before the last 1RM test and immediately after exhaustion ($n=9$ /group). (D) Evaluation of the counter-regulatory response induced by an acute injection of 0.5 mg/kg 2DG. (E) Area under the curve in the glycemia after 2DG administration ($n=10$ – 12 /group). (F and G) Serum ACTH (F) and corticosterone (G) levels in control ($n=12$) and SF1 Socs3 KO ($n=8$) mice 60 min after an i.p. injection of 2DG. Unpaired two-tailed Student's *t*-test was used in the statistical analyses.

important modulation in the training adaptations, and SF1-specific SOCS3 ablation might change the metabolic responses to exercise. Our results revealed that SF1 Socs3 KO mice exhibit histological and functional changes in the adrenal gland. The overall metabolic responses induced by resistance training remained unchanged in SF1 Socs3 KO mice. However, resistance training performance and glucose control during intense exercise were impaired in mice carrying SF1-specific SOCS3 ablation. Furthermore, a reduced counter-regulatory response to hypoglycemia was also observed in SF1 Socs3 KO mice. Therefore, our findings suggest a previously unrecognized participation of SOCS3 modulating several endocrine and metabolic aspects.

The main role of the adrenal gland is to maintain body homeostasis by secreting corticosteroids, such as corticosterone and aldosterone in rodents. Multiple signaling pathways have already been described to promote steroid hormone biosynthesis in steroidogenic cells, including cytokine-induced cAMP-independent pathways (Stocco *et al.* 2005). Our results show that SOCS3 ablation in SF1-positive cells apparently did not alter the adrenal gland function under basal conditions. For example, adrenal weight and basal corticosterone and ACTH level were not affected in SF1 Socs3 KO mice.

However, a reduction in the total amount of lipids along the adrenal cortex was observed in SF1 Socs3 KO mice, which suggests a lower capacity to produce steroid hormones, since steroidogenic cells do not store significant amounts of steroid hormones, and there is a need for a constant supply of cholesterol (including from lipid droplets) to synthesize new steroid hormones when the cell is stimulated (Shen *et al.* 2016). In accordance with these results, SF1 Socs3 KO mice showed a blunted adrenal steroidogenic capacity when corticosterone secretion was evaluated during an ACTH stimulation test. Therefore, these findings indicate that although SF1 Socs3 KO mice are able to maintain normal adrenal function during basal conditions, their maximal adrenal steroidogenic capacity seems to be limited.

One of the most intriguing phenotypes observed in SF1 Socs3 KO mice was the retention of the fetal adrenal X-zone in adult animals. In rodents, a transient cell layer, named the X-zone, is located in the corticomedullary junction and regresses at puberty in males and at the first pregnancy in females (Holmes & Dickson 1971). Interestingly, even with the presence of this zone in SF1 Socs3 KO mice, the atrophy of the adult cortex resulted in an adrenal weight equivalent to control animals. Knockout mice for the *Stat5a* and *Stat5b* genes

have no profound effect on X-zone morphology or on the expression of 20 α -hydroxysteroid dehydrogenase, which is a steroidogenic enzyme expressed in X-zone cells (Hershkovitz *et al.* 2007). Although the molecular mechanisms that drive fetal zone regression and terminal differentiation of the adrenal cortex are not yet clearly defined, our findings suggest that SOCS3 is a previously unrecognized factor required for the terminal differentiation of the adrenal gland.

The beneficial metabolic effects of exercise are well established (Strasser *et al.* 2012, Golbidi & Laher 2014, Sarvas *et al.* 2015). Chronic exercise improves energy and glucose homeostasis (Laing *et al.* 2016). Interestingly, several studies reported increased SOCS3 expression in response to exercise in the skeletal muscle (Spangenburg *et al.* 2006, Trenerry *et al.* 2007, Guerra *et al.* 2011, Begue *et al.* 2013) or hypothalamus (Pereira *et al.* 2015). Although these results seem to be conflicting since increased SOCS3 levels are associated with metabolic imbalances, such as obesity and insulin resistance (Bjorbaek *et al.* 1998, Mori *et al.* 2004, Howard & Flier 2006, Jorgensen *et al.* 2013), an increased SOCS3 expression may simply indicate an acute effect of exercise caused by cytokine/hormone secretion. In fact, chronic exercise causes no changes or even a reduction in the SOCS3 expression in different tissues, which may help to explain the higher insulin and leptin sensitivity observed in trained animals (Steinberg *et al.* 2004, Yaspelkis *et al.* 2010, Guerra *et al.* 2011, Olmedillas *et al.* 2011, Kang *et al.* 2013, Sarvas *et al.* 2015).

Previous studies have shown improved body adiposity and glucose homeostasis after SOCS3 ablation (Mori *et al.* 2004, Pedroso *et al.* 2014, 2016, Zampieri *et al.* 2015, Bohlen *et al.* 2016). Zhang *et al.* (2008) also studied mice carrying SF1-specific SOCS3 deletion. These authors observed no changes in body weight and adiposity in mutant mice exposed to normal chow or high-fat diet. However, different from our results, they found improved glucose homeostasis and lower energy expenditure in SF1 Socs3 KO mice (Zhang *et al.* 2008). Comparing control and SF1 Socs3 KO mice, independently of the training status, we only found very small metabolic differences. For example, we observed a lower food and water intake in SF1 Socs3 KO mice during the pre-training period. A previous study also showed that SOCS3 deletion in SF1 cells reduces food intake in mice, an effect attributed to increased leptin sensitivity in the VMH (Zhang *et al.* 2008). The reduced water intake was probably secondary to a lower food intake, since water intake is important for swallowing the dry pelleted feed. Furthermore, our data

confirmed some expected metabolic effects induced by exercise. Trained animals showed a lower body weight and adiposity, and an improved glucose tolerance compared to sedentary groups. Regarding the acute effects induced by the training session, we observed a significant effect of exercise during the post-training period by increasing VO₂, probably representing the excess post-exercise oxygen consumption (Maehlum *et al.* 1986) and by reducing RER and locomotor activity, which may indicate, respectively, a higher fat oxidation and a tiredness state. Water intake also increased in the dark period possibly indicating a post-exercise rehydration. However, none of these parameters were affected by SF1-specific SOCS3 ablation.

SF1 Socs3 KO mice showed a remarkably lower performance during the last weeks of training. The lack of changes in the first weeks of training may indicate that at lower intensities, SF1-specific SOCS3 ablation causes no significant biological effects for the exercise adaptations. However, when the intensity progressively increased, mutant mice were not able to lift similar loads during training sessions or 1RM test, compared to control animals. Interestingly, SF1 Socs3 KO mice were also unable to maintain circulating glucose levels during the last 1RM test. An incapacity to control glycemia at situations that consume high amounts of glucose is certainly a factor that can impair exercise performance and thereby training adaptations (Coyle 1999). A reduced adrenal capacity to respond to stress-secreting glucocorticoids could impair high-intensity exercise training by disrupting glycemia control.

Defects in VMH neurons could also affect glucose homeostasis and other metabolic changes. It is well established that VMH neurons control the energy balance, glucose homeostasis and autonomic nervous system (Brobeck 1946, Powley 1977, Ikeda *et al.* 1995, Majdic *et al.* 2002, Seoane-Collazo *et al.* 2015). Pharmacological manipulations in VMH neurons can decrease plasma glucose levels, hepatic glycogenolysis, lipolysis and fatty acid oxidation during exercise, leading to a reduced performance (Vissing *et al.* 1989, Balkan *et al.* 1991, Wanner *et al.* 2010, Miyaki *et al.* 2011). Exercise also increases noradrenergic and dopaminergic tonus to VMH neurons (Kitaoka *et al.* 2010), which in turn modulate the autonomic nervous system in order to produce the required adjustments in the cardiovascular system, nutrient mobilization and glucose homeostasis (Ball 2015, Seoane-Collazo *et al.* 2015). Accordingly, VMH SF1-positive neurons send projections to autonomic sympathetic and parasympathetic centers of the

hypothalamus and hindbrain, which innervate white and brown adipose tissue, heart, liver, pancreas and skeletal muscles (Lindberg *et al.* 2013, Seoane-Collazo *et al.* 2015). VMH SF1-positive neurons are also fundamental components of the neurocircuitry that regulates counter-regulatory responses to hypoglycemia, ensuring a rapid and efficient recovery of glucose levels (Flak *et al.* 2014, Verberne *et al.* 2014, Meek *et al.* 2016). In the present study, a lower glucoregulatory response to 2DG was observed in SF1 Socs3 KO mice. Since VMH neurons trigger the counter-regulatory response evoked by 2DG and the reduced counter-regulatory response occurred despite a similar activation of the HPA axis by 2DG, these results suggest a possible involvement of VMH SF1 neurons in the metabolic defects observed in SF1 Socs3 KO mice.

In conclusion, our study showed for the first time that SOCS3 expression in SF1 cells is required to maintain training performance and glucose control during intense resistance exercise. Additionally, SF1-specific SOCS3 ablation decreased testis weight, although it did not affect the fertility, and caused histological and functional changes in the adrenal cortex. Furthermore, an attenuated counter-regulatory response to hypoglycemia was also observed in SF1 Socs3 KO mice. Therefore, our results revealed a novel participation of SOCS3 regulating several endocrine and metabolic aspects. These findings provide new insights into how cytokine signaling can modulate multiple biological functions, especially regarding physical exercise and the terminal differentiation of the adrenal gland.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J A B P and J D designed the study; J A B P, I T, V L P, L B L, I C F and D N B performed the exercise training and *in vivo* analyses; M A S F did the muscle analysis; P O R M, T B A, I C C and C F P L did the adrenal analysis; A M R L performed the brain histology; J A B P, M A S F, P O R M, T B A, I C C, C F P L and J D analyzed the data; J A B P, P O R M and J D wrote the paper. All authors read and approved the final version of the manuscript.

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