ABSTRACT: In animal studies, high dosages of corticosteroids cause changes in diaphragm structure and function. The present study was designed to investigate the effects of long-term low-dose methylprednisolone (MP) administration on rat diaphragm contractile properties and morphology. Thirty adult rats were treated with saline or MP (0.2 mg/kg/day SC) during 6 months. Contractile properties of isolated diaphragm strips, immunohistochemical characteristics analyzed by means of antibodies reactive with myosin heavy chain isoforms, and enzyme activities were determined in the diaphragm muscle. MP significantly reduced diaphragm force generation by ~15% over a wide range of stimulation frequencies. The number of type Iib fibers was reduced by MP. There was a mild but significant decrease in type I and Iia fiber cross-sectional area (CSA), whereas type Ix and Iib CSA did not change. These changes resulted in a reduction in the relative contribution of type Iib fibers to total diaphragm muscle area. Biochemically, MP decreased glycogenolytic activity, while fatty acid oxidation and oxidative capacity were increased. In conclusion, long-term low-dose MP administration caused a marked impairment in diaphragm function. This is accompanied by changes in diaphragm muscle morphology and enzyme capacity. © 1997 John Wiley & Sons, Inc.

Key words: corticosteroids; respiratory muscles; contractile properties; morphology; biochemistry

THE EFFECTS OF LONG-TERM LOW-DOSE METHYLPREDNISOLONE ON RAT DIAPHRAGM FUNCTION AND STRUCTURE

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Treatment with corticosteroids may cause peripheral and respiratory muscle dysfunction. Two types of steroid-induced myopathies have been described in humans, depending on the extent and duration of steroid treatment: acute and, more often found in clinical practice, chronic steroid myopathy.4 Weakness of the respiratory muscles following low doses of methylprednisolone (MP) (average daily dose ranging from 1.4 to 21.3 mg during 6 months) was recently reported in patients with chronic obstructive pulmonary disease (COPD).5

The mechanisms by which nonfluorinated steroids cause myopathy are partly unknown. In animal studies, increased variation in fiber dimensions and excess of connective tissue were observed after administration of prednisolone 5 mg/kg/day during 4 weeks.7 Cortisone acetate 10 mg/kg/day during 3 weeks resulted in myonecrosis, vacuolization, and fiber atrophy.9 Changes in myosin heavy chain composition in the muscle fibers may contribute, since myosin heavy chain turnover rate in muscle cells was decreased following dexamethasone therapy.26 These myosin heavy chains determine different levels of myosin adenosine triphosphatase (ATPase) activity depending on their type (I, Iia, Ix, Iib) and are therefore partly responsible for the differences
in contractile properties between the different fibers.\textsuperscript{19,24} Since the fast and most powerful fibers (i.e., types IIx and IIb) are expected to be most sensitive to the side effects of corticosteroids,\textsuperscript{20} we speculated that the contribution of fast fibers to total muscle area declines and maximal force generation decreases following treatment with corticosteroids. Changes in energy substrate and enzyme activities have also been reported before,\textsuperscript{20,31} but to what extent this influences diaphragm contractile properties is unclear.

Most of the above-mentioned animal studies were performed using relatively high dosages during short periods of time. The present study was designed to investigate if lower, clinically relevant doses of corticosteroids also affect rat diaphragm function. We therefore examined functional changes in rat diaphragm in response to administration of MP 0.2 mg/kg/day SC for 6 months. Morphological and biochemical parameters were determined to gain insight into possible underlying mechanisms.

**METHODS**

**Study Design, Animals, and Treatment.** Adult male outbred Wistar rats (n = 30), aged 18–20 weeks, weighing 380 ± 25 g, were randomized into two groups: a control group (C), receiving saline 0.2 mL SC daily, and MP group, receiving methylprednisolone hemisuccinate (Sigma Chemicals, Bornem, Belgium) 0.2 mg/kg SC daily (7 days a week) for 6 months. With each injection all animals received a similar volume (~0.20 mL). The rats were fed ad libitum, kept on a 12/12-h light–dark regime, and weighed twice weekly. The animals were injected daily between 8:30 and 10:00 AM. Although daily food intake was not accurately quantified (animals were not kept in metabolic cages), food intake appeared to be similar in both groups. At the end of the treatment period, contractile properties, and immunohistochemical and biochemical characteristics of the diaphragm were examined. All MP-treated animals were investigated between 23 and 50 h after the last injection with MP. The study was approved by the Animal Experiments Committee of the University of Nijmegen and performed according to the Dutch National Guidelines of Animal Care.

**Contractile Properties.** At the end of the treatment period, the rats were anesthetized with sodium pentobarbital (70 mg/kg IP). A polyethylene cannula was inserted through a tracheotomy for mechanical ventilation (oxygen-enriched gas mixture, flow 0.5 mL/g body weight/min, respiration frequency 70/min, and a duty cycle of 50%). A combined laparotomy and thoracotomy was performed to remove the diaphragm. Immediately after excision the diaphragm was immersed in a cooled, oxygenated Krebs solution at a pH of 7.4. This solution consisted of (mmol/L): 137 NaCl, 4 KCl, 2 MgCl\textsubscript{2}, 1 KH\textsubscript{2}PO\textsubscript{4}, 24 NaHCO\textsubscript{3}, 2.7 CaCl\textsubscript{2}, and 7 glucose. D-tubocurarine chloride 25 µmol/L (Sigma Chemicals, The Netherlands) was added to prevent spontaneous neuromuscular activity. Contractile properties were measured on two small rectangular bundles, dissected from the middle part of the lateral costal region of each hemidiaphragm and parallel to the long axis of the muscle fibers. Silk sutures were firmly tied to both ends of the bundle to serve as anchoring points. Each bundle was placed in a tissue bath containing Krebs at 37°C and was oxygenated with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The central tendon insertion of the bundles was tied to a fixed point and the costal margin origin to an isometric force transducer (Sensotec, model 81/1437, Columbus, OH). Data acquisition and storage were performed using a Dash-16 interface and Twist-Trigger software (I.D.-electronics, University of Nijmegen, The Netherlands). The stimulator (I.D.-electronics, University of Nijmegen) was activated by a personal computer. The muscle strips were stimulated with two large platinum electrodes on both sides of the muscle. To ensure supramaximal stimulation, subsequent stimulations were performed 20% above the voltage at which maximal forces were obtained (~6 V). The pulse duration was set on 0.2 ms. Twitch stimuli were used to determine the optimal length (Lo), followed by a 15-min thermoequilibration period. The following measurements were made:

**Twitch Characteristics.** Two twitches were recorded at Lo to obtain maximal twitch force (P\textsubscript{T}), contraction time (CT), and half relaxation time (%RT). The averages were used for further analysis.\textsuperscript{7}

**Maximal Tetanic Contraction.** Two maximal tetanic stimuli (with a frequency of 160 Hz and a train duration of 250 ms) were generated to obtain maximal tetanic force (P\textsubscript{T}).\textsuperscript{7}

**Force–Frequency Protocol.** Muscle bundles were stimulated every 2 min at the following frequencies: 25, 50, 80, 120, and 160 Hz (train duration 250 ms).

The generated force was expressed per cross-sectional area (N/cm\textsuperscript{2}). Cross-sectional area (CSA) was measured by dividing diaphragm bundle weight by muscle density (1.056 mg/mm\textsuperscript{3}) and bundle length.\textsuperscript{21}

**Histological and Immunohistochemical Procedures.** Muscle strips obtained from the costal part of the right hemidiaphragm were embedded in Tis-
Subsequently, these specimens were quickly frozen in isopentane cooled in liquid N₂ followed by further freezing in liquid N₂. During this procedure, the diaphragm muscle bundles were not fixed at optimal length. Serial cross sections were cut at 7 μm with a cryostat kept at -30°C. Diaphragm sections were taken from each group for routine hematoxylin and eosin (H&E) staining.

Antimyosin heavy chain antibodies (Regeneron Pharmaceuticals, New York, NY) were used for morphometric examination of serial diaphragm sections. The following antibodies were used: BA-D5 reactive with type I myosin heavy chains (MHCs), SC-71 reactive with type Ila MHCs, BP-35 reactive with type I, Ila, and Iib but not with type Ix MHCs, and BF-F3 reactive with type Iib MHCs. Incubation with myosin heavy chain antibodies was performed at room temperature for 1 h. Antibodies were subsequently labeled with ultrasmall immunogold reagent, followed by silver enhancement (Aurion, Wageningen, The Netherlands). Of each diaphragm a minimum of 350 fibers was analyzed using a Spryut-based, PC-image digital analysis system (Bos Inc., Waddinxveen, The Netherlands). Fiber type distribution and CSA were analyzed for type I, Ila, Ibx, and Iib diaphragm muscle fibers. The relative contribution to total diaphragm muscle area per fiber type was calculated as the product of the mean CSA and fiber distribution in the diaphragm.

Biochemistry. Parameters of the bioenergetic capacity of the diaphragm included the activities of the glycolytic enzyme phosphofructokinase, the mitochondrial enzymes 3-hydroxyacyl-CoA dehydrogenase (HADH), a marker for the fatty acid oxidation capacity, and citrate synthase (CS), an index of citric acid cycle activity.

After dissection of the diaphragm, fat and tendon were removed from what was left of both hemidiaphragms. These diaphragm parts were quickly frozen in liquid N₂ and stored at -80°C. Segments of fresh frozen diaphragm were thawed in ice-cold buffer containing 250 mmol/L sucrose, 2 mmol/L, edetic acid, and 10 mmol/L Tris-HCl (pH 7.4). In this buffer muscle homogenates (5% wt/vol) were prepared by hand homogenization, using a Potter-Elvehjem glass-tellon homogenizer.

Total phosphorylase (a + b) activity was assayed at 37°C according to the method described by Jacobs et al., and was expressed as pmol NADPH formed/min per g tissue. HADH activity, assessed at 50 μmol/L acetoacetyl-CoA at 37°C, was expressed in nmol HADH oxidized/min per g tissue. Citrate synthase activity was determined at 25°C and was expressed as μmol coenzyme A formed/min per g tissue. The assays for metabolic enzymes were performed spectrophotometrically in duplicate. The coefficient of variation for the assays applied was ~5%.

Data Analysis. The SPSS/PC+ package V5.0.1 (Chicago, IL) was used for statistical analysis. Data of contractile properties of the two bundles obtained from 1 rat were averaged and compared using Student’s t-test. Repeated-measures analysis of variance was used for force-frequency and growth curve analysis. Morphometric analysis was performed using an average per fiber type per animal, which was utilized as a single value in the statistical analysis. Results were considered significant at P < 0.05. All data were expressed as mean ± SE.

RESULTS

Body Weight. No differences in body weight were observed at the start of the study (375 ± 7 mg in saline vs. 385 ± 4 mg in MP). At the end of the 6-month treatment period, body weight in the MP-treated animals was 5% lower compared to the saline-treated rats (500 ± 5 mg in MP vs. 529 ± 9 mg in saline). Repeated measurements showed a significant effect of treatment on body growth during the 6-month study period.

Contractile Properties. Diaphragm bundle dimensions were equal in both groups (saline vs. MP: length 21.7 ± 0.3 mm vs. 21.3 ± 0.3 mm, thickness 0.62 ± 0.01 mm vs. 0.62 ± 0.01 mm, width 2.13 ± 0.06 mm vs. 2.12 ± 0.05 mm, and weight 31.2 ± 1.1 mg vs. 31.5 ± 0.8 mg).

Both P₀, and P₀ decreased by ~14% following MP administration (P < 0.001) (Table 1). No changes were observed in P₀/P₀ ratio, CT, or %RT (Table 1).

The force-frequency curves, expressed in N/cm², showed a significant reduction in force generation at all stimulation frequencies in the MP group (Fig. 1). When normalized for P₀, forces were similar in the two groups (data not shown).

Histology and Immunohistochemistry. Histological examination of H&E-stained slides of the diaphragm showed a normal muscular pattern in both groups. No signs of myogenic alterations such as an increase in the number of nuclei, excessive variations in fiber dimensions, and excess of connective tissue were found.

Morphometric analysis of the immunohistochemically stained slides showed a significant reduc-
Table 1. Diaphragm contractile properties.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$P_t$ (N/cm²)</th>
<th>CT (ms)</th>
<th>$\frac{1}{2}RT$ (ms)</th>
<th>$P_0$ (N/cm²)</th>
<th>$P/P_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.7 ± 0.02</td>
<td>25.7 ± 0.5</td>
<td>23.1 ± 0.3</td>
<td>27.1 ± 0.06</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>MP</td>
<td>6.6 ± 0.01*</td>
<td>26.0 ± 0.5</td>
<td>22.9 ± 0.3</td>
<td>23.4 ± 0.06*</td>
<td>0.29 ± 0.01</td>
</tr>
</tbody>
</table>

Means ± SE.

$P_t$: twitch force; CT: contraction time; $\frac{1}{2}RT$: half relaxation time; $P_0$: maximal tetanic force.

*P < 0.001 compared to control.

Small but significant reductions in type I and IIa fiber CSA were observed in the MP group. In contrast, no changes were found in type IIx and IIb fiber CSA (Table 2). The distribution of fiber CSA per fiber type is shown in Figure 2. The histogram for type IIb fibers illustrates that the MP-induced decrease in number of IIb fibers occurred without preference for fiber size. This explains the similarity in fiber CSA between MP and control. As a result of the changes in number and CSA of the different fiber types, the relative contribution of type IIb fibers to total diaphragm muscle areas was reduced in the MP group, while the contribution of type IIx fibers was increased (Table 2).

Biochemistry. MP administration caused a significant reduction in glycogenolytic activity, as measured by phosphorylase ($P < 0.01$). Both fatty acid oxidation capacity, measured by HADH, and oxidative capacity, indicated by CS activity, increased in the MP group ($P < 0.01$) (Table 3).

DISCUSSION

The aim of the present study was to evaluate the kind and extent of changes in rat diaphragm caused by low-dose administration of MP during 6 months. Our data show that MP significantly reduced diaphragm force generation over a wide range of stimulation frequencies. This was accompanied by a marked reduction in the number of type IIb fibers, and slight but significant type I and IIa fiber atrophy. The combined effect of these morphological alterations was a reduction in the relative contribution of type IIb fiber CSA to total diaphragm CSA and, conversely, an increase in the relative contribution of type IIx fibers. In line with these data, there was a reduction in phosphorylase activity, combined with an increase in markers of oxidative capacity, confirming a shift toward slower fibers. However, these changes in muscle morphology and biochemistry, although statistically significant, were subtle and at
Table 2. Fiber type distribution, cross-sectional area, and relative fiber type contribution to the total diaphragm muscle area.

<table>
<thead>
<tr>
<th>Fiber type distribution (%)</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.6 ± 1.0</td>
<td>27.1 ± 1.1</td>
<td>26.4 ± 0.64</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>MP</td>
<td>43.5 ± 1.6</td>
<td>29.0 ± 1.3</td>
<td>25.7 ± 1.0</td>
<td>1.8 ± 0.4*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fiber CSA (µm²)</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1164 ± 95</td>
<td>1403 ± 140</td>
<td>3528 ± 421</td>
<td>5828 ± 599</td>
</tr>
<tr>
<td>MP</td>
<td>1041 ± 88*</td>
<td>1214 ± 104*</td>
<td>3532 ± 415</td>
<td>5926 ± 557</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fiber type contribution to total diaphragm area (%)</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.2 ± 0.6</td>
<td>18.4 ± 0.4</td>
<td>45.2 ± 1.3</td>
<td>13.2 ± 1.3</td>
</tr>
<tr>
<td>MP</td>
<td>24.2 ± 0.9</td>
<td>19.8 ± 0.8</td>
<td>50.2 ± 0.7*</td>
<td>5.8 ± 1.3*</td>
</tr>
</tbody>
</table>

Means ± SE.

*P < 0.05 compared to control.

most only partly explained the reduction in diaphragm force generation.

Our intention was to evaluate the effects of a low dose of a nonfluorinated steroid (MP) comparable to the dose that is occasionally used in chronic treatment of patients with COPD. The MP dose used in the present study was based upon the following considerations. Anti-inflammatory potency and metabo-

FIGURE 2. Histograms showing the distribution of fiber CSA. (A) type I fibers, (B) type IIA fibers, (C) type IIX fibers, and (D) type IIB fiber.
Treatment | Phosphorylase (U/g) | CS (U/g) | HADH (U/g)
---|---|---|---
Control | 42.7 ± 0.9 | 26.2 ± 0.8 | 6.19 ± 0.3
MP | 38.6 ± 0.8* | 31.5 ± 1.1* | 7.32 ± 0.3*

Enzyme activities of the diaphragm (means ± SE). CS, citrate synthase; HADH, 3-hydroxyacyl-CoA dehydrogenase.
*P < 0.01 compared to control.

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libers increased. This might be of functional significance, since type IIx and IIb MHCs possess differences in contractile properties. Maximum velocity of shortening and resistance to fatigue in type IIx muscle fibers are intermediate between those of type IIa and IIb fibers. In addition, twitch and tetanic are higher in IIb compared to IIa and IIx motor units. However, it must be stressed that the morphological changes in the present study were small. Consequently, it is unlikely that these alterations completely explain the changes in diaphragm contractile properties.

A methodological point of consideration is that diaphragm muscle morphology in the present study may have been influenced by the fact that the muscle strips were not fixed at optimal length before freezing. The excised diaphragm bundle was therefore allowed to assume its equilibrium length, resulting in shortening of the muscle. The degree of shortening is associated with loss of passive tension present in vivo. In our study this passive muscle tension was similar in the control and the MP group (0.038 ± 0.01 and 0.037 ± 0.01 N). As a consequence, the degree of muscle shortening (and thus the change in fiber CSA) is not likely to be different between control and MP. This, however, does not exclude the possibility of a disproportion in degree of shortening between fiber types. In addition, the differences in CSA between type I, IIa, IIx, and IIb fibers in the control group were in proportion to the differences in CSA when muscle strips were fixed at optimal length. Thus, the physiological differences in size between the different fiber types did not appear to be affected by muscle shortening in the present study.

In line with the small shift toward slower fiber types, glycolytic activity decreased and oxidative capacity increased following MP treatment. The increase in glycogen storage in the diaphragm muscle following steroid administration found by previous investigators is the result of a decrease in glycogen breakdown or an increase in glycogen production, or both. This reduction in glycolytic activity may result in an increase in diaphragm muscle dependence on fatty acid oxidation capacity of fatty acids to provide acetyl-CoA for mitochondrial oxidation. Indeed, HADH activity increased in the MP group, confirming this increase in fatty acid oxidation capacity, although 10 days of prednisolone (5 mg/kg/day SC) or 8 weeks of MP (1 mg/kg/day) did not change HADH activity. The increase in oxidative capacity in this study corresponds with our morphometrical observations, since type IIx muscle fibers are known to have a rich mitochondrial content in contrast to type IIb fibers. This is in line with the increase in oxidative staining reaction in skeletal muscle reported previously. An increase in CS activity was also found following MP 1 mg/kg/day. In contrast to these observations, CS activity in rat diaphragm was reduced following 5 mg/kg prednisolone per day for 10 days. Yet, no changes in CS activity were reported following 0.5, 1, or 2 mg/kg per day, suggesting that CS activity was only reduced after administration of high doses of prednisolone.

Besides depending on dose and duration of the steroid, changes in metabolism due to corticosteroids may also be related to fiber type composition of the muscle. The resistance of different fiber types is believed to depend on their ability to compensate the steroid-induced deficiency of the glycolytic route by converting to oxidative metabolism. It remains unclear if these biochemical changes are also responsible for the changes in fiber types or, in turn, whether this shift is responsible for the biochemical changes.

Since the changes in muscle morphology and biochemical capacity in the present study are likely to be responsible for part of the reduction in muscle force generation, other alterations are presumably also involved. For example, protein degeneration, caused by corticosteroids, may lead to a reduction in myofibrillar protein density. This is likely to reduce the number of cross-bridges available for interaction with actin, which will lead to a reduction in force generation. Further studies are required to explore these potential changes.

The observed reduction in force generation following MP administration in this study may be of clinical significance in patients with severe COPD, since in these patients diaphragm function may be compromised as result of hyperinflation, malnutrition, inactivity, disturbances in blood gases, and cardiac failure. Indeed, it has recently been shown that in these patients administration of low doses of corticosteroids compromises diaphragm function even more. The present study shows that these functional alterations are accompanied by biochemical and structural changes in the diaphragm.

We thank Ms. Yvette Brom for her expert biotechnical assistance.
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