ADAPTAÇÕES MORFOFUNCIONAIS E RESPOSTAS MOLECULARES DO MÚSCULO ESQUELÉTICO DE RATOS SUBMETIDOS AO TREINAMENTO RESISTIDO

ANDREO FERNANDO AGUIAR

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, área de concentração em Biologia Celular, Estrutura e Funcional

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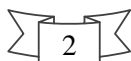
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Dedicatória

"Dedico este trabalho a minha esposa Rachel Colauto Milanezi Aguiar que sempre me apoiou para que este objetivo fosse concretizado. Sou imensamente grato por todo amor, carinho e companheirismo que tem me dado todos estes anos que estamos juntos. Esta dedicatória significa muito pouco, perto de todo esforço que você tem feito para que nossa família esteja sempre em harmonia e na paz de Deus. Obrigado por tudo..."

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Agradeço primeiramente a Deus, pois sem ele nada neste mundo é concebido. A verdade esta em Deus, o amor esta em Deus, a paz esta em Deus, a família esta em Deus. Tudo esta sob o seu controle, é por isto que conseguimos nos levantar a cada queda, nos preparar para as situações mais difíceis e acreditar que não estamos aqui por acaso. "Tudo posso naquele que me fortalece"

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Resumo

Embora fortes evidências demonstrem que os fatores de regulação miogênica (MRFs) e o fator de crescimento semelhante à insulina (IGF-I) apresentem um importante papel na resposta hipertrófica após treinamento resistido (TR) agudo, permanece desconhecido se a resposta dos MRFs e IGF-I também ocorre durante a adaptação ao TR a longo-prazo. Portanto, o objetivo deste estudo foi testar a hipótese de que a resposta hipertrófica e modulação das fibras do músculo esquelético após TR a longo-prazo poderia estar associada ao aumento na expressão gênica dos MRFs e IGF-I. Ratos Wistar (80 dias de idade, 250-300 g) foram divididos em quatro grupos: Controle 8 semana (C8, $n = 8$), Treinado 8 semanas (T8, $n = 8$), Controle 12 semanas (C12, $n = 8$) e Treinado 12 semanas (T12, $n = 8$). Os grupos T8 e T12 foram submetidos a um programa de TR progressivo (3 dias/semana) durante 8 e 12 semanas, respectivamente. O protocolo de treinamento consistiu de quatro séries de 10-12 repetições, com um período de descanso de 40 segundos entre cada série, realizado a 65-75% de uma repetição máxima (1RM). Ao término do experimento, os animais foram sacrificados e o músculo plantar coletado para as análises morfológica e molecular. O TR durante 8 e 12 semanas não promoveu qualquer alteração ($p > 0,05$) significante no ganho de peso corporal e consumo alimentar dos grupos T8 e T12 em relação aos grupos C8 e C12, respectivamente. Após 8 e 12 semanas de TR, a força absoluta (T8: 69,7% and T12: 126,0%, $p < 0,05$) e relativa (T8: 36,1% and T12: 57,7%, $p < 0,05$) foi significantemente elevada nos grupos T8 e T12, em comparação aos seus respectivos controles. No entanto, houve um similar aumento da área de secção transversal (AST) das fibras musculares (T8: 29% vs. T12: 35%, $p > 0,05$) entre os grupos T8 e T12, comparados aos grupos C8 e C12, respectivamente. Esta estagnação hipertrófica entre 8 e 12 semanas foi consistente com o similar aumento na expressão de mRNA para miogenina (T8: 44,8% vs. T12: 37,7%, $p > 0,05$) e MyoD (T8: 22,9% vs. T12: 22,3%, $p > 0,05$) nos grupos T8 e T12. Em adição, a expressão gênica do IGF-I aumentou 30,1% após 8 semanas de TR (T8 x C8, $p < 0,05$), mas retornou aos níveis basais após 12 semanas (T12 x C12, $p > 0,05$). A modulação das isoformas de MHCIIx/d para MHCIIa e recíproca transição das fibras IIX/IId para IIA foi evidente somente após 12 semanas de TR (T12 x C12, $p < 0,05$). Os dados demonstram que a estagnação hipertrófica observada durante o programa de TR a longo-prazo foi associada a um limite máximo de expressão gênica da miogenina e MyoD, e reciprocamente, com o retorno da

expressão gênica do IGF-I para os valores basais. Além disso, o aumento da expressão de miogenina, MyoD e IGF-I após 8 semanas de TR não foi associada a alterações no conteúdo das isoformas de MHC e freqüência dos tipos de fibras. Portanto, os resultados indicam uma possível interação entre os MRFs e IGF-I no controle da hipertrofia muscular em resposta ao TR a longo-prazo, sugerindo que esses fatores estejam mais envolvidos no controle da massa muscular do que na modulação dos tipos de fibras.

Abstract

Although strong evidence show that the myogenic regulatory factors (MRFs) and insulin-like growth factor (IGF-I) have important roles in the hypertrophy response after acute resistance training, it is still unclear if response of MRFs and IGF-I also occurs during the adaptation to prolonged periods of resistance training (RT). Therefore, the purpose of this study was to test the hypothesis that fiber-types transition and hypertrophy during long-term RT could be associated with increased MRFs and IGF-I mRNA expression in the skeletal muscle. Male *Wistar* rats (80 days old, 250-300 g) were divided into four groups: 8 weeks control (C8, *n* = 8), 8-weeks trained (T8, *n* = 8), 12-weeks control (C12, *n* = 8), 12-weeks trained (T12, *n* = 8). T8 and T12 groups were submitted to a progressive RT program (3 day/week) for 8 and 12 weeks, respectively. The training protocol consisted of four sets of 10–12 repetitions, with a 40 s rest period between each set, performed at 65–75% of one repetition maximum (1RM). At the end of the experiment, animals were sacrificed and the plantaris muscle collected for morphological and molecular analysis. The RT did not change ($p > 0.05$) in body weight gain and food intake in the T8 and T12 compared to the C8 and C12 groups, respectively. After 8 and 12 weeks of RT, the absolute (T8: 69.7% vs. T12: 126.0%; $p < 0.05$) and relative (T8: 36.1% vs. T12: 57.7%; $p < 0.05$) strength (relative 1RM) was significantly elevated in the T8 e T12 groups, compared to respective control groups. RT for 8 and 12 weeks induced similar increase in myogenin (T8: 44.8% vs. T12: 37.7%; $p > 0.05$), MyoD (T8: 22.9% vs. T12: 22.3%; $p > 0.05$) and muscle fiber cross-sectional area (CSA) (T8: 29% vs. T12: 35%; $p > 0.05$) in the T8 and T12, compared to C8 and C12 groups, respectively. After 8 weeks of RT, IGF-I increased in 30.1% in the T8 compared to C8 group, but returned to baseline after 12 weeks of RT. The modulation of MHCIIx/d-to-MHCIIa isoforms and fiber IIX/D-to-IIA transition was evident only after 12 weeks of RT. The data show that the hypertrophic stagnation during long-term RT was associated with a maximal limit of myogenin and MyoD mRNA expression and with the return of IGF-I mRNA levels to baseline. In addition, the increase in myogenin, MyoD and e IGF-I expression after 8 weeks of RT was not associated with changes in the MHC content and fiber-types frequency. Therefore, the results indicate a possible interaction between MRFs and IGF-I in the control of muscle hypertrophy during long-term resistance training and suggest that these factors are more involved in the control of muscle mass than in fiber-type transitions.

I - INTRODUÇÃO

1. Características Gerais das Fibras Musculares

O músculo estriado esquelético é constituído por células multinucleadas especializadas, as fibras musculares, que apresentam mionúcleos localizados na região periférica da fibra, abaixo da membrana plasmática (figura 1A). Morfologicamente, as fibras musculares são constituídas por unidades contráteis repetidas, os sarcômeros (58). Cada sarcômero é formado por várias proteínas, das quais se destacam as proteínas contráteis miosina (filamento grosso formado pela polimerização de 200 a 300 moléculas de miosina da classe II) e actina (filamento fino associado às proteínas reguladoras troponina e tropomiosina). A miosina é um hexâmero formado por seis polipeptídeos: duas cadeias pesadas de miosina (MHC), enroladas em α-hélice e quatro cadeias leves de miosina (*myosin light chain*, MLC) (76, 131, 39) (Figura 1B). Cada MHC pode ser separada em duas porções: meromiosina leve (*light meromyosin*, LMM), em forma de bastão, e meromiosina pesada (*heavy meromyosin*, HMM), conhecida como porção globular da miosina, que contém um sítio de interação com a actina e uma região de ligação com a molécula de ATP (atividade ATPásica) (58, 76) (Figura 1B). A contração muscular ocorre através da interação entre os filamentos finos (actina) e grossos (porção globular da molécula de miosina) do sarcômero (58), após a hidrólise do ATP, pela miosina ATPase (mATPase) (76). A porção globular da MHC representa o componente essencial para o mecanismo de geração de força do músculo. Segundo Talmadge e Roy (122), a velocidade de contração é diretamente proporcional a atividade da mATPase, na porção globular da MHC. Tal evento foi demonstrado em análises de fibras isoladas, que revelaram uma alta correlação entre o tipo de fibra, baseado na atividade da mATPase, com a especificidade da MHC (14, 91).
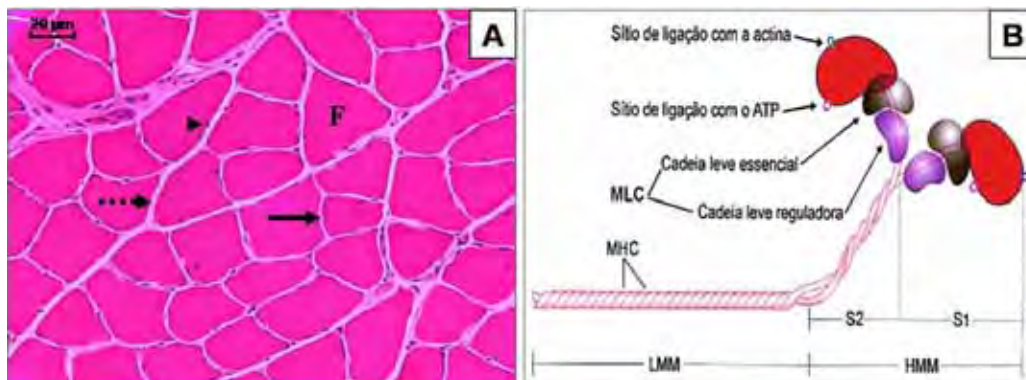
Figura 1. A: Corte transversal do músculo sóleo de rato submetido a coloração HE. Fibras musculares (F); Perimísio (seta descontínua); Endomísio (seta contínua) e Mionúcleos (ponta da seta). B: Esquema da molécula de miosina da classe II. Cada molécula de miosina é composta por duas cadeias pesadas de miosina (MHC) e quatro cadeias leves de miosina (MLC). As MHC podem ser clivadas em meromiosina leves (LMM) e pesadas (HMM). As HMM são formadas pela porção globosa S1 e porção α hélice S2. As MLC estão dispostas na proporção de duas cadeias (uma essencial e uma reguladora) para cada subfragmento S1.

Os músculos são constituídos por diferentes proporções de distintas fibras que conferem a este tecido uma ampla diversidade estrutural, metabólica e funcional (91, 106). Inicialmente, as fibras musculares foram divididas em três discretas categorias: 1- fibras oxidativas de contração lenta (tipo I), 2- fibras oxidativas/glicolíticas de contração rápida (tipo IIA) e 3- fibras glicolíticas de contração rápida (tipo IIB), de acordo com o padrão de coloração histoquímica para a reação da mATPase (20). No entanto, evidências mais recentes baseadas nas características bioquímicas e imunohistoquímicas das fibras musculares, demonstraram uma estreita relação entre a classificação histoquímica das fibras do tipo I, IIA e IIB, e a expressão das isoformas de MHC expressas por essas fibras (100). Neste contexto, Pette e Staron (92), classificaram as fibras musculares de mamíferos em quatro distintos tipos: fibras de contração lenta (tipo I), expressando MHCI; e fibras de contração rápida (tipo II), subdivididas em tipo IIA, IID e IIB, que expressam as isoformas de MHCIIa, MHCIId e MHCIIb, respectivamente. Os autores classificaram essas fibras como puras, uma vez que, expressavam uma única isoforma de MHC. As fibras do tipo IID (MHCIId) se equivalem as fibras IIX (MHCIIx), descritas em ratos (106). Vale ressaltar que as fibras musculares humanas não expressam a MHCIIb, a classificação prévia de fibras IIB se equivale às fibras do tipo IIX ou IID (MHCIIx ou MHCIId), de acordo com a

similaridade da MHC descrita em ratos (114). No entanto, a literatura comumente denomina as fibras do tipo IIX/D como fibras do tipo IIB.

Além das fibras puras, existem as fibras "híbridas", que expressam duas ou mais isoformas de miosina. Estas fibras foram classificadas em Tipo IC (MHCI > MHCIIa), IIC (MHCIIa > MHCI), IIAD (MHCIIa > MHCIId), IIDA (MHCIId > MHCIIa), IIDB (MHCIId > MHCIIb) e IIBD (MHCIIb > MHCIId) (91, 92). A associação entre as fibras puras e híbridas representa um contínuo de fibras lentas em direção a fibras mais rápidas: I → I/IIA → IIA/I → IIA → IIA/D → IID/A → IID → IID/B → IIB/D → IIB (90). Deste modo, assume-se uma organização seqüencial das fibras puras, as quais são intermediadas por fibras híbridas (I ↔ IC ↔ IIC ↔ IIA ↔ IIAD ↔ IID ↔ IIDB ↔ IIB) (figura 2).
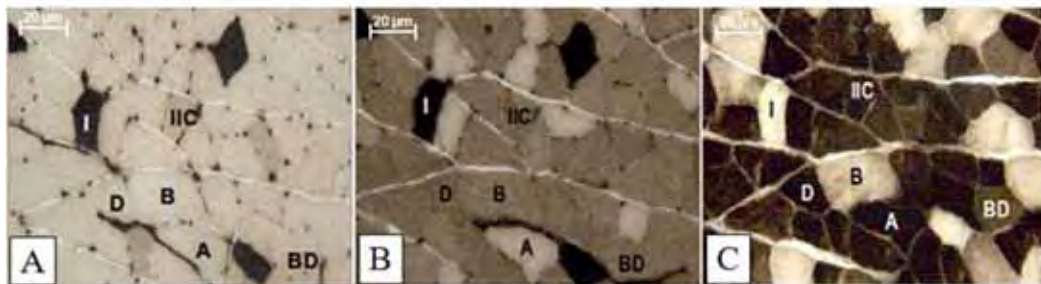
Figura 2. Cortes transversais seriados do músculo plantar de rato. Fibras musculares puras (I, IIA, IID e IIB) e híbridas (IIC e IIBD). Análise histoquímica da mATPase em pHs 4.2 (A), 4.5 (B) e 10.6 (C).

As características funcionais e metabólicas dos diferentes tipos de fibras tem sido investigadas em estudos que correlacionaram a expressão de diferentes isoformas de MHC, com as propriedades metabólicas e funcionais das fibras musculares (91, 98, 100). Rivero et al. (100), utilzando-se de métodos histoquímicos, investigaram as interrelações entre a atividade da mATPase, a atividade das enzimas oxidativas (*succinato desidrogenase*, SDH) e glicolíticas (*α-glicerolfosfatase desidrogenase*, GPD) e a área de secção transversal (AST) das fibras musculares do músculo gastrocnêmio de ratos. Este estudo evidenciou um padrão de atividade das enzimas glicolíticas mATPase e GPD, de acordo com o tipo de fibra: IIB> IID/X> IIA>I. Por outro lado, a atividade da enzima oxidativa SDH foi maior nas fibras IIA e I. Os autores também demonstraram uma maior atividade da GPD nas fibras com maior AST (IIB e IID/X), enquanto as fibras com menor AST (I e IIA) apresentaram maior atividade da SDH. Os resultados

deste estudo apontam uma estreita relação entre a expressão das isoformas de MHC, a AST e as propriedades metabólicas dos diferentes tipos de fibras musculares.

A expressão diferencial das isoformas de MHC nos diferentes tipos de fibras é refletida em suas respostas funcionais, tais como as características contráteis (exemplo: velocidade de contração e produção de potência) e metabólicas (exemplo: resistência à fadiga). Tal fato confere ao músculo esquelético uma ampla capacidade para realizar uma variedade de demandas funcionais (22). Além disso, as fibras musculares exibem extensa plasticidade, o que habilita este tecido alterar suas características morfológicas, metabólicas e funcionais, em resposta a estímulos específicos (42, 79, 91), como a intervenção nutricional, a carga mecânica, os fatores hormonais, o envelhecimento e o treinamento/exercício físico (113, 43, 3, 53, 9). Tem sido relatado que o músculo esquelético pode se adaptar através de um mecanismo quantitativo, baseado nas mudanças da massa muscular e área da fibra, e por um mecanismo qualitativo, baseado nas alterações dos tipos de fibras e conteúdo das isoformas de MHC (22). Neste contexto, vários estudos demonstraram que ambos os treinamentos resistido e aeróbio podem promover alterações significantes na distribuição das fibras e no conteúdo de MHC dos diferentes músculos (1, 22, 36). Essas modificações fenotípicas são necessárias para suprir as necessidades funcionais e metabólicas do organismo e, conseqüentemente, otimizar o desempenho físico.

2. Modulação das Fibras Musculares ao Treinamento Resistido

O treinamento resistido (TR) tem sido divulgado como um potente estímulo, capaz de promover adaptações funcionais, estruturais e metabólicas no músculo esquelético (50, 112). Inúmeros estudos envolvendo sujeitos humanos (22, 81, 112, 135) e animais (70, 123) demonstram um aumento da massa (hipertrofia), força e área muscular após TR a longo prazo. A magnitude das respostas musculares a este modelo de treinamento é dependente da intensidade, do número de séries e repetições, do período de descanso entre as séries e exercícios, da freqüência de treinamento e da velocidade das repetições (68). Campos et al. (22), em um estudo com indivíduos jovens (22,5 ± 5,8 anos), analisaram os efeitos de três regimes de TR (G1: *baixa repetição*, 4 séries de 3 a 5 RM; G2: *intermediária repetição*, 3 séries de 9 a 11 RM e G3: *alta repetição*, 2 séries de 20 a 28 RM) durante 12 semanas, sobre a AST das fibras musculares, a força dinâmica máxima (FDM), o percentual relativo dos tipos de fibras, o conteúdo das isoformas de MHC e a resistência muscular localizada (RML). Após o

período de treinamento, o grupo *baixa repetição* apresentou maiores ganhos de FDM, enquanto o grupo *alta repetição* apresentou maior RML. Além disso, houve um maior aumento na AST das fibras do tipo I, IIA e IID nos grupos *baixa* e *intermediária repetição* comparado ao grupo *alta repetição*, o que confirma a efetividade do TR de alta intensidade e baixo volume, para promover uma maior resposta hipertrófica das fibras musculares. Os resultados desse estudo revelam uma estreita relação entre as adaptações morfofuncionais e a especificidade do estímulo aplicado sobre o tecido muscular.

Interessantemente, as adaptações morfofuncionais do tecido muscular em resposta ao TR têm sido intimamente relacionadas com alterações no perfil fenotípico das fibras musculares (24, 116). Tal fato foi corroborado em estudos com indivíduos jovens de ambos os gêneros submetidos ao TR progressivo, na qual demonstraram um aumento da força e área do músculo em conjunto com alterações na proporção de fibras musculares (22, 117). Os achados mais consistentes relatam redução na distribuição das fibras do tipo IID com recíproco aumento das fibras do tipo IIA (54, 116, 118). Consistente com o ajuste das fibras do tipo IID para IIA, a análise eletroforética (SDS-PAGE) das isoformas de MHC evidenciou redução no conteúdo de MHCIId com concomitante aumento da MHCIIa no músculo vasto lateral humano submetido ao TR (1, 22). Em adição, Harber et al. (53), analisando os efeitos de 10 semanas de TR em indivíduos jovens (21-25 anos), observaram um aumento da força dinâmica máxima e da AST das fibras do tipo IIA, com recíproco aumento na expressão da MHCIIa e redução da MHCIId. Reciprocamente, as alterações observadas no conteúdo das isoformas de MHC não foram acompanhadas de alterações significantes no percentual dos tipos de fibras musculares (mATPase). Os autores sugerem um ajuste nas isoformas de MHC, precedente a modulação das fibras musculares, similar ao observado em indivíduos jovens (119) e idosos (136), após 2 e 12 semanas de TR progressivo, respectivamente.

De fato, tem sido amplamente demonstrado um ajuste das isoformas de MHCIId para MHCIIa em indivíduos jovens submetidos a diferentes modelos e protocolos de TR progressivo (1, 24, 63). Resultados similares foram observados em idosos (60-75 anos) após 24 semanas de TR, na qual se evidenciou um aumento da força dinâmica máxima, associada à transição das isoformas de MHCIId para MHCIIa (112). Os autores relatam que o ajuste fenotípico da isoformas de MHCIId em direção a MHCIIa, ocorre devido a plasticidade da MHCIId para ajustar-se em direção a fibras mais oxidativas, em

conseqüência do recrutamento das fibras de alto limiar de estimulação durante a progressão do TR. O aumento das isoformas de MHCIIa demonstra uma adaptação benéfica das fibras musculares em direção a um fenótipo mais eficiente, sugerindo que ocorra preservação da plasticidade em músculos de indivíduos idosos submetidos ao treinamento TR progressivo. O trabalho de Kesidis et al. (67) confirma a efetividade do TR para promover ajuste entre a população de fibras rápidas (IID → IIA). Os autores observaram uma alta proporção de fibras que expressavam as isoformas de MHCI/IIa e MHCIIa, com recíproca ausência da isoforma MHCIId no músculo vasto lateral de *bodybuilders* (tempo de TCR: 8,5 ± 5 anos), comparado a indivíduos fisicamente ativos. Interessantemente, a transição das isoformas de MHC (MHCIId→MHCIIa) observada em *bodybuilders* foi similar ao observado em indivíduos saudáveis submetidos a diferentes programas de TR a médio-prazo (8-19 semanas) (1, 44, 69). Os dados demonstram que a transição das isoformas de MHC induzidas pelo TR progressivo abrange apenas as isoformas rápidas de MHC (MHCIId/x→MHCIIa).

Evidências recentes sugerem que o mecanismo de ajuste/transição das fibras musculares em resposta ao TR ocorra de forma gradual, na qual as fibras híbridas podem representar um estado transicional em direção a fibras mais estáveis. Williamson et al. (137) demonstraram uma transição da MHCIId para MHCIIa, com recíproca redução da proporção de fibras híbridas (I/IIA + I/IIA/IIX + IIA/IIX), no músculo vasto lateral de indivíduos jovens submetidos a 12 semanas de TR progressivo. Segundo os autores, a redução da proporção de fibras híbridas contribuiu para o aumento da MHCIIa, sugerindo um processo de transição gradual em meio as isoformas rápidas de MHC (MHCIId→MHCIIa). Em adição, Campos et al. (22) observaram um aumento da proporção de fibras híbridas IIAD e redução das fibras puras IID do músculo vasto lateral de indivíduos jovens, após 8 semanas de TR progressivo. Reciprocamente, os autores relataram um aumento do conteúdo das isoformas de MHCIIa e redução das isoformas de MHCIId. Embora o TR tenha induzido a modulação das isoformas de MHC, a transição das fibras ocorreu parcialmente, abrangendo somente a transição das fibras IID para IIAD. Os resultados suportam a idéia de que a modulação das fibras musculares ocorre de forma gradual na dependência do estímulo aplicado ao músculo. Consistente com estes achados, Andersen et al. (8) em análise do músculo vasto lateral de indivíduos em repouso no leito, demonstraram um aumento das fibras expressando tanto mRNA como proteína para MHCIIx e MHCIIa, sugerindo um estado transicional das fibras musculares. Considerando o papel fundamental das fibras híbridas na

transição dos tipos de fibras, torna-se indispensável uma análise acurada desta população em estudos sobre as adaptações fenotípicas do músculo esquelético. Embora as fibras híbridas freqüentemente representem uma negligente população, uma considerável presença dessas fibras tem sido identificada em músculo adulto normal (18, 45, 117, 121). Portanto, as fibras híbridas representam uma importante população que não deve ser ignorada.

Apesar da resposta mais comum para uma variedade de estímulos de TR indicar uma conversão das fibras do tipo IID (MHCIId) em direção as fibras IIA (MHCIIa), Andersen et al. (7) demonstraram uma redução significante do percentual de fibras contendo MHCI e aumento das fibras expressando MHCIIa no músculo vasto lateral de atletas do gênero masculino, após 3 meses de treinamento intervalado de alta intensidade. Além disso, as fibras que co-expressavam ambas as isoformas de MHCIIa e MHCIId foram reduzidas com o treinamento. Os resultados relatam um ajuste bi-direcional de ambas isoformas de MHCI e MHCIId em direção a MHCIIa em resposta ao treinamento de *sprint*. Em adição, Aguiar et al. (4) observaram um aumento da isoforma de MHCII com recíproca redução da MHCI no músculo sóleo de ratos, após 5 semanas de TR. O músculo sóleo é constituído predominantemente de 90% de fibras do tipo I (MHCI) e 10% de fibras do tipo II (MHCII). Os autores argumentam que em músculos oxidativos de ratos, que não expressam as isoformas de MHCIId ou MHCIIb, a transição das isoformas de MHCI para MHCII pode representar uma adaptação benéfica das fibras musculares em direção a um fenótipo mais eficiente para suportar a sobrecarga progressiva do exercício. Coletivamente, os resultados destes estudos revelam um ajuste bi-direcional de ambas isoformas de MHCI e MHCIId em direção a MHCIIa (MHCI→MHCIIa←MHCIb) em resposta ao TR de alta intensidade. No entanto, resultados contraditórios foram observados em *bodybuilders* do gênero masculino (26,1 ± 4,2 anos) treinados durante 8 anos (67) e indivíduos saudáveis do gênero masculino (36 ± 2 anos) submetidos a 19 semanas de TR, na qual foi observada uma transição das isoformas de MHCIId em direção a MHCIIa, sem qualquer alteração no percentual de MHCI no músculo vasto lateral (1). A discrepância dos resultados encontrados pode ser decorrente dos diferentes protocolos de TR, do músculo estudado e da espécie investigada.

Pesquisadores são encorajados a investigar a relação estímulo-resposta do músculo esquelético sob diferentes sobrecargas e modelos de treinamento de força. Embora muitas perguntas ainda persistam em relação às mudanças fenotípicas das fibras

musculares em humanos em resposta ao TR, a resposta mais plausível sugere um ajuste entre a população de fibras rápidas (tipo IIX/D→tipo IIA). Tal evento pode contribuir para uma maior eficiência funcional e capacidade oxidativa das fibras musculares. Todavia, outros modelos de treinamento físico (por exemplo: o treinamento aeróbico) podem ocasionar respostas adaptativas semelhantes às observadas no TR, porém, a magnitude destas respostas parece ser diferente, devido ao padrão de estímulo nervoso e recrutamento das fibras musculares.

3. Mecanismos Moleculares Envolvidos na Modulação das Fibras Musculares

A transição dos tipos de fibras não envolve apenas mudanças na expressão das isoformas de miosina, mas incluem também alterações no perfil de várias proteínas saroméricas (91). A magnitude e especificidade das mudanças no perfil fenotípico muscular são dependentes de alterações na expressão gênica (conteúdo de mRNA) e na tradução de proteínas musculares (95, 105). A expressão de genes músculo-específicos pode ser aumentada ou diminuída pela ação de fatores transcricionais e diferentes vias intracelulares, que podem ser ativadas ou reprimidas em conseqüência de diferentes estímulos extrínsecos (por exemplo: estimulação elétrica, carga mecânica, estiramento) e intrínsecos (por exemplo: níveis de glicogênio, ATP, K^+, H^+ e Ca^{+2} intracelular; hipóxia e estado redox) que afetam o tecido muscular. Em adição, diferentes padrões de atividade do nervo motor podem ativar distintos programas da transcrição de genes músculo-específicos, estabelecendo assim um alto grau de especialização metabólica e fisiológica entre os subtipos de fibras musculares (138). Assim, o determinante primário das alterações fenotípicas das fibras musculares é a interação das vias moleculares intracelulares que regulam a expressão de múltiplos genes e, conseqüentemente, a taxa de síntese e degradação de proteínas.

Evidências recentes apontam as vias moleculares sensíveis a oscilações na [Ca^{+2}] intracelular como importantes reguladores das mudanças fenotípicas dos tipos de fibras musculares. O aumento da [Ca^{+2}] intracelular, em resposta ao padrão estímulo nervoso que atinge a fibra muscular, pode atuar como segundo mensageiro na transdução dos sinais mecânicos em sinais químicos, desencadeando uma cascata de eventos intracelulares que modificam a função de fatores transcricionais. Dentre as vias moleculares reguladas pelo Ca^{+2} destaca-se a via da Calcineurina (CaN)-NFAT (29). Evidências recentes apontam a CaN como importante mediadora da expressão gênica em fibras musculares rápida e lentas (85, 87). A CaN atua como um sensor molecular de

atividade contrátil, sendo seletivamente ativada em resposta as elevações sustentadas na $[Ca^{+2}]$ intracelular, após a ligação entre o Ca^{+2} e a proteína Calmodulina (Ca^{+2}-Calmodulina) (28). A CaN ativa será responsável pela retirada do fosfato ligado ao substrato NFAT (*nuclear factor of activated T cells*, fator nuclear de células T ativadas), que em condições basais encontra-se no sarcoplasma (28). Após a retirada do fosfato, o NFAT é translocado para a região promotora nuclear (DNA) (96) e irá promover a ativação de genes músculo-específicos (28, 38). Outros importantes achados demonstram a sinalização *cruzada* de outras proteínas (CAMKs, p38MAPK e AMPK) e fatores transcricionais (PGC-1, MEF2, ATF2 e PPARs), com a via CaN-NFAT (13, 74, 82, 102, 130, 139, 141). A multiplicidade de genes que são regulados pelas vias dependentes de Ca^{+2} incluem o GLUT4 (*glucose transporter 4*), SERCA 1 (*SR Ca^{+2} ATPase*), MHC (*myosin heavy chain*) e enzimas oxidativas (6, 86).

Em linfócitos T, a CaN responde preferencialmente a níveis de $[Ca^{+2}]$ sustentados de baixa amplitude, enquanto permanece insensível a níveis de $[Ca^{+2}]$ transitórios de alta amplitude (37). A habilidade da CaN para discriminar as flutuações na $[Ca^{+2}]$ intracelular, sugere que a mesma pode afetar a expressão de genes musculares em diferentes tipos de fibras. Em condições de repouso, a $[Ca^{+2}]$ analisada em fibras musculares isoladas varia entre 30-50 nM (138). Por outro lado, durante a contração, a $[Ca^{+2}]$ nas fibras de contração lenta comumente oscila entre 100-300 nM (27), enquanto as fibras rápidas geralmente apresentam elevações transitórias de alta amplitude, oscilando entre 1-2µM (134). Essa elevação transitória da $[Ca^{+2}]$ durante a atividade contrátil não permite a ativação da CaN (37), sugerindo que a sinalização molecular da CaN-NFAT seja modulada pela relação entre o estímulo e a $[Ca^{+2}]$ intracelular. Nesta linha de investigação, Liu et al. (75) demonstraram que a translocação do NFAT para o núcleo em cultura de fibras musculares, foi estimulada com padrões de atividade similar a ativação de fibras lentas, enquanto que o estímulo para ativação das fibras rápidas inibiu a translocação do NFAT. Os resultados demonstram que as oscilações na $[Ca^{+2}]$ podem ser um fator determinante para a manutenção fenotípica muscular, sugerindo que o padrão tônico de estimulação, similar ao observado em fibras lentas, pode controlar seletivamente a ativação da via CaN-NFAT. Neste contexto, Chin, (30) relata que a expressão dos diferentes genes ocorre de forma diferencial entre as fibras de contração rápida (tipo IID e IIA) e lenta (tipo I), suportando a idéia de que o nível da ativação muscular, como resultado da amplitude e duração das elevações na $[Ca^{+2}]$ intracelular, pode determinar o padrão e magnitude da expressão gênica nos diferentes tipos de fibra.

Evidências recentes mostraram um aumento da proporção de fibras rápidas do músculo sóleo de ratos tratados com ciclosporina A (CsA), um inibidor da CaN (Serrano et al., 2001). Em adição, Goy et al. (49), observaram o desenvolvimento de miopatia e perda da capacidade oxidativa do músculo esquelético em pacientes transplantados mantidos com inibidores de CaN (CsA e FK-506). Os resultados evidenciam o importante papel da CaN na regulação da função muscular, particularmente, do fenótipo lento da fibra muscular.

Em adição, Mitchell et al. (84) sugerem que a vias de sinalização mediada pela CaN no músculo esquelético podem não ser reguladas exclusivamente pelas mudanças na $[Ca^{+2}]$ intracelular, mas também pelos mecanismos que regulam a expressão de outras subunidades da CaN, como as subunidades catalítica A (CaNA) e reguladora B (CaNB). Os autores demonstraram que a expressão protéica da CaNB é maior em músculos lentos, comparado a músculos rápidos, enquanto a CaNA é mais expressa em músculos rápidos. Deste modo, a eficiência da CsA em um determinado músculo pode ser dependente da expressão da subunidade da CaN, sugerindo que em estudos envolvendo a contribuição da CaN nas alterações fenotípicas das fibras musculares, seja comprovado que ocorreu a inibição total da expressão da CaN no músculo investigado. Embora a imunossupressão da CaN com CsA seja uma ferramenta amplamente utilizada para investigar os efeitos da CaN no controle do fenótipo muscular, deve-se considerar que a inibição da CaN pode não apresentar uma relação direta de causa-efeito na indução da transformação dos tipos de fibras. Neste contexto, Chin et al. (28) relatou que o aumento da proporção de fibras rápidas no músculo sóleo de ratos administrados com CsA, pode não ter ocorrido exclusivamente devido ao efeito específico da droga sobre a ação da CaN, mas também pelo efeito inespecífico sobre outras proteínas que não estão associadas a inibição da CaN do músculo esquelético. Tal fato sugere que outras vias moleculares podem estar envolvidas na modulação dos diferentes tipos de fibras do músculo esquelético.

O avanço no entendimento dos eventos moleculares que mediam as alterações fenotípicas musculares, em particular atenção para as vias intracelulares mediadas pelas oscilações na $[Ca^{+2}]$ intracelular, pode ser de suma importância para o aprimoramento dos programas de exercício/treinamento físico, e futuras intervenções farmacológicas em situações de miopatia adquirida e congênita. Portanto, o papel da CaN no controle do fenótipo muscular sob diferentes padrões de ativação muscular tem despertado amplo interesse de pesquisadores no campo da saúde e do esporte. Chin et al. (28)

argumentam que medicamentos e terapia gênica capazes de modificar seletivamente a atividade da CaN no músculo esquelético poderia ser uma estratégia adequada para aumentar a capacidade aeróbia e reduzir os riscos de doenças potencialmente fatais em sujeitos humanos. Vale ressaltar que a identificação de uma única via molecular que regula a expressão de um único gene em um determinado tipo de fibra não deve ser descrito como o mecanismo chave para os múltiplos eventos moleculares que regulam as características fenotípicas musculares em resposta a atividade. Vários genes são expressos em um único tipo de fibra e os eventos que mediam a expressão múltipla de vários genes nos diferentes tipos de fibras permanece não esclarecido. Os pesquisadores são encorajados a esclarecer a interação entre as vias moleculares no controle de um único gene promotor, para caracterizar as mudanças dos tipos de fibras musculares em diferentes músculos durante diferentes estímulos. Simplificar o processo de sinalização celular para explicar que a inativação ou ativação de uma proteína específica pode não ser uma idéia favorável, visto que cada proteína da cascata de sinalização pode apresentar um papel diferente no controle da expressão gênica. Evidentemente, as diferentes vias de sinalização intracelular são ativadas de acordo com a especificidade das respostas funcionais, na qual múltiplos processos são necessários para regular a expressão de genes músculo-específicos responsáveis pelas alterações das propriedades contráteis e metabólicas das fibras musculares.

4. Mecanismos Envolvidos no Controle da Hipertrofia Muscular

O treinamento resistido (TR) tem sido divulgado como um potente estímulo, capaz de promover a hipertrofia e induzir à modulação dos tipos de fibras e isoformas de MHC, aumentando a força e a potência muscular (17, 22, 68). A magnitude e especificidade das adaptações musculares aos diversos modelos e protocolos de TR (freqüência, intensidade, duração), são dependentes de alterações na expressão gênica (conteúdo de mRNA) e na tradução de proteínas músculo-específicas (95, 105), que promovem o aumento da síntese de proteínas miofibrilares. O conteúdo de proteínas miofibrilares representa aproximadamente 85% do volume da fibra muscular (57). Assim, as alterações no balanço entre a síntese e degradação de proteínas miofibrilares podem contribuir para o aumento ou redução da massa muscular. Nesta linha de investigação, vários trabalhos apontam um aumento da síntese protéica, após uma única sessão de treinamento resistido, que pode permanece elevada por até 24, 36 e 48 h (78, 93, 99). Neste contexto, Laurent et al. (71) sugerem que os estágios iniciais de aumento

da síntese protéica, são mediados por uma maior eficiência traducional. Já, em níveis posteriores, o aumento de mRNA torna-se crítico para a continuidade deste processo (2).

Em adição, Booth et al. (19) argumentam que o fator determinante da taxa de síntese protéica pode não ser a concentração total de mRNA, mas sim uma maior eficiência no processo de tradução. Em humanos, Welle et al. (132), demonstraram que o exercício pode estimular a síntese protéica sem alterar as concentrações de RNA total e mRNA. Os autores argumentam que se houver uma maior disponibilidade de ribossomos e de fatores traducionais (fatores de iniciação e elongação) para traduzir os mRNAs, o aumento da concentração de mRNA pode levar ao aumento da síntese protéica. Neste sentido, Psilander et al. (95) sugerem que o aumento da síntese protéica seja resultado de uma melhora transcricional, de uma maior estabilidade das moléculas de mRNA, de uma maior taxa de tradução, ou a combinação desses processos. Consistente com o aumento da síntese protéica, Staron et al. (117), mostraram um aumento de 10 a 30% na área de secção transversal (AST) das fibras musculares de indivíduos sedentários, após 12 semanas de treinamento resistido. Em adição, vários trabalhos relatam que o treinamento resistido crônico pode afetar positivamente a resposta aguda de síntese protéica muscular, após uma única sessão de exercício resistido (94, 97), que favorece uma maior resposta anabólica muscular.

Teoricamente, o número de mionúcleos é o fator determinante da taxa de síntese protéica, uma vez que fornecem a quantidade necessária de DNA para suportar o aumento da transcrição. A quantidade de citoplasma (área da fibra) controlada por um único mionúcleo caracteriza o conceito de domínio mionuclear (26). Em humanos, Petrella et al. (88, 89) sugerem que até um limite moderado de hipertrofia, que alcance um tamanho máximo de ~ 2.000-2.250 μm^2 na área do domínio mionuclear, o aumento na AST das fibras é suportado, sem a necessidade de acrescentar novos mionúcleos. Tal fato ocorre devido à capacidade dos mionúcleos existentes na fibra de intensificar o processo de tradução e, assim, promover um aumento da síntese protéica. Porém, em modelos de treinamento resistido de longo prazo, que envolvem sessões repetidas de exercício, o aumento da AST das fibras pode exceder um volume citoplasmático suportável pelo mionúcleo. Neste caso, a adição de novos mionúcleos é necessária para suprir a hipertrofia das fibras musculares (5, 64).

Com base no conhecimento de que os mionúcleos das fibras musculares maduras são considerados pós-mitóticos (não apresentam capacidade de divisão), sugere-se que a

adição de novos mionúcleos seja realizada somente pelas células satélites (CS), também denominadas de células miossatélites. As CS são precursores miogênicos com intensa atividade mitogênica, que contribuem para o crescimento muscular pós-natal, para a regeneração das fibras musculares danificadas e para a manutenção do músculo esquelético adulto (111). O número de CS é dependente da espécie, da idade e do tipo de fibra considerado, e sua freqüência varia ao longo da fibra muscular, sendo 20% maior na região da junção mioneural e próximo de capilares sanguíneos (107). Além disso, os músculos de contração rápida de ratos apresentam menor porcentagem de CS, em relação aos músculos de contração lenta (46).

As CS são mitoticamente quiescentes no músculo adulto, mas em resposta a miotraumas severos, que sobrevenha a necrose da fibra (por ex: ação de toxinas ou distrofia muscular), ou miotraumas adaptativos (por ex: exercício resistido), estas células retomam o ciclo celular e proliferam-se para formar uma nova população de mioblastos (55). Uma pequena proporção de mioblastos regressa ao estado quiescente e restabelecem a população de CS (35), enquanto os mioblastos comprometidos com a regeneração migram para a região danificada e fundem-se na fibra muscular pré-existente para reparar o local do miotrauma e/ou adicionar núcleos para ampliar a taxa de síntese protéica, promovendo assim a hipertrofia muscular (Figura 3).
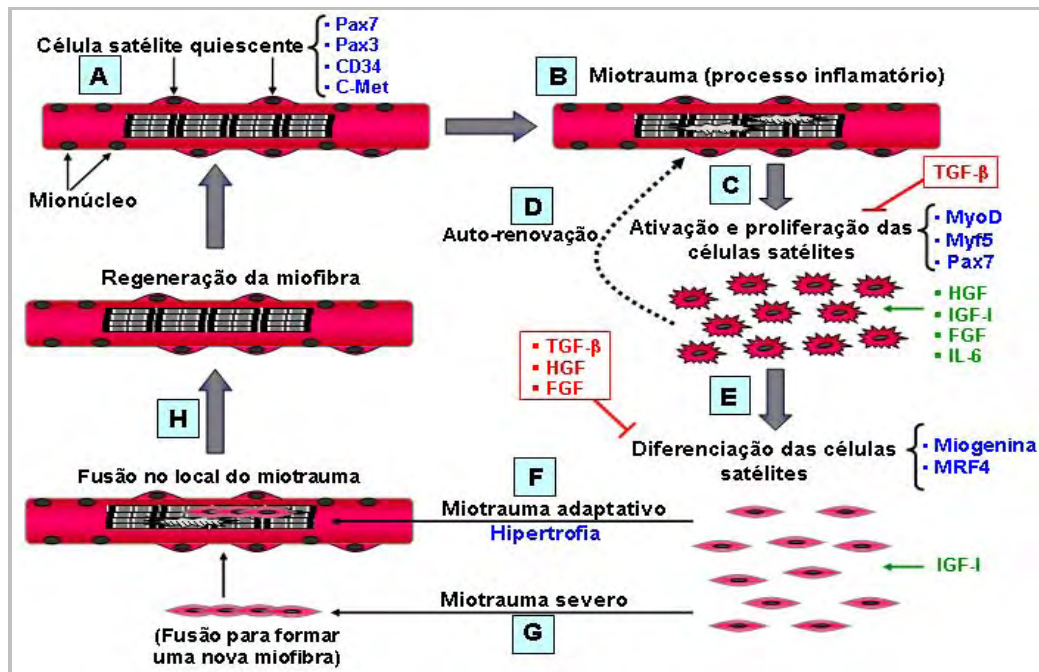
Figura 3. Regeneração Muscular. Fibra muscular normal com célula satélite quiescente e mionúcleo (A). Após um miotrauma (B), as células satélites quiescentes são ativadas, proliferam-se (C) e se diferenciam em mioblastos (E). No miotrauma adaptativo do exercício físico, os mioblastos migram para a região danificada e fundem-se à fibra muscular pré-existente para reparar o local da microlesão e/ou adicionar núcleos para ampliar a taxa síntese protéica (hipertrofia) (F). Porém, em situações de miotraumas severos que ocorra necrose das fibras (ação de toxinas e distrofia), os mioblastos poderão se alinhar e fundir-se entre si, para formar uma nova miofibra (G), e reparar o dano da fibra muscular (H). Durante o processo de regeneração, alguns mioblastos retornam ao estado quiescente e restabelecem a população de células satélites (D). A fase de ativação das células satélites é caracterizada pela alta expressão de MyoD e Myf5, e na diferenciação ocorre um aumento na expressão de Miogenina e MRF4 (Azul). Cada estágio do processo de regeneração é mediado por fatores de crescimento, que atuam como reguladores positivos (verde) ou reguladores negativos (vermelho). Adaptado de Charge e Rudnick, (25).

Consistente com a participação das CS no processo de hipertrofia, Kadi et al. (65) observaram em humanos um aumento do número de CS, após 30 (19%) e 90 (31 %) dias de treinamento resistido, porém, o número de mionúcleos permaneceu inalterável por todo período de treinamento. Os autores argumentam que as mudanças moderadas no tamanho (~ 20%) da fibra muscular podem ser alcançadas, sem a adição de novos mionúcleos. Já, em músculo humano suportando acentuada hipertrofia (AST da fibra > 25%), a adição de novos mionúcleos, via recrutamento de células satélites,

parece ser necessária para suportar o aumento da AST das fibras (89). Segundo os autores, a presença basal de CS é o fator determinante, em resposta ao treinamento, para aumentar a quantidade de CS, incorporar novos núcleos e atingir maior hipertrofia muscular.

O controle e ativação das CS podem ser influenciados pela expressão de vários fatores de crescimento, que podem atuar como reguladores positivos, como os hormônios (GH, *growth hormone*; insulina, *insulin* e testosterona, *testosterone*), os fatores de crescimento (IGF-I, *insulin-like growth factor;* HGF, *hepatocyte growth factor* e FGF, *fibroblast growth factor*) e as citocinas (IL-6, *interleukin-6* e IL-15, *interleukin-15*); e reguladores negativos, como o TGF-β (*Transforming growth factor beta*). Além disso, as CS tanto no estado de quiescência como no estado ativado, expressam marcadores miogênicos (55), que controlam as fases de ativação, proliferação e diferenciação celular durante o processo de reparo muscular (111). Entre os marcadores miogênicos destacam-se os fatores de regulação miogênica (MRFs, *myogenic regulatory factors*), que são proteínas pertencem à família dos fatores transcricionais "basic helix-loop-helix" (BHLH), da qual fazem parte a MyoD, Myf5, Miogenina e MRF4 (111).

Os MRFs compartilham um domínio altamente conservado, conhecido como "basic helix-loop-helix" (bHLH), que é necessário para a ligação com proteínas E, como E12 e E47. Os MRFs ligam-se a seqüências de DNA (5´-CANNTG-3´) conhecidas como Ebox, presentes na região promotora de vários genes músculo-específicos, como a desmina, a troponin-I e a cadeia leve da miosina (MLC, *myosin light chain*) (72, 73, 133). As fases de ativação e proliferação das CS é caracterizada pela alta expressão de MyoD e Myf5, enquanto na diferenciação ocorre um aumento na expressão de Miogenina e MRF4 (140). Juntos, os MRFs são comumente utilizados como marcadores das fases de proliferação e diferenciação das CS durante o processo de hipertrofia muscular. Psilander et al. (95), demonstraram um aumento significante nos níveis de mRNA para Miogenina, MyoD e MRF4 no músculo vasto lateral de indivíduos jovens (23,9 ± 2,2 anos) fisicamente ativos, após uma única sessão de treinamento resistido. Em adição, Bickel et al. (17), observaram um aumento transitório na expressão de mRNA para MyoD e miogenina, no músculo vasto lateral de sujeitos jovens (25 ± 4 anos), após uma única sessão de contração máxima, por meio de estimulação elétrica neuromuscular. Coletivamente, os resultados destes estudos suportam a idéia de que os MRFs estejam comprometidos com o processo de hipertrofia

muscular. Tal fato tem despertado o interesse de muitos pesquisadores para buscar compreender as possíveis interações existentes entre a expressão dos MRFs e ativação das vias moleculares promotoras da hipertrofia muscular, como a via do fator de crescimento IGF-I.

5. Controle Molecular da Síntese Protéica

Além do processo de hipertrofia, as adaptações fenotípicas musculares (por exemplo: a modulação dos tipos de fibras e isoformas de miosinas; a síntese de enzimas glicolíticas e oxidativas), observadas em diferentes modelos e protocolos de exercício/treinamento físico, são dependentes dos estímulos fisiológicos extrínsecos (estresse mecânico, estiramento) e intrínsecos (níveis de cálcio intracelular, hipóxia e estado redox), que afetam o tecido muscular (11, 129). Tais estímulos são transduzidos por receptores de superfície celular (moléculas transmembranas), ativando uma "cascata" de moléculas intracelulares que integram esta informação (129) e, assim, controlam as mudanças quantitativas e qualitativas no músculo, por meio da ativação ou repressão de genes músculo específicos (15). Dentre as vias moleculares, destaca-se a via da Calcineurina (proteína fosfatase dependente da Ca^{+2}/Calmodulina, *calmodulin-dependent protein phosphatase*).

Investigações recentes apontam a via da Calcineurina (CaN) como um sensor molecular de atividade contrátil, sendo ativada em resposta as elevações sustentadas na concentração de Ca^{+2} intracelular, após a ligação entre o Ca^{+2} e a proteína Calmodulina (Ca^{+2}- Calmodulina) (28). A CaN ativa será responsável pela retirada do fosfato ligado ao substrato NFAT (*nuclear factor of activated T cells*), que em condições basais encontra-se no sarcoplasma (28). Após a retirada do fosfato, o NFAT é translocado para a região promotora nuclear (DNA) (96) e irá promover a ativação de genes músculo-específicos (28, 38). Pesquisas recentes apontam à participação de várias outras vias moleculares no controle do fenótipo muscular, incluindo a via das proteínas kinases CaMKs, *Ca^{+2}/calmodulin-dependent protein kinases* (30) e AMPKs, *AMP-activated protein kinases* (66); a via do NF-kβ, *nuclear factor Kappa β* (77); a via do MEF2, *myocyte enhancer factor-2* (30), além dos hormônios insulina, *insulin* (30, 129) e IGF-I, *insulin-like growth factor* (126). As diferentes vias de sinalização intracelular são ativadas de acordo com a especificidade das respostas funcionais, na qual múltiplos processos são necessários para regular a expressão de genes músculo-específicos

responsáveis pelas alterações das propriedades contráteis e metabólicas das fibras musculares.

Nesta linha de investigação, vários estudos demonstram que o IGF-I pode atuar como potente sinal anabólico no tecido muscular (47, 48). A importância do IGF-I na promoção da hipertrofia muscular foi demonstrada em estudos que monitoraram o aumento da expressão do IGF-I em associação com o aumento da massa muscular. A expressão de IGF-I no músculo promove significante hipertrofia e concomitante aumento da produção de força (47). Contudo, o efeito anabólico do IGF-I pode ser potencializado pela carga mecânica, assim como observado nos modelos de treinamento resistido e hipertrofia compensatória induzida pela sobrecarga gerada após a retirada de músculos sinergistas (48). O IGF-I pode atuar de modo autócrino ou parácrino, e/ou através das vias miogênicas, pela ativação das células satélites (47). Em adição, os sinais mecânicos específicos que atingem as células musculares, como por exemplo, a perturbação mecânica ocasionada pela contração durante o exercício físico, induz à liberação do IGF-I, que se liga ao receptor na superfície celular e, conseqüentemente, ativa uma "cascata" de eventos intracelulares, por meio da via IRS (*insulin-receptor substrate*) →PI3K (*phosphatidylinositol 3-kinase*) →AKT (*serine/threonine kinase*) →mTOR (*mammalian target of rapamycin)* →p70S6K (*p70 S6 kinase*), que promove aumento da tradução protéica. Inicialmente, o IGF ligado ao receptor irá recrutar o IRS (*insulin response substrate*), promovendo a ativação da PI3K (*phosphatidyl-inositol-3 kinase*), que adiciona fosfato a AKT (*serine-threonine kinase)*, tornando-a ativa para fosforilar a mTOR (*mammalian target of rapamycin*) e, subseqüentemente, a p70S6K (*p70S6 Kinase*), que atua como potente estimulador da síntese protéica (47, 126).

Pesquisas recentes avançam no entendimento das respostas celulares e moleculares envolvidas no controle da expressão de genes relacionados ao processo de adaptação e modulação do perfil fenotípico muscular, na tentativa de responder uma questão fundamental no campo da ciência dos esportes: Como o músculo se adapta ao exercício físico? A compreensão dos eventos celulares e moleculares mediadores do processo de hipertrofia muscular podem fornecer estratégia para o desenvolvimento de fármacos terapêuticos eficazes para atenuar a perda progressiva da massa e força muscular decorrente de processos fisiológicos (por ex: envelhecimento) e patológicos (por ex: câncer, doenças neuromusculares, insuficiência respiratória e sepsia). Além disso, caracterizar o perfil de expressão gênica em resposta a hipertrofia compensatória do músculo esquelético provavelmente contribuirá para predizer as respostas individuais

de adaptação ao exercício/treinamento físico, possibilitando uma maior eficácia na prescrição do treinamento.

II – OBJETIVOS

Avaliar os efeitos do treinamento resistido a longo prazo sobre as adaptações morfológicas e expressão gênica dos fatores de regulação miogênica (MRFs) e do fator de crescimento semelhante à insulina (IGF-I) no músculo esquelético de ratos.

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IV - CÁPITULO

Effects of long-term resistance training on myogenin, MyoD and IGF-I mRNA expression in rat skeletal muscle

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ABSTRACT

Although strong evidence show that the myogenic regulatory factors (MRFs) and insulin-like growth factor (IGF-I) have important roles in the hypertrophy response after acute resistance training, it is still unclear if response of MRFs and IGF-I also occurs during the adaptation to prolonged periods of resistance training (RT). Therefore, the purpose of this study was to utilize an animal model to test the hypothesis that fiber-types transition and hypertrophy during long-term RT could be associated with increased MRFs and IGF-I mRNA expression in the skeletal muscle. Male *Wistar* rats (80 days old, 250-300 g) were divided into four groups: 8 weeks control (C8, $n = 8$), 8-weeks trained (T8, $n = 8$), 12-weeks control (C12, $n = 8$), 12-weeks trained (T12, $n = 8$). T8 and T12 groups were submitted to a progressive RT program (3 day/week) for 8 and 12 weeks, respectively. The training protocol consisted of four sets of 10–12 repetitions, with a 40 s rest period between each set, performed at 65–75% of one repetition maximum (1RM). At the end of the experiment, animals were sacrificed and the plantaris muscle collected for morphological and molecular analysis. The RT did not change ($p > 0.05$) in body weight gain and food intake in the T8 and T12 compared to the C8 and C12 groups, respectively. After 8 and 12 weeks of RT, the absolute (T8: 69.7% vs. T12: 126.0%; $p < 0.05$) and relative (T8: 36.1% vs. T12: 57.7%; $p < 0.05$) strength (relative 1RM) was significantly elevated in the T8 e T12 groups, compared to respective control groups. RT for 8 and 12 weeks induced similar increase in myogenin (T8: 44.8% vs. T12: 37.7%; $p > 0.05$), MyoD (T8: 22.9% vs. T12: 22.3%; $p > 0.05$) and muscle fiber cross-sectional area (CSA) (T8: 29% vs. T12: 35%; $p > 0.05$) in the T8 and T12, compared to C8 and C12 groups, respectively. After 8 weeks of RT, IGF-I

increased in 30.1% in the T8 compared to C8 group, but returned to baseline after 12 weeks of RT. The modulation of MHCIIx/d-to-MHCIIa isoforms and fiber IIX/D-to-IIA transition was evident only after 12 weeks of RT. The data show that the hypertrophic stagnation during long-term RT was associated with a maximal limit of myogenin and MyoD mRNA expression and with the return of IGF-I mRNA levels to baseline. In addition, the increase in myogenin, MyoD and e IGF-I expression after 8 weeks of RT was not associated with changes in the MHC content and fiber-types frequency. Therefore, the results indicate a possible interaction between MRFs and IGF-I in the control of muscle hypertrophy during long-term resistance training and suggest that these factors are more involved in the control of muscle mass than in fiber-type transitions.

Key Words: skeletal muscle; hypertrophy; muscle fibers; training; myogenic regulatory factors; IGF-I.

INTRODUCTION

Skeletal muscle displays a wide ability to alter the morphology and phenotypic features in response to specific stimuli, such as physical training (12). Whereas endurance training frequently leads to minor changes in muscle mass, strength training induces marked muscle hypertrophy (15). For example, the resistance training (RT) during 10 and 12 weeks promoted a 10 to 30% increase in muscle fiber cross-sectional area (CSA) in sedentary subjects (55); this rise reaching about 80% in weightlifting athletes (29). Muscle hypertrophy is directly associated with increased myofibrillar protein content (17) by increase in protein synthesis which enables new contractile filaments to be added to the pre-existing muscle fiber; this, in turn, enables the muscle to generate greater force (16). While the exact mechanisms involved in the increased expression of contractile proteins are unclear, two of the major molecular pathway in regulation of muscle hypertrophy are myogenic regulatory factors (MRFs) and insulin-like growth factor-I (IGF-I).

The MRFs are a superfamily of transcription factors that regulate the expression of several skeletal muscle-specific genes (44). The family is composed of four members: myogenin, MRF4, MyoD and Myf5. These proteins belong to a larger basic helix-loop-helix (bHLH) class of transcription factors that, after dimerization with a ubiquitous E protein, bind to an E-box domain and promote the expression of muscle-specific genes (11), thus potentially influencing hypertrophy. The MRFs are upregulated during muscle hypertrophy when satellite cells (SC) are required to repair the load-induced muscle damage. The steps associated with the proliferation, differentiation and fusion of SC to damaged myofibers (hypertrophy) are generally well established (for review see ref. 13). Briefly, MyoD is predominantly upregulated during the SC proliferation (54), which from new cells known as myoblasts. Myoblasts differentiation is marked by the upregulation of the MRF4 and Myogenin (54). Then, MRFs are often used as markers of myogenic cell differentiation in settings associated with muscle formation or muscle hypertrophy (22, 47).

Increased MyoD and myogenin gene expression has been shown in human and animal studies that investigated the acute response to RT (47, 8, 58). Psilander et al. (47) conducted an acute exercise protocol involving eight sets of 8–12 repetitions, and biopsy samples were collected serially (pre- and 0, 1, 2, 6, 24, and 48 h postexercise) after exercise. The authors showed that the Myogenin, MyoD and mRNA expression

were elevated by 100-400% 0–24 h postexercise, In addition, Bickel et al. (8) also observed a transient increase in myogenin (~3-fold) and MyoD (83%) mRNA expression in human muscle after a single bout of RT induced by neuromuscular electrical stimulation. The results of these studies support the hypothesis that the MRFs, particularly MyoD and myogenin, may be involved in regulating hypertrophy induced by acute RT.

MyoD and myogenin have also been implicated in regulating muscle-fiber type; myogenin has been found to accumulate in slow-twitch and MyoD in fast-twitch fibers (27, 60). Moreover, Mozdziak et al. (40) showed that myogenin and MyoD mRNA levels are more implicated with myosin heavy chain (MHC) isoforms composition changes than with muscle mass alterations. This hypothesis was confirmed by Willoughby and Nelson (62) that showed a positive correlation between the increase in gene expression of type I, IIA, and IIX MHC isoforms and MyoD and myogenin mRNA expression after a single session of RT. Although considerable evidence from time-course studies involving acute RT suggest that the MRFs may play an important role in controlling muscle hypertrophy and the fiber-type transition, remains unknown whether the hypertrophic and phenotypic response of skeletal muscle induced by long-term RT could be associated with the increase in MyoD and myogenin mRNA expression.

In addition to the role of MRFs in skeletal muscle hypertrophy, IGF-I has been characterized as a strong anabolic agent by acting in two different ways during load-induced muscle hypertrophy, 1- by stimulating SC to proliferate and differentiate during the process of compensatory hypertrophy (1), and/or 2- by activating a cascade of signaling pathways *via* phosphatidylinositol 3-kinase (PI3K)/Akt/ mammalian target of rapamycinm (mTOR)/ p70 S6 kinase (p70S6K), resulting in the downstream activation of targets which are required for protein synthesis (10, 16). The "mature" IGF-I gene comprises exons 3 and 4 of the IGF-I gene; during gene processing, alternative splicing occur resulting in three IGF-I transcripts (IGF-IEa, IGF-IEb and IGF-IEc, also known as MGF). Some authors have shown that IGF-I transcripts gene expression are upregulated following acute high-intensity resistance exercise, albeit with different time courses (47, 9, 5). However, contradictory results have been observed in different species and RT protocols (63, 39, 5, 23, 47). According to Psilander et al. (47) the differential expression of the IGF-I transcripts may be associated with different stimulus that affect skeletal muscle. While discrepancy results have been found on IGF-I

transcripts (IGF-IEa, IGF-IEb, and MGF) expression, an increase in "mature" IGF-I gene expression during the acute RT has been well reported (5).

The importance of IGF-I in regulating muscle hypertrophy was demonstrated in studies that monitored the increased IGF-I expression in combination with increased muscle mass (18, 46). For example, transgenic mice in which IGF-1 expression is increased using a muscle-specific promoter have muscles that are at least twofold greater in mass when compared with wild-type mice (41). In addition, increased muscle loading in augmented IGF-1 expression has been shown in both human and animal models (14, 5), resulting in increase of muscle mass. Although several lines of evidence support a strong association between increased muscle mass and gene expression of IGF-I and MRFs, our current knowledge on the possible effects of IGF-I and MRFs on muscle hypertrophy induced by long-term RT remains to be elucidated. Considering the close relationship of IGF-I with the initial increase in muscle mass during acute RT, and the important role of MRFs in the control of CS during the muscle repair of local tissue injury, we speculate that myogenin, MyoD and IGF-I gene expression could be a determinant factor for increased muscle mass and phenotypic changes during long-term RT. Therefore, the aim of the present study was to test the hypothesis that hypertrophy and fiber-types transition during long-term RT could be associated with an increase in myogenin, MyoD and IGF-I mRNA expression in the skeletal muscle. To our knowledge, the present study provides the first data on the plantaris muscle MRFs and IGF-I mRNA expression during long-term RT.

METHODS

Research design. An animal model was used to test the hypothesis that hypertrophic and phenotypic response of skeletal muscle induced by long-term RT could be associated with an increase in MyoD, myogenin and IGF-I gene expression. To this purpose, the animals were submitted to a progressive RT program during 8 or 12 weeks using a weight lifted model well established, that transcribes the traditional human squat exercise (57). To ensure the same training intensity throughout the experiment period, overload adjustments were made biweekly, through the test of one-repetition maximum (1RM). The relative intensity (65-75% of 1RM) and volume (four sets of 10-12 repetition) of training were the same in the in all trained rats, ensuring the same stimulus intensity to the recruited muscles. We ensured that this protocol provided an effective manner to investigate the effects of RT on the muscle morphological and molecular parameters. At the end of the experiment, the plantaris muscle of right and left legs was removed and its weight was normalized to body weight (muscle weight/body weight ratio). The middle portion of right muscle was collected for morphometrical, histochemical and biochemical analysis. The left muscle was collected for molecular analysis. Samples were kept at -80ºC until use. The animal model provided the unique and accurate way to isolate single muscles and perform analysis on whole muscle preparations, reflecting the total muscle response. In addition, our animal model ensures total control over the variables (e.g., food intake, lifestyle, motivation, and technique of movement) that can affect the phenomenon investigated, allowing a reliable response when these same relationships are tested in human subjects. We investigated the plantaris muscle because it is highly recruited in our model of RT and it possesses a mixed fast-twitch fibers-based phenotype, indicating a significant capability to support the increased overload during the long-term RT.

Animals and experimental groups. Thirty-two male *Wistar* rats (80 days old, 250-300 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, São Paulo, Brazil). They were housed in collective polypropylene cages (4 animals per cage) covered with metallic grids, in a temperature-controlled room (22-24ºC) under a 12-hour light–dark cycle, and provided with unlimited access to standard rat chow and water. We used the independent variable (training) to examine its effects on muscle parameters (dependent variables). For this purpose, rats were randomly divided into four groups: 8 weeks control (C8, *n* = 8), 8-

weeks trained (T8, $n = 8$), 12-weeks control (C12, $n = 8$), 12-weeks trained (T12, $n = 8$). This experiment was approved by the Biosciences Institute Ethics Committee, UNESP, Botucatu, SP, Brazil (Protocol No. 73/07-CEEA) and was conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Training protocol. All rats performed progressive RT program on a squat apparatus as described by Tamaki et al. (57) (Fig. 1). Rats fitted with a canvas jacket were able to regulate the twisting and flexion of their torsos and were suspended in a standard position on their hind limbs. An electrical stimulation (1 Hz/ 1 ms duration / active cycle 2:4 s/ current intensity ranging from 4 a 15 mA) was applied to the rat's tail through a surface electrode. As a result, the rats flexed their legs repeatedly, which lifted the weight-arm of the training apparatus. Rats remained placed in the squat apparatus until completion of the resistance exercise protocol. To ensure the same training intensity throughout the experiment period, overload adjustments were made biweekly, through the test of one repetition maximum (1RM). Each rat was electrically stimulated to perform as many repetitions as possible with a pre-established weight, and based on the number of repetitions achieved by each rat the weight was adjusting with increments of 100, 50, 25 and/or 10 g, until the animal perform 1RM. No more than three 1RM attempts were performed for each rat within this study. Five minutes of rest were given between maximal lifts. The 1RM was defined as the highest weight lifted from squat to standing position. The T8 and T12 groups performed four sets of 10-12 repetitions, with a 40 s rest period between each set, at 65–75% of one repetition maximum (1RM) three times per week. The C8 and C12 control group's rats were placed in the squat apparatus for the same time period that the trained groups, but did not receive any electrical stimulation or perform resistance exercise. We chose use a control group that received neither electrical stimulation nor exercise because previous research has indicated that electrical stimulation alone does not influence muscle hypertrophy (7, 57). Before the initial training program, animals performed a 2-week pretraining (once a day) to familiarize them with the squat apparatus and execution of exercise. In the 1st week, rats were placed in the squat apparatus during 20 min, without receiving electrical stimulation. In the 2st week, rats received electrical stimulation and performed two sets of 5-10 repetitions at 40-60% of body weight. All training sessions were performed between 2 and 3 pm in a dark room.
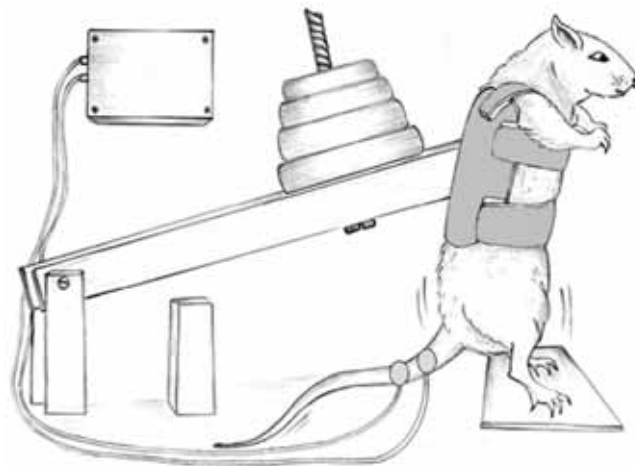
Fig. 1. Sketch of the resistance training apparatus. Adapted from Tamaki et al. (57)

Anatomical data. Throughout the experiment the body weight and food intake of animals were monitored daily. At the end of the respective training periods the animals were anesthetized with pentobarbital sodium (40 mg/kg IP) and sacrificed by decapitation. The plantaris muscle of right and left legs was removed and its weight was normalized to body weight (muscle weight/body weight ratio). The middle portion of right muscle was collected and frozen in isopentane cooled in liquid nitrogen (–156 °C) for morphometrical, histochemical and biochemical analysis. The left muscle was directly frozen in liquid nitrogen at –156ºC for molecular analysis. Samples were kept at –80ºC until use.

Morphometrical analysis. Plantaris histological sections (10μm thick) were obtained in a cryostat (JUNG CM1800, Leica, Germany) at –24°C and stained with hematoxylin and eosin (HE). The stained sections were used for photographic documentation of three random histological fields (20μm lens) of each animal. The images were obtained in a microscopy connected to a computer. Muscle hypertrophy was determined by measurement of cross-sectional area (CSA) of the ~ 400 muscle fibers of each animal (Fig. 4A, B, C and D). This corresponds to approximately 80% of amount plantaris muscle fibers. Cross-sectional area (CSA) measure was performed using an image analysis system (software Leika QWin Plus, Germany).

Histochemical analysis. Muscle samples previously stored in a freezer at –80°C were thawed to –20°C and sectioned serially (10μm thick) for histochemical analysis. To determine the muscle fiber-type percentages, myofibrillar adenosine triphosphatase

(mATPase) histochemistry was performed using preincubation at pH 4.2, 4.5 and 10.6. Analyses revealed pure (Type I, IIA, IID and IIB) and hybrid muscle fibers (Type IC and Type IIC) based on their staining intensities (56) (Fig. 2). Muscle fiber–type percentages were determined using Image Analysis System Software (LeicaQWin Plus, Germany).
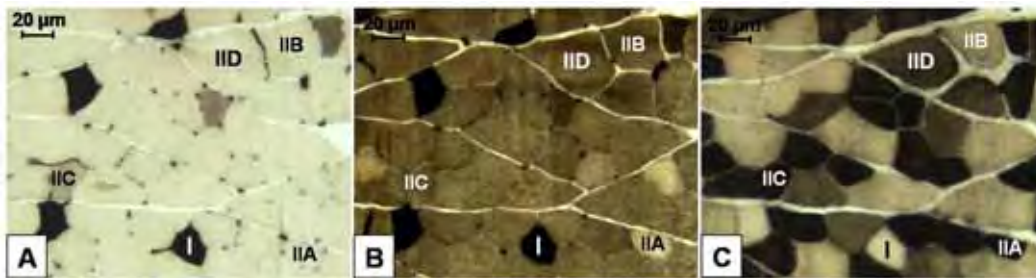
Fig. 2. Serial cross sections of plantaris muscle samples taken from a representative control animal showing fiber-type distribution using myofibrillar adenosine triphosphatase histochemistry after preincubation at pH 4.2 (A), 4.5 (B), and 10.6 (C). *I*, type I fiber; *IIC*, type IIC fiber; *IIA*, type IIA fiber; *IID*, type IID fiber; and *IIB*, type IIB fiber.

Biochemical analyses. Myosin heavy chain (MHC) isoforms analysis was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in duplicate (maximum 5% of variation). Eight histological sections (12-mm thick) were collected from each sample and placed in a solution (0.5 mL) containing glycerol 10% (w/vol), 2- mercaptoethanol 5% (vol/vol), and sodium dodecylsulfate (SDS) 2.3% (w/vol) in a Tris/HCl buffer 0.9% (pH 6.8) (w/vol). The final solution was shaken for 1 minute and heated for 10 minutes at 60ºC. Small amounts (8 μL) of the extracts were submitted to electrophoresis reaction (SDSPAGE 7–10%), using a 4% stacking gel, for 19 to 21 hours at 120V. The gels were stained with Coomassie Blue (6) and used to identify the MHC isoforms according to their molecular weight showing bands at the MHCI, MHCIIa, MHCIIx/d and MHCIIb levels (Figure 7*A*). The gels were photographed and images were captured by VDS Software (Pharmacia Biotech). Finally, densitometry was performed using Image Master VDS Software (version 3.0), which determined the relative MHC isoforms content.

Quantitative analyses of gene expression by RT-qPCR

RNA extraction. Total RNA was extracted from muscle samples with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized in 1 mL of TRIzol reagent. Total RNA was resuspended in RNase-free water, treated with DNase I (Life Technologies, Carlsbad, CA, USA) to remove any possible DNA present in sample. Total RNA was quantified using Nanodrop spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), measuring optical density (OD) at 260nm. RNA purity was ensured when OD260/280 ratio was around 2.0. And RNA integrity was verified by 1% agarose gel electrophoresis, which well defined bands were observed correspondents to 18S and 28S ribosomal RNAs.

cDNA synthesis. cDNA was synthesized from 2µg of total RNA using High Capacity cDNA archive kit (Life Technologies, Carlsbad, CA, USA). Each reaction had 10µL of 10X Reverse Transcription Buffer, 4µL of 25X dNTPs, 10µL of 10X random primers, 100 units of RNase inhibitor (Life Technologies, Carlsbad, CA, USA), 250 units of Reverse Transcriptase MultiScribe™, and the final volume was adjusted to 100µL with nuclease-free water. And cDNA synthesis reaction conditions were: 10 minutes at 25°C for primers annealing and 2 hours at 37°C for reverse transcription. Reaction control was made by omission of the reverse transcriptase enzyme. All cDNA samples were amplified by PCR to ensure that there was no contamination by DNA. The resulting cDNA samples were aliquoted and stored at -20°C. 2µL of cDNA, corresponding to 20ng of total RNA, were used as template in real time PCR reactions which were made in 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with equipment universal thermal cycling conditions: 95°C for 10min, 40 cycles of 95°C for 15s and 60°C for 1min. The reactions were performed in duplicate using 0.4 µM of each primer and 2X Power SYBR Green PCR master mix (Life Technologies, Carlsbad, CA, USA) with final volume of 25 µL.

Quantitative real-time RT-PCR. Series of five dilutions (10x) was constituted from PCR products diluted 500 times, for each gene, from an initial mixture containing equal amounts of cDNA from three extra samples used only for standardization, thus were generated a five-point standard curve for each primer set initially chosen for qPCR. qPCR linearity and efficiency were calculated from those standard curves slope,

generated by 7300 System SDS software (Life Technologies, Carlsbad, CA, USA) for each gene analyzed. The analysis of all standard curves showed high linearity (r2=0.99). PCR efficiency (Ex) was calculated from the equation Ex = 10^{-1}-1/slope-1. Slope of -3.32 implies in a reaction efficiency of 100%. All genes showed slope around -3.32 and estimates of efficiency were between 99.5% and 100.5%. Primers for all genes were obtained using Primer3 software, available at webpage http://frodo.wi.mit.edu/primer3/, from published sequences in GenBank (www.pubmed.com) and synthesized by Life Technologies (Carlsbad, CA, USA) (Table 1). Dissociation curves and agarose gel electrophoresis were performed to confirm the amplification of only one target sequence for each primer. Reaction controls were made without cDNA template to investigate possible contamination of reagents. Gene expression was compared between individual samples using the ΔΔCq method described by Livak and Schmittgen (36). It was also necessary to perform the correction of experimental variability between different samples, for example, amount of RNA and reverse transcription reaction efficiency, prior to the final quantification. Data normalization for at least three reference genes is the most accepted method to avoid such disparities (59). The choice of appropriate genes is crucial for reliable results, and the expression level of these genes must remain unchanged for different experimental conditions (59). Expression level of three genes, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and TATA box binding protein (TBP) (Table 1), was assessed through DataAssist software (Life Technologies, Carlsbad, CA, USA) and then used as reference genes for data results normalization.

Table 1. Oligonucleotide primers used for real-time PCR amplification of transverse

Genes	GenBank Accession No	Sequence (5' – 3')
IGF-I	NM178866	S: GCTATGGCTCCAGCATDCG
		A: TCCGGAAGCAACACTCATCC
MyoD	NM_176079	S: CCTACTACAGTGAGGCGTCCA
		A: GTGGAGATGCGCTCCACTAT
Myogenin	NM_017115	S: AGTGAATGCAACTCCCACA
		A: CGTAAGGGAGTGCAGGTTGT
TBP	NM_001075742	S: ATTTGCCAAGAAGGTGAACG
		A: CCGTAAGGCATCATTGGACT
HPRT	NM_001034035	S: CACTGGGAAGACAATGCAGA
		A: ACACTTCGAGGGGTCCTTTT
GAPDH	NM_001034034	S: AGATGGTGAAGGTCGGAGTG
		A: GAAGGTCAATGAAGGGGTCA

S: primer senso; A: primer antisenso

STATISTICAL ANALYSIS

Statistical analyses were performed using a software package (SPSS for Windows, version 13.0). To ensure that the data were stable, the statistic procedure was accomplished after the preliminary study of the variable related to normality and equality of variance among all groups, with statistical power of 80% for the comparisons performed. Fiber-type frequency data were analyzed using the Goodman Test for contrasts intermultinomial and intramultinomial populations (20, 21) to assess differences among all groups. Statistical comparisons among the groups were made using analysis of variance (ANOVA) for the 1-factor model (65) for body weight, food intake, muscle weight, mRNA expression and MHC isoforms content values. When significant main effects were revealed, specific differences were assessed using Tukey's post hoc comparisons. Data are expressed as mean ± SD. Differences were considered significant with a *p* value of < 0.05.

RESULTS

Body weight and food intake. Throughout the experiment, the body weight evolution of the groups and the final average body weight are shown in Fig. 3 and Table 2, respectively. Confirming that animals initiated the experiment with similar health status, no significant ($p > 0.05$) difference was observed in the initial body weight among the groups (Table 2). After 8 and 12 weeks of experiment, all groups showed significant ($p < 0.05$) body weight gain ($\Delta\%$) (Fig. 3 and Table 2); the values were 21.7%, 26.2%, 30.8% and 33.5% in the T8, C8, T12 and C12 groups, respectively (Table 2). These values were not statistically ($p > 0.05$) different among all groups.

**Table 2.** Initial and final body weight, and body weight gain ($\Delta\%$) in experimental groups.

Groups	Initial body weight (g)	Final body weight (g)	Δ %
T8	336.3 ± 28.6	$429.6 \pm 49.9*$	21.7
C8	308.9 ± 27.1	$418.6 \pm 37.7*$	26.2
T12	326.0 ± 27.8	$471.4 \pm 34.9*$	30.8
C12	302.8 ± 32.3	$455.1 \pm 61.8*$	33.5

Values are means ± SD. *n*= 8 rats per group. C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. * Significant difference compared to their initial body weight at $p < 0.05$.

The similar progression of PC in both trained and control groups demonstrated that the training model used, although intense, did not subject the animals to state of overtraining. The data show that the increase in body weight reflected only the somatic growth of animals, given that they began the experiment in the young phase. Consistent with no significant ($p > 0.05$) change in body weight, resistance training did not promote significant ($p > 0.05$) alteration in food intake in the T8 and T12 groups compared to the C8 and C12 groups, respectively (Figure x).
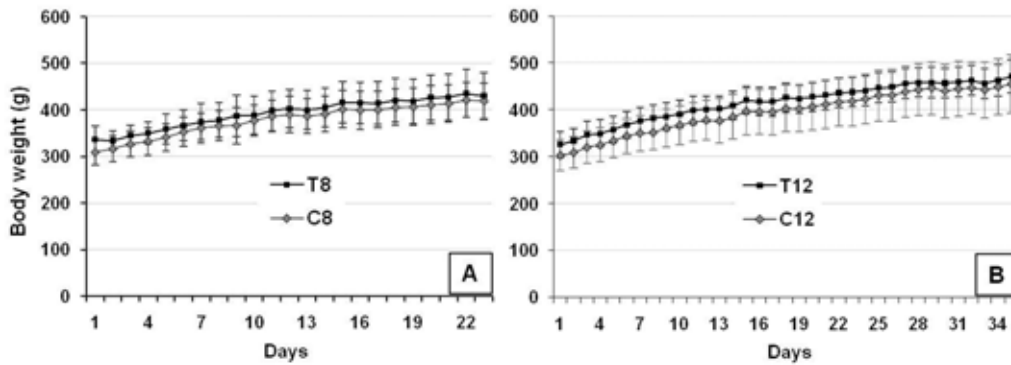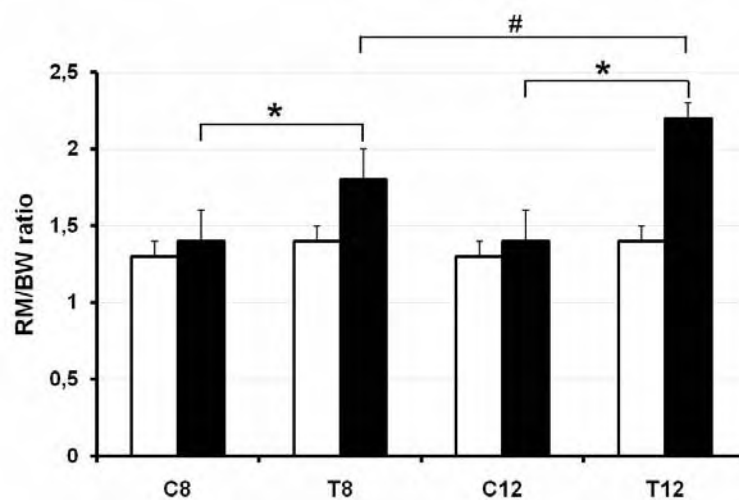
Fig. 3. Body weight evolution along 8 (A) and 12 (B) weeks of experiment. The measurements were undertaken on the first day of the first week following the 2 weeks adaptation period until the last day of training. C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. Values are means ± SD. *n*= 8 rats per group. No significant differences among groups were observed; p > 0.05 (ANOVA + Tukey test).
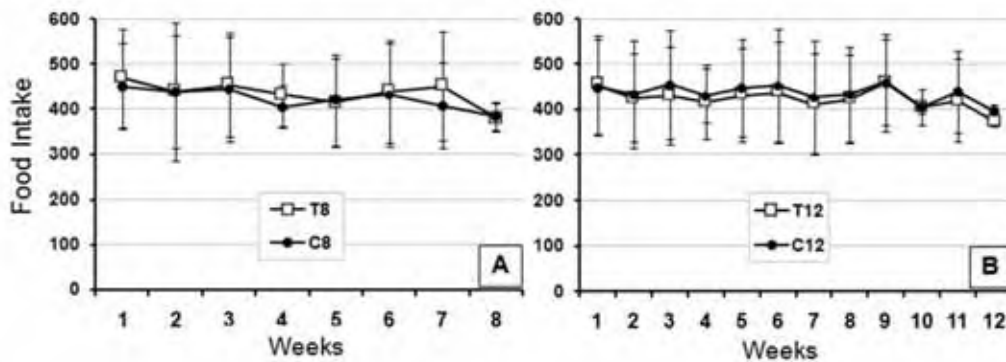
Fig. 4. Food intake along 8 (A) and 12 (B) weeks of experiment. The measurements were undertaken on the first day of the first week following the 2 weeks adaptation period until the last day of training. C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. Values are means ± SD. *n*= 8 rats per group. No significant differences among groups were observed; p > 0.05 (ANOVA + Tukey test).

Maximal strength. The representative sketch of the apparatus used in the training and 1RM test is shown in Fig. 1, and the corresponding data are summarized in Fig. 5. All groups began the training protocol with similar (p > 0.05) absolute 1RM (C8: 450.0 ± 12,9g; T8: 463.0 ± 28.1g; C12: 423.8 ± 30.7g; and T12: 444.8 ± 19.1g), indicating similar levels of maximal force between all groups. However, after the respective training periods the absolute 1RM increased significantly only in the T8 e T12 groups (C8: 28.3% and C12: 33.9% both p > 0.05; T8: 69.7% and T12: 126.0%, both p < 0.05).

Considering that the body weight may influence the maximal strength, 1RM values were normalized by body weight (RM/body weight ratio) (Fig. 5). After 8 and 12 weeks of training there was a significant ($p < 0.05$) increase in relative 1RM in the T8 e T12 groups, while no statistical ($p > 0.05$) difference was observed in their respective control groups (Fig. 5). The relative 1RM in the last week were 36.1% and 57.7% higher in the T8 e T12 groups, compared to C8 e C12 groups, respectively. In addition, the training during 12 weeks promoted an increase ($p < 0.05$) significant of relative 1RM in the T12 group compared to T8 group (Fig. 5).


Fig. 5. Repetition maximum (1RM) relative to bodyweight (RM/BW ratio) of all groups before (□) and after (■) of resistance training. The measurements were undertaken on the first day of the first week following the 2 weeks adaptation period and on the last day of training. C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. Values are means ± SD. *n*= 8 rats per group. * Significant difference of T8 and T12 groups compared to values obtained in their respective controls at level of $p < 0.05$; # Significant difference between T8 and T12 groups at $p < 0.05$ (ANOVA + Tukey test).

Muscle fiber cross-sectional area (CSA) and muscle weight. A representative Hematoxylin and Eosin (HE) staining used to measure the muscle fiber cross-sectional area (CSA) is shown in Fig. 6*A-D* and the corresponding data are presented in Figure 6*E*. After 8 and 12 weeks of the training there was a significant ($p < 0.05$) increase in muscle fiber CSA in the T8 and T12 groups, compared to C8 and C12 groups, respectively (Fig. 6*E*). In fact, there was a gradual increase in plantaris muscle fibers CSA throughout the training period. A direct statistical comparison revealed a

significant ($p < 0.05$) increase of 29 and 35% in plantaris muscle fibers CSA of the groups T8 and T12, compared with their respective controls. In addition, no statistical difference ($p > 0.05$) was observed between C8 and C12 groups (Fig. 6*E*). Despite the continuance of training promoted a higher increase in muscle fiber CSA in T12 comparing to T8 group, the values did not differ statistically ($p > 0.05$) with each other (Fig. 6*E*). Consistent with the muscle fiber CSA values, the plantaris muscle weight and muscle weight/body weight ratio were statistically ($p < 0.05$) higher in T8 and T12, compared to C8 and C12 groups, respectively. However, T8 and T12 groups did not differ significantly ($p > 0.05$) with each other (data not shown).
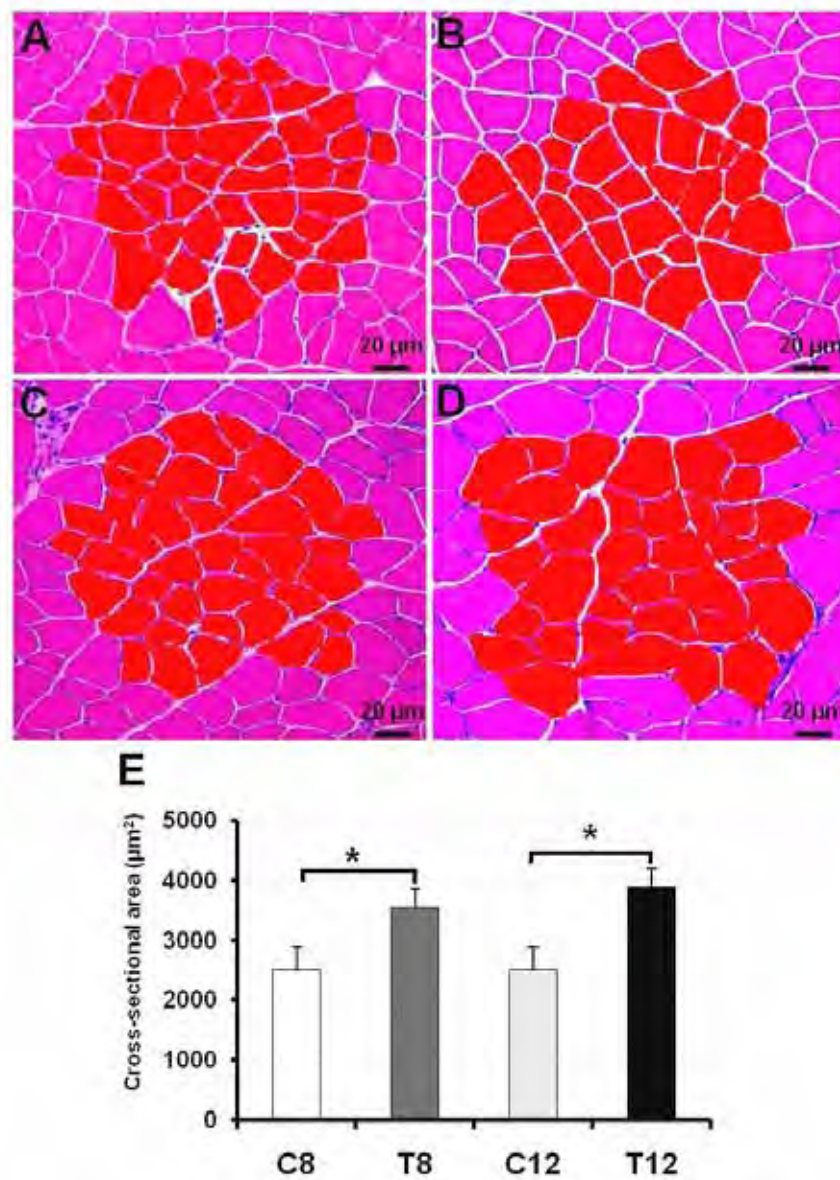
Fig. 6. Histological sections (A, B, C, and D) of plantaris muscle from one representative animal of each group: C8, 8 weeks control (A); T8, 8 weeks trained (B); C12, 12 weeks control (C); and T12, 12 weeks trained (D). Hematoxylin and Eosin stain. Note in fibers marked in red that resistance training during 8 and 12 weeks promoted an increase in muscle fibers cross-sectional area (CSA) in T8 and T12 groups (B and D) compared to C8 and C12 groups (A and C), respectively. (E) Corresponding data of Muscle fibers CSA. Values are means ± SD. *n*= 8 rats per group. * Significant difference between trained and control groups at p < 0.05 (ANOVA + Tukey test).

MHC content and fiber-type frequency. The representative SDS-PAGE gel used to quantify MHC isoforms is shown in Fig. 7*A*, and the corresponding data are summarized in Table 3. Resistance training during 12 weeks promoted a significant ($p < 0.05$) decrease in MHCIIx/d content with reciprocal increase in MHCIIa in the T12 group, compared to the C12 group (Table 3). On the other hand, no significant difference ($p > 0.05$) in the MHCIIa and MHCIIx/d content was observed in T8 group, in relation to the C8 group (Table 3). The training during 8 and 12 weeks did not promote any significant alteration in MHCI and MHCIIb content in the T8 and T12 groups, compared to the C8 and C12 groups respectively (Table 3).

Table 3. Relative myosin heavy chain (MHC) isoforms percentages of plantaris muscle samples determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Groups	MHCI	MHCIIA	MHCIID	MHCIIB
C8	6,3 ± 1,1	12,0 ± 1,0	48,2 ± 3,0	33,6 ± 1,1
T8	4,7 ± 1,6	13,6 ± 2,7	47,2 ± 4,6	34,4 ± 1,6
C12	4,5 ± 3,1	12,5 ± 1,4	48,1 ± 3,6	35,0 ± 3,1
T12	6,1 ± 1,3	16,3 ± 0,5*	41,7 ± 2,7*	36,0 ± 1,3

Values are means ± SD. *n*= 8 rats per group. C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. * Significant difference compared to C12 group at $p < 0.05$ (ANOVA + Tukey test)

The advantages of our study compared with previous work in this area include the full range of histochemical fiber types and relative MHC content (Fig. 7*A*) to validate the histochemical data. Fiber-type frequency for each group is shown in Fig.7*C*, and the corresponding mATPase histochemistry used for fiber-types characterization is shown in Fig. 2 and 8*A*. The significant ($p < 0.05$) increase of MHCIIa content and reduction of MHCIIx/d in the T12 group (Table 3) reflected in a significant ($p < 0.05$) increase in percentages of type IIA fibers and reduction of type IIX/D fibers compared to the C12 group (Fig. 7*C*). In agreement with no significant ($p > 0.05$) change in MHC isoforms content, the training during 8 weeks did not promote any significant ($p > 0.05$) alteration in the frequency of type IIA and IIX/D fibers in the T8 group compared to the C8 group (Fig. 7*C*). No statistical difference ($p > 0.05$) was observed in the frequency of type I and IIB fibers among all groups (Fig. 7*C*).
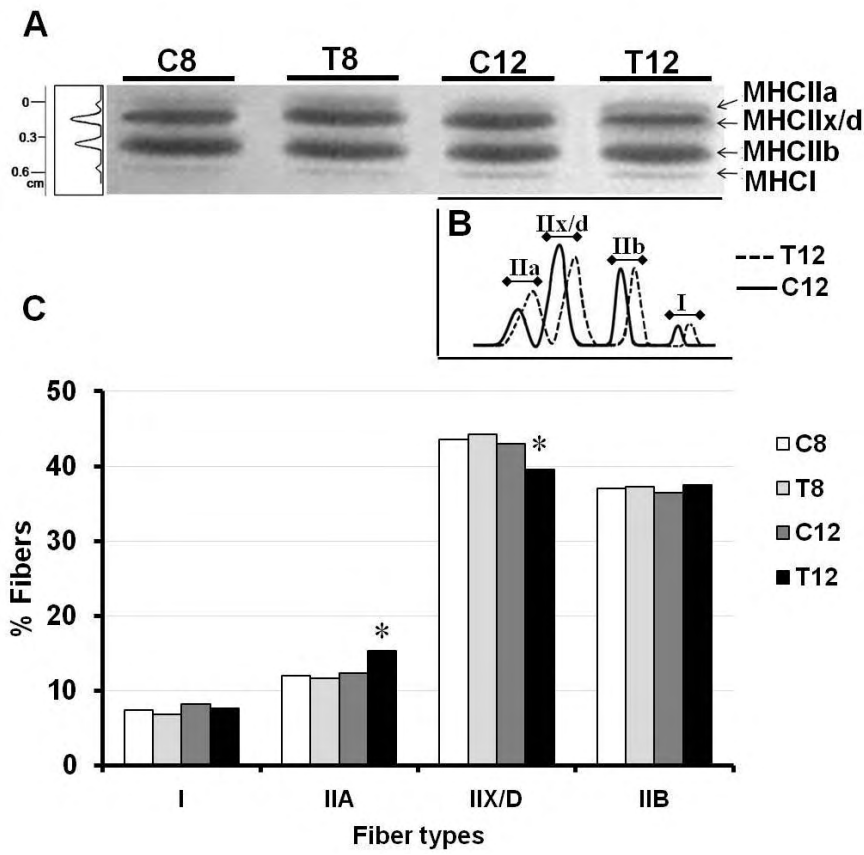
Fig. 7. (A) Electrophoretic separation of myosin heavy chain (MHC) isoforms of plantaris muscle from one representative animal in each group: C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. (B) Densitometric tracings that accompanies the separated MHC isoforms in the C12 (continuous tracing) and T12 (dotted tracing) groups. Arbitrary units were used. Note that training during 12 weeks induced a decrease in MHCIIx/d isoforms content and reciprocal increase in MHCIIa. (C) Plantaris muscle fiber I, IIA, IIX/D and IIB frequency in the groups studied. Values are means ± SD. *n*= 8 rats per group. Interestingly, the MHCIIx/d-to-MHCIIa modulation after 12 weeks of resistance training reflected in the IIX-to-IIA muscle fibers type transition. * Significant difference compared to all other groups at p < 0.05 (Goodman test).
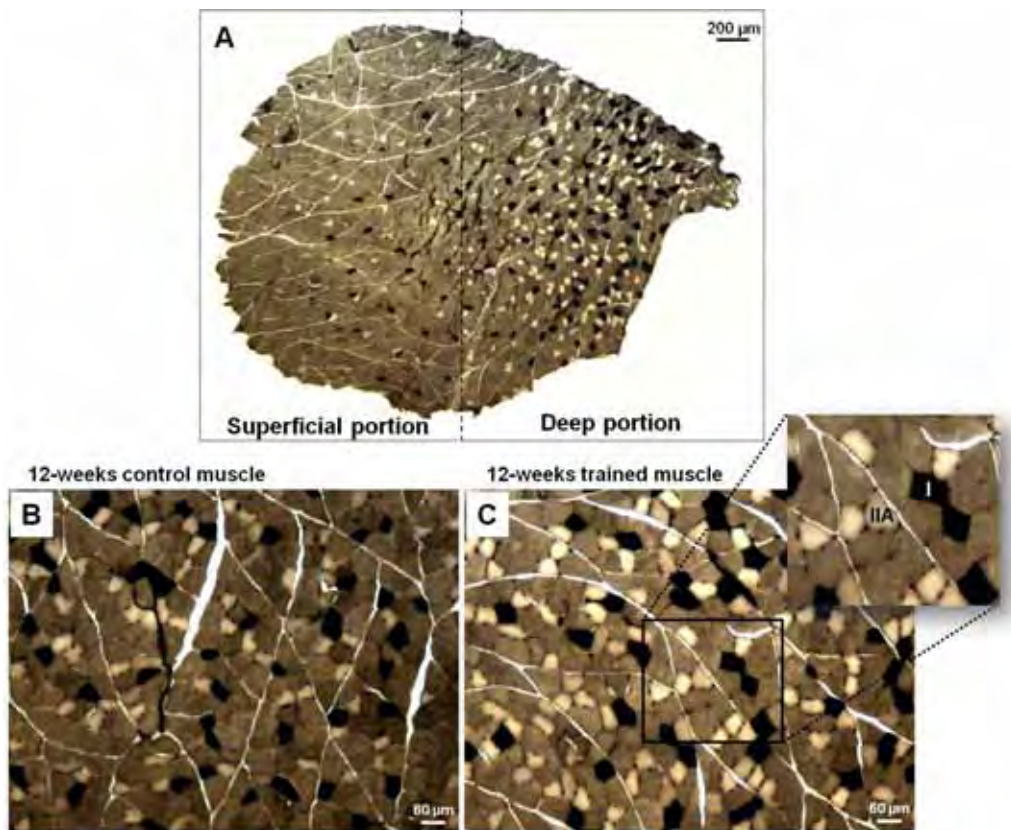
Fig. 8. (A) Cross section of plantaris muscle sample from a representative T12 group animal, demonstrating type I (black) and IIA (white) muscle fibers using myofibrillar adenosine triphosphatase (mATPase) histochemistry after preincubation at pH 4.5. Observe the highest concentration of type I and IIA muscle fiber in the plantaris deep portion; the IID and IIB muscle fibers are more frequent in the superficial portion, although it is not possible to distinguish the types IID and IIB at pH 4.5. (B and C) Cross sections of plantaris deep portion from a 12-week control (B) and trained (C) animal. Note a significant increase in the type IIA fibers percentage after 12 weeks of resistance training. The amplified region in C shows more clearly the muscle fibers type I and IIA.

MyoD, Myogenin and IGF-I mRNA expression. Resistance training during 8 and 12 weeks induced a significant (p < 0.05) increase in MyoD and myogenin mRNA expression in the T8 and T12 groups, compared with C8 and C12 groups, respectively (Fig. 9*B* and *C*). Myogenin showed an increase of 44.8% and 37.7% in the T8 and T12 groups, compared to C8 and C12 groups, respectively (Fig. 9*C*). In addition, MyoD increased in 22.9% and 22.3% in the T8 and T12 groups, compared to their respective controls (Fig. 9*B*). No statistical (p > 0.05) change was observed in MyoD and myogenin mRNA expression between C8 and C12 groups (Fig. 9*B* and *C*). As for IGF-I mRNA expression, there was a significant (p < 0.05) increase of 30.1% in the T8 group compared to C8 group, while no statistical change was observed in T12 group compared to C12 group. In addition, there were no significant changes in IGF-I mRNA expression between C8 and C12 groups (Fig. 9*A*)
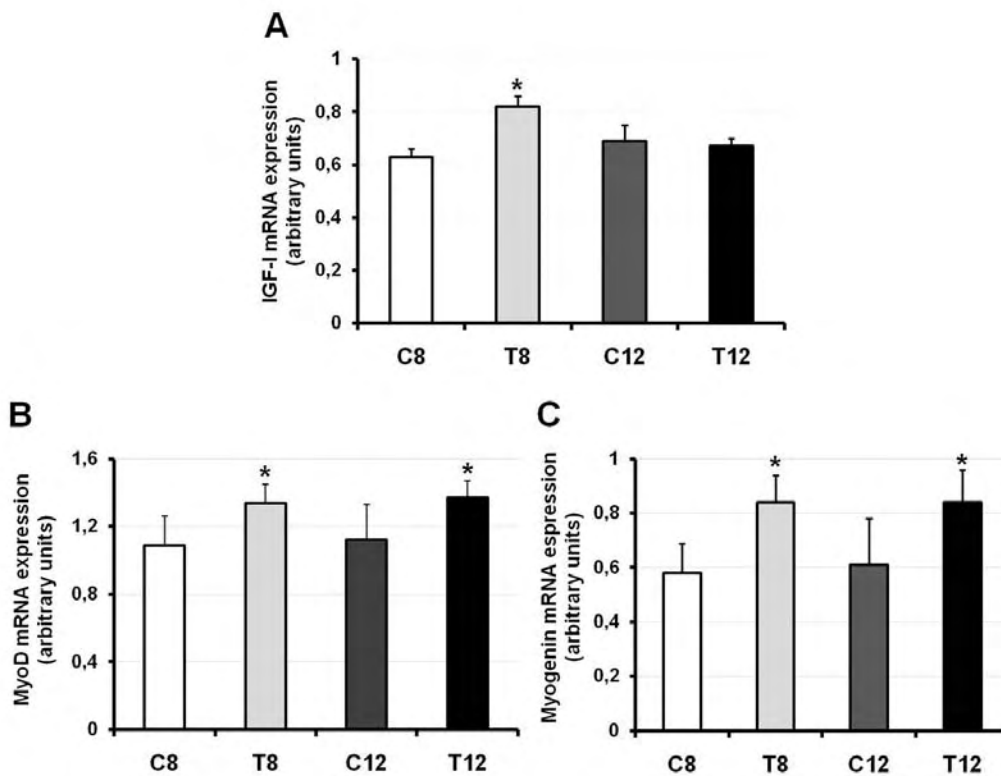
Fig. 9. IGF-I (A), MyoD (B) and myogenin (C) mRNA expression in the plantaris muscle of all experimental groups: C8, 8 weeks control; T8, 8 weeks trained; C12, 12 weeks control; and T12, 12 weeks trained. Values are means ± SD. *n*= 8 rats per group. * Significant difference compared to their respective control at p < 0.05) (ANOVA + Tukey test).

DISCUSSION

Although it is easy to study resistance training (RT) in humans, it is difficult to determine the phenotypic muscle responses to this training. This limitation is primarily a result of the invasive nature of muscle biopsies and the risks inherent in using human subjects. Considering the heterogeneity of muscle fibers in different muscle regions, a small muscle sample cannot accurately reflect the total muscle response. In addition, human studies may be influenced by motivation, movement technique during training and physical tests, and lifestyle subjects. To circumvent these problems, numerous animal models have been used to induce skeletal muscle hypertrophy, such as removal of synergist muscles (4), ladder climbing (34) and water jump (2). Here, we used a model of weightlifting well established that transcribes the traditional squat exercise in humans (57), in progressive training protocol similar to that used to induce hypertrophy in humans, according to the guidelines of the college American sports medicine. This protocol emphasizes multiple series of high-volume (10-12 repetitions) and moderate intensity (65-75% 1RM), with short (1 min) rest period between sets.

The animal model provided the unique and accurate way to isolate single muscles and perform analysis on whole muscle preparations, reflecting the total muscle response. Furthermore, the use of an animal model provides a training method independent of the motivation of subjects and ensures complete control over environmental conditions, food intake, movement technique, or any other psychological parameters. With these variables controlled, the purpose of our study was to test the hypothesis that hypertrophic and phenotypic response of skeletal muscle during long-term RT could be associated with increased in myogenin, MyoD and IGF-I mRNA expression. The major findings of this study were that: 1- RT during 12 weeks induced a decrease in MHCIIx/d isoform content and reciprocal increase in MHCIIa content; this MHCIIx/d-to-IIa modulation was consistent with type IIX/D-to-IIA muscle fibers transition; 2- Myogenin and MyoD expression was similarly elevated between 8 and 12 weeks of RT, while IGF-I mRNA levels was only increased during 8 weeks, but returned to baseline after 12 weeks. Interestingly, the similar increase in myogenin and MyoD mRNA expression and reciprocal return of IGF-I to baseline was associated with the stagnation of hypertrophy between 8 and 12 week; and 3- The increase in myogenin, MyoD and IGF-I mRNA expression after 8 weeks of RT was not associated with changes in the MHC content and fiber-types frequency.

MHC and fiber-type. Similar to previous resistance training studies (31, 12, 52, 25), our experiment found a fiber IIX/D-to-IIA type and MHCIIx/d-to-IIa conversion after 12 weeks of RT (Table 3 and Fig. 7*C*). Sharman et al. (52) argue that the MHCIIx/d-to-MHCIIa isoforms conversion occurs due to the plasticity of MHCIIx/d to adjust toward more oxidative fibers. This is due to recruitment of fibers with high-threshold stimulation during strength training. The increase in MHCIIa isoforms content show a beneficial adaptation of the muscle fibers towards a phenotype functionally and metabolically more efficient during training. Interestingly, the muscle fiber IIX/D (MHCIIx/d)-to-IIA (MHCIIa) transition has been similarly demonstrated in bodybuilders (30) and untrained individuals undergoing RT (31), indicating that the magnitude of fiber-types changes is not directly related to the level of training. Collectively, our results together with of others (30, 31) suggest that there is a maximal limit of fiber-type transition in response to resistance training, which occurs only among the fast isoforms (IIx/d toward IIa). In addition, the increase of muscle fibers CSA during 8 weeks (Fig. 6*E*) was not associated with changes in fiber frequency (Fig. 7*C*) and MHC content (Table 3), indicating that different stimulus of training are required to induce muscle fibers hypertrophy and transition. Thus, the data strongly suggest that the stimulus (e.g., intensity and/or duration) of training required to induce muscle fiber-type transition need to be greater than that to promote hypertrophy.

While the morphologic and phenotypic responses to RT have been well described, the exact molecular mechanisms underlying fiber-type transition during long-term RT are not completely understood. Previous acute-resistance training studies have shown a positive correlation between MHC isoforms, MRFs and IGF-I mRNA expression (40, 62, 61), suggesting that the expression pattern of different MHC isoforms can be influenced by the MRFs and IGF gene expression. In our study, the upregulation in myogenin, MyoD and IGF-I mRNA expression after 8 weeks of training (Fig. 9*B* and *C*) was not associated with changes in muscle fibers-type frequency (Fig. 7*C*) and MHC content (Table 3). Contrary to other studies (40, 62, 61) our data show that the increased in myogenin, MyoD and IGF-I mRNA expression was not associated with MHC modulation and muscle fiber-type transition. Corroborating our findings, Rudnicki et al. (50) showed that the expression of slow and fast fiber types was not prevented in mice by a knockout of MyoD gene. In addition, electrical stimulation studies in fast muscle were not accompanied by conspicuous changes in MyoD and myogenin mRNA levels (42, 32). So, the function of MRFs controlling the MHC

expression patterns in skeletal muscle is still under discussion. Our data suggest that other cellular and/or molecular mechanisms (e.g., nervous system, growth factors e/or others transcription factors) may be involved in MHC isoforms modulation and fiber-types transitions during chronic resistance training. This implies that the increased myogenin, MyoD and IGF-I expression may be more associated with control of muscle mass than with muscle fiber-type transitions.

MRFs. We evaluated two transcription factors important in myogenic processes (myogenin and MyoD) and found that RT during 8 and 12 weeks induced a marked increase in myogenin (T8: 44.8% and T12: 37,7%) and MyoD (T8: 22,9% and T12: 22,3%) mRNA expression (Fig. 9*B* and *C*) This increase is not surprising considering previous human (47, 8) and animal (45) studies that examined the acute myogenic response to resistance exercise; these studies show an increase in myogenin and MyoD mRNA expression after a single bout of RT, suggesting that these MRFs may be involved in initial hypertrophic response to RT. However, most of these studies examined the molecular responses on a scale of hours, to establish a time course for the activation of myogenic mechanisms. Thus, it is still unclear if response of MRFs occurs during the adaptation to long-term RT.

The important novel observation of our study was that both myogenin and MyoD mRNA expression were upregulated during 8 and 12 weeks of RT; this increase was accompanied by an increase in muscle fibers CSA (Fig. 6*E*) The increase in myogenin and MyoD mRNA expression is consistent with the important role of MRFs in hypertrophy mediated by activation of satellite cells (SC) (13). Several studies have shown that muscle injury induces satellite cell activation and MRFs expression (33, 38). The MRFs regulates the expression of muscle-specific genes and are involved in proliferation (MyoD, myf-5) and differentiation (myogenin, myf-6) of SCs in response to load-induced activation (9, 22). It has been demonstrated that myogenin and MyoD expression is essential for successful skeletal muscle hypertrophy (28, 37). Considering that proliferation, differentiation, and fusion of CS to preexisting fibers are necessary if the hypertrophy response is to continue during longer periods of mechanical overload (37, 48), it seems reasonable to assume that myogenin and MyoD are involved in regulating long-term muscle hypertrophy.

IGF-I. In addition to important role of MRFs in load-induced hypertrophy, the IGF-I has been indicated as a potential mediator of hypertrophy in response to load-induced muscle injury. Previous study report an increase in muscle IGF-I expression in

several models of injury and repair. IGF-I mRNA expression in SCs and proliferating myoblasts was increased in EDL muscle within 24 h of ischemia/reperfusion injury and peaked at 3 days (35). In humans, eccentric contraction, which produces greater muscle damage than concentric contraction, led to greater increases in muscle IGF-I mRNA expression (5). Although it has been well reported the important role of IGF-I in activation of the CSs, it remains unknown whether the IGF-I contributes to muscle hypertrophy induced by long-term RT. In our study, the training during 8 weeks induced a 30.1% increase in IGF-I mRNA expression, but this increase returned to normal levels after 12 weeks of training (Fig. 9*A*).

The return of IGF-I mRNA expression to baseline after 12 weeks of RT is consistent with previous studies that showed a decrease in circulating IGF-I levels at 11 and 15 weeks of intense training in untrained healthy subjects (49, 51). However, studies that analyzed the local IGF-I expression during long-term resistance training demonstrated an increase in IGF-I at both the mRNA (24) and protein (53) levels. Although contradictory results have been obtained in previous studies, our findings together with others (49, 51, 53) raise important questions concerning the relative contributions of the systemic vs. local IGF-I system to adaptations induced by chronic RT. Zanconato et al. (64) reported increase in local IGF-I gene expression despite no changes in circulating IGF-I was observed after exercise training in rats. In addition, Nindl et al. (43) suggest that the circulating IGF-I levels can be influenced by the degree of tissue sequestration of IGF-I from the systemic circulation. Despite the decreased IGF-I mRNA expression in the current study, it is important to note that the long-term RT induced improvements in physical performance outcomes (i.e., increase of strength, fiber IIX/D-to-IIA types transition and increase of muscle fibers CSA). Thus, it appears that favorable neuromuscular anabolic adaptations can occur despite of IGF-I gene expression returned to baseline during long-term RT More studies, particularly in healthy subjects, are needed to concomitantly quantify both local and systemic IGF-I in order to clarify the relative roles of IGF-I in mediating long-term RT adaptations.

Hypertrophy stagnation. In the current study, the hypertrophy response showed stagnation between 8 and 12 weeks of training (Fig. 6*E*), while the absolute and relative muscle strength was markedly increased (Fig. 5). Considering that the MRFs were upregulated during RT program, why hypertrophic response showed stagnation during the time course of resistance training program? There are two possibilities that might explain this paradox. First, if the myogenin and MyoD regulate the hypertrophy, the

reason for the stagnation of hypertrophy could be due to the fact that myogenin and MyoD expression were also similar between 8 and 12 weeks of training (Fig. 9*B* and *C*). Thus, the stagnation of muscle mass may be associated with a maximum limit of myogenin and MyoD expression during prolonged RT; this possibility is supported by studies that showed a positive correlation between the MRFs expression and muscle hypertrophy. For example, Tamaki et al. (58) demonstrated that the loss of satellite cells activation potential in muscle of the elderly, caused by the reduced expression of MyoD protein, can be a critical factor for the reduction of skeletal muscle regenerative capacity and function. In addition, Always et al. (3) showed that hypertrophy was attenuated in old compared with young rats due to a lower MRFs expression. Together, the results of these studies suggest that the upregulation in MRFs expression may be a determinant factor to promote increased muscle mass. Therefore, the stagnation of hypertrophy observed in our study may be due to a tendency of MRFs expression to become constant during the time course of RT. The exacts mechanism that explain this condition remains unknown, but it seems reasonable to speculate that the interindividual variability of the hypertrophic responses may be linked with the ability to express the MRFs during the long-term training progression.

Second, the stagnation of hypertrophy response between 8 and 12 weeks of training could be related to the fact that the IGF-I expression increased only during 8 weeks, but returned to baseline after 12 weeks (Fig. 9*A*). Although the expression of MRFs remained high between 8 and 12 weeks, the return of IGF-I expression levels to baseline may have influenced the evolution of hypertrophy. This hypothesis is consistent with previous studies that showed increased in IGF-I expression together with an increased in muscle mass (18, 46). Indeed, IGF-I has been described as a potent anabolic agent for muscle tissue, stimulating hypertrophy in different physiological conditions (26, 19) However, it has not been conclusively established whether the increased IGF-I mRNA expression is necessary for the evolution of hypertrophy in response to long-term resistance training; one possibility is that the MRFs alone are not sufficient to control the hypertrophy evolution, and an increase in IGF-I expression could be an essential factor for hypertrophy during long-term resistance training. While it remains unknown whether the stabilization of muscle hypertrophy in response to long-term resistance training occurs due to the return of IGF-I mRNA expression to baseline or by maximal limit of MRFs expression during prolonged period of resistance

training, our results raise the question about a possible interaction between MRFs and IGF-I to promote hypertrophy during long-term resistance training.

In conclusion, the present study shows that the training protocol used is efficient to promote functional, hypertrophic and phenotypic adaptations in rat plantaris muscle, similar to that observed in human muscles. Myogenin and MyoD expression was similarly elevated between 8 and 12 weeks of RT, while IGF-I mRNA levels was only increased during 8 weeks, but returned to baseline after 12 weeks. This fact was associated with the hypertrophy stagnation between 8 and 12 weeks of training. According to the results, two hypotheses could be formulated to explain this hypertrophy stagnation: first, due to the return of IGF-I mRNA levels to baseline, and/or second, by maximal limit of MRFs expression during prolonged period of RT. While these possible hypothesis remains to be tested, our results raise the question of a possible interaction between MRFs and IGF-I to promote hypertrophy during long-term resistance training. In addition, the increase in myogenin, MyoD and IGF-I mRNA expression after 8 weeks of RT was not associated with changes in the MHC isoforms content and fiber-types frequency. This implies that these MRFs are more involved in the control of muscle mass than in muscle fiber-type transitions.

ACKNOWLEDGMENTS

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