Corticosteroid treatment and nutritional deprivation cause a different pattern of atrophy in rat diaphragm

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Dekhuijzen, P. N. Richard, Ghislaine Gayan-Ramirez, Anja Bisschop, Vera De Bock, René Dom, and Marc Decramer. Corticosteroid treatment and nutritional deprivation cause a different pattern of atrophy in rat diaphragm. *J. Appl. Physiol.* 78(2): 629—637, 1995.—Triamcinolone (TR), a fluorinated steroid, caused myogenic alterations in the rat diaphragm. Type Ila fiber atrophy in the rat diaphragm, which is associated with changes in contractile properties. We investigated whether this is a direct effect of TR or the result of an accompanying loss of body and diaphragm weights. For 6 wk, adult rats received saline intramuscularly, TR (0.5 mg/kg im), or nutritional depletion (ND) that resulted in a similar (~40%) reduction in body weight as TR. In these animals, the half-relaxation time of the diaphragm bundles increased, the force-frequency relationship shifted leftward, and the resistance to fatigue was increased. No histological changes were found in the ND diaphragm, in contrast to severe myogenic alterations in the TR diaphragm. Type Ila fiber cross-sectional area (CSA) in the TR diaphragm was reduced by 51%, whereas type I and Ila CSAs were unaffected. In the ND animals, the CSAs of type I, Ila, and Iib fibers were reduced by 31, 35, and 52%, respectively. Similar changes occurred in the deep part of the m. gastrocnemius. In conclusion, myogenic changes and selective type Ila fiber atrophy were caused by TR, whereas ND induced generalized fiber type atrophy without histological changes.

**METHODS**

**Study Design, Animals, and Treatment**

Thirty adult male Wistar rats, aged 14 wk and weighing 350—400 g, were randomized in triplets into one of three treatment groups: control (C; 0.05 ml/day of saline im), TR (0.5 mg·kg\(^{-1}\)·day\(^{-1}\) of TR diacetate im), and ND (0.05 ml/day of saline im and food restriction that resulted in a similar weight loss as TR treatment).

For 6 wk, the animals were injected daily in the left hind-limb. The C and TR groups were fed ad libitum. The ND animals received ~25% of rat chow compared with the C animals because preliminary experiments had shown that this volume of food intake resulted in a similar rate of loss of body weight compared with the TR animals. No accurate measurements of food intake in the TR animals were made, but it appeared to be less than in the C animals. All animals were weighed thrice weekly. The animals were housed in individual cages in a temperature-controlled room. They had free access to water. Veterinary oversight, including regular observations of the health of the animals, was performed according to the Belgian National Guidelines of Animal Care.

After the treatment period, the contractile properties of the DIA and the histological and morphological characteristics of the DIA and gastrocnemius (GA) were examined.

**Contractile Properties**

Twenty-four hours after the last injection, the rats were anesthetized with pentobarbital sodium (Nembutal; 60 mg/kg ip). The animals were tracheotomized, and a tracheal cannula (polyethylene tubing PE-200) was inserted. The animals were mechanically ventilated with an O\(_2\)-enriched gas mixture (tidal volume 5 ml, respiratory frequency 40 breaths/min; Harvard pump respirator, South Natick, MA).

The DIA was quickly removed through a laparotomy and immediately immersed in a cooled oxygenated Krebs solution containing (in mM) 137 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 1 KH\(_2\)PO\(_4\), 12 NaHCO\(_3\), and 6.5 glucose. To avoid regional differences in cross-sectional area (CSA) or fiber type proportions as much as possible, two small rectangular bundles from the middle part of the lateral costal region were obtained.

**RESULTS**

In these studies, severe loss of body and DIA weights occurred, up to ~30—40%. This degree of wasting on itself, e.g., induced by nutritional depletion (ND), may cause structural changes in the DIA, accompanied by abnormalities in contractile properties (14, 15, 18, 30). These studies, however, were discrepant with regard to the fiber types affected. Some studies showed selective type IIB fiber atrophy (14, 15). Conversely, in other studies, both type I and type IIB fibers were affected by ND (18, 30). In the latter studies, a distinction between type Ila and IIB fibers was not made. Lewis et al. (16) made a direct comparison of the changes induced by weight loss and by treatment with dexamethasone, another fluorinated steroid, and a pair-weight group in hamsters. Dexamethasone (7.5 mg/kg sc daily for 3 wk) was accompanied by type II fiber atrophy in the DIA, but a differentiation between type Ila and IIB fibers was not made. In addition, histo-
by dissection parallel to the long axis of the fibers. Silk sutures were tied to both ends of the bundle to serve as anchoring points.

Each bundle was then placed within the external chamber of a jacketed tissue bath containing Krebs solution, was maintained at 37°C, and was perfused with a 95% O2-5% CO2 mixture. The Krebs solution was changed with each new bundle. One end of the bundle was tied to a rigid support, and the other end was fastened to an isometric force transducer mounted to a micrometer. The muscle was placed between two large platinum stimulating electrodes.

The bundles were placed at their optimal length (L0), defined as the length at which the peak twitch force was obtained. This was followed by a 15-min theremoequilibration period. Stimulation were delivered through a Harbor 50–5016 stimulator (Edenbridge, Kent, UK) connected in series to a power amplifier from power one model HS24–4.8 (R. J. Evans, University of Virginia). Stimuli were applied with a pulse duration of 0.2 ms and a train duration of 250 ms. When the maximum twitch force (Pt) was achieved, the voltage was then increased to 120% (80–100 V) to ensure supramaximal stimulation. This voltage was subsequently used during all stimulations. Isometric force was measured by means of a force transducer (Maywood, Hampshire, UK). The signal was amplified and recorded on a computer via analog-to-digital conversion (DT2802-A) with Labdat software (Labdat/Andatat, RHT-InfoDat, Montreal, Quebec, Canada). Signal analysis was done with Anadat.

The following four measurements were performed.

Twitch characteristics. Two twitches were recorded at L0 to determine Pt, contraction time (CT), and half-relaxation time (RT50). Average values were used for further analysis.

Maximal tetanic force (P0). Bundles were stimulated twice tetanically at 160 Hz for 250 ms to obtain a clear plateau in maximal force generation during the force-frequency protocol always occurs.

Fatigue properties. Fatigability was assessed in two different ways. First, the force output at 160 Hz after each stimulus frequency during the force-frequency curve was measured. Second, bundles were fatigued by means of 330-ms stimulations repeated every 2 s at 25 Hz for 5 min (modified after the method of Burke et al. [2]).

After these measurements, each muscle bundle was removed from the bath while its L0 was kept similar to the experimental condition. Subsequently, its L0 thickness, and width were measured. The bundle was blotted dry and weighed. CSA was calculated by dividing weight by specific density (1.056) and muscle L0. Pt and P0 were expressed per unit CSA (23, 29). The twitch-to-tetanic ratio (Pt/P0) was calculated for each muscle bundle.

Finally, the remaining DIA tissue was trimmed, blotted, and weighed. The parasternal muscles (including the sternum and chondral parts of the ribs), right medial scalene muscle, and GA and soleus muscles from the right hindlimb were dissected, trimmed, blotted, and weighed, as were the two adrenal glands.

Histological and Histochemical Procedures

Muscle strips obtained from the costal region of the DIA and transverse sections from the midbelly of the GA of the right hindlimb were prepared for histopathological examination. Muscle samples were put into "tissue glue" (Tissue-Tek, Elkhard, IN) on a cork holder with the muscle fibers oriented perpendicularly to the surface of the cork. Proper orientation of the bundles was controlled by using magnifying glasses. Subsequently, these specimens were quickly frozen in isopentane cooled with liquid N2. Serial cross sections, parallel to the cork, were cut at 10-μm thickness with a cryostat kept at −20°C. Sections of each DIA and GA were taken for routine hematoxylin and eosin staining.

The other serial sections were stained for myofibrillar adenosinetriphosphatase after alkaline (pH 9.3) and acid (pH 4.5) preincubation. Muscle fibers were classified as type I, IIA, or III fibers (7). Slides preincubated at pH 4.5 offered the best separation of different fiber types and were subsequently used for further analysis.

Morphometric examination was carried out with a Leitz microscope (Wetzlar, Germany) connected to a digitizing board (Numonics 2207, Montgomeryville, PA) at ×25 magnification. Areas in which fiber orientation was not transverse to the long axis were not analyzed. The boundaries of individual muscle fibers were delineated, and the fiber CSA was determined from the number of pixels within the outlined fiber. At least 100 fibers of each DIA and deep (red) part of the GA were used to calculate the mean CSA of all fiber types.

Data Analysis

Two DIA bundles were obtained from each animal. Because variation between animals was as large as between animals, all bundles were used for statistical analysis as independent cases. Data from the different treatment groups were compared by using two-way analysis of variance. Differences between means were assessed by using Duncan’s multiple range test. Statistical significance was set at P < 0.05. All analyses were performed by using the SPSS/PC+ package. Values are means ± SD, unless specified otherwise.

RESULTS

Body and Muscle Weight

Body weight at the start of the study was not different between the three groups (Fig. 1). In the C group,
TABLE 1. Weight of muscles and adrenal glands

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diaphragm</th>
<th>Parasternals</th>
<th>Scalene</th>
<th>Gastrocnemius</th>
<th>Soleus</th>
<th>Adrenal Glands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>663±67</td>
<td>3,233±311</td>
<td>578±56</td>
<td>2,238±179</td>
<td>163±17</td>
<td>45±3</td>
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<tr>
<td>Triamcinolone</td>
<td>391±57*</td>
<td>2,068±291*</td>
<td>111±65†</td>
<td>908±199†</td>
<td>140±8†</td>
<td>15±3†</td>
</tr>
<tr>
<td>Nutritional</td>
<td>369±55*</td>
<td>2,099±217*</td>
<td>298±44*</td>
<td>1,228±134*</td>
<td>120±19*</td>
<td>35±3*</td>
</tr>
<tr>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD in mg. * P < 0.05 compared with control. † P < 0.05 compared with nutritional depletion.

TABLE 2. Diaphragm bundle dimensions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length, mm</th>
<th>Thickness, mm</th>
<th>Width, mm</th>
<th>Weight, mg</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.2±1.9</td>
<td>0.51±0.13</td>
<td>1.62±0.20</td>
<td>28.5±6.2</td>
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<td>Triamcinolone</td>
<td>19.6±2.8</td>
<td>0.39±0.10*</td>
<td>1.31±0.24*</td>
<td>17.5±5.3*</td>
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<tr>
<td>Nutritional</td>
<td>19.5±2.6*</td>
<td>0.41±0.15*</td>
<td>1.18±0.29*</td>
<td>16.1±4.1*</td>
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<tr>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. * P < 0.05 compared with control.

Diaphragmatic Contractile Properties

**DIA bundle dimensions.** Bundle dimensions are shown in Table 2. The TR and ND bundles were smaller, resulting in a lower bundle weight (P < 0.05).

**Twitch characteristics and P₀.** The data of the contractile properties of the DIA are summarized in Table 3. P₀ and CT were similar in the three groups. RT₁/₂ in the TR and ND groups was significantly prolonged compared with that in the C group (P < 0.05). P₀ was not significantly different among the three groups. P₀/P₀ in the ND group was significantly higher than in C and TR groups (P < 0.05).

**Force-frequency curve.** The response of DIA strips to increasing stimulus frequencies is shown in Fig. 2. When expressed in absolute values (in kg/cm²), no differences were observed among the three groups. When expressed as a percentage of the 160-Hz stimulations before and after each stimulus frequency, however, a significant leftward shift of the TR and ND bundles at 25, 50, and 80 Hz was observed (P < 0.05).

**Fatigue properties.** Decline in P₀ during the force-frequency protocol. In all groups, the force generated at 160 Hz during the force-frequency stimulation procedure decreased significantly. In the C, TR, and ND groups, the percentage decreases relative to the initial P₀ were 8.0 ± 1.9 (SE), 5.0 ± 1.2, and 7.0 ± 3.4%, respectively. There were no significant differences among the three groups.

**Fatigue run.** During low-frequency fatigue run, force generation in the TR and ND bundles was higher than that in the control group (P < 0.05; Fig. 3A). A similar pattern was observed when values were expressed as percentage of initial forces (P < 0.05; Fig. 3B).

Histopathology

Histological examination of hematoxylin- and eosin-stained slides showed a normal muscular pattern in the DIA and GA of the C animals. No histological changes were observed in the DIA and GA of the ND group, but all fiber types appeared smaller than in the C group. In the TR group, however, profound myogenic changes were noticed, with an increased variation in the diameter of all fiber types, an increased amount of connective tissue, atrophy of type Iib fibers, and an excess of nuclei (Fig. 4).

Morphometry

Fiber type distribution was not changed by different treatments. In general, the DIA consisted of 40% type I fibers, 30% type Ila fibers, and 30% type Iib fibers. The red deeper part of the GA consisted of ~30% type I fibers, ~25% type IIA fibers, and ~45% type IIB fibers.

Muscle fiber dimensions in both the DIA and GA were affected differently in the TR and ND groups. In the TR DIA, type IIB fiber CSA was reduced by 51% (P < 0.05), whereas type I and IIA fiber CSAs were unaffected (Figs. 5, bottom, left and 6A). In the ND DIA, mean CSAs of type I, IIA, and IIB fibers were decreased by 31, 35, and 52%, respectively (P < 0.05; twist-to-tetanic ratio. * P < 0.05 compared with control.

TABLE 3. Diaphragmatic contractile properties

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P₀, kg/cm²</th>
<th>CT, ms</th>
<th>RT₁/₂, ms</th>
<th>P₀, kg/cm²</th>
<th>P₀/P₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.72±0.19</td>
<td>19±2</td>
<td>24±2</td>
<td>2.57±0.45</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>0.65±0.13</td>
<td>20±2</td>
<td>28±6*</td>
<td>2.28±0.53</td>
<td>0.29±0.06*</td>
</tr>
<tr>
<td>Nutritional</td>
<td>0.79±0.24</td>
<td>20±2</td>
<td>28±4*</td>
<td>2.33±0.52</td>
<td>0.34±0.06*</td>
</tr>
<tr>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Values are means ± SD. P₀, maximal twitch force; CT, contraction time; RT₁/₂, half-relaxation time; P₀, maximal tetanic force; P₀/P₀, twitch-to-tetanic ratio. * P < 0.05 compared with control.
CORTICOSTEROIDS, NUTRITIONAL DEPLETION, AND RAT DIAPHRAGM

**Fig. 2.** A: force-frequency curve of diaphragm bundles. B: force-frequency curve of diaphragm bundles expressed as percentage of force generated during 160-Hz stimulations (%P160Hz). Symbols as in Fig. 1. ★ Significant difference for triamcinolone and nutritional depletion groups compared with control group, $P < 0.05$.

Figs. 5, middle left, and 6A). Similar changes were observed in the GA (Figs. 5, bottom right, and 6B).

The relative contributions of the different fiber types to the total CSA in the DIA are presented in Fig. 7. An increased percentage of total CSA was taken up by type I fibers in the ND group (34%) and in the TR DIA (35%) compared with 26% in the C group.

**DISCUSSION**

The present data show that TR and ND affect the rat DIA differently. TR treatment caused selective type IIb fiber atrophy in the DIA. The prolonged $RT_{1/2}$, the leftward shift of the force-frequency curve, and the relative resistance to fatigue during the force-frequency protocol were all consistent with type IIb fiber atrophy. Similar changes in contractile properties were observed in the ND DIA. However, the morphological changes were clearly different from the TR-induced abnormalities and consisted of atrophy of all fiber types. These differences in the pattern of fiber atrophy were confirmed by the changes in weight of the other muscles investigated. The weights of the scalene muscle and total GA, both consisting predominantly of type IIb fibers, were lower in the TR group than in the ND group. In contrast, the weight of the soleus muscle (consisting mainly of type I fibers) was lower after ND than after TR treatment. This implies that the observed differences in the pattern of muscle fiber atrophy are generalized and consequently affect both respiratory and peripheral skeletal muscles.

Despite these distinct morphological changes induced by ND and TR, no significant differences in diaphragmatic contractile properties were found between these two groups. This may be explained by the fact that the contribution of the different fiber types to the total CSA was affected similarly in both groups (Fig. 7).

The atrophy of all fiber types in the ND DIA in the present study is in line with some earlier reports (18, 30) but was not found by other investigators (14, 15). In the study by Kelsen et al. (14), a 4-wk period of ND in hamsters resulted in a loss of body weight and DIA weight of 25% of initial weight. Type IIa and IIb fiber CSAs decreased by ~20%, whereas type I fiber CSA did not change. After a similar loss of body weight in
FIG. 4. Hematoxylin and eosin staining of diaphragm in control animal (A), nutritional-depletion animal (B), and triamcinolone animal (C). In nutritional-depletion diaphragm, all fiber types appear smaller than in control diaphragm. Note variations of diameters of all fiber types, increased amount of connective tissue (CT), similar dimensions of fibers (caused by type IIb fiber atrophy), and excess of nuclei (arrows) in the diaphragm of triamcinolone animal. Magnification, ×25.
9 wk (with no changes in body weight occurring in the last 4 wk), Lanz et al. (15) found no changes in type I fiber CSA in rats. The discrepancy with the present study might be explained by a more severe loss of body weight resulting in type I fiber atrophy. This would imply that the morphological changes induced by ND depend on both the severity and the rate of weight loss. In line with this concept, Lewis and Sieck (17) found that acute nutritional deprivation for 90 h resulted in a decrease of 20% in body weight but did not cause changes in DIA weight or CSA of any fiber type. In contrast, Prezant et al. (25) noted a decrease of type II fibers after 4.5 days of ND in rats, resulting in a loss of 18% of costal DIA weight. Type I fibers were not affected. Similar results were obtained by Dureuil et al. (8).
With respect to peripheral skeletal muscles, several studies have reported skeletal muscle atrophy in all fast-twitch types [e.g., Gardiner and co-workers (10, 11)]. However, direct comparisons between the response of fast-twitch oxidative-glycolytic and fast-twitch glycolytic fibers or between type IIa and IIb fibers have not been made. In recent studies, changes in the DIA were compared with changes in peripheral muscles. Wilcox et al. (33) studied the effects of TR (3 mg·kg⁻¹·day⁻¹) for 4 wk in hamsters. They found selective type IIb fiber atrophy in the DIA; type IIb and, to a lesser extent, type IIa fiber atrophy in the extensor digitalis longus muscle (mainly composed of type IIb fibers); and type IIa fiber atrophy in the soleus muscle (mainly composed of type I fibers). In the study by Ferguson et al. (9), the effects of cortisol acetate (10 mg·kg⁻¹·day⁻¹) for 3 wk in rabbits were evaluated. These investigators noticed atrophy of type I, IIa, and IIb fibers in the DIA; type IIa and IIb fiber atrophy in the extensor digitalis longus; and no changes in the soleus muscle. The effects of dexamethasone (7.5 mg·kg⁻¹·day⁻¹) for 3 wk in hamsters were studied by Lewis et al. (16). They found a reduction in the CSA of type II fibers (a differentiation between type IIa and IIb fibers was not made), no significant decrease in type I fiber CSA, and atrophy of both type I and II fibers in the red GA. When considering these studies, it appears that the effects of glucocorticoid treatment on skeletal muscle morphology vary with the type and dosage of the steroid administered and the animal model used.

A pair-fed control group was not used in the present study. In the study by Moore et al. (23), no significant differences were found in the DIA mass and contractile properties between a pair-fed and an ad libitum-fed group. Similarly, in the GA, the effects of TR and a pair-fed group were clearly different with regard to contractile properties and fiber sizes, with the pair-fed group closely resembling the ad libitum-fed group (12).

Although morphometric analysis in the present study was performed by using a standardized procedure, fiber dimensions depend on the degree of shortening when the DIA is excised. Indeed, an excised DIA bundle will assume its equilibrium length, which may imply a shortening up to ~40% in dogs, associated with the loss of passive tension present in vivo (31). This, however, affects all fiber types and cannot be responsible for the differences in the patterns of fiber atrophy noticed in the present study.

The functional and morphological changes induced by TR are in line with previous reports regarding the effects of fluorinated steroids on the DIA (6, 32, 33). In these studies, higher doses were administered during shorter periods [TR: 3 mg/kg for 3 (33), 1.2 mg/kg for 8 days (32), or 1 mg/kg for 4 wk (6); or dexamethasone: 1 and 4 mg/kg for 2 wk and 1 mg/kg for 7 wk (29)]. In the present study, a lower dose of TR (0.5 mg/kg for 6 wk) resulted in similar changes, underlining the potential hazards of this drug for the DIA.

Although twitch and tetanic forces, when corrected...
for fiber CSA, were similar among the three groups, total force generation by the DIA was decreased because the DIA weight was reduced by 40–45% in the TR and ND groups. A similar reduction in DIA weight (44%) was reported by Nachazel and Palecek (24), who studied the effects of hydrocortisone (60 mg/kg im for 8 days) on the breathing pattern in normal rats. Breathing pattern and arterial PCO2 at rest were unaltered, but the response to an increased ventilatory load was diminished in the steroid-treated animals. Further studies are required to investigate the occurrence of hypercapnia due to respiratory muscle weakness induced by corticosteroid treatment and by ND.

The mechanism of the effects of chronic and acute nutritional depletion on DIA morphology is, at present, unclear. Muscle fibers with a mainly oxidative metabolic pathway are relatively resistant to ND-induced catabolism (28, 80). This may explain why, in the present study, ND affected type I and IIA fibers less than type IIb fibers. Similarly, a clear explanation for the selective type IIb fiber atrophy in the DIA is not available. Fluorination of the skeletal muscle enhances all biological activities. The exact mechanism, however, by which fluorinated steroids cause extensive muscle damage compared with nonfluorinated steroids is not known (6). A difference in binding of TR and cortisol to cytoplasmatic proteins may contribute (21).

Because of its continuous activity, the DIA was believed to be relatively spared with regard to the negative effects of both acute ND (13, 19) and corticosteroids (13), in contrast to peripheral muscles such as the GA. In chronic ND, as in the present study, DIA morphology changed in a similar way as the GA. We did not examine the contractile properties of the GA, but, in a previous study by Gardiner et al. (12), it appeared that changes similar to those observed in the DIA occurred in this muscle after a 6-wk period of food restriction.

ND is frequently present in patients with chronic obstructive pulmonary disease (COPD) (1, 27) and may affect both respiratory and peripheral muscle functions (20, 27). Besides ND, other factors such as hyperinflation, disturbances in blood gases, and cardiac failure may affect respiratory muscle function in COPD patients. Treatment with corticosteroids may also affect respiratory muscle function (3–5). An accurate assessment of the contribution of ND and treatment with steroids to a further loss of function may be necessary under several circumstances to treat these patients appropriately. The present study suggests that muscle biopsy and histological and histochemical analyses may add to an accurate diagnosis of the cause of muscle weakness. Moreover, if the patterns of fiber atrophy produced by ND and steroid treatment are entirely different, their effects on gene expression are likely to be different as well. This needs further study.

In conclusion, the present study shows that ND and TR affect the DIA in different ways. ND induced generalized fiber atrophy in the DIA, whereas selective type IIb fiber atrophy was induced by TR. Apparently, the steroid-associated histological changes are not induced by the loss of body weight or DIA weight alone. Similar changes occurred in the GA, indicating that in patients with COPD examination of a peripheral muscle biopsy may add to the differentiation between ND and steroid-induced respiratory and peripheral muscle weaknesses.

The authors thank N. Plaisance-Buts for expert technical assistance. This work was supported by grants from the Dutch Asthma Foundation and Fonds voor Geneeskundig Wetenschappelijk Onderzoek. Address for reprint requests: M. Decramer, Respiratory Div., Univ. Hospital, Herestraat 49, B-3000 Leuven, Belgium.

Received 28 February 1994; accepted in final form 28 September 1994.

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