Apopotosis and Id2 expression in diaphragm and soleus muscle from the emphysematous hamster

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THE PREVALENCE OF CHRONIC obstructive pulmonary disease (COPD) is rising worldwide and is a leading cause of death. The primary cause of death is respiratory failure (4) and inspiratory muscle weakness is an independent predictor of survival of those patients (11). Respiratory muscle weakness also contributes to the exercise intolerance of patients with COPD, which is further compounded by remodeling and wasting of peripheral muscles (10, 44). Recently, we found that diaphragm dysfunction is already evident in myocytes from patients with mild COPD and normal body mass index (27).

The mechanisms of diaphragm and peripheral muscle adaptations during COPD are not entirely clear. Many chronic diseases, including COPD, are associated with systemic inflammation (10, 41). In COPD the lung is a source of inflammatory cytokines, but also resistive breathing induces cytokine expression in the diaphragm, among them TNF-α (42). These local elevations in TNF-α may cause a rise in plasma TNF-α levels and, via a positive feedback loop, induce the expression of TNF-α in the muscle tissue itself (18), ultimately contributing to diaphragm and peripheral muscle wasting (42). Indeed, systemic inflammation and elevated TNF-α levels play an important role in skeletal muscle wasting and remodeling during chronic heart failure (22) and in aging TNF-α expression within myocytes is negatively related to muscle mass (12).

Apopotosis, triggered by TNF-α, plays an important role in skeletal muscle wasting during chronic heart failure (22), and recent evidence for apoptosis in skeletal muscle from patients with COPD has been obtained (1). Since apoptosis only occurred in COPD patients with a low body mass index (1), it is possible that only in patients with severe COPD and cytokine levels above a threshold apoptosis is induced. The diaphragm, however, may be an exception, since even in patients with mild COPD and a normal body mass index the caspase 3 activity was significantly elevated (26). Little work, however, has so far been done on the pathways of apoptosis that contribute to muscle remodeling and wasting during COPD.

Mice treated with TNF-α exhibited an impaired regenerative capacity of skeletal muscle that was related to deactivation of MyoD (20). The deactivation of MyoD appeared to be mediated by breakdown of MyoD via the ubiquitin proteasome pathway (20, 31), which in turn might be activated by the p65 subunit of NF-κB (20). These observations and the decrement in specific tension and power output in diaphragm of MyoD−/− mice (35) strongly resemble the changes that occur during COPD (10, 44), suggesting that alterations in the activity and/or expression of MyoD may contribute to the alterations in skeletal muscle during COPD.

An elevated expression of inhibitors of differentiation (Id) proteins may, via dimerization with MyoD, not only inhibit the

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binding of MyoD to DNA (36), but also make MyoD more vulnerable to breakdown via the ubiquitin proteasome pathway (30). Id2 appears to be essential for both regulating cell proliferation and apoptosis (8, 45). Where the DNA-binding domain-negative helix-loop-helix motif promotes cell division, the NH2-terminal region of Id2 has been implicated in mediating the proapoptotic cascade which is probably related to mitochondria-associated apoptotic signaling (8, 45). Indeed, high levels of Id2 have been identified under conditions where apoptosis contributes to muscle wasting (2, 3).

Both ubiquitination of MyoD and increased nuclear apoptosis would hamper the differentiation of satellite cells and thus the regenerative capacity, which ultimately would result in muscle wasting. Moreover, transgenic overexpression of Id proteins leads to muscle fiber atrophy, while fibers within the same muscle that do not overexpress these proteins exhibit (compensatory) hypertrophy (13), suggesting that these proteins indeed play an important role in the regulation of muscle mass. Although it is not yet verified in muscle, in astrocytes TNF-α induces the expression of Id proteins (39). Therefore, we hypothesize that during emphysema the expression of these proteins in the diaphragm and peripheral muscles is elevated and contributes to the muscle adaptations during COPD.

Deconditioning induces muscle wasting and remodeling, and it is therefore not surprising that at least part of the muscle wasting during COPD has been attributed to skeletal muscle disuse (10, 44). Also medications commonly used by patients with COPD (e.g., corticosteroids) may in themselves cause muscle wasting (7), and it is thus difficult to separate these factors from the effects of COPD per se (44). The emphysematous hamster has regularly been used as a model for COPD (24, 40) and does not exhibit a reduction in activity level (24). Therefore, it is a suitable model to study the effects of emphysema per se on skeletal muscle adaptations without the bias related to disuse and medication.

The aim of the present study was to determine whether apoptosis and altered MyoD and Id2 expression may play a role in muscle remodeling during COPD. We used the emphysematous hamster to examine the expression of TNF-α, MyoD, Id2, the DNA binding activity of the p65 subunit of NF-κB and alterations in the expression and activity of markers of apoptotic pathways in the diaphragm and several calf muscles.

**MATERIALS AND METHODS**

*Animals and induction of emphysema.* Male Syrian hamsters (7–9 wk old) were obtained from Harlan Labs (Indianapolis, IN) and kept one to a cage at a 12:12-h light-dark cycle with food and water provided ad libitum. The animals were anesthetized with ketamine/xylazine (0.2 ml of 50 mg/ml ketamine with 0.02 ml of 50 mg/ml xylazine). The animals were placed supine on a 45° slant board, supported by a metal wire below their incisors and a strap across the abdomen. A 24-gauge feeding needle was inserted into the trachea under direct observation. In coordination with the spontaneous inspirations, instillation of either 0.9% sterile saline (0.3 ml/100 g body mass) or porcine elastase (40 IU Sigma E-6883 in 0.3 ml sterile normal saline/100 g body mass) was performed in less than 30 s. This dosage has been shown to produce significant and stable increases in lung volume as early as 16 days after instillation (15). The animal was then removed from the slant board and rotated in a head-up position.
for 3–5 min to distribute the liquid and returned to its cage in a head-up position to recover. This typically took < 5 min.

**Determination of physical activity.** The hamsters were given access to a 1-m circumference running wheel (Mini Miter, Bend, OR) once a week for a period of 24 h. The total number of rotations was counted and was used as an indicator for the activity level of the animal.

**Terminal experiment.** One to five months after instillation, the animals were killed with carbon dioxide inhalation. The diaphragm, soleus, plantaris, and gastrocnemius muscles were excised, blotted dry, and, in the case of calf muscles, weighed. All muscles were frozen in isopentane cooled in liquid nitrogen and stored at −80°C until use. The degree of emphysema was determined from saline displacement of formalin-fixed lungs and a measure of alveolar intercept density in histological sections (24, 38). For the determination of alveolar intercepts, three sections were taken at equally spaced intervals from the peripheral, midlung and central portions of the right and left lung of each animal and imaged at ×20 magnification. Digital images were printed and an overlay grid 1 cm × 1 cm was placed onto the photos. Ten vertical 1-cm lines were randomly selected for each image, and the number of times an alveolar wall intercepted the line was counted (alveolar wall intercepts). The data are presented as the average of the alveolar wall intercepts of the 10 vertical lines. All procedures were approved by the Animal Care and Use Committee of West Virginia University. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Dept. of Health and Human Services and proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279).

**Fiber-type composition.** Sections (10 μm) of the soleus, plantaris, and gastrocnemius muscles were cut on a cryostat and stained for myosin ATPase after preincubation at pH 4.58 for 3.5 min to classify fibers into types I, IIA, or IIB and to determine their fiber cross-sectional area (FCSA) using Scion software (Scion Image, Scion, Fredrick, MD). In the diaphragm, however, the staining intensity was low and no further subdivision of type II fibers into type IIA and IIB fibers was made. Staining of serial sections of several diaphragm muscles with antibodies against type I or type II myosin (data not shown) confirmed that the type I and type II fibers were identified correctly.

**Isolation of mRNA and protein.** RNA was isolated from each muscle tissue using Tri-reagent (Molecular Research Center, Cincinnati, OH), treated with DnaseI (Ambion, Austin TX) to remove any contamination with DNA and reverse transcribed to generate cDNA, as described previously (2, 3). To obtain total muscle protein, muscles were homogenized on ice in T-PER tissue protein extraction buffer (1:20 w/vol) and quantified in duplicate using bicinchoninic acid reagent (Pierce, Rockford, IL) (2, 3).

**Separation of cytosolic and nuclear protein fraction.** Cytoplasmic and nuclear protein extracts were obtained from muscle tissue as described previously (33). Briefly, muscle tissue was homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM diithiothreitol, pH 7.4) and centrifuged at 1,000 rpm for 1 min at 4°C. The supernatant contained the cytosolic fraction and was separated into two aliquots, one containing a protease inhibitor cocktail [4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin; cat. no. P8340, Sigma-Aldrich, St. Louis, MO] and the other part without the protease inhibitor. The nuclear pellet was resuspended in 360 μl of lysis buffer with 39.8 μl of 5 M NaCl, rotated for 1 h at 4°C and centrifuged for 15 min at 14,000 rpm at 4°C. Protease inhibitor cocktail was added to the supernatant containing the nuclear proteins. The protein content of each fraction was quantified in duplicate using bicinchoninic acid reagent (Pierce, Rockford, IL) (2, 3).

**Myosin heavy chain composition.** The myosin heavy chain (MHC) composition was determined with SDS-PAGE as described previously (3). Thereto, about 1.5 μg of cytosolic protein was loaded on a 4% (wt/vol) stacking gel and 7% (wt/vol) separating gel containing 30% (vol/vol) glycerol. The gel was run at 120 V for 27 h at 15°C, and myosin isoforms identified based on the relative migration distance.

**Nuclear binding activity of NF-κB.** The nuclear binding activity of the p65 subunit of NF-κB was determined with a transcription factor assay kit according to the instructions of the manufacturer (Active Motif, Carlsbad, CA). Briefly, 2.5 μg nuclear extract was loaded in the wells of a 96-well plate. The wells are coated with oligonucleotides containing the NF-κB consensus site. Then the wells were incubated with an antibody against the p65 subunit of NF-κB, followed by incubation of a peroxidase-conjugated secondary antibody. The data were expressed as the optical density at 450 nm.

We also tried to assess the nuclear binding activity of the p50 subunit of NF-κB. However, despite loading 15 μg of nuclear extract and letting the reaction proceed for more than 30 min, no signal was observed. Most likely, the primary antibody is not suitable for the hamster, as the positive control did react, or the p50 subunit is not abundant in the muscles we studied.

**Caspase 3 activity and cell death.** Caspase 3 activity and cell death detection were performed on the protease-inhibitor-free cytosolic protein fraction. The caspase 3 activity was determined with a commercial caspase assay kit according to the instructions of the manufacturer (APO-54A-019-K101; Apotech, Switzerland). Briefly, 50 μl of extract were incubated in 50 μM of 7-amino-4-trifluoromethyl coumarin-conjugated substrate at 37°C for 2 h. We have previously confirmed the specificity of the assay by coinoculation of extracts

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**Table 2. Fiber type composition of the soleus, plantaris, and gastrocnemius muscles from control and emphysematous hamsters**

<table>
<thead>
<tr>
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<th>n</th>
<th>I</th>
<th>IIA</th>
<th>IIB</th>
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<tr>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
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<td>91.3±8.0</td>
<td>7.1±8.7</td>
<td>1.6±2.8</td>
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<td>Emphysematous</td>
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<td>87.8±5.7</td>
<td>8.6±4.7</td>
<td>3.6±3.6</td>
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<tr>
<td><strong>Plantaris</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>20.2±6.8</td>
<td>58.5±5.4</td>
<td>21.3±7.9</td>
</tr>
<tr>
<td>Emphysematous</td>
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<td>13.9±5.2*</td>
<td>67.2±10.1</td>
<td>18.9±9.0</td>
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<tr>
<td><strong>Gastrocnemius</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>26.0±23.5</td>
<td>66.1±26.5</td>
<td>7.9±11.3</td>
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<tr>
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<td>48.5±15.6</td>
<td>21.6±25.3</td>
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<tr>
<td><strong>Diaphragm</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
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<td>24.4±3.6</td>
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<td>28.2±2.9*</td>
<td>71.8±2.9*</td>
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Values are means ± SD; n = number of muscles in the diaphragm no subdivision between types IIA and IIB fibers is made (see MATERIALS AND METHODS for explanation). *Significantly different from control at P < 0.05.
with a caspase-specific inhibitor (33). The change in fluorescence between the onset of incubation and after 2 h was determined with a spectrofluorometer with an excitation and emission wavelength of 390 and 530 nm, respectively. Caspase activity was given as the change in arbitrary fluorescence units normalized per milligram protein.

To assess the degree of cell death, we applied a Cell Death Detection ELISA following the instructions of the manufacturer (Roche Diagnostics, Penzberg, Germany). Briefly, cytosolic extracts were loaded into the wells of a microtiter plate coated with a histone antibody. Subsequently, a secondary anti-DNA peroxidase-conjugated antibody was added. The amount of peroxidase in the immunocomplex was determined by incubation with 2.2’-azino-di-[3-ethylbenzthiazoline sulfonate (6)] as a substrate. The absorbance at 405 nm was measured. The data are presented as the optical density at 405 nm normalized to the amount of protein in the sample.

RT-PCR estimates of mRNA. The mRNA levels of TNF-α, apoptosis-inducing factor (AIF), BAX, BCL-2, caspase 9, MyoD, and inhibitor of differentiation protein-2 (Id2) were determined with semiquantitative PCR as described previously (2, 3, 33). Briefly, 100 ng of frozen muscles were minced and then mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA was solubilized in RNase-free H2O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was determined by examining the OD260/OD280 ratio. Two micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 μl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). A control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR by using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 μM deoxyribonucleotide triphosphates (dNTPs), 1 × PCR buffer, and 2.5 units Taq DNA polymerase (USB, Cleveland, OH) in a final volume of 50 μl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). Primers for the various genes and reaction conditions have been described previously (2, 3, 29, 33, 34). The number of cycles was kept within the linear range of amplification for each gene. All amplifications for a particular gene were performed simultaneously to prevent bias related to different reaction conditions. Ten microliters of the product of the PCR reaction was then immediately electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. The images were captured with and signals quantified by using a Kodak 1D image analysis system (Eastman Kodak, Rochester, NY). A semiquantitative measure of the expression of the gene of interest was obtained by normalization of the signal of the gene of interest to the corresponding 18S signal. The product was verified by determination of the size of the fragments of the product after digestion with restriction enzymes.

Determination of protein levels. In whole muscle homogenates, the abundance of MyoD, Id2, BAX, and BCL-2 protein levels were determined with Western blotting as described previously (3). Briefly, 20–40 μg of protein were loaded on a 10 or 12% poly-acrylamide gel and run for 1.5 h at 20°C. The gels were blotted to polyvinylide fluoride membranes (Bio-Rad, Hercules, CA) and stained with Ponceau S (Sigma) to confirm similar loading and transfer in each lane. The membranes were probed with antibodies against MyoD, Id2, BAX, and BCL-2 (PharMingen, San Diego, CA), at a concentration of 2 μg/ml in Tris-buffered saline, 0.5% Tween 20 in 2% milk. The secondary antibody was an horseradish peroxidase-conjugated antimouse (Chemicon International, CA). The signals were developed by chemiluminescence (Boehringer-Roche) and the membranes were then exposed to X-ray films. The resulting bands were quantified as optical density times band area with Kodak 1D imaging software (Eastman Kodak) and expressed in arbitrary units.

Statistics. Data are presented as means ± SD. Differences between emphysematous and control hamsters were determined with a two-tailed unpaired t-test. When conditions for a t-test were violated, a nonparametric Mann-Whitney U-test was applied; this only applied to the MyoD and Id2 mRNA and protein levels. Differences were considered significant at P ≤ 0.05.

RESULTS

Although the age and duration of emphysema were not identical for all hamsters, we did not observe age-related changes in the control group in any of the parameters of interest, except for an increase in the FCSA of type II fibers in the soleus, diaphragm, and gastrocnemius muscles. Similarly, we did not observe significant differences in any of the parameters of interest between 1, 3, and 5 mo of emphysema. Therefore, the data were pooled in a control and emphysematous group.

Fig. 2. Myosin heavy chain (MHC) composition in the diaphragm (A), plantaris (B), and gastrocnemius (C) muscle of control (C) and emphysematous (■) hamsters. Control, n = 4; emphysematous hamsters, n = 11. Values are means ± SE.
Animal and muscle characteristics. Instillation of elastase led to a significant ($P < 0.01$) increase in lung volume in the emphysematous (E) compared with the control (C) animals ($C = 3.95 \pm 0.51$ ml, $E = 5.11 \pm 1.53$ ml). Further evidence for emphysema was the significantly reduced (one-sided $t$-test, $P < 0.05$) alveolar intercept density ($C = 19.33 \pm 7.03$, $E = 14.50 \pm 4.06$), indicating that the induction of emphysema was successful. The physical activity level was similar in emphysematous ($18,671 \pm 3,791$ m/24 h) and control hamsters ($17,938 \pm 3,243$ m/24 h). The body mass of the emphysematous hamsters was similar to that of control hamsters. Also the mass of the soleus, gastrocnemius, and plantaris muscles were similar, both in absolute terms and in terms of muscle mass-to-body mass ratio (Table 1).

Histochemistry and MHC composition. In line with the absence of changes in muscle weight during emphysema, also the FCSA of each fiber type was similar in the diaphragm, soleus, plantaris, and gastrocnemius muscles (Fig. 1). Only the FCSA of type IIa fibers in the soleus muscle was significantly elevated during emphysema. This is, however, a minor proportion of the total number of fibers (Fig. 1). The fiber-type composition (Table 2) and, in line with it, the MHC composition (Fig. 2) of the plantaris and gastrocnemius muscles did not differ significantly between control and emphysematous hamsters. A decrease in the percentage of type I fibers in the plantaris and an increase in the proportion of type I fibers in the diaphragm muscle of emphysematous hamsters (Table 1) were not reflected by a change in MHC composition (Fig. 2).

NF-κB nuclear binding activity. The DNA binding activity of the p65 subunit of NF-κB was not significantly altered during emphysema in any of the muscles (Fig. 3).

Markers of apoptosis. Although we did not observe a significant increase in caspase 3 activity in any of the muscles (Fig. 4).
muscles (Fig. 4A), there was a significantly increased DNA fragmentation, as indicated by the increased signal in the Cell Death Detection ELISA, in the diaphragm and soleus, but not in the plantaris and gastrocnemius muscles (Fig. 4B). Therefore, we further explored the expression of apoptotic signals in the diaphragm and soleus muscles. It appeared that the mRNA levels of the proapoptotic marker BAX were higher in the soleus and diaphragm of the emphysematous hamster than in control hamsters (Fig. 5A). This, and the lower mRNA levels of antiapoptotic BCL-2 mRNA in the soleus and diaphragm of the emphysematous hamster than in the muscles of the control hamsters (Fig. 5B), resulted in an elevated BAX-to-BCL-2 ratio in the muscles of the emphysematous hamsters (Fig. 5C). These changes in mRNA expression were accompanied by similar changes in BAX and BCL-2 protein expression (Fig. 5, A–C). Also the mRNA level of caspase 9 was higher in the soleus and diaphragm of emphysematous than those of control hamsters (Fig. 5D). Emphysema was accompanied by an elevated level of AIF mRNA in both muscles, but in contrast to the other apoptotic markers, AIF mRNA levels were elevated more in the soleus than in the diaphragm during emphysema (Fig. 6A).

Expression of TNF-α in the soleus and diaphragm. Emphysema resulted in elevated mRNA levels of TNF-α in both the soleus and diaphragm muscles. The interaction between emphysema and muscle was reflected by a larger increase in TNF-α in the diaphragm than in the soleus muscle (Fig. 6B).

MyoD and Id2 expression. Although the MyoD mRNA levels in the soleus and diaphragm did not differ significantly between control and emphysematous hamsters (Fig. 7A), the MyoD protein levels were lower in the muscles from the emphysematous hamsters (Fig. 7B). Both Id2 mRNA (Fig. 7C) and protein levels (Fig. 7D) were higher in the soleus and diaphragm muscles of the emphysematous hamsters than in control hamsters.

Fig. 5. Expression of BAX (A), BCL-2 (B), the BAX/BCL-2 ratio at the mRNA and protein level (C) and caspase 9 mRNA (D) in the soleus (SOL) and diaphragm (DIA) of control (C; □) and emphysematous (E; ■) hamsters. Below the graphs are representative gels from the PCR-product and Western blot analysis. The signal of the mRNA of interest is normalized to the corresponding 18S signal; number of hamsters for each data point: n = 4; *different from control at P < 0.05.
The main finding of the present study is that emphysema was associated with apoptosis in the diaphragm and soleus muscle, but not in the plantaris and gastrocnemius muscles. Although the apoptosis was accompanied by a rise in the mRNA levels of markers of the mitochondrial pathway of apoptosis, there was no change in caspase 3 activity. The elevated expression of AIF, a factor that may contribute to apoptosis in the absence of caspase activation (25), and TNF-α mRNA suggests that primarily caspase-independent apoptosis occurred during emphysema. Furthermore, the reduced expression of MyoD and increased expression of Id2 protein may cause an impaired transcriptional regulation of muscle-specific gene transcription and regenerative capacity of the muscle. These changes could not be attributed to medication or a decline in physical activity, indicating that emphysema itself, possibly via altered expression of TNF-α, induces apoptosis and an impaired regenerative capacity of skeletal muscle.

Validation of the model. All hamsters treated with elastase developed emphysema as reflected by an increase in lung volume and loss of alveolar intercepts (38). In agreement with previous studies (24), the level of voluntary physical activity was similar in emphysematous and control hamsters. This supports the notion that the emphysematous hamster is an appropriate model to study the effects of emphysema per se not compounded by the effects of disuse.

Apoptosis. The diaphragm and soleus muscle of the emphysematous hamster exhibited a significant increase in apoptosis, as indicated by increased DNA fragmentation, while there was no evidence for increased apoptosis in the fast plantaris and gastrocnemius muscles. This suggests that muscles with different fiber-type composition may respond differently. We speculate that because muscles with a significant proportion of (slow)-oxidative fibers have a greater mitochondrial volume, their nuclei may be more susceptible to mitochondrial-associated apoptosis, particularly during enhanced oxygen demands. Opposite to what we found during emphysema, however, the slow soleus muscle exhibited less apoptosis than the fast vastus lateralis and plantaris muscles during aging (28, 29). However, in line with our data in the emphysematous hamster, also in COPD patients with a normal body mass index, no evidence for apoptosis was found in the quadriceps muscle (1, 9), while there was evidence for apoptosis in the diaphragm (26). Where during aging the level of physical activity declines, this was not the case for the emphysematous hamster. At least for the diaphragm the load and thus the oxygen demand are even chronically elevated as a result of emphysema. Since AIF may play a role in the assembly and maintenance of the respiratory chain and protect against oxidative damage (25), it is possible that at least in the diaphragm the increased expression of AIF mRNA during emphysema may be a response to the increased strain on the respiratory chain and radical formation that would follow the increased work of breathing.

Although the mRNA and protein levels of markers of the mitochondrial pathway of apoptosis were elevated in the diaphragm and soleus this was not accompanied by an increase in caspase 3 activity or a loss of muscle mass. An unaltered muscle mass and caspase 3 activity in the presence of apoptosis has also been observed in aging human muscles (43) and in old rat muscles following hind limb suspension (21). The activation of the mitochondrial apoptotic pathway may result in an environment that sensitizes myonuclei to apoptosis, making the muscle more vulnerable to apoptotic stimuli. This then may result in the occurrence of muscle wasting during, for instance, exacerbations or exercise. Nevertheless, it seems that caspase-independent pathways resulted in apoptosis, as has been observed during hind limb suspension (21). One of the caspase-independent pathways or factors is AIF, which by binding to and causing condensation of chromatin induces apoptosis (25). Although we did not specifically determine the nuclear content.
Fig. 7. Expression of MyoD mRNA (A) and protein (B), and inhibitor of differentiation protein 2 (Id2) mRNA (C) and protein (D) in the soleus and diaphragm of control (□) and emphysematous (■) hamsters. The signal of the mRNA of interest is normalized to the corresponding 18S signal. Protein expression was determined as the intensity of the band of the protein of interest × volume, normalized to the amount of protein loaded. Representative gels for RT-PCR and Western blot analysis are shown left. The number of hamsters for each data point: n = 8, except for emphysematous diaphragm where n = 9. *Different from control at P < 0.001; @diaphragm differs from soleus at P < 0.015; §significant muscle × emphysema interaction at P = 0.004.
of this proapoptotic factor, the elevated AIF mRNA expression in our model could have contributed to nuclear fragmentation independent of the caspase pathway. The release of AIF from the mitochondria is stimulated by BAX, while BCL-2 inhibits this release. Furthermore, the notion that Id2 can induce apoptosis (8) is supported by the observation that an increase in Id2 expression in aging rat muscles was accompanied by apoptosis (3). It is thus possible that the elevated expression of AIF mRNA and BAX protein and the decline in BCL-2 protein, as well as the elevated expression of Id2 proteins, may underlie the observed apoptosis in the soleus and diaphragm of the emphysematous hamster.

It is thought that in COPD not only the lung, but also the diaphragm that is subjected to a chronically increased load during normal breathing, are important sources of inflammatory cytokines, including TNF-α, spilling over into the circulation (42). If this is true, this spillover may then, via positive feedback, induce the expression of TNF-α within skeletal muscle (18). In line with this, we observed elevated TNF-α mRNA levels in the diaphragm and soleus of the emphysematous hamster. As TNF-α-induced apoptosis in human oligodendrocytes is mediated by AIF (17) it is possible that TNF-α induced the elevated AIF expression in the diaphragm and soleus muscle during emphysema.

It might appear puzzling that there is no loss of muscle mass, while apoptosis is significantly elevated. One possible explanation is that apoptosis primarily occurs in myosatellite cells, rather than in the myonuclei. Indeed, myoblasts appear to be more vulnerable to apoptosis than differentiated myocytes (14, 19). This vulnerability of the satellite cells to TNF-α-induced apoptosis may play an important role in the decline in regenerative capacity and hence, in the long run, muscle wasting. It is possible that the degree of systemic inflammation in the emphysematous hamsters was too low to significantly affect skeletal muscle mass, and that only when the muscle is subjected to a challenge, the impaired regenerative capacity becomes evident.

Alterations in MyoD and Id2 expression. As far as we know there is only one other study that reports changes in the expression of Id proteins and MyoD in muscles from emphysematous hamsters (32). Similar to our study, they report unaltered MyoD mRNA levels. However, in contrast to the increased level of Id2 mRNA we observed, they reported no significant change. The cause of this discrepancy is not clear. However, we also studied protein levels and observed that they were, similar to mRNA levels, also elevated.

Another possible explanation for the absence of atrophy in the presence of apoptosis is an increased activation/recruitment of myosatellite cells. This may be reflected by the upregulation of Id2, which induces satellite cell proliferation (45). At the same time, however, the expression of MyoD, which plays an important role in differentiation of the satellite cell (5), is reduced. This corresponds with the impaired cell-cycle exit and differentiation in regenerating muscle of mice treated with TNF-α (14, 20).

While in the normal situation the muscle mass of the emphysematous hamster is apparently maintained, it is possible that during a challenge the impaired regenerative capacity appears inadequate. Indeed, mice treated with, or overexpressing, TNF-α exhibited a decreased regenerative capacity, which was accompanied by a decline in MyoD protein levels without a change in MyoD mRNA (18, 20). Our data are largely in line with these observations where we observed unaltered MyoD mRNA levels and significantly reduced MyoD protein expression.

The discrepancy between mRNA and protein levels may be related to the breakdown of MyoD by the ubiquitin proteasome pathway. It has been reported that transcription of MyoD mRNA and destabilization of MyoD protein requires the transcriptional activity of the p65 subunit of NF-κB (14, 19), which may cause the activation of the ubiquitin proteasome pathway (16). Yet, in our study the DNA binding activity of the p65 subunit of NF-κB was unaltered. One possibility is that the specific activity of the ubiquitin-proteasome pathway is elevated without an increase in the abundance of the proteasome, as has been observed in the diaphragm of patients with COPD (26). Another explanation for the decline in MyoD protein in the absence of a decrease in mRNA levels is the elevated susceptibility to breakdown via the ubiquitin proteasome pathway when MyoD is heterodimerized with Id proteins (30). Clearly, the chance of heterodimerization is elevated with the increased protein levels of Id2 proteins.

Functional implications. Despite the fact that we observed significant apoptosis and altered expression of MyoD and Id2 proteins, there was no loss of muscle mass. Recently, however, evidence has been obtained that caspase activation may cause a loss of muscle function without atrophy (26, 27, 37). Clearly, we did not find caspase activation, but we contend that it is possible that caspase-independent apoptosis may have similar effects. In line with this, it has been observed that diaphragm strength is reduced in the emphysematous hamster (40), while the strength of the gastrocnemius muscle, where no apoptosis occurred, is normal (23). This corresponds closely with the normal function of the quadriceps muscle in patients with COPD with a normal physical activity level and fat free mass (6), while diaphragm function may be affected (27). Finally, the increased apoptosis and the altered expression of Id2 and MyoD may result in an impaired regenerative capacity.

In conclusion, here we have shown that the emphysematous hamster, which shows no decline in physical activity levels, exhibits no muscle wasting or shifts in fiber-type composition. Nevertheless, in the diaphragm and the soleus muscles, but not in the fast plantaris and gastrocnemius muscles, there is significant caspase-independent apoptosis. In the diaphragm and soleus muscles the reduced expression of MyoD and elevated expression of Id2 protein will most likely cause an impaired transcriptional regulation of muscle-specific gene expression and regenerative capacity. If similar changes occur during COPD then these alterations may contribute to the skeletal muscle wasting and remodeling that is often observed during COPD.

REFERENCES


