UNIVERSIDADE ESTADUAL DE CAMPINAS

Francis da Silva Lopes

EXPRESSÃO DOS FATORES DE REGULAÇÃO MIOGÊNICA NO MÚSCULO DIAFRAGMA DE RATOS COM INSUFICIÊNCIA CARDÍACA

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) FRANCIS DA SILVA LOPES

e aprovada pela Comissão Julgadora.

Maeli Dal Pai Silva

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

Orientadora: Profa. Dra. Maeli Dal Pai Silva

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BANCA EXAMINADORA

Profa. Dra. Maeli Dal Pai Silva (Orientadora)

Assinatura

Profa. Dra. Maria Alice da Cruz Höfling

Assinatura

Profa. Dra. Marina Politi Okoshi

Assinatura

Prof. Dr. Sérgio Luís Felisbino

Assinatura

Prof. Dr. Antonio de Castro Rodrigues

Assinatura

Dedico este trabalho...

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1. RESUMO

A insuficiência cardíaca (IC) é caracterizada pela intolerância ao exercício físico, devido à fadiga precoce e à dispnéia. Entre as alterações que podem contribuir para a dispnéia nesta síndrome, tem sido descrita a miopatia diafragmática com atrofia e mudanças na miosina de cadeia pesada (MHC) do tipo II "rápida" para a do tipo I "lenta". Entretanto, os mecanismos regulatórios músculos específicos que alteram a expressão das isoformas de miosinas no diafragma durante a IC não são conhecidos. O objetivo do presente trabalho foi determinar, no músculo diafragma (DIA) de ratos Wistar com IC induzida por monocrotalina, se a transição da expressão protéica das MHCs está associada com alterações na expressão do RNAm dos fatores de regulação miogênicos (MRF). A região costal do diafragma de ratos Wistar machos (3-4 semanas de idade; 80-100g) dos grupos IC (n= 09) e Controle (CT; n= 06) foi estudada quando os sinais de IC estavam evidentes, aproximadamente 22 dias após a administração da monocrotalina nos animais IC. A expressão de MyoD e miogenina foi determinada através da técnica de RT-PCR enquanto que a expressão das MHCs foi estudada através de eletroforese por gel de poliacrilamida. Os animais com IC apresentaram diminuição tanto na expressão protéica da MHC IIa/IIx quanto na expressão gênica de MyoD, sem alterar a expressão das MHCs I e IIb e da miogenina. Em conclusão, na IC, a alteração na expressão do RNAm da MyoD pode, em parte, explicar a alteração na expressão protéica da MHC IIa/IIx.

2. ABSTRACT

Heart failure (HF) is characterized by a reduced tolerance to exercise due to early fatigue and dyspnea. Alterations that may contribute to dyspnea in this syndrome are diaphragmatic myopathy with atrophy and shift from type II "fast" to type I "slow" myosin heavy chain (MHC). However, the skeletal muscle-specific molecular regulatory mechanisms that alter MHC expression in the diaphragm during heart failure have not been defined. The purpose of this investigation was to determine whether myosin heavy chain expression during heart failure is associated with changes in myogenic regulatory factors (MRFs) mRNA expression in Wistar rat diaphragm with heart failure (HF) induced by monocrotaline. Costal diaphragm (DIA) muscle from HF (n=09) and control (n=06) 3-4 week old, 80-100g male Wistar rats was studied when overt HF had developed in the HF animals 22 days after monocrotaline administration. MyoD and myogenin expression were determined by RT-PCR, MHC isoform expression was determined by polyacrylamide gel electrophoresis. HF animals presented decreased myosin heavy chain IIa/IIx protein isoform and MyoD gene expression content, without altering MHC I & IIb and myogenin expression. In summary, our results show that in HF, alterations in MyoD mRNA expression may, in part, explain alterations in MHC IIa/IIx content.

3. INTRODUÇÂO

3.1. Músculo estriado esquelético: caracterização morfológica e embriogênese

O tecido muscular estriado esquelético é formado por células especializadas na contração muscular, as fibras musculares, que são multinucleadas e os núcleos estão localizados na periferia da fibra, abaixo da membrana plasmática. As fibras musculares estão imersas em uma matriz extracelular rica em carboidratos e proteínas, que constituem o tecido conjuntivo do músculo. Esse tecido está organizado em três bainhas distintas: o epimísio, que circunda todo o músculo; o perimísio, que divide o músculo em fascículos e o endomísio, que circunda cada fibra muscular (Craig, 1994; Sanes, 1994).

O desenvolvimento do tecido muscular ocorre a partir de células precursoras dos somitos do embrião. O controle da diferenciação dessas células ocorre pela ação dos fatores indutores, (Shh-sonic heghog, Wnts e Noggin) ou inibidores (BMPs), secretados pela notocorda e pelo tubo neural (para revisão ver Charge and Rudnichi, 2004). Um grupo de fatores transcricionais músculo-específicos também está envolvido no processo de diferenciação muscular (Olson, 1990; Rudnicki *et al.*, 1993; Megeney and Rudnick, 1995; para revisão ver Parker *et al.*, 2003). Dos fatores de transcrição, os membros da família MyoD têm papel central na diferenciação do músculo esquelético de vertebrados. Coletivamente chamados de fatores de regulação miogênica (MRF), nos vertebrados, são conhecidos quatro tipos: MyoD, myf-5, miogenina e MRF4, sendo encontrados

apenas em células musculares e em seus precursores imediatos, onde ativam genes específicos do músculo (para revisão ver Watabe, 2001). 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).

Outros importantes reguladores da diferenciação muscular são os fatores de transcrição da família Pax (paired-box), e os da família MEF2, que cooperam na ativação da transcrição de genes músculo-específicos (Rhodes and Konieczny, 1989; Olson, 1992; Daston *et al.*, 1996; para revisão ver Parker *et al.*, 2003 e Charge and Rudnicki, 2004).

As células embrionárias com potencial para se tornarem células musculares são denominadas células progenitoras (ou precursores miogênicos). Essas células originam os mioblastos, capazes de se fundir e de sintetizar proteínas contráteis específicas do músculo, formando os miotubos ou fibras musculares imaturas e multinucleadas (sincício) (Okazaki and Holtzer, 1966, Kelly and Zacks, 1969; Moss and Strohman, 1976). Existem diferentes tipos de mioblastos, cujas populações variam durante o desenvolvimento do tecido muscular e todos expressam pelo menos um dos quatro genes para os MRFs. Os primeiros MRFs expressos são a MyoD e Myf-5, importantes para a proliferação dos mioblastos, enquanto a miogenina e o MRF4 são expressos posteriormente e regulam a diferenciação da fibra muscular (Megeney and Rudnick, 1995).

Na miogênese, ocorrem dois eventos distintos temporalmente para a formação dos miotubos. Inicialmente, ocorre a formação dos miotubos primários que apresentam núcleos centrais e miofibrilas localizadas na região periférica.

Esses miotubos fornecem um suporte para a posterior formação dos miotubos secundários a partir da diferenciação e fusão de mioblastos adjacentes aos miotubos primários. 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 e as miofibrilas passam a ocupar todo o sarcoplasma. Inicialmente, os miotubos primários, os mioblastos e miotubos secundários associados, compartilham a mesma lâmina basal (Ross *et al.*, 1987). Após a diferenciação, cada fibra muscular é envolvida por uma lâmina basal e as mesmas formam um tecido pós mitótico (Franzini-Armstrong and Fischman, 1994; Schmalbruch and Lewis, 2000).

Durante a diferenciação dos miotubos, ocorre a organização das proteínas no sarcoplasma formando o sarcômero, unidade contrátil fundamental da fibra muscular (Huxley, 1969). O sarcômero é constituído por várias proteínas estruturais e contráteis. As proteínas estruturais mantêm a organização do sarcômero e a sua integridade funcional. Entre as proteínas contráteis destaca-se a miosina da classe II, hexâmero formado por duas cadeias pesadas de miosina (MHC) enroladas em α-hélice e quatro cadeias leves de miosina (MLC). A cadeia pesada da miosina apresenta uma porção globular com atividade ATPásica. Várias moléculas de miosina formam o filamento espesso do sarcômero (Lowey *et al.*, 1969).

A proteína actina é o principal componente dos filamentos finos do sarcômero além das proteínas reguladoras, tropomiosina e troponina (Schiaffino and Reggiani, 1996). A contração muscular depende da interação dos filamentos

finos e grossos, que deslizam uns sobre os outros, após a neurotransmissão (Huxley, 1969; 1971; 1983).

Durante a diferenciação dos miotubos, os sarcômeros se organizam da periferia em direção ao centro, e os núcleos migram do centro para a periferia (Okasaki and Holtzer, 1996). Simultaneamente, há o desenvolvimento de um sistema de membranas que também está envolvido com a contração muscular. Ao final desses processos, o miotubo passa a ser chamado de fibra muscular adulta (Schiaffino and Margreth, 1969; Kelly, 1971; Flucher *et al.,* 1992).

Durante o desenvolvimento muscular, uma população distinta de mioblastos não se diferencia e permanece em estado quiescente associada à superfície da fibra muscular, entre a lâmina basal e a membrana plasmática, sendo denominadas de células satélites (Mauro, 1961). Estas células podem ser ativadas por diversos estímulos como traumas, estresse induzido, denervação ou exercício físico e são responsáveis pelo crescimento, reparo e manutenção do músculo esquelético pós-natal (Bischoff, 1994; Seale and Rudnicki, 2000; Charge and Rudinicki, 2004).

Os músculos estriados esqueléticos são constituídos por vários tipos de fibras musculares, e a sua distribuição e prevalência confere aos diferentes músculos uma diversidade estrutural e funcional (Schiaffino and Reggiani, 1994).

3.2. Tipos de fibras musculares

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 genéticas, morfológicas, bioquímicas e fisiológicas distintas (Dubowitz and 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 miosina (mATPase) (Brooke and Kaiser, 1970). Ashmore and Doerr (1971), utilizando a combinação das reações para a mATPase e para a enzima succinato desidrogenase (SDH), classificaram as fibras musculares como βRed, αRed e αWhite. 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 mATPase e NADH-TR (Tetrazolium Reductase).

Estudos envolvendo a microdissecção de fibras, associando a reação histoquímica mATPase com a técnica da eletroforese, possibilitaram a separação de quatro isoformas de miosina de cadeia pesada (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 e mobilidade eletroforética

intermediária entre a IIa e IIb (Termin *et al.* 1989). As fibras IID apresentam características histoquímicas e bioquímicas similares às fibras 2X descritas em ratos (Larsson *et al.*, 1991), camundongos e coelhos (Hämäläinem and Pette, 1993). As fibras do tipo I, IIA, IID/X e IIB são classificadas como fibras puras (Pette and Staron, 1997; Staron *et al.*, 1999).

Além das fibras puras, que expressam apenas um tipo de RNAm para a miosina, há fibras que co-expressam diferentes genes para a MHC (Aigner *et al*, 1993; Schiaffino and 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 (Pette and Staron, 1993; DI Maso *et al.*,2000).

A natureza dinâmica dos tipos de fibras nos músculos de mamíferos, demonstra que cada tipo de fibra apresenta diferentes atividades das enzimas metabólicas. A análise histoquímica do músculo para as atividades de enzimas mitocondriais e mATPase permite a identificação das fibras como de contração lenta e metabolismo oxidativo; de contração rápida e metabolismo glicolítico e de contração rápida e metabolismo oxidativo e glicolítico (Peter *et al.*, 1972).

As fibras com metabolismo oxidativo apresentam, muitas mitocôndrias, pouco glicogênio e maior número de capilares por fibra. Recebem maior teor de oxigênio e metabólitos, possuem elevada atividade de fosforilação oxidativa e a remoção dos produtos do metabolismo é mais rápida. As fibras com metabolismo glicolítico apresentam poucas mitocôndrias, muito glicogênio e o número de

capilares por fibras é menor, sendo o aporte de oxigênio e metabólitos mais reduzido (Silau and Branchero, 1978; Gray *et al.*, 1983; Sanger and Stoiber, 2001).

Como os músculos estriados são constituídos por diferentes tipos de fibras, o conhecimento das características morfofisiológicas dos diferentes músculos é de fundamental importância, tendo em vista que esse tecido pode apresentar variações nos seus parâmetros metabólicos e contráteis na dependência das condições intrínsecas ou extrínsecas que esse músculo é submetido.

3.3. Plasticidade Muscular

O músculo estriado é um tecido dinâmico e pode alterar suas características morfológicas, metabólicas e funcionais em resposta a estímulos como hipóxia, estimulação elétrica, imobilização, exercício físico e condições patológicas (Robbins and Fahin, 1985; Armstrong *et al.*, 1993; Sullivan *et al.*, 1990; Fluck, 1996; Simonini *et al.*, 1996; Sandri *et al.* 1997).

A insuficiência cardíaca é uma dessas condições patológicas que pode induzir adaptações qualitativas e quantitativas nas propriedades musculares frente a sobrecargas funcionais. Dependendo do grau de acometimento da patologia pode-se observar uma redução da atividade locomotora, cansaço, dispnéia e intolerância para realização de atividades da vida diária. Todas essas alterações implicam em uma piora da qualidade de vida.

3.4. Insuficiência Cardíaca

A insuficiência cardíaca (IC) constitui um importante problema clínico devido à gravidade de suas manifestações e à sua grande 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 (Cowie *et al.,* 1997). Resulta em aproximadamente um milhão de hospitalizações por ano, sendo o diagnóstico mais comum nos pacientes hospitalizados acima de 65 anos. No Brasil não existem estudos epidemiológicos envolvendo a incidência de insuficiência cardíaca; porém, de acordo com outros países pode-se estimar que até 6,4 milhões de brasileiros sofram de insuficiência cardíaca (Guimarães *et al.,* 2002). Conforme dados publicados pelo Ministério da Saúde, foram realizadas nos primeiros sete meses de 2003, 203.893 internações por insuficiência cardíaca, com ocorrência de 14 mil óbitos e taxa de mortalidade de 14,7. A IC encontra-se entre as principais causas de internação do Sistema Único de Saúde (Albanesi Filho, 1998; Rossi Neto, 2004).

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. Essa patologia pode progredir e alterar rins, sistema nervoso autônomo, vasos periféricos e músculo esquelético (Lindsay *et al.*, 1996; Braunwald *et al.*, 2001).

A redução da atividade locomotora e/ou a intolerância aos exercícios, que ocorrem na IC, estão associados a dois de seus principais sintomas: dispnéia e

fadiga muscular (Coats *et al.*, 1994; Poole-Wilson and Ferrari, 1996; Wilson, 1996).

3.5. Insuficiência cardíaca e disfunção diafragmática

O sintoma da dispnéia observado na IC é em parte decorrente do aumento da pressão venosa pulmonar e de outros fatores como a atuação de quimioreceptores periféricos, a atividade neuronal aferente e alterações nos volumes pulmonares e nos músculos respiratórios (Buller and Poole-Wilson, 1990; Macfarlane, 2000; Meyer *et al.*, 2000).

Dentre os músculos respiratórios, o diafragma é o mais importante músculo inspiratório dos mamíferos, e talvez por isso um dos mais acometido na IC (Macklem, 1980; Lindsay *et al.*, 1996; Walsh *et al.,*1996, De Sousa et al., 2001).

O diafragma é dividido em duas regiões, crural e costal, com diferenças na distribuição dos tipos de fibras e na capacidade oxidativa (Sugiura *et al.*, 1992). Em ratos, utilizando-se técnicas histoquímicas, foram identificados três tipos de fibras musculares nesse músculo, com base nas características contráteis: I, IIA e IIB (George and Susheela, 1961; Sieck *et al.*, 1983; Metzger *et al.*, 1985). Bär and Pette, 1988, utilizando a técnica de eletroforese em gel de poliacrilamida (SDS-PAGE), demonstrou, no diafragma de ratos quatro tipos de MHCs: MHC I, MHC IIa, MHC IIb, além de um outro tipo de MHC rápida com padrão de migração eletroforética entre a MHC IIa e MHC IIb, que foi designada de MHC IId (Termin *et al.* 1989; Schiaffino and Reggiani 1994). Larsson *et al.* (1991) demonstraram que, no músculo tibial anterior de ratos, a atividade da enzima succcinato

desidrogenase (SDH) nas fibras que expressam MHC IIx, é idêntica à atividade dessa enzima nas fibras que expressam MHCIId evidenciada no diafragma. Portanto, alguns autores utilizam a classificação MHC IIx, em vez de MHC IId, ou de MHC IId/x (De Sousa *et al* 2001; Staib *et al.*, 2002; Rácz *et al.*, 2003). Na região costal do diafragma de ratos há maior proporção das MHC I e MHC IId/x, quando comparada à região crural desse músculo, e, metabolicamente a região costal possui capacidade oxidativa superior à crural (Sugiura *et al.,* 1992).

Caiozzo *et al.* (2003), através de eletroforese de fibra única, observaram alto grau de polimorfismo do músculo diafragma em ratos, com a presença de fibras que co-expressam MHCs I/IIx. Nas diferentes espécies animais, a proporção dos tipos de fibras do diafragma é variável e essa heterogeneidade na distribuição das fibras, neste músculo, reflete as suas adaptações funcionais (Gauthier and Padykula, 1966).

Estudos demonstraram que a dispnéia na IC está associada com a miopatia diafragmática. Várias alterações morfológicas, funcionais e moleculares têm sido descritas no músculo diafragma de humanos e em modelos animais de IC, e estas alterações são mais proeminentes neste músculo, quando comparadas com aquelas observadas nos músculos esqueléticos dos membros (Lindsay *et al.*, 1996; Walsh *et al.,*1996, De Sousa et al., 2001). Howell *et al.* (1995), observaram atrofia das fibras e diminuição de força de contração no músculo diafragma de cobaias, em modelo de IC induzida por taquicardia supraventricular. Esta diminuição de força diafragmática foi observada também em humanos com IC (Hughes *et al.,* 1999, Meyer *et al.*, 2000) e em cães com IC induzida por da taquicardia por marcapasso (Supinski *et al.*, 1994). MacFarlane *et al.* (2000),

identificaram um decréscimo da contratilidade no diafragma por estimulação do nervo frênico, com queda da frequência tetânica de 100 HZ para 25 HZ, paralelamente à redução da amplitude do trânsito de cálcio. Os autores sugerem que a alteração na liberação de cálcio do retículo sarcoplasmático contribuiu para a disfunção diafragmática.

Outro fator responsável pela disfunção diafragmática na IC é a alteração na freqüência dos tipos de fibras, onde ocorre aumento na freqüência das fibras tipo I, de contração lenta, e diminuição na proporção de fibras do tipo II, de contração rápida (Howell *et al.*, 1995). A IC induz mudança nas isoformas de MHC de contração rápida para a isoforma lenta, no músculo diafragma (Tikunov *et al.*, 1996; De Souza *et al.*, 2001), contrário ao que ocorre nos músculos dos membros, onde há a mudança do fenótipo do músculo para um padrão mais rápido (Simonini *et al.*, 1996; Vescovo *et al.,* 1998; 2001; De Sousa *et al.,* 2000; Bernocchi *et al.,* 2003, Carvalho *et al.*, 2003).

A adaptação do diafragma para um fenótipo mais lento na IC é, provavelmente, resultante do aumento da sobrecarga respiratória, e está associada com aumento da capacidade oxidativa (Howell et al., 1995; Tikunov *et al.*, 1996). Entretanto, De Sousa *et al.*, 2001, observaram alteração na atividade mitocondrial e diminuição da capacidade oxidativa no diafragma na IC, que pode estar relacionada à maior susceptibilidade do músculo à fadiga (Walsh *et al.*, 1992; Mancini *et al.*, 1994; Supinski *et al.*, 1994).

Embora as causas da mudança dos tipos de fibras musculares no diafragma na IC não estejam esclarecidas, é provável que a imposição de sobrecarga funcional neste músculo favoreça uma adaptação semelhante à que

ocorre nos músculos dos membros quando submetidos ao exercício de moderada intensidade (Tikunov et al., 1996). O que contribui para o aumento do trabalho respiratório na IC é a alteração da função pulmonar em decorrência de congestão venosa pulmonar, edema intersticial, restrição ao fluxo aéreo, que é acompanhada por decréscimo da complacência pulmonar (Mancini *et al.*, 1995; Buller and Poole-Wilson, 1990).

A hipótese deste trabalho é que a mudança no fenótipo do músculo diafragma, de rápido para lento, que ocorre na IC, estaria relacionada com alterações na expressão dos fatores de regulação miogênica, que controlam a expressão de vários genes músculo-específicos. Essa família é constituída por quatro membros: MyoD, myogenin, Myf5 and MRF4. A Miogenina e a MyoD também estão envolvidas na manutenção do fenótipo da fibra muscular adulta, rápida ou lenta; 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 com a expressão das isoformas de miosina de cadeia pesada rápidas, dos tipos IIx e IIb (Hughes *et al.,* 1993; 1997, Mozdiziak *et al.,* 1998; 1999; Seward *et al.*, 2001). Foi demonstrado, no músculo diafragma de camundongos que, na ausência da expressão da MyoD, não ocorre a expressão da MHC IIb, modificando o fenótipo do músculo (Seward *et al.*, 2001).

Não há informações na literatura sobre a relação entre a expressão dos fatores de regulação miogênica e das MHCs no músculo diafragma de ratos na IC.

4. OBJETIVO

O objetivo do presente trabalho foi determinar, no músculo diafragma de ratos Wistar jovens com IC induzida por monocrotalina, se a expressão protéica das MHCs está associada com a expressão do RNAm dos Fatores de Regulação Miogênicos (MRF).

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6. Heart failure affects MyoD and myosin heavy chain expression in rat diaphragm muscle

F.S. LOPES, R.F. CARVALHO, G.E.R. CAMPOS, M.M. SUGIZAKI, C.R. PADOVANI, C.R. NOGUEIRA, A.C. CICOGNA and M. DAL PAI-SILVA . (artigo a ser submetido para publicação no International Journal of Cardiology).

Heart failure affects MyoD and myosin heavy chain expression in rat diaphragm muscle

FRANCIS DA SILVA LOPES[2,5], ROBSON FRANCISCO CARVALHO[1,2], GERSON EDUARDO ROCHA CAMPOS[4], MARIO MATHEUS SUGIZAKI[3], CARLOS ROBERTO PADOVANI[6], CÉLIA REGINA NOGUEIRA[4], ANTONIO CARLOS CICOGNA[4] and MAELI DAL PAI-SILVA[1]

[1] Departamento de Morfologia, UNESP, CEP 18618-000, Botucatu; [2] Departamento de Biologia Celular, UNICAMP, CEP 13084-971, Campinas; [3] Departamento de Clínica Médica, UNESP, CEP 18618-000, Botucatu; [4] Departamento de Anatomia, UNICAMP, CEP 13084-971, Campinas; [5] Departamento de Fisioterapia, UNOESTE, CEP 19050-900, Presidente Prudente; [6] Departamento de Bioestatística, UNESP, CEP 18618-000, Botucatu, Brasil.

Correspondence: Maeli Dal Pai-Silva, Departamento de Morfologia, UNESP, Botucatu, 18618-000, São Paulo, Brasil. E-mail: dpsilva@ibb.unesp,br

Summary. Dyspnea is a common symptom experienced by patients with chronic heart failure (HF) and is responsible for an intolerance to exercise. Specific diaphragm myopathy with atrophy and increased expression of slow fibers and type I myosin heavy chains (MHC) have been described. The pathways that regulate expression of skeletal myosin heavy chain during heart failure have not been described. Myogenic regulatory factors (MRFs), a family of transcription factors that control the expression of several skeletal muscle-specific genes, may be related to these alterations. The purpose of this investigation was to determine whether myosin heavy chain expression during heart failure is associated with changes in MRF mRNA expression levels in the diaphragm of Wistar rat with heart failure (HF) induced by monocrotaline. Diaphragm (DIA) muscle from both HF and control Wistar rats was studied when overt HF had developed in HF animals, usually twenty-two days after monocrotaline administration. MyoD and myogenin content were determined by using RT-PCR, and MHC isoforms were separated by polyacrylamide gel electrophoresis. HF animals presented decreased myosin heavy chain IIa/IIx protein isoform and MyoD gene expression content, without altering MHC I & IIb and myogenin expression. In summary, our results show that in HF, alterations in MyoD mRNA expression may, in part, explain alterations in MHC IIa/IIx content.

Key words: Heart failure, myogenic regulatory factors, myosin heavy chain, diaphragm, Wistar rats.

Introduction

Chronic heart failure (HF) is characterized by a decreased exercise capacity due to early fatigue and dyspnea. Limb-muscle fatigue has been partly attributed to alterations in morphological and intrinsic biochemical skeletal muscle properties (Lipkin *et al.*, 1988; Drexler *et al.*, 1992; Wilson *et al* 1993; Mancini *et al.*, 1993 Levy *et al.*, 1996; Simonini *et al.*, 1996; Vescovo *et al.*, 1998; De Sousa *et al.*, 2000; Gosker *et al.*, 2000; Levy *et al.,* 2000; Bernochi *et al.*, 2003; Carvalho *et al.*, 2003). There are several molecular, histological and functional abnormalities in diaphragm muscle that may contribute to dyspnea and reduced exercise tolerance (Tikunov *et al.,* 1997; MacFarlane *et al.,* 2000; De Sousa *et al.,* 2001).

Alterations that may contribute to dyspnea in this syndrome: diaphragmatic myopathy with atrophy and shift from myosin heavy chain (MHC) II "fast" to I "slow" have been described (Howell *et al.,* 1995; De Sousa *et al.*, 2001). HF induces diaphragm myosin heavy chain isoform expression toward the slow isoform (Tikunov *et al.*, 1996; De Sousa *et al.*, 2001). However, the skeletal muscle-specific molecular regulatory mechanisms that alter MHC expression in the diaphragm during heart failure are not known.

Different pathways regulate the expression of the skeletal myosin heavy chain (Allen *et al.*, 2001). Four regulatory proteins found in skeletal muscle have been identified as important regulators of muscle-specific gene expression: MyoD, miogenin, Myf-5, and MRF4 (Hughes *et al.*, 1993; Hughes *et al.*, 1999). The primary function of these myogenic regulatory factors (MRFs) appears to involve

muscle determination and development, including activation of muscle-specific genes (Parker *et al.*, 1993).

Previous studies have suggested that myogenin and MyoD may also be involved in establishing and maintaining the mature muscle fiber phenotype, slow or fast; 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, Mozdiziak *et al.,* 1998; Mozdiziak *et al.,* 1999, Seward *et al.*, 2001). In our laboratory, we have shown that MyoD is decreased in the limb muscles of young Wistar rats with monocrotaline-induced heart failure (Carvalho *et al.*, 2005). In at experiment, we did not observe changes in MHC isoform perhaps because monocrotaline induced severe heart failure within a few weeks (twenty-two days). However, during HF, the work of the diaphragm tends to increase both at rest and during exercise, whereas limb muscle work tends to decrease. As reviewed recently, diaphragm dysfunction may exceed loss of function in peripheral muscles (Coats 1996; Lindsay *et al.*, 1996; Stassijns *et al.,* 1996; Harrington *et al.*, 1997).

The objective of this study was to analyze the potential relationships between MRF mRNA expression and myosin heavy chain isoforms in the diaphragm of Wistar rat with monocrotaline induced heart failure.

Materials and Methods

1. Experimental model

Fifteen 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 nine rats (HF group) by a single intra-peritoneal injection of 30mg/kg monocrotaline (MCT), a largely accepted heart failure model (Vescovo *et al.*, 1998; 2002; Dalla Libera *et al.*, 1999; 2001b; 2004; Bernocchi *et al.*, 2003;). MCT is a pyrrolizidine alkaloid that induces pulmonary hypertension with severe right ventricle hypertrophy and failure (Vescovo *et al.*, 1989, Reindel *et al.*, 1990), without producing direct changes in skeletal muscle MHC composition (Vescovo *et al.*, 1998; Dalla Libera *et al.*, 1999; Dalla Libera *et al.*, 2001b; 2004; Vescovo *et al.*, 2002; Bernocchi *et al.*, 2003). MCT-treated rats were allowed to eat freely from a supply of standard rat chow. Six controls rats (CT group) were injected with saline and were fed the same amount of food consumed on the previous day by the HF rats. HF and CT rats were studied after the development of tachypnea and labored respiration in the HF group, which occurred twenty-two days after monocrotaline administration. HF was documented at sacrifice by the presence of pleural, pericardial, and peritoneal effusions, and severe atrial and ventricular hypertrophy and dilatation. At time of the study, all animals were anaesthetized with sodium pentobarbital (50 mg/Kg), and killed. Body weight (BW) was measured. The left and right portions of the costal diaphragm were isolated and immediately frozen in liquid nitrogen and stored at –80°C. Left (LVW) and right (RVW) ventricle weight

normalized to BW (LVW/ BW and RVW/ BW, respectively) were used as indexes of ventricular hypertrophy.

This experiment was approved by the Ethics Committee of Bioscience Institute, UNESP, Botucatu, SP, Brazil (protocol n° 16/05-CEEA, IB - UNESP - Botucatu).

2. Morphometric analysis

Transverse sections approximately 10µm thick of frozen left costal diaphragm were cut in a cryostat at –20°C. Sections were stained by haematoxylin and eosin (Bancroft and Stevens, 1991) and fiber diameters were measured by compound microscope attached to a computerized imaging analysis system (Qwin, Leica, Nussloch, Germany). The cross-sectional area of at least 100 fibers from each muscle was measured and expressed in μm^2.

3. 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) of frozen left costal diaphragm were placed in 450µL of a solution containing 10% (wt/vol) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (wt/vol) SDS and 0.9% (wt/vol) Tris-HCl (pH6.8) for 10min at 60°C. Small quantities 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.

4. RT- PCR analysis of mRNA for MRF

Total RNA was extracted from right portions of the costal diaphragm 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 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 at 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 done in which the RT enzyme was omitted. The Control No RT reactions were PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of cDNA was then amplified using 1µM of each primer (Table I), 1X PCR buffer minus Mg, 5mM $MgCl_2$, 1mM deoxyribonucleotide triphosphates, 2 units of Platinum® Taq DNA Polymerase in a final volume of 25µL. Primer pairs for MyoD were designed from sequences published in GenBank; myogenin primer sequences were as per Smith *et al.*, 1994. Preliminary experiments were conducted with each gene to determine the number of PCR cycles that represented the linear amplification range. All PCR products were verified by restriction digestion or sequencing. The cDNA from each muscle for both CT and HF groups were amplified simultaneously by using aliquots from the same PCR mixture. After PCR amplification, 10µL of each reaction were

electrophoresed on 1.0% agarose gel and stained with ethidium bromide. The images were captured and the bands corresponding to each gene were quantified by densitometry as Integrated Optical Density (IOD). The PCR products were run in duplicate on different gel for each gene, and results averaged. The size (number of base pairs) of each band corresponds to the quantity of processed mRNA. The PCR products were normalized to the housekeeping gene cyclophilin (Always *et al.*, 2002, Siu *et al.*, 2004).

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

luct	Accession N°	Sequence	Start Position	T_A, °C	Cycles	PCR Length, bp	Restriction Enzyme	Restriction products, bp
)D	M84176	5′ - GACGGCTCTCTC TGCTCCTT	259	60	32	544	Sequenced	
		3′- GTCTGAGTCGCCGCTGTAGT	782					
enin	M24393	5′ - TGCCACAAGCCAGACTACCCACC	827	63	31	246	*Pst I*	234, 80, 65
		3′- CGGGGCACTCACTGTCTCTCAA	1050					
)hylin	M19533	5′ - ACGCCGCTGTCTCTTTTC	9	57.7	32	440	*HindII*	40, 400
		3′- TGCCTTCTTTCACCTTCC	431					

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

Statistical methods

Data are expressed as mean ± standard error (SE). The comparisons were made between CT and HF groups. Student's unpaired t-test was used in the analysis of anatomical data, mean fiber area, and MRF mRNA content. MHC isoform percentages were submitted to multivariate statistical analysis. The level of significance was $p<0.05$.

Results

Anatomical Data

Table II shows the anatomical data of the Control (CT) and HF groups. At 22 days, all monocrotaline-treated rats showed the signs of heart failure at post-mortem examination such as atrium hypertrophy, right ventricular hypertrophy (RVW/BW $\geq$ 0.80), ascite, pleural and pericardial effusions, and congestion of the liver. No alterations were found in the control rats.

There was no significant difference in BW between HF and CT groups. Heart weight was greater in HF than CT group, as demonstrated by LVW, RVW, and atrium (AT) and heart hypertrophy indexes (LVW/BW, RVW/BW, and ATW/BW). There was no significant difference in Lung wet/dry ratio between CT and HF groups. The wet RV, LV, AT, and liver wet/dry ratio were greater in HF than CT group.

Morphometric analysis

Cross-sections of diaphragm muscle from Control and HF stained with haematoxilyn and eosin showed normal morphology. There were no differences in fiber cross-sectional area between HF and CT groups (790.27 ± 72.91μm^2 in CT vs. 757.02 ± 23.85μm^2 in HF, p= 0.6) (Table II).

Table II. Anatomical data and mean fiber area of diaphragm muscle in the experimental groups.

	Experimental groups	
	CT (*n*= 6)	*HF* (*n*= 9)
BW (g)	158.4 ± 3.5	154.6 ± 8.4
LV wt (g)	0.37 ± 0.006	0.45 ± 0.02 *
RV wt (g)	0.10 ± 0.007	0.34 ± 0.02 +
AT wt (g)	0.06 ± 0.005	0.1 ± 0.01 **
LV/Body wt (mg/g)	2.35 ± 0.04	2.99 ± 0.14 *
RV/Body wt (mg/g)	0.65 ± 0.05	2.22 ± 0.08 +
AT/Body wt (mg/g)	0.38 ± 0.03	0.65 ± 0.01 *
LV w/d	4.12 ± 0.04	4.69 ± 0.054 +
RV w/d	4.14 ± 0.021	4.78 ± 0.07 *
AT w/d	3.4 ± 0.22	0.02 ± 0.002 +
Liver w/d	3.16 ± 0.07	3.59 ± 0.036 +
Lung w/d	4.83 ± 0.18	5.18 ± 0.17
Area (μm^2)	790.3 ± 72.9	757.0 ± 23.9

Values are means ± SE; n, number of animals; CT: control group; HF: heart failure group; BW: body weight; LV: left ventricle weight; RV: right ventricle weight; AT: atrium weight; and w/d: wet-to-dry weight. * $p<0.05$, ** $p<0.001$, + $p<0.0001$: statistical significance vs. control group.

MHCs electrophoretic pattern

In diaphragm muscle, four MHC isoforms were separated, MHC I, MHC IIa, MHC IIx and MHC IIb. The difference in electrophoretic migration between MHC IIa and MHC IIx was very small, and it was not possible to quantify them separately. Consequently, we considered the sum of MHC IIa and MHC IIx since from a metabolical point of view, both isoforms are glycolitic and moderately oxidative (Bernochi *et al.*, 2003). MHC I and MHC IIb were not different between groups (CT= 23.97 ± 2.96% vs. HF = 27.86 ± 1.27% and CT = 4.24 ± 2.02% vs. HF = 4.93 ± 1.87%, respectively). MHC IIa/IIx decreased in the HF group compared to the CT group (CT= 71.79 ± 0.97% vs. HF = 67.21 ± 1.36%, $p<0.05$; Fig 1).

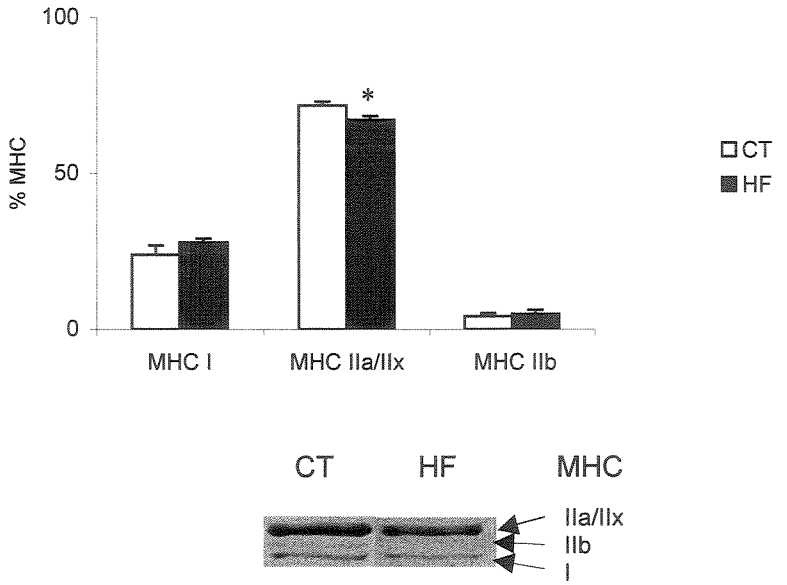

Fig 1. Percentage distribution of myosin heavy chain (MHC), MHC I, MHC IIa/IIx and MHC IIb in the diaphragm muscle of control (CT) and heart failure (HF) groups. * $p<0.05$ vs CT group $P<0.05$.

MRF mRNA levels in diaphragm estimated by semi-quantitative RT-PCR

MyoD mRNA levels were lower in the HF than CT group (CT = 2.63 ± 0.63 vs. HF = 0.88 ± 0.19, $p<0.05$; Fig. 2). Myogenin expression was similar in both groups (CT = 1.0 ± 0.21 vs. HF = 0.74 ± 0.08). The MyoD to Myogenin mRNA ratio was less in the HF than CT group (CT = 2.90 ± 0.79 vs. HF = 1.30 ± 0.31, $p<0.05$).

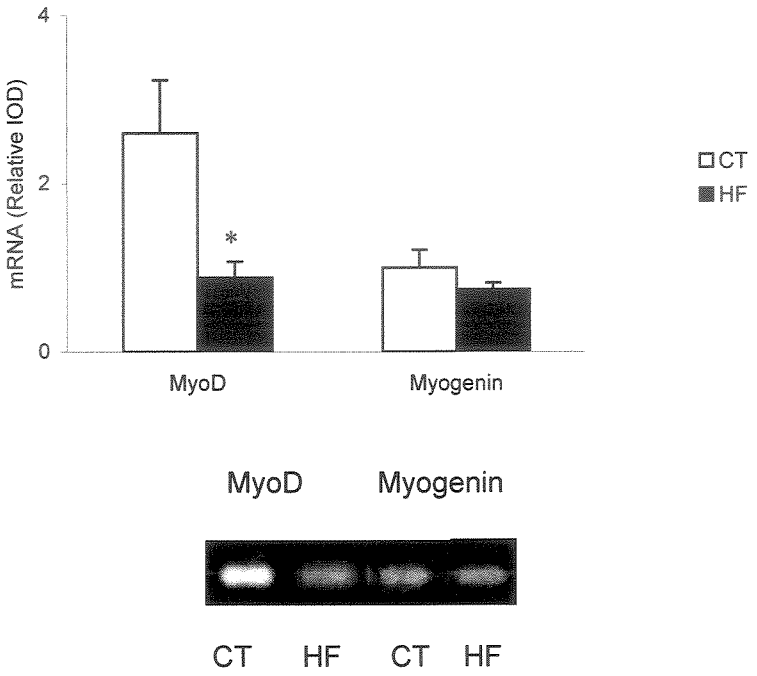

Figure 2. MyoD and myogenin mRNA content estimated by RT-PCR in Control (CT) and Heart Failure (HF) groups from rat diaphragm muscle. Data were run in duplicate on different gels for each gene, and results averaged. PCR products were visualized with ethidium bromide staining. PCR quantification was by densitometric analysis of the product as Integrated Optical Density (IOD). The expressions of genes were normalized to the cyclophilin signal from the same RT product. Normalized data are expressed as means ± SE. * Data are significantly different from CT group at $p<0.05$.

Discussion

The objective of this study was to analyze the potential relationships between myogenic regulatory factors (MRFs) mRNA expression and myosin heavy chain isoforms (MHC) in Wistar rat diaphragm with heart failure induced by monocrotaline. This is the first study showing that heart failure (HF) changes the relative mRNA level of the myogenic regulatory factor (MyoD) in association with an alteration in MHC IIa/IIx protein isoform in the costal diaphragm muscle. We did not observe any change in MHC I and MHC IIb protein isoforms or in mRNA relative expression of myogenin.

The pattern of phenotype alterations such as increased MHC I and decreased MHC IIb in the diaphragm muscle has been shown in humans with chronic congestive heart failure (Tikunov *et al.*, 1996). De Souza *et al.,* 2001 observed an increase in MHC I and/or MHC IIa in the diaphragm of rats with HF induced by aortic stenosis. In these chronic HF models, there was reorientation in the diaphragm toward slow pattern. In our investigation, the change in MHC IIa/IIx only, may be that in young Wistar rats (3-4 weeks) monocrotaline induces severe heart failure within a few weeks (twenty-two days). However, the MHC I isoform tended to increase.

These findings are different from those observed in the locomotor muscles of patients and in other HF models where the levels of slow MHC are decreased and fast MHC levels are increased (Simonini *et al.*, 1996; De Sousa *et al.*, 2000; Spangenburg *et al.*, 2002; Bernochi *et al.*, 2003; Carvalho *et al.*, 2003).

The skeletal muscle is able to adapt to a variety of injuries. The modulation in MHC that occurred in the diaphragm muscle of monocrotaline induced HF, may be related to increased workload, as observed by labored respiration, which frequently occurs in heart failure patients (Mancini *et al.*, 1994; Lindsay *et al.*, 1996; De Sousa *et al.*, 2001). The increase in respiratory load occurs in response to elevated lung vascular resistance and interstitial edema, reduced lung compliance, and possibly increased airway resistance; a more negative pleural pressure is required in HF to inflate the lungs. This suggests increased work for breathing (Mackelem 1980; Meyer *et al.*, 2000). In fact, the changes observed in MHC expression in HF rat diaphragm muscle are consistent with those seen in endurance exercise of normal subject limb muscles (Tikunov *et al.*, 1997).

Numerous signaling pathways and molecular mechanisms are currently being examined as candidates that may govern differential MHC gene expression in skeletal muscle. There is substantial evidence that the myogenic regulatory factors (MRFs), MyoD, Myf5, Myogenin, and MRF4 are important regulators in the expression of muscle-specific proteins (Murre *et al.*, 1989; Hughes *et al.*, 1993, Megeney *et al.*, 1995; Hughes *et al.*, 1997). 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 expression of fast type IIX and IIB MHC isoforms (Hughes *et al.,* 1993; Hughes *et al.,* 1997, Mozdiziak *et al.,* 1998; Mozdiziak *et al.,* 1999, Seward *et al.*, 2001).

Alterations in MRFs expression may be directly involved in controlling fiber type-specific gene expression in response to external signals such as hypothyroidism, chronic low-level frequency stimulation, cross-reinnervation,

denervation, and hindlimb suspension (Eftimie *et al.,* 1991; Mozdiziak *et al.*, 1998; Carlsen *et al.*, 2000).

In this study, the reduction in MyoD mRNA expression provides evidence of its role in the change of MHC protein isoform expression in diaphragm muscle during heart failure. Staib *et al.*, 2002 also showed change of MHC protein isoform expression in costal diaphragm of mice MyoD -/-. MyoD deletion was associated with a downward shift in the diaphragm force-frequency relationship and a decrease in the maximal tetanic force, and resulted in a MHC phenotype fast-to-slow shift. This supports the view in our study, that down-regulation of MyoD in heart failure may have contributed to the previously reported reduction in diaphragm function. Thus, the decrease in MyoD mRNA and MHC IIa/IIx suggest a phenotypic adaptation in the diaphragm toward a slower profile. Similarly, a study in diaphragm muscle of rats subjected to mechanical ventilation showed an association between decreased MyoD mRNA level and diminished fast MHC IIa and IIb mRNA expression; the authors suggest muscle phenotypic adaptation toward a slower profile considering the reduction in the MyoD/myogenin ratio, similar to that observed in our study (Racz *et al.*, 2003).

The causes of down-regulation in MyoD mRNA expression during heart failure are still unknown; however, neurohormones and cytokine activation may contribute (Anker *et al.*, 1999). This last point has undergone considerable debate because tumor necrosis factor-alpha (TNF-α) is markedly increased in human and animals with chronic heart failure (Levine *et al.*, 1990; MucMurray *et al.*, 1991, Dalla Libera *et al.*, 2001a). Li *et al.* (2000) demonstrated that elevated circulating

levels of TNF-α provoke contractile dysfunction in the diaphragm through an endocrine mechanism is thought to be mediated by oxidative stress. One hallmark of TNF-α action is the activation of the nuclear factor Kappa B (NFκB), a ubiquitous transcription factor normally inactive and sequestered in the cytoplasm through association with IκB (Guttridge *et al.,* 2000). TNF-α exposure leads to the degradation of IκB, allowing NFκB translocation to the nucleus where it down-regulates MyoD mRNA at a post-transcriptional level (Israel, 2000). This mechanism may partially explain the down-regulation of MyoD that we found in diaphragm muscle.

In spite of the phenotypic adaptation tendency of HF rat diaphragm toward a slower profile, mRNA levels of myogenin did not change. Although myogenin has been observed to be overexpressed in mature slow muscle (Hughes *et al.*, 1993, Voytik *et al.*, 1993), it has been shown that this MRF is also involved with oxidative gene expression and metabolic enzyme activity (Hughes *et al.*, 1999; Ekmark *et al.*, 2003; Siu *et al.*, 2004). Although we did not analyze these metabolic parameters in our study, possibly they were unchanged since the mRNA relative expression of myogenin was not altered. On the other hand, myogenin may be involved in the metabolic profile maintenance of skeletal muscle without changes in fiber type-specific myosin heavy chain expression (Hughes *et al.* 1999).

Concerning the mean fiber area we did not observe diaphragm fiber atrophy in HF rats induced by monocrotaline. Variable results related to this parameter have been reported for different HF models (Mancini *et al.*, 1989; Sullivan *et al.*, 1990; Brunotte *et al.*, 1995; Howell *et al.*, 1995).

In summary, our results show that in HF, the alterations in MyoD mRNA expression protein may in part, explain the alterations in MHC IIa/IIx content. These changes are likely to contribute to the diaphragm dysfunction caused by heart failure. Further studies are needed to map the complexity of this phenomenon to develop strategies to minimize the effects of heart failure on diaphragm function.

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7. CONCUSÕES

Os animais com insuficiência cardíaca induzida pela monocrotalina desenvolveram miopatia diafragmática, com diminuição na expressão protéica da miosina de cadeia pesada rápida do tipo IIa/IIx e tendência no aumento da expressão das miosinas lentas, sem atrofia muscular. Houve diminuição na expressão do mRNA do fator regulador miogênico MyoD sem alteração na miogenina.

Na IC ocorreu uma alteração fenotípica do músculo diafragma tornando-o mais lento associada à diminuição na expressão da MyoD. Nossos dados corroboram a hipótese da associação entre a expressão das miosinas de cadeia pesada e dos fatores reguladores miogênicos na IC.