Influence of corticosteroids on myonuclear domain size in the rat diaphragm muscle

A. Jeroen Verheul,1 Carlos B. Mantilla,1 Wen-Zhi Zhan,2 Miguel Bernal,2 P. N. Richard Dekhuijzen,2 and Gary C. Sieck1,2

Departments of 1Anesthesiology and 2Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota 55905; and 3Department of Pulmonary Diseases, University Medical Center Nijmegen, 6500 HB Nijmegen, The Netherlands

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To investigate the effects of low- and high-dose CoS treatment, 40 adult male Sprague-Dawley rats were divided equally into five groups: 1) age-matched unoperated control (CTL-AgeM), 2) ...
age-matched sham-operated (SHAM-AgeM), 3) weight-matched control (CTL-WtM), 4) low-dose CoS-treated (CoS-Low), and 5) high-dose CoS-treated (CoS-High). All animals were housed in separate cages under a 12:12-h light-dark cycle; Purina rat chow and water were provided ad libitum. The Institutional Animal Care and Use Committee of the Mayo Clinic approved all experimental procedures. Initial and final body weights are shown in Table 1. Animals in SHAM-AgeM, CoS-Low, and CoS-High were implanted with a subcutaneous osmotic minipump for sustained infusion of saline (SHAM-AgeM) or CoS for 11 days.

Experimental treatment. For CoS-Low and CoS-High animals, continuous CoS administration was provided during 11 days by an osmotic minipump (model 2ML4, Alzet, Palo Alto, CA). Under ketamine (60 mg/kg) and xylazine (2.5 mg/kg) anesthesia, a pump, filled with 4 mg/ml (CoS-Low) or 35 mg/ml (CoS-High) methylprednisolone sodium succinate (Solu-Medrol, Pharmacia & Upjohn, Kalamazoo, MI), was implanted subcutaneously at the dorsum of each animal. At a flow rate of 2.5 μl/h, the pump provided a daily dose of 1 and 8 mg/kg methylprednisolone in the CoS-Low and CoS-High groups, respectively. An identical pump filled with saline was placed in the SHAM-AgeM animals.

At termination of the treatment period, the animals were anesthetized by intramuscular injection of ketamine (60 mg/kg) and xylazine (2.5 mg/kg), and the costal DIAM was rapidly excised and weighed. Although the costal and crural DIAM may differ in MHC isoform distribution, we did not determine regional effects of CoS treatment. Midcostal DIAM segments were cut into strips, stretched to optimal fiber length, and pinned on a piece of cork. Subsequently, muscle strips were placed in a relaxing solution consisting of (in mM) 59.0 potassium acetate, 6.7 magnesium acetate, 5.6 NaATP, 10 EGTA, 2.0 dithiothreitol, and 50 imidazole. The total ionic strength of this solution was 200 mM at pH 7.0 at 4°C (all reagents were obtained from Sigma-Aldrich). After 24 h, the fibers were stored at −20°C in 50% glycerol-50% relaxing solution for ≤3 wk before single-fiber dissection.

Verification of systemic treatment. Blood samples (~2.5 ml) were also collected at the terminal experiment to measure circulating levels of steroid and thyroid hormones [3, 5, 3′,5′-triiodothyronine (T3) and thyroxine (T4)]. Methylprednisolone levels were determined by liquid chromatography-tandem mass spectrophotometry. T3 and T4 levels were measured with an IMx microparticle enzyme immunoassay and IMx fluorescence polarization immunoassay, respectively.

To verify adequate drug delivery, the remaining amount of methylprednisolone solution in the pumps was also measured. On the basis of the set delivery rate of the pump and initial filling concentration and volume (2,200 μl), we calculated the total administered dose.

Single-fiber dissection and fluorescent labeling. Muscle bundles were pinned on a Sylgard-coated culture dish containing cooled 100% relaxing solution. From each DIAM, 20–30 fibers were dissected using a dissecting microscope (Leica StereoZoom 4). Single muscle fibers were subsequently placed in a 0.1% Triton X-100 relaxing solution for 5 min, and aluminum clips were attached to the ends. Fibers were carefully placed on a glass slide, fixed in 2% paraformaldehyde for 3 min, and washed in 0.1% PBS.

Fibers were then incubated for 2 h with a mouse IgG monoclonal anti-α-actinin antibody (1:500; Sigma Chemical, St. Louis, MO) in 0.1% Tris-buffered saline (TBS) containing 2% normal donkey serum and 0.3% Triton X-100, thereby staining the sarcomeric pattern. After they were washed in PBS, the fibers were incubated for 1 h with Cy5-conjugated donkey anti-mouse IgG (1:500; Jackson Immunoresearch, West Grove, PA) in 0.1% TBS containing 0.3% Triton X-100. After a third wash, fibers were incubated for 5 min with 0.2 mM propidium iodide (Sigma Chemical) to stain the myonuclei. After a final wash in PBS, the fibers were mounted in glycerol gelatin (Sigma Chemical) and covered by a coverslip. Compression of the fiber by the coverslip was minimized by the use of specifically prepared aluminum clips (height ~50 μm), which served as struts.

Single-fiber imaging and MND size determination. Fibers were imaged using an Olympus Fluoview confocal microscope mounted on a BX50WI microscope (Olympus America, Melville, NY). An Olympus DAPI ×40/1.3 NA oil-immersion objective was used for imaging. Serial confocal optical sections were obtained by moving the stage in only one direction, thus eliminating backlash error in the stepper motor. Each optical section was digitized and stored in arrays of 800 × 600 pixels. Pixel dimensions were calibrated using a stage micrometer and were found to be 0.5 × 0.5 μm for the xy-plane (parallel to the microscope stage, by convention). The calculated thickness of optical sections was matched to this dimension, such that each voxel was 0.25 μm3. Optical distortions in the xy- and z-axes were estimated empirically by imaging 10- and 15-μm fluorescein-labeled microspheres (FluoSpheres, Molecular Probes, Eugene, OR). Distortion in the xy-plane was estimated to be <1%; in the z-axis, an average distortion was ~9%. Muscle fiber cross-sectional area (CSA) and volume were estimated on the basis of optical sections obtained at three different positions along a randomly selected 300-μm length of the fiber. At each of these locations, the number of myonuclei in each fiber segment was counted and the sarcomeric spacing was determined. The total number of myonuclei per fiber was determined from the average number of myonuclei per micrometer (after adjustment for a sarcomeric length of 2.5 μm) and normalized for a 2-mm fiber. The average fiber volume per myonucleus (MND) was calculated and expressed as micrometers cubed per myonucleus.

Exclusion of nonmuscle nuclei. Although previous studies showed that in mechanically isolated fibers the number of myonuclei is not biased by accidental inclusion of nonmuscle nuclei (fibroblasts and other nonmyogenic cells) (3, 5, 29, 36, 53), we analyzed a subset of fibers to verify exclusion of these nonmyogenic nuclei. Single DIAM fibers were dissected as described above. Exclusive sarcolemmal staining was achieved by incubation of the fibers for 1 h with a mouse IgG antidystrophin antibody (1:100; Novoceastra Laboratories). After a wash in 0.1% PBS, the fibers were incubated for 1 h with a Cy5-conjugated donkey anti-mouse IgG (1:500) in 0.1% TBS containing 2% normal donkey serum. Finally, the nuclei were stained with 0.2 mM propidium iodide. The fibers were imaged using a confocal microscope as described above.

Single-fiber electrophoresis. After fiber dissection, a small segment of each fiber was cut to determine MHC content. This segment was dissolved directly in 25 μl of SDS sample buffer consisting of 62.5

Table 1. Body weight and diaphragm muscle weight

<table>
<thead>
<tr>
<th></th>
<th>Body wt, g</th>
<th>Diaphragm muscle wt, mg</th>
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<tbody>
<tr>
<td>Initial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTL-WtM</td>
<td>224 ± 5*</td>
<td>783 ± 8</td>
</tr>
<tr>
<td>CTL-AgeM</td>
<td>273 ± 1</td>
<td>901 ± 8</td>
</tr>
<tr>
<td>SHAM-AgeM</td>
<td>275 ± 3</td>
<td>890 ± 7</td>
</tr>
<tr>
<td>CoS-Low</td>
<td>269 ± 4†</td>
<td>648 ± 6†</td>
</tr>
<tr>
<td>CoS-High</td>
<td>286 ± 9†‡</td>
<td>556 ± 5†‡</td>
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<tr>
<td>Final</td>
<td></td>
<td></td>
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<tr>
<td>CTL-WtM</td>
<td>267 ± 6*</td>
<td>738 ± 6*</td>
</tr>
<tr>
<td>CTL-AgeM</td>
<td>320 ± 2</td>
<td>901 ± 8</td>
</tr>
<tr>
<td>SHAM-AgeM</td>
<td>318 ± 5</td>
<td>890 ± 7</td>
</tr>
<tr>
<td>CoS-Low</td>
<td>262 ± 7*</td>
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</tr>
<tr>
<td>CoS-High</td>
<td>251 ± 9*</td>
<td>556 ± 5†‡</td>
</tr>
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</table>

Values are means ± SE. CTL-WtM, weight-matched control; CTL-AgeM, age-matched control; SHAM-AgeM, age-matched sham-operated; CoS-Low and CoS-High, low-dose (1 mg/kg x1 day−1) and high-dose (8 mg/kg x1 day−1) corticosteroid-treated for 11 days. * Significantly different from CTL-AgeM and SHAM-AgeM (P < 0.05). †Significantly different from CTL-WtM (P < 0.05). ‡Significantly different from CoS-Low (P < 0.05).

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mM Tris·HCl, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromphenol blue at pH 6.8. The samples were boiled for 2 min and run on SDS-PAGE, as previously described (18). The stacking gel contained 3.5% acrylamide (pH 6.8), and the separating gel contained 5–8% acrylamide (pH 8.8) with 25% glycerol. Gels (8 × 10 cm, 0.75 mm thick; Hoefer SE250) were run overnight at a constant current of 20 mA and, subsequently, silver stained according to the procedure described by Oakley et al. (41). The expression of MHC isoforms in each single-fiber segment was determined on the basis of their migration pattern using myosin standards. Because MHC2B is frequently coexpressed with MHC2X, DIAm mass as percentage of body weight was significantly reduced after CoS treatment in a dose-dependent manner (0.25 and 0.22% for CoS-Low and CoS-High groups, respectively, P < 0.05). CSA at MHC2X and MHC2X/2B fibers was observed in CoS-Low compared with CTL-AgeM and SHAM-AgeM groups (Fig. 1). Compared with CTL-AgeM and SHAM-AgeM groups, the CoS-High group displayed significantly decreased fibrer CSA across all fiber types, ranging from ~35% for MHCslow fibers to ~45% for MHC2X/2B fibers (P < 0.05). Only at MHCslow and MHC2A fibers was CSA significantly smaller (~35%) in CoS-Low than in CTL-AgeM and SHAM-AgeM animals (P < 0.05). However, compared with CTL-WtM fibers, there was no significant effect on CSA of fibers containing MHCslow and MHC2A in CoS-Low or CoS-High groups.

RESULTS

Body weight and DIAm mass. Mean initial and final body weights are shown in Table 1. Animals in the three control groups (CTL-WtM, CTL-AgeM, and SHAM-AgeM) showed expected weight gain of 35% in CoS-Low and CoS-High groups (P < 0.05). However, compared with CTL-WtM animals, a significant reduction in CSA only at MHC2X/2B fibers (26%; P < 0.05) was observed in CoS-Low compared with CTL-AgeM and SHAM-AgeM groups. In contrast, compared with CTL-WtM animals, a significant reduction in CSA only at MHC2X/2B fibers (26%; P < 0.05) was observed in CoS-Low compared with CTL-AgeM and SHAM-AgeM groups.

Table 2. Number of diaphragm muscle fibers

<table>
<thead>
<tr>
<th></th>
<th>CTL-WtM</th>
<th>CTL-AgeM</th>
<th>SHAM-AgeM</th>
<th>CoS-Low</th>
<th>CoS-High</th>
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<tbody>
<tr>
<td>MHCslow</td>
<td>52</td>
<td>67</td>
<td>47</td>
<td>37</td>
<td>47</td>
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<tr>
<td>MHC2X</td>
<td>50</td>
<td>66</td>
<td>67</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>MHC2X/2B</td>
<td>59</td>
<td>24</td>
<td>42</td>
<td>61</td>
<td>63</td>
</tr>
<tr>
<td>MHC2X/2B</td>
<td>49</td>
<td>12</td>
<td>10</td>
<td>28</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>210</td>
<td>169</td>
<td>166</td>
<td>160</td>
<td>191</td>
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</table>

MHC, myosin heavy chain.

Verification of myonuclear staining. In DIAm fibers stained with antidystrophin and propidium iodide, the number of nuclei outside the fiber when visualized in three dimensions from the confocal stack were measured. These extrasarcosomal nuclei were found only sporadically, comprising <1% of total nuclei in a fiber segment. Thus we are confident that the majority of propidium iodide-stained nuclei represented myonuclei at each single DIAm fiber.

DIAm fiber dimensions. The CSA of all DIAm fiber types was not different between the CTL-AgeM and SHAM-AgeM groups (Fig. 1). In agreement with previous studies (34, 50), the CSA of fibers containing MHC2X or MHC2B was considerably larger than that of MHCslow fibers in the CTL-AgeM, SHAM-AgeM, and CTL-WtM groups (range ~55 to ~260%, P < 0.05). CSA was significantly larger in the CTL-AgeM and SHAM-AgeM groups than in the CTL-WtM group only at fibers containing MHCslow and MHC2A (~50%, P < 0.05), but not MHC2X or MHC2B (Fig. 1). Subcutaneous administration of CoS resulted in a dose-dependent and fiber type-specific reduction of fiber CSA (Fig. 1). Compared with CTL-AgeM and SHAM-AgeM groups, the CoS-High group displayed significantly decreased fiber CSA across all fiber types, ranging from ~35% for MHCslow fibers to ~45% for MHC2X/2B fibers (P < 0.05). Only at MHCslow and MHC2A fibers was CSA significantly smaller (~35%) in CoS-Low than in CTL-AgeM and SHAM-AgeM animals (P < 0.05). However, compared with CTL-WtM fibers, there was no significant effect on CSA of fibers containing MHCslow and MHC2A in CoS-Low or CoS-High groups. A significant reduction in CSA only at MHC2X/2B fibers (26%; P < 0.05) was observed in CoS-Low compared with CTL-WtM animals. In contrast, a significant decrease in CSA at MHC2X and MHC2X/2B DIAm fibers was observed in CoS-High compared with CTL-WtM animals (38 and 57%, respectively, P < 0.05). Fibers containing MHC2X and
MHC\textsubscript{2X2B} were also significantly smaller in CoS-High than in CoS-Low animals ($P < 0.05$).

**Number of myonuclei.** Generally, ~20–30 myonuclei were measured in a ~300-μm-long fiber segment. The number of myonuclei per fiber according to experimental condition and MHC fiber type is shown in Fig. 2. In CTL-WtM animals, the number of myonuclei was significantly higher in fibers expressing MHC\textsubscript{2X} or MHC\textsubscript{2X2B} [95% confidence interval (CI) = 1.608–1.871 and 1.781–2.070, respectively] than in fibers expressing MHC\textsubscript{slow} and MHC\textsubscript{2A} (95% CI = 1.239–1.519 and 1.101–1.387, respectively, $P < 0.05$). There were no differences in myonuclear number between CTL-AgeM and SHAM-AgeM animals at any DIam fiber type: 95% CI = 1.186–1.344 and 1.209–1.458 for MHC\textsubscript{slow}, 1.191–1.351 and 1.106–1.315 for MHC\textsubscript{2A}, 1.260–1.525 and 1.309–1.585 for MHC\textsubscript{2X}, and 1.406–1.781 and 1.407–1.973 for MHC\textsubscript{2X2B}, respectively. In CTL-AgeM and SHAM-AgeM animals, the number of myonuclei per fiber was greater in MHC\textsubscript{2X2B} than in MHC\textsubscript{slow} and MHC\textsubscript{2A} fibers ($P < 0.05$). In the SHAM-AgeM group, MHC\textsubscript{2X} fibers also displayed more myonuclei than MHC\textsubscript{slow} and MHC\textsubscript{2A} fibers ($P < 0.05$). Compared with the CTL-WtM group, fibers in the CTL-AgeM and SHAM-AgeM groups displayed a significant decrease in myonuclear number only at fibers expressing MHC\textsubscript{2X2B} (Fig. 2).

In CoS-Low animals, the number of myonuclei was significantly higher in fibers containing MHC\textsubscript{2X} or MHC\textsubscript{2X2B} (95% CI = 1.793–2.100 and 1.618–2.071, respectively) than in fibers containing MHC\textsubscript{2A} (95% CI = 1.200–1.612) but not MHC\textsubscript{slow} (95% CI = 1.428–1.822) fibers. In the CoS-High group, the number of myonuclei was significantly higher in fibers containing MHC\textsubscript{2X} or MHC\textsubscript{2X2B} (95% CI = 1.544–1.785 and 1.459–1.793, respectively) than in fibers containing MHC\textsubscript{slow} (95% CI = 1.165–1.444, $P < 0.05$), but not MHC\textsubscript{2A} (95% CI = 1.300–1.576), fibers.

No differences in myonuclear number at fibers containing MHC\textsubscript{slow}, MHC\textsubscript{2A}, or MHC\textsubscript{2X2B} were evident between the CoS-Low and CoS-High groups. At MHC\textsubscript{2X} fibers, CoS-High animals displayed a reduced number of myonuclei per fiber compared with CoS-Low animals ($P < 0.05$). At DIam fibers containing MHC\textsubscript{slow} or MHC\textsubscript{2A}, no significant differences in myonuclear number were observed between CoS-Low and CoS-High animals and CTL-WtM, CTL-AgeM, and SHAM-AgeM animals ($P > 0.05$, all comparisons). Compared with CTL-AgeM and SHAM-AgeM animals, DIam fibers containing MHC\textsubscript{2X} displayed a significant increase in myonuclear number ($P < 0.05$) in CoS-Low, but not CoS-High, animals. Compared with CTL-WtM animals, DIam fibers expressing MHC\textsubscript{2X2B} displayed a significant decrease (11%) in the number of myonuclei in CoS-High, but not CoS-Low, animals.

**MND size.** Figure 3 presents the MND size for all MHC isoforms and experimental groups. MND size did not differ between the CTL-AgeM and SHAM-AgeM groups for all MHC isoforms. However, MND at fibers containing MHC\textsubscript{2X} or MHC\textsubscript{2X2B} was significantly larger than at MHC\textsubscript{slow} or MHC\textsubscript{2A} fibers (~33 and 78%, respectively, $P < 0.05$). Compared with the CTL-WtM group, fibers in the CTL-AgeM and SHAM-AgeM groups displayed a significant increase in MND at MHC\textsubscript{slow}, MHC\textsubscript{2A}, and MHC\textsubscript{2X} fibers (~36, 67, and 33%, respectively, $P < 0.05$), but not at DIam fibers expressing MHC\textsubscript{2X2B} (Fig. 3). In the CTL-WtM group, MHC\textsubscript{2X2B} fibers showed significantly larger MND than MHC\textsubscript{2X} fibers, which in turn had a significantly larger MND than MHC\textsubscript{slow} and MHC\textsubscript{2A} fibers ($P < 0.05$).

Systemic CoS treatment resulted in a dose-dependent decrease in MND size (Fig. 3). Compared with CTL-AgeM and SHAM-AgeM animals, DIam fibers in CoS-Low and CoS-High animals displayed significantly decreased MND across all fiber types, ranging from ~36% for MHC\textsubscript{slow} to ~55% for MHC\textsubscript{2X2B} ($P < 0.05$). Compared with the CTL-WtM group, MND size at fibers containing MHC\textsubscript{slow} and MHC\textsubscript{2A} was not significantly different in the CoS-Low and CoS-High groups. However, MHC\textsubscript{2X} and MHC\textsubscript{2X2B} fibers in the CoS-High group displayed significantly decreased MND sizes compared with the CTL-WtM group ($P < 0.05$). In the CoS-Low group, MND size was significantly reduced at DIam fibers expressing...
MHC slow, MHC 2A, MHC 2X, and MHC 2X/2B. Values are means ± SE. *Significantly different from CTL-AgeM and SHAM-AgeM (P < 0.05). †Significantly different from CTL-WtM (P < 0.05). ‡Significantly different from CoS-Low (P < 0.05).

The relative change in MND size and fiber CSA in the CoS-treated groups was expressed as a percentage of the pooled MND and CSA of fibers in the CTL-AgeM and SHAM-AgeM groups and was used to determine the correlation between these variables. Indeed, at DIAm fibers containing MHCslow and MHC 2A, the relative change in MND and CSA was highly correlated, with a slope of 0.87 (r² = 0.99, P = 0.004). In contrast, the relative change in MND and CSA at fibers expressing MHC 2X and MHC 2X/2B was not correlated (r² = 0.75, P = 0.14), indicating that MND is likely not regulated in these fibers in response to CoS treatment. However, when the relative change in MND and CSA was expressed as a percentage of fibers in the CTL-WtM group, the results were highly correlated for all MHC fiber types (r² = 0.88, P = 0.01 and r² = 0.86, P = 0.01, for fibers containing MHCslow/MHC 2A and MHC 2X/MHC 2B, respectively).

DISCUSSION

To the best of our knowledge, the present study is the first describing MND size in DIAm fibers and the effect of CoS treatment on MND size. Previous studies investigating MND size during atrophy induced by other conditions have yielded conflicting results. For instance, MND size decreased after atrophy of hindlimb muscles (soleus and plantaris) induced by limb suspension (4, 24, 25) and spaceflight (7, 24). However, it was also reported that MND size was relatively preserved in type I and type II limb muscle fibers atrophied by spaceflight (22). There are several possible reasons for these differences. 1) The experimental conditions used to induce muscle atrophy could impose distinct adaptations in MND size. For example, after limb immobilization, denervation, and microgravity, neural influences are clearly different and may influence the number of myonuclei before atrophy occurs. In contrast, CoS are known to induce muscle atrophy associated with increased protein catabolism, which could precede myonuclear loss. 2) It is likely that the effects on MND size depend on the specific muscle. In this regard, the results of our study clearly suggest fiber type-specific differences, even within an individual mixed muscle. 3) Duration of the intervention can determine the myonuclear adaptations to fiber atrophy. It is unknown whether a longer treatment with CoS would result in myonuclear loss and preservation of MND at rat DIAm fibers.

CoS treatment. The present study shows that CoS treatment results in significantly smaller MND at rat DIAm fibers expressing MHC 2X or MHC 2B, whereas MND size in fibers expressing MHCslow and MHC 2A is unaffected compared with weight-matched animals. The effects of CoS on MND size were dose dependent; i.e., a larger decrease in CSA and MND size was observed in CoS-High than in CoS-Low animals at fibers containing MHC 2X or MHC 2B. Compared with weight-matched animals, CoS treatment caused selective atrophy of fibers expressing MHC 2X and MHC 2X/2B, without affecting the CSA of fibers expressing MHCslow and MHC 2A. In contrast, all MHC fiber types showed atrophy and reduced MND compared with age-matched animals. Despite the CoS-induced atrophy of fibers expressing MHC 2X and MHC 2X/2B, the number of myonuclei was not proportionately reduced to maintain MND size. Thus MND size does not appear to be regulated after CoS treatment.

Methylprednisolone, a nonfluorinated CoS, was selected, because nonfluorinated CoS are frequently given systemically in pulmonary medicine (43, 52). Moreover, the low (1 mg·kg⁻¹·day⁻¹) and high (8 mg·kg⁻¹·day⁻¹) doses of CoS administered in our study are equivalent to doses prescribed for several days to patients with lung disease (14, 30, 40, 54) provided a ~60% absorption of subcutaneous administered CoS (16, 37).

Although side effects related to steroid administration occur less frequently after treatment with nonfluorinated than with fluorinated CoS treatment (10, 15, 27), significant DIAm atrophy has been reported after nonfluorinated (35, 40, 56) and fluorinated (11, 12, 40, 58) CoS treatment. The observation of a selective CoS-induced atrophy of fibers expressing MHC 2X and MHC 2X/2B in this study is in accordance with previous reports (11, 35, 54, 56, 59). However, others did not find an effect of prednisolone (12) or methylprednisolone (10) on fiber CSA. In addition, selective atrophy of fibers containing MHCslow or MHC 2A was also reported (55). Differences in fiber-specific atrophy after CoS treatment may relate, at least in part, to differences in drug selection, dose, duration of treatment, and/or mode of administration (51, 55).

Myonuclear number and fiber dimensions. The observed differences in MND size across fiber types in the rat DIAm are in general agreement with previous studies, which reported a larger MND size at type IIX/Iib fibers than at type I and Iia fibers in hindlimb muscles (4, 7, 46). However, MND of fibers containing MHCslow and MHC 2A was much smaller in rat DIAm than in rat plantaris (46) but only slightly smaller than in rat soleus fibers (4, 7). Clearly, some of the differences in MND may be the result of differences between muscles. Dissecting techniques and fiber swelling may alter fiber volume and, thus, MND measurements. However, our measurements of CSA are consistent with multiple previous studies.
using alternative techniques in whole DIAm (33, 34) and also similar to those reported by Roy et al. (46).

Several authors proposed that, during muscle atrophy, loss of myonuclei can occur via apoptosis or related mechanisms (3, 5–7, 25, 31, 32). Allen et al. (3) showed that the number of apoptotic myonuclei increases in atrophied rat soleus muscle after hindlimb unloading. Lee et al. (31) showed that myonuclear apoptosis occurs during high-dose CoS-induced hindlimb muscle atrophy. Although the number of myonuclei remained relatively constant after CoS treatment, apoptosis of myonuclei could have occurred in isolation or in combination with the addition of new myonuclei (via satellite cell fusion with existing myofibers).

Mechanisms of CoS-induced muscle atrophy. Alterations in muscle protein content appear to be the major cause of CoS-induced muscle atrophy (17). Indeed, CoS treatment is associated with a reduction of myofibrillar and sarcoplasmic protein concentration (35). However, the mechanisms by which CoS induces muscle wasting are not entirely clear. CoS inhibits protein synthesis and may increase intracellular proteolysis of myofibrillar and soluble proteins (20, 26, 45, 47–49). Interestingly, CoS-induced myopathy is associated with apoptosis of myonuclei (31, 32). A decrease in the number of myonuclei has been suggested to play a role in reduced protein transcription in atrophying muscle fibers (3). We did not directly assess whether CoS-induced atrophy of DIAm fibers resulted from changes in transcription or translational or posttranslational regulation. However, the results of the present study do not support a reduction in myonuclei as underlying DIAm atrophy induced by CoS. Kasper and Xun (25) suggested that a decrease in MND size in atrophic muscle fibers is necessary to achieve shorter mRNA diffusion distance from myonuclei to sarcoplasm. Alternatively, myonuclei in atrophic muscle fibers may downregulate transcriptional control, thereby maintaining a reduced MND size. This hypothesis is consistent with our findings but remains to be tested directly.

In recent studies, insulin-like growth factor I (IGF-I) has become an attractive factor for investigation in regulation of the effects of CoS-induced muscle atrophy, specifically in terms of the regulation of MND size. For instance, after 5 days of CoS treatment, IGF-I mRNA decreased in DIAm (17). Exogenous administration of IGF-I for 14 days partially prevented extensor digitorum longus muscle atrophy in rats (23). IGF-I is one of the key factors stimulating satellite cells, thereby regulating MND. After hindlimb suspension, IGF prevented myonuclear loss in the rat soleus (4), preserving MND. It would be interesting to evaluate the effect of exogenous IGF administration on CoS-induced DIAm atrophy. However, because we did not find evidence of an increased MND after CoS treatment in the DIAm, it is likely that distinct mechanisms may be present in the DIAm compared with hindlimb muscles. In addition, it is possible that the reported decrease in IGF mRNA does not directly result in decreased IGF protein expression, at least in the DIAm.

Although it is possible that DIAm atrophy induced by CoS treatment resulted from malnutrition (11, 34, 50), we previously observed a nonselective, generalized atrophy of all DIAm fiber types in animals that are subjected to food restriction (51). We found a fiber type-specific and dose-dependent effect of CoS treatment, suggesting mechanisms different from those involved in undernutrition-induced DIAm atrophy.

Age- vs. weight-based comparisons. During normal DIAm growth, we documented an increase in dimensions at fibers containing MHC slow and MHC2A, but not MHC2X or MHC2B. Myonuclear numbers were maintained, and MND size increased at all fibers, except those expressing MHC2X2B. To the best of our knowledge, these are novel findings regarding the regulation of MND during normal growth of the DIAm. We found blunting of the normal growth in CSA and MND at DIAm fibers containing MHC slow and MHC2A after CoS treatment, whereas frank atrophy was observed in MHC2X and MHC2X2B fibers, especially with the high-dose CoS regimen.

In accordance with previous reports (2, 44), implantation of a saline-filled pump did not affect animal body weight, DIAm mass, fiber dimensions, myonuclear number, or MND. Thus the control animals could be divided into age- vs. weight-matched groups. However, interpretation of the effects of CoS treatment differed depending on the control group used. Fiber-specific mechanisms might play a role in maintenance of MND size, inasmuch as fibers containing MHC slow or MHC2A showed a high degree of correlation between the changes in MND and CSA compared with age- and weight-matched controls. In contrast, fibers containing MHC2X or MHC2B displayed a correlation between these changes only compared with weight-matched animals. Thus our results are consistent with the notion that animal body weight is critical for the examination of muscle fiber adaptations. These findings suggest a complex interplay of CoS on myofibrillar loss, fiber atrophy, and systemic effects of treatment, which depend on fiber type, even within an active, mixed muscle such as the DIAm.

In conclusion, CoS treatment causes selective atrophy of DIAm muscle fibers expressing MHC2X and/or MHC2B. In these atrophied fibers, MND size was smaller, indicating that MND size is not regulated during CoS-induced DIAm atrophy. However, the mechanisms underlying CoS-induced muscle atrophy and regulation of MND remain to be explored.

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