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Recovery of Corticosteroid-induced Changes in Contractile Properties and Morphology of Rat Diaphragm

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Treatment with the fluorinated steroid triamcinolone (TR) induced type IIb fiber atrophy and the contractile profile of a slow muscle in rat diaphragm. In contrast, the nonfluorinated steroid prednisolone (PR) caused myogenic changes without fiber atrophy, and increased fatigability. The aim of the present study was to investigate the extent to which these changes were reversed 2 mo after discontinuation of treatment. Adult rats were randomly assigned to receive saline, PR 1.25 or 5 mg/kg, or TR 0.25, 0.5, or 1 mg/kg, intramuscularly daily during 4 wk. Administration of TR resulted in severe loss of body weight and dose-dependent mortality. During recovery, body weight in the TR groups increased gradually, still remaining reduced compared with the other groups. Two months after discontinuation of treatment, diaphragm weight was increased in proportion to body weight. Twitch characteristics, maximal tetanic force, force-frequency curve, and fatigue resistance of isolated diaphragm bundles were similar in all groups. Histologic examination of the diaphragm revealed no gross abnormalities in the PR and TR groups. Mild but significant type IIb fiber atrophy was still present in the diaphragm and gastrocnemius muscle of all TR-treated animals. In conclusion, recovery of alterations in morphology of respiratory and peripheral skeletal muscles induced by administration of TR is prolonged. Dekhuijzen PNR, Gayan-Ramirez G, Bisschop A, de Bock V, Dom R, Decramer M. Recovery of corticosteroid-induced changes in contractile properties and morphology of rat diaphragm.

were measured with a hand micrometer. The bundle was blotted dry and the bath, keeping its length at L₀. Bundle length was read from the two

ad). Signal analysis was done with Anadat.

770

transducer (Maywood Ltd., Hampshire, UK). The signal was amplified

tations. Isometric force was measured by means of a Maywood force

and a train duration of 250 ms. Maximum twitch force was achieved

using Labdat software (Labdat/Anadat; RHT-InfoDat, Montreal, Can­

imal stimulation. This voltage was subsequently used during all stimu­

lar bundles from the middle part of the lateral costal region were ob­

were tied to both ends of the bundle to serve as anchoring points. This

for contraction. The bundles were placed at their optimal length (L₀), defined as the

length at which peak twitch force was obtained. This was followed by

for the power amplifier (power one model HS24-4.8; R. J. Evans, Univer­

mixture. Krebs solution was changed with each new bundle.

Krebs solution, maintained at 37° C and perfused with a 95% O₂ and

5% CO₂ mixture. Krebs solution was maintained at 37° C and perfused with a 95% O₂ and 5% CO₂ mixture. Krebs solution was changed with each new bundle.

One end of the bundle was tied to a rigid support, while the other was

fastened to an isometric force transducer. Both anchoring points were

mounted to two separate micrometers. The muscle was placed in be­

tween two large platinum stimulating electrodes.

The bundles were placed at their optimal length (L₀), defined as the

length at which peak twitch force was obtained. This was followed by a

13-min thermoequilibration period. Stimulations were delivered through a

Harvard 50-5016 stimulator (Edenbridge, Kent, UK), connected in se­

ries to a power amplifier (power one model HS24-4.8; R. J. Evans, Univer­

sity of Virginia). Stimuli were applied with a pulse duration of 0.2 ms

and a train duration of 250 ms. Maximum twitch force was achieved at ± 34 V. The voltage was then increased by 20% to ensure supra­

minal stimulation. This voltage was subsequently used during all stimu­

lations. Isometric force was measured by means of a Maywood force
transducer (Maywood Ltd., Hampshire, UK). The signal was amplified

and recorded on computer via analog to digital conversion (DT2801-A)

using Labdat software (Labdat/Anadat; RHT-InfoDat, Montreal, Can­

ada). Signal analysis was done with Anadat.

The following measurements were performed:

Twitch characteristics: Two twitches were recorded to determine maximal twitch force (P₀), time to peak tension (TPT), and half-

relaxation time (1/2RT). Average values were used for further analysis. Maximal tetanic force (Pₘ): Bundles were stimulated twice tetanically at 160 Hz, during 250 ms in order to obtain a clear plateau in force generation (13, 14).

Force-frequency curve: Bundles were stimulated at the following frequencies: 25, 160, 50, 160, 120, and 160 Hz (15). Each stimu­

lus was separated by a 2-min interval.

Fatigue properties: Fatigability was assessed in two different ways. First, force output at 160 Hz was measured after each stimulus frequency during the force-frequency curve (see above). This protocol allowed calculation of the actual force generation as a percentage of the maximal force generation at that instance (15). Second, bundles were fatigued by means of 330-ms stimulations repeated every second at 25 Hz during 5 min (modified after Burke and coworkers [16]).

The whole sequence of this contractile protocol took approximately 1 h. After these measurements, each muscle bundle was removed from the bath, keeping its length at L₀. Bundle length was read from the two micrometers to which the bundle was mounted; its thickness and width were measured with a hand micrometer. The bundle was blotted dry and weighed. Cross-sectional area (CSA) was calculated by dividing weight by specific density (1.056) and muscle length. Forces were expressed per

unit CSA (3, 15). Twitch-to-tetanus ratio (Pₜ/P₀) was calculated for each muscle bundle.

Finally, the remaining diaphragm tissue was trimmed, blotted, and weighed. Parasternal muscles (including sternum and chondral parts of the ribs), right mediastinal scaple, and the muscles gastronemius and soleus from the right hindlimb were dissected, trimmed, blotted, and weighed.

Histologic and Histochemical Procedures

Muscle strips obtained from the costal region of the diaphragm and from the midbelly of the gastrocnemius were prepared for histopathologic ex­
amination. Muscle samples were put into "tissue glue" (Tissue-Tek, Elkh­
hard, 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 diaphragm and gastrocnemius were

taken for routine hematoxylin-eosin staining.

The other serial sections were stained for myofibrillar adenosine triphosphatase after alkaline (pH 9.3) and acid (pH 4.5) preincubation. Muscle fibers were classified as type I (slow-twitch), type IIa (fast-twitch oxidative), or type IIb (fast-twitch glycolytic) fibers (17). Slides prein­
cubated 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 Leica microscope (Leitz Laborlux S., Wetzlar, Germany) at ×20 magnification, connected to a computerized image system (Quantimet 500; Leica, Cambridge Ltd., UK). Areas in which fiber orientation was not transverse to the long axis were not analyzed. Boundaries of individual muscle fibers were delineated, and fiber CSA was determined from the number of pixels within the outlined fiber. At least 150 fibers of each diaphragm and deep (red) part of the gastrocnemius were used to calculate mean CSA of all fiber types.

Data Analysis

Two diaphragm bundles were obtained from each animal for measure­ments of contractile properties. Mean values from each animal were taken for statistical analysis. Data from the different treatment groups were

compared, using one-way analysis of variance. Differences between means were assessed using Duncan’s multiple range test. Statistical significance was set at p < 0.05. All analyses were performed using the SPSS/PC+ package (18). Means ± SD are represented in text, tables, and figures, unless otherwise specified.

RESULTS

Body and Muscle Weight

Body weight increased in both control and prednisolone groups during the treatment period (Table 1). In contrast, it decreased in all triamcinolone groups (p < 0.001). Mortality was observed only in the triamcinolone groups, and was 3 of 15 (20%), 9 of

<table>
<thead>
<tr>
<th>Treatment (A)</th>
<th>Baseline</th>
<th>After 4 Wk</th>
<th>After 13 Wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (A)</td>
<td>311 ± 20</td>
<td>345 ± 27 (+11%)</td>
<td>399 ± 32 (+28%)</td>
</tr>
<tr>
<td>Prednisolone 1.25 mg/kg</td>
<td>311 ± 14</td>
<td>335 ± 15 (+8%)</td>
<td>396 ± 23 (+27%)</td>
</tr>
<tr>
<td>Prednisolone 5 mg/kg</td>
<td>316 ± 22</td>
<td>333 ± 22 (+5%)</td>
<td>398 ± 32 (+26%)</td>
</tr>
<tr>
<td>Triamcinolone 1 mg/kg</td>
<td>312 ± 18</td>
<td>376 ± 20 (+44%)</td>
<td>329 ± 18 (+59%)</td>
</tr>
<tr>
<td>Triamcinolone 0.25 mg/kg</td>
<td>358 ± 18</td>
<td>381 ± 16 (+6%)</td>
<td>439 ± 24 (+23%)</td>
</tr>
<tr>
<td>Triamcinolone 0.5 mg/kg</td>
<td>354 ± 17</td>
<td>324 ± 14 (-33%)</td>
<td>381 ± 26 (+8%)</td>
</tr>
<tr>
<td>Triamcinolone 1 mg/kg</td>
<td>353 ± 19</td>
<td>220 ± 16 (-37%)</td>
<td>357 ± 26 (+1%)</td>
</tr>
</tbody>
</table>

* Body weight is expressed in g (mean ± SD) and as percentage of baseline. 
† Control (A): control group in the first series (A) of experiments; Control (B): control group in the second series (B) of experiments.
§ p < 0.001 compared with baseline.
¶ p < 0.0001 compared with baseline.
|| p < 0.001 compared with control group (A).
### | Triamcinolone 1 mg/kg | 312 ± 17 | 376 ± 20 (+44%) | 329 ± 18 (+59%) |
| Triamcinolone 0.25 mg/kg | 358 ± 18 | 381 ± 16 (+6%) | 439 ± 24 (+23%) |
| Triamcinolone 0.5 mg/kg | 354 ± 17 | 324 ± 14 (-33%) | 381 ± 26 (+8%) |
| Triamcinolone 1 mg/kg | 353 ± 19 | 220 ± 16 (-37%) | 357 ± 26 (+1%) |
15 (60%), and 12 of 15 (80%) in the TR 0.25, 0.5, and 1 mg/kg groups, respectively. Mortality occurred throughout the period of administration of triamcinolone; only one animal died (one day) after the last administration of TR 0.5 mg/kg. Postmortem examination of these animals was not performed. After discontinuation of steroid treatment, body weight in the triamcinolone groups increased gradually, reaching a final body weight approximately 1 to 8% above starting body weight. No change in growth occurred in the prednisolone groups after discontinuation of treatment.

Respiratory and peripheral muscle weight are shown in Table 2. All muscle weights were clearly reduced in the triamcinolone-treated animals, except for soleus weight. When corrected for body weight, however, muscle weights were similar in all groups. Physical activity of the animals was not measured in a standardized fashion. Roughly estimated, no changes occurred during the treatment and recovery period.

**Diaphragmatic Contractile Properties**

**Bundle dimensions.** Bundle dimensions were similar in all groups (Table 3). Bundle weight was slightly lowered in the TR 0.5 and in the TR 1.0 mg/kg groups.

**Twitch characteristics and maximal tetanic force.** No significant differences were found in twitch characteristics among the different treatment groups (Table 4). The prolonged 1/2RT previously found after treatment with triamcinolone was not present after recovery.

**Force-frequency curve.** The response of diaphragm strips to increasing stimulus frequencies is shown in Figure 1A, B. Both when expressed in absolute values and corrected for actual maximal tetanic force (data not shown), no differences were observed between the different treatment groups.

**Fatigue properties.** Decline in maximal tetanic force during force-frequency protocol. In all groups force generated at 160 Hz during the force-frequency stimulation procedure decreased. The percentage decreases in the first series of experiments were [mean (SEM)] 10.6 (1.1), 11.4 (1.2), 12.9 (1.3) and 6.7 (0.8)%, in control, PR 1.25 mg/kg, PR 5 mg/kg, and TR 1 mg/kg, respectively. The TR group showed the smallest decline (p < 0.05 compared with PR 5 mg/kg). In the second series of experiments, these percentage decreases in maximal tetanic force were 9.0 (1.1), 9.1 (1.2), and 9.0 (1.0), in control, TR 0.25 mg/kg and TR 0.5 mg/kg, respectively.

**Fatigue run.** During the low-frequency fatigue run, force generation in all groups was similar, both when expressed in absolute values (Figure 2A, B) or as a percentage of initial values (data not shown).

**Histopathology and Morphometry**

Histologic examination of hematoxylin-eosin stained slides showed a normal muscular pattern in all treatment groups.

Fiber type distribution was not changed by different treatments. In general, the diaphragm consisted of ~40% type I fibers, ~30% type IIa fibers, and ~30% type IIb fibers. In the gastrocnemius muscle, these percentages were ~30, ~25, and ~45%, respectively. Diaphragm fiber dimensions were affected differently according to the type of steroid administered. Mean CSA of type I, IIa, and IIb fibers were similar in both control and prednisolone groups (Figure 3A, B). In all TR groups, however,
TABLE 4
DIAPHRAGMATIC CONTRACTILE PROPERTIES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$P_t$ (kg/cm²)</th>
<th>TPT (ms)</th>
<th>$%RT$ (ms)</th>
<th>$P_0$ (kg/cm²)</th>
<th>$P_t/P_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (A)†</td>
<td>0.476 ± 0.128</td>
<td>25.6 ± 5.3</td>
<td>24.8 ± 5.1</td>
<td>2.23 ± 0.54</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Prednisolone 1.25 mg/kg</td>
<td>0.448 ± 0.126</td>
<td>25.0 ± 5.5</td>
<td>24.3 ± 5.5</td>
<td>2.27 ± 0.61</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Prednisolone 5 mg/kg</td>
<td>0.511 ± 0.196</td>
<td>25.0 ± 5.5</td>
<td>24.7 ± 5.6</td>
<td>2.23 ± 0.54</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Triamcinolone 1 mg/kg</td>
<td>0.492 ± 0.056</td>
<td>25.0 ± 6.5</td>
<td>23.8 ± 4.6</td>
<td>2.23 ± 0.61</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Control (B)†</td>
<td>0.596 ± 0.130</td>
<td>22.5 ± 2.0</td>
<td>23.2 ± 5.3</td>
<td>2.43 ± 0.50</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Triamcinolone 0.25 mg/kg</td>
<td>0.578 ± 0.155</td>
<td>22.8 ± 2.1</td>
<td>26.1 ± 6.0</td>
<td>2.42 ± 0.55</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Triamcinolone 0.5 mg/kg</td>
<td>0.575 ± 0.196</td>
<td>21.8 ± 2.1</td>
<td>24.1 ± 6.4</td>
<td>2.44 ± 0.71</td>
<td>0.23 ± 0.02</td>
</tr>
</tbody>
</table>

Definition of abbreviations: $P_t$ = twitch force; TPT = time to peak tension; $\%RT$ = half-relaxation time; $P_0$ = tetanic force; $P_t/P_0$ = twitch/tetanic ratio.

* Values are expressed as mean ± SD.
† Control (A): control group in the first series (A) of experiments; Control (B): control group in the second series (B) of experiments.

Figure 1. (A) Force-frequency curve in the first series of experiments. Data are expressed in kg/cm². Closed circles: control; closed squares: prednisolone 1.25 mg/kg; open squares: prednisolone 5 mg/kg; open circles: triamcinolone 1 mg/kg; dashed line: pooled SD. (B) Force-frequency curve in the second series of experiments. Data are expressed in kg/cm². Closed circles: control; closed squares: triamcinolone 0.25 mg/kg; open squares: triamcinolone 0.5 mg/kg; dashed line: pooled SD.

Figure 2. (A) Fatigue curve in the first series of experiments. Data are expressed in kg/cm². Same conventions as in Figure 1A. *TR 0.25 mg/kg versus control: p < 0.05. (B) Fatigue curve in the second series of experiments. Data are expressed in kg/cm². Same conventions as in Figure 1B.
FIGURE 3. (A) Diaphragm fiber CSA in the first series of experiments (means + SD). Open bars: control; hatched bars: prednisolone 1.25 mg/kg; solid bars: prednisolone 5 mg/kg; cross-hatched bars: triamcinolone 1 mg/kg. *p < 0.05 compared with all other groups. (B) Diaphragm fiber CSA in the second series of experiments (means + SD). Open bars: control; hatched bars: triamcinolone 0.25 mg/kg; solid bars: triamcinolone 0.5 mg/kg. *p < 0.05 compared with control.

dose-related. The cause of this mortality is not clear. Severe (respiratory) muscle wasting may possibly lead to respiratory failure, although we did not investigate this possibility. In addition, a reduction in the function of the immune system might play a role.

DISCUSSION
The present study shows the differences in recovery of steroid-induced changes in morphology and contractile properties of rat diaphragm when administering triamcinolone and prednisolone. Two months after discontinuation of administration of all dosages of triamcinolone studied, mild but significant selective type IIb fiber atrophy of the diaphragm is still present. Functional differences are not present among the groups. Similar morphologic changes are found in the gastrocnemius. In contrast, no histologic or functional changes are observed after complete withdrawal of prednisolone, which is in accord with the subtle changes associated with acute administration of this steroid.

Mortality induced by triamcinolone appeared to be high and type IIb CSA was significantly reduced compared with the respective control groups (p < 0.05). Type I and IIa diameters were unaffected by treatment with TR. A similar pattern of isolated type IIb fiber atrophy was observed in the gastrocnemius in all TR groups (p < 0.05) (Figure 4A, B).

FIGURE 4. (A) Gastrocnemius fiber CSA in the first series of experiments (means + SD). Open bars: control; hatched bars: prednisolone 1.25 mg/kg; solid bars: prednisolone 5 mg/kg; cross-hatched bars: triamcinolone 1 mg/kg. *p < 0.05 compared with all other groups. (B) Gastrocnemius fiber CSA in the second series of experiments (means + SD). Open bars: control; hatched bars: triamcinolone 0.25 mg/kg; solid bars: triamcinolone 0.5 mg/kg. *p < 0.05 compared with control.
administration of triamcinolone 1 mg/kg/d (5). It is conceivable that similar changes would have occurred after a treatment period of 4 wk. Although we did not study the acute effects of treatment during 4 wk with triamcinolone 0.25 mg/kg/d, the residual type IIb fiber atrophy in this group after the follow-up period would suggest that more pronounced type IIb fiber atrophy has occurred after the acute treatment period. A recent study showed that this dose of triamcinolone caused a reduction in body weight of ~26% after a 2-wk treatment period (19). This was accompanied by an increase in glutamine and phenylalanine efflux from the diaphragm, indicating an increased diaphragm protein degradation rate. The changes in body and muscle weight occurring in the present study further support the presence of alterations in muscle structure after triamcinolone treatment. In conclusion, although we did not study the acute effects of 4 wk treatment with triamcinolone 0.5. and 0.25 mg/kg/d, the data from previous studies and the changes in body and muscle weight in the present study support the contention that acute changes (i.e., type IIb fiber atrophy) were likely to be present after 4 wk of treatment, and that persistent atrophy of these fibers present after the 2-mo follow-up period indicates an incomplete structural recovery.

Changes induced by nonfluorinated steroids appear to be different, depending on the amount of steroid administered. High doses of (hydro)cortisone acetate (10 to 100 mg/kg intramuscularly) also reduce diaphragm weight by approximately 30 to 40% (15, 20–22). Atrophy of all fiber types was found in rabbit diaphragm (21), whereas no morphometry was performed in the other studies. In contrast, prednisolone, administered intramuscularly in a dose of 5 mg/kg/d during 4 wk, caused myogenic changes in the diaphragm, consisting of greater than normal variation of the diameter of all fiber types, excess of nuclei, and increased amount of connective tissue (5). Generalized or selective atrophy did not occur. Functionally, these changes were accompanied by an increased tendency toward fatigue.

Bundle dimensions themselves may affect contractile properties. Segal and Faulkner (23) studied the effect of muscle thickness and incubation temperature on the contractile properties of rat skeletal muscles. The critical radius for O₂ diffusion (i.e., the distance into a muscle at which O₂ tension declined to zero) was ~0.60 mm at 37°C. This is clearly above the radius of the bundles in our study, which was ~0.25 mm. It seems therefore unlikely that the small differences in bundle weight and consequent differences in oxygen diffusion would account for the observed changes in contractile properties.

The pattern of recovery after cessation of fluorinated and nonfluorinated steroids was not studied before in animal models. In the present study, 2 mo after discontinuation of triamcinolone, residual type IIb fiber atrophy was still present. Apparently, the degree of decrease in type IIb fiber size was too small to result in pronounced changes in contractile properties. In contrast, no histologic or functional changes were observed after complete withdrawal of prednisolone. The latter finding is in agreement with the small changes associated with acute treatment.

In most of the abovementioned animal studies high or even massive doses of steroids were administered. Consequently, acute atrophy and rhabdomyolysis might have been induced in these studies. This has also been observed in patients with acute airway obstruction, treated with massive doses of corticosteroids (8, 24–26). This pattern of acute generalized muscle atrophy and weakness, however, is distinctly different from the clinical pattern observed during chronic steroid myopathy. The latter occurs after prolonged administration of steroids, and is characterized by the insidious onset of muscle weakness predominantly localized in the proximal extremities (9).

In patients, recovery of both types of steroid-induced muscu- lar changes appears to be slow. After acute steroid myopathy, electromyographic examination of peripheral skeletal muscle still showed abnormalities 6 mo after complete withdrawal of steroid treatment (8). Similarly, in chronic steroid myopathy recovery of respiratory and peripheral muscle strength may take up to 3 to 6 mo (9, 10). This was confirmed in a recent prospective study by Weiner and coworkers (11). These investigators studied the effects of prednisone 1 to 1.5 mg/kg/d administered during 8 wk in patients with no underlying lung diseases. Treatment with prednisone clearly reduced inspiratory muscle strength and endurance. Subsequent tapering down of the dosage to complete withdrawal within 6 wk resulted in a gradual improvement of respiratory muscle function. This improvement was continued even 6 mo after complete withdrawal (11). Histologic data with regard to morphologic features in respiratory or peripheral skeletal muscles several months after discontinuation of steroids in patients are not available.

Steroid-induced changes in structure and function of the diaphragm are of specific clinical interest in patients with COPD. Treatment with systemic steroids is frequently indicated in these patients. Diaphragm function in these patients, however, is compromised by malnutrition (27, 28), hyperinflation (29), disturbances in blood gases (30), and cardiac failure (31, 32). In addition, age itself may affect respiratory muscle strength (33). It may be expected that the effects of steroids on the already compromised diaphragm may be more pronounced (34). The unfavorable effects of steroids on the diaphragm might then be enhanced if a subsequent course of steroids would be administered before the diaphragm has recovered completely from the previous treatment with steroids. Physical inactivity, which may accompany recurrent exacerbations, leads to an increase in the number of steroid receptors on skeletal muscle (35). If a similar mechanism would also occur in the diaphragm and the other respiratory muscles, this would enhance the steroid-induced effects even further.

In conclusion, the present study shows that discontinuation of triamcinolone treatment for a period of 2 mo does not allow the diaphragm to regain its normal structure completely. The acute effects of prednisolone on the diaphragm, however, appear to have resolved at the time of examination.

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References