UNIVERSIDADE ESTADUAL DE CAMPINAS

Robson Francisco Carvalho

EXPRESSÃO DE CADEIA PESADA DE MIOSINA E ATROFIA NO MÚSCULO ESTRIADO ESQUELÉTICO DE RATOS DURANTE A TRANSIÇÃO DE HIPERTROFIA VENTRICULAR PARA INSUFICIÊNCIA CARDÍACA

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

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Maeli Dal Pai Silva

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Dedico este trabalho...

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Índice

1. RESUMO

A Insuficiência cardíaca (IC) é caracterizada por uma reduzida tolerância ao exercício, devido a uma miopatia do músculo esquelético, com atrofia e mudança de fibras de contração lenta para de contração rápida. A IC induz a expressão de isoformas de cadeia pesada de miosina (*myosin heavy chain*, MHC) em direção à isoforma rápida. O objetivo do presente trabalho foi determinar, no músculo sóleo, possíveis mudanças na expressão das isoformas de MHC, a freqüência e o diâmetro dos tipos de fibras e o índice de atrofia muscular, durante a transição de hipertrofia ventricular para IC, induzida por estenose aórtica (EAo). Adicionalmente foi estudado o papel da apoptose na atrofia muscular, durante a fase de IC. Foram utilizados ratos Wistar machos (idade de 3 a 4 semanas; 80-100 g), divididos em dois grupos: com EAo - grupo EAo, e sem EAo - grupo controle. Os ratos do grupo EAo foram comparados com os ratos do grupo controle após 12 e 18 semanas e quando os animais com EAo apresentavam sinais visíveis de IC, 28 semanas após a cirurgia. Os animais com estenose aórtica desenvolveram uma miopatia no músculo sóleo, caracterizada por diminuição da MHC1 e da freqüência das fibras do tipo I e aumento da MHC2a e da freqüência das fibras do tipo IIa, na hipertrofia cardíaca (após 18 semanas) e na IC (após 28 semanas). A atrofia das fibras do tipo IIa ocorreu durante a IC. Não foi possível estabelecerem-se correlações entre a atrofia muscular e apoptose no músculo esquelético, como demonstrado pela marcação da fragmentação do DNA. Nossos dados demonstram que as alterações no fenótipo do músculo esquelético têm inicio na fase de hipertrofia cardíaca e sugerem que atenção seja dada na determinação

das mudanças do fenótipo do músculo esquelético antes que se tornem claros os sintomas de IC.

2. ABSTRACT

Chronic heart failure (CHF) is characterized by a reduced tolerance to exercise, which may be due to a skeletal muscle myopathy, with atrophy and a shift from the slow to the fast fibers. CHF induces skeletal muscle myosin heavy chain (MHC) isoform expression toward the fast isoform. The purpose of this investigation was to determine whether changes in MHC expression and atrophy of skeletal muscle are observed during the transition from cardiac hypertrophy to CHF induced by aortic stenosis (AS). We also investigated the possible role of apoptosis in skeletal muscle atrophy during CHF. AS and control animals were studied 12 and 18 weeks after the surgery and when overt CHF had developed in AS animals, 28 weeks after the surgery. The following parameters were observed in the soleus muscle: the index of muscle atrophy (soleus weight/body weight), muscle fibers diameter and frequency, and MHCs expression. AS animals presented decreases in both MHC1 and type I fibers as well as increases in both MHC2a and type IIa fibers during late cardiac hypertrophy and CHF. Type IIa fiber atrophy occurred during CHF and was not associated with skeletal muscle apoptosis as demonstrated by DNA nick-end labelling. In conclusion, our data show that skeletal muscle phenotype changes occur in both late cardiac hypertrophy and heart failure and suggest that attention should be placed on determining skeletal muscle phenotype changes prior to overt symptoms of heart failure.

3. INTRODUÇÃO

3.1. Considerações sobre o tecido muscular estriado esquelético

O tecido muscular estriado esquelético constitui o maior tecido do corpo dos mamíferos, sendo formado por células multinucleadas especializadas, as fibras musculares, que possuem a capacidade de contração. Essa é uma propriedade fundamental de todas as células animais, que alcança sua maior expressão nas fibras musculares.

O desenvolvimento do tecido muscular ocorre a partir do mesoderma somático, onde células se diferenciam em células miogênicas, que posteriormente constituirão os mioblastos, os quais podem se fundir para formar os miotubos (Kelly e Zacks 1969; Ontell e Koseka 1984; Ross et al. 1987). Alguns mioblastos não se fundem e permanecem quiescentes entre a membrana plasmática da fibra muscular e a lâmina basal, sendo denominados de células satélites (Mauro 1961).

Nos miotubos, ocorre a organização das proteínas que irão constituir a unidade contrátil, o sarcômero (Huxley 1969). O sarcômero é constituído principalmente pelos filamentos grossos; compostos pelas cadeias de miosina e pelos filamentos finos; compostos pelas proteínas actina, troponina e tropomiosina. A contração muscular depende do deslizamento dos filamentos finos sobre os filamentos grossos (Huxley 1969; Huxley 1971; Huxley 1983).

Durante a diferenciação dos miotubos ocorre a organização dos sarcômeros da periferia em direção ao centro do miotubo, enquanto que os núcleos migram do centro para a periferia (Okasaki e Holtzer 1966). Paralelamente, ocorre o desenvolvimento de um sistema de membranas, que

também está envolvido no mecanismo de contração muscular (Schiaffino e Margreth 1969; Kelly 1971; Flucher et al., 1992). Ao final desses processos, o miotubo passa a ser chamado de fibra muscular adulta.

Os músculos esqueléticos dos mamíferos são constituídos por diferentes tipos de fibras que apresentam características metabólicas e funcionais distintas (Ogata 1958).

3.2. Tipos de fibras musculares

Três tipos principais de fibras musculares, denominadas de fibras tipos I, IIA e IIB, foram descritos inicialmente por Brooke e Kaiser (1970), de acordo com o padrão de reatividade para a atividade da ATPase da porção globular da miosina (ATPase miofibrilar ou mATPase). A molécula de miosina é um hexâmero formado por duas miosinas de 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 e Lowey 1971; Elliot e Offer 1978; Warrick e 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, que apresenta um 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).

Posteriormente, com a utilização de vários métodos histoquímicos para a identificação da atividade da mATPase, as fibras musculares do tipo II de camundongos, coelhos e ratos foram subdivididas em fibras dos tipos IIA, IIX (ou

IID) e IIB (Hamäläinen e Pette 1993). Entre esses três tipos também podem ser identificadas fibras musculares com intensidade de reação intermediária (Pette e Staron 1993). Termin et al. (1989) desenvolveram um método de eletroforese combinado com histoquímica para mATPase e com microdissecção de fibras individuais que possibilitou separar quatro principais isoformas de MHC. Esses autores determinaram que as fibras do tipo I contêm MHCI, enquanto as fibras dos tipos IIX, IIA e IIB contêm MHCIIx, MHCIIa e MHC IIb, respectivamente. As fibras musculares com intensidade de reação intermediária entre esses três tipos são fibras transitórias, contendo dois tipos de MHC (Aigner et al. 1993). Essas fibras são classificadas de acordo com o tipo de MHC predominante, assim, fibras como as do tipo IIC possuem uma grande quantidade de MHC2a, porém menor quantidade de MHC1, já nas fibras do tipo IC ocorre o oposto (Pette e Staron 1993).

A velocidade de contração de uma fibra muscular está diretamente relacionada com o tipo de MHC (Talmadge e Roy 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 são encontradas nas fibras do tipo I, de contração lenta (Kelly e Rubinstein 1994). Guth e Yellin (1971) descreveram a natureza dinâmica dos tipos de fibras nos músculos de mamíferos, demonstrando que cada tipo de fibra, definido pela reação para a mATPase, mostra 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 de metabolismo

oxidativo e glicolítico (Peter et al. 1972). A identificação das características metabólicas e 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.

3.3. Plasticidade muscular

O músculo esquelético possui uma alta plasticidade podendo alterar suas características morfológicas, metabólicas e funcionais, em resposta a mudanças na atividade neuromuscular, idade, programas de treinamento ou em condições patológicas (Armstrong et al. 1983; Robbins e Fahin 1985; Sullivan et al., 1990; Simonini et al., 1996; Sandri et al., 1997; Silva et al., 2000). A insuficiência cardíaca é uma dessas condições patológicas que pode induzir adaptações qualitativas e quantitativas nas propriedades do músculo frente a novas demandas funcionais, e em algumas circunstâncias, pode ocasionar redução da atividade locomotora e intolerância para a realização de exercícios físicos.

3.4. 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 (Cowie et al. 1997). 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 1998).

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 (Braunwald et al. 2001). 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.

A redução da atividade locomotora e/ou a intolerância aos exercícios físicos, que ocorrem na IC, estão associados a dois de seus principais sintomas: dispnéia e fadiga muscular (Poole-Wilson e Ferrari 1996; Wilson 1996).

3.5. Origem dos sintomas da insuficiência cardíaca: a hipótese muscular

Imaginava-se, inicialmente, que a fadiga muscular presente na IC era resultante da diminuição da perfusão do músculo esquelético, com conseqüente acidose intramuscular devido à inabilidade do coração em gerar um rendimento cardíaco adequado (Weber et al. 1982; Wilson et al. 1983; Wilson et al. 1984). Porém, Wilson et al. (1993) demonstraram que a fadiga ao esforço físico é resultado de disfunção do músculo esquelético ao invés de uma redução do fluxo sanguíneo.

O desenvolvimento da disfunção muscular nos pacientes com IC é decorrente, em parte, da presença de atrofia da musculatura esquelética, observada em 68% dos pacientes com essa síndrome (Mancini et al. 1992; Harrington et al. 1997; Poehman 1999). Outro fator responsável é a alteração na freqüência dos tipos de fibras nos músculos esqueléticos, com diminuição na proporção das fibras do tipo I, de contração lenta, e aumento na proporção das

fibras do tipo II, de contração rápida (Lipkin et al. 1988; Sullivan et al. 1990; Mancini et al. 1992; De Sousa et al. 2000). A IC induz mudança nas isoformas de MHC de contração lenta para a isoforma rápida (Vescovo et al. 1998a; Simonini et al. 1996) a qual é diretamente proporcional à severidade da IC (Spangenburg et al. 2002).

Coats et al. (1994) propuseram que a redução da função do ventrículo esquerdo determina, por mecanismos ainda não esclarecidos, as alterações intrínsecas no músculo esquelético, as quais são detectadas por ergoreceptores. Esses receptores possuem a capacidade de mediar respostas simpato-excitatórias e vaso-constritoras ao exercício físico (Abboud et al. 1976), propriedades capazes de vincular as alterações do músculo esquelético à fadiga, dispnéia, vaso-constrição e excessiva resposta ventilatória característicos da IC (Vescovo et al. 2001). Esse sistema poderia eventualmente levar à progressiva debilitação do ventrículo esquerdo e causar mais alterações para o músculo esquelético (Coats 1996).

3.6. Mecanismos responsáveis pelas mudanças dos tipos de fibras musculares na insuficiência cardíaca

Embora as causas das mudanças dos tipos de fibras musculares ainda não estejam esclarecidas, é provável que a ativação de citocinas e perda de função anabólica (McMurray et al. 1991), disfunção de ergoreceptores (Coats 1996), mudanças no fluxo sangüíneo (Wilson et al. 1984), redução nas atividades neuromusculares (Talmadge 2000) e apoptose do músculo esquelético possam ser importantes. Esse último ponto tem sido amplamente debatido. Os núcleos das

células intersticiais, principalmente os núcleos das células endoteliais, sofrem apoptose na IC juntamente com os núcleos das fibras musculares, porém em maior intensidade (Allen et al. 1997; Vescovo et al. 1998b; Dalla Libera et al. 1999). De acordo com Vescovo et al. (1998b), a apoptose surge com o desenvolvimento da insuficiência cardíaca e é paralela ao aumento nos níveis de TNF-α circulante, o qual poderia induzir a apoptose das células endoteliais. A apoptose dessas células, mesmo na ausência de mudanças no fluxo sanguíneo, poderia alterar a nutrição das fibras musculares e induzir relativa isquemia para as quais as fibras se adaptariam mudando para isoformas de miosina rápida (Vescovo et al. 1998b).

Outro ponto, que será futuramente analisado em nosso laboratório, é a provável participação de fatores de transcrição pertencentes à família "basic helix-loop-helix" (bHLH), da qual fazem parte a MyoD, Miogenina, Myf5 e o MFR4, conhecidos como fatores de regulação miogênica, nas mudanças dos tipos de fibras. 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 MFR4 são apenas expressos em células no final da fase de diferenciação (Megeney e 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). A Miogenina e a MyoD também podem estar 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; Hughes et al. 1997; Mozdiziak et al. 1998; Mozdiziak et al. 1999). Portanto, a razão entre os fatores de transcrição miogênica pode ser importante na transição fenotípica dos tipos de fibras musculares que ocorre na IC.

3.7. Atrofia muscular na insuficiência cardíaca

Atrofia muscular significa redução no tamanho dos músculos. Não significa necessariamente redução do comprimento do músculo, do seu peso ou do seu volume, porém, ao longo do processo ocorre diminuição da área ou do diâmetro das fibras e diminuição do número de núcleos da fibra muscular (Allen et al. 1999). Estudos recentes têm mostrado que, na IC, a atrofia do músculo esquelético pode estar associada a apoptose dos núcleos das fibras musculares (Allen et al. 1997; Vescovo, et al. 1998b; Dalla Libera et al. 1999). De fato, Vescovo et al. (2000) mostrou uma correlação inversa entre o número de núcleos apoptóticos das fibras musculares e o grau de atrofia. Porém, a apoptose é um evento breve, durando apenas algumas horas (Allen et al. 1997; Sandri et al., 1995) e a morte de pequenas frações de núcleos por dia pode ser significante apenas após um longo período de tempo (Vescovo et al. 1998b; Dalla Libera et al. 1999).

Tem sido aventado que pelo fato da fibra muscular ser multinucleada, a apoptose mionuclear pode não resultar em imediata morte da fibra e, além disso, a

regeneração do tecido muscular a partir de células satélites poderia contrabalançar, durante um período, a morte dos núcleos das fibras musculares e disfarçar a perda de massa muscular (Sandri et al. 1995). Nesse caso, a atrofia muscular, que ocorre na IC, provavelmente aparece quando a capacidade de proliferação das células satélites está esgotada ou for suplantada pela morte celular, como ocorre nas distrofias musculares (Sandri et al. 1995; Sandri et al. 1997; Vescovo et al. 1998b). Porém, outros mecanismos podem colaborar para a atrofia muscular, como a via de perda de proteínas induzida pelo TNF-α (Llovera et al., 1997), uma vez que o nível circulante dessa citocina encontra-se elevado na IC (Vescovo et al. 1998b).

3.8. Alterações musculares na fase de hipertrofia ventricular

Embora bem descritas durante a fase de IC, poucos estudos avaliaram a expressão das isoformas de MHC e atrofia no músculo esquelético durante a fase de hipertrofia ventricular sem IC (Vescovo et al.,1998a; Coirault et al. 1999). De acordo com Vescovo et al. (1998a), a hipertrofia ventricular direita compensada não é acompanhada por alterações morfológicas e bioquímicas do músculo esquelético. Entretanto alterações no metabolismo (Chati et al. 1994), diminuição da resistência à fadiga (Levy et al. 1996) e diminuição da performance mecânica (Coirault et al. 1999) da musculatura esquelética foram descritos na sobrecarga de volume cardíaco, sem IC, o que aumenta a possibilidade de que a miopatia do músculo esquelético possa se desenvolver durante a fase de hipertrofia cardíaca.

Isso também confirmaria o fato de que as alterações bioquímicas são anteriores à perda de massa muscular (Vescovo et al. 1998b).

4. OBJETIVOS

O objetivo do presente trabalho foi determinar se são observadas mudanças na expressão das isoformas de cadeia pesada de miosina e atrofia da musculatura estriada esquelética durante a transição de hipertrofia ventricular para insuficiência cardíaca induzida por estenose aórtica em ratos. Nós também investigamos o possível papel da apoptose na atrofia da musculatura esquelética durante a fase de insuficiência cardíaca.

5. ARTIGO: Myosin heavy chain expression and atrophy in skeletal muscle during the transition from cardiac hypertrophy to heart failure (artigo submetido à revista European Journal of Applied Physiology)

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Myosin heavy chain expression and atrophy in skeletal muscle during the transition from cardiac hypertrophy to heart failure

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Abstract Chronic heart failure (CHF) is characterized by a reduced tolerance to exercise, which may be due to a skeletal muscle myopathy, with atrophy and a shift from the slow to the fast fibers. CHF induces skeletal muscle myosin heavy chain (MHC) isoform expression toward the fast isoform. The purpose of this investigation was to determine whether changes in MHC expression and atrophy of skeletal muscle are observed during the transition from cardiac hypertrophy to CHF induced by aortic stenosis (AS). We also investigated the possible role of apoptosis in skeletal muscle atrophy during CHF. AS and control animals were studied 12 and 18 weeks after the surgery and when overt CHF had developed in AS animals, 28 weeks after the surgery. The following parameters were observed in the soleus muscle: the index of muscle atrophy (soleus weight/body weight), muscle fibers diameter and frequency, and MHCs expression. AS animals presented decreases in both MHC1 and type I fibers as well as increases in both MHC2a and type IIa fibers during late cardiac hypertrophy and CHF. Type IIa fiber atrophy occurred during CHF and was not associated with skeletal muscle apoptosis as demonstrated by DNA nick-end labelling. In conclusion, our data show that skeletal muscle phenotype changes occur in both late cardiac hypertrophy and heart failure and suggest that attention should be placed on determining skeletal muscle phenotype changes prior to overt symptoms of heart failure.

Key words: Heart disease, Muscle fiber types, Myosin heavy chains, Aortic stenosis

Introduction

Chronic heart failure (CHF) is characterized by a reduced tolerance to exercise due the early occurrence of fatigue and dyspnea. It has been suggested that these symptoms are in part due to skeletal muscle myopathy, with atrophy and shift from type I "slow" fibers to type II "fast" fibers (Lipkin et al. 1988; Sullivan et al. 1990; Mancini et al. 1992; De Sousa et al. 2000). CHF induces skeletal muscle myosin heavy chain (MHC) isoform expression toward the fast isoform (Vescovo et al. 1998a; Simonini et al. 1996), which is related to the severity of CHF (Spangenburg et al. 2002).

Although well described during CHF, there are few investigations that have studied the skeletal muscle MHC isoform expression and atrophy during cardiac hypertrophy and heart failure (Vescovo et al. 1998a; Coirault et al. 1999). According to Vescovo et al. (1998a), compensated right ventricle hypertrophy is not accompanied by biochemical or morphological skeletal muscle changes. However, alterations in metabolism (Chati et al. 1994) and impaired in both fatigue resistance (Levy et al. 1996) and performance (Coirault et al. 1999) of limb skeletal muscle have been reported in experimental cardiac volume overload, without failure; raising the possibility that skeletal muscle myopathy may develop during cardiac hypertrophy. This could also confirm the fact that biochemical changes occur prior to muscle atrophy, which may be caused by cell death via apoptosis (Vescovo et al. 1998b).

The purpose of this investigation was to determine whether changes in MHC expression and atrophy of skeletal muscle are observed during the transition from

cardiac hypertrophy to CHF failure induced by aortic stenosis. We also investigated the possible role of apoptosis in skeletal muscle atrophy during heart failure.

Methods

Experimental model

Forty weaned male Wistar rats (3-4 weeks; 80-100 g) were obtained from the Central Animal House at the State University of São Paulo. Aortic stenosis was created in twenty rats by placing a stainless-silver hemoclip of 0.6 mm internal diameter on the ascending aorta via thoracic incision (AS group), according to Feldman et al. (1993). Twenty age–matched control rats underwent the same procedure without clip placement (C group). Aortic-banded rats and aged-matched sham-operated control rats were studied in three different periods: 12 and 18 weeks after the surgery and when overt heart failure had developed in AS group. The rats subjected to aortic stenosis presented tachypnea and labored respiration 28 weeks after the surgery and they were studied 1 to 2 weeks after the development of heart failure. This experimental model of heart failure has been well characterized and is widely accepted (Feldman et al. 1993; De Sousa et al. 2000; Ding et al. 2000).

After anesthesia with intraperitoneal sodium pentobarbital (50 mg/Kg), the animals were killed and the body weight (BW) and soleus weight (Sol) were evaluated. The ratio Sol/BW was taken as an index of muscle atrophy. Muscles were immediately frozen in liquid nitrogen and stored at – 80 °C. Left ventricle weight (LVW) and right ventricle weight (RVW) normalized by body weight (LVW/BW and RVW/BW, respectively) were used as indexes of ventricular hypertrophy.

Histochemistry

The midbelly regions of frozen soleus were mounted vertically on a cryostat chuck in Tissue Freezing Medium (Jung, Germany). Transverse cryosections approximately 10 μm thick were cut in a cryostat cooled to – 20 °C. Sections were stained histochemically for myofibrillar ATPase after acid preincubations (pH 4.32 and pH 4.5) (Brooke and Kaiser 1970) and fibers were classified as types I, Ic/IIc and IIa.

Fibers cross-section diameter was measured using a compound microscope attached to a computerized imaging analysis system (Qwin, Leica, Germany). To estimate fiber cross-section diameter (μm), at least 400 fibers of each muscle were measured using the smallest diameter method (Dubowitz 1985) and their frequency was expressed as the number of fibers for each type relative to the total number of fibers measured.

Electrophoretic separation of MHCs

MHC isoform analysis was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Six to ten serial cross sections (12-μm thick) were placed in 250 μl 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 for 10 min at 60°C. Small amounts of the extracts (8 μl) were loaded on a 7-10 % SDS-PAGE separating gel with a 4% stacking gel, run overnight (19-21 h) at 120 V, and stained with Coomassie Blue. MHC isoforms were identified according to their molecular mass, and the relative percentage of each was quantified by

densitometry. Two MHC isoforms (MHC1 and MHC 2a) were separated on the basis of their relative mobility in rat soleus muscle.

In situ DNA nick-end labelling (TUNEL)

Tissue cryosections from soleus of AS group with heart failure and its respective C group were cut and collected in poly-L-lysine-precoated slides. *In situ* nick-end labelling of fragmented DNA (TdT-FragEl, Amersham-Phamacia) was performed using terminal deoxynucleotidyl transferase (TdT) and biotin-conjugated dUTP, according to the manufacturer's instructions. An incubation step with peroxidase-conjugated streptavidin was completed and the TUNEL reaction products visualized with DAB. Negative control slides were prepared by substituting distilled water from the TdT enzyme and continuing with the staining procedure.

Statistical methods

Data are expressed as mean $\pm$ SD. Anatomical data were compared using ANOVA, with the Tukey multiple comparison test used to localize differences when appropriate. The comparisons of skeletal muscle fibers frequency and diameter, as well as the percentage distribution of myosin heavy chain between the control and AS groups were made by repeated measures analysis (multivariate analysis–mean profile). Differences were considered to be significant when $P<0.05$.

Results

Clinical and anatomical data

Criteria for heart failure were based on previous studies, in which animals with evidence of heart failure had findings that included labored respiration, left atrial thrombi, pleural and pericardial effusions, congested liver and right ventricular hypertrophy (Feldman et al. 1993; Cicogna et al. 1999; Ding et al. 2000). In this study, all animals with heart failure had labored respiration, pleural and/or pericardial effusion and right ventricle hypertrophy (RVW/BW > 0.80). None of the aortic-banded animals studied 12 or 18 weeks after the surgery exhibited any of these clinical or pathological features.

Anatomical data are presented in Table 1. There was no significant difference in BW and Sol weights between AS and C groups. Similar findings were present in the Sol/BW, except 18 weeks after the surgery, when was larger in the AS group than in the C group. LVW and LVW/BW were always greater in the AS groups than in the C groups. The RVW and the RVW/BW were larger in the AS group than in the C group only during heart failure.

After 28 weeks of the experimental period, the C group (C28) presented BW, LVW and Sol weight higher than those of the other two C groups (C12 and C18). The BW was different among the AS groups. The AS group with heart failure (AS28) presented LVW, RVW and RVW/BW higher than the other AS groups (AS12 and AS18). RVW/BW was higher in C12 than in C28 and it was similar between C12 and C18. The RVW, LVW/BW and Sol/BW were equal in the three C groups. The Sol/BW was higher in AS 18 compared to AS12 and AS28. The LVW/BW was similar in the three AS groups.

Table 1. Anatomical data

		Experimental Periods (Weeks)		
		12 (n=7)	18 (n=7)	28 (n=6)
BW (g)	C	425 ± 34 $_{a}$	494 ± 37 $_{a}$	629 ± 88 $_{b}$
	AS	434 ± 53 $_{a}$	504 ± 58 $_{b}$	589 ± 80 $_{c}$
LVW (g)	C	0.80 ± 0.09 $_{a}$	0.87 ± 0.06 $_{a}$	1.05 ± 0.09 $_{b}$
	AS	1.32 ± 0.15 $_{a}$ *	1.42 ± 0.17 $_{a}$ *	1.72 ± 0.12 $_{b}$ *
RVW (g)	C	0.26 ± 0.03 $_{a}$	0.29 ± 0.04 $_{a}$	0.29 ± 0.04 $_{a}$
	AS	0.29 ± 0.07 $_{a}$	0.30 ± 0.06 $_{a}$	0.60 ± 0.05 $_{b}$ *
LVW/BW	C	1.89 ± 0.15 $_{a}$	1.77 ± 0.09 $_{a}$	1.68 ± 0.12 $_{a}$
(mg/g)	AS	3.07 ± 0.39 $_{a}$ *	2.83 ± 0.29 $_{a}$ *	2.99 ± 0.50 $_{a}$ *
RVW/BW	C	0.61 ± 0.10 $_{b}$	0.58 ± 0.05 $_{ab}$	0.46 ± 0.03 $_{a}$
(mg/g)	AS	0.66 ± 0.11 $_{a}$	0.61 ± 0.11 $_{a}$	1.03 ± 0.16 $_{b}$ *
Sol	C	208 ± 20 $_{a}$	247 ± 19 $_{a}$	303 ± 36 $_{b}$
(mg)	AS	209 ± 26 $_{a}$	278 ± 49 $_{b}$	282 ± 23 $_{b}$
Sol/BW	C	0.49 ± 0.02 $_{a}$	0.50 ± 0.04 $_{a}$	0.49 ± 0.09 $_{a}$
(mg/g)	AS	0.48 ± 0.03 $_{a}$	0.55 ± 0.05 $_{b}$ *	0.48 ± 0.05 $_{a}$

Values are mean ± SD; n, number of animals; C: control group; AS: aortic stenosis group; BW: body weight; LVW: left ventricle weight; RVW: right ventricle weight; Sol: soleus weight. $_{a, b, c}$: experimental periods that do not share a common letter are statistically different ($p<0,05$); *: $p<0.05$ vs. C.

Histochemistry, morphometric analysis and fiber type frequency

Using the myofibrillar ATPase, after acid preincubations, three fibers types were identified in C and AS groups: dark staining fibers (type I), medium staining fibers (type Ic/IIc) and pale staining fibers (Type IIa) (Figure 1).

The morphometric analysis and fiber type frequency data are presented in Table 2. Fibers cross-section diameter and frequency were similar between AS group and its corresponding C group 12 weeks after the surgery. There were a decreased frequency of fiber type I and an increased frequency of fiber type IIa in the AS group, compared with its corresponding C group, 18 and 28 weeks after the surgery. There were no significant changes in fiber cross-section diameter 18 weeks after the surgery between C and AS groups. Fiber type IIa cross-section diameter decreased in the AS group, in relation to its corresponding C group, 28 weeks after the surgery.

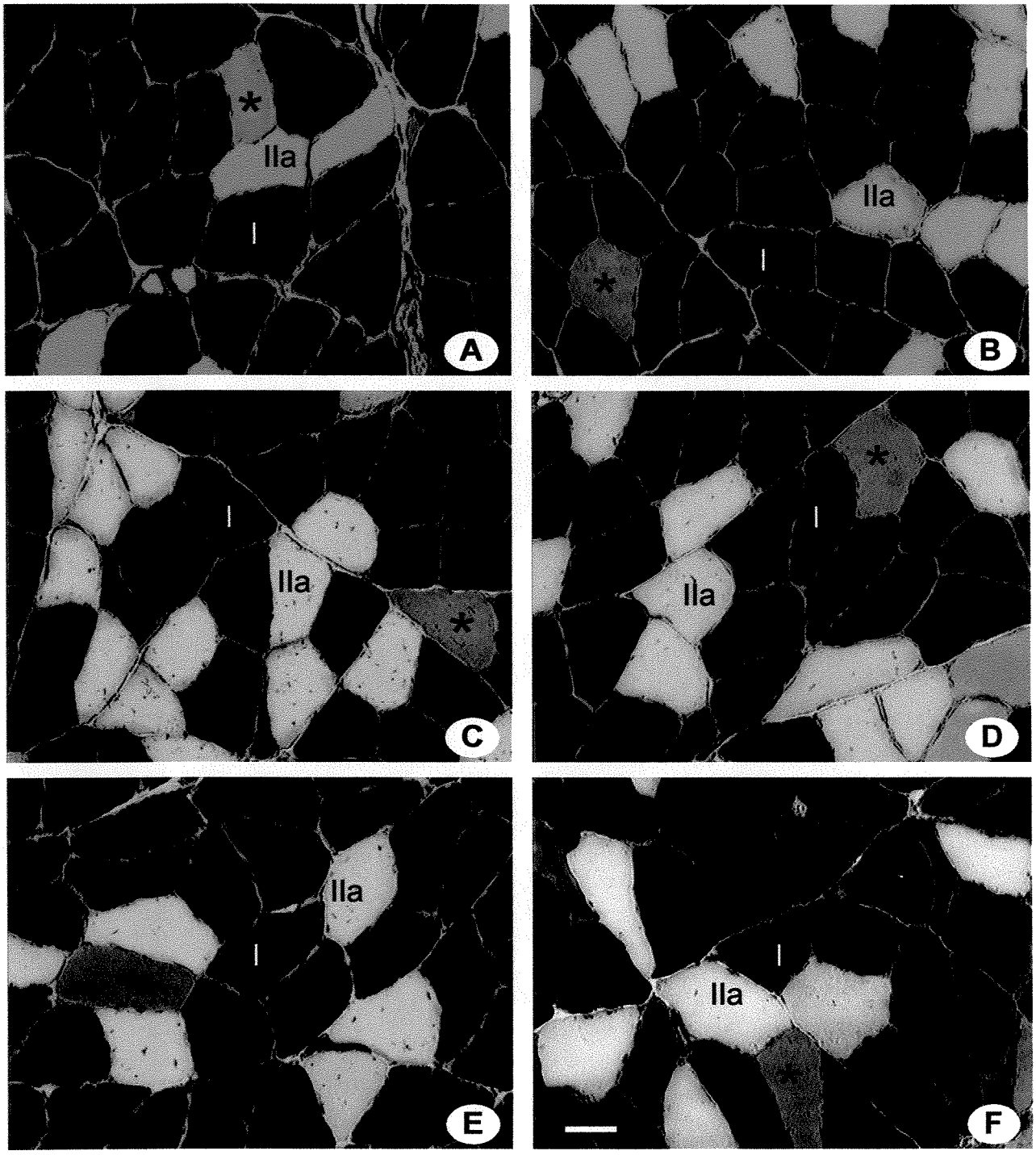

Figure 1: Cross-sections of rat soleus muscle from C12 (A), AS12 (B), C18 (C), AS18 (D), C28 (E) and AS28 (F) stained for myofibrillar ATPase pH 4.32. Fiber types I (I), IIa (IIa) and Ic/IIc. Bar = 50 μm.

Table 2. Characteristics of fibers from soleus muscle

Experimental	*Fiber*	*Frequency (%)*		*Fiber Diameter (μm)*	
Periods (W)	*Type*	*C*	*AS*	*C*	*AS*
12	I	68.5 ± 4.6	69.0 ± 5.2	51.8 ± 5.7	48.5 ± 3.7
	Ic/IIc	3.0 ± 1.9	2.6 ± 1.5	49.0 ± 6.4	41.7 ± 8.6
	IIa	28.6 ± 5.1	28.5 ± 6.2	48.8 ± 5.4	46.6 ± 3.4
18	I	80.5 ± 5.0	71.8 ± 8.2 *	49.0 ± 2.7	48.9 ± 2.8
	Ic/IIc	3.1 ± 1.8	2.1 ± 1.9	44.2 ± 9.2	42.3 ± 7.1
	IIa	16.4 ± 5.5	26.1 ± 8.6 *	48.2 ± 2.5	47.4 ± 3.6
28	I	79.0 ± 7.9	66.0 ± 6.3 *	54.3 ± 1.6	51.1 ± 3.1
	Ic/IIc	3.1 ± 2.6	1.2 ± 1.5	48.9 ± 3.2	38.4 ± 14.0
	IIa	17.9 ± 7.5	32.8 ± 6.7 *	56.8 ± 4.1	51.3 ± 2.7 *

Values are mean ± SD; W: weeks; C: control group; AS: aortic stenosis group; *: $p<0.05$ vs. C.

MHCs electrophoretic pattern

The percentage distributions of MHCs in the soleus muscle are presented in Figures 2 and 3. MHC1 and MHC2a expressions were similar between AS group and its corresponding C group 12 weeks after the surgery (AS = 90.1 ± 8.8% vs. C = 85.5 ± 8.4% and AS = 9.9 ± 8.8% vs. C = 14.5 ± 8.4%, respectively). Eighteen weeks after the surgery, MHC1 decreased (AS = 87.2 ± 6.1% vs. C = 96.3 ± 2.1%, $p<0.05$) and MHC2a increased (AS = 12.8 ± 6.2% vs. C = 3.7 ± 2.1%, $p<0.05$) in AS group in relation to its C group. Twenty-eight weeks after the surgery, the AS group also presented a shift toward the fast isoform compared with its corresponding C group; MHC1 decreased (AS = 77.9 ± 7.3% vs. C = 93.5 ± 4.3%, $p<0.05$) whereas MHC2a increased (AS = 22.1 ± 7.3% vs. C = 6.5 ± 4.3%, $p<0.05$).

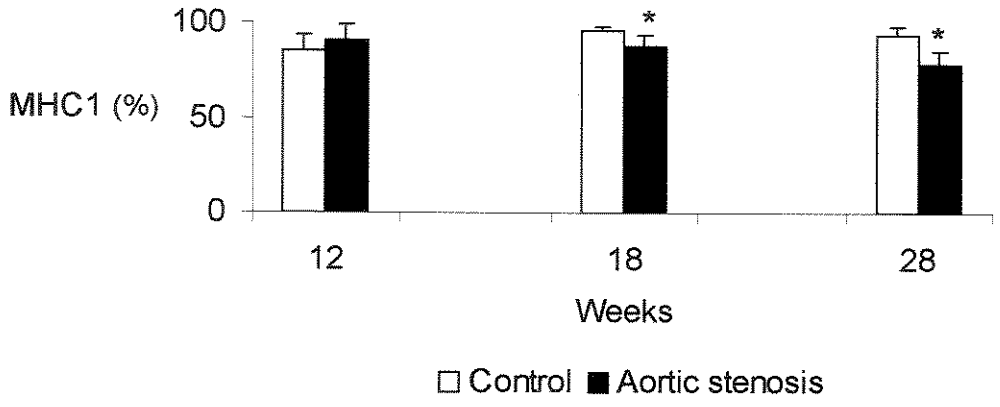

Figure 2: Percentage distribution of myosin heavy chain (MHC1) in the soleus muscle of control and aortic stenosis groups, during the experimental periods. (*: $p<0.05$ vs. control group)

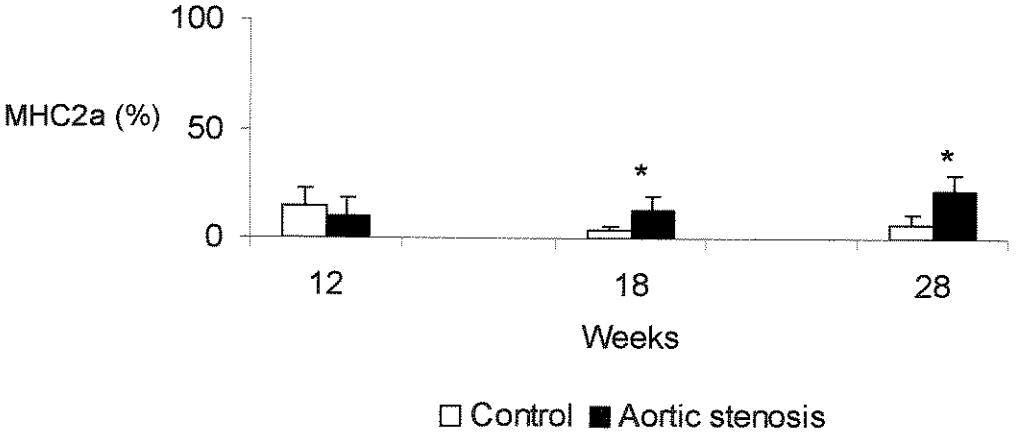

Figure 3: Percentage distribution of myosin heavy chain (MHC2a) in the soleus muscle of control and aortic stenosis groups, during the experimental periods. (*: $p<0.05$ vs. control group)

In situ DNA nick-end labelling (TUNEL)

Positive nuclei were detected in all animals with heart failure and their corresponding C group, and the number of these nuclei was less than 1% of all myonuclei. As it is quite difficult to determine whether these positive nuclei were myonuclei or nuclei of interstitial cells, they were not distinguished.

Discussion

The purpose of the present investigation was to study the morphological and biochemical changes in the skeletal muscle during the transition from cardiac hypertrophy to heart failure induced by aortic stenosis.

The major finding of this study was an increase in MHC2a and in type IIa fibers and a decrease in MHC1 and in type I fibers in the soleus muscle, during late cardiac hypertrophy without heart failure (AS 18). This result diverges from that of Coirault et al. (1999), who did not observe changes in the myosin heavy chain composition of soleus in early stages of pressure cardiac overload induced by subtotal constriction of the suprarenal abdominal aorta in New Zealand rabbits. Vescovo et al. (1998a), by means of an alkaloid that induces severe hypertension, right ventricle hypertrophy and heart failure in Sprague-Dawley rats, also reported that right ventricular hypertrophy is not accompanied by changes in myosin heavy chain composition of soleus. These disparities in results may be due to the diverse experimental models used. The data obtained in our model, a chronic left ventricular pressure overload, indicate that skeletal muscle weakness can occur early in the course of this cardiopathy due to biochemical changes and not on account of muscle atrophy, once the latter was only observed during heart failure.

The biochemical changes found during left ventricular hypertrophy were also observed during the heart failure, and, although we could not detect loss of soleus muscle bulk, as indicated by Sol/BW, the animals with cardiac failure presented type IIa fiber atrophy. The occurrence of skeletal muscle myophaty accompanied by alterations in the MHC pattern that reflect changes in fiber types and atrophy is a well-described phenomenon during heart failure (Sullivan et al. 1990; Simonini et

al. 1996; Drexler et al. 1992; Vescovo et al. 1998a; De Sousa et al. 2000; Spangenburg et al. 2002).

The possible causes of the changes in skeletal muscle myosin heavy chain during heart failure are still unknown. However, it has been hypothesized that cytokine activation and loss of anabolic function (McMurray et al. 1991), ergo-metaboloreceptor dysfunction (Coats 1996), changes in blood flow (Wilson et al. 1984), reduction in neuromuscular activity (Talmadge 2000) and skeletal muscle apoptosis (Vescovo et al. 1998a) may be relevant. The latter point has been considerably debated. According to Vescovo et al. (1998b), endothelial apoptosis, even in the absence of changes in skeletal muscle blood flow, could alter myofibers nutrition and induce relative ischemia to which muscle fibers adapt by shifting toward the fast myosin isoforms. It has also been suggested that apoptosis and atrophy are tightly linked once myonuclei undergo apoptosis in heart failure together with endothelial cells (Allen et al. 1997; Vescovo et al. 1998b; Dalla Libera et al. 1999). In fact, Vescovo et al. (2000) showed an inverse correlation between the number of apoptotic myonuclei and the degree of muscle atrophy. In this investigation we did not find apoptosis in the soleus of rats with heart failure. Therefore, we believe that apoptosis may not be primarily responsible for the changes in myosin isoforms and atrophy. However, we cannot discard the role of apoptosis in muscle atrophy due to the fact that apoptosis is a transient event lasting perhaps only a few hours (Allen et al. 1997; Sandri et al. 1995) and the death of such a small fraction of nuclei per day may be significant only over a long period of time (Vescovo et al. 1998b; Dalla Libera et al. 1999). Studies with more

sensitive procedures and different periods of analyses during heart failure are required for further clarification of the processes involved in muscle atrophy.

The mechanisms involved in the skeletal muscle adaptations that occurred in rats with 18 weeks of aortic stenosis without signs of heart failure need to be determined. However, observation has indicated that alterations in skeletal muscle in heart failure are, after all, consequences of impaired cardiac function. The degree and/or the duration of cardiac dysfunction may influence structural and biochemical damages to the skeletal muscle (Coirault et al. 1999). In our laboratory, Bregagnollo et al. (2003) demonstrated that after 6 weeks of aortic stenosis, the animals consistently presented significant increase in left ventricle end diastolic pressure; the authors also showed that after 21 weeks of aortic stenosis, the rats without signals of heart failure also presented significantly systolic dysfunction evaluated by hemodynamics and echocardiograph methods. Therefore, although the animals did not present heart failure, they showed important signs of left ventricle dysfunction. These data obtained in rats with 21 weeks of aortic stenosis, probably were present in our animals with 18 weeks of aortic stenosis and may have induced the alterations in the muscle fiber phenotype observed in this experiment

In summary, the aortic banding animals develop a myopathy in the soleus muscle with decreases in both MHC1 and type I fibers as well as increases in both MHC2a and type IIa fibers during both late cardiac hypertrophy and heart failure. Type IIa fiber atrophy occurs during heart failure and is not associated with skeletal muscle apoptosis. If analogous phenomena occur in clinical setting of cardiac

hypertrophy and subsequent failure, attention should be given on determining skeletal muscle phenotype changes prior to overt symptoms of heart failure.

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6. CONCLUSÕES

Os animais com estenose aórtica desenvolveram uma miopatia no músculo sóleo, caracterizada por diminuição da MHC1 e da freqüência das fibras do tipo I e aumento da MHC2a e da freqüência das fibras do tipo IIa, na hipertrofia cardíaca e na insuficiência cardíaca. A atrofia das fibras do tipo IIa ocorreu durante a insuficiência cardíaca. Não foi possível estabelecerem-se correlações entre a atrofia muscular e apoptose no músculo esquelético. Nossos dados demonstram que as alterações no fenótipo do músculo esquelético têm inicio na fase de hipertrofia cardíaca e sugerem que atenção seja dada na determinação das mudanças do fenótipo do músculo esquelético antes que se tornem claros os sintomas de insuficiência cardíaca.

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