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Rat diaphragm contractility and histopathology are affected differently by low dose treatment with methylprednisolone and deflazacort

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ABSTRACT: The extent to which treatment with low doses of the nonfluorinated steroid methylprednisolone affects diaphragm contractility and morphology is unknown. In the present study, we compared the effects of equipotent doses of methylprednisolone and deflazacort, an oxazoline derivate of prednisolone with less systemic side-effects on bone structure and carbohydrate metabolism.

Twenty six male adult rats were randomized to receive daily saline (control), methylprednisolone 0.4 mg·kg^{-1} or deflazacort 0.5 mg·kg^{-1} *i.m.* Contractile properties and histopathology were measured after a 6 week treatment period.

During treatment, body weight increased in control and methylprednisolone-treated animals, but decreased by 4.2±1.1% (mean±SD) in the deflazacort group. Similarly, diaphragm mass in the deflazacort group was decreased compared to control and methylprednisolone groups. Twitch tension and twitch characteristics of isolated diaphragm bundles were similar in the three groups. Maximal tetanic tension was decreased in the deflazacort group. The force-frequency curve of the deflazacort bundles shifted downwards compared to control. Fatigue occurring during this protocol was greatest in the methylprednisolone- and deflazacort-treated animals. Microscopic examination revealed no gross abnormalities in the three groups. Histochemical analysis after staining for myosin adenosine triphosphatase (ATP-ase) showed that in the deflazacort group cross-sectional area of type I, IIa and IIb fibres were decreased.

We conclude that low doses of methylprednisolone caused subtle and negligible changes in rat diaphragm contractile properties without affecting fibre dimensions, while deflazacort at an equipotent dose induced generalized fibre atrophy and changes in diaphragm contractility.
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Recent animal studies have demonstrated that administration of corticosteroids affects diaphragm structure and function [1–6]. Fluorinated steroids (*e.g.* triamcinolone 0.5–3 mg·kg^{-1} *i.m.* and dexamethasone 1–4 mg·kg^{-1} *i.m.* administered during 1–7 weeks) caused severe loss of body weight and muscle mass up to 40%. This was accompanied by type IIb fibre atrophy in the diaphragm as well as in peripheral skeletal muscles [1–5]. These morphological alterations were reflected in changes in the physiological properties of isolated diaphragm bundles, which showed a shift towards the contractile profile of slow muscle. In contrast, the nonfluorinated steroid prednisolone, administered in a dose of 5 mg·kg^{-1} *i.m.* daily during 4 weeks, did not cause loss of body mass [4]. Histological examination revealed myogenic changes in the diaphragm, including greater than normal variation of the diameter of all fibre types, scattered necrotic fibres, excess nuclei, and an increased amount of connective tissue, but without fibre atrophy. In line with these alterations, an increased tendency towards fatigue was observed when examining the contractile characteristics of the diaphragm bundles.

In the above mentioned studies, moderate to high doses of corticosteroids were administered. It remains unclear whether lower doses may cause alterations in animal diaphragm. In patients with chronic obstructive pulmonary disease (COPD) there is evidence to suggest that treatment with low doses of methylprednisolone (MP) affects respiratory and peripheral muscle force [7].

The aim of the present study, therefore, was to investigate whether in rats contractile properties of the diaphragm (DIA) and morphological characteristics of DIA and gastrocnemius muscle (GA) were affected by treatment with a low dose (0.4 mg·kg^{-1} *i.m.* daily) of MP, theoretically comparable to doses commonly used for therapeutic purposes in humans. In addition, we studied

the effects of an equipotent dose of deflazacort (DF), 0.5 mg·kg^{-1} *i.m.* daily, an oxazoline derivate of prednisolone with similar anti-inflammatory and immunosuppressive activity [8], but less side-effects on bone structure and carbohydrate metabolism compared to prednisolone [9, 10].

Methods

Study design, animals and treatment

Twenty six adult male Wistar rats, aged 18 weeks, weighing 350–400 g, were randomized into one of three treatment groups: 1) control (C), (n=10), saline 0.05 ml daily *i.m.*; 2) methylprednisolone (MP), (n=10), 0.4 mg·kg^{-1} daily *i.m.*; and 3) deflazacort (DF), (n=6), 0.5 mg·kg^{-1} daily *i.m.*

The dosages of MP and DF were chosen in order to administer an equivalent anti-inflammatory dose to the animals. During 6 weeks, the animals were injected daily in the left hind limb. They were fed *ad libitum.* All animals were weighed thrice weekly. After the treatment period, contractile properties of DIA and histological and histochemical characteristics of DIA and GA were examined.

Contractile properties

Twenty four hours after the last injection, rats were anaesthetized with sodium pentobarbital (Nembutal), 60 mg·kg^{-1} *i.p.* Animals were tracheotomized and a tracheal cannula (polyethylene tubing PE-200) was inserted. They were mechanically ventilated with an O_2 enriched gas mixture (Harvard pump respirator, South Natick, Ma, USA).

The diaphragm was quickly removed through a laparotomy, and immediately immersed in a cooled, oxygenated Krebs solution containing (in mM·L^{-1}): NaCl 137, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 12, glucose 6.5. To avoid regional differences in cross-sectional area or fibre type proportions as much as possible, two small rectangular bundles from the middle part of the lateral costal region were obtained by dissection parallel to the long axis of the fibres. 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, maintained at 37°C and perfused with a 95% O_2 and 5% CO_2 mixture. One end of the bundle was tied to a rigid support, while the other was fastened to an isometric force transducer mounted to a micrometer. The muscle was placed in between two large platinum stimulating electrodes.

The bundles were placed at their optimal length (L_o), defined as the length at which peak twitch force was obtained. This was followed by a 15 min thermoequilibration period. Stimulations were delivered through a Harvard 50-5016 stimulator (Edenbridge, Kent, UK), connected in series to a power amplifier from power one model HS24-4.8. 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 supramaximal stimulation. This voltage was subsequently used during all stimulations. Isometric force was measured by means of a Maywood force transducer (Maywood Ltd, Hampshire, UK). The signal was amplified and recorded on computer *via* analogue to digital conversion (DT2801-A) using Labdat software (Labdat/Anadat, RHT-InfoDat, Montreal, Canada). Signal analysis was performed with Anadat.

The following measurements were carried out: 1) Twitch characteristics - two twitches were recorded at L_o to determine maximal twitch tension (P_t), time to peak tension (TPT) and half-relaxation time (1/2RT). Average values were used for further analysis; 2) Maximal tetanic tension (P_o): bundles were stimulated twice tetanically at 160 Hz, during 250 ms, in order to obtain a clear plateau in force generation [11, 12]; 3) Force-frequency curve - bundles were stimulated at the following frequencies: 25, 160, 50, 160, 80, 160, 120 and 160 Hz. Each stimulus was separated by a 2 min interval; and 4) Fatigue properties: fatigability was assessed in two different ways. Firstly, force output at 160 Hz after each stimulus frequency during the force-frequency curve was measured. Secondly, bundles were fatigued by means of 330 ms stimulations repeated every 2 s at 25 Hz during 5 min (modified after Burke *et al.* [13]).

After these measurements, each muscle bundle was removed from the bath and its length, thickness and width was measured at L_o. 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, 14]. Twitch-to-tetanus ratio (P_t/P_o) was calculated for each muscle bundle.

Finally, the remaining diaphragmatic tissue was trimmed, blotted and weighed. Parasternal muscles (including sternum and chondral parts of the ribs), the right medial scalene muscle, and the gastrocnemius and soleus muscles from the right hind limb were dissected, trimmed, blotted and weighed, as well as the two adrenal glands.

Histological and histochemical procedures

Muscle strips obtained from the costal region of the diaphragm and from the mid-belly of the GA of the right hind limb were prepared for histopathological examination. Muscle samples were put into "tissue glue" (Tissue-Tek, Elkhard, IN, USA) on a cork holder, with the muscle fibres orientated 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 N_2. Serial cross-sections were cut at 10 μm thickness with a cryostat kept at -20°C, parallel to the cork. Sections of each DIA and GA were taken for routine haematoxylin and eosin staining.

The other serial sections were stained for myofibrillar adenosine triphosphatase (ATPase) after alkaline (pH 9.3) and acid (pH 4.5) preincubation. Muscle fibres were classified as type I (slow-twitch), type IIa (fast-twitch

oxidative), or type IIb (fast-twitch glycolytic) fibres [15]. Slides preincubated at pH 4.5 offered the best separation of different fibre types, and were subsequently used for further analysis.

Morphometric examination was carried out with a Leitz microscope (Wetzlar, Germany) at ×25 magnification, connected to a digitizing board (Numonics 2207; Montgomeryville, PA, USA). Areas in which fibre orientation was not transverse to the long axis of the fibre were not analysed. Boundaries of individual muscle fibres were delineated, and fibre CSA was determined from the number of pixels within the outlined fibre. At least 150 fibres of each DIA and deep (red) part of the GA were used to calculate mean CSA of all fibre types.

Data analysis

Two diaphragm bundles were obtained from each animal. Since variation between animals was as large as within animals, all bundles were used for statistical analysis as independent cases. Data from the different treatment groups were compared, using two-way analysis of variance. Differences between means were assessed using Duncan's multiple range test. A chi-squared likelihood ratio test was used to detect differences in the distribution of maximal tetanic tensions between the three treatment groups. Statistical significance was set at a value of p less than 0.05. All analyses were performed using the statistical Package for the Social Sciences (SPSS)/PC+ package [16]. Means±SD are represented in text, tables and figures, unless otherwise specified.

Results

Body, muscle and adrenal weight

Body weight at the start of the study was not different between the three groups (fig. 1). During the treatment period, weight increased by 10.9±1.7% in C, and by 6.6±1.4% in MP-treated animals, whereas it decreased by 4.2±1.1% in the DF group (p<0.05 compared to C).

Muscle masses in the DF group were significantly reduced compared to C (DIA, scalenus and GA) and MP (scalenus and GA) (table 1). No changes occurred in the parasternal muscles, probably because the major part of the weight consisted of the ribs. In addition, the mass of the adrenal glands was decreased compared to C and MP. The ratio of DIA mass to total body weight was similar among the three groups (C 0.16±0.03%, MP: 0.15±0.02%, DF: 0.17±0.02%).

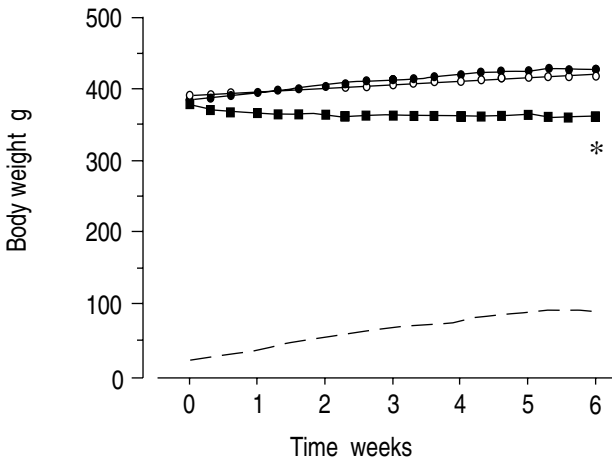

Fig. 1. – Body weight *vs* time during the 6 week treatment period. —●— : control; —○— : methylprednisolone; —■— : deflazacort; ——— : pooled SD. *: deflazacort compared to control; p<0.05 for all data points, except for time 0.

Diaphragmatic contractile properties

Bundle dimensions. Bundle dimensions are shown in table 2. The DIA bundles in the DF group tended to be smaller, but no significant differences were found compared to the other groups.

Twitch characteristics and maximal tetanic tension. The data of the contractile properties of the DIA are summarized in table 3. Pt, TPT, 1/2RT and Pt/Po were similar in the three groups. Po (expressed both as absolute values (kg) and corrected per CSA ($kg \cdot cm^{-2}$)) was decreased in the DF group (p<0.05). When the values were divided in two classes (Po <2.0 and >2.0 $kg \cdot cm^{-2}$), more bundles in the DF-treated animals generated tetanic tensions below 2.0 $kg \cdot cm^{-2}$ (37%, compared to 10% in the other two groups; χ^2=8.712, p<0.05).

Force-frequency curve. The response of diaphragm strips to increasing stimulus frequencies is shown in figure 2. When expressed in absolute values ($kg \cdot cm^{-2}$), a significant

Table 1. – Muscle and adrenal masses

Treatment	Diaphragm (DIA) mg	Parasternals mg	Scalenus mg	Gastrocnemius (GA) mg	Soleus mg	Adrenal glands mg
Control (C)	663 (67)	3233 (311)	578 (56)	2238 (179)	163 (17)	45 (3)
Methylprednisolone (MP)	626 (40)	3171 (167)	580 (53)	2053 (137)*	165 (12)	42 (6)*
Deflazacort (DF)	597 (39)*	3176 (299)	504 (46)*#	1872 (132)*#	155 (15)	29 (7)*#

Data are presented as mean, and SD in parenthesis. *: p<0.05 compared to control; #: p<0.05 compared to methylprednisolone.

Table 2. – Diaphragm bundle dimensions

Treatment	Length mm	Thickness mm	Width mm	Weight mg
Control	21.2 (1.9)	0.51 (0.13)	1.62 (0.20)	28.5 (6.2)
Methyl-prednisolone	20.3 (2.2)	0.53 (0.12)	1.62 (0.24)	27.1 (5.3)
Deflazacort	19.8 (2.5)	0.46 (0.11)	1.60 (0.28)	25.2 (7.1)

Data are presented as mean.

downward shift of the DF bundles at 50, 80 and 120 Hz was observed ($p<0.05$) (fig. 2a). A nonsignificant decrease in force generation was observed in the MP bundles. When expressed as a percentage of the 160 Hz stimulations before and after each stimulus frequency, no differences were observed among the three groups (fig. 2b).

Fatigue properties. In all groups, force generated at 160 Hz decreased during the force-frequency stimulation procedure. In the C, MP and DF groups, the values for percentage decrease relative to initial P_o were 8.0±1.9, 15.4 ±2.6, and 19.6±5.8 (mean±SE), respectively. This decrease in generated force in the MP and DF groups was significantly higher than in C ($p<0.05$.)

During the low-frequency fatigue run, stress generation in absolute values ($kg \cdot cm^{-2}$) was similar in all groups (fig. 3a). When expressed as a percentage of initial stresses, this ratio (fatigue/fresh, a dimensionless index) was higher in the DF bundles (fig. 3b), ($p<0.05$).

Histology and histochemistry

Histological examination of haematoxylin and eosin stained slides showed a normal muscular pattern of DIA and GA in the three groups.

Analysis of ATPase stainings showed that fibre type distribution was not changed by the different treatments. For groups taken together, the DIA consisted of 40.4± 5.3% type I fibres, 29.7±6.1% type IIa fibres, and 29.9± 4.2% type IIb fibres. The red (deeper) part of the GA consisted of 30.1±4.5% type I fibres, 25.3±5.1% type IIa fibres, and 44.6±6.5% type IIb fibres. Dimensions of the three fibre types in both DIA and GA were similar in C and MP groups (fig. 4). Treatment with DF, however, was accompanied by a decrease of diaphragmatic type I, IIa, and IIb fibre cross sectional area ($p<0.05$ compared to the other groups) (fig. 4a). Similar changes were observed in the GA (fig. 4b).

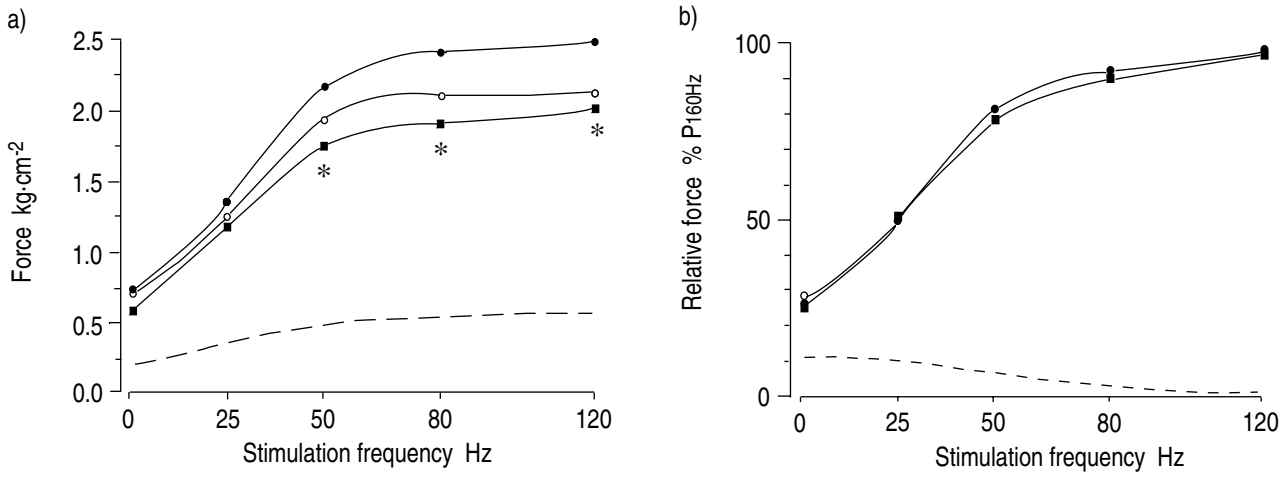

Fig. 2. – a) Force-frequency curve of diaphragm bundles. b) Force-frequency curve of diaphragm bundles, expressed as percentage of interspersed 160 Hz stimulations. —●— : control; —○— : methylprednisolone; —■— : deflazacort; ——: pooled SD; *: $p<0.05$ deflazacort compared to control.

Table 3. – Diaphragmatic contractile properties

Treatment	P_t $kg \cdot cm^{-2}$	TPT ms	1/2RT ms	P_o kg	P_o $kg \cdot cm^{-2}$	P_t/P_o
Control	0.72 (0.19)	19 (2)	24 (2)	0.033 (0.009)	2.57 (0.45)	0.28 (0.04)
Methylprednisolone	0.70 (0.15)	20 (2)	24 (4)	0.031 (0.009)	2.46 (0.33)	0.28 (0.05)
Deflazacort	0.62 (0.16)	19 (2)	25 (2)	0.028 (0.008)*	2.32 (0.45)*	0.27 (0.06)

Data are presented as mean (SD). P_t: twitch tension; TPT: time to peak tension; 1/2RT: half-relaxation time; P_o: tetanic tension; P_t/P_o: twitch/tetanic ratio. *: $p<0.05$ compared to control.

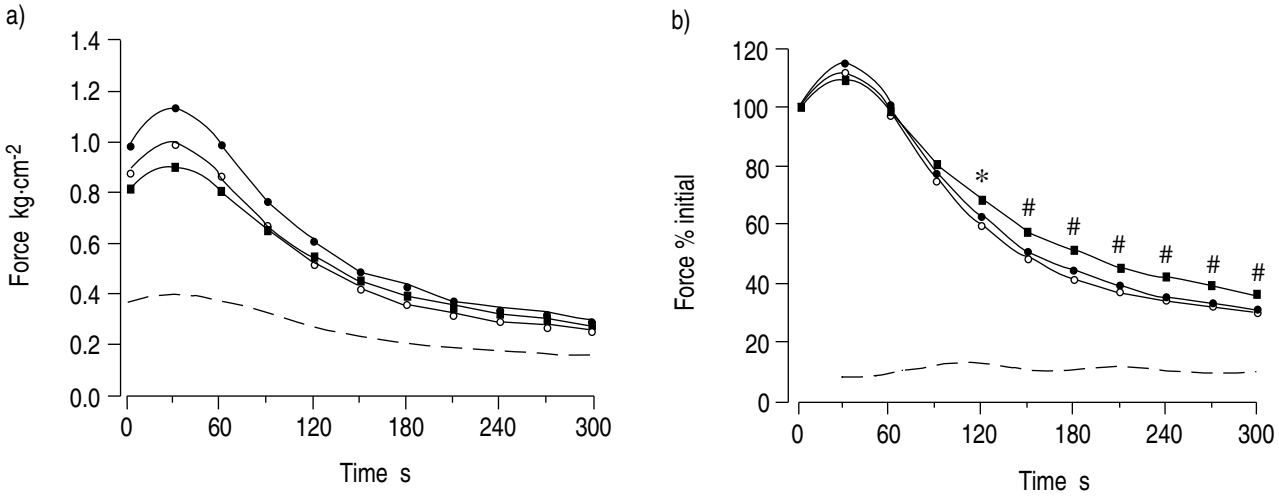

Fig. 3. – a) Fatigue curve of diaphragm bundles. b) Fatigue curve of diaphragm bundles, expressed as a percentage of initial value. —●— : control; —○— : methylprednisolone; —■— : deflazacort; ——: pooled SD. *: deflazacort compared to control; $p<0.05$; #: $p<0.05$ deflazacort compared to control and methylprednisolone.

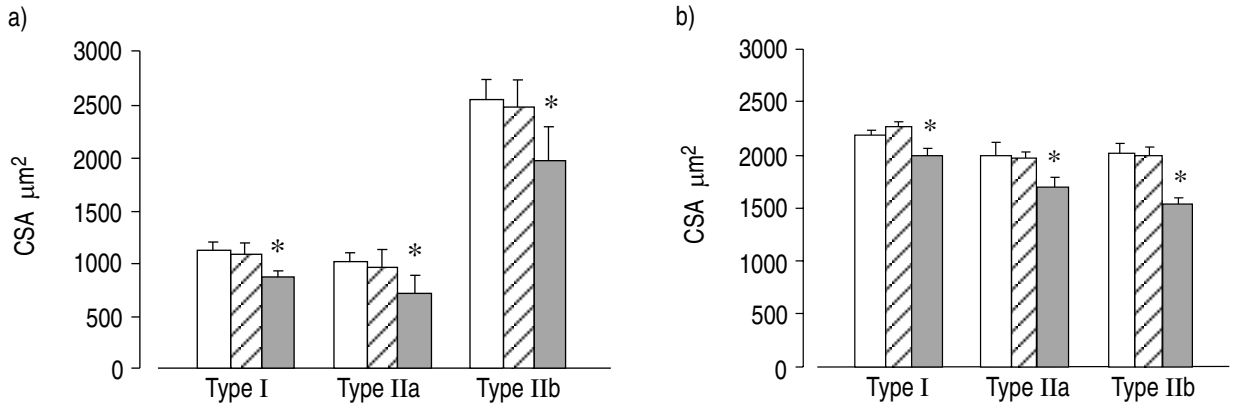

Fig. 4. – a) Fibre cross-sectional area in diaphragm; and b) gastrocnemius. □ : control; ▨ : methylprednisolone; ■ : deflazacort. *: $p<0.05$ deflazacort compared to control and methylprednisolone.

Discussion

The present study shows that low dose MP administered during 6 weeks hardly affects the contractile properties of the rat diaphragm, if at all. Only subtle changes were found, such as slightly increased fatigability during high-frequency stimulation and a nonsignificant downward shift of the force-frequency curve. MP did not alter diaphragm or gastrocnemius morphology. Treatment with DF, however, was associated with more pronounced changes in the contractile characteristics of the diaphragm. These alterations consisted of a decrease in maximal tetanic tension, a downward shift of the force-frequency curve and decreased maximal tension generation during this protocol. In contrast to MP, DF caused mild atrophy of all fibre types, both in the diaphragm and in the gastrocnemius. In line with these findings, treatment with DF was accompanied by slight loss of body and muscle mass.

Interpretation of the results

Side-effects, such as changes in bone and carbohydrate metabolism, associated with the use of DF appear to be less than with prednisolone [17–20]. Comparisons between DF and MP have not been made to our knowledge, nor have animal studies been published regarding the effects of DF on respiratory and peripheral skeletal muscles.

One early study has been published regarding the effects of MP on skeletal muscles in dogs [21]. In this study, the effects of (among other steroids) prednisolone 25 mg *i.m.* and MP 20 mg *i.m.* daily during 5 weeks were compared. Body weight decreased by ±10% in both groups. Prednisolone caused mild atrophy (not specified for different fibre types) in the quadriceps muscle, whereas MP did not change fibre size. This is in line with the morphological findings in the present study.

The pattern of fibre atrophy associated with DF in the

present study is clearly different from the selective type IIb fibre atrophy caused by fluorinated steroids [1–5]. Instead, this pattern of generalized fibre atrophy resembles the changes accompanying loss of body and diaphragm mass induced by nutritional depletion [5, 22, 23]. In a previous study, we showed that 44% loss of diaphragm mass induced by nutritional restriction, was accompanied by a decrease in CSA of type I, IIa, and IIb fibres of 31, 35 and 52%, respectively [5]. In comparison, treatment with DF in the present study resulted in a loss of diaphragm mass of 10%, associated with decreases in CSA of 23, 31 and 23%, respectively. These changes indicate that diaphragm fibre CSA is very sensitive to changes in body mass.

The changes in contractile properties induced by DF, however, were different from those induced by nutritional depletion. The latter is associated with a shift towards the functional profile of a slow muscle with increased half-relaxation time and an upward shift of the force-frequency curve [5]. In contrast, the decrease in maximal tetanic tension and the downward shift of the force-frequency curve in the DF group suggest that, besides atrophy, DF also caused a decrease in force generation (myopathy). These findings are similar to those found after treatment with prednisolone, 5 $mg \cdot kg^{-1}$ *i.m.* daily during 4 weeks [4]. The increased ratio fatigued/fresh in the DF bundles at the end of the fatigue run (fig. 3b) may be due to the initial decrease of force during the force-frequency protocol. This resulted in a lower force at the start of the fatigue protocol, but the eventual force generation was similar to the other groups.

It might be argued that variation in bundle dimensions may be responsible for the alterations in contractile properties, by changes in diffusion of substrates and oxygen. SEGAL and FAULKNER [24] studied the influence of muscle thickness and incubation temperature on the contractile properties of rat skeletal muscles. They showed that the critical radius for O_2 diffusion (*i.e.* the distance into a muscle at which O_2 tension declined to zero) was ~0.6 mm at 37°C. This is clearly above the radius of the bundles in our study, which varied from 0.23 (DF group) to 0.27 mm (MP group). If the reduced dimensions of the bundles in the DF group would, nevertheless, alter force generation, these bundles would be expected to be more resistant to fatigue compared to larger bundles, and not less resistant as was observed.

Mechanisms of steroid-induced changes in striated muscle

The mechanism of steroid-induced changes in striated muscle is complex and in part unknown. Protein wasting is due to a reduction in protein synthesis and to an increase in intracellular proteolysis. Inhibition of protein synthesis occurs primarily in type II muscle fibres, mainly by affecting the activity of factors involved in peptide initiation [25]. Increased muscle cytoplasmatic protease activity, associated with steroid treatment, results in myofibrillar destruction [26]. Carbohydrate metabolism within muscle fibres is also affected by corticosteroids. Muscle glycogen content is increased by increased muscle glycogen synthetase activity and decreased glycogen utilization [27]. Mitochondrial alterations in muscles affected by steroids have suggested that impaired oxidative respiration may also be an important factor in pathogenesis [28].

There is no clear explanation for the difference in response of striated muscle to different subgroups of glucocorticosteroids, such as fluorinated and nonfluorinated steroids. A difference in binding of triamcinolone, dexamethasone, and cortisol to cytoplasmatic proteins may play a role [26]. In the case of DF, another mechanism has been proposed, associated with its chemical structure. DF is characterized by an oxazoline group at C-17, which is believed to sterically hinder reactivity of the side chain [29]. This results in a reduced lipid solubility compared to prednisolone. Therefore, it has been postulated that DF is less extractable from serum by cells with a limited degree of exposure to the circulation, such as osteoblasts, accounting for its favourable effects on bone metabolism [8]. Such a mechanism, however, may be expected to protect organs with an abundant blood supply, such as the dia-phragm, less, as suggested by the findings in the present study. ASSANDRI *et al.* [30] showed that the concentration of deflazacort in striated muscle in rats after oral administration of 5 $mg \cdot kg^{-1}$ deflazacort was low. Data on the diaphragm were not presented.

Potential clinical significance of steroid-induced alterations

In previous studies, the doses of steroids administered were high compared to current use in clinical practice. In the present study, MP and DF were administered in low doses, comparable with the dose sometimes administered to patients with asthma or COPD. In these patients, diaphragm function is compromized by malnutrition [31, 32], hyperinflation [33], disturbances in blood gases [34], and cardiac failure [35, 36]. It may be speculated that the effects of steroids on already impaired diaphragmatic function, as in patients with COPD, might be more pronounced.

ZANOTTI *et al.* [37] showed that respiratory muscle strength was similar in patients with COPD treated with DF, 15 mg daily for at least four consecutive months, compared to COPD patients matched for age and severity of airflow obstruction. Firm conclusions from this study, however, are precluded since it was performed in a retrospective and cross-sectional design.

In a recent study, eight out of 21 consecutive patients who were admitted to hospital with acute exacerbations of COPD or asthma, suffered from generalized muscle weakness [7]. In seven of these eight patients the average daily dose of methylprednisolone during the last 6 months exceeded 4 mg, whilst this was the case in only three of the 13 patients with normal muscle strength. Although the therapeutic efficacy of corticosteroids in COPD is at least controversial [38], this observation indicates the potential effect of low-dose treatment with methylprednisolone on muscle strength.

In summary, this study shows that low dose of MP cause only subtle changes in contractile properties of rat diaphragm, without alterations in morphology. In contrast, more pronounced abnormalities in contractile

properties are observed in the DF treated bundles, which are accompanied by generalized muscle fibre atrophy in both diaphragm and gastrocnemius.

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