Apoptosis in Skeletal Myocytes of Patients With Chronic Heart Failure Is Associated With Exercise Intolerance

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OBJECTIVES
The purpose of the study was to investigate if apoptosis occurs in skeletal muscle myocytes and its relation to exercise intolerance in patients with chronic heart failure (CHF).

BACKGROUND
Intrinsic abnormalities of skeletal muscle frequently limit exercise tolerance in CHF patients. Recently, apoptosis has been detected in cardiac myocytes of patients with CHF, suggesting that apoptosis may contribute to the reduced contractile force. The presence and regulation of apoptosis in skeletal myocytes of patients with CHF remains to be defined.

METHODS
Skeletal muscle biopsies (m. vastus lateralis) of 34 CHF patients (New York Heart Association functional class II–III) and eight age-matched healthy control subjects were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling for the presence of apoptosis, and by immunohistochemistry and videodensitometrical quantification for inducible nitric oxide synthase (iNOS) and Bcl-2 expression. Maximal oxygen consumption (VO₂max) was determined by ergospirometry.

RESULTS
Apoptosis was detected in 16/34 (47%) patients with CHF and in none of the healthy subjects. Patients with apoptosis-positive skeletal muscle myocytes exhibited a significantly lower VO₂max (12.0 ± 3.7 vs. 18.2 ± 4.4 ml/kg/min; p = 0.0005), a higher iNOS expression (6.8 ± 3.6 vs. 3.7 ± 2.6% iNOS-positive stained tissue area; p = 0.015) and a lower Bcl-2 expression (1.0 ± 0.3 vs. 1.4 ± 0.4% Bcl-2-positive tissue area; p = 0.03) as compared with patients with apoptosis-negative biopsies.

CONCLUSIONS
These results indicate that apoptosis is frequently found in skeletal muscle obtained from CHF patients, which is associated with significant impairment of functional work capacity. In skeletal muscle of these patients, iNOS and Bcl-2 are possibly involved in the regulation of apoptosis. (J Am Coll Cardiol 1999;33:959–65) © 1999 by the American College of Cardiology

Chronic heart failure (CHF) is not only associated with an increased mortality, but also with exercise intolerance and early fatigue. It has been frequently pointed out, however, that the level of activity tolerated by individual patients could not be predicted by classical parameters of left ventricular performance (1,2). Therefore, considerable attention has been focused on the role of peripheral factors, such as regional blood flow and skeletal muscle, as determinants of work capacity. Intrinsic abnormalities of skeletal muscle including atrophy (3), impaired function (4), change in fiber type composition (5–7) and altered metabolism (8–10) have been implicated. These intrinsic muscle alterations are thought to be partially responsible for the exercise intolerance associated with CHF, although the pathophysiologic link to compromised left ventricular performance has escaped definition so far.

Recently apoptotic cells were detected in myocardial biopsies obtained from patients with arrhythmogenic right ventricular dysplasia (11) or end-stage heart failure (12,13). The authors concluded that the loss of myocytes due to apoptosis results in a loss of contractile function and therefore may contribute to the progressive myocardial dysfunction in these patients. Dysregulation of the apoptotic process can lead to a spectrum of defects ranging from embryonic lethality to disintegration of tissues by loss of cells (14). Therefore, tight regulation of this process is essential, and several genes have been identified to play a crucial role in its regulation. In particular, dominant oncogenes such as c-myc, Bcl-2 and tumor suppressor genes such as p53 have been shown to be potent regulators of apoptosis (15,16). Beside these oncogenes, nitric oxide (NO) has been reported to function as a pro- and anti-
apoptotic factor (reviewed in [17]). The contradicting effects of NO in apoptosis regulation can be rationalized by a dose-dependent phenomenon, with low concentrations acting antiapoptotic, and high concentrations being proapoptotic (17). Particularly, conditions associated with high levels of NO elaborated by inducible NO synthase (iNOS) seem to favor apoptosis (18,19).

On the basis of these observations and the knowledge that the expression of iNOS is significantly increased in skeletal myocytes of patients with CHF (20), we hypothesized that apoptosis may not only be involved in myocardial but also in skeletal muscle dysfunction in patients with CHF.

METHODS

Patients. Thirty-four male patients ≤70 years old with CHF (New York Heart Association functional class II to III) as a result of dilated cardiomyopathy or ischemic heart disease and eight sedentary normal individuals were studied. Healthy subjects had a normal physical examination, electrocardiogram, chest-X-ray, M-mode and two-dimensional echocardiographic evaluation. All patients had clinical, radiologic and echocardiographic signs of CHF and a reduced left ventricular ejection fraction (≤40%), as assessed by angiography. Exclusion criteria were diabetes mellitus, chronic alcohol abuse, immunosuppressive therapy, renal failure, chronic lung disease, primary valvular disease, hereditary or acquired neuromuscular disorders or recent myocardial infarction (less than three months). To determine the onset of disease, patients were asked to identify the time point of their first clinical symptoms.

The protocol of this study was approved by the Ethics Committee of the University of Leipzig, and written informed consent was obtained from all subjects before the beginning of the study.

Exercise testing and respiratory gas exchange measurements. All individuals underwent a baseline exercise test to exclude myocardial ischemia and significant ventricular tachyarrhythmias and to familiarize the subjects with ergospirometry. Exercise testing was performed on a calibrated, electronically braked bicycle in an upright position. Work load was increased progressively every 3 min in steps of 25 W beginning at 25 W. Exercise was terminated when patients were physically exhausted or developed severe dyspnea or dizziness. Respiratory gas exchange data were determined continuously throughout the exercise test as recently described (21,22).

Skeletal muscle biopsy. Percutaneous needle biopsies were obtained from the middle part of the m. vastus lateralis under local anesthesia as described in detail by Bergström (23). The biopsies were fixed with 4% buffered formaldehyde and subsequently paraffin embedded.

In situ detection of apoptotic cells. For the in situ detection of apoptotic cells, the method of terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate nick end-labeling (TUNEL) with several modifications was applied. Paraffin sections (3 μm) of the tissue material were dewaxed with xylene (2 × 5 min at room temperature [RT]) and rehydrated with graded dilutions of ethanol in water (100%; 75%; 50%, water). After rehydration, the tissue was treated with proteinase K (40 μg/ml; 5 min at RT) followed by several washing steps (3 × 5 min water at RT). To block the internal peroxidase activity the slides were incubated for 20 min with 0.6% hydrogen peroxide in 100% methanol. Before the addition of the TdT labeling mixture (25 U TdT [Promega, Madison, Wisconsin], 1 nmol digoxigenin-11-deoxyuridine-triphosphate [Boehringer Mannheim, Mannheim, Germany] in 400 μl of equilibration buffer [200 mmol/L cacodylat, 25 mmol/L Tris–HCl pH 6.6, 250 μg/ml bovine serum albumin and 2.5 mmol/L cobalt chloride]) the sections were incubated for 15 min at RT in equilibration buffer. After 1 h incubation at 37°C with the TdT labeling mixture the sections were rinsed (3 × 5 min at RT) in Tris–buffer (50 mmol/L Tris pH 7.6, 150 mmol/L NaCl) to remove the unincorporated digoxigenin label. To detect the digoxigenin-labeled DNA the following steps were performed: blocking of unspecific binding sides with 2% fetal calf serum in Tris–buffer (15 min at RT), 30-min incubation with an antidigoxigenin antibody at dilution 1:1,000 (Boehringer Mannheim, Mannheim, Germany), extensive rinsing (3 × 5 min at RT) with Tris–buffer to remove the free antibody and incubation of the slides for 30 min at RT with Tris–buffer (15 min at RT) containing 1:1,000 (Boehringer Mannheim, Mannheim, Germany) rabbit antisheep peroxidase–conjugated antibody at dilution 1:500 in Tris–buffer with 2% fat free milk powder (Dianova GmbH, Hamburg, Germany). After a final washing step (3 × 5 min with Tris–buffer) the sections were developed using 3-amino-9-ethylcarbazole as substrate for peroxidase and counterstained with hemotoxylin. As negative control reaction an in situ detection was performed as described above but without TdT in the labeling mixture. Positive control samples were prepared by incubating sections with 10 U/ml deoxyribonuclease I for 20 min at 37°C before treatment with TdT (24). Four sections of each biopsy were screened for apoptotic nuclei. A sample with at least two positive nuclei per section was classified as positive. To estimate the apoptotic index the percentage of positive stained nuclei was calcu-
lated in relation to unstained apoptosis-negative nuclei in a subset of apoptosis-positive skeletal muscle biopsies. The types of cells staining positive for DNA fragmentation were further characterized with a monoclonal antiactin antibody (HFF 35, Dako GmbH, Hamburg, Germany) as described elsewhere (12).

Detection of iNOS and Bcl-2 by immunohistochemistry. The expression of iNOS was visualized and quantitated using an iNOS-specific polyclonal antibody (Transduction Laboratories, Lexington, Kentucky) and biotinylated tyramid as recently described in detail (20). The specificity of the antibody is described elsewhere (25,26).

To detect Bcl-2 on paraffin sections from skeletal muscle biopsies, a specific monoclonal antibody (antihuman Bcl-2, clone 124; Dako GmbH, Hamburg, Germany) was used. The immunohistochemical detection was performed as follows: after deparaffinization and rehydration the tissue sections (4 μm thick) were boiled for 10 min in 10 mmol/L citrate buffer, pH 6.0 (27). To prevent unspecific binding of the primary antibody the sections were blocked for 20 min with blocking solution (1.5% normal horse serum in 4% nonfat dry milk solubilized in phosphate-buffered saline). After this blocking step the primary anti-Bcl-2 antibody was applied (1:40 dilution in blocking solution) onto the sections, and the reaction was carried out at 37°C for 2 h in a humified chamber. After rinsing in phosphate-buffered saline (3 × 5 min at RT) the bound primary antibody was visualized using the alkaline phosphatase–antialkaline phosphatase method (Dako Diagnostika GmbH, Hamburg, Germany) as described by the company. As negative control the primary antibody was omitted and replaced by blocking solution.

Statistical analysis. Values are given as mean ± SD for all variables. Single comparisons were performed by nonparametric tests (Mann–Whitney U test or Wilcoxon test), and a p value of less than 0.05 was considered statistically significant.

RESULTS

Study group. In 24 patients, dilated cardiomyopathy was diagnosed based on cardiac catheterization with global hypokinesis and normal coronary arteries (Table 1). Coronary artery disease was the underlying cause in 10 patients. All patients received angiotensin-converting enzyme inhibitors, and 26 patients (76.4%) were on digitalis. Thirty-one patients (91.1%) were treated with diuretics and eight patients (23.5%) with amiodarone. Twenty-one patients (61.7%) had a history of at least one episode of left heart decompensation.

Left ventricular ejection fraction (19.6 ± 8.0% vs. 70.8 ± 5.2%; p < 0.001) and maximal oxygen uptake (15.1 ± 5.1 vs. 26.0 ± 2.2 ml/kg/min; p = 0.0001) were significantly impaired, and a lower body weight (79.7 ± 13.7 vs. 92.0 ± 3.7 kg; p = 0.04) was detected as compared with the healthy control subjects.

Table 1. Clinical Parameters of Study Apoptosis in CHF

<table>
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<tr>
<th>Etiology</th>
<th>CHF Patients (n = 34)</th>
<th>Control Patients (n = 8)</th>
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<tr>
<td>CHF</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DCM</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>ICM</td>
<td>10</td>
<td>0</td>
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<tr>
<td>Age (yr)</td>
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<td>Weight (kg)</td>
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<tr>
<td>Ejection fraction (%)</td>
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<tr>
<td>VO_2max (ml/kg/min)</td>
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<td>Duration (months)</td>
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</tr>
<tr>
<td>Diuretics (%)</td>
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</tr>
<tr>
<td>Digitalis (%)</td>
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</tr>
<tr>
<td>Amiodarone (%)</td>
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</table>

Data presented are mean value ± SD or percent of patients.
ACE = angiotensin-converting enzyme; CHF = chronic heart failure; DCM = dilated cardiomyopathy; ICM = ischemic cardiomyopathy; VO_2max = maximal oxygen consumption.

Apoptosis in skeletal myocytes and its correlation to exercise intolerance. Apoptosis could be detected in 16 out of 34 patients with CHF (47%) by TUNEL staining (Fig. 1A), whereas no apoptose labeling could be observed in negative controls (omission of terminal transferase in labeling mixture). Furthermore, in biopsies of healthy control patients no staining for apoptosis was observed (Fig. 1B). Positive apoptotic staining could be observed in all nuclei by TUNEL after the pretreatment of the sections with deoxyribonuclease I (Fig. 1C), whereas no staining after deoxyribonuclease digestion was detected when TdT was omitted in the TUNEL reaction (Fig. 1D). Staining of TUNEL-treated sections with a monoclonal antiactin antibody showed that the TUNEL-positive nuclei are myocyte nuclei (Fig. 1E and F). Evaluation of the apoptosis-positive specimens revealed that less than 0.7 ± 0.4% of skeletal myocytes were apoptosis positive. Peak oxygen consumption was significantly reduced in patients with apoptosis as compared with patients without apoptosis in skeletal myocytes (12.0 ± 3.7 vs. 18.2 ± 4.4 ml/kg/min; p = 0.0005) (Table 2). Furthermore, patients with apoptosis-positive skeletal muscle biopsies had a significantly longer history of the illness (64.3 ± 60.2 vs. 25.8 ± 42.7 months; p = 0.02). No significant relation between the incidence of apoptosis and other clinical parameters like age, body weight, left ventricular ejection fraction and cardiac decompensation events could be detected (Table 2).

Detection of iNOS and Bcl-2 expression and its relation to apoptosis. Inducible NO synthase protein expression was detected in all skeletal muscle biopsies of patients with CHF, whereas in the healthy control subjects no staining
could be observed (Fig. 2, A and B, Table 2). Quantitative analysis revealed a significantly higher expression in comparison with normal healthy individuals (5.1 ± 3.4 vs. 0.8 ± 0.7% positive tissue area; p = 0.0001). Dividing the CHF patients into an apoptosis-positive and an apoptosis-negative group, the patients exhibiting apoptosis-positive skeletal muscle myocytes showed a significantly higher iNOS expression compared with apoptosis-negative patients (6.8 ± 3.6 vs. 3.7 ± 2.6% positive tissue area; p = 0.015) (Table 2).

The antiapoptotic factor Bcl-2 could be detected in the cytosol of skeletal myocytes (Fig. 2C) and showed a 50% higher expression level in the healthy control group when compared with CHF patients (1.8 ± 0.6 vs. 1.2 ± 0.4% positive tissue area; p = 0.017). In the negative control reaction no staining could be observed (Fig. 2D). A subgroup analysis of CHF patients revealed that apoptosis-positive patients had a 40% lower level of Bcl-2 expression than patients with apoptotic-negative skeletal myocytes (1.0 ± 0.3 vs. 1.4 ± 0.4% positive stained tissue area; p = 0.03) (Table 2).

**Figure 1.** Representative images (×200) of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling reactions or antiactin staining of skeletal muscle biopsies. Nuclei with fragmented deoxyribonucleic acid stained red (arrows) (A) whereas cells with normal nuclei stained blue (immunoperoxidase staining with hematoxylin counterstaining). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining of a skeletal muscle biopsy from a (A) chronic heart failure (CHF) patient; (B) normal control subject; (C) CHF patient after deoxyribonuclease I pretreatment; (D) CHF patient after deoxyribonuclease I pretreatment but with the omission of deoxynucleotidyl transferase in the labeling reaction; (E) CHF patient, and (F) the same as in (E) but counterstained with antiactin is shown. The identical nuclei in E and F are marked with asterisks.
DISCUSSION

Three important findings emerge from this study: 1) apoptosis occurs not only in the myocardium but also in skeletal muscle of patients with chronic heart failure, 2) apoptosis in skeletal myocytes is associated with a lower exercise capacity, and 3) iNOS expression is increased and the expression of Bcl-2 is reduced in patients with apoptosis-positive myocytes. These results suggest that iNOS and Bcl-2 may be involved in the regulatory mechanisms of apoptosis. Particularly in patients with a long history of CHF, apoptosis may represent another factor contributing to impaired exercise tolerance.

**Apoptosis in skeletal myocytes of patients with CHF.**

The concept that accumulation or loss of cells is due to uncontrolled cell proliferation or abnormal cell death rates has been amended. The realization that cell death is a normal process in development and cellular homeostasis has opened a new avenue for exploration of the causes and treatment of various diseases (14). In addition to the beneficial effects of apoptosis, the inappropriate activation of

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**Table 2. Clinical Parameters in Apoptosis-Positive or -Negative Patients**

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<thead>
<tr>
<th></th>
<th>Apo. pos.</th>
<th>Apo. neg.</th>
<th>p</th>
</tr>
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<tr>
<td>Etiology</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DCM</td>
<td>10</td>
<td>14</td>
<td>0.20</td>
</tr>
<tr>
<td>ICM</td>
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<td>4</td>
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<tr>
<td>Age (yr)</td>
<td>60.1 ± 7.1</td>
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<td>0.20</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.4 ± 14.1</td>
<td>82.6 ± 12.7</td>
<td>0.21</td>
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<tr>
<td>LVEF (%)</td>
<td>19.6 ± 7.9</td>
<td>19.6 ± 8.1</td>
<td>0.90</td>
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<tr>
<td>VO₂ max (ml/kg/min)</td>
<td>12.0 ± 3.7</td>
<td>18.2 ± 4.4</td>
<td>0.0005*</td>
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<tr>
<td>Duration (months)</td>
<td>64.3 ± 60.2</td>
<td>25.8 ± 42.7</td>
<td>0.02*</td>
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<tr>
<td>Decompensations</td>
<td>1.1 ± 1.4</td>
<td>0.8 ± 1.0</td>
<td>0.60</td>
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</table>

*Statistically significant. Data presented are mean value ± SD or percent of patients. Apo. = apoptosis; LVEF = left ventricular ejection fraction; neg. = negative; pos. = positive. Other abbreviations as in Table 1.

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**Figure 2.** Representative images of the immunohistochemical detection of inducible nitric oxide synthase (A, B) or Bcl-2 (C, D) of a skeletal muscle biopsy from a chronic heart failure patient (A, C) and a normal control subject (C, D) (×200).
programmed cell death may cause or contribute to a variety of diseases including neurodegenerative disorders and ischemic stroke (28,29), acquired immunodeficiency syndrome (30,31) and ischemic injury (24). Apoptosis is highly regulated, controlled by many different distinct signaling pathways, which ultimately converge to activate a common apoptotic program (14,32).

Terminally differentiated cells, such as myocardial/skeletal muscle or neuronal cells, are not likely to undergo apoptosis under natural conditions. Recently, however, three studies demonstrated histochemical evidence for an increase of apoptosis in myocardial myocytes of patients with heart failure (12,13) and reperfusion injury (33). These authors speculated that apoptosis may lead to a progressive deterioration in myocardial function, culminating in chronic cardiomyopathy and end-stage heart failure. One might hypothesize that apoptosis may contribute to the reduced aerobic capacity of skeletal muscle in patients with CHF. This assumption is supported by our finding that programmed cell death is found in 47% of our CHF patients, and that its presence is associated with a reduced exercise capacity. The majority of the apoptotic nuclei are, based on antiactin staining, skeletal muscle myocytes and not endothelial or satellite cells. This finding further strengthens the idea that an apoptotic loss of myocytes in patients with CHF may lead to a reduced contractile force, and therefore to a reduced functional work capacity. Furthermore, the finding that patients with apoptosis-positive skeletal myocytes exhibited a longer duration of the disease goes along with the clinical observation that exercise capacity decreases with progression of CHF.

At first glance, the observation that only a very small fraction (0.7 ± 0.4%) of cells are apoptotic in skeletal muscle biopsies of CHF patients seems to be too insignificant to impair functioning of the skeletal muscle. It is unlikely that we underestimated the number of apoptotic-positive nuclei in skeletal muscle biopsies of CHF patients, because 100% of the nuclei stained TUNEL-positive after deoxyribonuclease I pretreatment. However, as pointed out by Colucci (34,35), apoptosis is a transient event, lasting perhaps only a few hours, and the death of even such a small fraction of nuclei per day could, over a period of months, or perhaps only a few hours, and the death of even such a small fraction (0.7 ± 0.4%) of cells are apoptotic in skeletal muscle biopsies of CHF patients, and that its presence is associated with a reduced exercise capacity. The majority of the apoptotic nuclei are, based on antiactin staining, skeletal muscle myocytes and not endothelial or satellite cells. This finding further strengthens the idea that an apoptotic loss of myocytes in patients with CHF may lead to a reduced contractile force, and therefore to a reduced functional work capacity. Furthermore, the finding that patients with apoptosis-positive skeletal myocytes exhibited a longer duration of the disease goes along with the clinical observation that exercise capacity decreases with progression of CHF.

Regulatory mechanisms for the induction of apoptosis. In the current literature a variety of agents are reported to act either pro- or antiapoptotically (reviewed in [14]). The Bcl-2 protein, originally discovered by virtue of its involvement in the t(14;18) chromosomal translocation, acts as an antiapoptotic factor, by preventing the collapse of the mitochondrial membrane potential or by complexing the proapoptotic factor Bax (reviewed in [16]). Nitric oxide represents another factor that, when produced in high amounts by iNOS, is capable of inducing the apoptotic process (reviewed in [17]). Analyzing the skeletal muscle biopsies of patients with chronic heart failure and dividing the samples into two groups—apoptosis positive or apoptosis negative—a higher iNOS expression and a reduced expression of Bcl-2 were detected in apoptosis-positive specimens. On the basis of these results, one might speculate that the increased expression of iNOS and the reduction of the antiapoptotic factor Bcl-2 are involved in the regulation of the apoptotic process in skeletal muscle of patients with CHF.

Analyzing the iNOS immunohistochemical staining pattern, a diffuse positive staining of the biopsy was observed. Proceeding from this diffuse staining pattern and the small tissue area analyzed, one may hypothesize that an ischemic insult or a cytokine-mediated process is the pathophysiological mechanism for the increased expression of iNOS. This assumption is supported by our finding that apoptosis is associated with a diminished exercise capacity of these patients.

In conclusion, this study demonstrates that apoptosis is present in about 50% of skeletal muscle biopsies obtained from patients with CHF. The increased expression of iNOS, as well as the reduced expression of Bcl-2, are possible factors for the induction of the apoptotic process in skeletal muscle myocytes. Most important, the occurrence of apoptosis is associated with a diminished exercise capacity of these patients.

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REFERENCES


