UNIVERSIDADE ESTADUAL DE CAMPINAS

Robson Francisco Carvalho

EXPRESSÃO DE FATORES DE REGULAÇÃO MIOGÊNICA E

METALOPROTEINASES NO MÚSCULO ESTRIADO

ESQUELÉTICO DE RATOS COM INSUFICIÊNCIA CARDÍACA

Este exemplar corresponde à redação final
da tese defendida pelo(a) candidato (a)
Robson Francisco Carvalho

e aprovada pela Comissão Julgadora.

Maeli Dal Pai Silva

Tese apresentada ao Instituto de
Biologia para obtenção do Título de
Doutor em Biologia Celular e Estrutural,
na área de Histologia.

Orientadora: Profa. Dra. Maeli Dal Pai Silva

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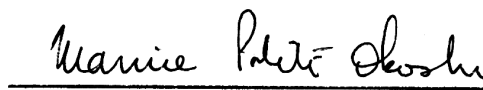
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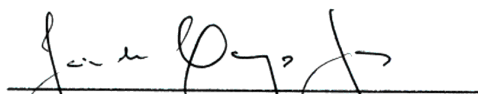
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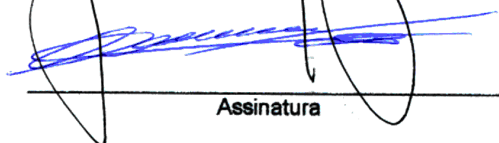
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Dedico este trabalho...

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Índice

1. RESUMO

Introdução: A Insuficiência Cardíaca (IC) está associada a uma miopatia do músculo esquelético com aumento da expressão das isoformas rápidas da cadeia pesada de miosina (MHC) e alterações na matriz extracelular (MEC). Os mecanismos moleculares que controlam a expressão de MHC durante a IC ainda não foram descritos. Os fatores de regulação miogênica (MRF), uma família de fatores transcricionais que controlam vários genes músculo-específicos, podem estar relacionados com essa miopatia. As alterações da MEC podem estar associadas a um aumento na expressão de RNA mensageiro e na atividade das metaloproteinases da MEC (MMP), uma família de endopeptidases dependentes de zinco que degradam a maioria dos componentes da MEC e que são indispensáveis para a remodelação do tecido conjuntivo ao redor das fibras musculares.

Objetivos: Analisar no músculo esquelético de ratos Wistar com IC induzida pela monocrotalina: 1) a expressão de RNA mensageiro para os MRF, as isoformas proteicas de MHC e a atrofia nos músculos Sóleo (SOL) e Extensor Longo dos Dedos (EDL); 2) a expressão de RNA mensageiro e a atividade das MMP nos músculos SOL, EDL e diafragma (DIA).

Métodos: A expressão do RNA mensageiro para MyoD, miogenina, MRF4, MMP2 e MMP9 foi determinada por RT-PCR; as isoformas de MHC foram separadas por eletroforese em gel de poliacrilamida e a atividade das MMP, por eletroforese em gel de poliacrilaminda contendo gelatina na presença de SDS em condições não redutoras.

Resultados: 1) Embora a composição de MHC do músculo esquelético de ratos com IC não tenha sido alterada, a expressão relativa do RNA mensageiro para a MyoD nos músculos SOL e EDL, e a de MRF4 no músculo SOL foi significativamente diminuída,

enquanto que a expressão relativa de RNA mensageiro para a miogenina não se alterou em ambos os músculos. A diminuição na expressão relativa de RNA mensageiro para o MRF4 está associada à atrofia do SOL em resposta à IC. 2) A IC aumentou a expressão de RNA mensageiro e a atividade da MMP9 nos músculos SOL, EDL e DIA, e aumentou a expressão de RNA mensageiro da MMP2 no músculo DIA.

Conclusão: Nossos resultados sugerem um potencial papel para os MRF e para as MMP na miopatia do músculo esquelético na IC.

2. ABSTRACT

Background: Heart failure (HF) is associated with a skeletal muscle myopathy with increased expression of fast myosin heavy chains (MHC) and extracellular matrix (ECM) alterations. The skeletal muscle-specific molecular regulatory mechanisms controlling MHC expression during HF have not been described. Myogenic regulatory factors (MRF), a family of transcriptional factors that control the expression of several skeletal muscle-specific genes, may be related to these alterations. The ECM alterations may be associated with enhanced mRNA expression and activity of matrix metalloproteinases (MMP), a family of zinc-dependent endopeptidases that degrade most ECM components and appear indispensable for the breakdown of the connective tissue surrounding muscle fibers.

Objectives: This investigation was undertaken in order to examine in Wistar rat skeletal muscle with monocrotaline-induced HF: 1) potential relationships between MRF mRNA expression and MHC protein isoforms and atrophy in Soleus (SOL) and extensor digitorum longus (EDL) muscles; 2) MMP mRNA expression and their potential relationships with changes in MMP activity in SOL, EDL, and diaphragm (DIA) muscles.

Methods: MyoD, myogenin, MRF4, MMP2, and MMP9 were determined by using RT-PCR; MHC isoforms were separated by using polyacrylamide gel electrophoresis, and MMP activity by electrophoresis in gelatin-containing polyacrylamide gel in the presence of SDS under nonreducing conditions.

Results: 1) Despite no change in MHC composition of Wistar rat skeletal muscles with HF, the mRNA relative expression of MyoD in SOL and EDL muscles and that of MRF4 in SOL muscle were significantly reduced, whereas myogenin was not changed in both

muscles. This down-regulation in the mRNA relative expression of MRF4 in SOL was associated with atrophy in response to HF. 2) HF increased MMP9 mRNA expression and activity in SOL, EDL, and DIA and MMP2 mRNA expression in DIA.

Conclusion: Taken together; our results show a potential role for MRF and MMP in skeletal muscle myopathy during HF.

3. INTRODUÇÃO

3.1. Desenvolvimento Embrionário do Músculo Esquelético

A maioria dos músculos esqueléticos de aves e mamíferos, com exceção de alguns músculos craniofaciais e esofágicos, são formados a partir dos somitos; condensações transitórias do mesoderma paraxial originadas em cada lado do tudo neural do embrião (revisados em Summerbell & Rigby, 2000; Pourquie, 2001; Pownall *et al.*, 2002). Os somitos diferenciam-se ao longo do eixo dorso-ventral do embrião e originam o dermomiótomo, localizado dorsalmente, e o esclerótomo, localizado ventralmente (Figura 1). O dermomiótomo origina a derme e a musculatura esquelética dos membros e tronco, enquanto que o esclerótomo origina a cartilagem e os ossos das vértebras e costelas.

A porção epaxial do dermomiótomo origina a musculatura profunda do dorso, a partir de células progenitoras da borda medial dorsal (BMD) que migram profundamente do dermomiótomo, saem do ciclo celular, se alongam e diferenciam-se em fibras musculares (Christ & Ordahl, 1995; Amthor *et al.*, 1999) (Figura 1). Um padrão similar de eventos induz a formação da borda lateral ventral (BLV) para estabelecer o dermomiótomo hipoaxial não migratório, o qual originará a musculatura lateral do tronco. Algumas células da BLV se separam do dermomiótomo e migram ventralmente para regiões de desenvolvimento dos músculos esqueléticos da parede ventral do corpo, da língua, dos membros e do diafragma.

A regulação do processo de formação dos músculos esqueléticos envolve a apropriada ativação, proliferação e diferenciação de várias linhagens de células miogênicas e depende da expressão e atividade de fatores transcricionais, conhecidos como fatores de regulação miogênica.
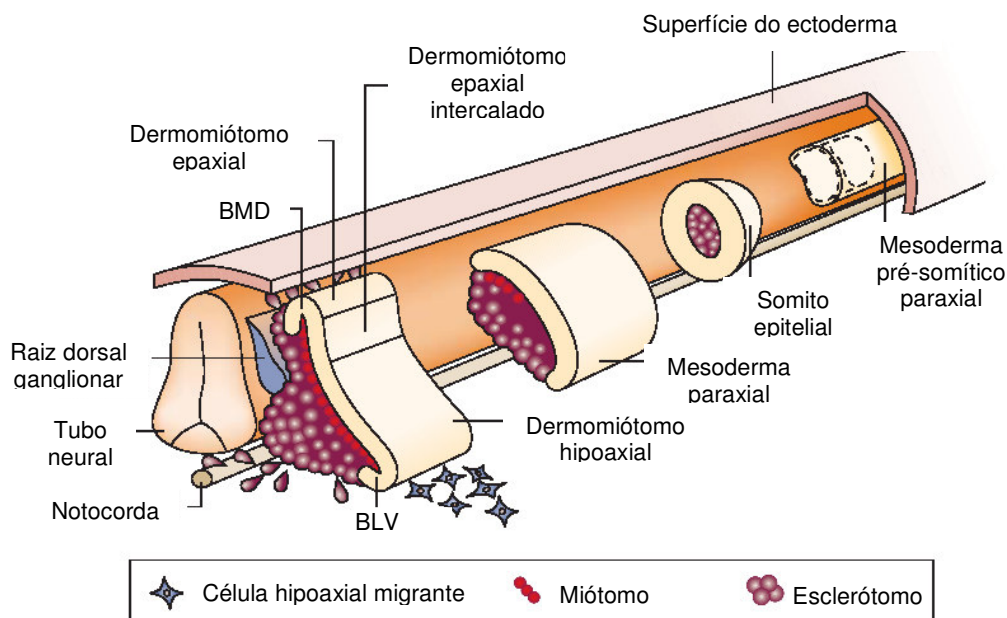
Figura 1. Origem embrionária dos músculos do tronco e dos membros. O mesoderma paraxial pré-somítico está localizado em cada lado da notocorda. A segmentação do mesoderma paraxial em somitos ocorre ao longo do eixo dorso-ventral e em uma direção cefálica – caudal do embrião. Em resposta a sinais da notocorda e do tubo neural, os somitos se diferenciam e subdividem-se para originar o dermomiótomo e o esclerótomo. Células da borda medial dorsal (BMD) do dermomiótomo migram profundamente do dermomiótomo para formar o dermomiótomo epaxial. Um evento semelhante ocorre na borda lateral ventral (BLV) do dermomiótomo, para a formação do dermomiótomo hipoaxial. Algumas células da BLV se separam do dermomiótomo e migram para regiões de desenvolvimento dos músculos da parede ventral do corpo, da língua, dos membros e do diafragma (células hipoaxiais migrantes) (adaptado de Parker *et al.*, 2003).

3.2. Fatores de Regulação Miogênica

Durante o desenvolvimento embrionário, o comprometimento das células somíticas do mesoderma com a linhagem miogênica depende inicialmente de sinais positivos [Wnts, Sonic hedgehog (Shh), *Noggin*] ou negativos (BMP4) oriundos de tecidos circundantes, tais como a notocorda e o tubo neural (revisado em Chargé & Rudnicki, 2004). Esses sinais irão ativar os genes capazes de transformar células não musculares em células com um fenótipo muscular.

Os genes responsáveis por essa transformação são membros da família dos fatores transcricionais "basic helix-loop-helix" (bHLH), da qual fazem parte a MyoD, Miogenina, Myf5 e o MRF4; coletivamente chamados de fatores de regulação miogênica (do inglês, my*ogenic regulatory factors* ou MRFs). Os MRFs compartilham um domínio homólogo bHLH, que é necessário para a ligação com o DNA e para a dimerização com fatores transcricionais da família da proteína E. Os heterodímeros MRF-proteína E e os monômeros de MRFs ligam-se a seqüências de DNA (5´-CANNTG-3´), conhecidas como *Ebox,* presentes na região promotora de vários genes músculo – específicos, levando à expressão dos mesmos (Murre *et al.*, 1989; Lassar *et al.*, 1991) (Figura 2).
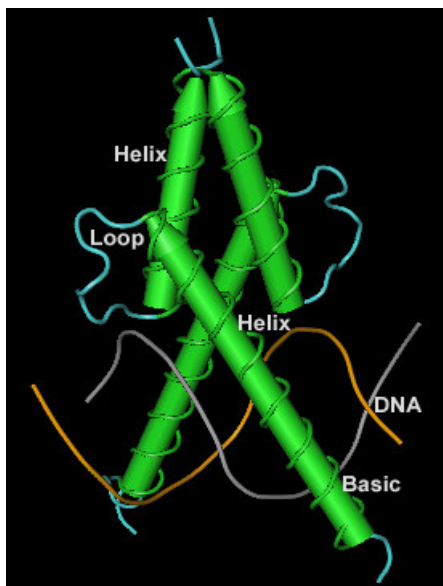
Figura 2. Estrutura cristalográfica do complexo formado pelo dímero do fator transcricional da família "basic Helix-Loop-Helix" (bHLH) MyoD e o DNA (adaptado de Ma *et al.*, 1994).

Assim como os MRFs, a família de fatores transcricionais MEF2 (do inglês, *myocyte enhancer factor-2*) também está envolvida na ativação de genes músculo - específicos (revisado em Naya & Olson, 1999). Os MEF2 são expressos em muitos tecidos, mas é apenas durante o desenvolvimento dos músculos cardíaco, liso e estriado que esses fatores ativam a transcrição (Naya *et al.*, 1999). Estudos demonstram uma ação interdependente entre a família MEF2 e os MRFs no controle da diferenciação do músculo esquelético (Naidu *et al.*, 1995; Novitch *et al.*, 1996; Novitch *et al.*, 1999; Ridgeway *et al.*, 2000)

Na diferenciação do músculo esquelético, o comprometimento das células somíticas do mesoderma com a linhagem miogênica é marcado pela expressão dos MRFs Myf5 e MyoD (Figura 3). Isso é demonstrado pela total ausência de tecido muscular em camundongos duplo *Knockout MyoD:Myf5* e pela observação de que, nesses animais, as supostas células progenitoras musculares permanecem multipotentes e contribuem para tecidos não musculares do tronco e dos membros desses camundongos (Rudnicki *et al.*, 1993; Kablar *et al* 1998; Palmer & Rudnicki, 2001). As células da linhagem miogênica em proliferação, positivas para Myf5 e/ou MyoD, são então denominadas de mioblastos (Megeney & Rudnicki 1995).

Embora a MyoD e o Myf5 definam a identidade dos mioblastos, as células precursoras somíticas devem ser "pré-comprometidas" com a linhagem miogênica antes da expressão dos MRFs. No embrião, esse "pré-comprometimento" é realizado pelo fator transcricional Pax3, da família Pax (do inglês, *paired-box*), o qual é expresso em células do mesoderma pré-somítico e dos primeiros somitos epiteliais (Goulding *et al.*, 1994; Williams & Ordahl, 1994). Já no dermomiótomo, as células precursoras, que apresentam expressão de Pax3 induzida por sinais secretados pelo mesoderma da placa lateral e pelo ectoderma superficial, são mantidas como uma população não diferenciada e em proliferação; contribuindo assim para a expansão das células da linhagem miogênica (Amthor *et al.*, 1999) (Figura 3).

Os mioblastos que saem do ciclo celular, positivos para Myf5 e MyoD, tornam-se miócitos diferenciados e iniciam a expressão dos MRFs miogenina e MRF4, os quais regulam a diferenciação dessas células em fibras musculares (Figura 3) (Megeney & Rudnicki 1995). Embriões deficientes em miogenina morrem no período perinatal

devido à deficiência na diferenciação dos miócitos, evidenciada pela quase total ausência de fibras musculares nesses mutantes (Hasty *et al.*, 1993; Nabeshina *et al.*, 1993). Similarmente, camundongos deficientes em MRF4 demonstram uma série de fenótipos consistentes com seu papel na diferenciação dos miócitos durante a miogênese (Patapoutian *et al.*, 1995; Rawls *et al.*, 1995; Zhang *et al.*, 1995, Yoon *et al.*, 1997).

Finalmente, no processo de miogênese, os miócitos mononucleados se fundem para formar os miotubos (Figura 3) e, no animal adulto, o músculo esquelético torna-se um tecido estável, caracterizado por fibras musculares multinucleadas (Decary *et al.*, 1997; Schmalbruch & Lewis, 2000).
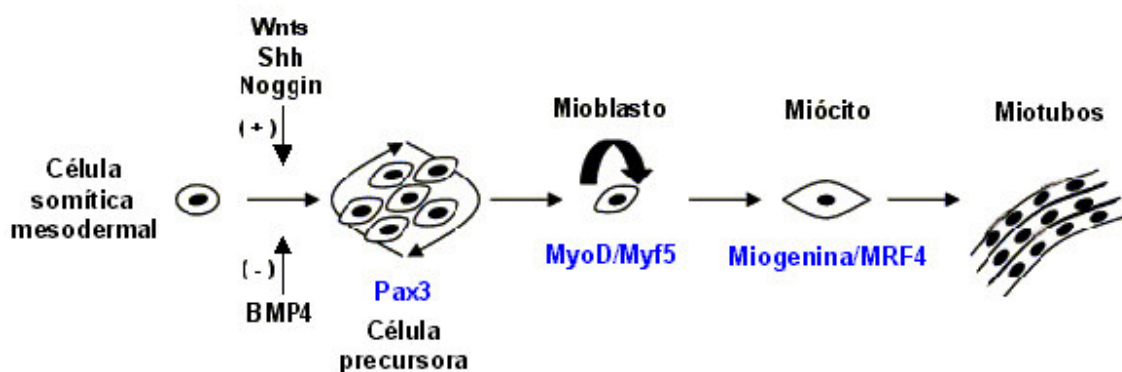
Figura 3. Células somíticas mesodermais recebem sinais de tecidos circundantes os quais podem induzir [Wnts, Sonic hedgehog (Shh), Noggin] ou inibir (BMP4) a expressão de Myf5 e MyoD. A expressão de Pax3 nas células precursoras contribui para a expansão das células miogênicas. Após a indução de Myf5 e/ou MyoD, as células somíticas mesodermais são comprometidas com a linhagem miogênica (mioblastos). A expressão de miogenina e MRF4 induz a diferenciação dos mioblastos em miócitos. Posteriormente, os miócitos se fundem para originar os miotubos.

3.3. Eventos celulares na formação dos Músculos Esqueléticos

A formação dos músculos dos membros e do diafragma envolve pelo menos duas populações de mioblastos, os primários e os secundários (Hauschka, 1994) (Figura 4). A caracterização *in vitro* dessas duas populações de células demonstra uma distinção quanto à morfologia dos clones e às necessidades de meio de cultura (White *et al.*, 1975, Rutz *et al.*, 1982; Seed & Hauschka, 1984). Além disso, há uma diferença na expressão das isoformas de cadeia pesada de miosina entre essas duas populações de células, sugerindo que os mioblastos primários são destinados a originar fibras lentas, enquanto que os secundários são destinados a originar fibras rápidas (para uma revisão, Stockdale, 1992). Entretanto, experimentos demonstram a importância de outros fatores, como a inervação, na determinação dos tipos de fibras musculares (Hughes & Blau, 1992; Pin & Merrifield, 1997).

A formação dos miotubos a partir dos mioblastos ocorre a partir de dois eventos distintos temporalmente (Figura 4). Inicialmente, ocorre a formação dos miotubos primários, os quais apresentam núcleos localizados na região central, e miofibrilas, na região periférica do sarcoplasma (Kelly & Zacks, 1969; Ontell & Kozeka, 1984). Esses miotubos fornecem um suporte (andaime) para a posterior formação dos miotubos secundários a partir da proliferação e fusão de mioblastos adjacentes aos miotubos primários (Ontell & Kozeka, 1984; Ross *et al.*, 1987). Posteriormente, ocorre a separação dos miotubos primários e secundários e a diferenciação em fibras primárias e secundárias; os núcleos migram para a região periférica e as miofibrilas passam a ocupar todo o sarcoplasma (Ontell & Kozeka, 1984; Ross *et al.*, 1987).

Durante o processo da miogênese, uma distinta população de mioblastos não se diferencia e permanece quiescente entre a membrana plasmática da fibra muscular em desenvolvimento e a lâmina basal, sendo denominados de células satélites ou mioblastos indiferenciados (Mauro, 1961). As células satélites são as responsáveis pela regeneração e crescimento pós-natal do músculo esquelético (para uma revisão, Chargé & Rudnicki, 2004) (Figura 4).
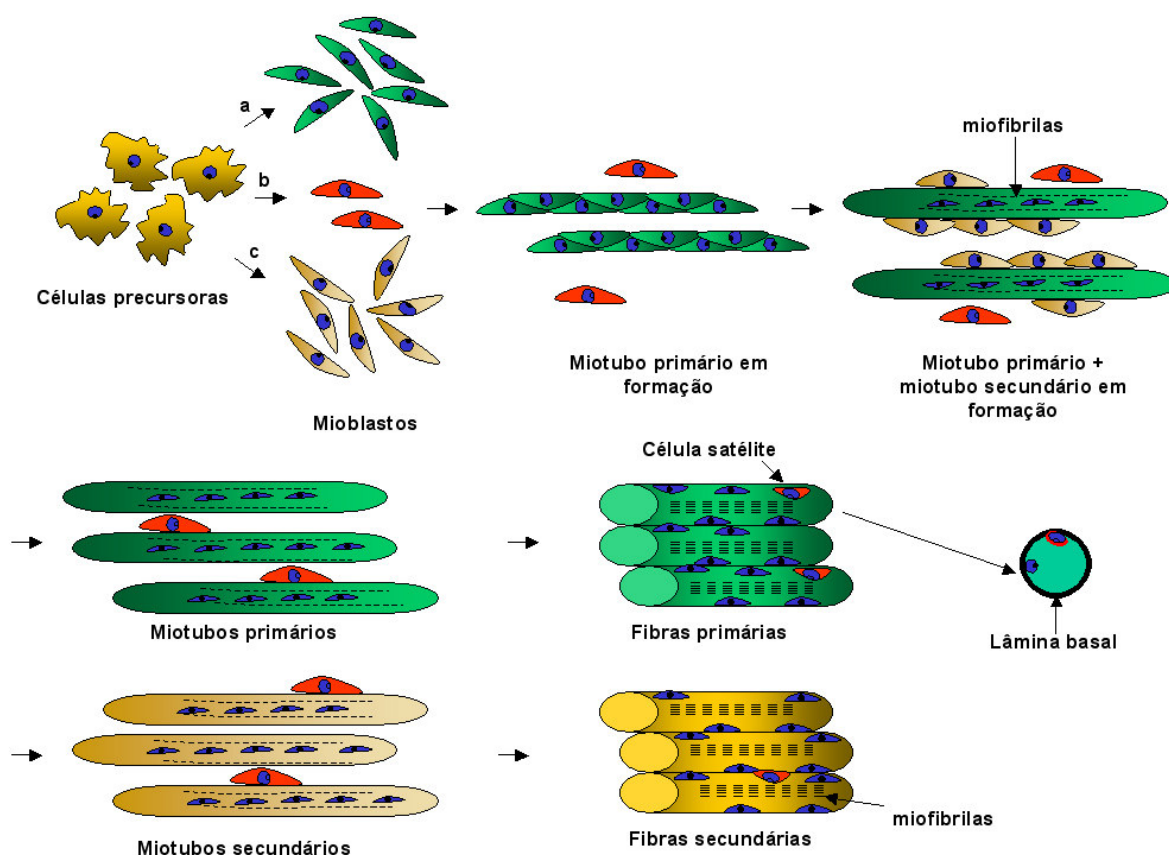
Figura 4. Formação das fibras musculares estriadas. As células precursoras originam diferentes populações de mioblastos (a,b e c). Alguns mioblastos (a) se alinham e fundem-se formando os miotubos primários, que apresentam núcleos na região central e, iniciam a síntese de miofibrilas que ocupam a região periférica no sarcoplasma do miotubo. Os miotubos primários fornecem o suporte para a subseqüente formação dos miotubos secundários, a partir de outra população de mioblastos (c). Posteriormente ocorre a separação dos miotubos primários e secundários e a diferenciação em fibras primárias e secundárias. Os núcleos das fibras migram para a região periférica da fibra e as miofibrilas passam a ocupar todo o sarcoplasma. Alguns mioblastos (b), denominados de células satélites, não se fundem e permanecem quiescentes entre a membrana plasmática da fibra e a lâmina basal (Dal Pai-Silva *et al.*, 2005).

3.4. Características das Fibras Musculares Esqueléticas Adultas

Os primeiros estudos envolvendo o tecido muscular classificavam os músculos em "vermelhos" ou "brancos" (Ranvier, 1873). A cor vermelha está relacionada com a presença do pigmento mioglobina e com o grau de vascularização do músculo. Com a utilização de técnicas histoquímicas, observou-se que a maioria dos músculos estriados dos mamíferos é constituída por uma população heterogênea de fibras, que apresentam características morfológicas, bioquímicas e fisiológicas distintas (Dubowitz & Pearse, 1960). Inicialmente, as fibras musculares foram classificadas em vermelhas, intermediárias e brancas (Ogata, 1958). Posteriormente, três tipos principais de fibras musculares foram descritas, sendo denominadas de fibras dos tipos I, IIA e IIB, de acordo com o padrão de reação para a atividade da ATPase da porção globular da cadeia pesada da miosina (ATPase miofibrilar ou m-ATPase) (Brooke & Kaiser, 1970).

A molécula de miosina é um hexâmero formado por duas cadeias pesadas de miosina (do inglês, *myosin heavy chain* ou MHC), enroladas em α-hélice, e quatro cadeias leves de miosina (do inglês, *myosin light chain* ou MLC) (Lowey *et al.* 1969; Weeds & Lowey, 1971; Elliot & Offer, 1978; Warrick & Spudich, 1987). Cada cadeia pesada pode ser separada em duas porções: meromiosina leve, em forma de bastão, e meromiosina pesada, conhecida como porção globosa da miosina, a qual apresenta o sítio de ligação com a actina e a região capaz de ligar-se à molécula de ATP e hidrolisá-la (atividade ATPásica) (Huxley 1969; Lowey *et al.* 1969) (Figura 4).
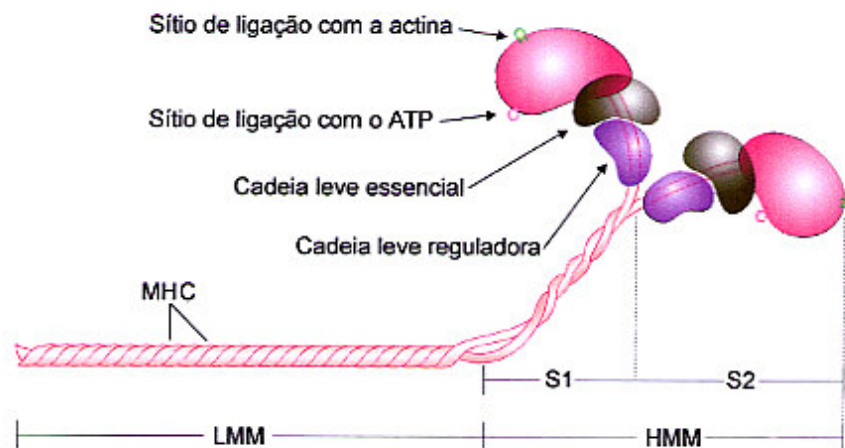
Figura 4 – Esquema da molécula de miosina da classe II. Cada molécula de miosina é composta por duas cadeias pesadas de miosina (MHC) e quatro cadeias leves de miosina (MLC). As MHC podem ser clivadas e gerar as meromiosina leves (LMM) e meromiosina pesadas (HMM). As HMM são compostas pela porção globosa S1 e pela porção α hélice em forma de bastão, S2. As MLC estão dispostas na proporção de duas cadeias (uma essencial e uma reguladora) para cada subfragmento S1 (Dal Pai-Silva *et al.*, 2005).

Ashmore & Doerr (1971), utilizando a combinação das reações histoquímicas para detecção da atividade das enzimas m-ATPase e succinato desidrogenase (SDH), classificaram as fibras musculares como βRed, αRed e αWhite. Posteriormente, Peter *et al.*, (1972), classificaram as fibras musculares em SO (*slow oxidative*), FOG (*Fast oxidative glycolytic*) e FG (*Fast glycolytic*), baseando-se na combinação das reações histoquímicas e na detecção da atividade das enzimas m-ATPase e NADH tetrazólio redutase (NADH-TR).

Estudos mais recentes, envolvendo a microdissecção de fibras e, associando a reação histoquímica m-ATPase com a técnica da eletroforese, possibilitaram a separação de quatro isoformas de cadeia pesada de miosina (MHC) presentes nas fibras musculares: fibras do tipo I, com MHCI, fibras do tipo IIA, com MHC IIa, fibras do tipo IIB, com MHC IIb e fibras do tipo IID com MHC IId (Termin *et al.* 1989). A MHCIId

está presente nos músculos de pequenos mamíferos e possui uma velocidade de contração intermediaria entre as MHCIIa e MHCIIb (Hilber *et al.*, 1999). As fibras IID apresentam características histoquímicas e bioquímicas similares às fibras 2X descritas em ratos (Larsson *et al.*, 1991), camundongos e coelhos (Hämäläinen & Pette, 1993), sendo também denominadas de fibras IID/IIX (para uma revisão ver Scott *et al.*, 2001). Baseado em vários tipos de evidências e na análise de seqüências de DNA, a MHC riginalmente identificada em humanos como MHCIIb é na verdade homóloga à MHCIId/IIx presente nas fibras IID/IIX de pequenos mamíferos (Pette & Staron, 1997). Portanto, os humanos expressam as seguintes isoformas de MHC (da mais lenta para a mais rápida): MHCI, MHCIIa e MHCIIx/d (Staron, 1997); e não expressam a mais rápida isoforma de todas as MHC, a MHCIIb (Hilber *et al.*, 1999).

As fibras do tipo I, IIA, IID/X e IIB são classificadas como fibras puras (Pette & Staron, 1997; Staron *et al.*, 1999). Porém, além das fibras puras, que expressam apenas um tipo de RNA mensageiro para a MHC, há fibras que co-expressam diferentes genes para a MHC (Biral *et al.*, 1988; Aigner *et al*, 1993; Schiaffino & Reggiani, 1994; Caiozzo *et al.*, 2003). Essas fibras são classificadas de acordo com o tipo de MHC predominante: (IC=MHCI>MHCIIa, IIC=MHCIIa>MHCI, IIAD=MHCIIa>MHCIId, IIBD=MHCIIb>MHCIId), sendo denominadas de fibras híbridas ou polimórficas (Staron & Pette, 1993; Di Maso *et al.*, 2000).

A velocidade de contração de uma fibra muscular está diretamente relacionada com o tipo de MHC (revisado em Talmadge *et al.*, 1993). A MHC capaz de rápida hidrólise do ATP é característica das fibras do tipo II, que são fibras de contração rápida. Já a MHC de baixa atividade ATPásica é encontrada nas fibras do tipo I, de contração lenta (Kelly & Rubinstein, 1994).

A identificação das características contráteis das fibras musculares é importante, pois como os músculos são compostos por vários tipos de fibras musculares, suas propriedades refletem a soma das características das fibras que o constituem. Isso é possível pois a estrutura da matriz extracelular do músculo esquelético permite a combinação das forças geradas pela contração de várias fibras musculares, a qual é transformada em movimento via junções miotendinosas, onde as fibras musculares se aderem ao esqueleto pelos tendões (revisado em Kjaer 2004)

3.5. Matriz extracelular do músculo esquelético

As fibras musculares estão envoltas por uma matriz extracelular rica em carboidratos e proteínas, que constituem o tecido conjuntivo do músculo; organizado em três bainhas: epimísio, que circunda todo o músculo; perimísio, que divide o músculo em fascículos e endomísio, que circunda cada fibra muscular (para uma revisão ver Sanes, 2003 e Kjaer, 2004) (Figura 5).
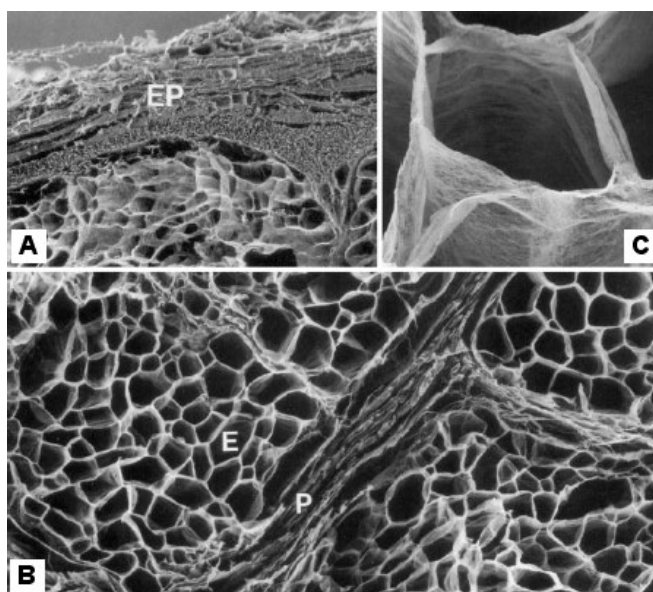
Figura 5. Estrutura da matriz extracelular do músculo esquelético. Músculo semitendinoso bovino após remoção das proteínas das fibras musculares (microscopia eletrônica de varredura). A: Epimísio (EP), B: Perimísio (P) e Endomísio (E). C: Detalhe do endomísio circundando uma fibra muscular esquelética. (adaptado de Kjaer, 2004)

Estudos de microscopia óptica revelaram inicialmente que cada fibra muscular está envolvida por um delicado tubo, denominado sarcolema, formado por 3 componentes: fibrilas reticulares, que seguem um curso em espiral ao redor da fibra (Borg & Caulfield, 1980), membrana basal (Zacks et al., 1973; Borg & Caulfield, 1980) e membrana plasmática da fibra muscular. A membrana basal é formada por duas discretas camadas: a lâmina reticular e a lâmina basal (Mauro & Adams, 1961). Posteriormente, estudos de microscopia eletrônica demonstraram que a lâmina basal é

ainda subdividida em lâmina densa (10 a 15 nm de espessura) e lâmina rara (2 a 5 nm de espessura), adjacente à membrana plasmática (Inoue, 1989). Os principais componentes da membrana basal são: laminina, fibronectina, entactina, heparam sulfato e os colágenos dos tipos I, III, IV, V e VI (Duance *et al.*, 1977; Duance *et al.*, 1980; Walsh *et al.*, 1981; Foidart *et al.*, 1981; Sanes, 1982; Stephens *et al.*, 1982; Bayne *et al.*, 1984; Linsenmayer *et al.*, 1986; Eldridge *et al.*, 1986; Lehto *et al.*, 1988). Na superfície externa da membrana plasmática, observa-se uma camada de glicoproteínas, o glicocálix, que se continua com a lâmina basal.

Poucas moléculas têm sido localizadas no epimísio e no perimísio (Bailey & Sims, 1977; Duance *et al.*, 1977; Duance *et al.*, 1980; Foidart *et al.*, 1981; Sanes, 1982; Stephens *et al.*, 1982; Linsenmayer *et al.*, 1986; Lehto *et al.*, 1988; Light & Champion, 1984). A fibronectina está presente em ambas camadas, bem como os colágenos dos tipos V e VI. O colágeno do tipo I está concentrado no epimísio e o colágeno do tipo III no perimísio, ambos presentes em concentrações maiores no epi - e perimísio que no endomísio. Laminina e colágeno IV estão presentes no local de contato da lâmina basal com o perimísio, mas ausentes no perimísio e endomísio propriamente ditos.

3.6. Plasticidade do Músculo Esquelético

O músculo esquelético possui uma alta plasticidade, podendo alterar sua matriz extracelular e as características morfológicas, metabólicas, contráteis e funcionais de suas fibras musculares em diversas patologias. A insuficiência cardíaca é uma dessas condições patológicas que induz adaptações qualitativas e quantitativas nas propriedades do músculo esquelético.

3.7. Insuficiência Cardíaca

A insuficiência cardíaca (IC) constitui uma importante patologia devido à gravidade de suas manifestações e à sua prevalência. Dados obtidos nos Estados Unidos e na Europa mostram que a incidência média de IC é de 1 a 5 casos por 1000 habitantes/ano, e sua prevalência é de aproximadamente 1% a 2% da população (Givertz *et al.* 2005). No Brasil, conforme dados publicados pelo Ministério da Saúde, a

IC encontra-se entre as principais causas de internação do Sistema Único de Saúde (Albanesi Filho, 2005).

A IC é um estado fisiopatológico no qual o coração é incapaz de bombear sangue de acordo com as necessidades metabólicas teciduais, ou pode fazê-lo adequadamente às custas da elevação da pressão de enchimento ventricular (Givertz *et al.* 2005). De acordo com Cohn (1988), a IC é uma síndrome clínica associada à disfunção cardíaca, diminuição da expectativa de vida e intolerância aos exercícios físicos. Essa intolerância aos exercícios físicos, bem como a redução da atividade locomotora, estão associados aos principais sintomas dos pacientes com IC: a fadiga e a fraqueza muscular (Wilson *et al.*, 1993; Poole-Wilson & Ferrari, 1996; Wilson, 1996; Bigard *et al.,* 1998).

3.8. Alterações nas Fibras do Músculo Esquelético na IC

Embora vários fatores tenham sido descritos como responsáveis pelo desenvolvimento de fadiga e fraqueza muscular, a sua etiopatogenia ainda não está completamente esclarecida. Esse fenômeno é decorrente, em parte, da mudança no metabolismo de oxidativo para glicolítico (Bernocchi *et al.*, 1996) e da presença de alterações nos tipos de fibras musculares (Lipkin *et al.*, 1988; Sullivan *et al.*, 1990; Mancini *et al.*, 1992; De Sousa *et al.*, 2000; Carvalho *et al.*, 2003). A IC induz a expressão da isoforma de cadeia pesada de miosina (MHC) em direção a isoforma rápida (Simonini *et al.* 1996; Bigard *et al.,* 1998; Vescovo *et al.* 1998; Carvalho *et al.*, 2003), a qual está relacionada com a severidade da IC (Vescovo *et al.*, 1996; Spangenburg *et al.*, 2002). Dados do nosso laboratório também demonstraram mudanças na composição das isoformas da miosina e na proporção dos tipos de fibras durante a transição de hipertrofia ventricular para IC (Carvalho *et al.*, 2003).

3.9. Mecanismos Responsáveis pelas Mudanças dos Tipos de Fibras Musculares na Insuficiência Cardíaca

Os mecanismos responsáveis pelas alterações na composição das isoformas da miosina no músculo esquelético na IC não estão completamente definidos. É provável que os fatores de transcrição pertencentes à família "basic helix-loop-helix" (bHLH), da

qual fazem parte a MyoD, Miogenina, Myf5 e o MRF4, conhecidos como fatores de regulação miogênica, sejam responsáveis pelas mudanças nos tipos de fibras. Como descrito anteriormente, na miogênese, esses fatores transcricionais músculo-específicos regulam a ativação, proliferação e diferenciação de células miogênicas. A MyoD e a Myf5 são expressos em mioblastos na fase de proliferação, que antecede a de diferenciação, enquanto que a Miogenina e o MRF4 são expressos em células no final da fase de diferenciação (Megeney & Rudnicki, 1995). Esses fatores ligam-se a seqüências de DNA (5´-CANNTG-3´), conhecidas como *Ebox,* presentes na região promotora de vários genes músculo–específicos, levando à expressão dos mesmos (Murre *et al.*, 1989; Lassar *et al.*, 1991).

Na fibra muscular adulta, a Miogenina e a MyoD também podem estar envolvidas na manutenção do seu fenótipo, rápido ou lento; a Miogenina é expressa em níveis superiores aos da MyoD em músculos lentos, enquanto que o oposto é verdadeiro para músculos rápidos (Hughes *et al.,* 1993; Voytik *et al.,* 1993). Similarmente, a MyoD é associada à expressão das isoformas rápidas de miosina de cadeia pesada dos tipos IIX e IIB (Hughes *et al.,* 1993; Hughes *et al.,* 1997, Mozdziak *et al.,* 1998; Mozdziak *et al.,* 1999, Seward *et al.*, 2001). Como na IC existe uma transição das isoformas de miosina de lenta para rápida, é provável que essa alteração seja decorrente de uma mudança na expressão dos fatores de regulação miogênica, MyoD e miogenina.

Há poucas informações na literatura a respeito do papel dos fatores de regulação miogênica na transição das isoformas de cadeia pesada de miosina que ocorre nos portadores de insuficiência cardíaca. A primeira hipótese deste trabalho é que a transição das isoformas de cadeia pesada de miosina, de lenta para rápida, que existe na IC, é devida às mudanças na expressão dos fatores de regulação miogênica.

3.10. Alterações da Matriz Extracelular do Músculo Esquelético na IC

Outro fator responsabilizado pelo desenvolvimento de fadiga e fraqueza muscular na IC é a presença de atrofia da musculatura esquelética, observada em diferentes modelos animais (Simonini *et al.*, 1996; De Souza *et al.*, 2000; Vescovo *et al.*, 1998; Dalla Libera *et al.*, 1999; Carvalho *et al.*, 2003; Carvalho *et al.*, 2005) e em

pacientes com essa síndrome (Mancini *et al.,* 1992; Harrington *et al.,* 1997; Toth *et al.*, 1997; Poehlman, 1999; Anker *et al.*, 2003). A atrofia da musculatura esquelética na IC é acompanhada por alterações na matriz extracelular adjacente às fibras musculares e envolve, principalmente, apoptose de células intersticiais (Vescovo *et al.*, 1998; Dalla Libera *et al.*, 1999; Vescovo *et al.*, 2000), aumento no conteúdo de colágeno (Filippatos *et al.*, 2003) e da atividade de metaloproteinases (MMPs) (Schiotz Thorud *et al.*, 2005).

3.11. Metaloproteinases

As MMPs são endopeptidases dependentes de metais, principalmente zinco e cálcio, capazes de degradar os componentes da matriz extracelular, tais como colágeno, elastina, laminina, fibronectina e proteoglicanos (para uma revisão ver Matrisian, 1990). Estas enzimas estão envolvidas em processos fisiológicos normais, tais como embriogênese, ovulação, involução do útero e da mama, erupção dental, remodelação e renovação dos componentes da matriz extracelular (Heikinheimo & Salo, 1995; Bagavandoss, 1998; Tanney *et al.* 1998; Ishizuya-Oka *et al.* 2000; Quaranta, 2000).

As MMPs são sintetizadas na forma de um precursor latente, sendo clivadas e ativadas no espaço extracelular (Nagase, 1997). A clivagem e ativação do precursor latente das MMPs é realizada por uma classe de metaloproteinases denominadas Metaloproteinases de Membrana (MT-MMPs) (Nagase, 1997). Além das MT-MMPs, outras proteases tais como a plasmina, triptase e quimase, também são capazes de ativar as MMPs (Saarinen *et al.*, 1994; Johnson *et al.*, 1998). As MMPs também podem sofrer os processos de auto-ativação ou autoclivagem (Matrisian, 1990; Stamenkovik, 2000).

As células também secretam proteínas inibitórias da atividade das MMPs. Esses inibidores são conhecidos como Inibidores Teciduais de Metaloproteinases (TIMPs) (Woessner *et al.*, 1991). Assim, a atividade dessas enzimas é regulada por meio de um complexo mecanismo de síntese, ativação e inibição.

De acordo com o substrato que degradam, as MMPs são divididas em 5 grupos principais: 1) colagenases intersticiais; 2) gelatinases; 3) estromelisinas; 4)

metaloproteinases de membrana e 5) MMPs que não se enquadram nos grupos citados (revisado em Visse & Nagase, 2003).

3.12. Papel das MMPs na Remodelação da Matriz do Músculo Esquelético na IC

No músculo esquelético, as MMPs parecem ser indispensáveis para a remodelação da matriz extracelular ao redor das fibras musculares (Bernocchi *et al.*, 2003). As MMPs são secretadas por células presentes no músculo, tais como as células de Schwann (Yamada *et al.*, 1995), células satélites (Guerin *et al.*, 1995), fibroblastos (Scott *et al.*, 1998) e pelos axônios (Muir, 1994; Nordstrom *et al.*, 1995). Em condições patológicas que induzem a degradação e a remodelação da matriz extracelular há um aumento da expressão das gelatinases A (MMP2 ou colagenase do tipo IV de 72kDa) e B (MMP9 ou colagenase tipo IV de 92kDa) (Bar-Shai *et al.*, 2005; Giannelli *et al.*, 2005; Reznick *et al.*, 2003; Kherif *et al.*, 1999). A MMP2 possui a capacidade de degradar vários componentes do tecido conjuntivo incluindo colágenos fibrilares dos tipos I (Aimes & Quigley, 1995), II e III, e colágenos não fibrilares dos tipos IV e V (Okada *et al.*, 1990), entretanto, possui baixa atividade para degradar proteoglicanos e fibronectina. A MMP9 degrada os mesmos substratos e é produzida principalmente por células inflamatórias, incluindo leucócitos polimorfonucleares, macrófagos (Stahle-Backdahl *et al.*, 1994) e linfócitos (Montgomery et al., 1993). A atividade das MMPs está aumentada no sangue circulante e no músculo esquelético de ratos com IC (Schiotz Thorud *et al.*, 2005), o que poderia colaborar com as alterações celulares e estruturais do músculo esquelético induzidas pela IC. Entretanto, se o aumento na atividade das MMPs dos músculos esqueléticos na IC origina-se a partir de um aumento na expressão de moléculas de MMPs pelo próprio músculo ainda precisa ser determinado.

A segunda hipótese do presente trabalho é a de que o aumento na atividade das MMPs do músculo esquelético na IC seja decorrente de um aumento nos níveis de expressão do RNA mensageiro para as MMPs 2 e 9.

Objetivos

Avaliar no músculo esquelético de ratos Wistar jovens com IC induzida pela monocrotalina:

1. A expressão das miosinas de cadeia pesada e dos fatores de regulação miogênica MyoD, miogenina e MRF4.
2. A expressão e a atividade das metaloproteinases 2 e 9.

Heart failure alters MyoD and MRF4 expression in rat skeletal muscle

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Summary:

Heart failure (HF) is associated with a skeletal muscle myopathy with increased expression of fast myosin heavy chains (MHC). The skeletal muscle-specific molecular regulatory mechanisms controlling MHC expression during HF have not been described. Myogenic regulatory factors (MRFs), a family of transcriptional factors that control the expression of several skeletal muscle-specific genes, may be related to these alterations. This investigation was undertaken in order to examine potential relationships between MRF mRNA expression and MHC protein isoforms in Wistar rat skeletal muscle with monocrotaline-induced HF. We studied Soleus (SOL) and extensor digitorum longus (EDL) muscles from both HF and control Wistar rats. MyoD, myogenin, and MRF4 contents were determined by using RT-PCR while MHC isoforms were separated by using polyacrylamide gel electrophoresis. Despite no change in MHC composition of Wistar rat skeletal muscles with HF, the mRNA relative expression of MyoD in SOL and EDL muscles and that of MRF4 in SOL muscle were significantly reduced, whereas myogenin was not changed in both muscles. This down-regulation in the mRNA relative expression of MRF4 in SOL was associated with atrophy in response to HF while these alterations were not present in EDL muscle. Taken together, our results show a potential role for MRFs in skeletal muscle myopathy during HF.

Key words: Heart failure; myogenic regulatory factors; myosin heavy chain; skeletal muscle; Wistar rats.

Introduction

Heart failure (HF) is characterized by a reduced tolerance to exercise due to early fatigue and dyspnea; this may in part be due to skeletal muscle myopathy, with atrophy and shift from type I "slow" to type II "fast" fibers (Lipkin *et al*. 1988; Sullivan *et al*. 1990; Mancini *et al*. 1992; De Souza *et al*. 2000). HF induces skeletal muscle myosin heavy chain (MHC) isoform expression toward the fast isoform (Simonini *et al*. 1996; Vescovo *et al*. 1998), which is related to HF severity (Spangenburg *et al*. 2002; Carvalho *et al*. 2003).

Different pathways regulate skeletal muscle MHC expression (Allen *et al*. 2001), including myogenic regulatory factors (MRFs), a family of transcriptional factors that control the expression of several skeletal muscle specific genes. The family has four members: MyoD, myogenin, Myf5 and MRF4. MRFs form dimers with ubiquitous E proteins (e.g. E12 or E47), resulting in heterodimeric complexes that bind to the E-box consensus DNA sequence (5´-CANNTG-3´) that is found in the regulatory region of many muscle-specific genes (Murre *et al*. 1989), including the MHCIIB gene (Wheeler *et al*. 1999). During embryogenesis, MRFs are critical for establishing myogenic lineage and controlling terminal differentiation of myoblasts (for a review, see Parker *et al*. 2003). Previous studies have suggested that myogenin and MyoD may also be involved in establishing and maintaining slow and fast mature muscle fiber phenotype; myogenin is expressed at higher levels than MyoD in slow muscles, whereas the opposite is true for fast muscles (Hughes *et al*. 1993; Voytik *et al*. 1993). Similarly, MyoD is associated with the expression of fast type IIX and IIB MHC isoforms (Hughes *et al*. 1993; Hughes *et al*. 1997; Kraus and Pette 1997; Mozdziak *et al*. 1998; Seward *et al*. 2001).

Therefore, it is reasonable to suggest that MRFs contribute to mechanisms controlling skeletal muscle MHC expression during heart failure.

This investigation was undertaken to examine MRF mRNA expression and their potential relationships with changes in myosin heavy chain composition in young Wistar rat skeletal muscle with monocrotaline-induced heart failure. To compare muscles with different fiber type composition and function, analyses were made in the fast phasic extensor digitorum longus (EDL) and slow postural soleus (SOL) muscles.

Methods

Heart failure induction

Seventeen weaned male Wistar rats (3-4 weeks old; 80-100g) were obtained from the Central Animal House at São Paulo State University. HF was experimentally induced in 10 rats (HF group) by a single intra-peritoneal (ip, 30mg/kg) injection of monocrotaline (MCT), a widely accepted heart failure model (Vescovo *et al*. 1998; Dalla Libera *et al*. 1999; Dalla Libera *et al*. 2001; Leineweber *et al*. 2002). MCT is a pyrrolizidine alkaloid that induces pulmonary vascular disease with severe right ventricle hypertrophy and failure (Vescovo *et al*., 1989; Reindel *et al*. 1990) without itself producing changes in skeletal muscle MHC composition (Vescovo *et al*. 1998). Preliminary experiments revealed that 30 mg/kg ip is an appropriate dose of MCT for our animals with regard to survival and HF induction. MCT-treated rats were allowed to eat freely from a supply of standard rat cubes. Seven controls rats (CT group) were injected with saline and were given the same quantity of food as consumed on the previous day by the treated rats. HF and CT rats were studied 22 days after monocrotaline administration when the HF group had developed overt heart failure. After anesthesia with intraperitoneal sodium pentobarbital (50 mg/Kg), the animals were killed; body weight (BW), SOL weight, and EDL weight were evaluated. The SOL/BW and EDL/BW ratios were used as indexes of muscle atrophy. Muscles were immediately frozen in liquid nitrogen and stored at -80°C. Left ventricle weight (LVW), right ventricle weight (RVW) and atrium weight normalized by body weight (LVW/BW, RVW/BW and ATW/BW respectively) were used as indexes of heart hypertrophy. This experiment was approved by Ethics Committee of Instituto de Biociências, UNESP, Botucatu, SP, Brazil.

Semi-quantitative RT-PCR analyses of mRNA for MRF genes

Total RNA was extracted from SOL and EDL muscles with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized on ice in 1mL of ice-cold TRIzol reagent. Total RNA was solubilized in RNase-free H_2O, incubated in DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove any DNA present in the sample, and quantified by measuring the optical density (OD) at 260nm. RNA purity was ensured by obtaining a 260/280nm OD ratio of ~2.0. Two micrograms of RNA were reverse transcribed with random hexamer primers and Superscript II RT in a total volume of 21μL, according to standard methods (Invitrogen Life Technologies, Carlsbad, CA, USA). Control "No RT" reactions were performed by omitting the RT enzyme. These reactions were then PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of cDNA was then amplified using 1μM of each primer (Table 1), 1X PCR buffer minus Mg, 5mM $MgCl_2$, 1mM deoxyribonucleotide triphosphates, and 2 units of Platinum® Taq DNA Polymerase (Invitrogen Life Technologies, São Paulo, SP, Brazil) in a final volume of 25μL. Primer pairs for MyoD were designed from a sequence published in GenBank, myogenin and MRF4 primer sequences were those used by Smith *et al.*, 1994. Preliminary experiments were conducted with each gene to determine the number of PCR cycles that represented the linear range of amplification. All PCR products were verified by restriction digestion or by sequencing. The cDNA from each muscle for both CT and HF groups were amplified simultaneously by using aliquots from the same PCR mixture. After the PCR amplification, 10μL of each reaction underwent electrophoresis on 1.0% agarose gels

and stained with ethidium bromide. Images were captured and the bands corresponding to each gene were quantified by densitometry as Integrated Optical Density (IOD). PCR products were run in duplicate on different gel for each gene, and results averaged. The size (the number of base pairs) of each band corresponded to the size of processed mRNA. The PCR signs were normalized to the housekeeping gene cyclophilin (Alway *et al.* 2002a).

Table 1: Oligonucleotide primers used for PCR amplification of reverse transcribed RNA

Product	Accession N°	Sequence	Start Position	T_A, °C	Cycles	PCR Length, bp	Restriction Enzyme	Restriction products, bp
MyoD	M84176	5´ - GACGGCTCTCTC TGCTCCTT	259	60	32	544	Sequenced	
		3´- GTCTGAGTCGCCGCTGTAGT	782					
Myogenin	M24393	5´ - TGCCACAAGCCAGACTACCCACC	827	63	31	246	*Pst I*	234, 80, 65
		3´- CGGGGCACTCACTGTCTCTCAA	1050					
MRF4	M30499	5´ - AGAGACTGCCCAAGGTGGAGATTC	491	63	32	272	*Pst I*	118, 96, 57
		3´- AAGACTGCTGGAGGCTGAGGCATC	1344					
Cyclophylin	M19533	5´ - ACGCCGCTGTCTCTTTTC	9	57.7	32	440	*HindII*	40, 400
		3´- TGCCTTCTTTCACCTTCC	431					

Accession N°, GenBank accession number; T_A, annealing temperature.

Electrophoretic separation of MHC

MHC isoform analysis was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Six to ten serial cross sections (12μm thick) were placed in 450μL of a solution containing 10% (wt/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 2.3% (wt/vol) SDS, and 0.9% (wt/vol) Tris/HCl (pH6.8) for 10min at 60°C. Small amounts of the extracts (6μL) were loaded on a 7-10% SDS-PAGE separating gel with a 4% stacking gel, run overnight (19-21h) at 120V, and silver stained. MHC isoforms were identified according to molecular mass, and their relative percentages were quantified by densitometry.

Statistical methods

Data are expressed as mean ± SE. The comparisons between groups were performed using the Student's unpaired t-test and multivariate statistical analysis where appropriate. The level of significance was $p < 0.05$.

Results

Presence of heart failure in the monocrotaline-treated rats (Table 2)

After 22 days, all ten monocrotaline treated rats showed heart failure at post-mortem, confirmed by atrium and right ventricular hypertrophies, pleural and pericardial effusions, and congested liver. No alterations were found in the control rats.

There was no significant difference in BW between HF and CT groups. Heart weight was increased in HF compared to CT, as demonstrated by LVW, RVW, and ATW and by the heart hypertrophy indexes (LVW/BW, RVW/BW and ATW/BW).

RV, LV, AT and Liver wet/dry ratio were greater in HF than in CT. There was no significant difference in Lung wet/dry ratio between CT and HF groups.

Muscle weight and indexes of muscle atrophy (Table 3)

SOL weight and SOL/BW were significantly decreased in HF compared to CT group. Both EDL weight and EDL atrophy index (EDL/BW) did not differ between CT and HF.

Table 2: Anatomical data of CT and HF groups.

	Experimental Groups	
	CT (n=7)	HF (n=10)
BW (g)	179.7 ± 2.8	182.2 ± 3.9
LVW (g)	0.42 ± 0.02	0.48 ± 0.01 *
RVW (g)	0.14 ± 0.01	0.37 ± 0.01 ++
ATW (g)	0.06 ± 0.00	0.10 ± 0.01 +
LVW/BW (mg/g)	2.32 ± 0.07	2.63 ± 0.08 *
RVW/BW (mg/g)	0.77 ± 0.06	2.03 ± 0.07 ++
ATW/BW (mg/g)	0.32 ± 0.02	0.54 ± 0.03 +
RV W/D	4.16 ± 0.06	4.68 ± 0.03 ++
LV W/D	4.13 ± 0.04	4.53 ±0.02 ++
AT W/D	3.53 ± 0.23	5.24 ± 0.23 +
Liver W/D	3.37 ± 0.05	3.64 ± 0.07 *
Lung W/D	4.57 ± 0.27	4.79 ± 0.17

Values are means ± SE; n, number of animals; CT: control group; HF: heart failure group; BW: body weight; LVW: left ventricle weight; RVW: right ventricle weight; ATW: atrium weight; and W/D: wet-to-dry weight ratio. * $p<0.05$, + $p<0.001$, ++ $p<0.0001$: statistical significance vs. control group.

Table 3: SOL and EDL weight of CT and HF groups.

	Experimental Groups	
	CT	HF
	(n=7)	(n=10)
SOL (mg)	98.7 ± 5.1	86.0 ± 3.4 *
SOL/BW (mg/g)	0.55 ± 0.03	0.47 ± 0.01 *
EDL (mg)	81.0 ± 7.7	85.3 ± 3.2
EDL/BW (mg/g)	0.45 ± 0.04	0.47 ± 0.01

Values are mean ± SE; n, number of animals; CT: control group; HF: heart failure group; BW: body weight; SOL: soleus weight; EDL: extensor digitorum longus weight. * $p < 0.05$: statistical significance vs. control group.

MRF mRNA levels estimated by Semi-quantitative RT-PCR

Soleus: MyoD and MRF4 mRNA levels decreased in the HF group compared to its CT group (CT = 0.67 ± 0.04 vs. HF = 0.55 ± 0.04 and CT = 0.64 ± 0.05 vs. HF = 0.46 ± 0.04, respectively) ($p < 0.05$). Myogenin mRNA level was similar between HF and CT groups (CT = 0.53 ± 0.04 vs. HF = 0.46 ± 0.03) (Fig. 1A).

EDL: MyoD mRNA level decreased in the HF group compared to its CT group (CT = 1.83 ± 0.30 vs. HF = 0.80 ± 0.08, $p < 0.05$). Myogenin and MRF4 expression were similar between HF and CT groups (CT = 1.01 ± 0.24 vs. HF = 0.99 ± 0.12 and CT = 1.14 ± 0.20 vs. HF = 1.09 ± 0.12, respectively) (Fig 1B).
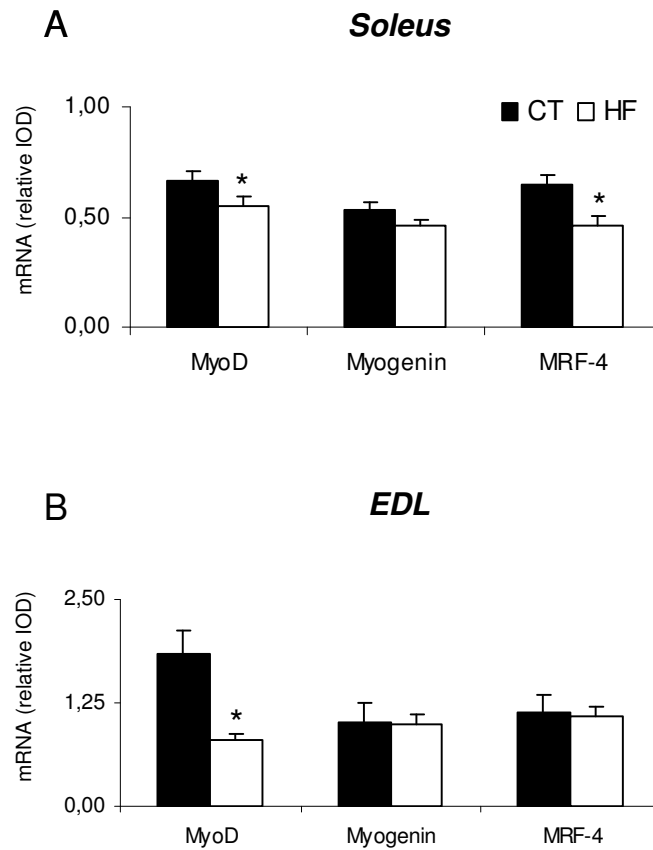
Fig. 1. MyoD, myogenin and MRF4 mRNA content estimated by RT-PCR in CT and HF groups from Soleus (A) and EDL (B) muscles. Data were run in duplicate on different gels for each gene, and the results were averaged. PCR products were visualized with ethidium bromide staining. Quantification of the PCR signal was obtained by densitometric analysis of the product as Integrated Optical Density (IOD). Gene expressions were normalized to the cyclophilin signal from the same RT product. Normalized data are expressed as means $\pm$ SE. * Data are significantly different from CT group at $p < 0.05$.

MHCs electrophoretic pattern

In soleus muscle, two MHC isoforms were separated, MHC1 and MHC2a; their expressions were not different between HF and CT groups (HF = 61.6 ± 1.6 % vs. CT = 63.3 ± 1.7% and HF = 38.4 ± 1.6 % vs. CT = 36.8 ± 1.7 %, respectively) (Fig. 2A). In EDL muscle, three MHC isoforms were separated (MHC 2a, MHC 2d and MHC 2b). The difference in electrophoretic migration between MHC 2a and MHC 2d is very small, and it was not possible to quantify them separately. Consequently, we considered the sum (MHC2a/2d) as both isoforms are metabolically similar. In EDL muscle, MHC2a/d and MHC2b were not different between HF and CT groups (HF = 44.5 ± 0.7 % vs. CT = 42.5 ± 1.2 % and HF = 55.5 ± 0.7 % vs. CT = 57.5 ± 1.2 %, respectively) (Fig. 2B).

A **Soleus**

B **EDL**
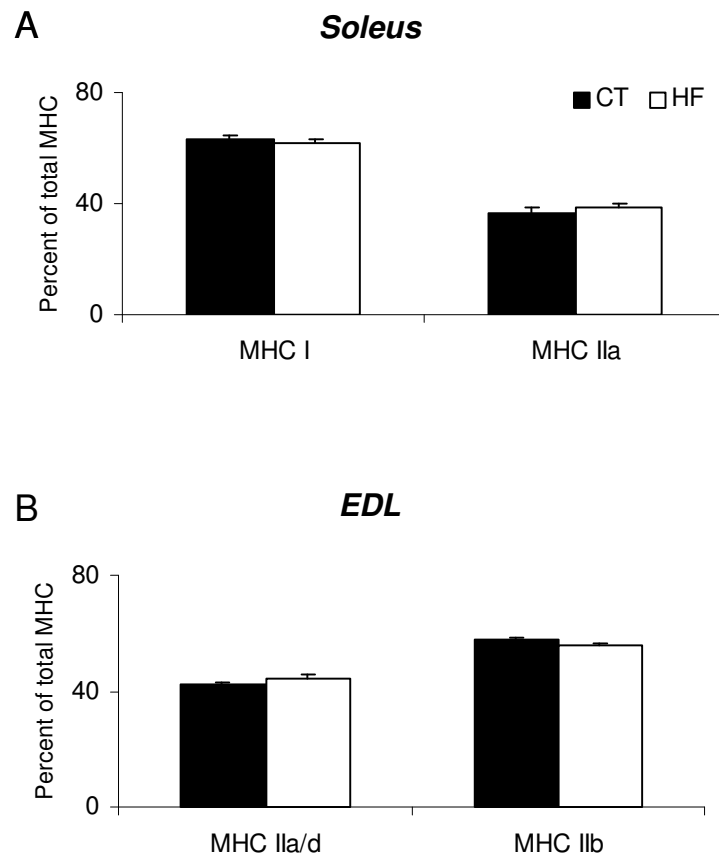
Fig. 2. Percentage distribution of myosin heavy chain (MHC) from soleus (A) and EDL (B) muscles from control (CT) and heart failure (HF) groups*.* Data are expressed as means ± SE.

Discussion

The major finding in this study was that despite there being no change in MHC composition of rat skeletal muscles with monocrotaline-induced heart failure, the mRNA relative expression of MyoD in SOL and EDL muscles and of MRF4 in SOL muscle were significantly reduced, whereas myogenin was not changed in either muscle.

The pattern of MHC expression during heart failure from this investigation diverges from previously published data that showed MHC isoform expression toward the fast isoform in chronic heart failure models such as myocardial infarction in rat (Simonini *et al.*, 1996) and rabbit (Spangenburg *et al.*, 2002), subtotal constriction of the suprarenal abdominal aorta in rabbit (Coirault *et al.*, 1999), and ascending aorta stenosis in rat (Carvalho *et al.*, 2003). The short duration of heart failure in our model could explain this. We could not push experiments further than 22 days because the severity of the monocrotaline-induced heart failure in young Wistar rats inevitably leads to death in this time frame. It is noteworthy that the heart failure period in our study was short and unable to induce change in MHC composition while in other studies, young Sprague-Dawley rats injected with the same dose of monocrotaline as in our experiment (30mg/kg ip) developed heart failure with consequent changes in MHC composition after 27-30 days (Vescovo *et al.* 1998; Dalla Libera *et al.* 1999; Dalla Libera *et al.* 2001).

The synthesis of muscle-specific proteins may be associated with myogenin and MyoD mRNA levels. Myogenin is expressed at higher levels than MyoD in predominantly slow muscle, whereas MyoD is expressed at higher levels than

myogenin in predominantly fast muscles of mature animals (Hughes *et al.*, 1993; Voytik *et al.*, 1993). MyoD is associated with the expression of fast type IIX and IIB MHC isoforms (Hughes *et al.*, 1993; Hughes *et al.* 1997; Kraus and Pette 1997; Mozdziak *et al.* 1998; Seward *et al.* 2001). Additionally to the role of myogenin in establishing mature slow muscle fiber phenotype (Hughes *et al.*, 1993; Voytik *et al.*, 1993), it is also involved with oxidative gene expression and metabolic enzyme activity (Coirault *et al.*, 1999; Hughes *et al.*, 1999; Ekmark *et al.*, 2003). Although we did not analyze these metabolic parameters in our study, they probably were not altered because the mRNA relative expression of myogenin was not changed. We also showed that heart failure induced a reduction in the mRNA relative expression of MyoD in both SOL and EDL muscles suggesting a MHC phenotypic adaptation toward a slower profile in both muscles, as the mRNA relative expression of myogenin was not changed. It is well known that MHC phenotypic adaptation in limb skeletal muscles during heart failure occurs toward a faster profile (Vescovo *et al.* 1998; Simonini *et al.* 1996; Spangenburg *et al.* 2002; Carvalho *et al.* 2003).

Interestingly, our results indicate that skeletal muscle fiber phenotype modulation during this syndrome is not exclusively dependent on the MRFs but may also be regulated by distinct pathways, such as those involving activated calcineurin and transcriptional factor nuclear factor of T cells (NFAT) (Chin *et al.*, 1998; McCullagh *et al.*, 2004), calcium-dependent CaM kinase (McKinsey *et al.*, 2000; Wu *et al.*, 2000), peroxisome proliferator-activated receptor-gamma

coativator 1 (PGC1α) (Lin *et al.*, 2002; Wu *et al.* 2001), Ras (Murgia *et al.*, 2000), and the transcriptional complex Six1/Eya1 (Grifone *et al.*, 2004).

What caused the down-regulation in MyoD mRNA expression during heart failure needs to be determined, however cytokine activation (Anker *et al.*, 1999) may have been involved. This point has been the subject of considerable debate given that tumor necrosis factor-alpha (TNF-α) is markedly increased in patients (Levine *et al.*, 1990; McMurray *et al.*, 1991) and Sprague-Dawley rats with heart failure (Dalla Libera *et al.*, 1999; Dalla Libera *et al.*, 2001). One hallmark of TNF-α is the activation of nuclear factor Kappa B (NFκB), a ubiquitous transcription factor normally inactive and sequestered in the cytoplasm through association with IκB (Baeuerle and Baltimore, 1988). TNF-α exposure leads to the degradation of IκB, allowing NFκB translocation to the nucleus (Israël, 2000) where it down-regulates MyoD mRNA at post-transcriptional level (Guttridge *et al.*, 2000). This mechanism may partially explain the down-regulation of MyoD that we found in both soleus and EDL muscles. However, further studies need to be conducted to demonstrate this mechanism during heart failure.

The analysis of MRF4 mRNA expression revealed that, during heart failure, MRF4 mRNA transcripts were down regulated in the slow SOL muscle but not in the fast EDL. MRF4 is expressed mainly after birth and is likely to have a role in the maintenance of skeletal muscles rather than the development, differentiation and regeneration processes which, may be controlled by MyoD, myogenin, and Myf-5 expression (Perry and Rudnick, 2000). The concept that MRF4 is involved in regulating maturation events and maintaining adult skeletal muscle is further

sustained by the fact that MRF4 is not expressed at substantial levels in limb until very late in fetal development (Hinterberger *et al.*, 1991) and is present at higher levels in postnatal muscle (Voytik *et al.*, 1993; Hughes *et al.*, 1997). Interestingly, in this investigation, a down-regulation in the mRNA relative expression of MRF4 in soleus was associated with atrophy in response to heart failure while these alterations were not present in EDL muscle. This soleus-specific down-regulation of MRF4 was also previously observed in response to disuse atrophy of skeletal muscle in adult rats (Loughna *et al.*, 1996) indicating a possible function for MRF4 in muscle atrophy. Additionally, it has been demonstrated that an increase in Id repressor protein expression, which forms heterodimers with MRFs and prevents their DNA binding (Benezra *et al.*, 1990), is related to muscle atrophy in conditions such as disuse (Gundersen *et al.*, 1994), hindlimb (Alway *et al.*, 2002a) and aging (Alway *et al.*, 2002b).

Taken together, these results show that the potential of MRFs in muscle atrophy clearly requires further examination. Furthermore, the knowledge of the mechanisms controlling MRF expression during heart failure may explain many alterations in skeletal muscle-specific gene and protein expression during this syndrome.

Acknowledgements

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Heart failure alters matrix metalloproteinase gene expression and activity in rat skeletal muscle

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Abstract

Heart failure (HF) is associated with a skeletal muscle myopathy with extracellular alterations. The hypothesis of this investigation is that these extracellular changes may be associated with enhanced mRNA expression and activity of matrix metalloproteinases (MMP). We examined MMP mRNA expression and their potential relationships with changes in MMP activity in young Wistar rats skeletal muscle with monocrotaline-induced heart failure (HF). We studied Soleus (SOL), extensor digitorum longus (EDL), and diaphragm (DIA) muscles from both HF and control Wistar rats. MMP2 and MMP9 mRNA contents were determined by RT-PCR, and MMP activity by electrophoresis in gelatin-containing polyacrylamide gels in the presence of SDS under nonreducing conditions. HF increased MMP9 mRNA expression and activity in SOL, EDL, and DIA and MMP2 mRNA expression in DIA. Taken together, our results show enhanced MMP gene expression which in turn may increase MMP activity, contributing to skeletal muscle myopathy during HF.

Introduction

Heart failure (HF) is characterized by a reduced tolerance to exercise due to early fatigue and dyspnea; this may in part be due to skeletal muscle myopathy with a shift from Type I "slow" to Type II "fast" fibers, atrophy, and reduced oxidative capacity (11,20,22,35). Several mechanisms have been proposed to explain the reduced tolerance to exercise during HF, focusing largely on myofibers intracellular alterations [for a review see Filippatos *et al.* (15)]. Changes in the extracellular matrix (ECM) around muscle fiber during HF mainly involve interstitial cell apoptosis (9,36,37), increased collagen content (14), and enhanced matrix metalloproteinase (MMP) activity (32).

The MMPs are members of a family of zinc-dependent endopeptidases that degrade most ECM components (24) and appear indispensable for the breakdown of the connective tissue surrounding muscle fibers (4). MMPs are synthesized in a latent form and sequentially activated in a cascade initiated by plasmin or membrane-type MMP (MT-MMP) (30). These proteases are secreted by cells present in muscle, such as Schwann cells (41), axons (28,29), satellite cells (16), and fibroblasts (33). MMP activity is increased in the circulating blood and skeletal muscle of HF rats (32), which could contribute with the cellular and structural changes in skeletal muscle induced by HF. However, whether the increased MMP activity is originated from an enhaced expression of skeletal muscle MMP mRNA molecules needs to be determined.

This investigation was undertaken to examine MMP mRNA expression and MMP activity in skeletal muscle of young Wistar rats with monocrotaline-induced HF. We compared muscles with different fiber type composition and function by

analyzing fast phasic extensor digitorum longus (EDL), slow postural soleus (SOL), and fast/slow respiratory diaphragm (DIA) muscles.

Methods

Heart failure induction

Fourteen weaned male Wistar rats (3-4 weeks old; 80-100 g) were obtained from the Central Animal House at São Paulo State University. HF was experimentally induced in 7 rats (HF group) by a single intra-peritoneal (IP, 30 mg/kg) injection of monocrotaline (MCT), a widely accepted heart failure model (3,8,9,37). MCT is a pyrrolizidine alkaloid that induces pulmonary vascular disease with severe right ventricle hypertrophy and failure (31,38) without itself producing changes in skeletal muscle MHC composition (37). Preliminary experiments revealed that 30 mg/kg IP was an appropriate MCT dose for our animals with regard to survival and HF induction. MCT-treated rats were allowed to eat freely from a supply of standard rat cubes. Seven controls rats (CT group) were injected with saline and given the same quantity of food consumed by treated rats on the previous day. HF and CT rats were studied 22 days after monocrotaline administration when the HF group had developed overt heart failure. After anesthesia with intraperitoneal sodium pentobarbital (50 mg/Kg), the animals were killed, and body weight (BW) was evaluated. Right and left SOL, EDL, and costal DIA were immediately frozen in liquid nitrogen and stored at –80 ℃. Left ventricle weight (LVW), right ventricle weight (RVW), and atrium weight normalized by body weight (LVW/BW, RVW/BW, and ATW/BW respectively) were used as indexes of heart hypertrophy. This experiment was approved by Ethics Committee of Instituto de Biociências, UNESP, Botucatu, SP, Brazil.

Semi-quantitative RT-PCR analyses of mRNA for MMP genes

Total RNA was extracted from right SOL, EDL, and costal DIA muscles with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized on ice in 1 mL of ice-cold TRIzol reagent. Total RNA was solubilized in RNase-free H_2O, incubated in DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove any DNA in the sample, and quantified by measuring the optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio of ~2.0. Two micrograms of RNA were reverse transcribed with random hexamer primers and Superscript II RT in a total volume of 21 µL, according to standard methods (Invitrogen Life Technologies, Carlsbad, CA, USA). Control "No RT" reactions were performed by omitting the RT enzyme. These reactions were then PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of cDNA was then amplified using 1 µM of each primer (Table 1), 1 X PCR buffer minus Mg, 5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphates, and 2 units of Platinum® Taq DNA Polymerase (Invitrogen Life Technologies, São Paulo, SP, Brazil) in a final volume of 25 µL. Primer pairs for MMP2 and MMP9 were designed from sequences published in GenBank. Preliminary experiments were conducted with each gene to determine the number of PCR cycles that represented the linear range of amplification. The cDNA from each muscle in both groups were amplified simultaneously using aliquots from the same PCR mixture. After PCR amplification, 10 µL of each reaction underwent electrophoresis on 1.0 % agarose gels and stained with ethidium bromide. Images

were captured and the bands corresponding to each gene were quantified by densitometry as Integrated Optical Density (IOD). PCR products were run in duplicate on different gel for each gene and results averaged. The size (the number of base pairs) of each of the bands corresponded to the size of the processed mRNA. PCR signals were normalized to the housekeeping gene cyclophilin (7).

Table 1: Oligonucleotide primers used for PCR amplification of reverse transcribed

RNA

Product	Accession N°	Sequence	Start Position	T$_A$, °C	Cycles	PCR Length, bp
MMP2	U65656	5` - GTGCTGAAGGACACCCTCAAGAAGA	496	60	32	604
		3` - TTGCCGTCCTTCTCAAAGTTGTACG	1076			
MMP9	U24441	5` - ACGGCAAGGATGGTCTACTG	548	58	32	480
		3` - AGTTGCCCCCAGTTACAGTG	1008			
Cyclophilin	M19533	5´ - ACGCCGCTGTCTCTTTTC	9	57.7	32	440
		3´- TGCCTTCTTTCACCTTCC	431			

Accession N°, GenBank accession number; T$_A$, annealing temperature; MMP, metalloproteinase.

Gelatin Zymography for MMP2 and MMP9

Aliquots (25 µg protein) from left SOL, EDL, and costal DIA muscle extracts were subjected to electrophoresis in gelatin-containing polyacrylamide (8% acrylamide) gels in the presence of SDS under nonreducing conditions as previously described (40). The gelatin substrate was present at 0.1 % final concentration in the gel. The gels (0.75 mm thick) were electrophoresed for 2 hours at 100 V, 4 °C, in a Bio-Rad MiniProtean II system (Bio-Rad Laboratories, Inc., Richmond, CA). Following electrophoresis, the gels were washed by gentle shaking at room temperature with 2.5 % Triton X-100 (2 changes) for 1 h. The gels were incubated overnight (18–20 h) in 50 mM Tris-HCl (pH 8.4) containing 5 mM $CaCl_2$ and 1 µM $ZnCl_2$ at 37 °C. Following incubation, gels were stained with Coomassie Blue. Areas of proteolysis appeared as clear zones against a blue background. Molecular mass determinations were made with reference to prestained protein standards (Bio-Rad Laboratories, Inc.) coelectrophoresed in the gels.

Statistical methods

Data are expressed as means $\pm$ SE. Comparisons between groups were performed using the Student's unpaired *t*-test. The level of significance was $p < 0.05$.

Results

Presence of heart failure in the monocrotaline-treated rats (Table 2)

After 22 days, all seven monocrotaline treated rats showed signs of heart failure at post-mortem examination, such as pleural and pericardial effusions, and congested liver. No alterations were found in the control rats.

BW of the HF group was significantly lower than the CT group. Heart weight was increased in HF compared to CT, as demonstrated by LVW, RVW, and ATW and heart hypertrophy indexes (LVW/BW, RVW/BW and ATW/BW).

RV, LV, AT and Liver wet/dry ratio were greater in HF than CT. There was no significant difference in Lung wet/dry ratio between groups.

Table 2: Anatomical data.

	Experimental Groups	
	CT (n=7)	HF (n=7)
BW (g)	176.5 ± 4.02	158.4 ± 2.9 *
LVW (g)	0.41 ± 0.02	0.46 ± 0.02
RVW (g)	0.13 ± 0.01	0.32 ± 0.01 ++
ATW (g)	0.05 ± 0.00	0.10 ± 0.00 ++
LVW/BW (mg/g)	2.33 ± 0.05	2.88 ± 0.13 +
RVW/BW (mg/g)	0.72 ± 0.02	2.03 ± 0.04 ++
ATW/BW (mg/g)	0.29 ± 0.01	0.63 ± 0.03 ++
RV W/D	3.87 ± 0.04	4.42 ± 0.03 ++
LV W/D	4.01 ± 0.02	4.42 ± 0.02 ++
AT W/D	3.86 ± 0.19	4.70 ± 0.09 *
Liver W/D	3.23 ± 0.02	3.59 ± 0.03 ++
Lung W/D	5.30 ± 0.33	5.03 ± 0.14

Values are means ± SE; n, number of animals; CT: control group; HF: heart failure group; BW: body weight; LVW: left ventricle weight; RVW: right ventricle weight; ATW: atrium weight; and W/D: wet-to-dry weight. * $P<0.05$, + $P<0.001$, ++ $P<0.0001$: statistical significance vs. control group.

MMPs mRNA levels estimated by Semi-quantitative RT-PCR (Figure 1)

SOL: MMP2 expression was similar between groups (HF = 1.75 $\pm$ 0.12 vs. CT = 1.39 $\pm$ 0.14). MMP9 mRNA levels increased in HF compared to CT (HF = 0.33 $\pm$ 0.11 vs. CT = not detectable).

EDL: MMP2 expression was similar between groups (HF = 1.39 $\pm$ 0.10 vs. CT = 1.42 $\pm$ 0.09). MMP9 mRNA level increased in HF compared to CT (HF = 0.14 $\pm$ 0.02 vs. CT = not detectable).

DIA: MMP2 expression increased in HF compared to CT (HF = 0.67 $\pm$ 0.11 vs. CT = 0.47 $\pm$ 0.08, p<0.05). MMP9 mRNA level increased in HF compared to CT (HF = 0.29 $\pm$ 0.08 vs. CT = not detectable).

Figure 1:

A

MMP2

B

MMP9

C

Figure 1. MMP2 (A) and MMP9 (B) content estimated by RT-PCR, and representative PCR result (C) from soleus (SOL), extensor digitorum longus (EDL), and diaphragm (DIA) muscles in control (CT, n=7) and heart failure (HF, n=7) groups. Data were run in duplicate on different gels for each gene, and the results were averaged. PCR products were visualized with ethidium bromide staining. Quantification of the PCR signal was obtained by densitometric analysis of the product as Integrated Optical Density (IOD). Gene expressions were normalized to the cyclophilin signal from the same RT product. Normalized data are expressed as means ± SE. * $p<0.05$ statistical significance vs. CT group. # Not detectable.

Gelatin Zymography for MMP2 and MMP9 (Figure 2)

The gelatinolytic activities of pro, inter, and active MMP2 (72, 64, and 57kDa, respectively) were not different between groups for SOL, EDL, and DIA. Heart failure increased the gelatinolytic activity of pre MMP9 (92kDa) in SOL, EDL, and DIA, and of active MMP9 (81kDa) in DIA.

Figure 2:
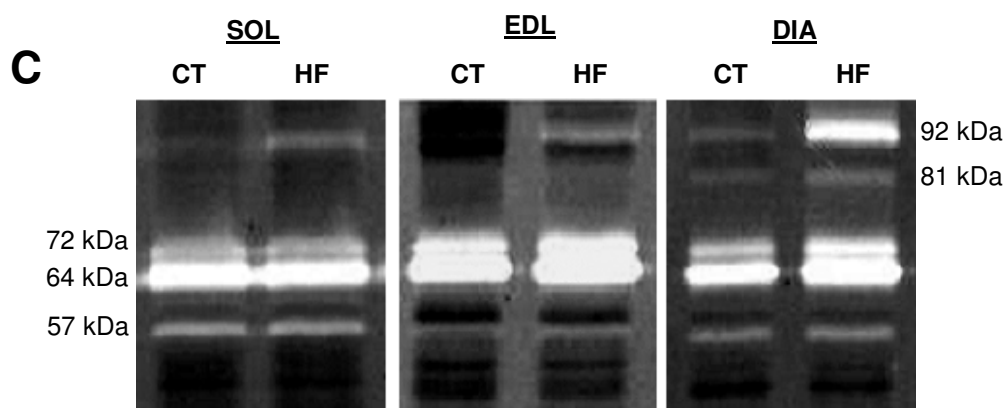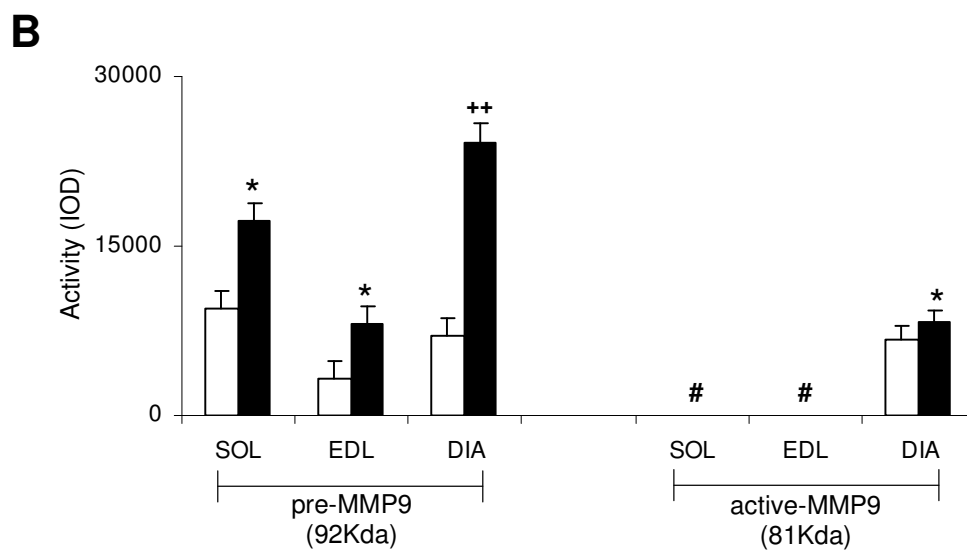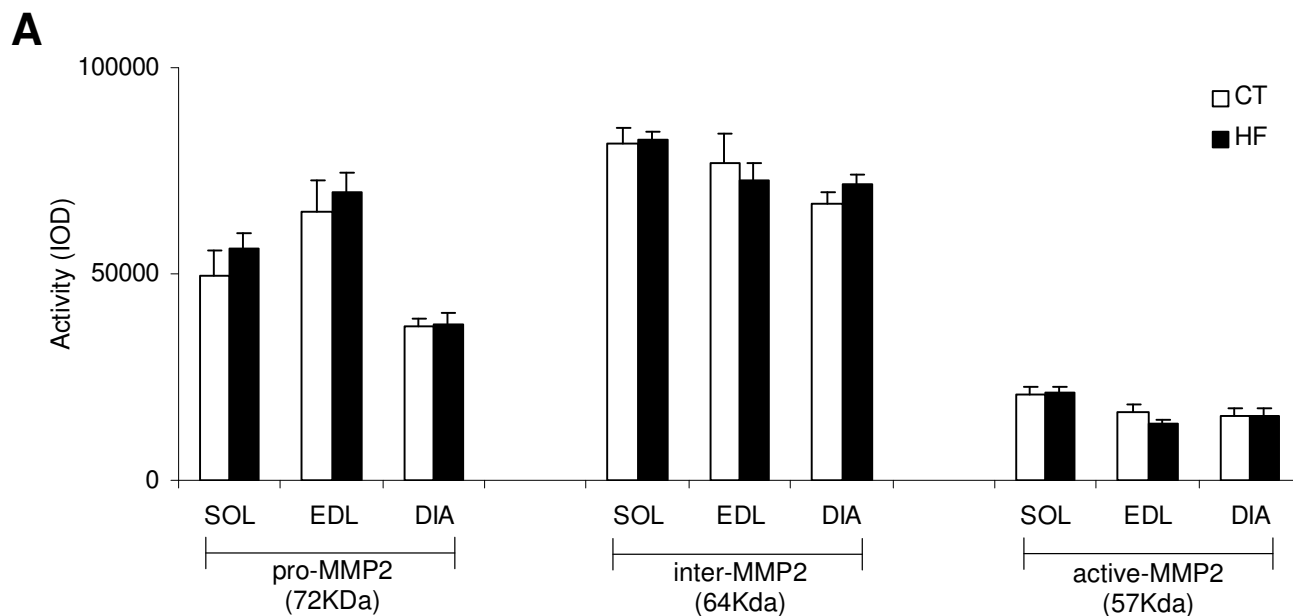
Figure 2. Densitometric analyses of pro-MMP2 (72kDa), inter-MMP2 (64kDa), and active-MMP2 (57kDa) (A), and pre-MMP9 (92kDa), and active-MMP9 (81kDa) (B), and representative gelatin gel zymography (C) from soleus (SOL), extensor digitorum longus (EDL), and diaphragm (DIA) muscles in control (CT, n=7) and heart failure (HF, n=7) groups. Data are expressed as means ± SE. * $p<0.05$, ++$p<0.0001$: statistical significance vs. CT group. # Not detectable.

Discussion

The purpose of this investigation was to determine metalloproteinases (MMP) mRNA expression and their potential relationship with changes in MMP activity in SOL, EDL, and DIA muscles of young Wistar rats with monocrotaline-induced heart failure (HF).

The novel finding in this study was that rats with monocrotaline-induced HF had increased MMP9 gene expression and activity in SOL, EDL, and DIA. This differs from Schiotz Thorud *et al*. (32), who studied SOL and EDL muscles but did not observe alterations in MMP mRNA levels despite changes in MMP activity in a chronic HF model induced by myocardial infarction in rat. These disparities may be due to the different models used. The data obtained in our model, right ventricular pressure overload, suggest that enhanced MMP gene expression may increase MMP activity, thereby contributing to cellular and extracellular alterations in skeletal muscle induced by HF.

The cause of the up-regulation in MMP9 mRNA expression and activity during HF still needs to be determined, however, cytokine activation (1) may have been involved. This point has been the subject of considerable debate given that tumor necrosis factor-alpha (TNF-α) is markedly increased in patients (18,25) and animals with HF (8,9). One hallmark of TNF-α is the activation of nuclear factor Kappa B (NFκB), a ubiquitous transcription factor normally inactive and sequestered in the cytoplasm through association with IκB (2). TNF-α exposure leads to the degradation of IκB, allowing NFκB translocation to the nucleus (17) acting as an important transcription factor in the regulation of the MMP9 gene (12).

Additionally, other researchers have demonstrated that gelatinase activity with a molecular mass of 92kDa (MMP-9) was induced in TNF-α-treated LI90 cells (27). Also, these authors have shown that TNF-α treatment did not affect the gelatinolytic activity with a molecular mass of 72kDa (MMP-2). This mechanism may partially explain the increased MMP9 expression and activity that we found in SOL, EDL, and DIA muscles. However, further studies are still needed to demonstrate this mechanism during HF.

To our knowledge, this is the first report demonstrating increased MMP2 mRNA expression in DIA muscle during HF. This alteration in MMP2 mRNA expression may be related to increased DIA workload, as observed by labored respiration, which frequently occurs in patients (10,19,23) and animals with HF (6,13). Increased respiratory load occurs in response to elevated lung vascular resistance and interstitial edema, reduced lung compliance, and possible increased airway resistance; a more negative pleural pressure is required in HF to inflate the lungs, suggesting increased work for breathing (21,26). Interestingly, the changes in rat DIA muscle MMP2 mRNA expression during HF in this experiment are consistent with Carmeli *et al*. (5) who observed that high-intensity exercise increased MMP2 mRNA levels in the gastrocnemius and quadriceps. Thus, increased MMP2 mRNA expression in the DIA during HF seem to be associated with enhanced muscle work not observed in limb muscles; during this syndrome, the work of DIA tends to increase both at rest and during exercise, whereas limb muscle work tends to decrease (19,34). Although we have not found increased MMP2 activity in this study, we cannot exclude its possible role in DIA muscle

changes during HF, since further regulation of MMP2 expression, probably at the post-transcriptional levels, may occur (39).

In summary, our results show enhaced MMP9 gene expression and activity in SOL, EDL and DIA muscles of rats with monocrotaline-induced heart failure. We also demonstrated an increased MMP2 gene expression in DIA muscle. Alterations in gene expression and activity of these colagenases may contribute to the heart failure induced myopathy.

Acknowledgements

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6. CONCLUSÕES GERAIS

A insuficiência cardíaca induzida pela monocrotalina em ratos Wistar promove:

1. Diminuição na expressão gênica do fator de regulação miogênica MyoD nos músculos sóleo e extensor longo dos dedos, sem alterar a expressão protéica das isoformas de miosina de cadeia pesada;

2. Atrofia do músculo sóleo associada a uma diminuição da expressão gênica do fator de regulação miogênica MRF4;

3. Aumento da expressão gênica e da atividade da metaloproteinase 9 nos músculos sóleo, extensor longo dos dedos e diafragma, e aumento na expressão gênica da metaloproteinase 2 no músculo diafragma.

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