Corticosteroid-induced myopathy of the animal diaphragm

Pathophysiology and interventions

Corticosteroid-induced myopathy of the animal diaphragm

Pathophysiology and interventions

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op maandag 14 september 1998
des namiddags om 1.30 precies

door

Roland Hubertus Henrikus van Balkom

geboren op 9 juni 1966 te Valkenswaard

Contents

CHAPTER 1

Introduction and outlines of the study

Introduction

Treatment with corticosteroids (CS) is frequently indicated in patients with obstructive pulmonary disease (asthma and COPD) Among the well-known side-effects of these drugs are myopathy and atrophy of skeletal muscles Continuously active muscles were believed to be relatively spared from this complication Recent animal studies (3,4) and clinical observations (2), however, have shown that the diaphragm may be involved in this process as well

The mechanisms by which CS cause myopathy are in part unknown Protein wasting due to a reduction in protein synthesis and an increase in intracellular proteolysis is believed to be an important factor in CS-induced myopathy (9) Animal studies were performed to obtain insight in the underlying mechanisms responsible for the corticosteroid-induced muscle impairment Fiber atrophy (5), changes in myosin heavy chain composition (10), in energy substrate and enzyme activities (8) have been reported So far, only high doses of steroids were administered for short periods This resembles acute steroid myopathy whereas chronic steroid myopathy is more often found in clinical practice Furthermore, there is an important difference in the effects of fluorinated and non-fluorinated steroids on striated muscles (3) In contrast to non-fluorinated steroids, fluorinated steroids cause severe body weight loss and muscle atrophy

Respiratory muscle function in patients with severe COPD has been shown to be affected by hyperinflation, hypoxemia, hypercapnia, malnutrition, chronic heart failure and physical inactivity This is of interest since COPD patients are occasionally treated with corticosteroids It is unknown, however, to what extent normal and emphysematous diaphragm structure and function are affected by clinically relevant dosages of a non-fluorinated CS We were also interested in the antagonistic potency of anabolic steroids on CS-induced changes in the diaphragm muscle Isotonic and isometric contractile properties, morphological and biochemical parameters, and structure and function of neuromuscular junctions were evaluated to obtain inside in the cellular adaptations to the drugs tested

Outlines of the study

Chapter 2 is a review of literature describing the differences between chronic and acute steroid myopathy and the clinical presentation of steroid myopathy in humans

The aim of Chapter 3 was to evaluate if different CS treatment regimens caused different changes in rat diaphragm muscle structure and function Alternate-day CS therapy is believed to reduce side effects without loss of efficacy since the anti-

inflammatory potency persists longer than the metabolic side-effects (1,6) In contrast, recovery of acute steroid myopathy, caused by short-time high-dose steroid administration, appears to take several months (7)

In Chapter 4 the effects of a non-fluorinated steroid, in a low clinically relevant dose, on rat diaphragm structure and function were evaluated. Rats were treated for six months with methylprednisolone (MP) in a dose comparable with 10-15 mg per day in humans

In Chapter 5 the effects of CS on diaphragm muscle neuromuscular junctions (NMJ) were studied NMJ morphology was assessed using a three-color immunocytochemical technique combined with confocal microscopy. Measurement of neuromuscular transmission fatigue was used to evaluate function of the NMJs.

The effects of CS on diaphragm muscle power and endurance were studied in Chapter 6 These measurements were determined from diaphragm isotonic contractile properties, using a load clamp technique Power was calculated as a product of velocity of shortening and force. The time at which power declined to zero was defined as the endurance time of the muscle

The aim of Chapter 7 was to evaluate the reversibility of MP-induced alterations in diaphragm function and structure by addition of the anabolic steroid nandrolone decanoate (ND). Both ND and MP were administered in clinically relevant dosages.

The interactive effects of emphysema and MP treatment are described in Chapter 8 In addition, in this chapter the antagonistic efficacy of ND on MP-induced changes in the emphysematous diaphragm were studied

References

1   Axelrod, L Glucocorticoid therapy Med Baltimore 55 39-65, 1976
2   Decramer, M , L M Lacquet, R Fagard, and P Rogiers. Corticosteroids contribute to muscle weakness in chronic airflow obstruction Am J Respir Crit Care Med 150 11-16, 1994
3   Dekhuijzen, P N R and M Decramer Steroid-induced myopathy and its significance to respiratory disease a known disease rediscovered Eur Respir J 5 997-1003, 1992
4   Dekhuijzen, P N R , G Gayan-Ramirez, V de Bock, R Dom, and M Decramer Triamcinolone and prednisolone affect contractile properties and histopathology of rat diaphragm differently J Clin Invest 92 1534-1542, 1993
5   Ferguson, G T , C G Irvin, and R M Cherniack Effects of corticosteroids on respiratory muscle histopathology Am Rev Respir Dis 142 1047-1052, 1990
6   Harter, J G , W J Reddy, and G W Thorn Studies on an intermittent corticosteroid dosage regimen N Eng J Med 269 591, 1963
7   Knox, A J , B H Mascie-Taylor, and M F Muers Acute hydrocortisone myopathy in acute severe asthma Thorax 41 411-412, 1986

8   Lieu, F K , S K Powers, R A Herb, D Criswell, D Martin, C Wood, W Stainsby, and C L Chen Exercise and glucocorticoid-induced diaphragmatic myopathy J Appl Physiol 75 763-771, 1993

9   Ruff, R L Endocrine myopathies Myology, edited by A G Engel and B Q Banker McGraw-Hill Book Company, 1986, 1871-1906

10   Seene, T Turnover of skeletal muscle contractile proteins in the glucocorticoid myopathy J Steroid Biochem 50 1-4, 1994

CHAPTER 2

Corticosteroid-induced myopathy of respiratory muscle, a review of literature

RHH van Balkom, HFM van der Heijden, CLA van Herwaarden, and
P.N.R. Dekhuijzen

Abstract

Corticosteroids may cause myopathy of both skeletal and respiratory muscles This is of specific clinical importance in patients with chronic obstructive pulmonary disease (COPD), who already have impaired respiratory muscle function After treatment with fluorinated steroids, side-effects occur more frequently and are worse compared to treatment with non-fluorinated steroids Acute myopathy and atrophy are caused by short-term high-dose corticosteroid administration, resulting in rhabdomyolysis, diffuse muscle weakness and severe dyspnoea In contrast, chronic myopathy occurs after prolonged treatment with corticosteroids, and results in proximal muscle weakness and type IIb fiber atrophy The pathophysiology of steroid myopathy is unknown, but reduction in protein synthesis and increased glycogen accumulation may play a major role Animal models have demonstrated weakening of the diaphragm and a decrease in body and diaphragm mass after corticosteroid administration In humans, a reduction in respiratory and peripheral muscle strength, an elevation of urinary creatine excretion and selective type IIb fiber atrophy may be observed Treatment of corticosteroid-induced myopathy consists of tapering the dose of steroids or switching to non-fluorinated steroids

Introduction

Treatment with systemic corticosteroids is frequently indicated in severe asthma, during exacerbations in chronic obstructive pulmonary disease (COPD), and in various other diseases In 1932, Cushing reported an association between endogenous corticosteroid excess and muscle weakness (1) Exogenously administered corticosteroids have been used since the 1940s and are associated with similar myogenic changes, affecting striated muscles of the limbs This especially occurs when fluorinated steroids such as triamcinolone and dexamethasone are used However, non-fluorinated steroids such as (methyl-) prednisolone and hydrocortisone are also associated with the occurrence of muscle weakness (2)

At first it was believed that only skeletal muscles were affected, as opposed to the respiratory muscles, which were thought to be spared because of their continuous activity Recent clinical reports and animal studies, however, showed changes in respiratory muscles induced by steroids This may occur in patients with pulmonary diseases, as well as in patients using systemic steroids for other disorders Steroid myopathy is of specific clinical importance in patients with COPD, since respiratory muscle function in these patients has already been affected by hyperinflation, hypoxaemia, hypercapnia,

malnutrition, chronic heart failure and physical inactivity.

The incidence of muscle weakness associated with chronic steroid use varies from 2.5 up to 21% (2). This may be an underestimation, since hip flexor weakness occurred in more than 50% of asthmatics using prednisone >40 mg· day^{-1} (3). In a series of 216 patients with brain tumors, clinically apparent steroid myopathy occurred in 10.6% after treatment with dexamethasone (4). This may also be an underestimation since EMG-examination was not performed. These findings suggest that myopathy is not an exceptional complication in systemic steroid therapy.

In this review steroid-induced abnormalities in both respiratory and peripheral skeletal muscles are discussed.

Clinical patterns

Two types of steroid-induced myopathy can be distinguished depending on height and duration of steroid treatment: acute and chronic steroid myopathy (5).

Acute myopathy
Short-term high-dose steroid administration as given during exacerbation in patients with asthma and COPD may cause acute myopathy. This complication has been observed in patients with acute severe asthma treated with hydrocortisone 3-4 g· day^{-1} IV (6,7). However, acute myopathy has also been reported in asthmatics who never received more than 1 g of intravenous hydrocortisone daily (8,9).

Pathological features include rhabdomyolysis, marked elevation of creatine kinase (CK), and focal or diffuse necrosis without predilection for a specific fiber type (7-12). Diffuse (proximal and distal) muscle weakness is the main clinical feature. In contrast to peripheral neuropathy, no changes in deep tendon reflexes or in the sensory component were observed. Respiratory muscle involvement may occur, resulting in severe dyspnoea and difficulty during weaning after mechanical ventilation (9). Recovery appears to be slow; electromyographic examination may show evidence of mild myopathy 6 months after clinical recovery (8).

Chronic myopathy
The classical pattern of steroid myopathy consists of proximal muscle weakness, occurring after prolonged treatment with corticosteroids (13-15). There is a wide variation in duration until symptoms of steroid myopathy appear and the correlation with either the total steroid dose or duration of therapy is poor. However, several authors reported muscle weakness after treatment with prednisolone 30-40 mg· day^{-1} (4,14-16).

Symptoms of this complication vary from complaining of muscle weakness with no objective fall in strength to inability to raise upper or lower limbs. Laboratory investigation shows normal or slightly elevated CK and increased urinary creatine excretion, while myoglobinuria and rhabdomyolysis are absent.

Biopsies from peripheral muscle show selective type IIb fiber atrophy (2), in contrast to the generalized fiber atrophy associated with acute steroid myopathy. Fiber type classification and distribution are summerized in table 1. The recovery of weakness after chronic use of steroids may take weeks or months (2).

Table 1. Fiber type classification and distribution

Histochemical type	Type I	Type IIa	Type IIx/b
Physiological nomenclature	slow twitch	fast twitch	fast twitch
	fatigue resistant	fatigue resistant	fatigue sensitive
Anatomical appearance	intermediate	red	white
Biochemical properties	oxidative	oxidative glycolytic	glycolytic
Fiber distribution			
Biceps & vastus lateralis	± 33%	± 33%	± 33%
Diaphragm	± 55%	± 21%	± 24%

Aetiology and pathogenesis

To improve the anti-inflammatory effect of the conventional glucocorticosteroids, a fluorine atom and a methyl group were added, resulting in the so-called fluorinated steroids. The fluorine atom increases both glucocorticoid and mineralocorticoid activity, while the methyl group decreases sodium-retaining properties. Fluorinated steroids such as triamcinolone and dexamethasone are more potent than non-fluorinated steroids. Similarly, steroidal side-effects occur more frequently and are worse after treatment with fluorinated steroids. The cause of these differences between the two steroid groups is unknown, as is the exact cause of steroid myopathy. Several mechanisms have been suggested (2).

Effect on proteins and enzymes. Steroid-induced protein wasting is due to a reduction in protein synthesis and to an increase in intracellular proteolysis. Glucocorticoids inhibit protein synthesis primarily in type II muscle fibers, mainly by regulating the activity of factors involved in peptide initiation (17) Increased muscle cytoplasmatic protease

activity, associated with steroid treatment, results in myofibrillar destruction (18).

Effect on muscle glycogen metabolism. Treatment with steroids increases muscle glycogen, probably by increasing muscle glycogen synthetase activity. Moreover, glycogen utilization is decreased (19).

Effect on mitochondrial function. Mitochondrial alterations in muscles affected by steroids have suggested that impaired oxidative respiration may be an important factor in pathogenesis. The severity of the corticosteroid-induced changes correlated with the percentage of type II glycolytic fibers (20).

In conclusion, steroid-induced myopathy may be caused by a complex of changes in the muscle cells, of which reduction in protein synthesis and glycogen accumulation play a major role.

Histological and functional changes in animal models

Animal models have been used to study respiratory muscle involvement in steroid myopathy (Table 2). The advantage of this is that isolated steroid effects can be observed. Interference with other factors affecting respiratory muscle function, such as the underlying disease, is thus excluded.

Fluorinated steroid administration affected type IIb fibers, whereas type I and type IIa muscle fibers were spared (21-23). Vacuolar changes may occur in all fiber types (22,24-27). Glycogen levels in muscle cells were elevated (24).

The above-mentioned histological alterations led to changes in contractile properties (21,22,28,29). As a result of the selective type IIb fiber atrophy, seen after fluorinated steroid administration, the cross-sectional area of the diaphragm contained more slow (type I and IIa) muscle fibers. Therefore, the diaphragm ended in having the same contractile abilities as slow muscles, such as prolonged contraction time, prolonged half relaxation time and a reduction in fatigability (23,28). On the other hand, the observed loss in diaphragm mass, which decreased in proportion to total body weight, also impaired diaphragm force. Non-fluorinated steroids, even in low doses (30), caused loss of function of the diaphragm without muscle fiber atrophy, resembling chronic steroid myopathy (22,29,30). These findings suggested that moderate and low doses of non-fluorinated steroids resulted in myopathy rather than in atrophy and affected the intrinsic contractile apparatus of the diaphragm.

From these animal models it can be concluded that glucocorticosteroid administration resulted in a reduction in total body and diaphragm mass. Type IIb fiber atrophy, which

Table 2. Steroid dose and duration and muscle strength in chronic steroid myopathy in humans

Reference	Disorder	No	Age (years)	Steroids dose/day, duration	PI_{max}	PE_{max}	Peripheral muscle strength
Wang et al (56)	healthy subjects	16	19-39	prednisolone 20 mg 2 weeks	=	=	-
Janssens et al (16)	connective tissue disease	2	50,58	prednisolone 30-50 mg 10 weeks	↓	↓	-
Askari et al (14)	connective tissue disease	8	10-80	prednisone 40-80 mg 2-18 weeks	-	-	↓
Vallet et al (15)	myasthenia gravis	1	37	prednisone 40 mg 104 weeks	-	-	↓
Decramer et al (13)	asthma, COPD	3	59-64	methylprednisolone 30-80 mg 2-12 weeks	↓	↓	↓
Bowyer et al (3)	asthma	60		prednisolone >40 mg <40 mg 2 weeks 'years'	↓ =	↓ (n=3) n p	↓ =
Picado et al (33)	asthma	34	58 ±7	prednisone 12 ±4 mg 8 ±4 years	=	=	=
Rogiers et al (31)	asthma, COPD	21	60 ±13	ADD during the last 6 months	r=-0 64*	r=-0 41*	r=-0 56*
Dropcho et al (4)	brain tumor	216	51 ±2	dexamethasone	-	-	↓ (10,6%)

PI_{max} maximal inspiratory pressure, PE_{max} maximal expiratory pressure, = no change, - not measured n p not published, ADD average daily dose, * correlation (r) with ADD

Table 3. Histological, histochemical and biochemical changes in the diaphragm in animal models

Animal n	Steroid dose, duration	Gross pathology	Histochemistry	Biochemistry	Reference
Rabbits 9	cortisone acetate *i m* 10 mg·kg⁻¹ *q d* 2 weeks	Vacuolization Fragmentation Myonecrosis	-	Glycogen ↑ Lactate ↑	Fergusson et al (24)
Rabbits 13	cortisone acetate *i m* 10 mg·kg⁻¹ *q d* 3 weeks	Vacuolization Myonecrosis	Fiber types = Fiber CSA ↓ of all fibers	Glycogen ↑ Lactate ↑	Fergusson et al (26)
Rats 15	prednisolone *i m* 1 25 mg·kg⁻¹ dialy	Normal	Fiber types =		Dekhuijzen et al (22)
	prednisolone *i m* 5 mg kg⁻¹ daily	Myonecrosis Vacuolization	Fiber types = IIb fiber size =		
	triamcinolone *i m* 1 mg·kg⁻¹ daily	Myonecrosis Number nuclei ↑	IIb fiber size ↓ CSA type IIb ↓		
	4 weeks				
Hamsters 4	triamcinolone *im* 3 mg·kg⁻¹ *q d* 3 weeks	More angulated muscle fibers	Fiber types =	Aggregate areas of SDH staining in type I and II fibers	Wilcox et al (23)
		No myonecrosis	IIb fiber size ↓		

CSA = cross-sectional area, SDH = succinate dehydrogenase

occurred after treatment with fluorinated steroids, converted the contractile properties of the diaphragm to those of slow muscles Loss of diaphragm mass without fiber atrophy, as observed after administration of low doses of non-fluorinated steroids, also affected diaphragm contractile properties Thus, besides causing loss of mass, corticosteroids also weaken the diaphragm through changes in the intrinsic contractile apparatus

Clinical findings

In recent years, several case reports have shown involvement of respiratory muscles in chronic steroid myopathy The clinical studies are summerized in table 2 Janssens and Decramer (16) reported two patients with connective tissue disease receiving prednisone 30-36 mg day ^{1}, who complained of muscle weakness and dyspnoea Pulmonary function tests showed decreases in maximal inspiratory mouth pressure (PI_{max}, 35-52% predicted) and maximal expiratory mouth pressure (PE_{max}, 40% predicted) PI_{max} and PE_{max} are used as a measure for inspiratory and expiratory muscle force After gradual reduction of the steroid dose, PI_{max} and PE_{max} markedly increased Similar changes in PI_{max} and PE_{max} were described during prolonged treatment with high doses of corticosteroids in two patients with asthma and one with COPD (Fig 1a and 1b) The quadriceps force was reduced in these three patients (Fig 1c) (13) In a study of 21 patients with asthma and COPD the corticosteroid dose, expressed as the average daily dose during the last six months (ADD), negatively correlated with PI_{max} ($r = -0$ 64, $p < 0$ 002) and with the quadriceps force ($r = -0$ 56, $p < 0$ 01) A similar tendency was found between ADD and PE_{max} ($r = 0$ 41, $p < 0$ 1) (31) These reports strongly suggest that treatment with corticosteroids contributed to the observed muscle weakness in patients with asthma and COPD

In several patients suspected of steroid myopathy, biopsies of striated skeletal muscles were performed to confirm the diagnosis There are histological differences between acute and chronic steroid myopathy

Acute steroid myopathy Biopsies showed atrophy of all fiber types (1,6,7,10), without a predilection for type IIb fibers Generally the degeneration of fibers was accompanied by diffuse necrosis (1,6,7,10,12) Lacomis et al (11) perceived myofibrillar disorganization and loss of both thick and thin myofilaments and Z bands

Chronic steroid myopathy Most biopsies showed abnormal variation in fiber size with selective atrophy of type IIb fibers (14,32) In some reports type II fiber atrophy was observed, while no differentiation was made between type IIa and IIb muscle fibers (15,33) No inflammatory cells were found (13,31) Muscle fiber necrosis was absent
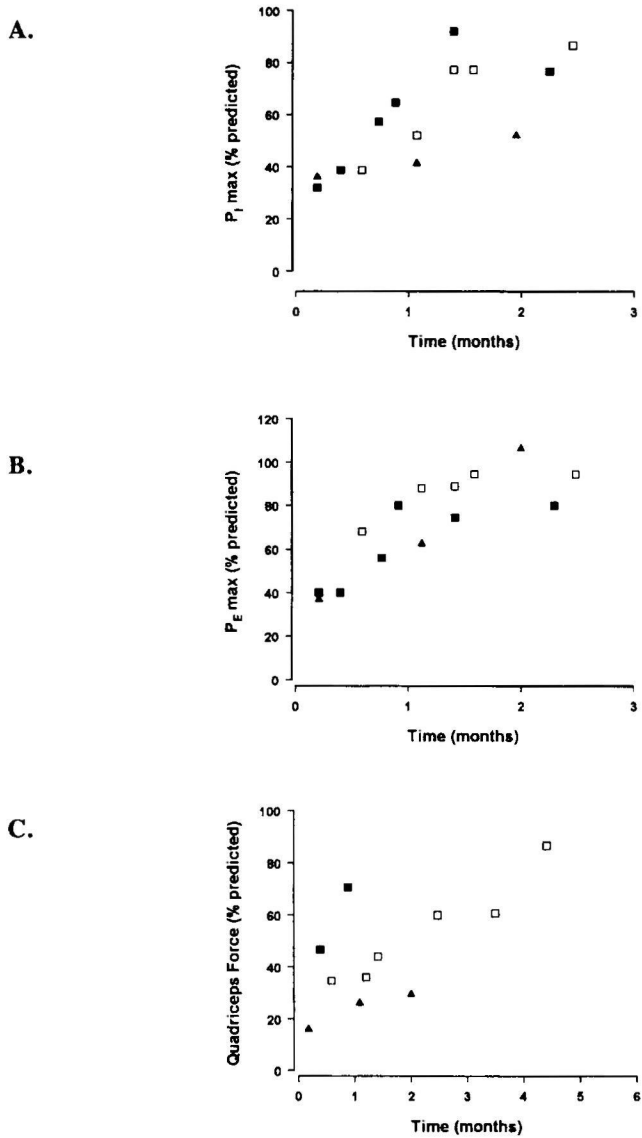
A.

B.

C.

Figure 1.   Maximal inspiratory pressure (PI_{max}, fig. A), maximal expiratory pressure (PE_{max}, fig. B), and quadriceps force (fig. C), expressed as % of predicted, versus time after gradual dose reduction in thee patients with COPD (open squares) and astma (triangles and solid squares); From ref (13), with permission from the Editor

(15,32)

Although of little importance in diagnosing steroid myopathy, a major electron microscopic feature in chronic (15) as well as in acute (7) steroid myopathy is the presence of large numbers of glycogen granules in the intermyofibrillar spaces

Corticosteroids are often administered to patients with COPD Steroid-induced respiratory muscle involvement may be more obvious and of more clinical importance in these patients since respiratory muscle function in COPD is already affected in several ways The high airway resistance increases the work load of these muscles and at the same time the work capacity is decreased by hyperinflation, malnutrition, hypercapnia, chronic heart failure, and physical inactivity (34)

Hyperinflation of the diaphragm results in a reduced number and length of sarcomeres Thus, the muscle fibers of the diaphragm continue to operate at an optimal length (35,36) However, as a result of this shorter length in COPD, the pressure generation of the diaphragm is reduced (37)

Beside this mechanical disadvantage, nutritional status is an important factor influencing respiratory muscle strength Malnutrition in animal models results in generalized fiber atrophy, also affecting the diaphragm (38,42) This is of clinical importance, since approximately one third of patients with COPD are significantly underweight (37) Rochester et al (37) reported a significant correlation between maximal inspiratory pressure and body weight in patients with COPD At necropsy they observed a substantial reduction in diaphragm dimensions in markedly underweight patients with COPD The degree of hypercapnia in COPD was inversely related to the maximal inspiratory pressure (37) Physical inactivity, as in severe COPD, further curtails respiratory muscle strength (38)

Diagnosis of chronic steroid myopathy

There is no specific test to diagnose steroid-induced myopathy However, the possibility of this diagnosis should be taken into account when the following abnormalities are found

Laboratory findings

In contrast to chronic steroid myopathy, high levels of CK (1,000 - 100,000 U l[1]) were measured in acute steroid myopathy This was associated with myoglobinuria, indicating rhabdomyolysis (7,10) In chronic myopathy, however, biochemical parameters like SGOT, aldolase and CK were not correlated with corticosteroid dose or muscle weakness (3,16) LDH may be within normal range (14) or slightly elevated (38) Myoglobinuria and rhabdomyolysis are absent Urinary creatine excretion seems to be the most sensitive

laboratory indicator for clinical diagnosis and for individual follow-up (14,16) However, changes in urinary excretion of creatine were of no help in confirming the diagnosis in a cross-sectional study (3)

Electromyographic examination (EMG)

The power of the EMG to discriminate between steroid myopathy and myopathy due to other causes is low (2) Most authors observed excessive numbers of easily recruited, short-duration, low-amplitude motor unit potentials, and occasional low-amplitude polyphasic motor units in predominantly the proximal muscles (8,11,12) The muscles were silent at rest and fibrillation potentials were absent, which is in contrast with findings in inflammatory myopathy (2) These changes are frequently detectable in some areas of the myopathic muscle, whereas other areas in the same muscle seem normal

Muscle strength testing (Table 3)

Respiratory muscle strength can be objectified by measurement of PI_{max} and PE_{max} at the mouth These values are frequently reduced in severe COPD patients Additional effects of corticosteroids may be detected by serial measurements, combined with changes in steroid dosages Peripheral muscle strength testing may reveal lowered force in extremities Bowyer et al (3) observed a good correlation between PI_{max} and hip flexor strength

Pulmonary function tests

Pulmonary function tests in patients with steroid myopathy are expected to be similar to those observed in patients with respiratory muscle weakness due to other causes (40) A reduced diffusion capacity for carbon monoxide may be observed because of microatelectases Lung compliance is reduced with low recoil pressures at total lung capacity (TLC) (40) This is in contrast to the pattern observed in interstitial lung disease in which elastic recoil pressure at TLC is increased (41) A decrease in lung volume occurs if respiratory muscle strength is lower than 50% of predicted (13,15)

Muscle biopsy

In a biopsy from an affected peripheral muscle, for example the deltoid muscle, type IIb fiber atrophy is prominent, without signs of inflammatory cells or myonecrosis A less specific finding is the presence of large numbers of glycogen granules in the intermyofibrillar spaces Muscle biopsy can differentiate between muscle weakness due to steroid myopathy and malnutrition, since malnutrition is associated with generalized muscle fiber atrophy (42) A muscle biopsy can be useful in strengthening the diagnose

Treatment of corticosteroid-induced myopathy

Steroid therapy is barely replaceable in present-day medical treatment As there is no definitive dose at which steroid myopathy occurs, it is difficult to give firm guidelines to prevent steroid myopathy

When steroid myopathy occurs during treatment with fluorinated steroids, the alternative is to switch to non-fluorinated steroids such as prednisolone or methylprednisolone (43) When non-fluorinated steroids are the cause of steroid myopathy, the dose should be tapered off If this is not possible, therapeutic interventions to antagonize steroid-induced myopathy may be considered, although they have not yet been confirmed by clinical studies

Exercise Several animal studies showed that exercise can impede steroid muscle atrophy (44,45), since it partially encounters the loss in muscle protein This may be explained by an increase in glucocorticoid receptors in immobilized muscles (46)

Anabolic steroids A reduction in corticosteroid induced weight loss due to the suppletion of anabolic steroids has been observed (18,47,48) This is possibly caused by an interaction on glucocorticoid receptor level between corticosteroids and anabolic steroids (18,49) In male rats anabolic steroids increased the mass of respiratory muscles in proportion to body weight (50), with an increase of specific tension generation in the diaphragm (50,51)

Growth hormone Growth hormone accelerated the recovery of the 'malnourished' diaphragm in rats (52) It may therefore be useful in refeeding malnourished patients with COPD, by improving their diaphragm function

β_2-agonists Agents such as clenbuterol and salbutamol have been used to manipulate growth and body composition These drugs reduce protein degradation, possibly accompanied by a simultaneous increase in protein synthesis Administration of clenbuterol, alone (53) or combined with growth hormone (54), increased the cross-sectional area of both type IIa and IIb fibers Martineau et al (55) observed an increase in peripheral muscle strength and inspiratory mouth pressure in humans after administration of 8 mg salbutamol twice daily for 3 weeks The effects of clenbuterol on diaphragm contractility and fatigue resistance are not clear yet

Conclusions

The incidence of involvement of the respiratory muscles in chronic steroid myopathy is unknown at present, yet may be quite common. Current therapy with positive inotropic drugs such as theophylline and inhaled β_2-agonists, might mask this complication. Especially in patients with COPD, who already have impaired respiratory muscle function, diagnosing steroid myopathy with respiratory muscle involvement is difficult but essential. If the diagnosis of chronic steroid myopathy is considered, peripheral and respiratory muscle strength, urinary creatine excretion, and serum muscle enzymes should be measured. Pulmonary function tests and a peripheral muscle biopsy may provide additional information. Treatment of corticosteroid-induced myopathy consists of tapering off the dose of steroids or switching to non-fluorinated steroids. Other therapeutic interventions are being studied.

References

1   Cushing H Basophic adenomas of pituitary body and their clinical manifestations (pituitary basophilism) Bull Johns Hopkins Hosp 1932,50 137
2   Ruff, RL Endocrine myopathies In Myology, edited by Engel, AG and Banker, BQ McGraw Hill Book Company 1986,1871-1906
3   Bowyer SL, La Mothe MP, Hollister JR Steroid myopathy incidence and detection in a population with asthma J Allergy Clin Immunol 1985,76 234-242
4   Dropcho, EJ, Soong S-J Steroid induced weakness in patients with primary brain tumors Neurology 1991,41 1235 1239
5   Dekhuijzen PNR, Decramer M Steroid-induced myopathy and its significance to respiratory disease a known disease rediscovered Eur Respir J 1992,5 997 1003
6   MacFarlane IA, Rosenthal FD Severe myopathy after status asthmaticus Lancet 1977,ii 615
7   van Marle W, Woods KL Acute hydrocortisone myopathy Br Med J 1980,281 271-272
8   Knox AJ, Mascie-Taylor BH, Muers MF Acute hydrocortisone myopathy in acute severe asthma Thorax 1986,41 411-412
9   Shee CD Risk factors for hydrocortisone myopathy in acute severe asthma Respir Med 1990,84 229-233
10   Williams TJ, O'Hehir RE, Czarny D, Horne M, Bowes G Acute myopathy in severe acute asthma treated with intravenously administered corticosteroids Am Rev Respir Dis 1988,137 460-463
11   Lacomis D, Smith TW, Chad DA Acute myopathy and neuropathy in status asthmaticus   case report and literature review Muscle & Nerve 1993,16 84 90
12   Griffin D, Fairman N, Coursin D, Rawsthorne L, Grossman JE Acute myopathy during treatment of status asthmaticus with corticosteroids and steroidal muscle relaxants Chest 1992,102 510-514
13   Decramer M, Stas KJ Corticosteroid-induced myopathy involving respiratory muscles in patients with COPD or asthma Am Rev Respir Dis 1992,146 800 802
14   Askari A, Vignos PJ, Moskowitz RW Steroid myopathy in connective tissue disease Am J Med 1976,61 485-492
15   Vallet B, Fourrier F, Hurtevent JF, Parent M, Chopin C Myasthenia gravis and steroid induced myopathy of the respiratory muscles Intensive Care Med 1992,18 424-426
16   Janssens S, Decramer M Corticosteroid-induced myopathy and the respiratory muscles Report of two cases Chest 1989,95 1160-1162

17.   Rannels SR, Rannels DE, Pegg AE, Jefferson LS. Glucocorticoid effects on peptide-chain initiation in skeletal muscle and heart. Am J Physiol 1978;235·E134-E139.

18   Mayer M, Rosen F. Interaction of glucocorticoids and androgens with skeletal muscle. Metabolism 1977;26·937-945.

19.   Shoji S, Takagi A, Sugita H, Toyokura Y. Muscle glycogen metabolism in steroid-induced myopathy of rabbits. Exp Neurol 1974;45:1-7.

20.   Vignos PJ, Greene R. Oxidative respiration of skeletal muscle in experimental corticosteroid myopathy. J Lab Clin Med 1973;81:365-378.

21.   Viires N, Pavlovic D, Pariente R, Aubier M. Effects of steroids on diaphragmatic function in rats. Am Rev Respir Dis 1990;124:34-38.

22.   Dekhuijzen PNR, Gayan-Ramirez G, Bisschop A, de Bock V, Decramer M. Prednisolone and triamcinolone affect contractile properties of rat diaphragm differently. J Clin Invest 1993;92:1534-1542.

23.   Wilcox PG, Hards JM, Bockhold K, Bressler B, Pardy RL. Pathologic changes and contractile properties of the diaphragm in corticosteroid myopathy in hamsters: comparison to peripheral muscle. Am J Respir Cell Mol Biol 1989;1:191-199.

24.   Ferguson GT, Irvin CG, Cherniack RM Effects of corticosteroids on diaphragm function and biochemistry in the rabbit. Am Rev Respir Dis 1990;141.156-163.

25.   Afifi AK, Bergman RA. Steroid myopathy. A study of the evolution of the muscle lesion in rabbits. John Hopkins Med J 1969,124:66-86.

26   Ferguson GT, Irvin CG, Cherniack RM. Effects of corticosteroids on respiratory muscle histopathology. Am Rev Respir Dis 1990;142:1047-1052.

27.   Smith B Histological and histochemical changes in the muscles of rabbits given the corticosteroid triamcinolone. Neurology 1964;14.857-863.

28.   Sasson L, Tarasiuk A, Heimer D, Bark H. Effect of dexamethasone on diaphragmatic and soleus muscle morphology and fatigability. Respir Physiol 1991;85·15-28.

29.   Dekhuijzen PNR, Gayan-Ramirez G, de Bock V, Decramer M Low-dose prednisolone treatment induces myopathy in rat diaphragm. Am Rev Respir Dis 1992;145:A670.

30.   Dekhuijzen PNR, Gayan-Ramirez G, Bisschop A, de Bock V, Dom R, Decramer M Rat diaphragm contractility and histopathology are affected by low dose treatment with methylprednisolone and deflazacort. Eur Respir J 1993;6:197s.

31.   Rogiers P, Lacquet LM, Decramer M. Steroid-induced myopathy in patients with COPD and asthma. Eur Respir J 1991;4:A346.

32.   Afifi AK, Bergman RA, Harvey JC. Steroid myopathy. Clinical, histologic and cytologic observations. John Hopkins Med J 1968;123:158-174.

33.   Picado C, Fiz AJ, Montserrat JM, Grau JM, Fernandez-Sola J, Luengo MT, Casademont J, Agusti-Vidal A. Respiratory and skeletal muscle function in steroid-dependent bronchial asthma. Am Rev Respir Dis 1990;141:14-20.

34.   Dekhuijzen PNR, Folgering HThM, van Herwaarden CLA. Chronic airflow obstruction, hyperinflation and the respiratory muscles. Neth J Med 1992;41:74-81.

35   Supinski GS, Kelsen SG. Effect of elastase-induced emphysema on the force-generating ability of the diaphragm. J Clin Invest 1982;70·978-988.

36.   Kelsen SG, Wolanski T, Supinski GS, Roessmann U The effect of elastase-induced emphysema on diaphragmatic muscle structure in hamsters. Am Rev Respir Dis 1983;127:330-334.

37.   Rochester DF, Braun NMT Determinants of maximal inspiratory pressure in chronic obstructive pulmonary disease. Am Rev Respir Dis 1985;132 42-47

38.   LoRusso TJ, Lewis MI, Zhan WZ, Sieck GC. Effects of chronic inactivity and malnutrition on diaphragm structure and function. Am Rev Respir Dis 1993;147(4).A962.

39.   Kanayama Y, Shiota K, Horiguchi T, Kato N, Ohe A, Inoue T. Correlation between steroid myopathy and serum lactic dehydrogenase in systemic lupus erythematosus. Arch Intern Med 1981;141·1176-1179

40   De Troyer A, Borenstein S, Cordier R. Analysis of lung volume restriction in patients with respiratory muscle weakness. Thorax 1980;35:603-610.

41   Yernault JC, De Jonghe M, De Coster A, Englert M. Pulmonary mechanics in diffuse fibrosing alveolitis Bull Europ Physiopath Resp 1975;11:231-234.

42   Dekhuijzen PNR, Gayan-Ramirez G, Bisschop A, de Bock V, Dom R, Decramer M The pattern of fiber atrophy induced by malnutrition and triamcinolone in rat diaphragm is different Am Rev Respir Dis 1993,147 A957

43   Williams RS Triamcinolone myopathy Lancet 1959,1 698-701

44   Hickson RC, Davis JR Partial prevention of glucocorticoid-induced muscle atrophy by endurance training Am J Physiol 1981,241 226-232

45   Seene T, Viru A The catabolic effect of glucocorticoids on different types of skeletal muscle fibers and its dependence upon muscle activity and interaction with anabolic steroids J Ster Biochem 1982,16 349-352

46   DuBois DC, Almon RR Disuse atrophy of skeletal muscle is associated with an increase in number of glucocorticoid receptors Endocrinology 1980,107 1649-1651

47   Reid MB, Wang N, Haack KE, Miller MJ Anabolic steroids protect skeletal muscles against cortisone-induced atrophy Faseb Journal 1990,4 A1068

48   Hickson RC, Czerwinski SM, Falduto MT, Young AP Glucocorticoid antagonism by exercise and androgenic-anabolic steroids Med Sci Sports Exerc 1990,22 331-340

49   Sharpe PM, Buttery PJ, Haynes NB The effects of manipulating growth in sheep by diet or anabolic agents on plasma cortisol and muscle glucocorticoid receptors Br J Nutr 1986,56 289-304

50   Bisschop APG, Dekhuijzen PNR, Gayan-Ramirez G, Buts N, Decramer M Effects of nandrolone decanoate on rat respiratory muscles Eur Respir J 1992,5 423S

51   deBoisblanc BP, Harris KY, Svec F, Summer WR Dietary supplementation with dehydroepiandrosterone increases specific diaphragmatic tension generation in normal female rats Am Rev Respir Dis 1993,147 A957

52   Lanz JK, Donahoe M, Rogers RM, Ontell M Effects of growth hormone on diaphragmatic recovery from malnutrition J Appl Physiol 1992,73 801 805

53   Zeman RJ, Ludemann R, Easton TG, Etlinger JD Slow to fast alterations in skeletal muscle fibers caused by clenbuterol, a β_2 receptor agonist Am J Physiol 1988,254 E726 E732

54   Maltin CA, Delday MI, Hay SM, Innes GM, Williams PEV Effects of bovine pituitary growth hormone alone or in combination with the β-agonist clenbuterol on muscle growth and composition in veal calves Br J Nutr 1990,63 535-545

55   Martineau L, Horan MA, Rothwell NJ, Little RA Salbutamol, a β_2-adrenoceptor agonist, increases skeletal muscle strength in young men Clin Science 1992,83 615-621

56   Wang Y, Zintel T, Vasques A, Gallagher CG Corticosteroid therapy and respiratory muscle function in humans Am Rev Respir Dis 1991,144 108-112

CHAPTER 3

Effects of different treatment regimens of methylprednisolone on rat diaphragm contractility, immunohistochemistry and biochemistry

RHH van Balkom, HFM van der Heijden, HTB van Moerkerk[a],
JH Veerkamp[a], JAM Fransen[b], LA Ginsel[b], HTM Folgering,
CLA van Herwaarden, and PNR Dekhuijzen

[a] Department of Biochemistry, and [b] Department of Cell Biology and Histology,
University of Nijmegen, The Netherlands

Abstract

Systemic corticosteroid therapy may affect diaphragm structure and function We postulated that functional, immunohistochemical and biochemical characteristics of rat diaphragm were less affected by alternate-day methylprednisolone (MP) administration, and more by repeated bursts of MP, in comparison to daily *s c* MP Sixty adult rats were randomized into 4 groups saline *s c* , MP continuously (MP-C, 1 mg kg^1 daily), MP alternate-day therapy (MP-A, 2 mg kg^1 every other day), or MP in bursts (MP-B, 2 mg kg^1 daily for 2 weeks, saline for 4 weeks, MP 2 mg kg^1 daily for 2 weeks) The total treatment period was 8 weeks Contractile properties of isolated diaphragm strips were measured Antibodies reactive with type I, IIa, IIx and IIb myosin heavy chains were used for immunohistochemical analysis Biochemical evaluation included markers of fast energy supply, glycogenolytic activity, ß-oxidation capacity and oxidative capacity The force-frequency curve was depressed in all MP groups Fiber type I, IIx and IIb cross sectional area (CSA) decreased in all MP groups Burst therapy decreased the contribution of type IIb CSA to total diaphragm CSA MP A affected glycogenolytic activity less than MP C Burst MP therapy reduced CK activity and ß-oxidation capacity compared to MP-C Oxidative capacity was increased in all MP groups In conclusion, although the MP treatment regimens affected diaphragm muscle morphology and bioenergetic enzyme activities in different ways, force generation decreased in all MP groups to the same extent

Introduction

Recently, animal and clinical studies have shown evidence of respiratory muscle dysfunction induced by treatment with corticosteroids (8,9) Morphological changes such as selective type IIb fiber atrophy were observed in animal studies following administration of fluorinated steroids (11,37) Non fluorinated steroids, however, caused loss of diaphragm function without muscle atrophy, suggesting myopathy (11)

   The mechanisms by which non-fluorinated steroids cause myopathy are in part unknown 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 (31) These myosin heavy chains determine myosin ATP-ase activity and the speed of shortening in the muscle fibers and are therefore in part responsible for contractile properties Polla *et al* (26) reported a complete disappearance of rat diaphragm muscle fibers containing IIb myosin heavy chains (MyHCs) following

cortisone acetate 100 mg·kg^{-1}·day^{-1} for eleven days. It is unknown if similar changes occur after administration of lower, clinically more relevant dosages.

Changes in energy substrate and enzyme activities may also contribute to the onset of steroid-induced myopathy. A consistent observation is the increase in glycogen content in the diaphragm (15,33). However, conflicting effects on diaphragm mitochondrial function were found following corticosteroid therapy (24,36). This may be explained by the differences in the dosage, duration and types of steroids studied.

In addition, the severity of structural and biochemical changes in the respiratory muscles may depend on the treatment regimens applied. Alternate-day glucocorticoid therapy may reduce side effects since anti-inflammatory potency appears to persist longer than the undesirable metabolic effects (4,17). The clinical efficacy of alternate-day therapy was similar in patients with COPD or stable asthma compared to daily treatment (2,6). Another treatment regimen, short-term high-dose steroid therapy is often applied during exacerbations of COPD or asthma. Repeated episodes of high-dose steroid administration, in this study referred to as bursts, may cause more severe side effects. This concept is supported by the observation that recovery of acute steroid myopathy, caused by short-time high-dose steroid administration, appears to take several months (21).

We postulate that the structure and function of the rat diaphragm (1) is less affected by alternate-day corticosteroid administration and (2) is more affected by repeated bursts, in comparison to daily administration. In order to test this hypothesis, we studied functional, immunohistochemical and biochemical changes in rat diaphragm induced by continuous, alternate-day and burst administration of the non-fluorinated steroid methylprednisolone.

Methods

Study design, animals, and treatment
Sixty adult male outbred Wistar rats, aged 18-20 weeks, mean (±SD) weight 525±34.8 g, were randomised into 4 groups:
- control (C): saline 0.2 ml·day^{-1} subcutaneously (*s.c.*)
- methylprednisolone (MP) continuously (MP-C): 1 mg·kg^{-1}·day^{-1} *s.c.*
- MP alternate-day therapy (MP-A): 2 mg·kg^{-1} *s.c.* every other day alternating with saline 0.2 ml *s.c.*
- MP in bursts (MP-B): MP 2 mg·kg^{-1}·day^{-1} *s.c.* for 2 weeks followed by saline 0.2 ml *s.c.* for 4 weeks and by MP 2 mg·kg^{-1}·day^{-1} *s.c.* for 2 weeks.

With each injection all animals received a similar volume (~0.20 ml). During 8 weeks the animals were *s.c.* injected daily between 8.30 and 10.00 a.m. in the neck. The total

dose of steroids administrated was equal in all treatment groups The rats were fed ad libitum, held on a 12/12 hour light-dark regime and weighed twice weekly At the end of the treatment period, contractile properties, immunohistochemical and biochemical characteristics of the diaphragm were examined All animals were investigated between 23 and 30 hours after the last injection Adrenal and diaphragm weights were measured immediately following dissection The study was approved by the local Animal Experiments Committee of the University of Nijmegen

Contractile properties

The rats were anaesthetized with sodium pentobarbital (70 mg kg^1 i p) and a poly-ethylene cannula was inserted through a tracheotomy The animals were mechanically ventilated with an oxygen-enriched gas mixture (flow 0 5 ml g^1 body weight min^1, respiration frequency 70 min^1 and a duty cycle of 50%) The diaphragm was quickly removed through a combined laparotomy and thoracotomy and was immediately immersed in a cooled, oxygenated Krebs solution at a pH of 7 4 This solution consisted of (mmol l^1) 137 NaCl, 4 KCL, 2 MgCl$_2$, 1 KH$_2$PO$_4$, 24 NaHCO$_3$, 2 7 CaCl$_2$, and 7 glucose D-tubocurarine chloride 25 μM (Sigma Chemicals, The Netherlands) was added to prevent spontaneous neuromuscular activity Two small rectangular bundles, parallel to the long axis of the muscle fibers, were dissected from the middle part of the lateral costal region of each hemidiaphragm Silk sutures were firmly tied to both ends of the bundle to serve as anchoring points Each bundle was placed in a tissue bath between two large platinum stimulating electrodes The tissue baths were filled with Krebs at 37°C and were oxygenated with 95% O$_2$ and 5% CO$_2$ The central tendon insertion of the bundles were tied to a fixed point and the costal margin origin to an isometric force transducer (Sensotec, model 31/1437, Columbus OH, USA) Data acquisition and storage were performed using a Dash-16 interface and Twist-Trigger software (I D -electronics, University of Nijmegen, The Netherlands) Stimulations were applied with a Grass S 48 stimulator (Quincy, MA, USA) Maximal twitch force was reached at 34 volt To ensure supramaximal stimulation, subsequent stimulations were performed with a 20% higher voltage (40 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 thermo-equilibration period (10) The following measurements were made

Twitch characteristics two twitches were recorded at Lo to obtain maximal twitch force (P$_t$), contraction time (CT), and half relaxation time (½RT) The averages were used for further analysis

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$_o$)

Force-frequency (FF) protocol muscle bundles were stimulated every 2 min with the following frequencies 25, 160, 50, 160, 80, 160, 120 and 160 Hz (train duration 250 ms) Forces were also expressed as percentage of the average force at 160 Hz before and after each stimulus (11,25)

The generated force was expressed per cross-sectional area (kg cm^2) Cross-sectional area (CSA) was measured by dividing diaphragm bundle weight by muscle density (1 056 mg mm^3) and bundle length

Immunohistochemical procedures

Muscle strips obtained from the costal part of the right hemidiaphragm were embedded in Tissue-Tek° in a plastic holder The muscle fibers were oriented parallel to the long side of the holder Subsequently, these specimens were quickly frozen in isopentane cooled in liquid N_2 followed by further freezing in liquid N_2 Serial cross sections were cut at 7 μm with a cryostat kept at 30°C Anti-myosin heavy chain antibodies (Regeneron Pharmaceuticals, New York, U S A) were used for morphometric examination The following antibodies were used BA-D5 reactive with type I MyHCs, SC 71 reactive with type IIa MyHCs, BF-35 reactive with type I, IIa and IIb but not with type IIx MyHCs, and BF-F3 reactive with type IIb MyHCs (30) Incubation with anti-myosin heavy chain antibodies was performed at room temperature for 1 hour Antibodies were subsequently labelled with ultra small immunogold reagent followed by silver enhancement (Aurion, Wageningen, The Netherlands) A minimum of 300 fibers were analysed from each diaphragm using a Sprynt-based, PC-Image digital analysis system (Bos B V , Waddinxveen, the Netherlands)

Biochemistry

Parameters of the bioenergetic capacity of the diaphragm included the activities of creatine kinase (CK), responsible for the fast energy supply, the glycogenolytic enzyme phosphorylase, and the mitochondrial enzymes 3-hydroxyacyl-CoA dehydrogenase (HADH), a marker for the ß-oxidation capacity, and citrate synthase (CS), an index of citric acid cycle activity

Fat and tendon were quickly removed from remainings of the left and right hemidiaphragm Subsequently, these diaphragm parts were frozen in liquid N_2 and stored at 80°C Segments of fresh frozen diaphragm were thawed in ice-cooled buffer containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7 4) In this buffer muscle homogenates (5% wt vol^1) were prepared by hand homogenization, using a Potter Elvehjem glass teflon homogenizer

CK activity was determined with the Boehringer CK-NAC activated kit (19) and

expressed in mmol NADPH formed min^1 g tissue Total phosphorylase (a+b) activity was assayed according to the method described by Jacobs *et al* (18) Phosphorylase activity was expressed as μmol NADPH formed min^1 g tissue HADH activity was assessed at 50 μM acetoacyl-CoA (5) and expressed in nmol HADH oxidized min^1 g tissue Citrate synthase activity was determined at 25°C (32) and was expressed as μmol coenzyme A formed min^1 g tissue All other above mentioned measurements were performed at 37°C The assays for metabolic enzymes were performed spectrophoto-metrically in duplicate The coefficient of variation for the assays applied was ~5%

Data analysis

Data of contractile properties of the two bundles obtained from one rat were averaged The SPSS/PC+ package V5 0 1 (Chicago Illinois, USA) was used for statistical analysis Data were compared using one-way analysis of variance followed by Duncan's multiple-range test Repeated measures analysis of variance was used for growth curve analysis and a two-way analysis of variance was used to detect treatment differences in force generation during the FF protocol Results were considered significant at $p < 0.05$ All data were expressed as mean$\pm$SD

Results

Body, muscle and adrenal weight

Figure 1 shows the body weight curve during the 8-week treatment period A significant effect of steroid treatment on growth curve was noted throughout this period (p = 0 039) Continuous MP administration affected body growth most Body weights of the MP-A animals closely tracked those of the control group Administration in bursts temporarily inhibited growth

No differences in absolute diaphragm weights were observed Diaphragm weight, normalized for body weight, was also similar in all groups (control 0 0132±0 001%, MP-C 0 0143±0 001%, MP-A 0 0136±0 001%, MP-B 0 0141±0 002%) Absolute adrenal weight was reduced in all MP treatment groups Adrenal weights in the treatment groups were reduced in proportion to body weight (control 0 0082±0 0014%, MP-C 0 0077±0 0008%, MP-A 0 0074±0 0007%, MP-B 0 0079±0 001%)

Contractile properties

Diaphragm bundle dimensions did not differ between the four groups (average length ~16 5 mm, thickness ~0 67 mm, width ~1 8 mm, and weight ~26 mg) P_t and P_o,
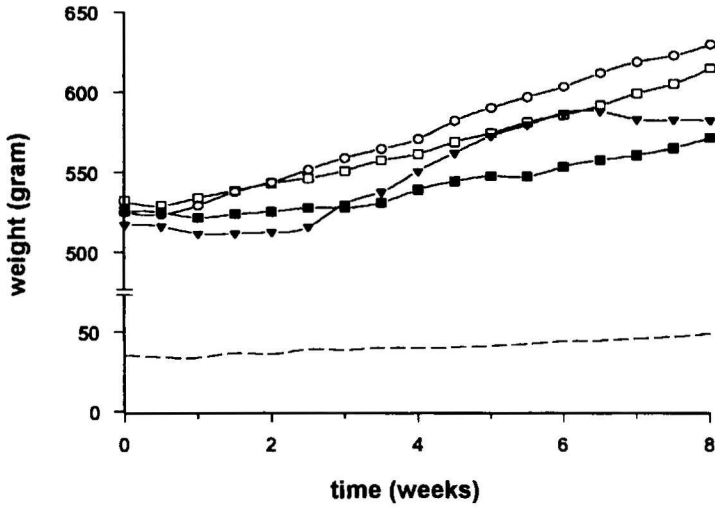
Figure 1.   Growth curve
Open circles: control; closed squares : MP-C; open squares:
MP-A; closed triangles: MP-B; dashed line: pooled SD; p=0.039
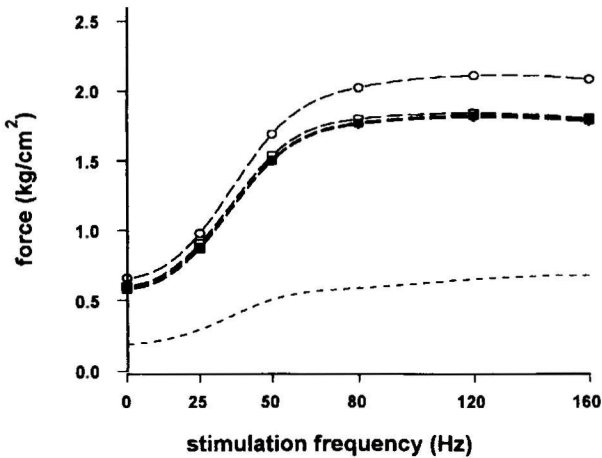
Figure 2.   Force-frequency curve
Open circles: control; closed squares: MP-C; open squares:
MP-A; closed triangles: MP-B; dashed line: pooled SD
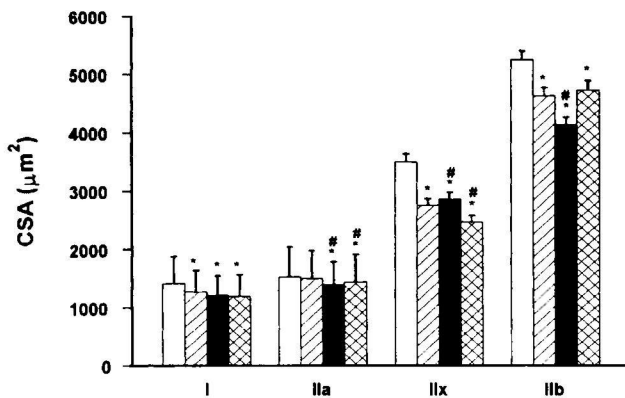
Figure 3. Fiber type cross sectional area
Open bars: control; hatched bars: MP-C; solid bars: MP-A;
cross hatched bars: MP-B;
* $p < 0.05$ compared to control; # $p < 0.05$ compared to MP-C
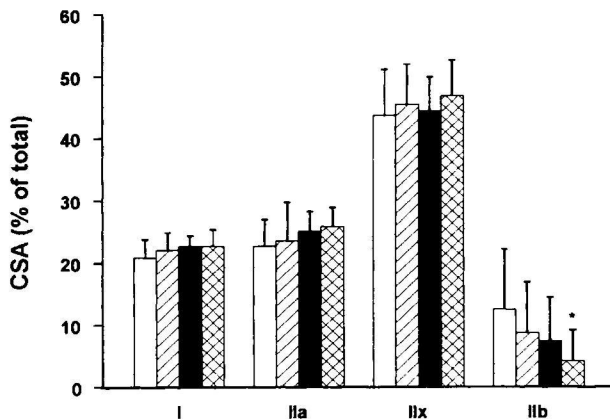
Figure 4. Relative fiber contribution to total diaphragm muscle area
Open bars: control; hatched bars: MP-C; solid bars: MP-A;
cross hatched bars: MP-B; * $p < 0.05$ compared to control

normalized for CSA, decreased by 7.0 to 9.5% in all MP groups (p = 0.07). Twitch characteristics did not differ between the groups (table 1).

The FF curves, expressed as absolute values, showed significant differences in force generation (p = 0.026) in all groups treated with MP. Force generation was equally reduced in all MP groups (fig. 2). MP treatment affected force generation at all stimulation frequencies in a similar way. The percentage decrease in P160 Hz during the FF protocol was similar in the three MP groups, and did not differ from control (MP-C 11.4±10.0%; MP-A 11.7±7.5%; MP-B 12.5±11.0%; control: 9.0±7.7%).

Immunohistochemistry

Methylprednisolone administrated in bursts (MP-B) caused a shift in fiber distribution from type IIb to type IIx in comparison to control (p < 0.01) (table 2).

Significant atrophy of type I, IIx and IIb fibers was observed in all MP groups (fig. 3). The differences in type IIa fiber CSA, as shown in figure 3, were significant but very small. The degree of type IIx atrophy was less following alternate-day in comparison to continuous MP therapy. In addition, type IIx CSA decreased in the MP-B group compared to MP-C. In other words, type IIx fiber atrophy was most pronounced following bursts and least affected by alternate-day steroid therapy compared to daily treatment (fig. 3). In contrast, the degree of type IIb fiber atrophy was greater in the MP-A animals compared to MP-C.

As a result of the decrease in number of type IIb fibers in the MP-B group, the relative contribution of type IIb fibers to the total muscle area of the diaphragm was decreased compared to control (fig. 4).

Biochemistry

The data on bioenergetic enzyme activities are shown in table 3. An approximately 30% increase in CS activity was found in all MP treatment groups, indicating an increased oxidative capacity (p < 0.01).

Treatment with the three MP regimens affected energy supply by different mechanisms. First, continuous steroid administration decreased glycogenolytic activity, indicated by a decrease in phosphorylase activity. Second, glycogenolytic activity was less affected following alternate-day steroid administration in comparison to daily treatment. Finally, burst MP administration affected CK activity and ß-oxidation capacity, as indicated by HADH, more in comparison to MP-C. Total phosphorylase activity, however, was higher in the MP-B group than in the MP-C group.

Table 1. Diaphragm contractile properties.

treatment	P_t	CT	½RT	P_o	P_t/P_o
	$kg \cdot cm^2$		ms	$kg \cdot cm^2$	
control	0.64 ± 0.18	29.3 ± 1.8	17.7 ± 1.3	2.23 ± 0.51	0.29 ± 0.03
MP-C	0.58 ± 0.20	$28\ 8 \pm 2.1$	17.9 ± 1	2.02 ± 0.65	0.29 ± 0.02
MP-A	0.60 ± 0.15	29.2 ± 2.3	$18\ 1 \pm 1.1$	2.07 ± 0.42	$0.29 \pm 0\ 03$
MP-B	0.59 ± 0.23	29.2 ± 2.6	18.1 ± 1.6	2.02 ± 0.74	0.29 ± 0.04

means $\pm$ SD; P_t: twitch force; CT: contraction time;
½RT: half relaxation time; P_o: maximal tetanic force

Table 2. Fiber type distribution

treatment	type I	type IIa	type IIx	type IIb
	%	%	%	%
control	32.7 ± 4.1	33.5 ± 4.2	28.3 ± 4.1	$5.5 \pm 4\ 3$
MP-C	33.4 ± 3.6	31.6 ± 6.1	31.4 ± 4.2	3.7 ± 3.7
MP-A	34.6 ± 3.5	33.4 ± 3.9	28.7 ± 3.5	3.3 ± 3.6
MP-B	33.6 ± 3.1	31.5 ± 3.5	$33.2 \pm 4.9^{\#}$	$1.8 \pm 2.0^{*}$

means $\pm$ SD; * $p < 0.05$ compared to control; # $p < 0.05$ compared to control and the MP-A group

Table 3. Biochemical analysis of the diaphragm.

treatment	CK $kU \cdot g^{-1}$	phosphorylase $U \cdot g^{-1}$	HADH $U \cdot g^{-1}$	CS $U \cdot g^{-1}$
control	2.18 ± 0.54	35.5 ± 3.3	2.86 ± 0.44	23.9 ± 2.3
MP-C	1.82 ± 0.64	$32.6 \pm 2.0^{\#}$	2.83 ± 0.66	$30.8 \pm 6.5^{*}$
MP-A	$1.48 \pm 0.32^{*}$	38.0 ± 3.2	2.84 ± 0.51	$31.2 \pm 4.0^{*}$
MP-B	$1.39 \pm 0.47^{*x}$	37.2 ± 4.0	$2.24 \pm 0.35^{\#}$	$30.6 \pm 4.8^{*}$

means $\pm$ SD; CK: creatine kinase, HADH: 3-hydroxyacyl-CoA dehydrogenase; CS: citrate synthase;
* $p < 0.01$ compared to control; x $p < 0.05$ compared to MP-C; # $p < 0.01$ compared to all other groups

Discussion

The question in the present study was whether there were differences in changes in rat diaphragm following different corticosteroid treatment regimens. The data showed that the MP treatment regimens affected body weight, diaphragm morphology and bioenergetic capacity in a different way. Force generation, however, decreased to a similar extent in all MP treatment regimens. This apparent discrepancy can be explained by the fact that the net result of these combined morphometric and biochemical changes, was an equal reduction in force generation.

Rationale of methylprednisolone dosage
The rationale for the dose of MP used in the present study was based on an absorption of 60% after *i m* injection (25) and the finding that the *s c* route may require higher doses to produce effects similar to *i m* administration (16). Similar anti-inflammatory potency and metabolism of methylprednisolone have been described in rats and humans (20,28). Therefore, 1 mg kg^1 of MP, as administrated in the MP-C group, may be equivalent to a dose of 35 mg day^1 in a 60 kg human. A daily dose of 35 mg methylprednisolone is not uncommon in the treatment of patients with COPD during an exacerbation (1).

The duration of biological effects of methylprednisolone in rat is difficult to analyse because of the nonlinear clearance (22). In addition, no close correlation was found between the circulating half-life of a glucocorticoid and its duration of action (4). However, the differences in growth curves observed in the present study indicates that there are differences in biological effects between continuous and alternate-day therapy.

Contractile properties
The reduction in diaphragm force generation following MP therapy, observed in this study during the FF protocol, cannot be explained by differences in oxygenation of the bundles, since their measures were similar in all groups. Since MP has little or no mineralocorticoid activity, an overestimation of the CSA due to an increase in extracellular fluid is unlikely. Glucocorticoids are known to cause protein wasting (27). A reduction in myofibrilar protein density would result in a reduction in the number of cross-bridges available for interaction with actin. This can lead to a reduction in force generation. Other plausible causes for the impairment in diaphragm force are the morphological and biochemical changes.

In previous studies no changes in twitch and maximal tetanic forces were found following 0 5 mg MP kg^1 day^1 for 6 weeks (10), or following 5 mg kg^1 day^1 of prednisolone for 4 weeks (11). Cortisone acetate 100 mg kg^1 day^1 for 10 days did not

lead to functional changes in the diaphragm either (25). The downward shift of the FF curve in the present study may be explained by differences in effects of dosage and duration of the steroid therapy on morphological and biochemical characteristics (see below).

Immunohistochemistry

Immunohistochemical differentiation between type IIx and IIb fibers, as performed in this study, may be of importance since type IIx and IIb MyHCs possess biochemical and functional differences. Maximum velocity of shortening (29) and resistance to fatigue (23) in type IIx muscle fibers are intermediate between those of type IIa and IIb fibers. Twitch and tetanic forces are higher in IIb in comparison with IIa and IIx motor units (23). Type IIx muscle fibers have a rich mitochondrial content, in contrast to type IIb fibers (30).

In line with our hypothesis, the degree of type IIx atrophy was less following alternate-day MP administration compared to continuous therapy. On the other hand, type IIb fiber atrophy was more pronounced in the alternate-day group. Cortisone acetate treatment in rabbits resulted in atrophy of all diaphragm fiber types (13), whereas no changes in fiber CSA were found in rats following prednisolone treatment (11). Muscle fiber atrophy can be the result of steroid-induced protein wasting due to a reduction in protein synthesis and an increase in intracellular proteolysis (27). However, muscle fiber atrophy may also occur as an attempt to increase oxygen delivery by decreasing the cell diameter, in this way creating a smaller diffusion distance (34).

Burst therapy reduced the number of type IIb fibers and decreased the contribution of type IIb CSA to total diaphragm CSA. Cortisone acetate administration (100 mg·kg^{-1}·day^{-1} for 11 days) even resulted in a complete disappearance of fibers containing IIb MyHCs, while fiber type distribution was not described (26). These changes were not detected using ATP-ase staining (11,13).

The cause of the shift from type IIb to type IIx fibers following burst therapy is not entirely clear. This shift may be caused by a disappearance of type IIb fibers combined with an appearance of new type IIx fibers. The following observations, however, support the occurrence of a transformation from type IIb to IIx fibers. Corticosteroids decrease the rate of amino acid incorporation into the MyHCs, resulting in a decreased turnover rate of the MyHCs in the muscle cell. As this decrease in turnover rate is most pronounced in the fast twitch muscle fibers (31), type IIb fibers are likely to be affected most. This may lead to a decrease in IIb MyHCs in type IIb fibers. Since genes of IIx MyHCs are coexpressed in a number of type IIb muscle fibers (12), an increase of IIx MyHCs in type IIb muscle fibers may occur to compensate for the decrease in type IIb MyHCs. To our knowledge, there is no evidence that type IIb fibers disappear while new

type IIx fibers are generated The shift from type IIb to type IIx fibers following MP burst therapy is probably too small to cause functional changes at different stimulation frequencies

Biochemistry

Treatment with MP resulted in a decrease in energy supply as indicated by a reduction in CK activity or in glycogenolytic activity This impairment in energy supply may have caused the increase in oxidative capacity in all MP groups To what extent these differences in biochemistry were influenced by the fact that animals in the MP-A and MP-B group received 2 mg kg^1 MP 23 to 30 hours before investigation, whereas, animals in the MP-C group only received 1 mg kg^1, is not clear

Creatine kinase rapidly rephosphorylates ADP from phosphocreatine in order to keep a constant ATP level in the muscle A decrease in CK activity may therefore directly lead to a reduction in fast energy supply CK activity decreased following steroid administration in bursts compared to MP-C In patients with bronchial asthma, CK activity in the deltoid muscle decreased following prednisone treatment (17 mg day^1 during 15 years) (15)

The increase in glycogen storage in the diaphragm muscle following steroid administration found by others (14) may be the result of a decrease in glycogen breakdown or an increase in glycogen production (33) or both (15) Our data show that alternate-day and burst therapy did not affect glycogenolytic capacity, measured by phosphorylase activity, in contrast to continuous steroid administration

Since the diaphragm muscle possesses a high ß-oxidation capacity (35), it is unclear if the 20% reduction in HADH activity, observed following steroid administration in bursts, has functional consequences Short-term (10 days) prednisolone treatment (5 mg kg^1 day^1 s c) did not change HADH activity in rat diaphragm (24) However, a decrease in HADH activity in vastus lateralis muscle of patients with rheumatoid arthritis occurred with low doses of prednisolone (7)

In the present study, administration of methylprednisolone resulted in an increase in oxidative capacity, as indicated by the increased CS activity, independent of the treatment regimen applied This is in line with the increase in oxidative staining reaction in skeletal muscle reported by others (36) Mitochondrial changes in rabbit diaphragm following glucocorticoid administration ranged from a numerical increase to the presence of enlarged and degenerated mitochondria (3) In contrast to these observations, CS activity in rat diaphragm was reduced following 5 mg kg^1 prednisolone per day for 10 days Yet, no changes in CS activity were reported following 0 5, 1 or 2 mg kg^1 per day (24), suggesting that enzyme activity was more affected using high dosages of prednisolone in

comparison to low dosages. Reactions of metabolism to corticosteroids may also depend on the fiber type composition of the muscle (14,24) because the resistance of different fiber types is believed to depend on their ability to compensate the steroid-induced deficiency of the glycogenolytic route by converting to oxidative metabolism (36).

In conclusion, force generation decreased in all MP groups to an equal extent, although the MP treatment regimens affected diaphragm muscle morphology and bioenergetic enzyme activities in a different way.

References

1. Anonymous Acute severe asthma in adults and children. Thorax 48· S12-S17, 1993.
2. Admoff, A.D. and J.R. Hollister. Steroid-induced fractures and bone loss in patients with asthma. N Engl J Med 309: 265-268, 1983.
3. Afifi, A.K and R.A. Bergman. Steroid myopathy. A study of the evolution of the muscle lesion in rabbits. John Hopkins Med J 124. 66-86, 1969.
4. Axelrod, L. Glucocorticoid therapy. Med Baltimore 55. 39-65, 1976
5. Bass, A , D. Brdicka, P Eyer, S. Hofer, and D. Pette. Metabolic differentiation of distinct muscle types at the level of enzymatic organization. Eur J Biochem 10: 198-206, 1969.
6. Blair, G.P and R.W. Light. Treatment of chronic obstructive pulmonary disease with corticosteroids. Comparison of daily vs alternate-day therapy. Chest 86: 524-528, 1984.
7   Danneskiold Samsoe, B and G. Grimby. The influence of prednisone on the muscle morphology and muscle enzymes in patients with rheumatoid arthritis. Clin Sci 71: 693-701, 1986.
8. Decramer, M., L M. Lacquet, R. Fagard, and P. Rogiers Corticosteroids contribute to muscle weakness in chronic airflow obstruction. Am J Respir Crit Care Med 150: 11-16, 1994.
9. Dekhuijzen, P.N.R. and M Decramer. Steroid-induced myopathy and its significance to respiratory disease: a known disease rediscovered. Eur Respir J 5: 997-1003, 1992.
10. Dekhuijzen, P.N.R., G. Gayan-Ramirez, A. Bisschop, V. de Bock, R. Dom, and M. Decramer. Different effects of methylprednisolone and deflazacort on contractile properties and histopathology of rat diaphragm. Eur Respir J 8· 824-830, 1995.
11. Dekhuijzen, P.N.R., G Gayan-Ramirez, V. de Bock, R. Dom, and M. Decramer. Triamcinolone and prednisolone affect contractile properties and histopathology of rat diaphragm differently. J Clin Invest 92. 1534-1542, 1993.
12. Denardi, C , S. Ausoni, P Moretti, L. Gorza, M. Velleca, M. Buckingham, and S Schiaffino. Type-2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. J Cell Biol 123· 823-835, 1993.
13 Ferguson, G.T , C.G. Irvin, and R.M. Cherniack. Effects of corticosteroids on respiratory muscle histopathology. Am Rev Respir Dis 142: 1047-1052, 1990.
14. Ferguson, G.T , C.G. Irvin, and R.M. Cherniack. Effects of corticosteroids on diaphragm function and biochemistry in the rabbit. Am Rev Respir Dis 141: 156-163, 1990
15 Fernandez-Sola, J., R. Cusso, C. Picado, M. Vernet, J M. Grau, and A. Urbano-Marquez Patients with chronic glucocorticoid treatment develop changes in muscle glycogen metabolism J Neurol Sci 117: 103-106, 1993
16. Frenkel, J K. and M.A. Havenhill. The corticoid sensitivity of golden hamsters, rats, and mice Lab Invest 12. 1204-1220, 1963.
17 Harter, J G., W J. Reddy, and G W. Thorn Studies on an intermittent corticosteroid dosage regimen. N Eng J Med 269: 591, 1963

18   Jacobs, A E M , A A G M Benders, A Oosterhof, and J H Veerkamp Effects of
      growth medium, electrical stimulation and paralysis on various enzyme activities in
      cultured rat muscle cells Comparison with rat muscles in vivo Int J Biochem 24
      751-758, 1992

19   Jacobs, A E M , A Oosterhof, and J H Veerkamp Palmitate oxidation and some
      enzymes of energy metabolism in human muscle and cultured muscle cells Int J Biochem
      19 1049-1054, 1987

20   Jasani, M K Anti-inflammatory steroids mode of action in rheumatoid arthritis and
      homograft reaction In Anti-inflammatory drugs, edited by J R Vane and S H Ferreira
      Berlin Springer-Verlag, 1979, p 598-660

21   Knox, A J , B H Mascie-Taylor, and M F Muers Acute hydrocortisone myopathy in
      acute severe asthma Thorax 41 411-412, 1986

22   Kong, A and W J Jusko Disposition of methylprednisolone and its sodium succinate
      prodrug in vivo and in perfused liver of rats nonlinear and sequential first-pass
      elimination J Pharma Sci 80 409-415, 1991

23   Larsson, L , L Edstrom, B Lindergren, L Gorza, and S Schiaffino MHC composition
      and enzyme-histochemical and physiological properties of a novel fast-twitch motor unit
      type Am J Physiol 261 C93 C101, 1991

24   Lieu, F K , S K Powers, R A Herb, D Criswell, D Martin, C Wood, W Stainsby,
      and C L Chen Exercise and glucocorticoid-induced diaphragmatic myopathy J Appl
      Physiol 75 763-771, 1993

25   Moore, B J , M J Miller, H A Feldman, and M B Reid Diaphragm atrophy and
      weakness in cortisone-treated rats J Appl Physiol 67 2420 2426, 1989

26   Polla, B , R Bottinelli, D Sandoli, C Sardi, and C Reggiani Cortisone-induced changes
      in myosin heavy chain distribution in respiratory and hindlimb muscles Acta Physiol
      Scand 151 353-361, 1994

27   Ruff, R L Endocrine myopathies In Myology, edited by A G Engel and B Q Banker
      McGraw-Hill Book Company, 1986, p 1871 1906

28   Sarett, L H , A A Patchett, and S L Steelman The effects of structural alteration on the
      anti-inflammatory properties of hydrocortisone Progr Drug Res 5 11-153, 1963

29   Schiaffino, S , S Ausoni, L Gorza, L Saggin, K Gundersen, and T Lomo Myosin
      heavy chain isoforms and velocity of shortening of type 2 skeletal muscle fibers Acta
      Physiol Scand 134 575-576, 1988

30   Schiaffino, S , L Gorza, S Sartore, L Saggin, S Ausoni, M Vianello, K Gundersen,
      and T Lomo Three myosin heavy chain isoforms in type 2 skeletal muscle fibers J Musc
      Res Cell Motility 10 197-205, 1989

31   Seene, T Turnover of skeletal muscle contractile proteins in the glucocorticoid myopathy
      J Steroid Biochem 50 1-4, 1994

32   Shepherd, D and P D Garland Citrate synthase from rat liver Meth Enzym 13 11-16,
      1969

33   Shoji, S , A Takagi, H Sugita, and Y Toyokura Muscle glycogen metabolism in
      steroid induced myopathy of rabbits Exp Neurol 45 1 7, 1974

34   Sweeney, H L The importance of creatine kinase reaction the concept of metabolic
      capacitance Med Sci Sports Exerc 26 30 36, 1994

35   Veerkamp, J H and H T B van Moerkerk Peroxisomal fatty acid oxidation in rat and
      human tissues Effect of nutritional state, clofibrate treatment and postnatal development in
      the rat Biochim Biophys Acta 875 301 310, 1986

36   Vignos, P J and R Greene Oxidative respiration of skeletal muscle in experimental
      corticosteroid myopathy J Lab Clin Med 81 365-378, 1973

37   Wilcox, P G , J M Hards, K Bockhold, B Bressler, and R L Pardy Pathologic changes
      and contractile properties of the diaphragm in corticosteroid myopathy in hamsters
      comparison to peripheral muscle Am J Respir Cell Mol Biol 1 191 199, 1989

CHAPTER 4

Effects of long-term low-dose methylprednisolone on rat diaphragm function and structure

R.H.H. van Balkom, P.N.R Dekhuijzen, H.T.M. Folgering,
J.H. Veerkamp[a], J.A.M. Fransen[b], and C.L.A. van Herwaarden

[a] Department of Biochemistry, and [b] Department of Cell Biology and Histology,
University of Nijmegen, The Netherlands

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 six months. Contractile properties of isolated diaphragm strips, immunohistochemical analysis using 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 IIx 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.

Introduction

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 was recently reported following low doses of methylprednisolone (MP) (average daily dose ranging from 1 4 to 21 3 mg during 6 months) in patients with chronic obstructive pulmonary disease (COPD) (3).

The mechanisms by which non-fluorinated steroids cause myopathy are in part 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 four weeks (7). Cortisone acetate 10 mg/kg/day during three 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 ATP-ase activity depending on their type (I, IIa, IIx, IIb) and are therefore in part responsible for the differences in contractile

properties between the different fibers (18,24) Since the fast and most powerful fibers (i e type IIx and IIb) are expected to be most sensitive to the side effects of corticosteroids (26), 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 previously (19,31), 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 investigation was designed to study 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 methylprednisolone (MP) 0 2 mg/kg/day sc for six months Morphological and biochemical parameters were determined to obtain insight in 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 a MP group, receiving methylprednisolone hemisuccinate (Sigma Chemicals, Bornem, Belgium) 0 2 mg/kg sc daily (7 days a week) for six months With each injection all animals received a similar volume (~0 20 ml) The rats were fed ad libitum, held on a 12/12 hour light-dark regime and weighed twice weekly The animals were daily injected between 8 30 and 10 00 a m Although daily food intake was not accurately quantified (animals were not held in metabolic cages), food intake appeared to be similar in both groups At the end of the treatment period, contractile properties, immunohistochemical and biochemical characteristics of the diaphragm were examined All MP-treated animals were investigated between 23 and 30 hours 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 anaesthetized with sodium pentobarbital (70 mg/kg *ip*) A poly-ethylene 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 $MgCl_2$, 1 KH_2PO_4, 24 $NaHCO_3$, 2.7 $CaCl_2$, and 7 glucose. D-tubocurarine chloride 25 μM (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 were oxygenated with 95% O_2 and 5% CO_2. The central tendon insertion of the bundles were tied to a fixed point and the costal margin origin to an isometric force transducer (Sensotec, model 31/1437, Columbus OH, USA). 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. thermo-equilibration period. The following measurements were made:

Twitch characteristics: two twitches were recorded at Lo to obtain maximal twitch force (P_t), contraction time (CT), and half relaxation time (½RT). The averages were used for further analysis (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_o) (7).

Force-frequency protocol: muscle bundles were stimulated every 2 min. with 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^2). Cross-sectional area (CSA) was measured by dividing diaphragm bundle weight by muscle density (1.056 mg/mm^3) and bundle length (20).

Histological and immunohistochemical procedures

Muscle strips obtained from the costal part of the right hemidiaphragm were embedded in Tissue-Tek● in a plastic holder. The muscle fibers were oriented parallel to the long side of the holder. Subsequently, these specimens were quickly frozen in isopentane cooled in liquid N_2 followed by further freezing in liquid N_2. 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 H&E staining

Anti myosin heavy chain antibodies (Regeneron Pharmaceuticals, New York, U S A) 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 IIa MHCs, BF-35 reactive with type I, IIa and IIb but not with type IIx MHCs, and BF-F3 reactive with type IIb MHCs (25) Incubation with myosin heavy chain antibodies was performed at room temperature for 1 hour Antibodies were subsequently labelled with ultra small immunogold reagent followed by silver enhancement (Aurion, Wageningen, The Netherlands) A minimum of 350 fibers were analysed from each diaphragm using a Sprynt-based, PC-Image digital analysis system (Bos Inc , Waddinxveen, the Netherlands) Fiber type distribution and cross sectional area (CSA) were analysed for type I, IIa, IIx 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 glycogenolytic enzyme phosphorylase, 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 remainings of the left and right hemidiaphragm These diaphragm parts were quickly frozen in liquid N_2 and stored at -80°C Segments of fresh frozen diaphragm were thawed in ice-cooled buffer containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7 4) In this buffer muscle homogenates (5% wt/vol) were prepared by hand homogenization, using a Potter-Elvehjem glass-teflon homogenizer

Total phosphorylase (a+b) activity was assayed at 37 °C according to the method described by Jacobs *et al* (16), and was expressed as μmol NADPH formed/min g tissue HADH activity, assessed at 50 μM acetoacyl-CoA at 37 °C (1), was expressed in nmol HADH oxidized/min g tissue Citrate synthase activity was determined at 25°C (27) and was expressed as μmol coenzyme A formed/min 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 Illinois, USA) was used for statistical analysis Data of contractile properties of the two bundles obtained from one rat were averaged and

compared using Student 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 $\pm SE$

Results

Body weight
No differences in body weight were observed at the start of the study (375 ± 7 g in saline vs 385 ± 4 g 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 g in MP vs 529 ± 9 g 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 P_t and P_o decreased by $\sim 14\%$ following MP administration ($p < 0.001$) (Table 1) No changes were observed in P_t/P_o ratio, CT or ½RT (Table 1)

The force-frequency curves, expressed in N/cm^2, showed a significant reduction in force generation at all stimulation frequencies in the MP group (Fig 1) When normalized for P_o, forces were similar in the two groups (data not shown)

Table 1 Diaphragm contractile properties

treatment	P_t	CT	½RT	P_o	P_t/P_o
	N/cm^2	ms		N/cm^2	
control	7.7 ± 0.02	25.7 ± 0.5	23.1 ± 0.3	27.1 ± 0.06	0.29 ± 0.01
MP	$6.6 \pm 0.01^*$	26.0 ± 0.5	22.9 ± 0.3	$23.4 \pm 0.06^*$	0.29 ± 0.01

means $\pm SE$, P_t twitch force, CT contraction time, ½RT half relaxation time, P_o maximal tetanic force
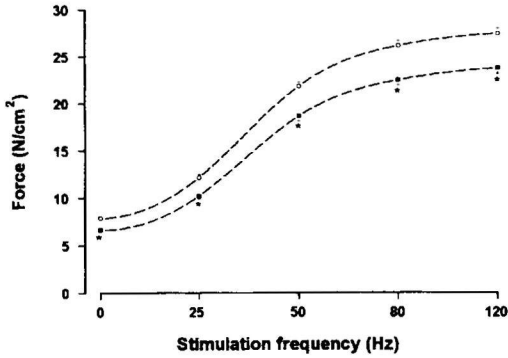* $p < 0.001$ compared to control

Figure 1. Force-frequency curve
Open circles: control; closed squares: MP;
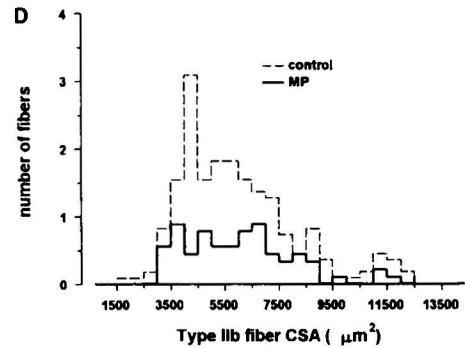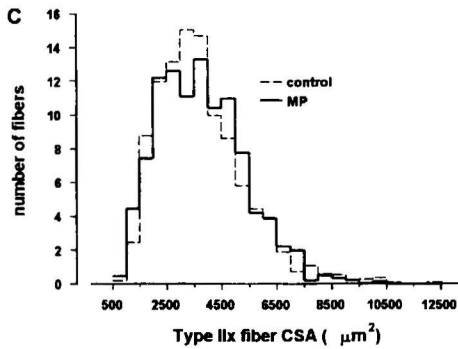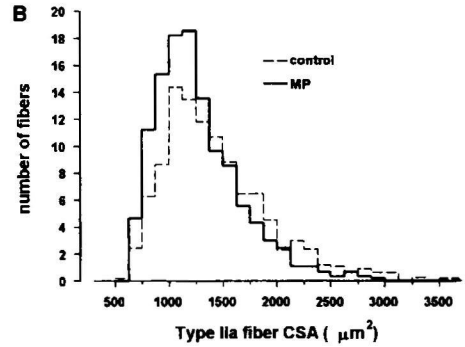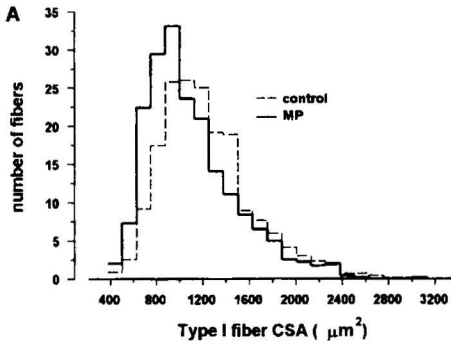dashed line: pooled SD;
* p < 0.01 compared to control

Figure 2. Histograms showing the distribution of fiber CSA
A: type I fibers, B: type IIa fibers, C: type IIx fibers, D: type IIb fibers

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 reduction in the percentage of type IIb fibers in the MP diaphragm, being $1.8 \pm 0.4\%$ compared to $4.8 \pm 0.6\%$ in the control group ($p < 0.05$) (Table 2). No significant changes were found in the numbers of type I, IIa and IIx fibers among the two groups.

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 area was reduced in the MP group, while the contribution of type IIx fibers was increased (Table 2).

Table 2. Fiber type distribution, cross sectional area, relative fiber type contribution to the total diaphragm muscle area

	type I	type IIa	type IIx	type IIb
fiber type distribution	%	%	%	%
control	41.6 ± 1.0	27.1 ± 1.1	26.4 ± 0.64	4.8 ± 0.6
MP	43.5 ± 1.6	29.0 ± 1.3	25.7 ± 1.0	$1.8 \pm 0.4^*$
fiber CSA	μm^2	μm^2	μm^2	μm^2
control	1164 ± 95	1403 ± 140	3528 ± 421	5828 ± 599
MP	$1041 \pm 88^*$	$1214 \pm 104^*$	3532 ± 415	5926 ± 557
fiber type contribution to total diaphragm area	%	%	%	%
control	23.2 ± 0.6	18.4 ± 0.4	45.2 ± 1.3	13.2 ± 1.3
MP	24.2 ± 0.9	19.8 ± 0.8	$50.2 \pm 0.7^*$	$5.8 \pm 1.3^*$

means $\pm$ SE; * $p < 0.05$ compared to control

Biochemistry

MP administration caused a significant reduction in glycogenolytic activity, as measured

by phosphorylase (p < 0.01). Fatty acid oxidation capacity, measured by HADH, and oxidative capacity, indicated by CS activity, both increased in the MP group (p < 0.01) (Table 3).

Table 3. Biochemical analysis

treatment	phosphorylase	CS	HADH
	U/g	*U/g*	*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*

means ±SE; CS: citrate synthase; HADH: 3-hydroxyacyl-CoA dehydrogenase;
* p < 0.01 compared to control

Discussion

The aim of the present study was to evaluate the kind and the extent of changes in rat diaphragm caused by low-dose administration of MP during six 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 towards slower fibers. However, these changes in muscle morphology and biochemistry, although statistically significant, were subtle and explained at most in part the reduction in diaphragm force generation.

Our intention was to evaluate the effects of a low dose of a non-fluorinated 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 metabolism of MP have been described as being similar in rats and humans (17,23). Assuming an absorption of 100%, 0.2 mg/kg of MP may be equivalent to a dose of ~14 mg/day in a 70 kg human. This is probably an overestimation since an absorption of 60% was found after im injection of cortisone acetate (20). In addition, the sc route requires higher doses to produce similar effects compared to im administration (12). Prolonged prednisolone administration in doses of 10-15 mg daily are no exception in the treatment of patients with COPD, although the

therapeutic efficacy of corticosteroids in COPD is at least controversial (15)

To our knowledge, no animal studies have been performed using low steroid dosages for a prolonged period In a previous study we found that 1 mg/kg/day MP for eight weeks depressed the force-frequency curve in rat diaphragm (31) The clear reduction in diaphragm force as observed in this study, was not found previously following 0 5 mg MP/kg/day for 6 weeks (5), or following 5 mg/kg/day of prednisolone for four weeks (7) Cortisone acetate in a dose of 100 mg/kg/day for 10 days did not induce functional changes either (20) The discrepancy with the present data suggests that diaphragm impairment is likely to occur following low dosages of corticosteroids, particularly if they are administrated for prolonged periods

At the end of the six month treatment period, body weight in the MP animals was 5% lower compared to the control group The question may arise to which extent this reduction in body weight growth may influence diaphragm force production Severe nutritional depletion (40% decrease in body weight over a period of 6 weeks) did not lead to a reduction in diaphragm force production in rats (6) In addition, in the same study, nutritional depletion caused a ~30% decrease in type I and IIa fiber CSA, while type IIb fiber CSA decreased by ~50% Since these finding are substantially different from the morphometric results in the present study following MP, the observed differences in force production are not likely to be the result of the small difference in final body weight

Since diaphragm bundle dimensions were similar in both groups, the reduced force generation of the MP treated diaphragm is unlikely to be caused by differences in oxygenation of these muscle strips An overestimation of the CSA due to increased extracellular fluid is unlikely since MP has little or no mineralocorticoid activity (14) An increase in connective tissue as noticed previously in the diaphragm following prednisolone treatment (5 mg/kg/day for four weeks) (7), may also lead to an overestimation of the muscle CSA and therefore reduce force per CSA However, differences in connective tissue proportion in the diaphragm muscle were not observed in the present study Pathologic features like myonecrosis (7,9), vacuolization (7,9), excess of internal nuclei and greater than normal variation of fiber type diameter (7) were described following administration of prednisolone 5 mg/kg/day for four weeks (7), and cortisone acetate 10 mg/kg daily for 3 weeks (9), respectively None of the above mentioned pathologic changes, however, occurred in the present study, possibly as a result of the low dose studied

Another explanation for the functional changes in this study may be a change in fiber distribution and size Indeed, our data show that MP administration caused small but significant changes in muscle morphometry First, a significant reduction in the percentage of type IIb fibers was found, whereas no changes occurred in the numbers of

type I, IIa and IIx fibers. This is in line with the data by Polla et al. (21) who even reported a total disappearance of type IIb fibers in rat diaphragm treated with cortisone acetate (100 mg/kg for 11 days). The cause of this phenomena is not entirely clear. Corticosteroids decrease the rate of amino acid incorporation into MHCs, resulting in a decreased turnover rate of MHCs in the muscle cell (26). This decrease in turnover rate is most pronounced in the fast twitch muscle fibers (26), thus type IIb fibers are likely to be affected most. This may lead to a decrease in IIb MHCs in type IIb fibers. Since genes of IIx MHCs are coexpressed in a number of type IIb muscle fibers (8), it may be speculated that an increase of IIx MHCs in type IIb muscle fibers may occur to compensate for the decrease in type IIb MHCs.

Second, significant but small reductions in type I and IIa fiber CSA were observed in the MP group, while type IIx and IIb fiber CSA were not altered. Previous studies using ATP-ase based fiber typing showed no effect of prednisolone (7) and MP (5) on rat diaphragm fiber size and composition. In another study, atrophy of type I, IIa and IIb fibers was observed after administration of cortisone acetate (10 mg/kg daily for 3 weeks) in rabbits (9). Dose and duration of treatment with steroids presumably explain these differences.

When combining the two above described types of alterations, the relative contribution of type IIb fibers to total diaphragm muscle area was reduced in the MP group while the contribution of type IIx fibers increased. This might be of functional significance, since type IIx and IIb MHCs possess differences in contractile properties. Maximum velocity of shortening (24) and resistance to fatigue (18) in type IIx muscle fibers are intermediate between those of type IIa and IIb fibers. In addition, twitch and tetanic forces are higher in IIb compared to IIa and IIx motor units (18). 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 degre of shortening is associated with loss of passive tension present in vivo (30). 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 strip were fixed at

optimal length (29). Thus, the physiological differences in size among the different fiber types did not appear to be affected by muscle shortening in the present study.

In line with the small shift towards slower fiber types, glycogenolytic 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 (10) is the result of a decrease in glycogen breakdown or an increase in glycogen production (28) or both (11). This reduction in glycogenolytic 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) (19) or 8 weeks of MP (1 mg/kg/day) (31) did not change HADH activity. The increase in oxidative capacity in this study matches with our morphometrical observations, since type IIx muscle fibers are known to have a rich mitochondrial content in contrast to type IIb fibers (25). This is in line with the increase in oxidative staining reaction in skeletal muscle reported previously (32). An increase in CS activity was also found following MP 1 mg/kg/day (31). In contrast to these observations, CS activity in rat diaphragm was reduced following 5 mg/kg prednisolone per day for 10 days (19). Yet, no changes in CS activity were reported following 0.5, 1 or 2 mg/kg per day (19), suggesting that CS activity was only reduced following high dosages of prednisolone.

Changes in metabolism due to corticosteroids may, besides depending on dose and duration of the steroid, also be related to fiber type composition of the muscle (10,19). The resistance of different fiber types is believed to depend on their ability to compensate the steroid-induced deficiency of the glycogenolytic route by converting to oxidative metabolism (32). 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 a part of the reduction in muscle force generation, other alterations are presumably involved. For example, protein degeneration, caused by corticosteroids, may lead to a reduction in myofibrillar protein density (22). 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 potenttial 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 compromized as result of hyperinflation, malnutrition,

inactivity, disturbances in blood gases and cardiac failure (13). Indeed, it has recently been shown that in these patients administration of low dose corticosteroids compromise diaphragm function even more (2). The present study shows that these functional alterations are accompanied by biochemical and structural changes in the diaphragm.

References

1. Bass, A., D. Brdicka, P. Eyer, S. Hofer, and D. Pette. Metabolic differentiation of distinct muscle types at the level of enzymatic organization. Eur J Biochem 10: 198-206, 1969.

2. Decramer, M., V. de Bock, and R. Dom. Functional and histologic picture of steroid-induced myopathy in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 153: 1958-1964, 1996.

3. Decramer, M., L.M. Lacquet, R. Fagard, and P. Rogiers. Corticosteroids contribute to muscle weakness in chronic airflow obstruction. Am J Respir Crit Care Med 150: 11-16, 1994.

4. Dekhuijzen, P.N.R. and M. Decramer. Steroid-induced myopathy and its significance to respiratory disease: a known disease rediscovered. Eur Respir J 5. 997-1003, 1992.

5. Dekhuijzen, P.N.R., G. Gayan-Ramirez, A. Bisschop, V. de Bock, R Dom, R. Bouillon, and M. Decramer. Rat diaphragm contractility and histopathology are affected differently by low dose treatment with methylprednisolone and deflazacort. Eur Respir J 8: 824-830, 1995.

6. Dekhuijzen, P.N.R., G. Gayan-Ramirez, A. Bisschop, V de Bock, R. Dom, and M. Decramer. Corticosteroid treatment and nutritional deprivation cause a different pattern of atrophy in rat diaphragm. J Appl Physiol 78: 629-637, 1995.

7. Dekhuijzen, P.N.R., G. Gayan-Ramirez, V. de Bock, R Dom, and M. Decramer. Triamcinolone and prednisolone affect contractile properties and histopathology of rat diaphragm differently J Clin Invest 92: 1534-1542, 1993.

8. Denardi, C., S. Ausoni, P. Moretti, L. Gorza, M. Velleca, M. Buckingham, and S Schiaffino. Type-2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene J Cell Biol 123: 823-835, 1993

9. Ferguson, G.T., C.G. Irvin, and R.M. Cherniack. Effects of corticosteroids on respiratory muscle histopathology Am Rev Respir Dis 142. 1047-1052, 1990.

10. Ferguson, G.T., C.G. Irvin, and R M Cherniack. Effects of corticosteroids on diaphragm function and biochemistry in the rabbit Am Rev Respir Dis 141 156-163, 1990.

11. Fernandez-Sola, J., R. Cusso, C. Picado, M. Vernet, J M. Grau, and A. Urbano-Marquez. Patients with chronic glucocorticoid treatment develop changes in muscle glycogen metabolism. J Neurol Sci 117. 103-106, 1993.

12. Frenkel, J K. and M A. Havenhill. The corticoid sensitivity of golden hamsters, rats, and mice. Lab Invest 12: 1204-1220, 1963

13 Gibson, J Respiratory muscles. In Chronic obstructive pulmonary disease, edited by P M.A. Calverley and N B. Pride. London: Chapman and Hall, 1995, 185-203

14. Haynes, R C., JR. Adrenocorticotropic hormone; Adrenocortical steroids and their synthetic analogs; Inhibitors of the synthesis and actions of adrenalcortical hormones. In: The pharmacological basis of therapeutics, edited by A. Goodman Gilman, T.W. Rall, A.S. Nies, and P. Taylor. New York: Pergamin Press, 1990, 1431-1462.

15 Hudson, L.D. and C.M. Monti. Rationale and use of corticosteroids in chronic obstructive pulmonary disease. Med Clin North Am 74: 661-690, 1990.

16. Jacobs, A.E.M., A.A.G.M. Benders, A. Oosterhof, and J.H. Veerkamp. Effects of growth medium, electrical stimulation and paralysis on various enzyme activities in cultured rat muscle cells. Comparison with rat muscles in vivo. Int J Biochem 24: 751-758, 1992.

17. Jasani, M.K Anti-inflammatory steroids: mode of action in rheumatoid arthritis and homograft reaction. In: Anti-inflammatory drugs, edited by J.R. Vane and S.H. Ferreira. Berlin: Springer-Verlag, 1979, 598-660.

18. Larsson, L., L Edstrom, B Lindergren, L Gorza, and S. Schiaffino. MHC composition and enzyme-histochemical and physiological properties of a novel fast-twitch motor unit type. Am J Physiol 261: C93-C101, 1991

19 Lieu, F K., S K. Powers, R.A. Herb, D. Criswell, D Martin, C. Wood, W Stainsby, and C.L Chen. Exercise and glucocorticoid-induced diaphragmatic myopathy. J Appl Physiol 75 763-771, 1993

20 Moore, B.J., M.J. Miller, H.A. Feldman, and M B. Reid. Diaphragm atrophy and weakness in cortisone-treated rats. J Appl Physiol 67: 2420-2426, 1989

21. Polla, B , R. Bottinelli, D. Sandoli, C Sardi, and C. Reggiani. Cortisone-induced changes in myosin heavy chain distribution in respiratory and hindlimb muscles. Acta Physiol Scand 151. 353-361, 1994.

22 Ruff, R L Endocrine myopathies. In: Myology, edited by A.G Engel and B.Q. Banker. McGraw-Hill Book Company, 1986, 1871-1906.

23 Sarett, L.H., A.A. Patchett, and S.L. Steelman. The effects of structural alteration on the anti-inflammatory properties of hydrocortisone. Progr Drug Res 5: 11-153, 1963.

24 Schiaffino, S., S. Ausoni, L Gorza, L. Saggin, K Gundersen, and T. Lomo. Myosin heavy chain isoforms and velocity of shortening of type 2 skeletal muscle fibers. Acta Physiol Scand 134: 575-576, 1988.

25. Schiaffino, S., L. Gorza, S. Sartore, L. Saggin, S. Ausoni, M. Vianello, K. Gundersen, and T. Lomo. Three myosin heavy chain isoforms in type 2 skeletal muscle fibers. J Musc Res Cell Motility 10: 197-205, 1989.

26. Seene, T. Turnover of skeletal muscle contractile proteins in the glucocorticoid myopathy. J Steroid Biochem 50: 1-4, 1994.

27. Shepherd, D. and P.D. Garland. Citrate synthase from rat liver. Meth Enzym 13: 11-16, 1969.

28 Shoji, S , A Takagi, H. Sugita, and Y. Toyokura. Muscle glycogen metabolism in steroid induced myopathy of rabbits. Exp Neurol. 45: 1-7, 1974.

29. Sieck, G C., W.Z. Zhan, Y.S Prakash, M.J. Daood, and J.F. Watchko. SDH and actomyosin ATPase activities of different fiber types in rat diaphragm muscle. J Appl Physiol 79. 1629-1639, 1995

CHAPTER 5

Corticosteroid effects on diaphragm neuromuscular junctions

G.C. Sieck[ab], R.H.H. van Balkom, Y.S. Prakash[a],
W.Z. Zhan[a], and P.N.R Dekhuijzen

Submitted

[a] Departments of Anesthesiology, and [b]Physiology and Biophysics
Mayo Clinic and Foundation, Rochester MN, USA

Abstract

The effects of corticosteroid (CS) treatment (6 mg/kg prednisolone; 3 weeks) on neuromuscular junction (NMJ) morphology and neuromuscular transmission in rat diaphragm muscle (DIAm) were compared to weight matched (SHAM) and *ad libitum* fed control (CTL) groups. Fibers were classified based on myosin heavy chain (MHC) isoform expression. CS treatment caused significant atrophy of fibers expressing MHC_{2X}, either alone or with MHC_{2B} (type IIx/b). Fibers expressing MHC_{Slow} (type I) and MHC_{2A} (type IIa) were unaffected by CS. The planar areas of nerve terminals and motor endplates at type IIx/b fibers were smaller in CS treated DIAm compared to SHAM and CTL. However, CS-induced atrophy of type IIx/b fibers exceeded changes in NMJ morphology. Thus, when normalized for fiber diameter, NMJs were relatively larger in the CS group compared to CTL. Neuromuscular transmission failure, assessed *in vitro* by comparing force loss during repetitive (40 Hz) nerve vs. direct muscle stimulation, was less in CS treated DIAm. These results indicate that alterations in NMJ morphology following CS treatment depend upon fiber type, and may contribute to improved neuromuscular transmission.

Introduction

Exogenously administered corticosteroids (CS) are used clinically to treat a variety of pulmonary conditions including asthma and severe chronic obstructive pulmonary disease. Several studies have reported atrophy of diaphragm muscle (DIAm) fibers and muscle weakness (20, 40, 42). In the DIAm, the effects of CS treatment, especially in high doses, appear to be manifest predominantly at type IIx/b fibers (20, 40, 42).

There are several previous studies which have reported that CS treatment induces morphological changes at the neuromuscular junction (NMJ) (7, 8, 18, 19, 39), but only a few studies (7, 8) have examined fiber type differences in CS-induced changes in NMJ morphology. In these studies, which were performed in limb muscles predominantly expressing a single fiber type, NMJ adaptations were more found to be more prominent at type I fibers. Previously, we reported that in the rat DIAm, the structure of pre- and postsynaptic elements of NMJs varied with fiber type, as determined by myosin heavy chain (MHC) isoform expression (27). The planar areas of nerve terminals and motor endplates of DIAm fibers expressing the MHC_{2X} isoform, either alone or in combination with the MHC_{2B} isoform (type IIx/b fibers), are larger and more complex than those at fibers expressing the MHC_{Slow} (type I fibers) and MHC_{2A} (type IIa fibers) isoforms. In the present study, we hypothesized that the selective atrophy of type IIx/b induced by CS treatment may also be reflected by a selective effect at NMJs on these fibers.

Several studies have demonstrated changes in the electrophysiological properties of CS-treated NMJs (2, 4-6, 24, 38, 43, 44). For example, van Wilgenburg et al. (38, 39)

reported that low doses of dexamethasone and prednisolone result in increased miniature endplate potential (mepp) amplitude at rat DIAm NMJs, while high doses decrease mepp amplitude Similar results at low CS doses were also reported by Dalkara and Onur (5) In the rat DIAm, Wilson et al (43) reported that prednisolone facilitated spontaneous release of ACh, manifest by a two to threefold increase in mepp frequency Furthermore, they found a reduction of mepp amplitude suggesting an additional postsynaptic effect Leeuwin et al (18) reported an increase in synaptic vesicle size, suggestive of an increase in quantal size for neuromuscular transmission If CS treatment leads to an increase in the quantal release of ACh at the NMJ, it might be expected that such an effect should improve neuromuscular transmission However, with repetitive stimulation, an increase in quantal release of ACh may lead to a more rapid depletion of transmitter stores and an impairment of neuromuscular transmission In previous studies, we found that type IIx/b DIAm fibers are more susceptible to neuromuscular transmission failure than type I and IIa fibers (15) To date, the effects of CS treatment on neuromuscular transmission failure during repetitive stimulation in the DIAm have not been examined The purpose of the present study was to examine the effects of three weeks of prednisolone treatment on the morphological properties of pre- and postsynaptic elements of the NMJ on type-identified fibers of the rat DIAm, and to evaluate DIAm neuromuscular transmission We hypothesized that the effects of prednisolone will be more pronounced on the NMJs of type IIx/b fibers

Methods

Twenty-four male Sprague-Dawley rats were randomly divided into 3 groups   1) Normal controls (CTL, n=8), 2) Surgical sham and weight-matched controls (SHAM, n=8), and 3) Corticosteroid-treated (CS, n=8) The animals were housed in separate cages under a 2-12 light dark cycle, fed with Purina Rat Chow, and provided with water *ad libitum* Animals in the CTL and CS groups were also provided food *ad libitum*, while the rats in the SHAM group were given limited quantities of food to match their weight growth curve with that of the CS group Body weights were monitored regularly

Animals were anesthetized with pentobarbital sodium (70 mg/kg), and the DIAm was rapidly excised Five muscle segments (2-3 mm wide) were dissected from the midcostal region of the right and left sides of the DIAm In one muscle strip from the right side, neuromuscular transmission failure was assessed by repetitive stimulation of the phrenic nerve (see below) In the remaining strips, resting (excised) muscle length was measured using digital calipers, and the strip was then stretched to 1 5 times this excised length, to approximate optimal length (L_o) for force production (25) before pinning to a sylgard-lined petri dish for immunocytochemical analysis

All procedures were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic, and were in strict accordance with the American Physiological Society Animal Care Guidelines Surgical procedures were performed under aseptic conditions

The recovery of animals from surgery was carefully monitored

Corticosteroid treatment

Surgical procedures were performed only on animals in the CS and SHAM groups Under ketamine (60 mg/kg) and xylazine (2 mg/kg) anesthesia, a miniosmotic pump (Alzet 2M4) filled with either 37 5 mg/ml prednisolone sodium succinate (Upjohn) in aqueous suspension (CS group), or sterile physiological saline (SHAM group) was implanted *sc* in the dorsum of each animal This concentration of prednisolone provided a final *sc* dose of 6 mg/kg at a flow rate of 2 5ml/h At the end of a three week treatment period, the remaining amount of solution in the pump was measured to ensure adequate drug delivery In addition, at the time of the terminal experiment, 3 ml of blood was collected to measure both steroid and thyroid hormone (T_3 and T_4) levels

Immunohistochemistry

Detailed descriptions of the three color fluorescent immunohistochemical technique used to label nerve terminals, motor endplates and muscle fibers have been recently published (27) Briefly, motor endplates were first labeled by incubation of each muscle strip in 5-10 mg/ml tetramethylrhodamine a-bungarotoxin (Molecular Probes Inc) in phosphate buffer (PB) The samples were then washed and immersion-fixed in 2% paraformaldehyde The fixed samples were blocked for non-specific staining using 4% normal donkey serum (NDS) in 0 1M Tris-buffered saline (0 15M NaCl) containing 0 3% Triton-X100 (TBS-Tx) The tissue was then incubated in a primary mixture of 1 200 donkey anti-protein gene product (Biogenesis Inc , to label axons and nerve terminals), and any one of the following antibodies specific to different MHC isoforms

1   1 400 mouse anti- MHC_{Slow} IgG (Novocastra)
2   1 200 mouse anti-MHC_{2A} IgG (14)
3   1 200 mouse anti-$MHC_{All\ 2X}$ IgG (31)
4   1 20 mouse anti-MHC_{2B} IgM (31)

Following incubation in these primary antibodies, the samples were washed and incubated further in a secondary cocktail of 1 100 fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) and 1 200 Cy5 conjugated donkey anti mouse IgG or IgM (Jackson Immunoresearch) All samples were finally washed, blotted dry, mounted on slides, and coverslipped with low-fluorescence immersion oil (Cargille Labs Inc , refractive index 1 515)

Confocal imaging and analysis

Detailed descriptions of the three-color confocal imaging and analysis procedures have also been recently published (27) Briefly, optical sections of labeled NMJs and muscle fibers were obtained using a Bio-Rad MRC500 confocal system mounted on an Olympus BH2 microscope and equipped with an Ar Kr laser and a Z axis focus controller A 40X 1 25 NA oil-immersion objective lens was used, and the step size for optical sectioning was set to 0 8 mm, matching the optical section thickness for the confocal system (29)

Morphometric and registration validations of the confocal imaging technique were performed using multicolor fluorescent latex beads, as described previously (27) 2D projection images were obtained by superimposing a stack of optical sections

A comprehensive image analysis software package (ANALYZE, Biomedical Imaging Resource, Mayo Foundation) running on a Sun 4/330 UNIX workstation was used to create 3D reconstructions of the digitized images and to make 2D and 3D morphometric measurements Grayscale images were converted to binary images using an intensity threshold An automated procedure was used to delineate the borders of nerve terminals and endplates Planar areas of nerve terminals and endplates were measured from the 2D projections, and normalized to muscle fiber diameter The extent of overlap between nerve terminal and endplate was estimated by subtracting the binary image of the nerve terminal from the binary image of the endplate The pattern of arborization of nerve terminals and endplates was quantified from optical sections using a tree-tracing tool in ANALYZE Details of this analysis technique have been previously published (27), and a schematic representation of the procedure is shown in Figure 1 The point of origin for each nerve terminal was defined as the first branch point of the axon The longest uninterrupted branches were classified as primary branches, and daughter branches arising from the primary branches, regardless of thickness, were classified as secondary branches Branch length was measured along the center of the branch The total number of branches and total branch length were determined The average distance between the occurrence of secondary branches along a primary branch (individual branch length) was used as an index of arborization

Assessment of neuromuscular transmission failure
The procedures for assessing neuromuscular transmission failure in the rat DIAm have been previously described (12, 15) A muscle strip from the right mid costal region, together with a 1-2 cm length of phrenic nerve, was mounted vertically in a glass chamber containing oxygenated (95% O_2 5% CO_2) Ringers' solution (137 mM Na^+, 5 mM K^+, 5 04 mM Ca^{2+}, 2mM Mg^{2+}, 121 mM Cl, 20 mM HCO_3, and 1 9 mM HPO_4^2, pH 7 4) maintained at 26°C The muscle was attached at one end to a calibrated force transducer and at the other end to a micromanipulator for adjustment of muscle length The muscle was stimulated directly (1 ms pulses) through platinum plate electrodes placed on either side of the muscle using a Grass stimulator and power amplifier (Section of Engineering, Mayo Clinic) Muscle fiber length was incrementally adjusted until maximal isometric twitch responses were obtained (L_o) The phrenic nerve was stimulated through a suction electrode using 0 2-ms duration pulses In both cases, stimulus intensity was increased until maximal twitch force responses were obtained and then set at 125% of this value (supramaximal intensity)

The phrenic nerve was stimulated repetitively at 40 Hz in 330-ms duration trains repeated every s (duty cycle 33%) for a 2 min period Direct muscle stimulation was

superimposed every 15s and the relative contribution of neuromuscular transmission failure to total fatigue was estimated using the following formula (1)

$$\% \text{ Neuromuscular Transmission Failure} = (DN\text{-}DM)/(1\text{-}DM)$$

where DN is force loss during nerve stimulation (normalized to initial muscle force) and DM is force loss during direct muscle stimulation (normalized to initial muscle force)

Statistical analysis

Muscle fibers were classified based on immunoreactivity for the different MHC antibodies Fibers classified as type I were immunoreactive only for the anti-MHC$_{Slow}$ antibody Similarly, type IIa fibers were reactive only for the anti-MHC$_{2A}$ antibody Approximately 22% of all DIAm fibers were not immunoreactive for the anti-MHC$_{All\ 2X}$ antibody, and were thus classified as type IIx Approximately 12% of all DIAm fibers were immunoreactive for the anti-MHC$_{2B}$ antibody, but previously we found that most of these fibers also expressed the MHC$_{2X}$ isoform (32) Given the relatively rare incidence of "true" type IIb fibers in the rat DIAm ($\sim 4\%$ (32)), and the fact that it was not possible to distinguish these fibers from those co-expressing the MHC$_{2X}$ isoform by immunohistochemistry, we combined the fibers expressing the MHC$_{2X}$ and MHC$_{2B}$ isoforms into a single group classified as type IIx/b Based upon a power analysis at b=0 8, and a=0 05, we determined that at least 15 fibers per type in each DIAm were required to establish a difference in NMJ architecture (27)

Means and standard errors were calculated for each parameter of interest Differences between groups were examined using a 2-way ANOVA, with experimental group and fiber type as grouping variables When justified, a Student's t-test was used for *post hoc* analysis Statistical significance was accepted at P < 0 05

Results

Prednisolone and thyroid hormone levels

Serum prednisolone levels were below the detectable range ($< 0\ 1\ \mu g/dl$) in the CTL and SHAM groups, while in the CS treated animals the prednisolone level was 4 9±1 μg/dl Serum T_3 and T_4 levels were not significantly affected by predisolone treatment (CTL T_3 46±3 ng/dl, T_4 4 0±0 2 mg/dl, SHAM T_3 48±6 ng/dl, T_4 4 2±0 4 mg/dl, and CS T_3 47±4 ng/dl, T_4 3 9±0 4 mg/dl) At the end of the 3-week period, body weights of the CS animals were significantly lower than CTL (P < 0 05) but comparable to the SHAM animals (final body weights, 327 2±8 8 g for CS, 337 5±9 5 g for SHAM and 397 3±3 4 g for CTL)

Muscle fiber diameters

In all three experimental groups, there were significant differences in DIAm fiber diameters across fiber types (Table 1) In CTL and SHAM groups, the diameters of type I DIAm fibers were the smallest followed in rank order by type IIa and IIx/b (P < 0 05, Table 1)

In the CS-treated animals, the diameters of type I and IIa fibers were not significantly different, although type IIx/b fibers remained larger (P < 0.05; Table 1). The diameter of type IIx/b fibers in the CS DIAm were significantly smaller than those of type IIx/b fibers in both CTL and SHAM animals (P < 0.05; Table 1). The diameters of type IIx/b fibers in the SHAM group were also smaller than those of CTL animals (P < 0.05; Table 1).

Nerve terminal morphology
In each experimental group, the planar (2D) area of nerve terminals innervating type I and IIa fibers were comparable, but smaller than that of nerve terminals innervating type IIx/b fibers (P < 0.05; Table 2; Fig. 2). The planar areas of nerve terminals innervating type I and IIa fibers were not significantly different across experimental groups, while the planar area of nerve terminals innervating type IIx/b fibers varied significantly. In SHAM animals, the planar area of nerve terminals innervating type IIx/b fibers was significantly larger than that of CTL (P < 0.05), whereas, in CS animals, type IIx/b fiber nerve terminal area was significantly smaller than both CTL and SHAM animals (P < 0.05; Table 2).

When normalized for fiber diameter, the planar areas of nerve terminals innervating different fiber types in CTL animals displayed a rank order, with type I > IIa > IIx/b (P < 0.05; Table 2). This rank order in normalized nerve terminal planar area was essentially reversed in both SHAM or CS animals where the normalized nerve terminal area of type IIx/b fibers was significantly greater than that of both type I and IIa fibers (P < 0.05; Table 2). Compared to CTL, the normalized nerve terminal area of type IIx/b fibers was significantly larger in SHAM and CS groups (P < 0.05; Table 2). However, in CS treated animals, this increase in normalized nerve terminal area was due to the disproportionate reduction of fiber diameter since nerve terminal area decreased (Tables 1 and 2). In contrast, in SHAM animals, the increase in normalized nerve terminal area resulted from both a decrease in fiber area as well as an increase in nerve terminal planar area (Tables 1 and 2).

In CTL and SHAM animals, the total number of nerve terminal branches displayed a rank order across different fiber types, with type I < IIa < IIx/b (P < 0.05; Table 2; Fig. 2). In CS animals, the total number of nerve terminal branches at type IIx/b fibers was also significantly greater than that at type I and IIa fibers (P < 0.05; Table 2), but there was no difference between type I and IIa fibers. In the CS group, the total number of nerve terminal branches on type IIx/b fibers was significantly lower, compared to CTL (P < 0.05; Table 2). In CTL animals, the total cumulative length of all nerve terminal branches displayed a rank order across different fiber types, with type I < IIa < IIx/b (P < 0.05; Table 2). In SHAM and CS groups, total nerve terminal branch length was not significantly different between type I and IIa fibers, but remained larger in type IIx/b fibers. In the CS group, the total cumulative length of type IIx/b nerve terminal branches was significantly reduced compared to both CTL and SHAM groups (P < 0.05; Table 2). In the CTL DIAm, the mean individual branch length of nerve terminals at type I fibers was greater than that

of nerve terminals at type IIa and IIx/b fibers (P < 0.05; Table 2). In the CS group, mean individual nerve terminal branch lengths at type I and IIa fibers were comparable, but remained significantly greater than that of nerve terminals at type IIx/b fibers (P < 0.05; Table 2). Within each fiber type, there was no significant difference between CTL, SHAM and CS groups in the mean individual branch length of nerve terminals.

Motor endplate morphology

In all experimental groups, the planar areas of motor endplates at different fiber types displayed a rank order with type I < IIa < IIx/b (P < 0.05; Table 3; Fig. 2). The planar areas of motor endplates at type I and IIa fibers were comparable between the three groups. However, the planar area of endplates at type IIx/b fibers of the CS DIAm was significantly smaller compared to that in both CTL and SHAM animals (P < 0.05; Table 3). When normalized for fiber diameter, the planar areas of motor endplates at different fiber types were comparable in the CTL DIAm (Table 3). In both SHAM and CS groups, the normalized endplate areas at type IIx/b fibers were significantly greater than those at type I and IIa fibers (P < 0.05; Table 3) which were comparable to each other in both groups. In the CS DIAm, the normalized endplate area at type IIx/b fibers was significantly greater than that in CTL (P < 0.05; Table 3). The normalized endplate areas at type I and IIa fibers were not different across experimental groups (Table 3).

In CTL and SHAM animals, the number of endplate branches displayed a rank order across different fiber types, with type I < IIa < IIx/b (P < 0.05; Table 3). In the CS group, the number of endplate branches was also greater at type IIx/b fibers compared to type I and IIa fibers (P < 0.05; Table 3). The number of endplate branches at type I and IIa fibers was comparable across the three experimental groups. However, the total number of endplate branches at type IIx/b fibers was significantly smaller in the CS group compared to CTL (P < 0.05; Table 3). In all experimental groups, the total cumulative length of endplate branches was comparable between type I and IIa fibers, but significantly greater at type IIx/b fibers (P < 0.05; Table 3). Total cumulative endplate branch length at type I and IIa fibers was comparable across the three experimental groups. However, the total cumulative branch length at type IIx/b fibers was significantly lower in the CS DIAm compared to CTL and SHAM animals (P < 0.05; Table 3). In the CTL DIAm, the mean individual branch length of endplates displayed a rank order across different fiber types, with type I > IIa > IIb (P < 0.05; Table 3). In SHAM animals, the mean individual branch length of endplates at type I fibers were longer than those at type IIa and IIx/b fibers (P < 0.05; Table 3). In the CS DIAm, there were differences in mean individual endplate branch lengths across the different fiber types (Table 3). Across the three experimental groups, there were no significant differences in mean individual branch lengths at any fiber type (Table 3).

Nerve Terminal

Branching Pattern

* **Origin**
━ **Primary Branch**
▬ **Secondary Branch**
— **Tertiary Branch**
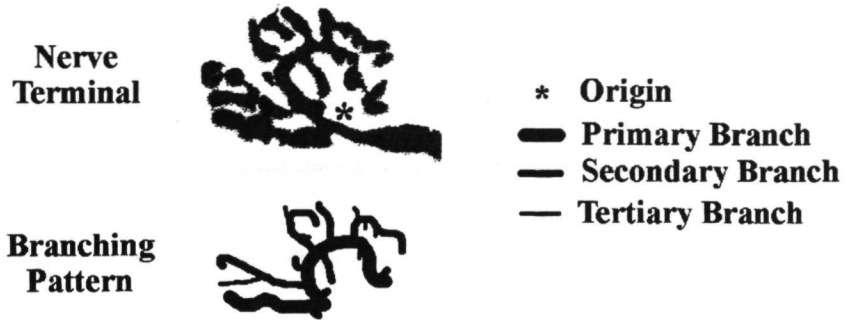
Figure 1: Schematic of the procedure used to characterize the branching patterns of nerve terminals and motor endplates. The origin of the NMJ was defined as the first point of branching. Primary branches were defined as the longest segments, secondary branches as those emanating from primary branches and so on.

Fiber Type

| | **I** | **IIa** | **IIx/b** |

Nerve Terminal

Motor Endplate
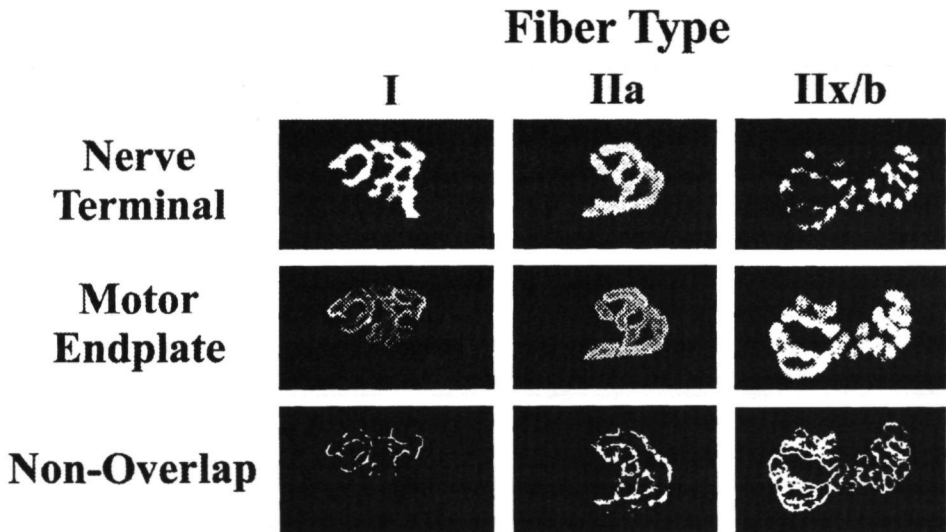
Non-Overlap

Figure 2: Grayscale images of nerve terminals and motor endplates on NMJs of type-identified DIAm muscle fibers. The extent of overlap of nerve terminal and motor endplate was determined from non-overlapping areas, obtained from a binary subtraction of the corresponding grayscale images.

Table 1: Effect of corticosteroid treatment on diaphragm muscle fiber diameter

Group	Fiber Diameter (mm)		
	I	IIa	IIx/b
CTL	26 2 ± 1 4	31 1 ± 1 1†	65 7 ± 2 7†‡
SHAM	21 4 ± 1 1*	27 5 ± 1 3†	46 4 ± 3 8*
CS	24 7 ± 1 8	28 5 ± 2 2	34 2 ± 3 5*§†‡

Values are means ± SE   * indicates significant difference from CTL § indicates significant difference from SHAM † indicates significant difference from type I, and ‡ indicates significant difference from type IIa Significance value is at P < 0 05

Table 2 Morphometric analysis of nerve terminals at different diaphragm muscle fiber types

	Group	Type I	Type IIa	Type IIx/b
Planar Area (μm^2)	_CTL_	343 ± 19	370 ± 12	656 ± 12†‡
	SHAM	322 ± 23	362 ± 22	737 ± 15*†‡
	_CS_	359 ± 23	410 ± 26	618 ± 11*§†‡
Normalized Planar Area (μm)	_CTL_	13 9 ± 0 6	12 4 ± 0 8†	10 1 ± 0 9†‡
	SHAM	15 3 ± 1 2	13 5 ± 1 2	17 0 ± 0 7*‡
	_CS_	14 5 ± 1 1	14 4 ± 1 4	18 1 ± 2 2*†‡
Total Number of Branches	_CTL_	6 ± 1	10 ± 1†	24 ± 2†‡
	SHAM	6 ± 2	10 ± 1†	21 ± 3†‡
	_CS_	7 ± 1	9 ± 1	17 ± 2*†‡
Total Branch Length (μm)	_CTL_	105 ± 7	123 ± 8†	224 ± 10†‡
	SHAM	99 ± 8	115 ± 8	235 ± 26†‡
	_CS_	105 ± 9	127 ± 15	168 ± 15*§†‡
Individual Branch Length (μm)	_CTL_	17 5 ± 1 4	13 2 ± 1 1†	9 5 ± 1 0†‡
	SHAM	16 2 ± 1 1	11 3 ± 1 1†	11 2 ± 1 3
	_CS_	14 8 ± 1 3	14 2 ± 1 2	9 9 ± 1 1†‡

Values are means ± SE * indicates significant difference from CTL § indicates significant difference from SHAM † indicates significant difference from type I ‡ indicates significant difference from type IIa Significance level is at P < 0 05

Extent of overlap between nerve terminals and motor endplates

In CTL animals, the extent of overlap between nerve terminals and motor endplates varied significantly across fiber types, with the overlap being ~95% at type I fibers, ~90% at type IIa fibers and ~80% at type IIx/b fibers (P<0 05, Fig 3) In CS animals, the extent of overlap between nerve terminals and motor endplates was unchanged at type I and IIa fibers, but was significantly greater at type IIx/b fibers, compared to CTL (P<0 05, Fig 3)

Table 3 Morphometric analysis of motor endplates at different diaphragm muscle fiber types

	Group	Type I	Type IIa	Type IIx/b
Planar Area (μm^2)	*CTL*	382 ± 18	420 ± 11†	830 ± 17 †‡
	SHAM	345 ± 21	394 ± 22†	808 ± 18†‡
	CS	383 ± 19	427 ± 21†	708 ± 19*§†‡
Normalized Planar Area (μm)	*CTL*	14 6 ± 0 8	14 0 ± 0 9	12 7 ± 1 4
	SHAM	16 4 ± 1 1	14 7 ± 1 2	17 7 ± 1 9*‡
	CS	15 8 ± 1 7	15 3 ± 2 1	20 9 ± 1 7*†‡
Total Number of Branches	*CTL*	6 ± 2	10 ± 1†	24 ± 3†‡
	SHAM	6 ± 2	10 ± 1†	22 ± 3†‡
	CS	7 ± 1	9 ± 1	16 ± 2*†‡
Total Branch Length (μm)	*CTL*	109 ± 13	132 ± 9	230 ± 10†‡
	SHAM	104 ± 8	125 ± 7	266 ± 19†‡
	CS	108 ± 8	128 ± 15	199 ± 14*§†‡
Individual Branch Length (μm)	*CTL*	18 3 ± 1 5	13 5 ± 1 2 †	9 8 ± 1 4 †‡
	SHAM	17 3 ± 1 4	12 4 ± 1 3†	12 2 ± 1 5†
	CS	15 7 ± 1 5	14 4 ± 1 4	12 6 ± 1 6

Values are means ± SE * indicates significant difference from CTL § indicates significant difference from SHAM † indicates significant difference from type I ‡ indicates significant difference from type IIa Significance level is at P < 0 05

Neuromuscular transmission failure

During the 2-min period of repetitive stimulation, the incidence of neuromuscular transmission failure progressively increased in all groups The relative extent of

neuromuscular transmission failure was significantly smaller in the CS DIAm compared to both CTL and SHAM animals (P < 0.05; Figure 4).
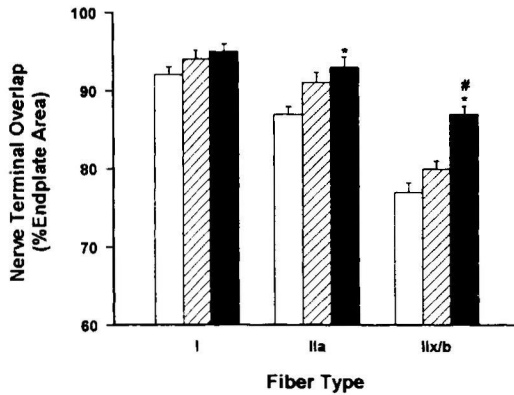

Figure 3: Effect of CS treatment on the extent of overlap of nerve terminal and motor endplate at type-identified fibers in the rat DIAm. In CTL animals, there was a rank order in the extent of overlap of pre- and postsynaptic elements among fiber types, with type I > IIa > IIx/b. The extent of overlap between nerve terminals and motor endplates was significantly increased at type IIx/b fibers of the CS-treated DIAm. * indicates significant difference from CTL (P < 0.05). # indicates significant difference from SHAM (P < 0.05).
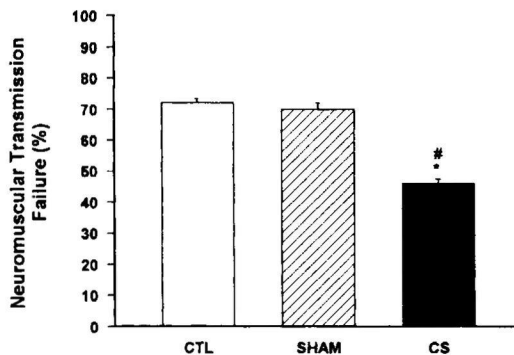

Figure 4: Effect of CS treatment on the extent of neuromuscular transmission failure during repetitive nerve stimulation. In the CS DIAm, neuromuscular transmission during repetitive nerve stimulation significantly improved compared to both CTL and SHAM animals. * indicates significant difference from CTL (P < 0.05). # indicates significant difference from SHAM (P < 0.05).

Discussion

The major observations of the present study were that 3 weeks of prednisolone treatment resulted in remodeling of pre- and postsynaptic elements of NMJs at type IIx/b DIAm fibers and an improvement in neuromuscular transmission. At type IIx/b fibers, nerve terminal and endplate areas decreased with CS treatment, while NMJs on type I and IIa fibers were largely unaffected. The reduction in NMJ area at type IIx/b fibers in the CS treated DIAm resulted from a decrease in the number of nerve terminal and endplate branches and a shorter mean branch length. However, the decrease in NMJ size at type IIx/b fibers with CS treatment was proportionately less than the fiber atrophy. Thus, with CS treatment, NMJ size normalized for fiber diameter actually increased at type IIx/b fibers compared to CTL. Furthermore, the extent of overlap between nerve terminal and motor endplate increased at type IIx/b fibers of the CS DIAm. Since type IIx/b fibers are normally more susceptible to neuromuscular transmission failure (15), these selective changes in NMJ morphology at type IIx/b fibers were consistent with the improvement in neuromuscular transmission observed in the CS treated DIAm.

Corticosteroid treatment regimen
In the present study, a dose of 0.3 mg/kg/day prednisolone was continuously infused via a miniosmotic pump. Although the prednisolone dose was comparable to that used in previous studies (37), the mode of CS administration via a miniosmotic pump was different. Undoubtedly, this mode of administration maintains a more stable serum CS level compared to bolus injections. The efficacy of the miniosmotic pump infusion was confirmed by measurement of elevated serum prednisolone levels. Furthermore, in the CS treated animals, there was an $\sim 20\%$ reduction in body weight, which was comparable to the weight loss observed in previous studies conducted over a 3 week period (11, 20, 42). Therefore, it is doubtful that the mode of CS administration significantly affected the results of the present study.

Effects of corticosteroids on diaphragm muscle fiber morphology
The observation that CS treatment was associated with a selective atrophy of type IIx/b fibers in the rat DIAm was consistent with several previous reports both in the DIAm (20, 36, 40, 42) as well as in hindlimb muscles (13, 40). However, the results of the present study contrast with those reported in the rabbit DIAm, where 3 months of cortisone treatment was found to be associated with a greater atrophy of type I fibers (11). This apparent discrepancy cannot be fully explained, but may reflect species differences in the response to CS treatment. Furthermore, there may be differences between the effects of prednisolone, a non-fluorinated CS, versus cortisone, which is fluorinated. Yet another factor that needs to be considered is the time course for CS effects, which may differ across species and/or the type of CS agent used. In the present study, CS effects on the rat DIAm were assessed only after 3 weeks.

The differential effect of CS treatment on type IIx/b fibers most likely reflects a selective inhibition of protein synthesis and/or an enhancement of protein degradation in these fibers (21). The underlying mechanisms for this selective catabolic effect remain unknown. One possibility is that fiber type differences exist in glucocorticoid receptor expression. However, in a previous study, it was reported that muscles predominantly composed of type I fibers have a higher concentration of glucocorticoid receptors, compared to muscles predominantly composed of type II fibers (16). However, fiber type differences in glucocorticoid receptor expression in the rat DIAm have not yet been characterized.

Effects of corticosteroids on NMJ morphology

In the CTL DIAm, the planar areas of nerve terminals and motor endplates at type I and IIa fibers were significantly smaller than the planar area of NMJs at type IIx/b fibers. This result is consistent with our previous observations in the rat DIAm (26, 27), and with other studies in the DIAm (41) as well as limb muscles (10, 35, 41). Also consistent with our previous observations, we found that NMJ size normalized for muscle fiber diameter was actually greater at type I and IIa fibers compared to type IIx/b fibers. Moreover, as reported earlier (26, 27), we found that the branching patterns of NMJs at type I and IIa fibers were relatively simple and compact compared to the more rosette and elaborate patterns of NMJs at type IIx/b fibers.

Previous studies in various muscles have also demonstrated a correlation between muscle fiber diameter and NMJ size (22, 23, 27). In fact, it has been suggested that muscle fiber size is a major determinant of NMJ size, and that changes in muscle fiber size actually trigger changes in NMJ morphology (22, 23, 27). Our previous study in the rat DIAm demonstrated that the correlation between muscle fiber diameter and NMJ size is valid only within a fiber type, hence the differences in normalized NMJ size across fiber types (41). However, we observed that changes in NMJ size were not always associated with changes in muscle fiber diameter (30). For example, 2 weeks of DIAm hemiparalysis induced by cervical spinal cord hemisection was associated with a significant expansion of NMJ size at type IIx/b fibers in the absence of any significant change in muscle fiber size (30). Conversely, 2 weeks of DIAm hemiparalysis induced by tetrodotoxin blockade of phrenic nerve axonal conduction resulted in substantial atrophy of type IIx/b fibers and hypertrophy of type I and IIa fibers without concomitant changes in NMJ morphology (28). In the present study, the selective atrophy of type IIx/b fibers induced by CS treatment was associated with a reduction in NMJ size at these fibers. However, the reduction in NMJ size did not match the atrophy of type IIx/b fibers in the CS-treated DIAm. Indeed, type IIx/b NMJ size normalized for muscle fiber diameter was actually greater compared to CTL. In previous studies by Fahim and colleagues (7, 8) fiber type differences in the effects of CS treatment on NMJ morphology were evaluated by comparing NMJs at fibers in the soleus (type I fibers) versus extensor digitorum longus (type II fibers) muscles. In

one study (8), it was reported that 3 months of cortisone treatment resulted in an enlargement of nerve terminals at type I fibers in the soleus muscle, with little or no structural changes in nerve terminals at type II fibers in the extensor digitorum longus muscle. However, in a subsequent study (7), these investigators reported that 3 months of cortisone treatment resulted in a reduction in nerve terminal size, which was greater at type II fibers in the extensor digitorum longus muscle. It was suggested that the apparent discrepancy between the results of these two studies could be attributed to the dynamic nature of NMJ remodeling, with simultaneous degeneration and regeneration of nerve terminals taking place (7), akin to that seen with aging (9).

It is likely that in the CS treated DIAm the macroscopic changes in NMJ morphology at type IIx/b fibers were also accompanied by a reduction in the number of nerve terminal active zones and/or the number of synaptic vesicles. However, these ultrastructural changes could not be resolved at the light microscopic level. Previous ultrastructural studies have reported that CS treatment is associated with changes in synaptic vesicle size, vesicular density and synaptic cleft width (7, 18, 19, 39). Glucocorticoids have also been reported to increase acetylcholinesterase levels at NMJs on innervated, cultured human skeletal muscle (3). Furthermore, electrophysiological studies have reported changes in mepp amplitude following CS administration (43). Thus, it appears that CS treatment induces both gross and ultrastructural changes at the NMJ that may impact neuromuscular transmission.

Effect of corticosteroids on diaphragm muscle neuromuscular transmission

By comparing the decrement in force during repetitive nerve stimulation to the force induced by direct muscle stimulation, we found that the extent of neuromuscular transmission failure was lower in the CS treated DIAm compared to CTL. Neuromuscular transmission failure during repetitive nerve stimulation has been attributed to either a failure in the axonal propagation of action potentials, primarily at axonal branch points, or a failure of synaptic transmission, at either pre- or postsynaptic sites (12, 17, 33, 34). The improvement in neuromuscular transmission following CS treatment could be attributed to either mechanism. In the normal DIAm, type IIx/b fibers are more susceptible to neuromuscular transmission failure, compared to type I or IIa fibers (15). This is consistent with the morphology of NMJs at type IIx/b fibers which have a greater number of nerve terminal branches and less overlap between pre- and postsynaptic elements, compared to NMJs at type I and IIa fibers (27). Following CS treatment, the total number of nerve terminal branches was reduced at type IIx/b fibers. Thus, the probability of axonal branch point failure may have decreased at type IIx/b fibers. In addition, the extent of overlap between nerve terminals and motor endplates improved at type IIx/b fibers following CS treatment. In areas of the NMJ where nerve terminals do not overlap with the motor endplate, the ACh released at nerve terminals may be less effective in inducing postsynaptic membrane potential changes, as reflected by a lower mepp amplitude. With an improvement in the extent of overlap between pre- and postsynaptic elements, the ACh

released at nerve terminals would become more effective in inducing postsynaptic membrane potential changes, and mepp amplitude would increase. Accordingly, previous electrophysiological studies have reported that CS treatment results in an increase in mepp amplitude (2, 4-6, 24, 38, 43, 44), indicating an improvement in synaptic efficacy.

The disproportionate effect of CS treatment on type IIx/b fiber diameter versus NMJ size may have also contributed, at least in part, to the improvement in neuromuscular transmission. Larger muscle fibers have greater total capacitance, requiring greater synaptic current to generate a given change in membrane potential. Thus, the greater normalized size of NMJs at type I and IIa fibers ensures greater efficacy of synaptic input, compared to that at type IIx/b fibers. Following CS treatment, the normalized size of NMJs at type IIx/b fibers increased, and this should have improved the efficacy of synaptic input.

In conclusion, the present study found that CS administration leads to a selective atrophy of type IIx/b fibers and a disproportionate reduction in NMJ size at these fibers. The morphological alterations of NMJs at type IIx/b fibers would lead to lower probability of axonal branch point failure and greater synaptic efficacy. Accordingly, it was observed that CS treatment was associated with decreased neuromuscular transmission failure in the DIAm.

References

1. Aldrich, T K., A. Shander, I. Chaudhry, and H. Nagashima. Fatigue of isolated rat diaphragm· role of impaired neuromuscular transmission. J. Appl Physiol 61. 1077-83, 1986

2. Arts, W.F , and H.J Oosterhuis Effect of prednisolone on neuromuscular blocking in mice in vivo Neurology 25: 1088-90, 1975

3. Askanas, V., J McFerrin, Y.C Park-Matsumoto, C.S Lee, and W K. Engel Glucocorticoid increases acetylcholinesterase and organization of the postsynaptic membrane in innervated cultured human muscle. Exp. Neurol 115: 368-75, 1992

4. Baker, T., W.F. Riker, and E.D. Hall Effects of a single methylprednisolone dose on a facilitatory response of mammalian motor nerve. Arch Neurol 34· 349-55, 1977

5. Dalkara, T., and R. Onur. Facilitatory effects of dexamethasone on neuromuscular transmission Exp. Neurol. 95: 116-25, 1987

6. Dengler, R , R Rudel, J. Warelas, and K.L Birnberger. Corticosteroids and neuromuscular transmission· electrophysiological investigation of the effects of prednisolone on normal and anticholinesterase-treated neuromuscular junction. Pflugers Arch. 380. 145-51, 1979

7. Fahim, M.A Chronic corticosterone treatment-induced ultrastructural changes at rat neuromuscular junction. Anat. Rec. 242 424-431, 1995.

8. Fahim, M A., and M H Andonian. Effects of chronic corticosterone treatment on the morphology of rat neuromuscular junctions. Anat Rec. 227· 132-137, 1990

9. Fahim, M A., J.A Holley, and N. Robbins. Scanning and light microscopic study of age changes at a neuromuscular junction in the mouse. J Neurocytol. 12. 13-25, 1983.

10. Fahim, M.A., J.A Holley, and N Robbins Topographic comparison of neuromuscular junctions in mouse slow and fast twitch muscles. Neurosci. 13: 227-235, 1984

11. Ferguson, G T , C G Irvin, and R M Cherniack Effect of corticosteroids on diaphragm function and biochemistry in the rabbit Am. Rev. Respir. Dis. 141 156-63, 1990

12. Fournier, M , M Alula, and G C Sieck Neuromuscular transmission failure during postnatal development Neurosci. Lett 125 34-36, 1991

13. Gardiner, P F., G Montanaro, D R. Simpson, and V R Edgerton Effects of glucocorticoid treatment and food restriction on rat hindlimb muscles. Am J Physiol 238 E124-30, 1980

14. Hughes, S.M , and H M. Blau Muscle fiber pattern is independent of cell lineage in postnatal rodent development. Cell 68 659-671, 1992

15   Johnson, B D, and G C Sieck Differential susceptibility of diaphragm muscle fibers to neuromuscular transmission failure J Appl Physiol 75 341 348, 1993

16   Konagaya, M, P A Bernard, and S R Max Blockade of glucocorticoid receptor binding and inhibition of dexamethasone-induced muscle atrophy in the rat by RU38486, a potent glucocorticoid antagonist Endocrinology 119 375-80, 1986

17   Krnjevic, K, and R Miledi Failure of neuromuscular propagation in rats J Physiol 140 440-461, 1958

18   Leeuwin, R S, K D Njio, G A Belling, and S van den Hoven Glucocorticoid-induced changes in synaptic vesicles of rat phrenic nerve terminals Arch Int Pharmacodyn Ther 266 200-7, 1983

19   Leeuwin, R S, and E C Wolters Effect of corticosteroids on sciatic nerve-tibialis anterior muscle of rats treated with hemicholinium-3 An experimental approach to a possible mechanism of action of corticosteroids in myasthenia gravis Neurology 27 171-7, 1977

20   Lewis, M I, S A Monn, and G C Sieck Effect of corticosteroids on diaphragm fatigue, SDH activity, and muscle fiber size J Appl Physiol 72 293-301, 1992

21   McGrath, J A, and D F Goldspink Glucocorticoid action on protein synthesis and protein breakdown in isolated skeletal muscles Biochem J 206 641-5, 1982

22   Nudell, B M, and A D Grinnell Regulation of synaptic position, size and strength in anuran skeletal muscle J Neurosci 3 161-76, 1983

23   Nystrom, B Postnatal development of motor nerve terminals in "slow-red" and "fast-white" cat muscles Acta Neurol Scand 44 363 383, 1968

24   Patten, B M, K L Oliver, and W K Engel Adverse interaction between steroid hormones and anticholinesterase drugs Neurology 24 442-9, 1974

25   Prakash, Y S, M Fournier, and G C Sieck Effects of prenatal undernutrition on developing rat diaphragm J Appl Physiol 75 1044-1052, 1993

26   Prakash, Y S, L E Gosselin, W Z Zhan, and G C Sieck Alterations of diaphragm neuromuscular junctions with hypothyroidism J Appl Physiol 81 1240-1248, 1996

27   Prakash, Y S, S M Miller, M Huang, and G C Sieck Morphology of diaphragm neuromuscular junctions on different fibre types J Neurocytol 25 88-100, 1996

28   Prakash, Y S, H Miyata, W -Z Zhan, and G C Sieck Inactivity alters structural and functional properties of the neuromuscular junction In The Physiology and Pathophysiology of Exercise Tolerance (J M Steinacker, and S A Ward, eds) New York, NY Plenum Press, 1996, pp 59-66

29   Prakash, Y S, K G Smithson, and G C Sieck Measurements of motoneuron somal volumes using laser confocal microscopy Comparisons with shape based stereological estimations NeuroImage 1 95 107 1993

30   Prakash, Y S, W Z Zhan, H Miyata, and G C Sieck Adaptations of diaphragm neuromuscular junction following inactivity Acta Anat 154 147 161, 1995

31   Schiaffino, S, L Gorza, S Sartore, L Saggin, S Ausoni, M Vianello, K Gundersen, and T Lomo Three myosin heavy chain isoforms in type 2 skeletal muscle fibres J Muscle Res Cell Motil 10 197 205, 1989

32   Sieck, G C, M Fournier, Y S Prakash, and C E Blanco Myosin phenotype and SDH enzyme variability among motor unit fibers J Appl Physiol 80 2179-2189, 1996

33   Sieck, G C, and Y S Prakash Fatigue at the neuromuscular junction Branch point vs presynaptic vs postsynaptic mechanisms In Neural and Neuromuscular Aspects of Muscle Fatigue (D G Stuart, S Gandevia, R M Enoka, A J McComas, and C K Thomas, eds) New York, NY Plenum Press, 1995, pp 83-100

34   Smith, D O Mechanisms of action potential propagation failure at sites of axon branching J Physiol 301 243-259, 1980

35   Tomas, J, M Santafe, R Fenoll, E Mayayo, J Battle, A Lanuza, and V Piere Pattern of arborization of the motor nerve terminals in the fast and slow mammalian muscles Biol Cell 74 299 305, 1992

36   van Balkom, R H H, Y S Prakash, W Z Zhan, and G C Sieck Prednisolone alters isotonic properties of rat diaphragm Am J Respir Crit Care Med 153 A685, 1996

37   van Balkom, R H H, W -Z Zhan, Y S Prakash, P N R Dekhuijzen, and G C Sieck Corticosteroid effects on isotonic contractile properties of rat diaphragm muscle J Appl Physiol 83 1062 1067, 1997

38   Van Wilgenburg, H The effect of prednisolone on neuromuscular transmission in the rat diaphragm Eur J Pharmacol 55 355-61, 1979

39. Van Wilgenburg, H., K.D. Njio, G A. Belling, and S. Van den Hoven. Effects of corticosteroids on the myoneural junction A morphometric and electrophysiological study. Eur. J. Pharmacol. 84: 129-37, 1982.

40. Viires, N., D. Pavlovic, R. Pariente, and M. Aubier. Effects of steroids on diaphragmatic function in rats Am. Rev. Respir. Dis. 142: 34-8, 1990.

41. Waerhaug, O. Species specific morphology of mammalian motor nerve terminals. Anat. Embryol. 185: 125-130, 1992.

42. Wilcox, P.G., J.M. Hards, K. Bockhold, B. Bressler, and R.L. Pardy. Pathologic changes and contractile properties of the diaphragm in corticosteroid myopathy in hamsters: comparison to peripheral muscle. Am. J. Respir. Cell Mol Biol. 1: 191-9, 1989.

43. Wilson, R.W., M.D. Ward, and T.R. Johns Corticosteriods: A direct effect at the neuromuscular junction. Neurology 24: 1091-1095, 1974.

44 Wolters, M.J., and R.S Leeuwin. Effect of corticosteroids on the phrenic nerve-diaphragm of preparation treated with hemicholinium A possible model of myasthenia gravis. Neurology 26: 574-8, 1976.

CHAPTER 6

Corticosteroid effects on
isotonic properties of rat diaphragm muscle

R.H.H. van Balkom, W.Z. Zhan[a], Y.S. Prakash[a],
P.N.R Dekhuijzen, and G.C. Sieck[ab],

[a] Departments of Anesthesiology, and [b] Physiology and Biophysics
Mayo Clinic and Foundation, Rochester MN, USA

Abstract

The effects of corticosteroids (CS) on diaphragm muscle (DIAm) fiber morphology and contractile properties were evaluated in three groups of rats: controls (CTL), surgical sham and weight-matched controls (SHAM) and CS-treated (prednisolone 6 mg/kg/day at 2.5 ml/hr for 3 wks). In the CS treated DIAm, there was a selective atrophy of type IIx and IIb fibers, compared to a generalized atrophy of all fibers in the SHAM group. Maximum isometric force (Po) was reduced by 20% in the CS group compared to both CTL and SHAM. Maximum shortening velocity (Vmax) in the CS DIAm was slowed by ~20% compared to CTL and SHAM. Peak power output of the CS DIAm was only 60% of CTL and 70% of SHAM. Endurance to repeated isotonic contractions improved in the CS-treated DIAm compared to CTL. We conclude that the atrophy of type IIx and IIb fibers in the DIAm can only partially account for the CS-induced changes in isotonic contractile properties. Other factors such as reduced myofibrillar density or altered cross-bridge cycling kinetics are also likely to contribute to the effects of CS treatment.

Introduction

Corticosteroid (CS) treatment is common in the clinical setting, despite a variety of contraindications including skeletal muscle myopathy. Recently, considerable attention has focused on the possibility that CS treatment impairs diaphragm muscle (DIAm) function in patients with chronic obstructive pulmonary disease (COPD) (1). In these patients, CS treatment appears to contribute to DIAm weakness, further reducing their functional reserve capacity. To date, animals studies have examined only the effects of CS treatment on isometric properties of the DIAm. However, an examination of only the isometric properties of the DIAm may not reveal the true impact of CS treatment. The force/velocity relationship is an essential characteristic of DIAm contractile properties, and, to date, there is very little information concerning the effects of CS treatment on the ability of the DIAm to shorten. This may explain the equivocal results of animal studies reporting either no effect of CS treatment on maximum isometric specific force (Po; force normalized for muscle cross-sectional area) of the DIAm (2,3,10,13,22) or only a small reduction in specific force (20).

As in other skeletal muscles, the maximum shortening velocity (Vmax) of DIAm fibers displays a strong association with myosin heavy chain (MHC) isoform composition (8,18). In the DIAm, type IIx and IIb fibers, expressing the MHC_{2X} and MHC_{2B} isoforms, respectively (16,19), have a faster Vmax than type I and IIa fibers, expressing the

MHC_{Slow} and MHC_{2A} isoforms, respectively. An effect of CS treatment on the force/velocity relationship of the DIAm is suggested by the selective atrophy of type IIx and/or IIb fibers (2,3,12,14,20,22). Accordingly, we hypothesize that in the DIAm, CS treatment is associated with a slowing of Vmax.

Fiber type differences in Vmax also correspond to differences in power output, with type IIx and IIb fibers generating greater power than type I and IIa fibers (18,19). If CS treatment selectively affects the size of type IIx and IIb fibers, then the power output of the DIAm should be reduced. The increased power output of type IIx and IIb DIAm fibers is also associated with greater energetic demands compared to type I and IIa fibers (18). Thus, a reduction in the relative contribution of type IIx and IIb fibers to total DIAm mass should result in an overall reduction in energy requirements. If muscle fatigue is related to an imbalance between energy supply and energy demand, the effects of CS treatment may be reflected by an improvement in fatigue resistance (rate of force decline) or endurance (duration of sustained power output). Indeed, previous studies have reported an improvement in isometric fatigue resistance of the rat DIAm following CS treatment (13,22). However, since energy requirements increase with power output (4,18), the effects of CS treatment on improving endurance should be even more pronounced during repetitive isotonic shortening.

In the present study, we evaluated the effects of CS treatment on the isotonic contractile and endurance properties of the rat DIAm. We hypothesized that CS treatment induces a selective atrophy of type IIx and IIb DIAm fibers, and that as a result there is a slowing of Vmax, a decrease in power output, and an improvement in isotonic endurance.

Methods

Male Sprague-Dawley rats (initial body weights 315 $\pm$5 g) were divided into 3 groups: 1) Untreated controls (CTL; n=8); 2) Surgical sham and weight-matched controls (SHAM; n=8); and 3) CS-treated (CS; n=8). All animals were housed in separate cages under a 12h-12h light-dark cycle, fed with Purina Rat Chow, and provided with water *ad libitum*. Animals in the CTL and CS groups were provided food *ad libitum*, whereas rats in the SHAM group were food-restricted to match their weight growth curve with that of the CS group. Body weights were monitored daily in all groups.

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic, and were in strict accordance with the American Physiological Society Animal Care Guidelines. Surgical procedures were performed under aseptic conditions. The recovery of animals from surgery was carefully monitored.

CS Treatment

Animals were anesthetized by *im* administration of ketamine (60 mg/kg) and xylazine (2 5 mg/kg), and a miniosmotic pump (Alzet 2M4) was implanted *sc* in the neck In the CS group, the miniosmotic pump contained a 37 5 mg/ml aqueous suspension of prednisolone sodium succinate (Upjohn), while in the SHAM group, the pump contained a sterile physiological saline solution   Based on a flow rate of 2 5 μl/h for the osmotic pump, a dosage of 6 mg/kg prednisolone was provided continuously for a three-week period Measurements of the remaining amount of solution in the pump at the end the three-week treatment period was used to estimate total drug delivery At the terminal experiment, blood samples were obtained to measure prednisolone and T_3/T_4 levels

Fiber Type Composition and Morphology
Following the three-week treatment period, the rats were anesthetized with pentobarbital sodium (70 mg/kg), and the right DIAm was rapidly excised Muscle segments were dissected from the midcostal region, and the resting, excised length of the strip was measured using digital calipers The muscle strips were then stretched to 1 5 times this excised length (an approximation for Lo (15)), pinned on cork, and rapidly frozen in melting isopentane cooled to its melting point by liquid nitrogen

Transverse sections of muscle fibers were cut at 6μm using a cryostat (Reichert Jung 2000E) kept at -20°C The muscle sections were then reacted with antibodies to different MHC isoforms 1) mouse anti-MHC$_{Slow}$ IgG (Novocastra) for identification of type I fibers by positive immunoreactivity, 2) mouse anti-MHC$_{2A}$ IgG (7) for identification of type IIa fibers by positive immunoreactivity, 3) mouse anti-MHC$_{All\ 2x}$ IgG (16) for identification of type IIx fibers by negative immunoreactivity, and 4) mouse anti-MHC$_{2B}$ IgM (16) for identification of type IIb fibers by positive immunoreactivity Following a 2-3 h incubation with the primary antibody, the sections were washed in 0 1 M phosphate buffer and incubated further in Cy3-conjugated donkey anti-mouse IgG or IgM

The fluorescently stained sections were visualized using an Olympus BH-2 microscope Images of the stained muscle sections were digitized into a 1024x1024 array of picture elements (pixels) using a CCD camera attached to a calibrated image processing system (19) Using a 20X microscope objective, each pixel had a projected area of 0 15 μm^2 The cross-sectional area of individual muscle fibers was determined from the number of pixels within the delineated boundary of the fiber To determine fiber type proportions, ~ 500 muscle fibers were sampled from each DIAm Cross sectional areas were measured for at least 25 fibers of each type within a given muscle The relative contribution of each fiber type to the total area of the muscle segment (an estimate of total mass when Lo was similar) was calculated based on the proportion and average cross-sectional area of each fiber type

MHC Isoform Composition

The techniques for determination of MHC isoform composition of the rat DIAm have been previously described (8,19). Briefly, myosin was extracted from scissor-minced DIAm tissue and the extracts were centrifuged and supernatants recovered. Following overnight storage to allow precipitation of myosin filaments, the solution as centrifuged and the pellet was dissolved in a sample buffer, boiled and then stored frozen. Different MHC isoforms were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The identity of specific MHC bands in silver-stained gels had been previously determined using immunoblotting techniques (9,19). The relative composition of the different MHC isoforms was determined by densitometry, normalizing the average density of each band for the total peak densities for all the isoforms combined.

Contractile and Endurance Properties

Muscle strips ($\sim$3 mm wide) were dissected   from the midcostal region with fiber insertions at the costal margin and central tendon left intact. The muscle strip was mounted vertically in a glass tissue chamber containing oxygenated mammalian Ringers' solution with the following composition (mM): 135 Na^+, 5 K^+, 2 Ca^{2+}, 1 Mg^{2+}, 121 Cl^-, 25 HCO_3^-, 11 glucose, 0.3 glutamic acid, 0.4 glutamate, and N,N-bis(2-hydroxyethyl)-2-aminoethane-sulfonic (BES) acid buffer (pH=7.4). A 0.0008% solution of d-tubocurarine chloride was added to prevent neuromuscular transmission. The solution was oxygenated with 95% O_2 and 5% CO_2 and maintained at 26^oC. The origin of the muscle bundle along the costal margin was attached to a metal clamp mounted in series with a micromanipulator at the base of the tissue chamber. The central tendon was glued to a thin, stiff plastic rod that was firmly fixed to the lever arm of a dual-mode length-force servo control system (Cambridge Technologies, model 300B).

The muscle was stimulated directly using platinum plate electrodes placed in close apposition on either side of the muscle. Rectangular current pulses (0.5 ms duration) were generated using a Grass S88 stimulator, and amplified by a current amplifier (Mayo Foundation, Section of Engineering). The stimulus intensity producing the maximum twitch force response was determined, and the stimulus intensity was set at ~125% of this value for the remainder of the experiment (~220 mA). Muscle preload was adjusted using the micromanipulator until optimal fiber length (Lo) for maximal twitch force was achieved.

The Cambridge system was controlled using custom-built software (LabView), implemented on an IBM 486 personal computer. Length and force were independently controlled, allowing the Cambridge system to operate either in isometric or isotonic modes, respectively. Length and force outputs were digitized using a data acquisition

board (National Instruments) at a sampling frequency of 1 KHz

Peak isometric twitch force (Pt) and Po (600 ms duration train) were measured The force/velocity relationship of the DIAm was then determined While the muscle was maximally stimulated at 75 Hz for 330 ms, afterloads were clamped at values ranging from 3 to 100% of Po A shorter stimulus duration was used to accommodate the limited range of lever movement of the Cambridge system during muscle shortening At least 1 min intervened between each load level The velocity of shortening at each load clamp was calculated as the change in muscle length (normalized for Lo) during a 50 ms period To eliminate the dynamics of connective and other non-contractile tissue in the muscle, the time window for this measurement was set to begin at 25 ms following the first detectable change in length Vmax was calculated by fitting the force-velocity curve using the modified Hill equation and extrapolating the fitted curve to zero-load (21)

Power output during isotonic contraction was calculated as the product of force and velocity, and the load clamp level yielding maximum power was determined The load clamp was set to this value, and endurance was assessed during repetitive isotonic shortening induced by stimulating the muscle at 75 Hz in 330 ms duration trains repeated every s The time at which power output declined to zero (no detectable muscle shortening) was defined as endurance time

Following the experiment, the muscle was weighed, and cross-sectional area was estimated based on the following formula muscle weight (g)/[Lo (cm) 1 056 (g/cm^3)] Forces were then normalized for cross-sectional area of the muscle segments

Statistical Analysis

Data were compared using a 1-way analysis of variance (ANOVA) followed by Duncan's multiple-range test Repeated measures ANOVA were used for analysis of force-frequency, force-velocity and force-power relationships, as well as for the analysis of the decline in maximum power output during the isotonic fatigue test Statistical significance was tested at the 0 05 level All data were expressed as mean ±SE

Results

Efficacy of CS Treatment

Following three weeks of treatment, there was very little residual solution ($<5\%$ of total volume) remaining in the miniosmotic pumps Prednisolone levels measured in blood serum of CTL and SHAM animals were below detectable levels ($<0\ 5\ \mu g/dl$) In

contrast, the serum prednisolone level measured in the CS-treated animals at the time of the terminal experiment was 4.9 ± 1 $\mu g/dl$ Serum T3/T4 levels were not significantly different across the three experimental groups (CTL T3 46 ± 3 ng/dl, T4 4.0 ± 0.2 $\mu g/dl$, SHAM. T3 48 ± 6 ng/dl, T4 4.2 ± 0.4 $\mu g/dl$, and CS T3 47 ± 4 ng/dl, T4 3.9 ± 0.4 $\mu g/dl$)

Table 1. Effect of CS treatment on fiber type proportions, cross-sectional areas and relative contributions to total diaphragm muscle cross-sectional area

Treatment	Type I	Type IIa	Type IIx	Type IIb
Fiber Type Proportions (% of total)				
CTL	36.1 ± 0.5	32.5 ± 0.7	23.5 ± 1.5	6.8 ± 1.6
SHAM	37.4 ± 1.5	31.0 ± 1.2	24.1 ± 1.5	7.5 ± 1.4
CS	40.4 ± 2.2	29.9 ± 1.5	24.1 ± 1.9	5.5 ± 1.5
Fiber Cross-Sectional Area (μm^2)				
CTL	875 ± 28	821 ± 35	2666 ± 163	3388 ± 263
SHAM	$600 \pm 19^*$	$693 \pm 18^*$	$1710 \pm 75^*$	$2685 \pm 186^*$
CS	772 ± 62^f	770 ± 67^f	$1668 \pm 202^*$	$2284 \pm 307^*$
Relative Contribution to Total DIAm Area (% of total)				
CTL	21.7 ± 0.9	18.6 ± 0.6	44.2 ± 4.1	15.5 ± 3.8
SHAM	21.6 ± 1.6	20.6 ± 2.0	39.5 ± 3.3	19.2 ± 4.1
CS	$29.8 \pm 2.3^{*f}$	21.7 ± 1.4	37.2 ± 3.2	11.3 ± 3.1

Values are means $\pm$SE * $p < 0.05$ compared to CTL, ^{f} $p < 0.05$ compared to SHAM

Body Weights

Over the three-week experimental period, body weights of CTL animals increased by 26% (315 ± 7 g initial and 397 ± 9 g final body weight) In the CS and SHAM animals, body weight gain was significantly reduced compared to CTL ($p < 0.05$), increasing by only 6% and 4 %, respectively (CS 319 ± 5 g initial and 338 ± 9 g final body weight, SHAM 313 ± 7 g initial and 327 ± 9 g final body weight)

Table 2. Effect of CS treatment on MHC isoform composition of the diaphragm muscle (% total MHC)

Treatment	MHC_{Slow}	MHC_{2A}	MHC_{2X}	MHC_{2B}
CTL	22.5 ± 1.1	29.0 ± 1.2	34.1 ± 1.2	14.5 ± 1.9
SHAM	24.0 ± 2.3	30.2 ± 1.8	31.6 ± 2.0	14.2 ± 2.1
CS	25.3 ± 1.9	34.8 ± 1.5	33.4 ± 1.0	$6.5 \pm 1.5^{*f}$

Values are means $\pm$SE * $p < 0.05$ compared to CTL, ^{f} $p < 0.05$ compared to SHAM

Fiber Type Composition and Morphology

In all three experimental groups, fiber types could be readily classified by immunoreactivity for the different MHC antibodies. The incidence of co-expression of MHC isoforms appeared to be very low ($< 1\%$) in all three groups. However, it was not possible to detect co-expression of MHC_{2X} and MHC_{2B} isoforms by immunohistochemistry, and it is likely that such co-expression was more frequent (19). There were no differences across groups in proportions of different fiber types (Table 1).

In the CS-treated animals, cross-sectional areas of type IIx and IIb DIAm fibers were significantly smaller than those of type IIx and IIb fibers in CTL ($p < 0.05$; Table 1). In contrast, cross-sectional areas of type I and IIa fibers in the CS DIAm were comparable to similar fiber types in CTL animals. In the SHAM DIAm, there was a generalized atrophy of all fiber types compared to CTL ($p < 0.05$; Table 1). Type I fibers in the SHAM DIAm were also smaller than type I fibers in the CS DIAm ($p < 0.05$; Table 1). Cross-sectional areas of type IIa, IIx and IIb fibers in the CS DIAm were comparable to similar fiber types in SHAM animals.

In the CS DIAm, the relative contribution of type I fibers to total DIAm cross-sectional area increased ($p < 0.05$; Table 1). Otherwise, there were no differences across groups in the relative contribution of different fiber types to total DIAm cross-sectional area. However, the combined contribution of type IIx and IIb fiber areas was $\sim 60\%$ of total DIAm cross-sectional area in CTL and SHAM animals, but only $\sim 48\%$ in the CS group (Table 1).

MHC Isoform Composition

Based on electrophoretic separation, the relative expression of the MHC_{2B} isoform decreased in the CS-treated DIAm ($p < 0.05$; Table 2). The MHC isoform composition of CTL and SHAM DIAm were comparable (Table 2).

Contractile and Endurance Properties

Following three weeks of CS treatment, Pt and Po of the DIAm were reduced compared to both CTL and SHAM groups ($p < 0.05$, Table 3). Pt and Po were not different between CTL and SHAM animals. Compared to CTL and SHAM groups, the force-velocity relationships of the CS DIAm was shifted to the left ($p < 0.05$; Fig. 1A). The Vmax of the CS DIAm was significantly slower than that of both CTL and SHAM DIAm ($p < 0.05$, Fig. 1B). In all DIAm, peak power output occurred at $\sim 33\%$ of Po and 33% of Vmax (Fig. 2). Peak power output of the CS DIAm was significantly lower than that of both CTL and SHAM groups (Fig. 2; $p < 0.05$). The peak power output of the SHAM DIAm was also slightly lower than that of CTL animals (Fig. 2; $p < 0.05$).
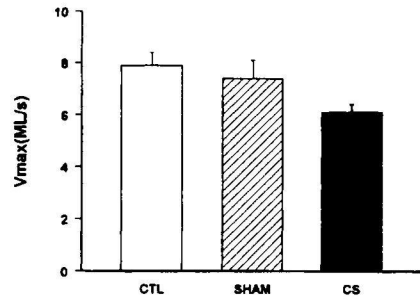
A.
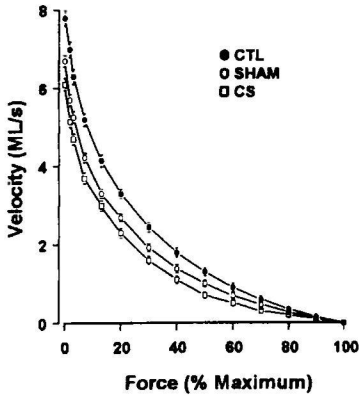
B.

Figure 1.   A. Force-velocity relationship of the CS DIAm was shifted leftward compared to both CTL and
SHAM animals (p < 0.05).
B. Vmax was slower in the CS DIAm compared to both CTL and SHAM groups (p < 0.05)

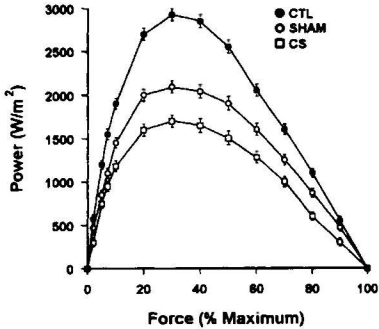**Figure 2.**   Power output at each load was reduced in
the CS an SHAM groups compared to
CTL (p < 0.05). In addition, the power
output of the CS DIAm was less than that
of the SHAM group (p < 0.05). Peak
power output was reduced in the CS and
SHAM groups compared to CTL
(p < 0.05). Peak power output of the CS
DIAm was lower than that of SHAM
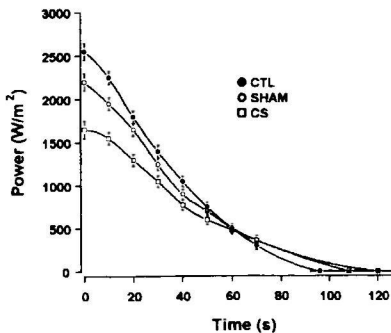(p < 0.05)

Figure 3.   During repetitive shortening at 30% Po,
power output of the DIAm rapidly declined
in all three groups (p < 0.05). The rate of
decline of power was slower in the CS
DIAm compared to CTL and SHAM
(p < 0.05). Endurance time of the CS
DIAm was prolonged compared to CTL
(p < 0.05), but not significantly different
from SHAM.

With repetitive shortening contractions, maximum power output of the DIAm rapidly declined in all three groups (Fig. 3, $p < 0.05$) After 60 s of repetitive contractions, DIAm power output was comparable in all three groups (Fig 3) However, given the differences in the initial peak power output of each group, the rate of decline in power was slower in the CS DIAm compared to both SHAM and CTL animals (Fig. 3; $p < 0.05$) Endurance time of the CS DIAm was 120 ± 6 s compared to 96 ± 4 s for CTL ($p < 0.05$) and 108 ± 7 s for SHAM (Fig 3)

Table 3. Effect of CS treatment on isometric contractile properties of the DIAm

Treatment	Pt (N/cm²)	Po (N/cm²)	Pt/Po
CTL	10 5 ± 0 7	21 1 ± 1 5	0 50 ± 0 02
SHAM	11 4 ± 0 7	20 3 ± 1 0	0 56 ± 0 02
CS	7 4 ± 0 6*¹	16 6 ± 0 9*¹	0 44 ± 0 02*¹

Values are means ±SE Pt peak twitch force, Po maximum tetanic force, ˙ $p < 0.05$ compared to CTL, ' $p < 0.05$ compared to SHAM

Discussion

The results of the present study support our hypotheses that CS treatment induces a selective atrophy of type IIx and IIb fibers in the rat DIAm, which is associated with a slowing of Vmax, a reduction in power output and an improvement in isotonic endurance However, the CS-induced changes in DIAm isotonic properties were disproportionately greater then the changes in type IIx and IIb fiber morphology and MHC isoform expression Therefore, we conclude that in addition to the selective atrophy of type IIx and IIb fibers, CS treatment exerts an influence on cross bridge cycling kinetics

Across the three-week period, the normal increase in body weight observed in CTL rats was blunted by CS treatment The final body weight of the CS-treated animals was ~ 15% lower than that of CTL rats Since alterations in nutritional status alone can affect morphology and function of the rat DIAm (2,11,17), interpretation of the direct effects of CS treatment is confounded However, the morphological and contractile adaptations of the DIAm in the SHAM group, where body weight was matched to that of the CS group by food restriction, were generally dissimilar to those observed in the CS-treated animals These results suggest that the effects of CS treatment on DIAm structural and functional properties cannot be solely attributed to a non-selective catabolic effect

The CS-induced selective atrophy of type IIx and IIb DIAm fibers observed in the present study is in general agreement with several previous studies (2,3,12,14,20,22)

However, these previous studies did not classify fiber types based on expression of different MHC isoform, nor did they distinguish between type IIx and IIb fibers Standard histochemical classification of fiber types based on the pH lability of myofibrillar ATPase staining, as used in these studies, cannot distinguish type IIx fibers, which are abundant in the rat DIAm (9,19) In addition, the MHC_{2B} isoform is often co-expressed with the MHC_{2X} isoform Therefore, it is not surprising that type IIx and IIb fibers displayed a similar pattern of atrophy in response to CS treatment In the SHAM DIAm there was a generalized atrophy of all fiber types which has also been previously observed (2,10,11,17,20,22) When combined, type IIx and IIb fibers comprised ~60% of both CTL and SHAM DIAm, but only ~48% of the CS DIAm In the CS-treated DIAm, there was a reduction in the relative expression of the MHC_{2B} isoform, while no changes in MHC isoform expression were observed in the SHAM group When combined, MHC_{2X} and MHC_{2B} isoforms comprised ~40% of the CS DIAm compared to ~49% of CTL and ~46% of SHAM DIAm The relatively modest change in MHC isoform composition of the CS DIAm was consistent with the normal T3/T4 levels of these animals Clearly, the CS-induced morphological adaptations of type IIx and IIb DIAm fibers and the alterations in MHC isoform expression were not as pronounced as the changes in isotonic contractile properties

Three weeks of CS treatment resulted in a 20% reduction in Po compared to both CTL and SHAM groups These results are in agreement with the previous report of van Balkom et al (20) but contrast with several other studies where no effect of CS treatment on DIAm Po was observed (2,3,10,13,22) The reasons for these discrepant results are unclear, but may relate to the type, dose and duration of CS treatment used It is unlikely that the reduction in specific force of the CS-treated DIAm observed in the present study was attributable only to the selective atrophy of type IIx and IIb fibers or the reduction in MHC_{2B} isoform expression A reduction in specific force could also arise from a number of alternative mechanisms, including a decrease in myofibrillar density and/or changes in cross bridge cycling kinetics Lieu and colleagues (12) reported that CS treatment is associated with a reduction of myofibrillar and sarcoplasmic protein concentration in the rat DIAm, albeit not as pronounced as in the plantaris muscle Such alterations in myofibrillar and sarcoplasmic protein concentration could reflect a decrease in the number of available cross bridges and/or changes in calcium handling

The force-velocity relationship of the DIAm was altered by CS treatment such that Vmax was slowed by ~20%, and peak power output was reduced by 40% compared to CTL animals The slowing of Vmax in the CS DIAm is generally consistent with the selective atrophy of type IIx and IIb fibers and the reduction in MHC_{2B} expression However, the slowing of Vmax induced by CS treatment was substantially greater than

that which would be predicted by the relatively modest reduction in MHC_{2B} expression Therefore, it is unlikely that the slowing of Vmax in the CS-treated DIAm was solely attributable to a selective atrophy of type IIx and IIb fibers and/or the reduction in MHC_{2B} expression In muscle fibers, Vmax is correlated with actomyosin ATPase activity (18) and cross bridge cycling rate Type I and IIa fibers have lower actomyosin ATPase activities than type IIx and IIb fibers (18,19), and as a result a slower Vmax It is possible that the slowing of Vmax in the CS treated DIAm reflects a decrease in actomyosin ATPase activity of muscle fibers independent of MHC isoform expression

In all groups, the DIAm displayed very rapid fatigue during repetitive isotonic contractions at a load corresponding to peak power output During shortening contractions, muscle fiber energy utilization increases (4,18), thus, the rapid fatigue may be related to an imbalance between energy utilization and energy production CS-treated animals displayed a slower rate of power decrement during repetitive isotonic contractions compared to CTL and SHAM groups and prolonged endurance time compared to CTL These results are in general agreement with the improved fatigue resistance during repetitive isometric contractions noted in previous studies (13,22) However, the results of the present study are in contrast to the report of Ferguson and colleagues (5) who found that CS-treated rabbits displayed less endurance to an incremental inspiratory threshold load However, DIAm fatigue was not directly verified in this study, and respiratory failure, used to define endurance, could have resulted from a number of mechanisms other than DIAm fatigue

The results of the present study suggest that CS treatment reduces energy utilization during repetitive isotonic contractions and thus improves the balance between energy supply and energy demand A reduction in energy utilization would result from the selective atrophy of type IIx and IIb fibers, which have higher actomyosin ATPase activities (18,19) In addition, as suggested above, CS treatment may directly reduce actomyosin ATPase activity independent of MHC isoform expression Other studies have also suggested that CS treatment impairs muscle energy utilization For example, following CS treatment, there is an accumulation of glycogen (5) and a reduction in creatine kinase activity (6) There may also be an effect of CS treatment on energy production For example, it has been reported that CS treatment reduces citrate synthase activity in the rat DIAm (12,20) However, no effect of CS treatment on succinate dehydrogenase activity was observed (10)

In conclusion, CS treatment causes a reduction in specific force, a slowing of Vmax, a decrease in power output, and an improvement in endurance during repetitive isotonic contractions These contractile adaptations are generally consistent with the selective atrophy of type IIx and IIb fibers and the reduction in MHC_{2B} expression that was

observed in the CS DIAm However, the contractile adaptations are disproportionately greater than the morphological changes induced by CS treatment Therefore, we conclude that the impairment of DIAm function associated with CS treatment involves additional mechanisms including a reduction in myofibrillar density and/or a slowing of cross bridge cycling kinetics

References

1   Decramer, M , L M Lacquet, R Fagard, and P Rogiers Corticosteroids contribute to muscle weakness in chronic airflow obstruction Am J Respir Crit Care Med 150 11-16, 1994

2   Dekhuijzen, P N R , G Gayan-Ramirez, A Bisschop, V de Bock, R Dom, and M Decramer Corticosteroid treatment and nutritional deprivation cause a different pattern of atrophy in rat diaphragm J Appl Physiol 78 629-637, 1995

3   Dekhuijzen, P N R , G Gayan-Ramirez, V de Bock, R Dom, and M Decramer Triamcinolone and prednisolone affect contractile properties and histopathology of rat diaphragm differently J Clin Invest 92 1534 1542, 1993

4   Fenn, W O A quantitative comparison between the energy liberated and the work performed by the isolated sartorius muscle of the frog J Physiol Lond 58 175-203, 1923

5   Ferguson, G T , C G Irvin, and R M Cherniack Effects of corticosteroids on diaphragm function and biochemistry in the rabbit Am Rev Respir Dis 141 156-163, 1990

6   Fernandez Sola, J , R Cusso, C Picado, M Vernet, J M Grau, and A Urbano-Marquez Patients with chronic glucocorticoid treatment develop changes in muscle glycogen metabolism J Neurol Sci 117 103-106, 1993

7   Hughes, S M and H M Blau Muscle fiber pattern is independent of cell lineage in postnatal rodent development Cell 68 659 671 1992

8   Johnson, B D , L E Wilson, W Z Zhan, J F Watchko, M J Daood, and G C Sieck Contractile properties of the developing diaphragm correlate with myosin heavy chain phenotype J Appl Physiol 77 481-487, 1994

9   LaFramboise, W A , M J Daood, R D Guthrie, S Schiaffino, P Moretti, B Brozanski, M P Ontell, G S Butler-Browne, R G Whalen, and M Ontell Emergence of the mature myosin phenotype in the rat diaphragm muscle Dev Biol 144 1-15, 1991

10   Lewis, M I , S A Monn, and G C Sieck Effect of corticosteroids on diaphragm fatigue, SDH activity, and muscle fiber size J Appl Physiol 72 293 301, 1992

11   Lewis, M I , G C Sieck, M Fournier, and M J Belman Effect of nutritional deprivation on diaphragm contractility and muscle fiber size J Appl Physiol 60 596-603, 1986

12   Lieu, F K , S K Powers, R A Herb, D Criswell, D Martin, C Wood, W Stainsby, and C L Chen Exercise and glucocorticoid-induced diaphragmatic myopathy J Appl Physiol 75 763-771, 1993

13   Moore, B J , M J Miller, H A Feldman, and M B Reid Diaphragm atrophy and weakness in cortisone treated rats J Appl Physiol 67 2420-2426, 1989

14   Polla, B , R Bottinelli, D Sandoli, C Sardi, and C Reggiani Cortisone induced changes in myosin heavy chain distribution in respiratory and hindlimb muscles Acta Physiol Scand 151 353 361 1994

15   Prakash, Y S , M Fournier, and G C Sieck Effects of prenatal undernutrition on developing rat diaphragm J Appl Physiol 75 1044 1052, 1993

16   Schiaffino, S , L Gorza, S Sartore, L Saggin, S Ausoni, M Vianello, K Gundersen, and T Lomo Three myosin heavy chain isoforms in type 2 skeletal muscle fibers J Musc Res Cell Motility 10 197 205, 1989

17   Sieck, G C , M I Lewis, and C E Blanco Effects of undernutrition on diaphragm fiber

size, SDH activity, and fatigue resistance   J Appl Physiol 66   2196 2205, 1989

18   Sieck, G C , and Y S Prakash Cross bridge kinetics in respiratory muscles Eur Respir J in press

19   Sieck, G C , W Z Zhan, Y S Prakash, M J Daood, and J F Watchko SDH and actomyosin ATPase activities of different fiber types in rat diaphragm muscle J Appl Physiol 79   1629-1639, 1995

20   van Balkom, R H H , H F M van der Heijden, H T B van Moerkerk, J H Veerkamp, J A M Fransen, L A Ginsel, H T M Folgering, C L A van Herwaarden, and P N R Dekhuijzen Effects of different treatment regimens of methylprednisolone on rat diaphragm contractility, immunohistochemistry and biochemistry Eur Respir J 9   1217 1223, 1996

21   Van Mastrigt, R Fitting the hill equation to experimental data IEEE Trans Biomed Eng 27   413-416, 1980

22   Wilcox, P G , J M Hards, K Bockhold, B Bressler, and R L Pardy Pathologic changes and contractile properties of the diaphragm in corticosteroid myopathy in hamsters comparison to peripheral muscle Am J Respir Cell Mol Biol 1   191 199, 1989

CHAPTER 7

Anabolic steroids in part reverse glucocorticoid-induced alterations in rat diaphragm

R.H.H. van Balkom, P.N.R. Dekhuijzen, H.T.M. Folgering,
J.H. Veerkamp[a], H.T. van Moerkerk[a], J.A.M. Fransen[b],
and C.L.A. van Herwaarden

[a] Department of Biochemistry, and [b] Department of Cell Biology and Histology,
University of Nijmegen, The Netherlands

Abstract

Animal and clinical studies have shown respiratory muscle dysfunction caused by treatment with glucocorticoids. The present study was designed to investigate whether anabolic steroids are able to antagonize the loss of diaphragm force induced by long-term low-dose methylprednisolone (MP) administration. Male adult rats were randomized to receive saline or MP 0.2 mg/kg/day *sc* during nine months, with or without nandrolone decanoate (ND) 1 mg/kg/wk *im* during the last 3 months. The ~10% reduction in force generation of isolated diaphragm bundles induced by MP was completely abolished by addition of ND. The MP-induced decrease in number of fibers expressing IIb myosin heavy chains (MHC) was not reversed by ND. MP slightly reduced type I, IIa and IIx fiber cross-sectional areas (CSA), but not type IIb fiber CSA. Addition of ND abolished the reduction in IIa and IIx fiber CSA. The MP-induced alterations in glycogenolytic activity and fatty acid oxidation capacity were not reversed by ND. In conclusion, the marked reduction in diaphragm force caused by long-term low-dose MP was completely abolished by addition of ND. ND in part also antagonized the effects of MP on diaphragm morphology but showed no beneficial effects on biochemical changes.

Introduction

Animal studies have shown evidence of respiratory muscle dysfunction induced by treatment with non-fluorinated glucocorticoids. Previously, high dosages were studied for short periods of time, resembling acute glucocorticoid myopathy (7). Subsequent studies, using lower dosages, showed no changes in rat diaphragm contractile properties following methylprednisolone (MP) 0.5 mg/kg/day for six weeks (8), or following 1.25 mg/kg/day of prednisolone for four weeks (10). In contrast, administration of a low dose of MP (0.2 mg/kg/day) for six months caused a significant (~15%) reduction in rat diaphragm force generation (38).

These observations in animal diaphragm following administration of low, clinically relevant dosages of MP for prolonged periods were recently confirmed in a clinical study in patients with chronic obstructive pulmonary disease (COPD) (6). In this study, a significant decrease in respiratory (and peripheral) muscle strength was observed after treatment with methylprednisolone 4.3 mg for 6 months (6). Since treatment with glucocorticoids is sometimes inevitable in these patients (21), interventions that attenuate or even abolish these alterations in respiratory muscles may be of importance.

In this respect, the use of anabolic steroids may be of interest. Anabolic steroids are able

to raise muscle protein by increasing protein synthesis (19). This is of special interest since the decrease in muscle protein caused by glucocorticoids is believed to be a major cause of glucocorticoid-induced muscle dysfunction (30). A negative nitrogen balance, indicating a catabolic condition, can be the result of glucocorticoids treatment or malnutrition, both not uncommon in patients with COPD (21,24). This may in part be reversed by anabolic steroids under the condition that protein intake is adequate (19). Indeed, Schols and coworkers (33) recently showed that anabolic steroids improved respiratory muscle function in undernourished COPD patients who were refeeded. However, the effects of anabolic steroids in clinically relevant dosages on existing glucocorticoid-induced myopathic changes in the diaphragm have not yet been reported to our knowledge.

Based on the metabolic effects of anabolic steroids described above, we hypothesized that anabolic steroids are able to reverse the loss in diaphragm force production observed following six months of glucocorticoid administration in clinically relevant dosages (38). To test this hypothesis, we examined in vitro contractile properties of the diaphragm of rats treated with nandrolone decanoate during the last three months of a nine month treatment period with a low dose of MP. Morphological and biochemical parameters were measured to evaluate cellular adaptations to the drugs tested.

Methods

Study design, animals, and treatment
Adult male outbred Wistar rats (n=30), aged 18-20 weeks, weighing (mean ±SE) 380 ±25 g, were randomized into three treatment groups:
-       Saline group: NaCl 0.9% 0.2 ml/day *sc* for 9 months
-       MP: methylprednisolone (MP): MP 0.2 mg/kg/day *sc* for 9 months
-       MP+ND: MP and nandrolone decanoate (ND): MP 0.2 mg/kg/day *sc* for 9 months, during the last 3 months combined with ND 1 mg/kg *im* every week.
The dose of MP (Sigma Chemicals, Bornem, Belgium) used in the present study was based upon the observation of similar anti-inflammatory potency and metabolism of MP in rats and humans (22). If an absorption of 100% is assumed, 0.2 mg/kg/day of MP would be equivalent to a dose of ~14 mg/day in a 70 kg human. However, the actual biologically available dose may be less since an absorption of only 60% was found following *i.m.* injections (27). In addition, the *s.c.* route requires higher doses to produce effects similar to *i.m.* administration (16). ND (Organon, Oss, The Netherlands) was selected as an anabolic agent because this drug is a long-acting steroid ester which is slowly hydrolyzed and therefore provides a constant tissue level. The dose used in the present study falls within the

range recommended by the manufacturer for humans (50 mg *im* once every 3 weeks and 200 mg *im* every week) and that has been proven to be effective in clinical studies (33)

With each *sc* injection (saline or MP) all animals received a similar volume ($\sim$ 0 20 ml) During 9 months the animals were daily *sc* injected in the neck between 8 30 and 10 00 a m ND was administered alternating in left and right upper hindlimb The rats were fed ad libitum, held on a 12/12 hour light-dark regime and were weighed once weekly Although daily food intake was not accurately quantified (animals were not held in metabolic cages), food intake appeared to be similar in all groups At the end of the treatment period, contractile properties, histological, morphometrical and biochemical characteristics of the diaphragm were examined The soleus (containing predominantly type I fibers) and extensor digitorum longus (EDL, containing predominantly type IIx/b fibers) were extracted and weighted All animals were investigated between 23 and 30 hours after the last *sc* injection Animals treated with ND were studied three to five days after the last ND injection The study was approved by the Animal Experiments Committee of the University of Nijmegen

Contractile properties

The rats were anaesthetized with sodium pentobarbital (70 mg/kg *ip*) and a poly-ethylene cannula was inserted through a tracheotomy The animals were mechanically ventilated with an oxygen-enriched gas mixture (flow 0 5 ml/g body weight/min, respiration frequency 70/min, duty cycle 50%) The diaphragm was quickly removed through a combined laparotomy and thoracotomy and was immediately immersed in a cooled, oxygenated Krebs solution at a pH of 7 4 This solution consisted of (mmol/l) 137 NaCl, 4 KCL, 2 $MgCl_2$, 1 KH_2PO_4, 24 $NaHCO_3$, 2 $CaCl_2$, and 7 glucose D-tubocurarine chloride 25 μM (Sigma Chemicals, The Netherlands) was added to prevent spontaneous neuromuscular activity Two small rectangular bundles, parallel to the long axis of the muscle fibers, were dissected from the middle part of the lateral costal region of each hemidiaphragm Silk sutures were firmly tied to both ends of the bundle to serve as anchoring points Each bundle was placed in a tissue bath between two large platinum stimulating electrodes The tissue baths were filled with Krebs at 37°C and were oxygenated with 95% O_2 and 5% CO_2 The central tendon insertion of the bundles were tied to a fixed point and the costal margin origin to an isometric force transducer (Sensotec, model 31/1437, Columbus OH, USA) Data acquisition and storage were performed using a Dash-16 interface and Twist-Trigger software (I D - electronics, University of Nijmegen) The stimulator (I D -electronics, University of Nijmegen) was activated by a personal computer To ensure supramaximal stimulation, subsequent stimulations were performed 20% above the voltage at which maximal forces were obtained The pulse duration was set on 0 2 ms Twitch stimuli were used to determine the optimal length (Lo), followed by a 15 min thermo-equilibration period The following

measurements were made:

Twitch characteristics: two twitches were recorded at Lo to obtain maximal twitch force (P_t), contraction time (CT), and half relaxation time ($\frac{1}{2}$RT). The averages were used for statistical analysis (10).

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_o) (10).

Force-frequency protocol: muscle bundles were stimulated every 2 min. with the following frequencies: 25, 50, 80, 120 and 160 Hz (train duration 250 ms). Data were expressed as absolute values (N/m^2) and as percentage of initial P_o).

The generated forces were expressed per cross-sectional area (N/cm^2). Cross-sectional area (CSA) was measured by dividing diaphragm bundle weight by muscle density (1.056 mg/mm^3) and bundle length.

Histological and Immunohistochemical procedures

Muscle strips obtained from the costal part of the right hemidiaphragm were embedded in Tissue-Tek° in a plastic holder. The muscle fibers were oriented parallel to the long side of the holder. Subsequently, these specimens were quickly frozen in isopentane cooled in liquid N_2, followed by further freezing in liquid N_2. 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 of five animals in each group were taken for routine H&E staining.

Anti-myosin heavy chain antibodies (Regeneron Pharmaceuticals, New York, U.S.A.) were used for morphometric examination of serial diaphragm sections. The following antibodies were used: BA-D5 reactive with type I MHCs, SC-71 reactive with type IIa MHCs, BF-35 reactive with type I, IIa and IIb but not with type IIx MHCs, and BF-F3 reactive with type IIb MHCs (32). Incubation with anti-myosin heavy chain antibodies was performed at room temperature for 1 hour. Antibodies were subsequently labelled with ultra small immunogold reagent followed by silver enhancement (Aurion, Wageningen, The Netherlands). A minimum of 300 fibers were analyzed from each diaphragm using a Sprynt-based, PC-Image digital analysis system (Bos Inc, Waddinxveen, the Netherlands). Fiber type distribution and CSA were analyzed for type I, IIa, IIx 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 glycogenolytic enzyme phosphorylase, the mitochondrial enzymes 3-hydroxyacyl-CoA

dehydrogenase (HADH), a marker for the fatty acid oxidation capacity, and citrate synthase (CS) as an index of citric acid cycle activity.

The procedures used to determine biochemical activities were recently described in detail (39). Briefly, remainings of the left and right hemidiaphragm were frozen in liquid N_2 and stored at -80°C. Segments of fresh frozen diaphragm were thawed in ice-cooled buffer containing 250 mM sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7.4). In this buffer muscle homogenates (5% wt/vol) were prepared using a Potter-Elvehjem glass-teflon homogenizer. Total phosphorylase (a+b) activity was assayed at 37°C and expressed as μmol NADPH formed/min g tissue. HADH activity, assessed at 50 μM acetoacyl-CoA at 37 °C, was expressed in nmol HADH oxidized/min g tissue. CS activity, determined at 25°C, was expressed as μmol coenzyme A formed/min g tissue. The assays for metabolic enzymes were performed spectrophotometrically in duplicate. The coefficient of variation for the assays applied was ~5%.

Data analysis
Data of contractile properties of the two bundles obtained from one rat were averaged and compared between groups using one-way analysis of variance followed by Duncan's multiple-range test. Repeated measures analysis of variance was used for 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. All tests were performed using the SPSS/PC+ package V5.0.1 (Chicago, Illinois, USA). Results were considered significant at $p < 0.05$. All data are expressed as mean $\pm$SE.

Results

Body and muscle weight
At the start of the study, body weight did not differ between the groups. Repeated measures analysis of variance showed a small but significant effect of treatment on rat body weight during the 9 month study period (Fig. 1). Body weight gain in saline, MP and MP+ND groups were 54% (from 384$\pm$7 g to 592$\pm$9 g), 44% (from 390$\pm$4 g to 564$\pm$7 g), and 41% (from 387$\pm$5 g to 546$\pm$9 g), respectively. Rat body length, measured as nose-anus as well as nose-tail length, was significantly reduced in both the MP and MP+ND groups compared to saline (Table 1). Total diaphragm muscle weight was not measured due to the speed of handling and the multiple purposes of the tissue.

Figure 1.   Growth curve
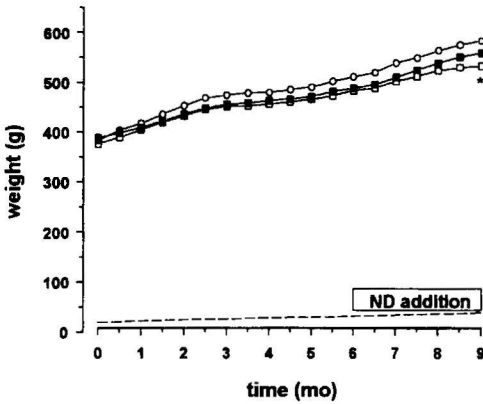Open circles: saline; closed squares: MP;
open squares: MP+ND;* p < 0.05 MP
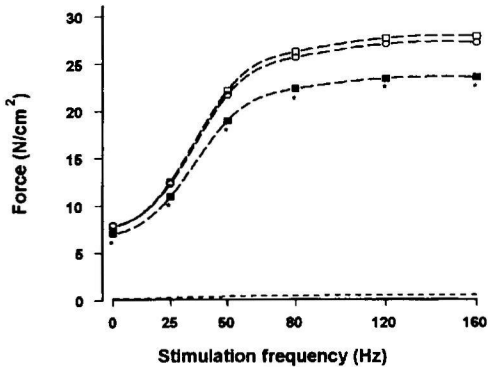and MP+ND compared to control

Figure 2.   Force-frequency curve
Open circles: saline; closed squares: MP;
open squares: MP+ND; * p < 0.05 MP
and MP+ND compared to control

Table 1. Body length

treatment	length (nose-anus)	length (nose-tail)
	cm	*cm*
saline	26.3±0.5	47.5±0.7
MP	25.8±0.5*	46.8±0.7*
MP+ND	25.9±0.5*	47.0±0.5*

means ±SE; * p<0.05 compared to saline

Diaphragm bundle dimensions and contractile properties

Diaphragm bundle dimensions were similar in all groups (data in the saline, MP and MP+ND groups: length 22.7±0.2, 22.6±0.3, and 22.9±0.2 mm; thickness 0.67±0.01, 0.67±0.01, and 0.68±0.01 mm).

Following MP treatment, P_t and P_o significantly decreased by 10 and 13%, respectively, in comparison with saline. This reduction in diaphragm force generation in the MP group was completely abolished by the addition of ND (Table 2). No changes were found in CT or ½RT. The P_t/P_o ratio was significantly lowered in the MP+ND group in comparison with MP, but both values did not differ from saline. The force-frequency curves, expressed in N/cm^2, showed a significant decrease in force generation in the MP group compared to saline. This downward shift was completely reversed by addition of ND to MP (Fig. 2). When expressed as percentage of initial P_o, no differences were observed between the three groups (data not shown)

Table 2. Contractile properties.

treatment	P_t	CT	½RT	P_o	P_t/P_o
	N/cm^2	*ms*		N/cm^2	
saline	7.9±0.1	26.4±0.4	23.5±0.4	27.1±0.3	0.29±0.02
MP	7.1±0.1*	26.8±0.4	24.3±0.5	23.2±0.5*	0.31±0.03
MP+ND	7.8±0.2	26.3±0.4	23.6±0.3	27.6±0.3	0.28±0.02†

means ±SE; P_t: twitch force; CT: contraction time; ½RT: half relaxation time; P_o: maximal tetanic force
* p<0.01 compared to saline and MP+ND; † p<0.05 compared to MP

Histology and immunohistochemistry

Histological examination of H&E stained slides showed a normal muscular pattern in all three groups. No signs of myogenic alterations such as an increase in the number of nuclei,
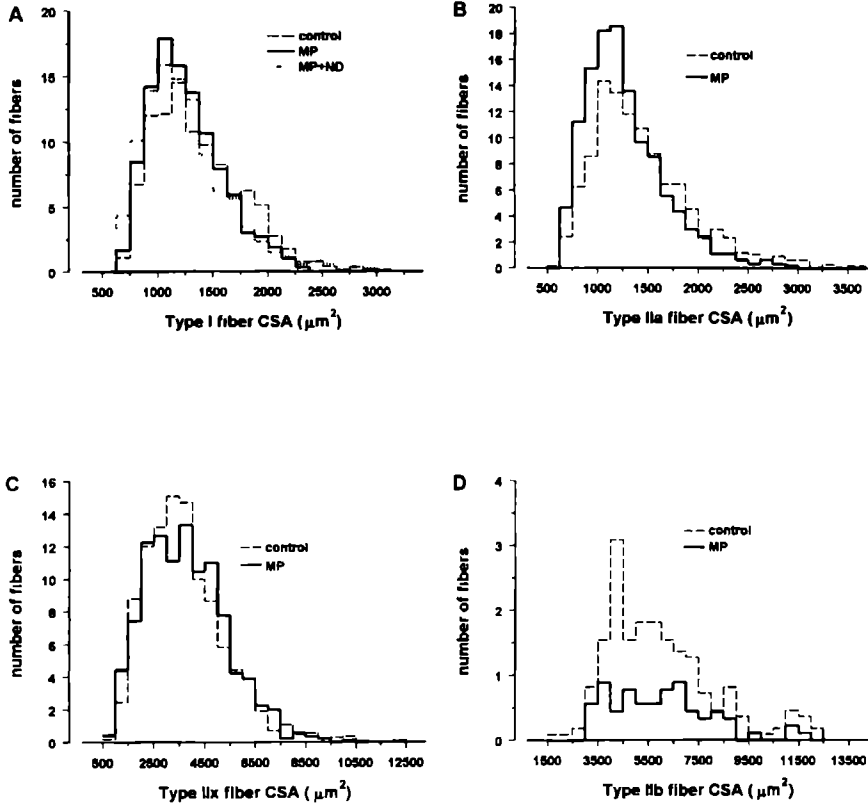
Figure 3.   Histograms showing the distribution of fibers cross sectional area
                   A. type I fibers, B: type IIa fibers, C: type IIx fibers, D: type IIb fibers

excessive variations in fiber dimensions nor excess of connective tissue were observed

Even though the proportions of the type IIb fibers in CTL animals were small, morphometric analysis of the immunohistochemically stained slides showed a significant reduction in the number of type IIb fibers (Table 3) induced by MP This reduction was not reversed by ND Type I, IIa and IIx fiber CSA significantly decreased following MP treatment In the MP+ND group, the reduction in type IIa and IIx fiber CSA was completely abolished, while ND had no effect on type I CSA (Table 3) Type IIb fiber CSA was not significantly changed by MP treatment Addition of ND to MP resulted in an increase in type IIb fiber size compared to saline The distribution of fiber CSA per fiber type is shown in figure 3 The histogram for type IIb fibers illustrates that the MP-induced decrease in number of IIb fibers occurred without preference for fiber size (Fig 3D)

Table 3. Fiber type distribution, CSA, and relative fiber type contribution to total diaphragm muscle area

treatment	type I	type IIa	type IIx	type IIb
fiber type distribution	%	%	%	%
saline	39 6±1 1	31 2±1 0	24 9±0 9	4 3±0 8
MP	41 6±1 5	30 4±1 6	27 1±1 2	0 9±0 9*
MP+ND	41 4±0 9	31 9±1 0	24 6±0 6	2 1±0 9*
fiber CSA	μm^2	μm^2	μm^2	μm^2
saline	1308±422	1519±160	4471±549	7667±935
MP	1197±335*	1451±125†	4177±481†	8650±894
MP+ND	1195±386*	1513±151	4716±580	9514±804*
fiber type contribution to total diaphragm area	%	%	%	%
saline	21 5±1 2	19 7±1	45 5±1	13 3±2 1
MP	23 2±0 7	20 7±0 9	52 7±0 9*	3 4±0 9*
MP+ND	21 2±0 6	20 9±0 7	49 5±0 6*	8 4±1

means ±SE, * p<0 05 compared to saline † compared to saline and MP+ND

As a result of the changes in number and CSA of the different fiber types, there was an increase in the relative contribution of type IIx fibers to total diaphragm muscle area following MP This increase was not reversed by administration of ND The reduced contribution of type IIb fibers in the MP group was in part reversed by addition of ND (Table 3)

Biochemistry

Glycogenolytic activity, measured by phosphorylase, was decreased in the diaphragm of the MP treated animals (p < 0 05) (Table 4) Addition of ND to MP further reduced phosphorylase HADH, a marker for ß-oxidation capacity, increased following MP treatment In the MP+ND group, HADH significantly decreased compared to MP MP alone did not change rat diaphragm oxidative capacity, indicated by CS activity Following addition of ND to MP, however, CS activity was reduced (p < 0 05)

Discussion

The present study was designed to investigate whether the anabolic steroid ND was able to reduce changes in rat diaphragm observed after six months of MP therapy, both in low, clinically relevant dosages The results show that, despite continuation of MP administration, the reduction in force generation was completely abolished by ND ND reversed the MP-induced atrophy of type IIa and IIx fibers but had no effect on type I fiber atrophy Both MP and MP+ND reduced the number of type IIb fibers in the diaphragm Biochemically, addition of ND to MP decreased oxidative capacity in the diaphragm muscle

Table 4 Biochemical analysis

treatment	phosphorylase	CS	HADH
	U/g	*U/g*	*U/g*
saline	44 3±1 3	29 4±1 3	7 97±0 4
MP	39 9±0 7**	28 0±0 7	8 83±0 22*
MP+ND	33 9±0 7†	24 8±0 7**‡	7 49±0 16‡‡

means ±SE, CK creatine kinase, CS citrate synthase HADH 3 hydroxyacyl CoA dehydrogenase * p < 0 05 compared to saline, ** p < 0 01 compared to saline, † p < 0 01 compared to saline and MP, ‡ p < 0 05 compared to MP, ‡‡ p < 0 01 compared to MP

Mechanism of action

The blunting capacity of ND on MP-induced changes, as observed in the present study, may be due to either a direct anabolic effect of ND on muscle fibers or to an antagonistic action at the receptor level of ND against glucocorticoids, or to a combination of these two actions

Regarding the first possibility, it is known that anabolic steroids can have an effect on normal skeletal muscles, i e independent of glucocorticoid treatment Anabolic steroids promote amino acid incorporation into muscle proteins, decrease amino acid catabolism, and

cause nitrogen retention and tissue growth (19) This results in an increase in muscle protein synthesis (18) and an increase in myosin and myofibrillar protein fraction (29) This may be important in the protection against glucocorticoid-induced fiber atrophy since glucocorticoids are known to reduce protein synthesis (30) The direct effect of anabolic steroids seems to be more pronounced in fast muscle fibers (11)

Second, several interactions between anabolic steroids and glucocorticoids have been described Anabolic steroids are believed to act via muscle glucocorticoid receptors rather than via muscle androgen receptors in antagonizing the catabolic effects of glucocorticoids (5) Mayer and Rosen (26) proposed a binding competition between androgens and glucocorticoids for the same side of the receptor responsible for mediating the catabolic action of glucocorticoids Inhibition of glucocorticoid action at the gene level (20) or downregulation of the glucocorticoid receptor content (34) were also reported as anabolic effects counteracting glucocorticoid-induced muscular changes

Besides these specific effects of anabolic steroids on muscle fibers, it has been shown that anabolic steroids increase capillary supply in the diaphragm resulting in an increase in endurance (12) In androgen-sensitive muscles like the levator ani, anabolic steroids may affect neuromuscular structure and function The density of acetyl choline receptors at the endplates is increased in the levator ani (3) probably as an adaptive adjustment to the androgen-induced increase in muscle fiber size This adaptation may be required to maintain a normal synaptic function If such mechanism also occurs in diaphragm muscle neuromuscular junctions is unknown

Body weight and muscle masses

The effects of anabolic steroids on body weight are gender related Anabolic steroids increased body weight gain in female animals (5,11), while in males a reduction (31) or similar (5) body weight gain was found compared to control When testosterone was combined with cortisone, body weight of male rabbits was higher in comparison to cortisone alone, but still significantly less than in control animals (14) In the present study, ND was not able to abolish the small reduction in weight gain caused by MP This discrepancy can be explained by the different starting points of the anabolic steroid intervention ND addition in this study was started following 6 months of MP treatment, while Ferguson (14) simultaneously started with testosterone and cortisone administration The small increase in relative EDL muscle weight in the MP+ND group compared to saline and MP, and the lack of changes in soleus muscle weight can be explained by a more pronounced effect of anabolic steroids on fast muscle fibers (11)

It has been suggested that malnutrition is in part responsible for the glucocorticoid-induced diaphragm impairment Indeed, in most animal studies, including the present one,

glucocorticoid treatment resulted in a reduction in body weight gain (10) It must be noted, however, that in the present study the differences in body weight gain between the saline and MP group were small (54, and 44% of initial weight in saline and MP animals, respectively) In addition, rat diaphragm muscle function was reduced following prednisolone administration while contractile properties were not affected in a pair weight control group (40) Short term triamcinolone administration and malnutrition had significantly different effects on rat diaphragm muscle (9) Although in that study a fluorinated steroid was used, we believe that there is enough evidence that corticosteroids and nutritional deprivation both affect diaphragm muscle in a different way This argument is supported by the observation that addition of ND to the MP treated animals further decreased body weight, while muscle contractile properties were improved This apparent discrepancy may in part be explained by the increase fat free body mass observed as result of anabolic steroid administration (2,33) However, we believe that this is subordinate to the effects of anabolic steroids on corticosteroid induced changes

Contractile properties
Previous studies showed that glucocorticoids can reduce diaphragm force generation, depending on the dose and duration of the therapy (10,39) In the present study we used a very low glucocorticoid dosage which was 2 5 times lower than the lowest dosage previously reported (8), but the period of administration was fourfold prolonged This confirms that the duration of administration is an important factor contributing to the onset of glucocorticoid-induced changes in contractile properties

The effects of anabolic steroids on skeletal muscle force generation are inconsistent (19) Administration of durabolin increased twitch force and improved fatigue resistance in the EDL muscle of female rats (11)

The MP-induced reduction in specific force in the present study was completely reversed by addition of ND to MP Testosterone administration was able to abolish the decrease in diaphragm muscle endurance observed following cortisone (10 mg/kg/day) treatment in male rabbits (14) The direct anabolic action of ND on muscle fibers as well as the antagonizing action of ND on MP, as described above, may be responsible for these drug-induced changes in diaphragm muscle function This direct effect of ND on muscle fibers results in an increase in muscle protein synthesis (18) and an increase in myosin and myofibrillar protein fraction (29) This may be an important mechanism in regaining force generation, since glucocorticoids are known to reduce these proteins resposible for muscle contraction (30)

Morphometry
In the present study, ND did not reverse the MP-induced decrease in number of fibers

expressing type IIb MHCs Such effect would not be expected, since ND did not alter the expression of the different MHC genes (37) Moreover, anabolic steroids are not believed to stimulate satellite cells in muscles (4) Thus, it is unlikely that new fibers are generated by ND

The type IIa and IIx fiber atrophy caused by MP, was completely abolished by ND In contrast, ND had no effect on MP-induced type I fiber atrophy The observation that the effects of anabolic steroids on fast fibers are more pronounced is in accordance with the findings by Egginton (11) This author found hypertrophy of fast fibers in the diaphragm muscle following nandrolone phenylpropionate treatment (1 mg/kg every other day for 5-6 weeks), while the CSA of slow fibers did not change

Compared to saline, MP treatment shifted the fiber contribution to total diaphragm area from type IIb to IIx fibers Addition of ND did reverse this reduction in type IIb contribution, while the increase in type IIx contribution to total diaphragm area compared to saline was still present Since fiber type distribution was similar in the MP and MP+ND groups, the changes in fiber contribution to total diaphragm muscle area are the result of the ND-induced changes in fiber CSA Although changes in type IIb fiber CSA and proportion were statistically significant between the treatment groups, it must be noted that this is probably of minor clinical relevance since the amount of type IIb fibers in the rat diaphragm is small

The morphometric data in the present study may have been influenced by the fact that muscle strips were not fixed at optimal length during 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 (36) In our study this passive muscle tension was similar in the saline, MP, and MP+ND groups (0 038±0 01 N, 0 037±0 01 N, and 0 038±0 01 N, respectively) As a consequence, the degree of muscle shortening (and thus the change in fiber CSA) is not likely to be different between the groups This, however, does not exclude the possibility of a disproportion in degree of shortening between fiber types The differences in CSA between type I, IIa, IIx and IIb fibers in the saline group, however, were in proportion to the differences in CSA when muscle strip were fixed at optimal length (35) Thus, the physiological differences in size among the different fiber types did not appear to be affected by muscle shortening in the present study

Biochemistry

Treatment with glucocorticoids alone has been shown to increase glycogen storage in rabbit diaphragm muscle (15) In the diaphragm of MP treated rats (1 mg/kg/day for 8 weeks) glycogenolytic activity decreased, oxidative capacity increased, and ß oxidation capacity

(HADH) remained unchanged in comparison to saline (39). Short-term (10 days) low-dose prednisolone (0.5, 1 and 2 mg/kg/day *sc*) administration did not change HADH enzyme capacity or CS activity in diaphragm muscle (25).

In soleus or superficial vastus muscle of male rats, nandrolone phenylpropionate (0.5 mg every second day) did not change CS activity or glycogen content (41). Methandrostenolone did not change glycolytic activity or oxidative capacity in the gastrocnemius muscle of male guinea pigs (13). Other investigators observed an increase in oxidative capacity in the EDL muscle of male rats, while no such change was found in the soleus muscle (23). Thus, anabolic steroids appear to cause little or no change in muscle biochemistry.

In the present study, addition of ND to MP had no beneficial effects on biochemical activities. ND addition even reduced glycogenolytic activity, ß-oxidation capacity and oxidative capacity compared to MP alone. Compared to cortisone treatment alone, the combination of cortisone and testosterone did not improve biochemical enzyme activities either (14). The mechanism of the additional negative effects on diaphragm biochemistry in the ND+MP group is unclear.

Clinical significance

The observed reduction in diaphragm force generation following MP administration in this study may be of clinical importance in patients with severe COPD. In these patients, respiratory muscle function may be compromised by factors like hyperinflation, malnutrition, physical inactivity, disturbances in blood gases and cardiac failure (17).

Regarding the effects of ND, Schols *et al.* provided evidence that anabolic steroids may be beneficial in regaining respiratory muscle strength in malnourished COPD patients (33). This was probably the result of an increase in muscle mass in patients receiving ND in addition to the nutritional support. A recent study by Bhasin and coworkers showed a beneficial effect of a high dose of testosterone (600 mg per week) on fat-free body mass, muscle size and peripheral muscle strength in normal men (2).

Other pharmacological interventions to reduce glucocorticoid-induced myopathic changes in skeletal muscles have been studied. Growth hormone did not prevent glucocorticoid-induced changes in rat diaphragm (28), while glucocorticoid-induced muscle atrophy was partly prevented by clenbuterol (4 mg/kg/day) (1) and by testosterone (20 mg/kg/day) (14). The clinical applicability of anabolic drugs in glucocorticoid-induced myopathy, has yet to be evaluated.

References

1   Agbenyega, E T and A C Wareham Effect of clenbuterol on skeletal muscle atrophy in mice induced by the glucocorticoid dexamethasone Comp Biochem Physiol 102A 141-145, 1992

2   Bhasin, S , T W Storer, N Berman, C Callegari, B Clevenger, J Phillips, T J Bunnell, R Tricker, A Shirazi, and R Casaburi The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men N Engl J Med 335 1-7, 1996

3   Bleisch, W V and A Harrelson Androgens modulate endplate size and ACh receptor density at synapses in rat levator ani muscle J Neurobiol 20 189-202, 1989

4   Danhaive, P A and G G Rousseau Binding of glucocorticoid antagonists to androgen and glucocorticoid hormone receptors in rat skeletal muscle J Steroid Biochem 24 481-487, 1986

5   Danhaive, P A and G G Rousseau Evidence for sex-dependent anabolic response to androgenic steroids mediated by muscle glucocorticoid receptors in the rat J Steroid Biochem 29 575-581, 1988

6   Decramer, M , L M Lacquet, R Fagard, and P Rogiers Corticosteroids contribute to muscle weakness in chronic airflow obstruction Am J Respir Crit Care Med 150 11-16, 1994

7   Dekhuijzen, P N R and M Decramer Steroid induced myopathy and its significance to respiratory disease a known disease rediscovered Eur Respir J 5 997-1003, 1992

8   Dekhuijzen, P N R , G Gayan-Ramirez, A Bisschop, V de Bock, R Dom, and M Decramer Different effects of methylprednisolone and deflazacort on contractile properties and histopathology of rat diaphragm Eur Respir J 8 824-830, 1995

9   Dekhuijzen, P N R , G Gayan-Ramirez, A Bisschop, V de Bock, R Dom, and M Decramer Corticosteroid treatment and nutritional deprivation cause a different pattern of atrophy in rat diaphragm J Appl Physiol 78 629 637, 1995

10 Dekhuijzen, P N R , G Gayan Ramirez, V de Bock, R Dom, and M Decramer Triamcinolone and prednisolone affect contractile properties and histopathology of rat diaphragm differently J Clin Invest 92 1534 1542, 1993

11 Egginton, S Effects of an anabolic hormone on striated muscle growth and performance Eur J Physiol 410 349 355, 1987

12 Egginton, S Effects of an anabolic hormone on aerobic capacity of rat striated muscle Eur J Physiol 410 356 361, 1987

13 Feraudi, M and H Weicker Effects of training and methandrostenolone (an anabolic steroid) on energy metabolism in the guinea pig changes in enzyme activities in gastrocnemius muscle and myocardium Int J Biochem 17 1191-1205, 1985

14 Ferguson, G T Effects of cortisone and testosterone on diaphragmatic function and biochemistry in the rabbit J Appl Physiol 78 1459 1468, 1995

15 Ferguson, G T , C G Irvin, and R M Cherniack Effects of corticosteroids on diaphragm function and biochemistry in the rabbit Am Rev Respir Dis 141 156-163, 1990

16 Frenkel, J K and M A Havenhill The corticoid sensitivity of golden hamsters, rats, and mice Lab Invest 12 1204-1220, 1963

17 Gibson, J Respiratory muscles In Chronic obstructive pulmonary disease, edited by P M A Calverley and N B Pride London Chapman and Hall, 1995, 185-203

18 Griggs, R C , W Kingston, R F Jozefowicz, B E Herr, G Forbes, and D Halliday Effect of testosterone on muscle mass and muscle protein synthesis J Appl Physiol 66 498 503, 1989

19 Haupt, H A and G D Rovere Anabolic steroids a review of the literature Am J Sports Med 12 469-484, 1984

20 Hickson, R C , S M Czerwinski, M T Falduto, and A P Young Glucocorticoid antagonism by exercise and androgenic-anabolic steroids Med Sci Sports Exerc 22 331 340, 1990

21 Hudson, L D and C M Monti Rationale and use of corticosteroids in chronic obstructive pulmonary disease Med Clin North Am 74 661-690, 1990

22   Jasani, M K Anti-inflammatory steroids mode of action in rheumatoid arthritis and homograft reaction In Anti-inflammatory drugs, edited by J R Vane and S H Ferreira Berlin Springer Verlag, 1979, 598-660

23   Kuipers, H , F M Peeze Binkhorst, F Hartgens, J A Wijnen, and H A Keizer Muscle ultrastructure after strength training with placebo or anabolic steroid Can J Appl Physiol 18 189-196, 1993

24   Laaban, J , B Kouchakji, M Dore, E Orvoen-Frija, P David, and J Rochemaure Nutritional status of patients with chronic obstructive pulmonary disease and acute respiratory failure Chest 103 1362-1368, 1993

25   Lieu, F K , S K Powers, R A Herb, D Criswell, D Martin, C Wood, W Stainsby, and C L Chen Exercise and glucocorticoid-induced diaphragmatic myopathy J Appl Physiol 75 763-771, 1993

26   Mayer, M and F Rosen Interaction of glucocorticoids and androgens with skeletal muscle Metabolism 26 937-945, 1977

27   Moore, B J , M J Miller, H A Feldman, and M B Reid Diaphragm atrophy and weakness in cortisone-treated rats J Appl Physiol 67 2420-2426, 1989

28   Petrof, B J , S B Gottfried, J Eby, J LaManca, and S Levine Growth hormone does not prevent corticosteroid-induced changes in rat diaphragm structure and function J Appl Physiol 79 1571-1577, 1995

29   Rogozkin, V A Anabolic steroid metabolism in skeletal muscle J Steroid Biochem 11 923-926, 1979

30   Ruff, R L Endocrine myopathies In Myology, edited by A G Engel and B Q Banker McGraw Hill Book Company, 1986, 1871-1906

31   Saborido, A , J Vila, F Molano, and A Megias Effect of anabolic steroids on mitochondria and sarcotubular system of skeletal muscle J Appl Physiol 70 1038-1043, 1991

32   Schiaffino, S , L Gorza, S Sartore, L Saggin, S Ausoni, M Vianello, K Gundersen, and T Lomo Three myosin heavy chain isoforms in type 2 skeletal muscle fibers J Musc Res Cell Motility 10 197-205, 1989

33   Schols, A M W J , P B Soeters, R Mostert, R J Pluymers, and E F M Wouters Physiologic effects of nutritional support and anabolic steroids in patients with chronic obstructive pulmonary disease - A placebo-controlled randomized trial Am J Respir Crit Care Med 152 1268-1274, 1995

34   Sharpe, P M , P J Buttery, and N B Haynes The effects of manipulating growth in sheep by diet or anabolic agents on plasma cortisol and muscle glucocorticoid receptors Br J Nutr 56 289-304, 1986

35   Sieck, G C , W Z Zhan, Y S Prakash, M J Daood, and J F Watchko SDH and actomyosin ATPase activities of different fiber types in rat diaphragm muscle J Appl Physiol 79 1629-1639, 1995

36   Tao, H and G A Farkas Predictability of ventilatory muscle optimal length based on excised dimensions J Appl Physiol 72 2024-2028, 1992

37   Tsika, R W , R E Herrick, and K M Baldwin Effect of anabolic steroids on skeletal muscle mass during hindlimb suspension J Appl Physiol 63 2122 2127, 1987

38   van Balkom, R H H , P N R Dekhuijzen, H T M Folgering, J H Veerkamp, H T B van Moerkerk, J A M Fransen, and C L A van Herwaarden Effects of long term low-dose methylprednisolone on rat diaphragm function and structure Muscle & Nerve 20 983-990, 1997

39   van Balkom, R H H , H F M van der Heijden, H T B van Moerkerk, J H Veerkamp, J A M Fransen, L A Ginsel, H T M Folgering, C L A van Herwaarden, and P N R Dekhuijzen Effects of different treatment regimens of methylprednisolone on rat diaphragm contractility, immunohistochemistry and biochemistry Eur Respir J 9 1217-1223, 1996

40   van Balkom, R H H , W Z Zhan, Y S Prakash, P N R Dekhuijzen, and G C Sieck Corticosteroid effects on isotonic contractile properties of rat diaphragm muscle J Appl Physiol 83 1062-1067, 1997

41   Van Zyl, C G , T D Noakes, and M I Lambert Anabolic androgenic steroid increases running endurance in rats Med Sci Sports Exerc 27 1385 1389, 1995

CHAPTER 8

Effects of anabolic steroids on diaphragm impairment induced by methylprednisolone in emphysematous hamsters

R.H.H. van Balkom, P.N.R. Dekhuijzen, H.F.M. van der Heijden,
H.T.M. Folgering, J.A.M. Fransen[a], and C.L.A. van Herwaarden

[a] Department of Cell Biology and Histology,
University of Nijmegen, The Netherlands

Abstract

This study was designed to investigate whether nandrolone decanoate (ND) was able to antagonize the loss in diaphragm function induced by long-term low-dose methylprednisolone (MP) administration in emphysematous hamsters Male hamsters were randomized into 1) control (CTL), 2) normal hamsters receiving MP (MP), 3) emphysema (EMPH), 4) EMPH receiving MP 0 2 mg/kg/day for nine months (EMPH+MP), and 5) EMPH receiving MP combined with ND 1 mg/kg/wk *im* during the last 3 months (EMPH+MP+ND) Contractile properties of isolated diaphragm strips and diaphragm muscle fiber composition, using antibodies reactive with type I, IIa, and IIx myosin heavy chains (MHC), were determined Force generating capacity was lowered by $\sim 12\%$ in the EMPH group compared to CTL and further decreased by $\sim 11\%$ in the EMPH+MP animals However, diaphragm force impairment was similar in the MP and EMPH+MP animals Addition of ND to the EMPH+MP hamsters improved force generation Force generation at low stimulation frequencies reached the level of CTL, while force generation at high frequencies improved to the level of the EMPH hamsters MP decreased type IIx fiber cross sectional area (CSA) compared to control Type IIa fiber CSA was reduced in the EMPH+MP group Addition of ND, however, prevented this reduction in type IIa fiber CSA MHC-IIx fiber CSA was reduced by $\sim 20\%$ in the EMPH and EMPH+MP groups Addition of ND restored type IIx fiber CSA to CTL values In conclusion, ND in part reversed the loss in diaphragm force generating capacity in emphysematous hamsters treated with MP, and reversed type IIa and IIx fiber atrophy completely

Introduction

Patients with severe chronic obstructive pulmonary disease (COPD) may suffer from respiratory muscle weakness due to hyperinflation, malnutrition, disturbances in blood gases and cardiac failure (13) In these patients, respiratory muscle function was recently reported to be further reduced by administration of a low-dose methylprednisolone (MP) (mean dosage 4 3 mg) daily for 6 months (3) Since respiratory muscle weakness may contribute to respiratory failure, interventions that attenuate or even abolish respiratory muscle impairment in these patients may be of clinical importance

With respect to the impairment caused by glucocorticoids, the use of anabolic steroids may be considered Anabolic steroids are able to raise skeletal muscle protein synthesis (15) This may be of relevance since the major cause of glucocorticoid-induced muscle

dysfunction is believed to be a reduction in muscle protein (32). Besides glucocorticoid treatment, malnutrition is also known to cause a catabolic condition in patients with COPD (17,21). This catabolic condition may in part be reversed by anabolic steroids in combination with adequate protein intake (15). Indeed, Schols and coworkers (35) recently showed that anabolic steroids combined with refeeding improved respiratory muscle function in undernourished COPD patients. Whether anabolic steroids are also able to counteract the glucocorticoid-induced impairment in human respiratory muscle function has not yet been studied.

Animal models have been used to obtain insight in the underlying mechanisms responsible for the changes in diaphragm muscle structure and function. The type and severity of alterations caused by glucocorticoids appear to depend on the type of steroid used, and the dosage and the duration of administration. In a previous study, we observed changes in contractile properties and morphology of the diaphragm from normal rats following long-term treatment with a low dose of methylprednisolone (MP; 0.2 mg/kg/day for 6 months) (39). Recent results from our lab showed that in normal rats anabolic steroids were able to reverse diaphragm function loss due to corticosteroid-induced myopathy (40).

The use of emphysematous animals to determine drug effects on the diaphragm may be of interest since this animal model resembles to some extent the functional alterations observed in COPD patients. Induction of emphysema by intratracheal instillation of elastase in hamsters resulted in a progressive increase in lung volumes and compliance (38), diaphragm muscle adaptation to hyperinflation of the lungs (7,23), and impairment in respiratory muscle function (7,23).

Therefore, we used this emphysematous model to investigate whether anabolic steroids in a clinically relevant dose are able to antagonize the impairment caused by long-term low-dose MP treatment on the diaphragm that adapted to emphysematous changes in the lung. Based on the metabolic effects of anabolic steroids described above, we hypothesized that anabolic steroids are able to reverse the alterations in diaphragm function and structure induced by MP. Therefore, contractile properties of the diaphragm of emphysematous hamsters treated with nandrolone decanoate during the last three months of a nine month treatment period with a low dose of MP were examined. Immunoreactivity to MHC antibodies was tested to evaluate the effects of MP and ND on diaphragm fiber composition.

Methods

Study design, induction of emphysema, and treatment

Adult male Golden hamsters (n=48), aged >40 weeks, weighing ~150 g, were lightly anaesthetized with a mixture of halothane and N_2O, vaporized in air A poly-ethylene cannula was inserted into the trachea with the tip located above the carina The hamsters received either a single instillation of NaCl 0 9% 0 5 ml/100 g body weight (n=12) or 25 IU porcine pancreatic elastase (PPE) (Sigma Chemicals, Bornem, Belgium) per 100 g body weight (n=36) PPE was dissolved in a concentration of 25 IU per 0 5 ml 0 9% NaCl to ensure similar fluid loads per animal To improve the distribution to the peripheral parts of the lung, 3 ml of room air was injected through the tube The hamsters were monitored carefully until spontaneous breathing

Six months following instillation, emphysematous hamsters were randomized into three groups (n=12 per group, fig 1)

normal hamsters

- controls (CTL) receiving saline 0 2 ml sc daily
- controls receiving methylprednisolone (MP) hemisuccinate (Sigma Chemicals, Bornem, Belgium) 0 2 mg/kg sc daily for nine months

emphysematous hamsters

- emphysematous hamsters (EMPH) receiving saline 0 2 ml sc daily
- emphysematous hamsters receiving MP 0 2 mg/kg sc daily for nine months (EMPH+MP)
- emphysematous hamsters receiving MP 0 2 mg/kg sc daily, during the last 3 months combined with nandrolone decanoate (ND) (Organon, Oss, The Netherlands) 1 mg/kg im once a week (EMPH+MP+ND)

Our intention was to evaluate the effects of a low dose of a non-fluorinated steroid (MP) comparable to the dose that is occasionally used in chronic treatment of patients with COPD We made a calculated estimation that 0 2 mg/kg of MP is equivalent to a dose of at most 14 mg/day in a 70 kg human This was based on similar anti-inflammatory potency and metabolism in rodents and humans (18,33), an absorption of only 60% after im injection of cortisone acetate (26), and the observation that the sc route requires higher doses to produce similar effects compared to im administration (12) Indeed, in a previous study, administration of 0 2 mg/kg/day of MP for 6 months in normal rats caused a 10-15% reduction in diaphragm twitch and maximum tetanic force generation (39) Although the therapeutic efficacy of glucocorticoids in COPD is at least controversial (17), prolonged prednisolone administration in doses of 10-15 mg daily are no exception in the treatment of these patients ND was administered in a dose used by others in clinical studies (35), and recommended for patient use by the manufacturer

During the nine month treatment period, all hamsters received a similar volume

(~ 0.20 ml) with each sc injection. The hamsters were fed ad libitum (RMHB, Hope Farms, Woerden, the Netherlands) with free access to drinking water, held on a 12/12 hour light-dark regime and weighed once every week. Although daily food intake was not precisely quantified (animals were not held in metabolic cages), food intake appeared to be similar in all groups (~ 10 g per day). In line with earlier studies (20,25), previous pilot experiments showed no change in body weight curve in normal and EMPH hamsters. Therefore, a pair-weight or pair-fed control group was not included.

Fifteen months after instillation and nine months following the start of the treatment with MP or saline, the animals were sacrificed to measure contractile properties and histological characteristics of the diaphragm. The diaphragm, musculus extensor digitorum longus (EDL) and the soleus muscle were weighed immediately after dissection and the lungs were removed to evaluate the extent of emphysema. 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.

Verification of emphysema

Five animals per group were used to evaluate the degree of emphysema induced by PPE. At the end of the treatment period, the hamsters were anaesthetized with sodium pentobarbital (70 mg/kg ip). A poly-ethylene 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%). After dissection, the lungs were inflated with 4% buffered formalin to a pressure of 25 cm H_2O. Minimal fixation time was 2 hours. Postfixation lung volume was determined by fluid displacement. The left lower lobe was embedded in paraffine and sagittal sections (6 μm thickness) were cut and stained with hematoxylin-eosin. Alveolar cross sectional area (CSA) was measured to determine the extent of emphysematous changes in the lung. These measurements were made using a Sprynt-based, PC-Image digital analysis system (Bos Inc, Waddinxveen, the Netherlands).

Contractile properties

Procedures for anaesthesia and intubation were performed as described above. 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, 1 $MgCl_2$, 1 KH_2PO_4, 24 $NaHCO_3$, 2 $CaCl_2$, and 7 glucose. D-tubocurarine chloride 25 μM (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_2 and 5% CO_2 The central tendon insertion of the bundles were tied to a fixed point and the costal margin origin to an isometric force transducer (Sensotec, model 31/1437, Columbus OH, USA) 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 The pulse duration was set on 0 2 ms Twitch stimuli were used to determine the optimal length (Lo), followed by a 15 min thermo-equilibration period The following measurements were made

Twitch characteristics two twitches were recorded at Lo to obtain maximal twitch force (P_t), contraction time (CT), and half relaxation time (½RT) The averages were used for further analysis

Maximal tetanic contraction two maximal tetanic stimuli (with a frequency of 160 Hz and a train duration of 400 ms) were generated to obtain maximal tetanic force (P_o)

Force-frequency protocol muscle bundles were stimulated every 2 min with the following frequencies 25, 50, 80, 120 and 160 Hz (train duration 400 ms)

The generated force was expressed per cross sectional area (N/cm^2) Cross-sectional area (CSA) was measured by dividing diaphragm bundle weight by muscle density (1 056 mg/mm^3) and bundle length

Morphometric evaluation of the diaphragm muscle

Resting (excised) muscle length of strips obtained from the costal part of the right hemidiaphragm were measured Before freezing, the strips were stretched to 1 5 times this excised length to approximate Lo (29), and pinned on cork These specimens were quickly frozen in isopentane cooled in liquid N_2 followed by further freezing in liquid N_2 Serial cross sections were cut at 7 μm with a cryostat kept at -30°C

Myosin heavy chain antibodies (DSM, Braunschweig, Germany) were used for morphometric examination of serial diaphragm sections The following antibodies were used BA-D5 reactive with MHC-I, SC-71 reactive with MHC-IIa, BF-35 reactive with MHC-I, MHC-IIa and MHC-IIb but not with MHC IIx, and BF-F3 reactive with MHC-IIb (34) Incubation with anti-myosin heavy chain antibodies was performed at room temperature for 1 hour Antibodies were subsequently labelled with ultra small
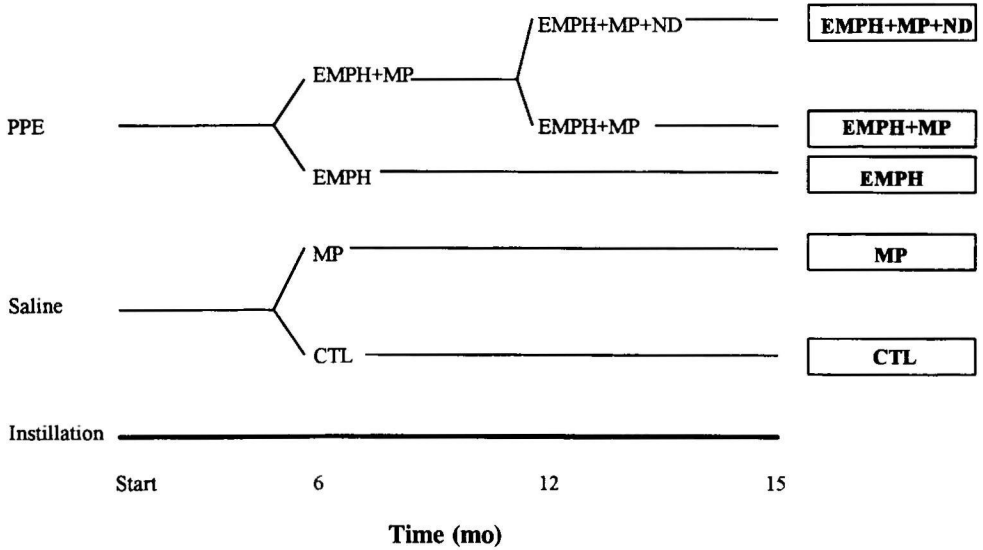
Figure 1.   Time course of intervention
                  PPE: porcine pancreatic elastase
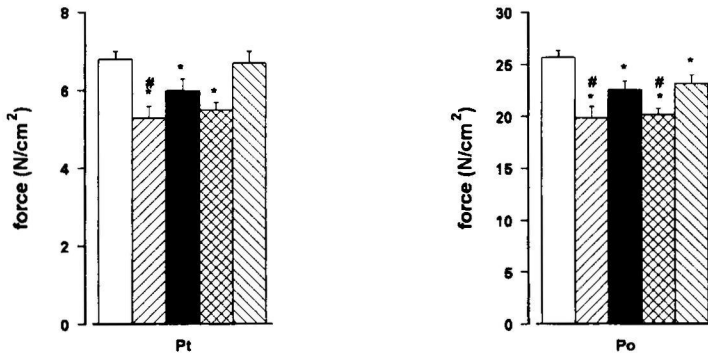

Figure 2.   Twitch force (left panel).
                  P_t: twitch force; * $p < 0.05$ compared to control and EMPH+MP+ND.
                  Maximal tetanic force (right panel).
                  P_o: maximal tetanic force; * $p < 0.05$ compared to control; #
                  $p < 0.05$ compared to EMPH+MP+ND

immunogold reagent followed by silver enhancement (Aurion, Wageningen, The Netherlands) The cross sectional area (CSA) of at least 250 fibers were analyzed from each diaphragm using a Sprynt-based, PC-Image digital analysis system (Bos Inc, Waddinxveen, the Netherlands)

Data analysis

Data of contractile properties of the two bundles obtained from one hamster were averaged The SPSS/PC+ package V 6 1 3 (Chicago Illinois, USA) was used for statistical analysis Data were compared using one-way analysis of variance followed by Duncan's multiple-range test Repeated measures ANOVA was used to analyze the force-frequency relationship Morphometric analysis was performed using the 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 $\pm$ SE

Results

Body and muscle weights

Growth curve was not significantly affected by drug treatment or by induction of emphysema Initial and final body weight were similar in all groups (table 1) No differences in diaphragm and soleus muscle weight were observed An increase in EDL muscle weight, normalized for body weight, was found in the EMPH+MP+ND group compared to CTL, EMPH, and EMPH+MP (table 1)

Table 1. Body and muscle weights

treatment	initial body weight	final body weight	diaphragm	EDL	soleus
	g	*g*	‰ *b w*	‰ *b w*	‰ *b w*
CTL	150±3	149±2	2 02±0 04	0 21±0 01	0 24±0 01
MP	151±4	145±3	1 94±0 03	0 22±0 01	0 24±0 01
EMPH	149±3	148±3	2 08±0 02	0 20±0 01	0 22±0 01
EMPH+MP	152±4	147±3	2 04±0 03	0 21±0 01	0 23±0 01
EMPH+MP+ND	149±4	145±3	2 07±0 04	0 23±0 01*	0 24±0 01

means $\pm$ SE, EDL extensor digitorum longus, *b w* body weight, * $p < 0.05$ compared to CTL, EMPH, and EMPH+MP

Degree of emphysema

The alveolar cross sectional area in the EMPH, EMPH+MP, and EMPH+MP+ND

hamsters was ~190% from CTL and MP hamsters (5061 ±319, 5132 ±328, and 5248 ±363 μm^2, respectively, versus 2740 ±86 μm^2 and 2675 ±79 μm^2 in CTL and MP) Fluid displacement lung volume increased by ~50 % from normal hamsters (9 5 ±0 8, 9 7 ±1 2, and 9 8 ±1 3 ml in EMPH, EMPH+MP, and EMPH+MP+ND versus 6 3 ±0 5 ml and 6 5 ±0 5 ml in CTL and MP) No differences were found between the three groups of emphysematous hamsters

Table 2. Diaphragm muscle strip dimensions

treatment	length	width	thickness	weight	weight normalized for strip dimension
	mm	*mm*	*mm*	*mg*	*mg/mm³*
CTL	15 6±0 3	2 1±0 03	0 6±0 01	15 3±0 7	0 8±0 02
MP	15 2±0 3	2 2±0 05	0 6±0 02	15 9±0 8	0 8±0 03
EMPH	14 2±0 2*	2 0±0 02	0 6±0 01	12 9±0 5*	0 8±0 03
EMPH+MP	14 5±0 1*	2 1±0 03	0 6±0 01	14 1±0 6	0 8±0 02
EMPH+MP+ND	14 6±0 3*	2 0±0 03	0 6±0 01	14 1±0 5	0 8±0 02

means ±SE, * p<0 05 compared to CTL and MP

Diaphragm bundle dimensions and contractile properties
Diaphragm muscle strip length was significantly reduced in all EMPH groups compared to CTL and MP (table 2) Muscle strip weight was reduced only in the EMPH group However, no differences were found between the groups when muscle strip weight was normalized for muscle strip dimension (table 2)

Table 3. Diaphragm contractile properties

treatment	CT	½RT	CT+½RT	Pt/Po
	ms	*ms*	*ms*	*%*
CTL	26 7±0 2	28 4±0 7	55 1±0 9	26 5±0 6
MP	26 1±0 3	28 1±0 7	55 2±1 0	27 2±0 9
EMPH	26 7±0 4	28 8±0 8	55 5±1 2	26 8±0 6
EMPH+MP	27 0±0 3	29 5±0 6	56 5±0 7	27 9±0 8
EMPH+MP+ND	27 5±0 4	30 8±1 0	58 3±1 2*	29 3±0 9†

means ±SE, CT contraction time, ½RT half relaxation time, P_t twitch force, P_o maximal tetanic force, * p<0 05 compared to CTL and MP, † p<0 05 compared to CTL and EMPH
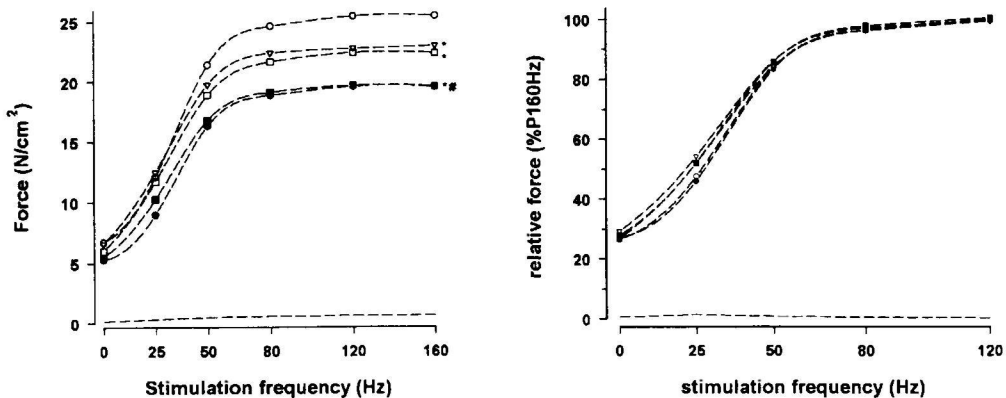
Figure 3A. Force-frequency curve (absolute force, left panel)
open circles: control; closed circles: MP; open squares: EMPH; closed squares: EMPH+MP; open triangles: EMPH+MP+ND
* $p < 0.05$ compared to control; # $p < 0.05$ compared to EMPH+MP+ND

3B. Force-frequency curve (normalized for P_o, right panel)
open circles: control; closed circles: MP; open squares: EMPH; closed squares: EMPH+MP; open triangles: EMPH+MP+ND
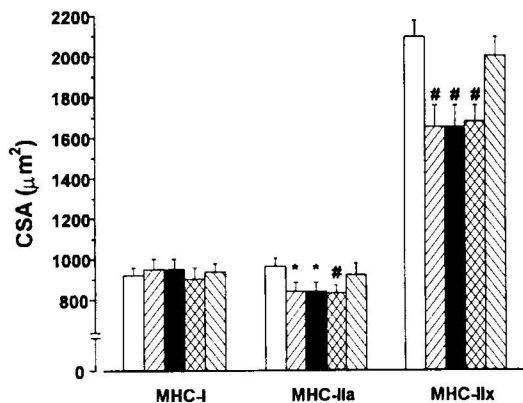

Figure 4. Fiber type cross-sectional area
open bars: control; hatched bars (/): MP; solid bars: EMPH; cross hatched bars: EMPH+MP; hatched bars (\): EMPH+MP+ND; * $p < 0.05$ compared to control; # $p < 0.05$ compared to control and EMPH+MP+ND

Pt was reduced in the diaphragm of the MP, EMPH and EMPH+MP animals compared to CTL and EMPH+MP+ND (fig 2) Twitch force in the MP animals was significantly lower compared to EMPH Addition of ND increased Pt to the level of CTL Po was significantly lower in all intervention groups No significant differences in contractile properties were found between MP and EMPH+MP Since Po decreased in the MP and EMPH+MP group compared to all other groups, the most pronounced reduction in maximum force was observed following MP treatment Administration of ND to the animals increased Po to the level of the EMPH group (fig 2) Induction of EMPH nor treatment with MP or ND caused changes in CT or 1/2RT However, since fusion of the contractions is dependent on the speed of contraction and relaxation, we also analyzed the sum of CT and 1/2RT ND addition significantly lengthened the sum of CT and 1/2RT compared to CTL and MP (table 3) This suggests earlier fusion of contractions at lower frequencies in the EMPH+MP+ND diaphragm muscle

Repeated measures ANOVA indicated a significant effect of treatment on absolute and normalized force-frequency relationship (fig 3 and 4) Force generation at 25 Hz was decreased in the MP and EMPH+MP group compared to CTL but not compared to EMPH (fig 3) At all stimulation frequencies above 25 Hz, no differences in force between the MP and EMPH+MP group were found, however, force production in the EMPH+MP animals was significantly lower compared to EMPH Addition of ND reversed this decrease in force to the level of the EMPH animals No differences were found between CTL, MP, EMPH, and EMPH+MP when forces were normalized for maximum tetanic tension (fig 4) However, ND addition seemed to have a more pronounced effect at lower frequencies as reflected by the significant increase in Pt/Po in the EMPH+MP+ND compared to CTL, MP, and EMPH (table 3) Normalized force production at 25 Hz was significantly increased in the EMPH+MP+ND group compared to CTL (fig 4)

Diaphragm muscle morphology

The number of fibers expressing predominantly type I MHC isoforms decreased in the diaphragm of the EMPH hamsters compared to CTL and EMPH+MP+ND (table 4) No significant changes in MHC-IIa and MHC-IIx fiber distribution were observed

MP reduced MHC-IIx CSA, whereas, EMPH and EMPH+MP both reduced CSA of MHC-IIa and MHC-IIx diaphragm muscle fibers compared to CTL (fig 5) However, no significant difference was found between CSA in the EMPH and the EMPH+MP groups ND addition reversed the reduction in both the MHC-IIa and MHC-IIx CSA in the EMPH+MP diaphragm to the level of CTL

Relative fiber type contribution to total diaphragm muscle area was not changed by

either treatment (table 4). No fibers reactive with type IIb MHCs were found.

Table 4. Fiber type distribution and relative fiber type contribution to the total diaphragm muscle area

treatment	MHC-I	MHC-IIa	MHC-IIx
fiber type distribution	%	%	%
CTL	26 5±0 9	41 4±2 4	32 0±1.9
MP	23 8±0 9	44 8±1 9	31 5±2 5
EMPH	22 7±1 2*	42 8±1 9	34 5±2 1
EMPH+MP	24 3±0 8	42 3±0 7	33 4±0 7
EMPH+MP+ND	26 4±0 8	41 4±1 2	32 2±1 1
fiber type contribution to total diaphragm area	%	%	%
CTL	17 4±0 7	32 2±2 3	50 4±2 5
MP	18 6±0 9	35 5±2 4	45 9±3 2
EMPH	17 5±1 0	33 5±2 4	49 0±2 6
EMPH+MP	18 1±0 7	32 7±0 7	49 2±1 0
EMPH+MP+ND	18 2±0 5	31 5±1 4	50 3±1 3

means ±SE, * $p < 0.05$ compared to CTL and EMPH+MP+ND

Discussion

Summary of the results

The present study was designed to evaluate diaphragm muscle impairment caused by nine months of low-dose MP administration in emphysematous hamsters. In addition, we investigated whether the anabolic steroid ND was able to antagonize the loss in diaphragm function caused by the combination of emphysema and long-term treatment with a low dose of MP

The results show that, despite the decrease in force generation in the diaphragm of emphysematous hamsters, MP reduced force generation in the diaphragm of emphysematous and normal hamsters to a similar extent. Second, our data show that diaphragm function in the MP treated EMPH hamsters improved following ND addition. At low stimulation frequencies, this improvement in force generation was to the level of CTL, while maximum force generation improved to the level of force production in the EMPH hamsters The MHC-IIa and MHC-IIx fiber atrophy in the EMPH+MP

diaphragm was completely reversed to the level of CTL by the addition of ND.

Effects of emphysema and MP on diaphragm function and structure
Hamsters have been frequently used to evaluate diaphragmatic adaptations to elastase-induced emphysema. The increase in alveolar CSA observed following induction of emphysema combined with an ~50% increase in fluid displacement in all three PPE instillated groups indicated the presence of emphysematous changes in this study. Induction of emphysema may cause changes in diaphragm force generation (7,22,23). The reduction in Pt and Po in the present study was in line with previous reports with this animal model (7,22,23). This reduction in specific force can be the result of an increase in diaphragm muscle load, as result of emphysematous changes in the lung (41). Force generating capacity was also reduced in models representing skeletal (19) and diaphragm muscle (6) overloading. In these overloaded muscles and also in the diaphragm of emphysematous hamster (23), the reduction in force was associated with fast fiber hypertrophy. However, in line with our findings, muscle overload (9) and induction of emphysema (8) can also decrease fiber CSA. Although emphysema did not change relative fiber type contribution to total diaphragm muscle area in the present or in previous studies (23), MHC-IIx fiber CSA decreased by ~20% in the diaphragm of the EMPH animals. The observation that in the emphysematous diaphragm a decrease in force generating capacity can be associated with fast muscle fiber hypertrophy and atrophy, indicates muscle changes at the ultrastructural level.

In the emphysematous diaphragm, Farkas and Roussos (8) observed an increased oxidative and decreased glycolytic activity, measured by citrate synthase and phosphofructokinase, respectively. These biochemical changes following emphysema were associated with a fast fiber atrophy. Besides these disadvantages, more subtle alterations like changes in muscle contractile protein composition, are also likely to be responsible for the decrease in force in the overloaded muscles.

Following MP administration, twitch and tetanic forces further decreased in the diaphragm of the EMPH animals. Morphometrically, type IIa fiber atrophy was found in the EMPH+MP group compared to CTL, but type IIx fiber size decreased to a similar extent in the EMPH and EMPH+MP animals. In a previous study, using the same dose of MP in normal rats, MP also decreased diaphragm force production without a decrease in type IIx fiber CSA while type I and IIa fibers mildly atrophied (39). In that study the decrease in force was in part explained by the reduction in the number of type IIb fibers. Other investigators, using ATP-ase based fiber typing showed no effect of prednisolone (5) and MP (4) on normal rat diaphragm fiber size and composition, whereas type I, IIa and IIb fiber atrophy was observed following administration of cortisone acetate (10

mg/kg daily for 3 weeks) in rabbits (11). Since changes in muscle fiber morphometry cannot explain the difference in force production between the EMPH and EMPH+MP groups, more subtle ultrastructural changes are likely to be responsible.

Effects of ND on MP treated emphysematous diaphragm

Several pharmacological interventions to prevent glucocorticoid-induced myopathic changes in skeletal muscles have been studied. Growth hormone treatment improved maximum inspiratory pressure and nitrogen balance in COPD patients (27). This, however, was a preliminary report on a non-placebo controlled trial. In rat diaphragm, glucocorticoid-induced changes were not prevented by growth hormone administration (28). Clenbuterol (4 mg/kg over a 21 day period) (1) and testosterone (20 mg/kg/day) (10) partly prevented glucocorticoid-induced muscle atrophy. In these studies, treatment with the anabolic drugs was started simultaneously with the glucocorticoid administration in order to evaluate the antagonistic potency of the anabolic drugs. Reid et al. (30) reported an increase in lean body mass and a decrease in body fat mass following testosterone replacement in prednisolone treated ($\sim$10 mg/day) hypogonadal men. In the present study, however, we were interested if MP-induced changes could be reversed. Since changes in diaphragm structure and function occurred following 6 months of MP treatment (0.2 mg/kg/day) (39), the hamsters in this study were treated with MP for 6 months before ND was added to the glucocorticoid treatment. ND was chosen because of the specific antagonistic action at the receptor level between synthetic anabolic steroids and glucocorticoids (2,37). Besides this antagonistic mechanism, anabolic steroids have proven to be beneficial in clinical practice (35).

Theoretically, the blunting capacity of ND on MP-induced changes may be due to a direct anabolic effect of ND on muscle fibers (14,15,31), or to an antagonistic action between ND and glucocorticoids. Addition of ND to EMPH+MP restored Pt to normal (CTL) values but increased Po only to the level of the EMPH animals. At physiological stimulation frequencies ($\sim$25 Hz), force generating capacity was similar in the EMPH+MP+ND, EMPH, and CTL groups. The increase in normalized force in the EMPH+MP+ND animals at 25 Hz can in part be the result of the increase in the sum of contraction and relaxation time of the muscle strips.

The reversibility of the loss in diaphragm force production in the EMPH+MP animals by addition of ND can be due to an anabolic effect on the emphysematous changes, to an antagonistic effect on the MP-induced changes, or a combination of both. In normal rats, however, ND completely abolished the MP-induced impairment in diaphragm force generating capacity (40). It is therefore most likely that ND predominantly antagonized the action of MP with at most a small effect on the changes induced by emphysema.

Several mechanisms might be responsible for the interaction between anabolic steroids and glucocorticoids. Anabolic steroids are believed to act via muscle glucocorticoid receptors rather than via muscle androgen receptors in antagonizing the catabolic effects of glucocorticoids (2). Mayer and Rosen (24) proposed a binding competition between androgens and glucocorticoids for the same site of the receptor responsible for mediating the catabolic action of glucocorticoids. Inhibition of glucocorticoid action at the gene level (16) or downregulation of the glucocorticoid receptor number (37) were also reported as anabolic effects counteracting glucocorticoid-induced muscular changes. The direct effects of anabolic steroids on normal skeletal muscles include promoting amino acid incorporation into muscle proteins, decreasing amino acid catabolism (15), and increasing myosin and myofibrillar protein fraction (31). These actions, however, apparently had at most a minor effect on EMPH-induced diaphragm function impairment.

No pair-weight or pair-fed control group was added in the present study, since EMPH and the drugs tested did not affect body weight and food intake in a pilot study. Indeed, at the end of the treatment period, no changes in body weight were found among the four groups. This confirms earlier data, showing no difference in body weight growth in adult hamsters (20,25). Therefore, functional and structural changes as described above can not be explained by differences in body weight. The viability of the in vitro muscle preparation is not likely to be influenced by the bath temperature of 37°C in this study. Segal and Faulkner (36) showed that the critical radius for O_2 diffusion was ~0.6 mm at 37°C. Apart from the fact that no differences in muscle strip thickness were observed, this radius is clearly above the radius of 0.3 mm found in our muscle strips.

In conclusion, the decrease in force generating capacity in the MP treated emphysematous diaphragm was partly antagonized by ND addition. ND only antagonized the effects of MP but did not influence diaphragmatic changes induced by emphysema. MHC-IIa and IIx diaphragm muscle fiber atrophy in the EMPH+MP group was completely reversed by ND. Since both MP and ND were administered in low, clinically applied dosages, these observations may support the rationale for the development of clinical trials to investigate if anabolic steroids have similar protective effects in COPD patients with steroid-induced respiratory muscle dysfunction.

References

1.   Agbenyega, E.T. and A.C. Wareham. Effect of clenbuterol on skeletal muscle atrophy in mice induced by the glucocorticoid dexamethasone. Comp Biochem Physiol 102A: 141-145, 1992.
2.   Danhaive, P.A. and G.G. Rousseau. Evidence for sex-dependent anabolic response to androgenic steroids mediated by muscle glucocorticoid receptors in the rat. J Steroid Biochem 29: 575-581, 1988.

3   Decramer, M , V de Bock, and R Dom Functional and histologic picture of steroid induced myopathy in chronic obstructive pulmonary disease Am J Respir Crit Care Med 153 1958-1964, 1996

4   Dekhuijzen, P N R , G Gayan-Ramirez, A Bisschop, V de Bock, R Dom, R Bouillon, and M Decramer Rat diaphragm contractility and histopathology are affected differently by low dose treatment with methylprednisolone and deflazacort Eur Respir J 8 824-830, 1995

5   Dekhuijzen, P N R , G Gayan Ramirez, V de Bock, R Dom, and M Decramer Triamcinolone and prednisolone affect contractile properties and histopathology of rat diaphragm differently J Clin Invest 92 1534-1542, 1993

6   Farkas, G A , L E Gosselin, W Z Zhan, E H Schlenker, and G C Sieck Histochemical and mechanical properties of diaphragm muscle in morbidly obese Zucker rats J Appl Physiol 77 2250-2259, 1994

7   Farkas, G A and C Roussos Adaptability of the hamster diaphragm to exercise and/or emphysema J Appl Physiol 53 1263-1272, 1982

8   Farkas, G A and C Roussos Histochemical and biochemical correlates of ventilatory muscle fatigue in emphysematous hamsters J Clin Invest 74 1214-1220, 1984

9   Faulkner, J A , L C Maxwell, D A Brook, and D A Lieberman Adaptation of guinea pig plantaris muscle fibers to endurance training Am J Physiol 221 291 297, 1971

10 Ferguson, G T Effects of cortisone and testosterone on diaphragmatic function and biochemistry in the rabbit J Appl Physiol 78 1459-1468, 1995

11 Ferguson, G T , C G Irvin, and R M Cherniack Effects of corticosteroids on respiratory muscle histopathology Am Rev Respir Dis 142 1047 1052, 1990

12 Frenkel, J K and M A Havenhill The corticoid sensitivity of golden hamsters, rats, and mice Lab Invest 12 1204-1220, 1963

13 Gibson, J Respiratory muscles In Chronic obstructive pulmonary disease, edited by P M A Calverley and N B Pride London Chapman and Hall, 1995, 185-203

14 Griggs, R C , W Kingston, R F Jozefowicz, B E Herr, G Forbes, and D Halliday Effect of testosterone on muscle mass and muscle protein synthesis J Appl Physiol 66 498-503, 1989

15 Haupt, H A and G D Rovere Anabolic steroids a review of the literature Am J Sports Med 12 469-484, 1984

16 Hickson, R C , S M Czerwinski, M T Falduto, and A P Young Glucocorticoid antagonism by exercise and androgenic-anabolic steroids Med Sci Sports Exerc 22 331-340, 1990

17 Hudson, L D and C M Monti Rationale and use of corticosteroids in chronic obstructive pulmonary disease Med Clin North Am 74 661-690, 1990

18 Jasani, M K Anti-inflammatory steroids mode of action in rheumatoid arthritis and homograft reaction In Anti-inflammatory drugs, edited by J R Vane and S H Ferreira Berlin Springer-Verlag, 1979, 598 660

19 Kandarian, S C and T P White Mechanical deficit persists during long-term muscle hypertrophy J Appl Physiol 69 861-867, 1990

20 Kelsen, S G , T Wolanski, G S Supinski, and U Roessmann The effect of elastase-induced emphysema on diaphragmatic muscle structure in hamsters Am Rev Respir Dis 127 330-334, 1983

21 Laaban, J , B Kouchakji, M Dore, E Orvoen Frija, P David, and J Rochemaure Nutritional status of patients with chronic obstructive pulmonary disease and acute respiratory failure Chest 103 1362-1368, 1993

22 Lewis, M I , S A Monn, W Z Zhan, and G C Sieck Interactive effects of emphysema and malnutrition on diaphragm structure and function J Appl Physiol 77 947-955, 1994

23 Lewis, M I , W Z Zhan, and G C Sieck Adaptations of the diaphragm in emphysema J Appl Physiol 72 934-943, 1992

24 Mayer, M and F Rosen Interaction of glucocorticoids and androgens with skeletal muscle Metabolism 26 937 945, 1977

25   Mercer, R R and J D Crapo Structural changes in elastic fibers after pancreatic elastase administration in hamsters J Appl Physiol 72 1473-1479, 1992

26   Moore, B J , M J Miller, H A Feldman, and M B Reid Diaphragm atrophy and weakness in cortisone-treated rats J Appl Physiol 67 2420 2426, 1989

27   Pape, G S , M Friedman, L E Underwood, and D R Clemmons The effect of growth hormone on weight gain and pulmonary function in patients with chronic obstructive lung disease Chest 99 1495-1500, 1991

28   Petrof, B J , S B Gottfried, J Eby, J LaManca, and S Levine Growth hormone does not prevent corticosteroid-induced changes in rat diaphragm structure and function J Appl Physiol 79 1571-1577, 1995

29   Prakash, Y S , M Fournier, and G C Sieck Effects of prenatal undernutrition on developing rat diaphragm J Appl Physiol 75 1044-1052, 1993

30   Reid, I R , D J Wattie, M C Evans, and J P Stapleton Testosterone therapy in glucocorticoid-treated men [see comments] Arch Intern Med 156 1173-1177, 1996

31   Rogozkin, V A Anabolic steroid metabolism in skeletal muscle J Steroid Biochem 11 923-926, 1979

32   Ruff, R L Endocrine myopathies In Myology, edited by A G Engel and B Q Banker McGraw-Hill Book Company, 1986, 1871-1906

33   Sarett, L H , A A Patchett, and S L Steelman The effects of structural alteration on the anti-inflammatory properties of hydrocortisone Progr Drug Res 5 11-153, 1963

34   Schiaffino, S , L Gorza, S Sartore, L Saggin, S Ausoni, M Vianello, K Gundersen, and T Lomo Three myosin heavy chain isoforms in type 2 skeletal muscle fibers J Musc Res Cell Motility 10 197-205, 1989

35   Schols, A M W J , P B Soeters, R Mostert, R J Pluymers, and E F M Wouters Physiologic effects of nutritional support and anabolic steroids in patients with chronic obstructive pulmonary disease - A placebo-controlled randomized trial Am J Respir Crit Care Med 152 1268-1274, 1995

36   Segal, S S and J A Faulkner Temperature-dependent physiological stability of rat skeletal muscle in vitro Am J Physiol 248 C265 C270, 1985

37   Sharpe, P M , P J Buttery, and N B Haynes The effects of manipulating growth in sheep by diet or anabolic agents on plasma cortisol and muscle glucocorticoid receptors Br J Nutr 56 289 304, 1986

38   Snider, G L and C B Sherter A one-year study of the evolution of elastase induced emphysema in hamsters J Appl Physiol 43 721 729, 1977

39   van Balkom, R H H , P N R Dekhuijzen, H T M Folgering, J H Veerkamp, H T B van Moerkerk, J A M Fransen, and C L A van Herwaarden Effects of long-term low-dose methylprednisolone on rat diaphragm function and structure Muscle & Nerve 20 983 990, 1997

40   van Balkom, R H H , P N R Dekhuijzen, H T M Folgering, J H Veerkamp, H T B van Moerkerk, J A M Fransen, and C L A van Herwaarden Anabolic steroids in part reverse glucocorticoid-induced alterations in rat diaphragm J Appl Physiol 1998 (in press)

41   Younes, M Load responses, dyspnea, and respiratory failure Chest 97 59S-68S, 1990

CHAPTER 9

Summary and conclusions
Samenvatting en conclusies

Summary

Weakness of the respiratory muscles was recently reported following low doses of methylprednisolone (MP) in patients with COPD (1). The mechanisms by which non-fluorinated steroids cause myopathy are in part unknown. Most of the previous animal studies were performed using relatively high dosages during short periods of time. This resembles acute steroid myopathy whereas chronic steroid myopathy is more often found in clinical practice. In patients with chronic obstructive pulmonary disease (COPD) a significant decrease in respiratory (and peripheral) muscle strength was observed after treatment with methylprednisolone 4.3 mg for 6 months (1). Since treatment with glucocorticoids is sometimes inevitable in these patients (2), interventions that attenuate or even abolish these alterations in respiratory muscles may be of importance. The aim of this thesis was to evaluate functional, histological, biochemical and neuromuscular effects of glucocorticoids on the diaphragm of normal rats and emphysematous hamsters. In addition, the antagonistic potency of anabolic steroids on glucocorticoid induced changes were evaluated.

Chapter 2 describes the differences between acute and chronic steroid myopathy. In addition, clinical presentation and diagnostic features in corticosteroid induced myopathic changes in patients were discussed.

Twitch force (Pt), maximum tetanic force (Po), force frequency relationships, velocity of shortening, muscle power output, and neuromuscular transmission fatigue from isolated diaphragm strips were evaluated *in vitro*. Antibodies reacting with type I, IIa, all but IIx and IIb myosin heavy chains were used for morphometrical analysis. Bioenergetic enzyme activities were measured on biopsies from the costal part of the diaphragm. Fiber type identified neuromuscular junction morphology was assessed using a three-color immunocytochemical technique.

In Chapter 3, three different treatment regimens of methylprednisolone (MP) in rats (MP 1 mg/kg/day; 2 mg/kg every other day; 2 mg/kg/day for 2 weeks, saline 4 weeks, 2 mg/kg/day for 2 weeks) were compared with saline. The total treatment period was 8 weeks. All MP treatment regimens studied reduced diaphragm force generating capacity. Alternate-day MP administration caused less biochemical impairment compared to continuous MP treatment. Administration in bursts resulted in a decrease in type IIb fiber proportion compared to control and caused a considerable impairment of diaphragm enzyme activities compared to continuous MP treatment. We therefore concluded that although the MP treatment regimens affected diaphragm muscle morphology and bioenergetic enzyme activities in different ways, force generation decreased in all MP groups to the same extent.

In Chapter 4, the effects of long-term low-dose MP (0 2 mg/kg/day during six months) were evaluated in rats. Diaphragm force generating capacity decreased by ~14% following MP administration. This impairment in functional capacity was, at least in part, explained by a shift towards slower fibers and a reduction in glycogenolytic activity

Chapter 5 reports the effects of prednisolone (6 mg/kg/day for 3 wks) on structure and function of rat diaphragm neuromuscular junctions (NMJ). Normalized for fiber dimension, prednisolone increased NMJs innervating type IIx/b fibers. Neuromuscular transmission failure was less in prednisolone treated diaphragm muscle. These results indicate that alterations in NMJ morphology following prednisolone depend upon fiber type, and may contribute to improved neuromuscular transmission

The effects of prednisolone on rat diaphragm muscle isotonic contractile properties were studied in Chapter 6. Prednisolone treatment resulted in a decreased maximum velocity of shortening and maximum power output (the product of force and velocity). This decreased power output of the prednisolone treated diaphragm did not prolong endurance to repeated isotonic contractions at peak power output

Chapter 7 describes the antagonistic potency of the anabolic steroid nandrolone decanoate (ND) on MP-induced changes in rat diaphragm. Addition of ND during the last 3 months of 9 months of low-dose MP treatment completely reversed the marked reduction in diaphragm force caused by long-term low-dose MP. This was associated with reversibility of the MP induced type IIa and IIx fiber atrophy, while ND addition showed no beneficial effects on biochemical changes

The interactive effects of elastase-induced emphysema (EMPH) and MP on hamster diaphragm function and structure were studied in Chapter 8. MP and EMPH+MP reduced diaphragm force generation to a similar extent, which was significantly more pronounced than EMPH alone. Therefore, despite the decrease in force generation, the diaphragm of emphysematous hamsters seemed less susceptible to MP treatment compared to the normal diaphragm. This chapter also describes the effects of ND addition to emphysematous hamsters treated with MP. The reduction in force in the MP treated EMPH animals was reversed to the level of the EMPH hamsters. Therefore, ND was able to antagonize the MP-induced impairment but had at most a minor effect on the changes induced by emphysema

Conclusions

In conclusion, prolonged administration of MP in low doses results in reduced force generation and structural changes in rat diaphragm. NMJ morphology changed and neuromuscular transmission failure decreased following prednisolone treatment

Prednisolone reduced diaphragm power output without prolongation of the endurance time. The MP-induced changes in muscle function and morphology are to a large extent reversed by anabolic steroids. MP and EMPH+MP reduced diaphragm force generation to a similar extent, suggesting that the emphysematous diaphragm is less susceptible to MP-induced impairment. Anabolic steroids improved diaphragm force production in the MP treated emphysematous hamsters to the level of force production in the emphysematous hamsters, which was significantly less compared to the control group.

The observations described in this thesis may have consequences for future treatment of patients with severe chronic obstructive pulmonary disease (COPD). Patients with COPD may suffer from respiratory muscle weakness due to hyperinflation, malnutrition, disturbances in blood gases and cardiac failure. In these patients, respiratory muscle function is further reduced by administration of corticosteroids. Since respiratory muscle weakness may contribute to respiratory failure, interventions that attenuate or even abolish respiratory muscle impairment in these patients may be of clinical importance. In this respect we believe that anabolic steroids can be of use in corticosteroid treated patients with severe COPD.

Recommendations for future research

In future research, measurements of growth factors and satellite cell activity in the diaphragm muscle may provide additional information on the mechanisms responsible for corticosteroid-induced muscle impairment.

Evaluation of the influence of corticosteroid on the amount of crossbridge attachments and the force production per crossbridge may be helpful clarifying the functional impairment caused by corticosteroid.

Additional information on corticosteroid-induced neuromuscular changes, as described in this thesis, can be obtained from measurements of the acetylcholine quantum and number of acetylcholine receptors on the post synaptic membrane.

The reversibility of the corticosteroid-induced myopathy of the respiratory muscles by addition of anabolic steroids, as reported in this thesis, will have to be confirmed in clinical randomized trials.

References

1. Decramer, M., L M. Lacquet, R Fagard, and P. Rogiers Corticosteroids contribute to muscle weakness in chronic airflow obstruction. Am J Respir Crit Care Med 150 11-16, 1994.

2   Hudson, L D and C. M Monti Rationale and use of corticosteroids in chronic obstructive pulmonary disease Med Clin North Am 74 661-690, 1990

Samenvatting

Patiënten met een ernstig COPD worden in sommige gevallen behandeld met systemische corticosteroïden (CS). In diermodellen werd aangetoond dat CS de structuur en de functie van perifere en ademhalingsspieren negatief beïnvloed. Hierbij werden relatief hoge doses CS in een korte tijd toegediend. Het doel van deze dierexperimentele studie was het evalueren van functionele en structurele veranderingen in het diafragma t.g.v het toedienen van een klinisch relevante dosis CS. Contractiele eigenschappen van het diafragma werden in vitro bepaald. Steroïd-geïnduceerde cellulaire adaptatie werd geëvalueerd door bepaling van biochemische veranderingen in enzymsystemen in combinatie met morfologische analyse op immunohistochemisch gekleurde spier preparaten. Hiervoor werden antilichamen tegen type I, IIa, IIx en IIb myosine zware ketens gebruikt.

Verschillende behandelingsschema's met methylprednisolon (MP) werden vergeleken. We hypothetiseerden dat het alternerend toedienen van MP minder en het toedienen van MP in stootkuren meer bijwerkingen op het diafragma zou hebben i.v.m. continue MP behandeling. De drie bovenbeschreven MP toedieningsschema's resulteerden in een zelfde mate van krachtsverlies. Morfologische en biochemische analyse lieten sterkere veranderingen na stootkuur toediening zien.

Vervolgens werden de effecten van chronische lage dosis MP behandeling, vergelijkbaar met 10-15 mg bij mensen, in het ratten diafragma geëvalueerd. Deze dosis MP veroorzaakte een 14% krachtsvermindering van het diafragma. Omdat deze krachtvermindering klinische relevant kan zijn, werd de antagonerende werking van anabole steroïden op de MP-geïnduceerde veranderingen in het diafragma bestudeerd. Nandrolone decanoate (ND), in een door de fabrikant aanbevolen dosis, werd gedurende de laatste drie maanden van een negen maanden durende MP behandeling toegediend. ND herstelde de afname in diafragma functie, veroorzaakt door MP, compleet.

Bij COPD patiënten kan de diafragma functie door hyperinflatie van de longen, malnutritie, hypoxie en inactiviteit reeds gestoord kan zijn. De invloed van MP op het reeds door emfyseem aangetaste diafragma is nog onbekend. Een studie hiernaar liet zien dat ondanks vermindering van krachtgeneratie in het emfysemateuze diafragma, de krachtproductie in het met MP behandelde emfysemateuze en normale diafragma tot het zelfde niveau afneemt. Hieruit concluderen we dat het diafragma van emfysemateuze dieren minder gevoelig is voor de effecten van MP. Dit zou het gevolg kunnen zijn van een verhoogde activiteit van het diafragma bij emfyseem.

De effecten van CS op isotonische contractiele eigenschappen van het diafragma, zoals power (produkt van snelheid van verkorting en kracht) en endurance (tijd totdat power nul

is), werden geanalyseerd CS behandeling verlaagde de maximale power output Ondanks een lagere power output (=minder werk belasting) was er geen verlenging van de endurance tijd na CS behandeling

CS veroorzaken electrofysiologische veranderingen in de neuromusculaire transmissie De effecten van CS op morfologie en functie van de neuromusculaire overgangen (NMJ) zijn nog onbekend Met drie-kleuren fluorescentie techniek werden gelijktijdig de zenuw-uiteinden (NT), motorische eindplaatjes (EP) en vezeltypes gelabeled Deze structuren werden geanalyseerd met 3D confocale microscopie Het neuromusculaire falen werd berekend uit het verschil in krachtafname gedurende herhaalde zenuw versus spierstimulaties Genormaliseerd voor vezel diameter induceerde CS een toename van de NMJ grootte van type IIx/b vezels Het neuromusculaire falen was minder uitgesproken na CS behandeling in vergelijking met controle

Conclusies Chronische toediening van lage dosis CS veroorzaakt dysfunctie van het normale en emfysemateuze diafragma De effecten van CS op het emfysemateuze diafragma zijn niet sterker uitgesproken Anabole steroiden antagoneren de negatieve effecten van CS op de functie van het normale en emfysemateuze diafragma CS verlaagt de power output van het diafragma en vermindert het falen van de neuromusculaire overdracht

Dankwoord

Mijn promotoren Prof. Dr. C.L.A. van Herwaarden, Prof. Dr. H.T.M. Folgering en Dr. P.N.R. Dekhuijzen dank ik voor hun vertrouwen. Toen Richard naar mij toe kwam met een concept voor een dierexperimenteel onderzoek was ik enerzijds erg opgetogen, anderzijds realiseerde ik mij dat ik mijn vrees voor 'knaagbeesten' zou moeten overwinnen. Beste Richard, veel heb ik aan jou te danken. Al tijdens mijn co-assistentschap interne geneeskunde maakte je me enthousiast voor het vak longziekten. En na mijn wetenschappelijke stage vertrouwde je mij een door het Nederlands Astma Fonds gesubsidieerd promotie onderzoek toe. Met je passie voor onderzoek, je tomeloze inzet en je gave om altijd de positieve kanten te benadrukken (ook al wist ik dat boven een gecorrigeerd manuscript 'prima start' net zoveel als 'lijkt nog nergens op' betekende) wist je me bijna altijd weer te motiveren. Mocht het echt tegenzitten dan waren de analyses over de prestaties van Ajax en *moppen* een welkome afwisseling. Beste Hans, als begeleider van mijn wetenschappelijke stage bracht je mij de beginselen van het onderzoeken bij (communiceren, discipline, inzet en roeien met de riemen die je hebt). Hierna heb ik veel profijt gehad van jouw analyserend vermogen en frisse kritische blik. Beste Professor van Herwaarden, u creëerde een perfect onderzoeks-klimaat door ons de ruimte te geven (en niet alleen in financieel opzicht) voor het opzetten van nieuwe onderzoeks-technieken en voor het uitbreiden van kennis en ervaring. Hierbij denk ik met name aan mijn onderzoeksperiode in de Mayo Clinic en aan de congres bezoeken. Tevens zorgde u ervoor dat we bij het interpreteren van de resultaten de klinische relevantie niet te ver uit het oog verloren.

De medewerkers van het Centraal Dieren Laboratorium: Yvette, Theo, Geert, Gerrie, Bianca, Wilma en Francien wil ik niet alleen bedanken voor hun professionele begeleiding van de proefdieren en van mij tijdens de studies, maar ook voor de gezelligheid en het feit dat al mijn vingers nog ongeperforeerd zijn. Guus Visser (afdeling fysiologie) wil ik bedanken voor het meedenken en meesleutelen aan de opstelling voor de contractiliteits-metingen en Mark Massa (instrumentele dienst) voor het schrijven van de benodigde software. Hein en Huib (afdeling celbiologie en histologie), bedankt voor het geduld dat jullie hadden om een arts de beginselen van antilichaam kleuringen bij te brengen. Ik weet dat jullie dit als een vrijwel onmogelijk taak beschouwden en daarom is mijn waardering voor jullie prestatie des te groter.

Prof. G.C. Sieck, dear Gary, I would like to thank you for the opportunity to work in your lab and for your support in every way. You showed me the American way of running a research laboratory and the technique of always creating a win

versus win situation. Prakash, your technical advise and assistance (especially in the confocal 'stuff') were indispensable. Fang and When-Zhi, besides your help in obtaining the histological and contractile data, I would like to thank you for your never ending smile and excellent cooking.

Mijn vrienden bij wie ik nooit tevergeefs aanklopte ook al was ik soms een periode onzichtbaar. Jullie waren er voor mij als het tegenzat en lieten mij zien dat er meer was dan onderzoek alleen. Mijn ouders en familie die mij de gelegenheid gaven om te studeren. Jullie stonden altijd achter mij en bekritiseerden mijn beslissingen nooit.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 9 juni 1966 te Valkenswaard. Na het behalen van het diploma Moderne Humaniora Wetenschappelijke A aan het Sint Hubertuscollege te Neerpelt (België), studeerde hij Geneeskunde aan de Katholieke Universiteit Nijmegen. Onder leiding van Prof. Dr. H.T.M. Folgering en Dr. J.M. Rooyackers werd zijn wetenschappelijke stage in het Universitair Longcentrum Dekkerswald te Groesbeek volbracht. Het artsexamen werd behaald in 1992. Van 1993 tot 1997 verrichtte hij wetenschappelijk onderzoek bij de afdeling Longziekten, Academische Ziekenhuis Nijmegen onder leiding van Prof. Dr. C.L.A. van Herwaarden, Prof. Dr. H.T.M. Folgering en Dr. P.N.R. Dekhuijzen. Gedurende deze periode was hij 5 maanden werkzaam bij de afdeling Anesthesiologie, Fysiologie en Biofysica, Mayo Clinic and Foundation, Rochester MN, USA (hoofd: Prof. Dr. G.C. Sieck).

In januari 1997 werd begonnen met de basisopleiding Interne Geneeskunde in het Rijnstate Ziekenhuis te Arnhem (opleider: Dr. L. Verschoor). In januari 1999 zal de opleiding tot longarts voortgezet worden bij de afdeling Longziekten, Universitair Longcentrum Nijmegen (opleider: Prof. Dr. C.L.A. van Herwaarden).

Stellingen

1   Het is een misverstand dat corticosteroiden alleen in een hoge dosis een myopathie induceren Ook na chronische behandeling met een lage dosis corticosteroiden vermindert de ademhalingsspierfunctie *(dit proefschrift)*

2   Een corticosteroid geinduceerde myopathie betekent niet alleen een vermindering van de maximale spierkracht. maar gaat ook gepaard met een afname in uithoudingsvermogen *(dit proefschrift)*

3   In een diermodel zijn anabole steroiden in staat de negatieve effecten van corticosteroiden op het diafragma volledig te antagoneren *(dit proefschrift)*

4   Het diafragma van emfysemateuze hamsters is minder gevoelig voor de bijwerkingen van corticosteroiden *(dit proefschrift)*

5   Corticosteroid-geinduceerde myopathie behoort een plaats te hebben in de differentiaal diagnose van progressieve dyspnoe bij patienten met COPD die orale corticosteroiden gebruiken

6   Of anabole steroiden naast een plaats in de prijzenkast ook een plaats in de medicijnkast kunnen verwerven dient nader onderzocht te worden

7   Het bedroevend lage aantal terugzendingen van de donorregistratie formulieren heeft weinig te maken met het vertrouwen in het eeuwige leven, maar weerspiegelt de tijdgeest

8   De levensvreugde van een Sumatraan met een karbouw bevestigt dat ontwikkelingssamenwerking niets met ontwikkeling, maar alles met samenwerking te maken heeft

9   Er is meer tussen hemel en aarde dan onderzoek Het is echter een wetenschappelijk gegeven dat het meeste daarvan lucht is

10   Leven is de juiste weg volgen, zonder te weten waarheen die leidt