Apoptosis and atrophy in rat slow skeletal muscles in chronic heart failure

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Apoptosis and atrophy in rat slow skeletal muscles in chronic heart failure. Am. J. Physiol. 277 (Cell Physiol. 46): C982–C986, 1999.—Congestive heart failure is characterized by a decreased exercise tolerance, which has been suggested to be due to a specific skeletal muscle myopathy, with muscle atrophy and shift from the fatigue-resistant type I fibers to the more fatigue-prone type II fibers (11, 15, 16, 25, 29, 30). Although reversible (27), both the origin and the pathophysiology of this myopathy are not yet fully understood. Cytokines, such as tumor necrosis factor-α (TNF-α) (17), growth factors, such as growth hormone and insulin growth factor I (1), and ergot metaboloreceptors (20) have been thought to play roles in the development of this myopathy. Apoptosis occurs in the compensated human heart (18, 19), in cardiomyopathies (14), contributing to cell loss and to the worsening of myocardial dysfunction, and in capillary muscles in hypertension (5). In skeletal muscle, apoptosis contributes to fiber atrophy both in disuse (1) and in dystrophic-deficient myopathies (23). We have investigated the possible role of skeletal muscle apoptosis in congestive heart failure (CHF). By using the monocrotaline-treated rat as a model of heart failure syndrome, we have demonstrated an increase in interstitial and myocyte apoptosis in tibialis anterior (TA) muscle (28).

We have also shown that myocyte apoptosis is ultimately responsible for muscle atrophy, which occurs simultaneously with the rise in the number of apoptotic cells. In the TA muscle, a typical fast-twitch muscle, apoptosis occurs nonselectively in both type IIa and IIb fiber, as demonstrated by triple staining with terminal transferase dUTP nick-end labeling (TUNEL), antibodies against myosin heavy chain (MHC) IIb and 2x and laminin. Neither apoptosis nor atrophy is, however, responsible for the shift in myosin isoforms that are observed far earlier than the occurrence of apoptosis and severe hemodynamic decompensation (26, 31). During the time course of apoptosis, the rise in number of apoptotic nuclei is paralleled by a dramatic increase in circulating levels of TNF-α, therefore suggesting that this cytokine is involved in triggering cell death.

This has been previously reported both in vitro for cardiac myocytes (9) and in vivo for the heart and vessels (6, 7). It has been also suggested that the role played by this cytokine may be more relevant in slow rather than in fast muscles (4, 10). Thus the present investigation aimed to evaluate whether in CHF slow muscles are more prone to develop apoptosis and atrophy than fast muscles and to speculate on the role of TNF-α and the proapoptotic and antiapoptotic proteins on programmed cell death in different muscle types. To this aim, we studied apoptosis in a typical slow muscle, the soleus, of rats that had CHF induced by monocrotaline, an alkaloid able to produce pulmonary hypertension, right ventricular hypertrophy, and eventually right ventricle failure. This model very closely mimics the neuroendocrine activation present in human CHF (3). A comparison with previously published data in the TA muscles of the same animals (31) was also made.

METHODS

Experimental Model and Time Course of Apoptosis

Male Sprague Dawley rats (80–100 g wt) with CHF induced by monocrotaline (8, 28) were studied. Monocrotaline was injected intraperitoneally in the rats at a dose of 30 mg/kg. Afterward, they were allowed to eat freely from a supply of standard rat cubes. We studied six animals at each of the following time points: 0, 17, 24, and 30 days after...
monocrotaline injection. Seven age- and diet-matched controls were also studied to show that monocrotaline-treated rats developed CHF.

When animals were killed, body weight as well as soleus weight were measured. The soleus weight-to-body weight ratio was taken as an index of muscle atrophy. Muscles, immediately after excision, were frozen in liquid nitrogen and stored at −80°C.

Experiments were approved by the Ethical Committee of the Interdepartmental Biological Building of the University of Padova and were carried out in accordance with Italian laws.

Electrophoretic Separation of MHCs

MHC composition was assessed in the day 0 and day 30 animals. The method used is an improvement of that published by Carraro and Catani (2) and is described in detail by Vescovo et al. (30). Briefly, muscles were homogenized and solubilized in 2.3% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, and 6.25 mM Tris·HCl, pH 6.8. Analytical SDS-PAGE of MHC was performed on 7% polyacrylamide slabs with 37.5% vol/vol glycerol. Separation of MHCs was achieved by using a constant current of 4 mA for 24 h. Gels were stained with Coomassie brilliant blue. Identification of individual MHCs was performed in a separate series of experiments by immunoblotting the gel bands with a panel of monoclonal antibodies against MHC1 (BA-D5, slow isoform), MHC2a (BF-71, fast oxidative), and MHC2b (D9, fast glycolytic) (all were a generous gift from Prof. Stefano Schiaffino). The percent distribution of the three different isoforms (MHC1, MHC2a, and MHC2b) was determined by densitometric scanning of the stained slab gels. A GS300 transmittance reflectance scanning densitometer (Hoeffer Scientific Instruments) connected to a Macintosh SE Apple computer was used. Data were processed with GS370 densitometry software. A linear response in terms of electrophoretic band area is attained with densitometry when 0.1–2 µg of individual MHC are analyzed (24). Quantitative densitometry was performed using internal MHC standards electrophoresed in the same gel (26, 30, 31). The coefficient of variation for inter- and intra-assay (same sample tested on different gels and the same sample tested on the same gel, respectively) was <2%.

Assessment of Apoptosis

In situ DNA nick-end labeling (TUNEL). Serial tissue cryosections from the soleus were cut and collected on polylysine-precoated slides. In situ nick-end labeling of fragmented DNA was performed using terminal deoxynucleotidyl transferase and fluorescein-conjugated nucleotides with the in situ cell death detection kit, peroxidase (Boehringer Mannheim), according to the manufacturer’s instructions. Negative control slides were prepared by substituting distilled water from the terminal deoxynucleotidyl transferase enzyme and continuing with the staining procedure as suggested by the manufacturer’s instructions. Labeled nuclei were identified from negative nuclei counterstained by Hoechst 33258 and counted after being photographed. The total number of positive nuclei was expressed as the percentage of the counted nuclei. At least 1,500 nuclei per field were counted according to the method described by Sandri et al. (21). The count was performed on at least 15 different fields (magnification of ×250) of each sample. TUNEL-positive myofiber nuclei and TUNEL-positive interstitial nuclei were distinguished on the basis of their location on sections stained with laminin, which selectively reacts with the basal lamina. Serial tissue cryosections from the soleus were cut and collected on polylysine-precoated slides. Slides were incubated with primary antibodies using rabbit anti-laminin (from Sigma Chemical, St. Louis, MO) diluted 1:10 in 1% BSA. The slides were then washed twice with PBS (5 min each) and incubated with rhodamine-conjugated goat anti-rabbit IgG (diluted 1:2,000 in 1% BSA) for 1 h at 37°C. Negative controls were performed by omitting the primary antibody. After slides were washed three times in PBS and after nuclei were stained with Hoechst 33258, slides were mounted in Elvanol and observed under an epifluorescence microscope (Zeiss).

TUNEL-positive nuclei within the muscle fiber basal lamina were taken to be myonuclei (1). It is possible that some of the nuclei reside in satellite cells, which lie adjacent to the muscle fibers and within the basal lamina. The presence of inflammatory infiltrates was excluded on serial histological sections stained with hematoxylin and eosin. Separate calculations were made for total TUNEL-positive nuclei, TUNEL-positive myonuclei, and interstitial nuclei. All data are expressed as means ± SD.

Western blot for Bcl-2, caspase-3, and ubiquitin. Fragments of the soleus were homogenized in SDS buffer. The amount of protein was determined by the Lowry method after precipitation with 10% TCA (13). The homogenates were analyzed by SDS-PAGE, 12.5% polyacrylamide, and Western blotting by loading 45 µg of protein per lane. The samples were assayed on the same day with the same reagents. The following conditions were used for binding the antibody: anti-caspase-3 CPP 32 (H-277) and anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:200 and 1:100, respectively, anti-CPP 32 (H-277) and anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:200 and 1:100, respectively, anti-ubiquitin and anti-rabbit alkaline phosphatase linked (Sigma Chemical, St. Louis, MO) were diluted 1:100 and 1:4,000, respectively. For caspase-3, we analyzed the band derived from the reaction between the caspase-3 antibody and the activated 20-kDa subunit. The absolute values were calculated on the blot bands with the densitometric system previously described (24). Values were expressed in arbitrary units (AU). The intra-assay coefficient of variation was 10%. Bcl-2, caspase-3, and ubiquitin were assessed in all the rats at days 0, 17, 24, and 30.

Statistical Analysis

Results are reported as means ± SD. Student’s t-test for unpaired data and ANOVA were used when appropriate. A 5% difference was considered statistically significant.

RESULTS

Degree of Muscle Atrophy

The index of soleus atrophy as measured by soleus weight-to-body weight ratio did not change throughout the study period (Fig. 1) and at day 30 was not different from that of the age-matched controls.

Electrophoretic Pattern of MHCs

At day 17, when no postmortem signs of heart failure were found, MHC1 was 91.2 ± 3.1%, whereas MHC2a was 8.8 ± 1.4% (P = not significant vs. controls). At day 30 in soleus muscle, when heart failure was evident, there was a shift from the slow to the fast MHCs. In
fact, MHC1 decreased from 93.18 \pm 3.36% to 87.37 \pm 2.76% (P = 0.02) and MHC2a increased from 6.76 \pm 3.38% to 12.55 \pm 2.78%, which was close to statistical significance.

Apoptosis

Count of in situ DNA nick-end labeling-positive nuclei. DNA nick-end labeling in the soleus showed, when CHF occurs, the presence of apoptotic nuclei in both myofibers and interstitial cells when CHF occurred. Interstitial and myocyte nuclei were further distinguished on the basis of laminin staining (Fig. 2). There was a progressive rise in the number of interstitial TUNEL-positive nuclei (Fig. 3) [0.025 \pm 0.013% at day 0, 0.075 \pm 0.064% at day 17, 0.072 \pm 0.052% at day 24, and 0.14 \pm 0.057% at day 30; this was statistically significant (P < 0.05 controls vs. day 30)]. Similarly, there was an increase in TUNEL-positive myonuclei (0.015 \pm 0.015% at day 0, 0.01 \pm 0.001% at day 17, 0.028 \pm 0.044% at day 24, and 0.062 \pm 0.03% at day 30; P < 0.01 controls vs. day 30) (Fig. 3).

Western blot for Bcl-2, caspase-3, and ubiquitin in soleus. There was a progressive increase in caspase-3 from 0–30 days, being 969 \pm 10 AU at day 0 and 1,893 \pm 481 AU at day 30 (P = 0.02). At the same time, Bcl-2 showed a significant drop from 2,490 \pm 36 to 1,335 \pm 67 AU (P < 0.001) (see Fig. 4).

We could not find any change in ubiquitin throughout the study period (2,640 \pm 284 vs. 2,813 \pm 48 AU, P = not significant) (Fig. 4).
we showed that, for the same levels of TNF-α in which the same batch of animals had been studied, the fast TA muscle. In a previously published paper (31) muscles. In the soleus muscle, the magnitude of myofiber apoptosis is much higher in TA muscle, in which 30 days after monocrotaline injection develop severe right heart failure, as demonstrated by the typical postmortem signs such as pleural and pericardial effusions and ascites (26, 31). In a previous paper in which the fast TA muscle was studied in the same animals (31), we found an increase in right ventricle/body weight, right ventricle mass/volume index, and β-atrial natriuretic peptide (3, 31). We also showed that CHF was paralleled by increased levels of circulating TNF-α (31). In the present study, we have found that, with the occurrence of CHF, the slow soleus muscle develops a myopathy with shift toward the fast MCH2a isoform. At the same time, in both myofibers and interstitial cells, TUNEL-positive apoptotic nuclei are shown. This phenomenon, as previously observed (31), was accompanied by increased soleus levels of caspase-3 and decreased soleus levels of Bcl-2. The magnitude of apoptosis is much higher in interstitial cells than in myofibers. At variance with TA muscle, in which 30 days after monocrotaline injection muscle atrophy appears, as demonstrated by the significant decrease in both the TA muscle weight-to-body weight ratio and fiber cross-sectional area (31), in soleus muscle, we did not observe any degree of muscle atrophy. This occurred despite the elevated levels of circulating TNF-α that were the same as those reported for the fast TA muscle (31). We can speculate that muscle atrophy appears late in CHF and in fast muscles. In the soleus muscle, the magnitude of myocyte apoptosis is in fact smaller compared with that of the fast TA muscle. In a previously published paper (31) in which the same batch of animals had been studied, we showed that, for the same levels of TNF-α, myocyte apoptosis at day 30 was 0.19 ± 0.055%, which is nearly three times higher than shown for the soleus muscle (0.06 ± 0.03%). It has been previously observed that the action of this cytokine may be more important in slow than in fast muscles (4), but our data do not confirm this hypothesis. TNF-α is known to induce apoptosis both in vivo (6, 7) and in vitro (9). Muscle atrophy in CHF can be determined by cell death occurring via apoptosis (31) or protein waste. Despite the first mechanism being active in this model, our results suggest that the TNF-α-induced protein waste pathway (12) is not activated, since ubiquitin tissue levels are not changed throughout the study period. It looks as if the muscle bulk loss in this model of CHF is only apoptosis dependent and atrophy occurs first in muscles like the TA, where apoptosis is more pronounced. Moreover, skeletal myocytes are multinucleated and the presence of an apoptotic nucleus does not ultimately lead to cell death. It is therefore conceivable that in the soleus we did not have time to observe muscle atrophy, which would have been likely to develop later on. It is conceivable that the small percentage of apoptotic nuclei found in our study could be relevant to account for muscle wasting. In fact, apoptosis is a transient event lasting perhaps only a few hours (22), and the death of such a small fraction of nuclei per day could be significant over a long period of time. The monocrotaline rat is in fact a model of subacute failure (26, 28), and animals inevitably die within 4–5 wk after the alkaloid injection.

Despite the limitations of these findings in an animal model of right ventricle failure, the different magnitude of apoptosis and the time course of atrophy between slow and fast muscles could have important clinical implications in the CHF myopathy in humans. We do not think that monocrotaline could have either a direct effect on the skeletal muscle composition or a proapoptotic effect. In fact, after 17 days of treatment, when CHF has not developed yet, there are neither changes in MHC composition nor changes in the number of TUNEL-positive myocyte nuclei.

In conclusion, the monocrotaline-treated rats develop a myopathy in the slow muscle, with shift from the slow to the fast fiber type. Myocyte and interstitial apoptosis rises with the development of CHF and is paralleled by an increase in circulating TNF-α. Apoptosis occurs in the slow muscle at a smaller degree than in fast ones. Muscle bulk loss in this model is only apoptosis dependent, since the TNF-α-mediated, ubiquitin-independent pathway is not activated.

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