Corticosteroid and nutrition induced muscle wasting in a rat model

Citation for published version (APA):

Koerts de Lang, E. (1999). *Corticosteroid and nutrition induced muscle wasting in a rat model*. [Doctoral Thesis, Maastricht University]. Maastricht University. <https://doi.org/10.26481/dis.19991209ek>

Document status and date:

Published: 01/01/1999

DOI:

[10.26481/dis.19991209ek](https://doi.org/10.26481/dis.19991209ek)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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Het verschijnen van dit proefschrift werd mede
mogelijk gemaakt met financiële steun van
de Stichting Pulmonologie,
het Nederlands Astma Fonds
en Nutricia Nederland B.V.

Corticosteroid and nutrition induced muscle wasting in a rat model.

The Graduate School

The study presented in this thesis was performed at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Druk: Datawyse | Universitaire Pers Maastricht

ISBN: 90 5278 261 x

Corticosteroid and nutrition induced muscle wasting in a rat model.

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 9 december 1999 om 12.00 uur

door

Esther Koerts-de Lang

geboren op 8 juni 1966 te Eindhoven.

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Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCAA	branched chain amino acids
BW	body weight
CHF	chronic heart failure
CK	creatine kinase
COPD	chronic obstructive pulmonary disease
Cr	creatine
CrP	creatine phosphate
CS	citrate synthase
CSA	cross-sectional area
DW	dry weight
EDL	extensor digitorum longus muscle
FF	free fed
F/F	force-frequency
Fm,a	plasma membrane rate from the arterial to the free amino acid muscle pool
Fv,a	plasma membrane rate from the arterial to the venous pool
Fv,m	plasma membrane rate from the free amino acid muscle to the venous pool
GA	glutaminase
GLN	glutamine
GLU	glutamate
GP	glycogen phosphatase
GR	glucocorticoid receptor
GS	glutamine synthetase
GS	glycogen synthase
HAD	3-hydroxyacyl coenzyme A dehydrogenase
LDH	lactate dehydrogenase
Lo	optimal length
MHC	myosin heavy chain
mRNA	messenger ribonucleic acid
ND	nutritional deprivation
p	p-value
PF	pair fed
PFK	phosphofructokinase
PHE	phenylalanine
Po	peak tetanic tension
PR	prednisolone
Pt	peak twitch tension
PW	pair weight
½ RT	half relaxation time
SEM	standard error of the mean
TAN	total adenine nucleotides
TCr	total creatine pool
TPT	time to peak tension
TR	triamcinolone
UDP	uridine diphosphate

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General introduction

1.1. Skeletal muscle dysfunction in chronic diseases

Many patients with chronic wasting diseases such as chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF) and renal failure are severely limited in daily functioning. Although progression of these disorders can be slowed down with medication, reversion can only be (partially) achieved through surgical interventions and transplantation. It now becomes more and more clear that besides primary organ failure, secondary factors contribute to the limitation in daily functioning. One of the major limiting factors in these conditions is exercise intolerance. Several recent reports have however revealed a poor relationship between measures of organ dysfunction and exercise capacity in cardiorespiratory diseases (1, 2). The fact that exercise intolerance in both disorders as well as in renal failure remains after surgical interventions or transplantation (3, 4), illustrates even more that secondary factors are involved in exercise limitation.

There is accumulating evidence that the skeletal muscles in these patient groups do not function normally and that this contributes to exercise intolerance (5). This is important because skeletal muscle dysfunction may well be a remediable source of exercise intolerance. Besides, muscle dysfunction is not limited to the peripheral skeletal muscles, but also affects the diaphragm and other respiratory muscles (6, 7). More specifically for respiratory muscle function a relationship with dyspnea was demonstrated (8) and furthermore it is well known that respiratory muscle fatigue predisposes to acute respiratory failure.

Recently it was shown that peripheral muscle wasting is an important predictor of peripheral muscle dysfunction (9, 10). However, as illustrated by the lower correlation between respiratory and peripheral muscle function in patients compared to healthy controls (11), intrinsic abnormalities in muscle might be involved. Analysis of muscle biopsies has indeed revealed alterations in key muscle enzymes involved in aerobic and anaerobic pathways as well as in muscle CSA and fibre type composition in COPD, CHF and renal failure (3, 12-14). Furthermore some studies have also reported myopathic features (15, 16). In order to extrapolate these observations to treatment strategies we need to know more about the underlying causes.

Nutritional depletion is recognised as an important factor in the treatment of these conditions since besides the functional consequences of muscle wasting, weight loss has been identified as an independent determinant of mortality (17). The reversibility of weight loss however is depending on the underlying mechanism: a decreased balance between energy intake and energy metabolism only, or involvement of altered anabolic and catabolic mediators. The former can be reversed by adequate nutritional support but the latter is yet more difficult to manage (18). Absence of an anabolic response to nutritional support or exercise has among others been associated to the use of systemic corticosteroid treatment as maintenance therapy or during acute exacerbations. Side effects of corticosteroids in chronic diseases in particular on muscle function has therefore received much attention in clinical studies (19-21). Furthermore one recent study also provides evidence for a contribution of systemic corticosteroids to increased mortality in chronic respiratory disease independent of weight loss (22).

1.2. Muscle function

Muscle activation is initiated by motoneurons, which form together with the muscle fibre the motor unit. Total muscle force depends on the number of motor units that are activated and on the force of separate motor units. Motor units can be classified on base of their mechanical and fatigue properties (23, 24), as shown in Table 1. In general, a motor unit type comprises muscle fibres of a single type, as classified by histochemical staining for myofibrillar ATPase (25, 26). Histochemical enzyme staining showed that the different

muscle fibres depended on different metabolic pathways (27). In recent studies it was shown that the different fibre types have different myosin heavy chain (MHC) isoforms.

Table 1. Motor unit and muscle fibre classification

Motor unit type	slow-twitch, fatigue resistant	fast-twitch, fatigue resistant	fast-twitch, fatigue intermediate	fast-twitch, fatigable
Muscle fibre type	I	IIa	IIx	IIb
Metabolic enzymes	oxidative	oxidative, glycolytic	?	glycolytic
MHC	MHC _{slow}	MHC _{2A}	MHC _{2X}	MHC _{2B}

Generally, the oxidative fibres are characterised by higher mitochondrial volume densities than fibres which are primarily glycolytic (28). The higher oxidative capacity may at least partially account for the greater fatigue resistance of these fibres (29, 30). However, on the other hand, the higher mitochondrial volume density would presumably be at the expense of a corresponding lower myofibrillar volume density, lower MHC content, and thus fewer cross-bridges in parallel for a given fibre cross-sectional area (31). This may explain the low tensions of oxidative fibres and the high tensions of glycolytic fibres.

Skeletal muscle mass comprises of about 60 to 80% of body cell mass and is therefore an important reserve system for the whole body protein pool. In catabolic conditions, it serves to supply amino acids for protein synthesis and energy metabolism elsewhere (32). Muscle protein is built of amino acids, which are derived from an active metabolic pool, originating from endogenous protein degradation and from dietary protein sources. Muscle wasting can occur by a depressed protein synthesis, an increased protein degradation or both. During muscle catabolism, degradation of muscle contractile protein is likely mediated via the ubiquitin-proteasome pathway (33). In this pathway the peptide ubiquitin is activated, which then covalently binds to protein substrates and this complex is finally degraded by an ATP-dependent protease (34). Lysosomes are however not involved in the degradation of myofibrillar proteins (35), and also Ca^{2+} -dependent proteinases were thought to play no major role in general intracellular protein turnover (36). However in recent experiments, both ubiquitin-proteasome proteolysis and calcium-dependent protein degradation were stimulated after corticosteroid administration (37).

ATP is the most important fuel for the cell, providing energy for cross-bridge cycling and active transport. At the onset of contractile activity, muscle fibres initially draw on a small pool of existing high-energy phosphates (39). Nevertheless, ATP levels do not change significantly because creatine phosphate (CrP) provides the substrate for rapid resynthesis catalysed by creatine kinase in the cytosol. The concentration of CrP in the cell is about four to six times higher than that of ATP. Therefore, CrP is considered as the high-energy phosphate reservoir. After only a few seconds of strenuous contractions, this energy source is depleted. The rates of glycogenolysis and glycolysis accelerate to provide a more sustainable energy source. As shown in Figure 1, during glycolysis, glucose is metabolised to pyruvate. Pyruvate can enter the mitochondria where it is converted into acetyl-CoA, which in turn enters the tricarboxylic acid cycle (TCA-cycle). Strong reducing agents such as NADH and FADH_2 formed during glycolysis and in the TCA-cycle, transfer their electrons via electron carriers to O_2 to form H_2O . During this so called respiratory chain reaction, protons are transported over the inner mitochondrial membrane into the intermembranous space where ATP is synthesised. Thus, via the proton gradient, this oxidative phosphorylation is coupled to the respiratory chain.

As shown in Figure 1, ATP can also be derived from oxidation of dietary protein and fat sources. Compared to anaerobic glycolysis, aerobic metabolism requires longer time to be activated. Nevertheless, it has two important advantages to working muscle fibres. It produces more energy; 38 ATP per mole of glucose versus 2 ATP from anaerobic glycolysis and it enables the cell to utilise lipid as a fuel via β -oxidation. These properties are critical for

muscle endurance and therefore mitochondrial content is a major determinant of muscle performance during prolonged exercise. In addition, other important determinants for muscle fatigue are the accumulation of inorganic phosphate, which is produced by ATP hydrolysis, and reactive oxygen species, produced by severe exercise, causing oxidative stress (40).

Figure 1. Nutrient oxidation and ATP production. Adapted from Pison (38).

1.3. Undernutrition

Depletion of muscle mass as a consequence of undernutrition may affect muscle morphology, contractility and metabolism. Relatively few studies have investigated the effects of undernutrition on the combination of these factors. In contrast, many studies have investigated the effects of starvation or restriction of a specific dietary component. In this paragraph, the available experimental animal studies that investigated the effects of varying

degrees and durations of undernutrition (i.e. semistarvation) on muscle morphology, metabolism and contractility are reviewed.

1.3.1. Effects of undernutrition on muscle mass, morphology and contractility

Muscle contractility can be assessed *in vivo* via nerve stimulation or *in vitro* with the use of a glass tissue chamber, in which the muscle nerve or the muscle itself can be stimulated directly with electrodes. Half-relaxation time ($\frac{1}{2}RT$) is obtained from baseline single twitch (P_t) and is defined as the time for tension to decay from maximum to half-maximum twitch tension. Force-frequency relationship can be examined by measuring the maximum tension occurring at increasing stimulation frequencies. Muscle fatigue can be measured during prolonged stimulation and is characterised by a fall in tension. At a high frequency, tetanic tension (P_o) can be assessed.

Table 2 summarises several studies on the effects of undernutrition on muscle mass, morphology and contractility. During undernutrition, muscle mass declined in a comparable extent as body weight. Muscle wasting of mixed muscles (like the diaphragm and the gastrocnemius) caused a generalised atrophic pattern after severe undernutrition whereas moderate undernutrition caused only type II fibre atrophy.

In general, muscle wasting caused a decreased muscle fatigability of the diaphragm muscle, but fatigability remarkably was found to be increased in the gastrocnemius and the soleus (mainly type I fibres) muscles. This could be caused by specific muscle characteristics, since the diaphragm is continuously active and the gastrocnemius and the soleus are relatively inactive after undernutrition. The differences in muscle fatigability between the diaphragm and the peripheral skeletal muscles is likely caused by different fatigue protocols, since diaphragm fatigue resistance occurred only at low stimulation frequencies (41, 42) and fatigue of the peripheral skeletal muscles was only found at high stimulation frequencies (43-45). In most studies, peak twitch and peak tetanic tensions were unchanged when expressed per amount of muscle mass. In the diaphragm and soleus muscles, half relaxation time was unchanged or increased, while in the gastrocnemius muscle, half relaxation time was found to be decreased. In general, force-frequency relationships were unchanged, but when expressed as percentage of tetanic force, it was found to be shifted to the left. This could be due to loss of force produced at high stimulation frequencies, while at low stimulation frequencies the force was maintained (43).

In summary, undernutrition induces muscle wasting, involving all fibre types, but predominantly type II fibres. It is not fully clear what the consequences are for the contractility of different muscles, since exercise protocols are not comparable. However, fatigue resistance is likely to be increased, since the relative cross-sectional area of oxidative fibres is increased. In addition, the results of *in vitro* and *in vivo* stimulation protocols have to be compared with caution, since whole body metabolism is affected after undernutrition, which in turn affects muscle performance *in vivo*, while *in vitro* muscle contractility is independent on whole body metabolism at time of contraction.

Table 2. Morphological and contractility changes in the muscle after undernutrition, in sequence of duration.

Reference (number)	Animal	Duration of undernutrition	Change in body weight		Studied muscle	Muscle reduction	Atrophy	fatigue			Contractility (in vitro)		
			of initial	of control				Pt	Po	$\frac{1}{2}$ RT			
Bissonnette <i>et al</i> 1997 (45)	rat	7 days	$\pm 20\% \downarrow$	$\pm 25\% \downarrow$	soleus	10% (DW)		$\uparrow$ (in vivo)	=		$\uparrow$ (in vivo)		
Barclay <i>et al</i> 1992 (44)	rat	10 days	$33\% \downarrow$	$21\% \downarrow$	EDL	17% (DW)		= (in vivo)	$\downarrow$		=		
Russell <i>et al</i> 1984 (43)	rat	21 days	$\pm 34\% \downarrow$	$49\% \downarrow$	soleus	=		$\uparrow$	$\uparrow$		$\uparrow$		
Sieck <i>et al</i> 1989 (47)	rat	± 4 weeks	$26\% \downarrow$	$49\% \downarrow$	gastro			$\uparrow$ (in vivo)			$\downarrow$		leftwards shift ¹
Lewis <i>et al</i> 1986 (41)	rat	5-6 weeks	$22\% \downarrow$	$50\% \downarrow$	diaphragm	51%	type I and II	$\downarrow$	=		=		leftwards shift ¹
Gardiner <i>et al</i> 1980 (71)	rat	6 weeks	=	$\pm 25\% \downarrow$	diaphragm		type I and II	=	=		=		
Dekhuijzen <i>et al</i> 1995 (42)	rat	6 weeks	$40\% \downarrow$	$\pm 50\% \downarrow$	soleus	$\pm 25\%$	type I	=	=		=		
					gastro	$\pm 25\%$	type IIa and IIb	=	=		$\downarrow$		
					diaphragm	44%	type I, IIa and IIb	=	=		=		leftwards shift ¹
					gastro	45%	type I, IIa and IIb	=	=		=		
					soleus	26%		=	=		=		
Kelsen <i>et al</i> 1985 (77)	hamster	4 weeks	$25\% \downarrow$	$28\% \downarrow$	diaphragm	27%	type IIa and IIb	=	=		=		
Prezant <i>et al</i> 1994 (78)	rat	10 weeks	$80\% \uparrow$	$26\% \downarrow$	gastro+soleus	20%		=	=		=		
Prezant <i>et al</i> 1998 (79)	rat	10 weeks	$72\% \uparrow$	$25\% \downarrow$	diaphragm	33%	type I and II	$\downarrow$	=		=		
						23%	type I and II	=	=		=		

=, no change; DW, dry weight; EDL, extensor digitorum longus; gastro, gastrocnemius; Po, tetanic tension; $\frac{1}{2}$ RT, half relaxation time; F/F, force-frequency; ¹, expressed as a percentage of tetanic stimulations

Table 3. Metabolic alterations in the muscle after undernutrition, in sequence of duration.

Reference (number)	Animal	Duration of undernutrition	Change in body weight		Studied muscle	Muscle reduction	Muscle energy and protein metabolism									
			of initial	of control			PFK	CS	SDH	cyt c	HAD	glyco	pyr	lact	GLN	ATP
Khan et al (49)	rat	2 days	± 10%↓	17%↓	gastro	12%									↑	
Rooyackers et al (46)	rat	6 days			gastro			=		↓						
Bissonnette et al (45)	rat	7 days	± 20%↓	± 25%↓	soleus EDL	10% (DW) 17% (DW)					↓	↓				
Pichard et al (51)	rat	7 days	25%↓	33%↓	gastro										=	↑
Mijan de la Torre et al (52)	rat	7 days	25%	39%	gastro							↑			=	↓
Layman et al (48)	rat	9 days	30%↓	30%↓	soleus EDL	10% 28% 22%			= ↓ ↓ ↓							
Russell et al (43)	rat	21 days	± 34%↓	49%↓	gastro		↓				↓	=	↑	↑	=	↓
Sieck et al (47)	rat	± 4 weeks	26%↓	49%↓	diaphragm deep gastro				= ↓							

=, no change; gastro, gastrocnemius; EDL, extensor digitorum longus; PFK, phosphofructokinase; CS, citrate synthase; SDH, succinate dehydrogenase; cyt c, cytochrome c oxidase; HAD, β-hydroxyacyl CoA-dehydrogenase; glyco, glycogen; pyr, pyruvate; lact, lactate; GLN, glutamine; ATP, adenosine triphosphate; CrP, creatine phosphate; ADP, adenosine diphosphate;

1.3.2. Effects of undernutrition on muscle metabolism

Remarkably, only a few studies have investigated muscle metabolism after nutritional deprivation (Table 3). A decreased phosphofructokinase (PFK) activity was reported in the gastrocnemius after 3 weeks of hypocaloric feeding (43), implying a decreased glycolytic activity. The activity of the TCA-cycle, as assessed by citrate synthase (CS) activity, was not changed after 6 days of undernutrition in the gastrocnemius muscle (46), but after 9 days or more, succinyl dehydrogenase (SDH) activity was reduced in this muscle (43, 47, 48). SDH activities of the soleus and the diaphragm on the other hand were unchanged (47, 48). Sieck and colleagues suggested that the continuous activity of the diaphragm may preserve SDH activity, but this theory cannot be applied to the soleus muscle. It is possible that muscle enzyme activities are maintained in the soleus muscle, because of the relatively low level of muscle wasting. The activity of cytochrome c oxidase, involved in the respiratory chain, was reduced after 6 days of undernutrition (46). The activity of β -hydroxyacyl-CoA-dehydrogenase (HAD), the rate limiting enzyme in the intramitochondrial β -oxidation of fatty acids, was reduced after 3 weeks of undernutrition (43). Seven days of undernutrition resulting in muscle wasting of both the soleus and EDL resulted in decreased intramuscular glycogen concentrations (45). This reduced glycogen concentration was not found in the gastrocnemius muscle after 3 weeks of undernutrition, but instead an increased pyruvate and lactate concentration was observed (43). Furthermore, protein synthesis was also found to be decreased after undernutrition (49, 50). A decrease of 12% in gastrocnemius muscle mass was already related to an increase in intramuscular glutamine concentration, independently of reductions in muscle protein synthesis (49). Pichard and colleagues (1988) have studied the effects of hypocaloric feeding on rat muscle energetics by nuclear magnetic resonance *in vivo*. During 7 days of undernutrition, rats lost 25% of their body weight and intramuscular creatine phosphate (CrP) was reduced as well as the CrP/Pi and CrP/ATP ratios in the gastrocnemius (51). ATP levels were maintained but ADP concentration was increased. The authors suggested that ATP production was disturbed by aerobic and anaerobic mechanisms. This concept of an impaired rephosphorylation of ADP in malnourished muscle was supported by other studies (43, 52).

In summary, it is difficult to compare various undernutrition studies, since the degree and/or duration of nutritional deprivation is different, as well as the studied muscles. One has to be cautious to compare different muscles because of differences in fibre types, activity patterns and metabolism. In general, undernutrition can suppress glycolytic, as well as glycogenolytic and oxidative metabolism in the muscle. This causes a disturbed ATP production, but ATP levels are maintained at the expense of CrP.

Table 4. Morphological and contractility changes in the muscle after corticosteroid treatment.

Reference (number)	Animal	Corticosteroid treatment	Change in body weight	Studied muscle	Muscle reduction	Atrophy	fatigue	Pt	Po	$\frac{1}{2}$ RT	Contractility (in vitro)	F/F relationship but ' =
Moore <i>et al</i> (80)	rat	cortisone acetate (100 mg/kg BW) i.m. 10 days	14%↓	diaphragm soleus	28% =		↓	=	=	=		downward shift,
Polla <i>et al</i> (81)	rat	cortisone acetate (100mg/kg BW) s.c. 11 days	25%↓	diaphragm soleus	35% =							
Ferguson <i>et al</i> (82)	rabbit	cortisone acetate (10 mg/kg BW) i.m. 3 weeks	11%↓	diaphragm soleus	28% =							
Van Balkom <i>et al</i> (58)	rat	methylprednisolone (1 mg/kg BW) s.c. 8 weeks	±12%↓	diaphragm	24% =	type IIa						
Van Balkom <i>et al</i> (56)	rat	methylprednisolone (0.2 mg/kg BW) s.c. 9 months	44%↑	diaphragm	23% =	type IIa,b,x						
Dodd <i>et al</i> (60)	rat	prednisolone (5 mg/kg BW) s.c. 10 days	22%↓	gastro+sol+ plant	42% =	type IIx						
Lieu <i>et al</i> (59)	rat	prednisolone (5.0 mg/kg BW) s.c. 10 days	22%↓	diaphragm plantaris	23% =	type I, IIa and IIb						
Van Balkom <i>et al</i> (57)	rat	prednisolone (6 mg/kg BW) s.c. 3 weeks	6%↑	diaphragm	20% =	type IIa and IIb						
Dekhuijzen <i>et al</i> (55)	rat	prednisolone (5 mg/kg BW) i.m. 4 weeks	=	diaphragm gastro	=	type I, IIx and IIb						
Lewis <i>et al</i> (61)	hamster	dexamethasone (7.5 mg/kg BW) s.c. 3 weeks	±20%↓	diaphragm gastro	=	type II (+myopathy)						

Reference (number)	Animal	Corticosteroid treatment	Change in body weight	Studied muscle	Muscle reduction	Atrophy	Contractility (<i>in vitro</i>)			
							fatigue	Pt	Po	F/F relationship
Sasson <i>et al</i> (62)	rat	dexamethasone (1 mg/kg BW) i.p. 7 weeks	25%↓ 38%↓	diaphragm soleus	28%		=	↑	↑	↓
Prezant <i>et al</i> (79)	rat	dexamethasone (2 mg/kg BW) i.m. 10 weeks	9%↑ 52%↓	diaphragm	51%	type I and II	↓	=	=	↑
Viires <i>et al</i> (83)	rat	triamcinolone (1.2 mg/kg BW) i.m. 8 days	30%↓ 29%↓ of pair fed	diaphragm soleus EDL	24% = 27%		=	=	↓	downward shift = downward shift
Petrof <i>et al</i> (84)	rat	triamcinolone (1 mg/kg BW) i.m. 10 days	25%↓ 27%↓ of pair fed	diaphragm	± 25%	type I and IIx/b	↓	=	=	leftward shift ¹
Gardiner <i>et al</i> (85)	rat	triamcinolone (2 mg/kg BW) s.c. 2 weeks	25%↓ 44%↓	gastro soleus	± 45% ± 35%		<i>in vivo</i> <i>in vivo</i>	↑ ↑	= =	leftward shift ¹ = =
Kanda <i>et al</i> (86)	rat	triamcinolone (5 mg/kg BW) s.c. 2 weeks	±20%↓ 33%	EDL soleus		type II type II				
Wilcox <i>et al</i> (87)	hamster	triamcinolone (3 mg/kg BW) i.m. 3 weeks	±25%↓ 27%	diaphragm EDL	45%	type IIb type IIa + IIb	↓	=	=	leftward shift ¹
Dekhuijzen <i>et al</i> (55)	rat	triamcinolone (1 mg/kg BW) i.m. 4 weeks	35%↓ 42%↓	soleus diaphragm gastro	= 39% 64%	type IIa type IIb(+myopathy)	↓	=	=	leftward shift ¹
Dekhuijzen <i>et al</i> (42)	rat	triamcinolone (0.5 mg/kg BW) i.m. 6 weeks	±40%↓ ±40%↓	diaphragm soleus	41% 14%	type IIb(+myopathy)	↓	=	=	leftward shift ¹
Gardiner <i>et al</i> (71)	rat	triamcinolone (1 mg/kg BW) s.c. 6 weeks	±25%↓ 51%↓	gastro gastro soleus plantaris	59% 57% 39% 52%	type IIb type I, IIa and IIb type I and IIa	<i>in vivo</i> <i>in vivo</i>	↑ ↑	= =	= leftward shift ¹

=, no change; BW, body weight; gastro, gastrocnemius; EDL, extensor digitorum longus; sol, soleus; Pt, twitch tension; Po, tetanic tension; 1/2RT, half relaxation time; F/F, force-frequency; s.c., subcutaneous; i.m., intramuscular; i.p., intraperitoneal; ↓, expressed as a percentage of tetanic stimulations

1.4. Corticosteroids

Cortisol is the major glucocorticoid secreted by the adrenal cortex and it protects the body during stressful situations. Glucocorticoids inhibit several aspects of the inflammatory process by modulating gene transcription in inflammatory cells present at the site of inflammation.

Exogenously administered corticosteroids have been used since 1940 in the treatment of several inflammatory diseases. From that moment on, a search for more ideal anti-inflammatory therapeutics has begun, which should be more potent than cortisone with less mineralocorticoid side effects. Synthetic glucocorticoid derivatives were developed instead of isolating cortisone from the adrenal glands. By introducing a fluoride group in the molecule, as in the fluorinated corticosteroids dexamethasone and triamcinolone, all biological activities (glucocorticoid and mineralocorticoid) of the corticosteroids were enhanced (53, 54). The anti-inflammatory effect of triamcinolone is 5 times that of cortisol and the anti-inflammatory effect of the non-fluorinated corticosteroid prednisolone is 4 times that of cortisol.

1.4.1. Effects of corticosteroids on muscle mass, morphology, and contractility

Besides its anti-inflammatory effectiveness, corticosteroids may induce several adverse side effects. Table 4 shows the effects of several corticosteroids on muscle wasting and morphology. Large doses of cortisone acetate induced wasting of both respiratory and peripheral skeletal muscle, except for the soleus. Body wasting did not occur after intramuscular administration of prednisolone (55), but prolonged subcutaneous administration of low to normal doses of (methyl)prednisolone showed a decrease in body weight (56-58) and muscle wasting (59, 60). It is likely that the route of prednisolone administration is of importance in inducing muscle wasting. Administration of the fluorinated corticosteroids dexamethasone and triamcinolone always caused muscle wasting, but the soleus muscle was less susceptible to it. In general, type II fibres were most prone to corticosteroid-induced muscle fibre atrophy, in particular the type IIx/b fibres. In some studies, also some degree of type I muscle fibre atrophy was found.

Concerning muscle contractility, corticosteroid treated diaphragm muscles seem less susceptible to fatigue. However, a few studies demonstrated an increased fatigability of the muscle after prednisolone (55) or dexamethasone treatment (61). The type of fatigue protocol cannot explain this discrepancy. Surprisingly, the fatigability of the soleus muscle (whether or not in combination with the gastrocnemius muscle) was increased after corticosteroid treatment (60, 62). Twitch and tetanic tensions were affected in various ways after corticosteroid treatment. Half relaxation time was unchanged or increased, except for the diaphragm in a study of Sasson and coworkers (1991), in which it was decreased. The force-frequency relationship was mostly shifted downward after corticosteroid treatment, but when tensions were expressed as a percentage of tetanic stimulations, the force-frequency relationship was shifted to the left after corticosteroid treatment.

In summary, corticosteroid treatment causes primarily muscle fibre type IIx/b atrophy, with or without type IIa and type I atrophy. Muscle contractility is mainly assessed in the diaphragm muscle under *in vitro* conditions. The diaphragm muscle is less fatigued after corticosteroid treatment. However *in vitro* studies of the soleus muscle showed that this muscle was more fatigable. Interestingly, this discrepancy is also noticed after undernutrition. As above mentioned, the results of *in vitro* and *in vivo* stimulation protocols have to be compared with caution, since whole body metabolism is affected after undernutrition, which in turn affects muscle performance *in vivo*, while *in vitro* muscle contractility is independent on whole body metabolism at time of contraction.

Table 5. Metabolic alterations in the muscle after corticosteroid treatment.

Reference (number)	Animal	Corticosteroid treatment	Change in body weight		Studied muscle	Muscle reduction	Muscle energy and protein metabolism														
			of initial	± 10%↓			of control	± 15%↓	glyc	GP	PFK	CS	HADH	pyr	lact	CK	GS	GA	GLN	GLU	Bcaa
Hickson <i>et al</i> (65)	rat	cortisol acetate (100 mg/kg BW) s.c. 7 days	± 10%↓	± 15%↓	plantaris	± 25%														↑	
Ferguson <i>et al</i> (63)	rab- bit	cortisone (10 mg/kg BW) i.m. 14 days	9%↓	12%↓	diaphragm EDL		↑									↑	↑				
Falduto <i>et al</i> (70)	rat	cortisol acetate (100 mg/kg BW) s.c. 15 days			deep quadriceps	± 40%													↑		
Van Balkom <i>et al</i> (58)	rat	methylprednisolone (1 mg/kg BW) s.c. 8 weeks		±12%↓	diaphragm	=		↓		↑	=								↓		
Van Balkom <i>et al</i> (56)	rat	methylprednisolone (0.2 mg/kg BW) s.c. 9 months	44%↑	5%↓	diaphragm			↓			=	↑									
Lieu <i>et al</i> (59)	rat	prednisolone (5.0 mg/kg BW) s.c. 10 days	22%↓	26%↓	diaphragm plantaris	14% 20%					=	↓	=								
Meynial- Denis <i>et al</i> (69)	rat	dexamethasone (0.8 mg/rat) oral 5 days	16%↓	14%↓ of pair fed	TA soleus	19% =												↑	↑		
Hundal <i>et al</i> (66)	rat	dexamethasone (0.44 mg/kg BW) s.c. 8 days	5%↓	11%↓	gastro	12%												=	=	↓	↑
																				efflux↑	influx=

Reference (number)	Animal	Corticosteroid treatment	Change in body weight		Studied muscle	Muscle reduction	Muscle energy and protein metabolism																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
			of initial	of control			glyc	GP	PFK	CS	HADH	pyr	lact	CK	GS	GA	GLN	GLU	Bcaa																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
Parry-Billings <i>et al</i> (68)	rat	dexamethasone (2.5 mg/kg BW) i.p. 8 days			gastro EDL soleus																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										

=, no change; BW, body weight; EDL, extensor digitorum longus; TA, tibialis anterior; gastro, gastrocnemius; epitro, epitrochlearis; glyc, glycogen; GP, glycogen phosphorylase; PFK, phosphofructokinase; CS, citrate synthase; HADH, 3-hydroxyacyl CoA dehydrogenase; pyr, pyruvate; lact, lactate; CK, creatine kinase; GS, glutamine synthetase; GA, glutamine; GLN, glutamine; BCAA, branched chain amino acids; SDH, succinyl dehydrogenase; s.c., subcutaneous; i.m., intramuscular; i.p., intraperitoneal

1.4.2. Effects of corticosteroids on muscle metabolism

As shown in Table 5, after cortisone treatment muscle glycogen was increased in the diaphragm but not in the EDL muscle (63). A possible explanation could be the observed decreased glycogen phosphorylase activity (56, 58). Lieu and colleagues (1993) suggested that a reduction in glycolytic capacity would likely increase the muscle's dependence on fat oxidation, resulting in a higher glycogen content. Indeed, both a decreased PFK and an (not significantly) increased HADH activity were found in the plantaris, but not in the diaphragm muscle. The oxidative capacity, as indicated by CS activity, was affected in several ways by (methyl)prednisolone treatment in the diaphragm, while it was unchanged in the plantaris muscle. Both the increased pyruvate and lactate levels in the diaphragm and peripheral skeletal muscles however, may indicate an increased glycolytic activity of the muscles. Furthermore, creatine kinase (CK) activity is decreased in the diaphragm after corticosteroid treatment, which implies a reduction in fast energy supply.

Protein metabolism, as reflected by amino acid metabolism, is also affected by corticosteroids. Glutamine, an important conditionally essential amino acid, has frequently been studied, because its an important nitrogen and ammonia carrier in interorgan metabolism (64). Glutamine efflux is reported to be increased (65-67). In most cases, this is accompanied by a decreased intramuscular glutamine concentration (65-68). Glutamine can be produced *de novo* from glutamate by glutamine synthetase (GS). GS activity and mRNA expression is often found to be increased after corticosteroid treatment (66, 67, 69, 70). An unchanged activity of glutaminase, which catalyses glutamate formation from glutamine, after corticosteroid treatment was shown (66, 67). Intramuscular glutamate concentration was found to be decreased after dexamethasone treatment in the epitrochlearis muscle (67) but increased in the gastrocnemius muscle, while glutamate influx was unchanged (66).

Dexamethasone treatment resulted also in most cases in an increased branched chain amino acid concentration in the peripheral skeletal muscle (66, 67).

In summary, glycogen stores can be increased after corticosteroid treatment, depending on the muscle. It is still unclear whether glycolytic and/or oxidative metabolism are decreased, unchanged or increased. However, it is consistently found that glutamine efflux is increased after corticosteroid treatment. It is difficult to compare these studies because of the different corticosteroid treatments and only a few parameters are investigated simultaneously in one muscle, which is different from the muscle in another study. To obtain a better insight in muscle metabolism after corticosteroid treatment, one has to study several aspects together in one muscle after administration of a given corticosteroid.

1.5. Outline of this thesis

Considering the important role of both corticosteroids and undernutrition on muscle dysfunctioning in chronically ill patients, the purpose of the studies described in this thesis was to discriminate between the effects of corticosteroids and undernutrition on muscle functioning, as determined by muscle mass, morphology and metabolism. It is not possible to investigate the individual effects of corticosteroids or of undernutrition on muscle function in patients, due to ethical reasons and intervening disease related factors. Therefore, an experimental rat model was chosen. The fluorinated corticosteroid triamcinolone was used because it is known to induce muscle wasting, caused by both undernutrition as well as by hypermetabolism (71). The triamcinolone-treated group could be compared with two nutritional-control groups. Firstly a pair fed group was introduced, to adjust for the effects of triamcinolone treatment on daily food intake. This pair fed group had a comparable daily food intake as the triamcinolone-treated group. Secondly, a pair weight group was included since the body weight of the rats was decreased to a greater extent than could be explained by the diminished food intake alone. To induce a comparable body weight as the triamcinolone-treated group, daily food intake was restricted severely. Finally a free fed control group was used, which had an *ad libitum* food intake and was a reference for normal untreated rats.

Food intake and body weight were registered daily and an acute and chronic response of triamcinolone treatment was shown. In our first study, we compared diaphragm muscle morphology and function after chronic triamcinolone treatment with (nutritional) control groups (**chapter 2**). This was performed in an isometric *in vitro* set-up. This means that a bundle of the diaphragm was placed in a glass tissue chamber and stimulated with an isometric force transducer. Since it was shown that the corticosteroid dose was too high (more than 50% mortality) and 2 weeks treatment was probably long enough to induce chronic muscle wasting (body weight was almost stable as shown in Figure 2B), we decided to adapt the model to a lower triamcinolone dose and a shorter treatment period. Additional to the effects on *in vitro* muscle function, diaphragm muscle metabolism was studied (**chapter 3**), especially the energy-rich phosphates, since alterations in energy metabolism may also reflect differences in muscle function.

In an *in vitro* experiment, muscle function is mainly dependent on substrates present in the muscle, which is muscle fibre dependent, and on diffusion for substrate metabolism and oxygen supply. *In situ* muscle function however, is also dependent on blood flow, which plays an important role in performing muscle function by supplying substrates and remove waste products. A peripheral skeletal muscle was studied. This is a relevant muscle since both peripheral skeletal muscle and respiratory muscle weakness is observed in chronically ill patients. In this *in situ* set-up, triamcinolone treatment was on the one hand compared with undernutrition and on the other hand with treatment of prednisolone in an equipotent anti-inflammatory dose (**chapter 4**). This non-fluorinated corticosteroid is more frequently used in clinical practice and did not induce muscle wasting after intramuscular administration. Subsequently, in the same groups, muscle enzyme activities and glycogen of the peripheral skeletal muscle was determined (**chapter 5**), since they can give more insight in the underlying pathophysiology.

Protein metabolism is obviously disturbed after corticosteroid treatment (72-74) and has been related to an increased glutamine release from the muscle (66-68). To investigate whether muscle wasting occurs during a chronic process or is mainly a reflection of acutely effects, muscle glutamine metabolism was studied after acute and chronic triamcinolone treatment (**chapter 6**). With the use of venous-arterial concentrations and stable isotope techniques muscle glutamine metabolism was studied. Glutamine is a conditionally essential amino acid and plays an important role during metabolic stressful conditions, such as after triamcinolone treatment. In all these described studies, triamcinolone and prednisolone were injected intramuscularly, which means that these corticosteroids diffuse in the circulation and may easily exert systemic effects in the whole body. Corticosteroids are often given locally instead of systemically, for instance in the form of inhalation corticosteroids given to patients with respiratory failure. A central role in the action of corticosteroids is played by the glucocorticoid receptors (GR). The presence of two functionally distinct receptors α and β GR mRNAs have been described in several human tissues (75, 76). In rats, no information is available about the existence of the β GR form in skeletal muscles and the regulation of the α and β form by corticosteroids. To investigate whether systemic effects could be found after corticosteroid inhalation, the expression of the α and β GR forms were investigated in a peripheral skeletal muscle (**chapter 7**). Finally, the major findings of these studies are summarised and discussed in **chapter 8**.

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Contractile properties and histochemical characteristics of the rat diaphragm after prolonged triamcinolone treatment and nutritional deprivation.

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Journal of Muscle Research and Cell Motility 19: 549-555 (1998)

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Abstract

The influence of decreased muscle mass and reduced food intake on diaphragm structure and contractility in male Wistar rats was determined after triamcinolone acetate treatment (TR: 0.5 mg/kg/day for 4 weeks) and two degrees of undernutrition (PW: pair-weight, which resulted in a similar (41%) reduction of body weight as TR; PF: pair-fed, which resulted in a moderate (13%) reduction of body weight and a free fed control group (FF, with an increase (9%) in body weight). Energy intake of TR decreased but based on daily measurements of food intake and body weight, energy expenditure of the TR rats was increased compared with the other groups. Body (BW) and muscle weights were reduced in proportion to the extent of undernutrition in the nutritionally deprived rats (i.e. BW and diaphragm weight of PF animals were reduced 2% and 16% respectively compared with FF, versus a 48% and 41% reduction in the PW group). Triamcinolone-induced atrophy was limited to type II fibres (30% of type IIa and 45% of type IIx/b, $p < 0.05$), while severe chronic undernutrition (PW) induced a generalised fibre type atrophy in the diaphragm (23% type I, 38% type IIa and 49% type IIx/b, $p < 0.05$) and moderate undernutrition (PF) caused only significant type IIa atrophy (20%, $p < 0.05$). A leftward shift of the diaphragmatic tension-frequency relationship and a decreased fatigability of the TR and PW bundles were observed ($p < 0.01$), while the PF bundles were not significantly different compared with FF. These results suggest that triamcinolone and severe undernutrition cause similar alterations in *in vitro* contractility of the diaphragm. The effects of triamcinolone treatment on diaphragm structure may be partly explained by the reduced food intake, but the atrophy pattern induced by severe undernutrition (PW) was different.

2.1. Introduction

Previous studies suggest that in patients with chronic obstructive pulmonary disease (COPD) muscle weakness may be the result of muscle wasting associated with weight loss (1, 2) and of corticosteroid treatment (3). Weight loss in patients with COPD appears to be primarily the result of increased energy requirements which cannot be restored by increased spontaneous dietary intake (4).

Experimental research in animals (mice, rats) indicates that particularly fluorinated steroids cause myopathies (5) or muscle weakness (6, 7), which are accompanied by decreased body weight and muscle mass. A decreased food intake of approximately 42% was observed with administration of the fluorinated corticosteroid triamcinolone, but the loss of body weight of 51% could not be only the result of this diminished food intake (6). In another study, Dekhuijzen and colleagues (8) concluded that triamcinolone treatment caused type IIb atrophy, while nutritional deprivation, resulting in a comparable body weight reduction, caused a generalised atrophy of all diaphragm fibre types, but these two treatments showed similar changes in *in vitro* diaphragm contractile properties. Based on this study, we questioned to what extent the effects of triamcinolone on muscle structure and *in vitro* function are related to the effects of nutritional deprivation *per se*, to the degree of muscle wasting induced by triamcinolone treatment, or to a combination of both. Therefore the present study was designed to compare the effects of triamcinolone treatment with two nutritionally deprived groups; a pair-fed group, with a similar food intake and a pair-weight group, with a comparable body weight, on diaphragm structure and *in vitro* contractility. Corticosteroids are used in low doses as maintenance therapy or in high doses with exacerbations in COPD. In this study, the dose of triamcinolone is within the range of chronic corticosteroid therapy in COPD patients. Furthermore, triamcinolone is used in the present study because muscle wasting occurs, which could be due to glucocorticoid effects or due to

chronic undernutrition. A possible differentiation between the effects of corticosteroids and chronic undernutrition on muscle structure and function may have clinical relevance in the assessment of (respiratory) muscle weakness in COPD patients.

2.2. Methods

Animals

After 4 days of acclimatisation in metabolic cages, 40 male Wistar rats (aged 14 weeks, body weight (BW) ranged from 287-339 g) were randomly divided into four groups to receive during 4 weeks: 1) Triamcinolone (TR: 0.5 mg*kg⁻¹ *day⁻¹ triamcinolone acetate i.m.), *ad libitum* food intake (n = 16 in order to take into account previously described premature death (8)); 2) Pair-weight (PW: 0.05 ml*day⁻¹ saline i.m.), restricted food intake, daily adjusted to achieve a similar weight loss as TR (n = 8); 3) Pair-fed (PF: 0.1 ml*day⁻¹ saline i.m. (BW > 300 g) or 0.05 ml (BW < 300 g)), restricted food intake, daily adjusted to the food intake of TR (n = 8); 4) Free fed (FF: 0.1 ml*day⁻¹ saline i.m.), *ad libitum* food intake (n = 8). The diet provided to all animals was Muracon-G chow (53% carbohydrate, 20% protein, 5% fat). Water was available *ad libitum* to all animals. The animals were housed in metabolic cages, at a temperature of 23°C, with 14 hours light and 10 hours dark. Food intake and body weight of all animals were registered daily. Veterinary oversight, including regular observation of the health of the animals was performed according to the Belgian National Guidelines of Animal Care.

In vitro determination of diaphragm contractility

Twenty-four hours after the last injection and after 16 hours fasting, the animals were anaesthetised with Nembutal (6 mg/100 g intraperitoneally), tracheostomized, and mechanically ventilated. Rectal temperature was monitored and adjusted with a heating lamp. The left part of the diaphragm was rapidly excised and two thin strips of muscle were cut from the central tendon to the ribs (in cooled oxygenated + 5% CO₂ Krebs solution). These diaphragm bundles were each placed in a glass tissue chamber containing oxygenated + 5% CO₂ Krebs solution, maintained at 37°C. One end of the bundle was tied to a rigid support and the other end was fastened to an isometric force transducer mounted to a micrometer. The optimal length (L_o) of the bundle was determined and thermoequilibrium was done for 15 minutes (to 37°C). The bundles were stimulated with a pulse duration of 0.2 ms and a train duration of 250 ms. From a series of single pulses, the peak twitch tension was determined. Other twitch characteristics, such as the time to peak tension (TPT) and the half-relaxation time (½ RT) were calculated. Maximal tetanic tension was determined at 160 Hz. Tension-frequency relationships were measured at 25, 160, 50, 160, 80, 160, 120, 160 Hz. Each stimulus was separated by a 2-min interval. Stimulation at 160 Hz was interposed between each stimulus frequency during the tension-frequency curve to relate tension output at a certain stimulus frequency to the maximal achievable tension output at that moment (9). Bundles were fatigued by means of 330 ms stimulations at 25 Hz and applied every 2 seconds during 5 minutes. Bundle thickness and length were measured at L_o. Afterwards it was blotted dry and weighed. Cross-sectional area (CSA) was calculated by dividing weight by specific density (1.056) and muscle L_o. All measured tensions were normalised for CSA of the bundle.

Muscle fibre histochemistry

The remaining part of the costal diaphragm was put into "tissue glue" (Tissue-Tek, Elkhard, IN) on a cork holder with the muscle fibres oriented perpendicularly to the surface of the cork, then quickly frozen in isopentane, cooled by liquid N₂. Serial cross sections were cut at 8 µm thickness by use of a cryostat kept at -20°C. Myofibrillar adenosinetriphosphatase was stained after alkaline (pH 9.3) and acid (pH 4.5) preincubation. Fibre analysis was done with a Leitz microscope, linked up to a digitising board (Quantimet 570, Leica, United Kingdom). Areas in which fibre orientation was not transverse to the long axis and areas with freezing artifacts, were not analysed. By the use of a computer program, the individual muscle fibres were selected and the fibre CSA was determined from the number of pixels within the outlined fibre. For the diaphragm, CSA was corrected for the shortening occurring from optimal length using a correction factor determined by ourselves in 32 rats, close to that found by Prakash and coworkers (10). At least 250 fibres of each rat were used to calculate the mean CSA of type I, IIa and IIx/b fibres.

Statistical analysis

Two diaphragm bundles were obtained from each animal. The mean of these two was determined and used for analysis. Differences between all groups were assessed using the Mann-Whitney U test, because of the non-normally distribution of the data. The level of significance was determined as $p \leq 0.05$. Analysis was performed using the SPSS/PC+ program. All data are presented as group means $\pm$ standard error of the mean (SEM). Only significant differences between groups are described.

2.3. Results

Animal food intake, body and muscle weights

During the 4 weeks of experiment, 7 rats of the TR group survived (one rat died after 12 days and eight after 20-27 days). In addition, 3 rats of the PW group matched to deceased TR rats and 1 of the PF group died. Triamcinolone treatment caused anorexia after the first injection. Thereafter appetite slowly recovered during the next 5 days, but remained below the *ad libitum* intake of the rats in group FF ($p < 0.05$). Food intake of PW was significantly lower than that of FF from day 1 onwards ($p < 0.01$) and significantly lower than that of TR from day 6 onwards ($p < 0.05$). Food intake between PF and TR was not significantly different.

Initial body weights of the groups were not significantly different (Figure 1A). During 4 weeks of experiment, the weights of the TR, PW and PF rats were reduced by 47%, 48% and 21% resp. compared with the FF group ($p < 0.01$), while those of the TR and PW group were significantly lower than those of the PF group ($p < 0.01$).

Adjusted for the differences in body weight, energy intake (food intake in kcal per 100 g BW) of the TR rats decreased and was significantly lower than FF from day 2 until day 8 (Fig 1B, $p < 0.05$), but was significantly increased from day 14 until day 26 (except for day 15, 21 and 22) compared with FF (Fig 1B, $p < 0.05$). Energy intake of the TR rats was significantly higher compared with PW (Fig 1B, $p < 0.05$) from day 2 onwards, except for day 4, 5, 6, 9, 10, 17, and compared with PF (Fig 1B, $p < 0.05$) from day 6 onwards, except for day 8, 15 and 16.

Muscle weight of the diaphragm was significantly decreased in the TR (0.39 ± 0.02 g, $p < 0.01$), PW (0.43 ± 0.01 g, $p < 0.01$) and PF (0.61 ± 0.03 g, $p < 0.05$) groups compared with the FF group (0.73 ± 0.04 g) and in the TR and PW groups compared with the PF group ($p < 0.01$). Both in absolute terms as well as relative to body weight, muscle mass was not significantly different between PW and TR.

Fig. 1. (A) Body weight of the groups as a result of triamcinolone treatment (●) or pair-weight (+), pair-fed (Δ) or ad libitum food intake (○). TR and PW are significantly different from FF ($p<0.01$) from day 2 onwards. PF is significant different from FF ($p<0.01$) from day 4 onwards. (B) Energy intake of the groups. See text for significance.

Peak twitch tension, peak tetanic tension and twitch characteristics

Tensions and twitch characteristics of the diaphragm are shown in Table 1. Peak twitch tension (P_t ; at 1Hz) of the PW group was significantly increased compared with PF ($p<0.05$) and FF ($p<0.01$). Peak tetanic (P_o ; at 160 Hz) was not significantly different between the groups. The P_t/P_o ratio was higher in the TR and PW groups compared with the FF group ($p<0.01$) and the PF group ($p<0.01$). Time to peak tension (TPT) of the PW group was prolonged compared with the PF and FF groups (Table 1, $p<0.05$). Half relaxation time ($\frac{1}{2}$ RT) was longer in the TR and PW groups compared with FF group (Table 1, $p<0.05$).

Table 1. Tensions, fatigue and twitch characteristics of the diaphragm

	Triamcinolone	Pair-weight	Pair-fed	Free-fed
Pt (kg/cm ²)	0.8 ± 0.04	0.9 ± .01 °#	0.7 ± 0.02	0.7 ± 0.02
Po (kg/cm ²)	2.6 ± 0.2	2.8 ± 0.1	2.7 ± 0.2	2.6 ± 0.2
Pt/Po	0.31 ± 0.02 ◊#	0.34 ± 0.02 ◊#	0.26 ± 0.01	0.25 ± 0.01
TPT (ms)	21.9 ± 0.3	22.8 ± 0.2 ◊*	20.9 ± 0.4	20.9 ± 0.4
½ RT (ms)	28.0 ± 2.1 *	25.8 ± 1.4 *	23.3 ± 0.5	22.6 ± 0.7

Values are means ± SEM. Pt, peak twitch tension; Po, peak tetanic tension; TPT, time to peak tension; ½ RT, half-relaxation time; ° $p < 0.05$ compared with pair-fed; ◊ $p < 0.01$ compared with pair-fed; * $p < 0.05$ compared with free-fed; # $p < 0.01$ compared with free-fed.

Tension-frequency relationships

Figure 2A depicts the absolute values of tensions, generated by the diaphragm bundles at different frequencies. The TR and PW group generated significantly higher tensions per CSA than the FF group at 25 Hz ($p < 0.05$ and $p < 0.01$ respectively). When the tensions were expressed as a percentage of interposed Po, the curves of the TR and PW groups were significantly shifted to the left compared with that of the FF group (Fig 2B, $p < 0.05$). The PF

Fig. 2. Tension-frequency relationships in TR (solid line), PW (dashed line), PF (dashed-dotted line) and FF (dotted line) groups. (A) Tension-frequency curves are represented as absolute tensions per cross-sectional area. (B) Tensions were determined as percentages of their maximal tensions. * $p < 0.05$ TR compared with FF; # $p < 0.01$ TR compared with FF; § $p < 0.001$ TR compared with FF.

group showed significantly higher relative tensions only at 25 Hz compared with the FF group ($p<0.05$). Fatigue of the interposed 160 Hz in the TR ($1.9 \pm 1.1\%$) and PW ($2.5 \pm 0.6\%$) rats was significantly less than that of the FF ($11.4 \pm 2.0\%$, $p<0.001$ resp. $p<0.01$) and PF ($7.0 \pm 1.2\%$, $p<0.01$ resp. $p<0.05$) rats.

Fatigue at 25 Hz

At 25 Hz, the bundles of the TR and PW rats generated significantly higher tensions than those of the FF and PF (after 120 seconds) rats, when expressed in absolute values (Fig 3A). Figure 3B depicts the tensions as percentage of their initial values, where TR and PW bundles showed significantly less fatigue than the FF group ($p<0.05$), and PW also compared with the PF group after 120 seconds ($p<0.05$).

Fig. 3. (A) Fatigue in the bundles of the TR (solid line), PW (dashed line), PF (dashed-dotted line) and FF (dotted line) groups. The absolute tensions per cross-sectional area are depicted. (B) The relative tension loss is depicted. * $p<0.05$ TR compared with FF; # $p<0.01$ TR compared with FF.

Diaphragm fibre cross-sectional area and fibre-type proportions

After triamcinolone treatment, the CSA of type IIa and IIx/b fibres decreased significantly compared with the FF group by 30% (Figure 4, $p<0.05$) and 45% ($p<0.01$) respectively. Severe undernutrition (PW) resulted in a significant reduction of CSA of type I fibres by 23% ($p<0.05$) and of both type II fibres by 38 resp. 48% (Fig 4, $p<0.01$). Moderate undernutrition (PF) had no effect on type I fibres but significantly decreased the CSA of IIa fibres by 20% (Fig 4, $p<0.05$). Type IIx/b fibres of TR and PW were significantly smaller than those of PF (Fig 4, $p<0.01$ and $p<0.05$ respectively). Significant differences were observed in CSAs of type I fibres between TR and PW rats (Fig 4, $p<0.05$).

Fig. 4. Fibre cross-sectional area (CSA) in the diaphragm of the different treatment groups. Values are means $\pm$ SEM.

* $p<0.05$, ** $p<0.01$.

Figure 5 shows the relative contribution of all fibre type CSAs to the total measured area. Relative type I CSA in TR (36.5%) and PW (33.8%) was increased compared with FF (22.7%, $p<0.01$) and PF (26.9%, $p<0.05$). The relative type IIx/b CSA of both the TR (38.3%) and PW (37.1%) groups were significantly reduced compared with the FF (51.4%, $p<0.01$) and PF groups (50.1%, $p<0.05$). Relative type IIa CSA of the PW group (29.1%) was increased compared with the PF group (23.0%, $p<0.05$).

All fibre type proportions remained the same after triamcinolone treatment. On the other hand, after severe chronic undernutrition (PW) a small significant increase in the percentage

of type IIa fibres was found compared with the PF group (33% vs 29%, $p=0.05$) and a decrease in the percentage of type IIx/b fibres compared with the FF group (29% vs 35%, $p<0.05$).

Fig. 5. Relative contributions of different fibre types to total CSA in diaphragm. Solid bars, type I fibres; hatched bars, type IIa fibres; open bars, type IIx/b fibres. * Significant different from FF $p<0.01$; # significant different from PF $p<0.05$.

Diaphragm bundle dimensions

The optimal length of the diaphragm bundles of the TR group (18.2 ± 0.6 mm) was significantly shorter than that of the FF group (19.9 ± 0.4 mm, $p<0.05$). The thickness of the bundles was not significantly different among the groups.

2.4. Discussion

The results of this study show a discrepancy between the balance of energy intake and body weight change after triamcinolone treatment, implying that weight loss is a consequence of increased energy expenditure not adequately restored by dietary intake. The diaphragm atrophy pattern of the TR rats was limited to type II fibres, whereas rats with comparable food intake (PF) only showed type IIa atrophy and rats with comparable body weight (PW) showed a generalised atrophy of all fibre types. This suggests a corticosteroid specific effect on diaphragm structure. TR and PW had similarly affected diaphragm muscle mass despite differences in muscle atrophy patterns between these groups, which may suggest atrophy of muscle fibres as well as non-fibre material after triamcinolone treatment. The effect of triamcinolone treatment and severe chronic undernutrition (PW) on diaphragm contractility was comparable, resulting in a decreased fatigue and higher tensions at 25Hz, but they differed from that of rats with comparable food intake. This suggests that the increased relative contribution of type I fibres was probably responsible for the alterations noted in contractility.

Chronic severe undernutrition (PW) in this present study showed atrophy of type I fibres (23%) but more pronounced atrophy of type IIa (38%) and IIx/b (48%) fibres. This is in agreement with earlier data on chronic undernutrition to a similar extent in rats, which resulted in 18% atrophy of type I diaphragmatic fibres and 50% type II atrophy (11), or 23% type I and 47% type II atrophy (12) but in these studies no differentiation was made between type IIa and IIx/b. With respect to the effects of undernutrition, the present study design allowed us to differentiate between the effects of moderate (PF) and severe undernutrition (PW) on muscle structure. As expected, moderate undernutrition (PF) caused less effect than severe undernutrition, resulting in only type IIa atrophy (20% vs FF).

In general, both triamcinolone and severe chronic undernutrition (PW) caused atrophy of type II diaphragm fibres, most pronounced for type IIx/b, while severe undernutrition also

caused type I atrophy. Diaphragm weight loss of the TR and PW group was comparable, but total atrophy of the diaphragmatic fibres in the TR group was less than in the PW group. This implies a smaller reduction of fibre material, and may suggest a greater reduction of non-fibre material like extracellular fat stores or connective tissue, after triamcinolone treatment. Moderate undernutrition (PF) resulted in atrophy of type IIa fibres approximately to the same extent as caused by triamcinolone, but no significant type IIx/b atrophy. This suggests that the alterations in diaphragm structure after triamcinolone treatment may only be partly explained by their diminished food intake. The atrophy pattern in PW was similar for type II fibres compared to TR, but more pronounced for type I fibres. A similar degree of muscle wasting induced by severe undernutrition resulted in generalised atrophy. Severe undernutrition and triamcinolone treatment resulted in type IIx/b atrophy to the same extent. The contractility of the diaphragm bundles showed similar alterations for the TR and PW groups compared with the PF and FF group, which were not mutually different. In this study, a prolongation of $\frac{1}{2}$ RT and a tendency for TPT to be elevated in the TR group was observed, which is in line with the data of Dekhuijzen and colleagues (8), showing a prolonged $\frac{1}{2}$ RT after six weeks triamcinolone treatment with the same dose, and a type II x/b fibre atrophy of 51%. After three weeks of dexamethasone treatment in hamsters, Lewis and coworkers (13) reported no change in contraction time and $\frac{1}{2}$ RT, with a type II atrophy of 33%. Petrof and coworkers (14), after 10 days of triamcinolone treatment in rats, observed a prolongation of the contraction time and a trend toward a prolonged $\frac{1}{2}$ RT, with a type IIx/b atrophy of 42%. Thus, it seems that the prolongation of $\frac{1}{2}$ RT after triamcinolone or dexamethasone treatment is related to the extent of type IIx/b atrophy. In the present study, the relative CSA of type I fibres was increased in the diaphragm after severe chronic undernutrition and after triamcinolone treatment, but only the TPT of the pair-weight group was increased, maybe because their type IIx/b atrophy was greater than that of the TR group. The results of the tension- frequency stimulation showed a shift to the left of the curves in the TR and PW rats. In earlier studies, this phenomenon was also demonstrated as a result of triamcinolone treatment in hamsters and rats (8, 15, 16). In the present study, the TR and PW groups demonstrated significantly less fatigue than the FF group, as observed by others in hamsters and rats after triamcinolone treatment (8, 15, 16). The increased fatigue resistance could be explained by the relatively higher proportion of type I fibres, which are less easily fatiguable than type II fibres.

It seems that the relative decrease in the type IIx/b CSA observed after both TR and PW was primarily responsible for similar alterations in diaphragm contractile properties. This does not exclude the possibility that the observed differences in chronic metabolic effects between TR and PW such as hyperglycaemia and hypermetabolism could well be reflected in *in vivo* contractility. It appears that the relative decrease in type IIx/b CSA as observed after both triamcinolone treatment and chronic undernutrition was responsible for similar alterations in diaphragm contractile properties. In conclusion, comparable loss of diaphragm muscle mass after triamcinolone treatment and after severe undernutrition resulted in different fibre-type atrophy patterns but similar changes in contractility. In contrast, comparable food intake during triamcinolone treatment and during moderate undernutrition (PF) resulted in differences in diaphragm contractility and structure, while the differences after moderate undernutrition (PF) were less pronounced but in the same direction as after triamcinolone treatment. This suggests that the alterations after triamcinolone treatment may be explained by the diminished food intake in addition to a corticosteroid-specific effect. Furthermore, a discrepancy between the extent of fibre atrophy and muscle mass reduction was observed after triamcinolone treatment and severe chronic undernutrition, which may be caused by a decrease of non-fibre material in the diaphragm muscle after triamcinolone administration.

Acknowledgements

The authors wish to express many thanks to N Plaisance-Buts for excellent technical assistance.

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3

Different effects of corticosteroid-induced muscle wasting compared with undernutrition on rat diaphragm energy metabolism.

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Abstract

An important adverse effect of corticosteroid treatment is respiratory muscle weakness and diaphragm muscle wasting, but little is known about the underlying pathophysiological processes. In order to differentiate between the effects of nutritional depletion and corticosteroids on diaphragm muscle metabolism, a study was performed to investigate the effects of triamcinolone (TR) for 2 weeks and of chronic undernutrition in a pair-weight (PW) group on structure and energy metabolism of the diaphragm in male Wistar rats compared with a free-fed (FF) group.

Diaphragm mass was reduced in TR and PW rats to a similar degree, but the extent of type IIx/b atrophy was more pronounced in TR rats than in PW rats. No myopathic features were observed after either treatment. ATP in absolute terms as well as ATP/ADP ratio, total adenine nucleotides, CrP and CrP/Cr ratio were decreased in the diaphragm of both TR and PW rats. In contrast to the PW group, total creatine pool was reduced and pyruvate and lactate levels were elevated in the diaphragm of the TR group compared with the FF group.

In conclusion, the results of this study indicate different metabolic alterations after undernutrition and triamcinolone treatment. Severe undernutrition causes a decrease in muscle energy status resulting in a new metabolic equilibrium while chronic low dose triamcinolone treatment causes a decreased muscle energy status together with a mismatch between glycolysis and oxidative metabolism.

3.1. Introduction

Weakness of both peripheral skeletal and respiratory muscles is frequently reported in patients with chronic obstructive pulmonary disease (COPD) (1, 2). Independent of the underlying lung function impairment, peripheral skeletal muscle dysfunction has been associated to exercise intolerance and a decreased health status (2). Weakness of the diaphragm and accessory respiratory muscles furthermore contributes to morbidity by dysfunction of the ventilatory pump and thus can predispose to respiratory failure. A strong relationship between muscle mass and peripheral muscle function has recently been demonstrated in patients with COPD (3). Muscle weakness associated with muscle atrophy in these patients may be a consequence of corticosteroid treatment (1), undernutrition (4) or inactivity. Studies in anorexia nervosa patients have clearly shown that the effects of muscle wasting are not limited to the peripheral skeletal muscles, but also affect respiratory muscle mass and strength (5).

Especially fluorinated corticosteroids, like triamcinolone and dexamethasone have been shown to induce muscle wasting. In experimental animal studies, triamcinolone-induced diaphragm muscle wasting comprised predominantly atrophy of muscle type IIx/b fibres and myopathic features like an increased amount of connective tissue and excess of nuclei. Furthermore, diaphragm contractility has frequently been studied *in vitro* and showed in general a decreased fatigue and prolonged contraction time and half-relaxation time of the diaphragm bundle after triamcinolone treatment (6-9). In view of the observed changes in diaphragm morphology and contractility, further analysis of metabolic alterations in muscle cell energy metabolism seems justified. Diaphragm muscle biochemistry has only occasionally been studied in relation to fluorinated corticosteroid treatment. After dexamethasone treatment, a reduction of succinyl dehydrogenase activity in the diaphragm was observed (10). Besides, muscle glycogen stores were found to be increased after triamcinolone treatment (11). To our knowledge, energy-rich phosphates of the diaphragm

have never been studied in relation to corticosteroid treatment in rats, while they may represent the functional consequences of altered enzyme activities and substrate levels.

The effects of chronic corticosteroid treatment on diaphragm structure and *in vitro* contractility were frequently compared with the effects of undernutrition, because both treatments may induce muscle wasting. However, the underlying mechanisms of muscle wasting are clearly different. Body and muscle weight reduction after triamcinolone treatment can not be explained by a diminished food intake alone, implying a hypermetabolic state (12). In contrast to triamcinolone treatment, previous studies showed that chronic undernutrition resulted in muscle atrophy which was not restricted to type IIx/b (6-9) and absence of myopathic features. *In vitro* contractility of diaphragm bundles of undernourished rats however, was comparable of that of rats with the same extent of body weight loss induced by triamcinolone treatment (6-9).

With the knowledge that in contrast to undernutrition, corticosteroids may induce hypermetabolism and hyperglycaemia (13, 14) and an increase in muscle glycogen, we hypothesise that triamcinolone treatment will induce other alterations in diaphragm muscle energy metabolism than undernutrition. This study was performed to better clarify the specific effects of triamcinolone treatment on diaphragm muscle, especially on energy metabolism and muscle structure in comparison to undernutrition. This information is important to consider for interpretation of *in vivo* diaphragm muscle performance and to provide adequate therapy to counteract the muscle wasting as potential side effect of these treatments.

3.2. Methods

Animals

Fourteen week old male Wistar rats were randomly divided into three groups for 14 days of treatment: 1) Triamcinolone treatment (TR: 0.25 mg/kg/day triamcinolone acetate i.m.), *ad libitum* food intake (n = 22); 2) Pair weight (PW: 0.05 ml/day saline i.m.), restricted food intake daily adjusted to achieve a similar weight loss as TR (n = 22); 3) Free fed (FF: 0.1 ml/day saline i.m.), *ad libitum* food intake (n = 40). Water was available *ad libitum* to all animals. The animals were individually housed in metabolic cages at an ambient temperature of 23°C with 14 hours light and 10 hours dark. Food intake and body weight of all animals were registered daily. Regular veterinary inspection of the animals took place to ensure the health and welfare was provided according to the Belgian and Dutch National Guidelines of Animal Care.

Muscle preparations

After overnight fasting, 10 rats of the TR and PW groups and 20 rats of the FF group were anaesthetised with ether. Rectal temperature was monitored and adjusted using a heating pad. Arterial blood was sampled from the carotid artery and collected in heparinized cups (Lithium-Heparin Micro-sample container CB100, Sarstedt, FRG) on ice.

For hematocrit determinations a micro-hematocrit tube (Modulohm 88302, Herlev, Denmark) was filled with heparinized blood and centrifuged at 10000 g at room temperature (Micro Hematocrit Centrifuge, Hawksley Ltd, 891481, UK). Hematocrit was read with a Micro Hematocrit Reader (Hawksley Ltd, UK).

Plasma was obtained by whole blood centrifugation at 8900 g at 4°C for 5 minutes. For plasma glucose determinations, plasma was vortexed with trichloroacetic acid (TCA, 10%) (10:1) and put into liquid nitrogen and stored at -80°C.

The remaining 44 rats were anaesthetised with Nembutal (6 mg/100 g intraperitoneally) tracheostomized and mechanically ventilated. Rectal temperature was monitored and body temperature was adjusted using a heating pad. The diaphragm muscle was dissected and weighed. One part was immediately freeze-clamped, frozen in liquid nitrogen and subsequently stored at -80°C until analysis of energy-rich phosphates, lactate and pyruvate.

The remaining part was put into "tissue glue" (Tissue-Tek, Elkhard, IN) on a cork holder with the muscle fibres oriented perpendicularly to the surface of the cork, then quickly frozen in isopentane and cooled by liquid nitrogen. Serial cross sections were cut at 8 mm thickness by use of a cryostat kept at -20°C . Sections were stained with haematoxylin and eosin to study myopathic changes. The sections were analysed for the presence or absence of the following morphological features: increased amount of central and subsarcolemmal nuclei, increased variation in fibre size, increased amount of split fibres, increased amount of vacuoles, increased amount of necrotic fibres, increased amount of abnormal mitochondria, and increased amount of connective tissue. The other section was stained for myofibrillar adenosine triphosphatase after alkaline (pH 9.3) and acid (pH 4.5) preincubation. Fibre analysis was done with a Leitz microscope, linked to a digitising board (Quantimet 570, Leica, UK). Areas in which fibre orientation was not transverse to the long axis and areas with freezing artifacts were not analysed. By the use of a computer program, the individual muscle fibres were selected and the fibre CSA was determined from the number of pixels within the outlined fibre. CSA was corrected for the shortening occurring from the optimal length using a correction factor determined by ourselves in 32 rats, close to that found by Prakash and colleagues (15). At least 250 fibres of each rat were used to calculate the mean CSA of type I, IIa and IIx/b fibres.

Biochemical analysis

Plasma glucose was determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (16).

To determine the dry/wet muscle weight ratio, approximately 200 mg pulverised tissue was freeze dried for 24 hours in a Speedvac (type SC200, Savant Instruments Inc., Farmingdale, NY, USA) connected with a refrigerated condensation trap (type RT 400, Savant Instruments Inc., Farmingdale, NY, USA).

For determination of muscle energy-rich phosphates, lactate and pyruvate, frozen muscle was pulverised using a mortar and pestle precooled in liquid nitrogen and homogenised and deproteinized in ice-cold 1M perchloric acid (1:5) using an OMNI 1000 mechanical homogeniser (OMNI international). The supernatant was neutralised with 2M potassium bicarbonate. ATP, ADP, AMP, creatine phosphate (CrP), creatine (Cr), lactate and pyruvate were measured as described by Harris and colleagues (17), using a centrifugal analyser (Cobas-Bio, Roche Diagnostics).

Statistical analysis

All biochemical measurements were performed in duplicate. Differences between the groups were assessed using the Kruskal Wallis test and the Mann-Whitney U test. Level of significance was determined as $p \leq 0.05$. Statistical analysis was performed using the SPSS/PC+ program (Wessex, Inc, Winnetka, IL, USA). Data are presented as group mean $\pm$ standard error of the mean (SEM).

3.3. Results

Animal food intake, body weight and muscle mass

After the first injection of triamcinolone, daily food intake of the TR rats significantly decreased ($p<0.01$) during four days from 18.4 ± 2 g to 7.3 ± 1 g and then increased slowly to approximately 16 g, but remained below the *ad libitum* food intake of the FF rats which was approximately 20 g ($p<0.05$). From day 6 on, daily food intake of the PW rats was significantly lower than that of the TR rats ($p<0.001$) and varied from 4 to 7 g to obtain a comparable body weight as the TR rats. Initial body weights of the groups were not significantly different, while the final body weights (BW) of the TR (290 ± 6 g) and PW (295 ± 5 g) rats were 21% respectively 20 % lower than of the FF rats (368 ± 8 g, $p<0.001$).

Diaphragm muscle mass of the TR rats (0.49 ± 0.02 g) and PW rats (0.48 ± 0.02 g) was significantly lower than that of the FF rats (0.63 ± 0.02 g, $p<0.001$), but diaphragm/body weight ratio was not different between the groups. Dry/wet ratio of the diaphragm was not significantly different between the groups.

Plasma glucose concentrations

Hematocrit levels were not different between the groups. Plasma glucose levels were significantly increased in the TR rats (19.9 ± 2.5 mM, $p<0.01$) compared with FF (11.8 ± 0.5 mM) and PW (10.7 ± 0.8 mM) rats.

Fig. 1 Cross- sectional area of all fibre types of the diaphragm of the triamcinolone group (TR), the pair-weight group (PW) and the free-fed group (FF). All values are means $\pm$ SEM. Significance * $p<0.05$, *** $p<0.001$.

Diaphragm fibre cross-sectional area and fibre-type proportions

As shown in Figure 1, triamcinolone resulted in a reduction of type IIx/b atrophy of the diaphragm of 43% compared with FF ($p<0.001$) and of 23.5% compared with PW ($p<0.05$). Related to this, fibre IIx/b diameter was reduced in the TR rats with 20% compared with FF ($p<0.001$) and 12% compared with PW ($p<0.01$). In the PW group, type IIx/b atrophy was observed compared with FF (25.1%; $p<0.05$), but fibre diameters were not changed. In the TR rats, the relative contribution of type I CSA (26%) and type IIa CSA (23.6%) were increased compared with FF (type I: 19.8%; $p<0.05$, type IIa: 17.6%; $p<0.05$) and the relative type IIx/b fibre area (50.4%) was decreased compared with FF (62.5%; $p<0.01$) and with PW (57.8%; $p<0.05$). No changes in relative fibre areas were noticed in the PW group compared with FF. Fibre type proportions were not altered in any group.

No evidence for myopathic changes were observed in any of the studied groups, as illustrated by Figure 2. Since, muscle atrophy was present in the diaphragm of the TR and PW rats, the fibre sizes were decreased.

Fig. 2 Haematoxylin and eosin staining of the diaphragm of the free-fed group (A), the pair-weight group (B) and the triamcinolone group (C). In the PW and TR group the fibres are smaller as a result of muscle atrophy.

Energy metabolites in diaphragm

Mutual comparisons between all 3 groups are shown in Table 1. Analysis of the pattern of changes revealed that both TR and PW caused a decrease in ATP in absolute terms as well as in ATP/ADP ratio compared with the FF group concentration ($p<0.01$ and $p<0.05$ resp.). Both groups showed a decreased CrP level of the diaphragm ($p<0.05$) compared with FF. Only the PW group showed a significantly increased intramuscular creatine level in the diaphragm compared with FF ($p<0.05$), whereas TR showed a reduced total creatine pool in the diaphragm ($p<0.05$) compared with FF. No significant differences were observed in energy-rich phosphates between TR and PW. In contrast to PW, TR demonstrated increased pyruvate and lactate concentrations compared with FF ($p<0.05$ resp. $p<0.001$).

Table 1. Concentrations of diaphragmatic energy-rich phosphates.

	Triamcinolone		Pair-weight		Free-fed
ATP ($\mu\text{mol/g ww}$)	4.2 $\pm$ 0.2	#	4.5 $\pm$ 0.2	*	5.0 $\pm$ 0.1
ADP ($\mu\text{mol/g ww}$)	1.03 $\pm$ 0.05		1.02 $\pm$ 0.02		1.01 $\pm$ 0.02
AMP ($\mu\text{mol/g ww}$)	0.074 $\pm$ 0.008		0.072 $\pm$ 0.004	*	0.061 $\pm$ 0.003
TAN ($\mu\text{mol/g ww}$)	5.3 $\pm$ 0.3	*	5.6 $\pm$ 0.3	*	6.1 $\pm$ 0.2
ATP/ADP	4.1 $\pm$ 0.2	#	4.4 $\pm$ 0.2	*	5.0 $\pm$ 0.1
CrP ($\mu\text{mol/g ww}$)	14.1 $\pm$ 1.3	*	16.1 $\pm$ 0.9	*	18.0 $\pm$ 0.6
Cr ($\mu\text{mol/g ww}$)	16.0 $\pm$ 0.8		16.8 $\pm$ 0.5	*	15.0 $\pm$ 0.5
TCr ($\mu\text{mol/g ww}$)	30.1 $\pm$ 1.2	*	32.9 $\pm$ 1.1		33.0 $\pm$ 0.7
CrP/Cr	0.9 $\pm$ 0.10	*	1.0 $\pm$ 0.06	#	1.2 $\pm$ 0.06
Pyruvate	236 $\pm$ 26	†*	162 $\pm$ 20		170 $\pm$ 8
Lactate	6.9 $\pm$ 0.7	†#	4.9 $\pm$ 0.4		4.4 $\pm$ 0.3

Values are means $\pm$ SEM. ww, wet weight; TAN, total adenine nucleotides; TCr, total creatine pool; CrP, creatine phosphate; Cr, creatine; pyruvate in nmol/g wet weight; lactate in $\mu\text{mol/g ww}$; * $p < 0.05$ compared with FF; # $p < 0.01$ compared with FF; † $p < 0.05$ compared with PW.

3.4. Discussion

The objective of this study was to differentiate between alterations in diaphragm muscle energy metabolism and muscle morphology after triamcinolone treatment and after a concomitant degree of body weight loss induced by restricted food intake. In spite of the fact that both treatments resulted in type IIx/b atrophy without other myopathic changes, differences in muscle energy metabolism were seen between the treatment groups.

Although both triamcinolone treatment and chronic undernutrition caused type IIx/b atrophy of the diaphragm, this was more pronounced in triamcinolone rats. Therefore, a relative increase of oxidative fibre area was only observed in the diaphragm of the triamcinolone rats. The same shift in relative cross-sectional area of the diaphragm was observed by Dekhuijzen and coworkers (6) after triamcinolone treatment but they also noticed myopathy, especially an increased amount of connective tissue and excess of nuclei. This could be caused by differences in treatment, since their triamcinolone dose was 2 times the dose of the present study and the duration of treatment was 3 times the duration of the present study. In the present study, the differences in muscle atrophy between the TR and PW groups were less than expected and differed from some other studies, in which a higher degree of undernutrition resulted in atrophy of all fibre types (7, 18, 19).

The type IIx/b atrophy in the present study in the TR and PW groups was accompanied by decreased ATP and CrP levels which is not surprising in view of the fact that fast-twitch muscles contain higher ATP and CrP levels than slow-twitch muscles. These reductions in energy-rich phosphates after triamcinolone treatment, are probably not only caused by alterations in muscle morphology but also by changes in muscle metabolism. In contrast to undernutrition, triamcinolone treatment resulted in reductions of both total adenine nucleotides and total creatine pool, implying metabolic impairment. The increased AMP levels suggests a contributing role of the adenylate kinase reaction to maintain a sufficiently high ATP level. The increased AMP concentration could also stimulate phosphofructokinase (PFK) activity, which was indeed found to be increased after triamcinolone treatment (11). Furthermore, the observed increased concentration of pyruvate in the diaphragm indicates an increased anaerobic glycolysis. The increased pyruvate concentration could indicate an incapability to oxidise pyruvate in the mitochondria. When pyruvate accumulates, glycolysis will be inhibited. To avoid this, lactate production is increased. Another possibility for an increased lactate level is an incapability to produce NAD⁺ in the mitochondria. This will lead to an excess of NADH, which can be reduced by formation of lactate. Thereby, the decreased CrP/Cr ratio implies a disbalance between the energy flux between ATP production and ATP consumption. These observations and those of an earlier triamcinolone

study, in which the ratio between PFK and citrate synthase activity was increased, suggest a mismatch between glycolysis and oxidative phosphorylation.

Chronic undernutrition however caused reductions in ATP and CrP levels because of a decreased energy supply as a result of the diminished food intake. In less severe undernourished rats, only CrP levels were observed to be reduced (20). In the present study total creatine pool was maintained after chronic undernutrition, suggesting the establishment of a new metabolic equilibrium with lower ATP and CrP levels. Most likely the decreased CrP/Cr ratio reflects an increased creatine and ATP production from CrP, in order to satisfy the need for ATP. Furthermore, as suggested by Pichard and colleagues (20), a transport problem from the cytoplasm to the mitochondria could be present.

Despite different metabolic mechanisms after triamcinolone treatment and after undernutrition, both treatments lead to similar alterations in diaphragm morphology and diaphragm energy-rich phosphates, namely, predominantly type IIx/b atrophy and a decrease in ATP and CrP levels. In order to provide adequate therapy to COPD patients with muscle wasting it is important to determine the underlying cause of it. Whereas caloric supply alone could be an adequate therapy for the nutritionally deprived patients to regain muscle mass, this will probably not be effective for patients treated with triamcinolone.

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4

The effects of corticosteroids and undernutrition on work capacity of skeletal muscle in an *in situ* rat model.

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Abstract

The effects of triamcinolone and prednisolone treatment in an equipotent anti-inflammatory dose were compared on *in situ* muscle function in rats, since muscle weakness often occurs with corticosteroid therapy. Furthermore, the effects of corticosteroid-induced muscle wasting were compared with those induced by undernutrition.

Muscle function was performed with a rat dynamometer in the following treatment groups ($n=10$): triamcinolone treatment ($0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 2 weeks), resulting in a (24%) reduction of body mass; nutritional deprivation (2 weeks at 30% of normal food intake) resulting in a similar (24%) decrease of body mass as triamcinolone; prednisolone treatment ($0.31 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 2 weeks), with a 9% increase in body mass; free-fed control group, with a 12% increase in body mass in 2 weeks. The dorsiflexor hindlimb muscles were tetanized supramaximally during two sessions of 60 isokinetic concentric contractions.

The decreased body mass after triamcinolone treatment was not only the result of a diminished food intake but implied an increased energy expenditure. In addition, plasma glucose level was increased after triamcinolone treatment ($p<0.01$), in contrast to the undernourished group. Muscle glycogen was decreased after undernutrition ($p<0.05$) and was increased both after triamcinolone ($p<0.01$) and prednisolone ($p<0.05$) treatment. During the first session, both triamcinolone and undernutrition showed a decline in absolute external work ($p<0.05$). Normalised initial external work (defined as external work per gram exercising muscle) of triamcinolone was higher than prednisolone ($p<0.05$) and fatigue (decline in external work) was less in triamcinolone compared to all other groups ($p<0.001$). Prednisolone and undernutrition did not affect normalised external work. During a 5 minute rest period in between the sessions, the triamcinolone and prednisolone rats fully recovered, in contrast to the undernourished and control rats. In conclusion, the decline in absolute muscle strength is related to muscle wasting independent of the underlying cause. The results show a corticosteroid type specific effect on muscle performance, but recovery of muscle performance was related to corticosteroid use *per se*.

4.1. Introduction

Glucocorticoids are commonly used as anti-inflammatory agents in the treatment of various acute and chronic diseases (1). This treatment is often accompanied by side-effects. The most important adverse effects observed after prolonged treatment with corticosteroids are muscle weakness and wasting (2). In addition, muscle weakness could also be the consequence of nutritional depletion *per se*, which is often shown in chronic diseases (3-5). The functional and clinical relevance of muscle weakness in chronic disease is shown in the fact that it contributes, independently of the severity of underlying disease, to a decreased exercise capacity and health status (6).

Triamcinolone, a fluorinated corticosteroid which induces also muscle wasting, has frequently been studied in relation to *in vitro* muscle contractility. It was shown that prolonged daily administration of 0.5 mg/kg triamcinolone increased fatigue resistance and half-relaxation time of respiratory muscles and caused a leftward shift of the force-frequency relationship (7, 8). A prolonged treatment of 0.2 mg/kg methylprednisolone caused also alterations in *in vitro* contractility of the diaphragm, like a downward shift of the force-frequency relationship and decreased twitch and tetanic tensions (9). Comparisons of the effects of triamcinolone and prednisolone however, are hampered by differences in dose and in administration protocols between studies. Many studies have investigated the effects of a higher dose of triamcinolone,

but the occurrence of premature death (7, 8) is likely to interfere with the results. Furthermore, in most of these studies muscle performance was investigated under *in vitro* conditions. An obvious disadvantage of *in vitro* studies upon administration of a drug is that the potential systemic effects that affect muscle contractile performance are surpassed. *In vitro* studying muscle performance is biased by the fact that muscles are studied in an incubation medium lacking the physiological interaction. Muscles studied during *in vitro* conditions rely on diffusion for substrate metabolism and oxygen supply, both of which will seriously be hampered if muscle size increases (according to Fick's law). *In vitro* conditions are therefore suboptimal compared to *in situ* situations, especially when the metabolic rate of the studied muscle is increased due to muscle contraction. Studying exercising muscles is therefore more appropriate in *in situ* conditions in which interaction with hormones, capillary flow and substrate availability is comparable to the physiological situation.

This study was performed in order to compare the effects of triamcinolone treatment in a non-lethal dose with a similar treatment of prednisolone in an equipotent glucocorticoid dose on *in situ* muscle work capacity by means of a rat dynamometer. This dose of triamcinolone has previously shown to cause muscle wasting, in contrast to prednisolone treatment in an equipotent dose (10). However this dose of prednisolone treatment has demonstrated to induce alterations in muscle protein metabolism, like an increased muscle glutamine *de novo* production (11). Muscle wasting can be a resultant of a diminished energy intake, an increased energy expenditure or both. The cause of weight loss after chronic triamcinolone treatment is primarily related to an increased metabolic rate (8). The second aim of the study was to compare the effects of muscle wasting induced by triamcinolone treatment with those induced by nutritional deprivation on *in situ* muscle performance.

4.2. Methods

Animals

Forty male Wistar rats, 12 weeks old, were treated during two weeks. The initial body mass of these animals ranged from 314 to 345 g. The animals were randomly allotted to four groups: 1) a triamcinolone treated group (TR: 0.25 mg*kg⁻¹*day⁻¹ triamcinolone acetate i.m.) (n = 10) with *ad libitum* food intake; 2) a nutritionally deprived group (ND: 0.05 ml*day⁻¹ saline i.m.), with a caloric food intake equivalent to 30% of normal intake, in order to achieve a similar weight loss as TR (n = 10); 3) a prednisolone treated group (PR: 0.31 mg*kg⁻¹*day⁻¹ prednisolone i.m.) (n = 10) with *ad libitum* food intake; 4) a control group (FF: 0.05 ml*day⁻¹ saline i.m.) (n = 10) with *ad libitum* food intake. All animals were injected in the right hindlimb. The diet provided to all animals was SRM-A 1210 pellets (Hopefarms, Woerden, NL). The animals were housed in metabolic cages, at a temperature of 23°C, with a 12 hour dark light cycle. Food intake and body mass of all animals were registered daily. Veterinary oversight, including regular observation of the health of the animals, was performed according to the Dutch National Guidelines of Animal Care. The experimental protocol was approved by the Ethical Committee of Animal Research of the Maastricht University.

In vivo determination of muscle function of the hindlimb dorsiflexor muscles

Rats were anaesthetised with Nembutal (6 mg*100g⁻¹, intraperitoneally) after short term (<20 s) CO₂ sedation. This CO₂ sedation may prevent excessive rise in plasma catecholamine levels and thus affecting energy metabolism. In order to electrically stimulate the hindlimb dorsiflexor muscles (m. tibialis anterior and m. extensor digitorum longus), an incision of the skin was made just distal to the caput fibulae at the lateral side of the left hindlimb and an electrode was placed on the common peroneal nerve. The positive pole of the electrode was placed percutaneously, distal to the cathode at the ventral side of the tibialis anterior muscle. The experimental set-up (Figure 1) for assessment of external work performed by the exercised muscles consists of 4 main units: a custom built rat dynamometer (RDM), a control

unit to adjust RDM settings (e.g., rotational velocity and range of motion) as desired; an Apple Macintosh 7100 PowerPC® with an 8 channel, 12 bits Lab-NB analog-to-digital conversion board (National Instruments®) programmed with LabVIEW® 3.1, and a pulse generator for purpose of electrical stimulation and triggering of the RDM (HSE 215/IZ, Freiburg, Germany). The RDM consists of a platform with 6 degrees of freedom, an adjustable femur fixation, a rotational footplate, a linear motor and a potentiometer.

Fig. 1. Overview of the experimental set-up. The upper panel shows a rat in the rat dynamometer (RDM). The lower panel shows the knee fixation and the rotational footplate.

The anaesthetised rat is placed in supine position on the heated (37°C) platform. After positioning the animal in the horizontal plane, the foot is secured to the footplate with two-sided adhesive tape while the instep is covered with a narrow fitting thin aluminum plate. The femur is fixed distally with a hinge fixation at a ~90° flexion in the knee. To induce rotation in the ankle joint, the footplate is secured to the axis of the linear motor.

Fixation of the femur to prevent lateral movement of the knee is achieved by two conical pieces placed on the skin between the femoral and tibial condyles. To prevent force transmission through the tibia towards the femur fixation unit, the femur fixation unit was connected to the body of the RDM by a hinge fixation, enabling small movements parallel to the tibia. The ankle rotation axis was aligned with the rotation axis of the RDM by translation of the footplate relative to the rotation axis of the RDM over 2 mm in 2 perpendicular directions. Inappropriate alignment of the ankle axis causes visible movement of the femur fixation unit which can be reduced by translation of the footplate. The footplate is rotated using a linear motor with an adjustable isokinetic velocity between 1°s^{-1} and $1000^{\circ}\text{s}^{-1}$. Axis angle, representing the actual ankle angle, is monitored with a precision potentiometer. Active muscle torque is assessed from the voltage through the linear motor of the RDM. External work of the active muscle group is calculated on-line as the integration of active torque over rotation angle.

The exercise protocol was preceded by determining, for each rat, supramaximal frequency (110-150 Hz at square waves of 0.4 ms) and voltage (0.7-2.0 V) during 4-7 contractions, followed by a 4 min rest. Two sessions of 60 isokinetic tetanic concentric contractions were performed, separated by a 5 min recovery period. This exercise protocol was chosen because of the possibility to determine absolute and normalised external work and to study fatigue of the muscles during 60 contractions. Furthermore, recovery of the muscle performance between successive series of 60 contractions can be observed. This intense exercise protocol was chosen since it heavily relies on type IIx/b fibres, known to be atrophied upon corticosteroid treatment (12-15) and undernutrition (7, 16). Therefore, we considered this protocol to be sensitive to detect differences between the treatment groups of this study. Whether there are differences between the groups, these will surely be observed during supramaximally exercise. The extensor digitorum longus (EDL) and the tibialis anterior (TA) were tetanized supramaximally every 3 s for 350 ms, of which the first 200 ms were isometric followed by 100 ms rotation (from 30° plantar flexion to 20° dorsal flexion at 500°s^{-1} , which is in the plateau of the muscle complex's force-length curve) during which the external work was recorded. With an angle velocity of 500°s^{-1} , muscle fibre shortening velocity approximates 1.4 fibre lengths per s (17). Initial work was defined as the external work during the first contraction of each session. End work was defined as the external work during the final contraction of each session. Fatigue was defined as the decline in external work as percentage of the initial external work of each session. Immediately after the exercise bout, arterial blood was sampled from the aorta and collected in heparinized cups (Lithium-Heparin Micro-sample container CB100, Sarstedt, FRG) on ice. Finally, the dorsiflexor muscles of the hindlimb were excised and muscle mass was determined in order to normalise external work on exercising muscle mass. Absolute external work is defined as the external work of the whole dorsiflexor muscles, while normalised external work is defined as the external work per gram exercising muscle mass. Recovery during the 5 minute rest period was assessed by comparing end external work of the first session with initial external work from the second session.

Biochemical analysis

Immediately after weighing the tibialis anterior muscle, it was freeze clamped in liquid nitrogen and stored at -80°C until analysis. Muscle was homogenized (10% w/v) in SET-buffer by use of an Ultra-Turrax T25 tissue homogenizer (Janke & Kunkel, GmbH & CO KG) for four 5 s intervals. At 0°C , the homogenization process was completed by treatment with ultrasonic sound (Soniprep 150, MSE) for four 15 s intervals. Total protein was assayed from this homogenate by means of bicinchoninic acid as described earlier (18, 19). Part of the muscle was dissolved for 1h in 1M NaOH (37°), precipitating glycogen with 96% ethanol (10 min at 80°C followed by overnight precipitation at 4°C). Subsequently the pellet was hydrolyzed using 1M HCL at 100°C for 3h after which HCL was neutralized with a KCl saturated KOH/Tris (2.1 M/ 0.12 M) buffer. Glycogen derived glycosyl units were determined using a glucose kit (hexokinase method, Roche) for a centrifugal analyzer. Glycogen was analyzed on a centrifugal analyzer (Cobas Fara, Hoffman La-Roche, Basle, Switzerland) and expressed as μmol glycosyl units per g wet weight.

Plasma was obtained by whole blood centrifugation at 8900 g at 4°C for 5 minutes. For plasma glucose determinations, plasma was vortexed with trichloroacetic acid (TCA, 10%) (10:1) and put into liquid nitrogen and stored at -80°C . Plasma glucose was determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (20).

Statistical analysis

Differences between all groups were first detected with the Kruskal Wallis test and then assessed using the Mann-Whitney U test. Level of significance was determined as $p \leq 0.05$. Correlation was assessed using the Pearson correlation test. Analyses were performed using the SPSS/PC+ program. Data are presented as group mean $\pm$ standard error of the mean (SEM).

4.3. Results

Animal food intake, body and muscle mass

Figure 2A depicts the daily food intake of the different groups. Food intake of the FF and the PR rats remained stable during the experiment and was not mutually different.

Fig. 2 (A) Mean daily food intake $\pm$ SEM of the triamcinolone ($\bullet$), nutritionally deprived (+), prednisolone (Δ) and free-fed ($\circ$) groups during experimental period. (B) Mean body mass $\pm$ SEM of the groups as a result of triamcinolone ($\bullet$) or prednisolone (Δ) treatment or nutritional depletion (+) or ad libitum food intake ($\circ$).

Food intake of the TR rats declined during two days (at day 3: $p < 0.001$) and increased gradually until it was not significantly different from FF at day 12. Daily food intake of the ND rats remained constantly at approximately 7 g per day ($p < 0.001$), in order to assess as comparable body mass reduction as the TR group.

As shown in Figure 2B, body mass of the FF and PR groups increased, while body mass of the TR and ND groups decreased. During the muscle test, the body mass of the TR (66% of FF) and ND (69% of FF) rats were significantly lower than the body mass of the FF and PR rats ($p < 0.001$) and were mutually not significantly different. Body mass of the PR rats was slightly but significantly lower than that of the FF rats (97% of FF, $p < 0.05$).

Table 1. Muscle weights

	Triamcinolone		Nutritional deprived		Prednisolone		Free-fed
TA + EDL (mg)	562 $\pm$ 13	†§#	701 $\pm$ 11	§#	931 $\pm$ 18	*	895 $\pm$ 13
% muscle mass	0.23 $\pm$ 0.003	†§	0.28 $\pm$ 0.003	‡#	0.26 $\pm$ 0.006	*	0.24 $\pm$ 0.003

Values are means $\pm$ SEM. TA, tibialis anterior; EDL, extensor digitorum longus; % muscle mass, muscle mass/body mass $\times$ 100; †, $p < 0.001$ compared to ND; ‡, §, $p < 0.05$ and $p < 0.001$ compared to PR;

Muscle mass of the EDL and TA was significantly decreased in the TR and ND groups compared to the FF and PR groups (Table 1, $p<0.001$). It was significantly lower in the TR group compared to the ND group ($p<0.001$). Muscle mass as a percentage of body mass was significantly decreased in the TR group compared to the PR and ND groups ($p<0.001$), increased in the PR group compared to the FF group ($p<0.05$) and even further elevated in the ND group compared to the FF and PR groups ($p<0.001$ and $p<0.05$ resp.).

Fig. 3 Mean glycogen content (SEM) in the tibialis anterior muscle of the triamcinolone (TR), prednisolone (PR), nutritional deprived (ND) and free-fed (FF) group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Muscle protein and glycogen content

Protein contents per gram muscle wet weight were not significantly different between the groups for the dorsiflexor muscles. Glycogen levels in TR and PR were higher than in FF and ND (Figure 3, $p<0.05$), while glycogen content in TR was significantly higher than in PR ($p<0.05$). Glycogen content in the ND group was significantly lower than in all other groups ($p<0.05$). Plasma glucose level was significantly increased in the TR group (33 ± 4 mM) compared to FF (11 ± 1 mM), PF (11 ± 1 mM) and PR (12 ± 3 mM) ($p<0.01$).

Fig. 4 Correlation between active muscle mass (EDL+TA) and its absolute initial external work during the first session for triamcinolone (●), nutritionally deprived (+), prednisolone (Δ) and free-fed (○) groups.

External muscle work of tibialis anterior and extensor digitorum longus

Muscle mass (EDL+TA) and absolute initial external work during the first session showed a correlation of 0.73, with $p<0.001$ (Fig.4). Figure 5A depicts the pattern of normalised external work ($\text{mJ}\cdot\text{g}^{-1}$ muscle) for all animal groups during the first session of stimulation. In the FF rats, external work gradually declined during the first exercise session. The patterns of normalised external work of the prednisolone and nutritionally deprived rats resembled that of the FF rats, while that of the triamcinolone rats declined much less.

During the second session, external work of the FF, ND and PR rats declined but reached a plateau after approximately 20 contractions (Fig. 5B). Normalised initial and end external work of the TR rats were higher than the other groups. In Table 2, external work of dorsiflexors is quantified at different moments of the exercise bout. TR rats showed significantly higher external work compared to PR rats when normalised on the mass of the exercising skeletal muscles ($p<0.05$). The TR rats showed significantly higher normalised initial external work compared to the FF, ND ($p<0.001$) and PR rats ($p<0.01$) during the second session. Normalised end external work of the TR rats was significantly higher than the other groups (Table 2, $p<0.001$) in each session. Fatigability, defined as the decline in external work as percentage of the initial external work, was significantly smaller in the TR group compared to all other groups (Table 2, $p<0.001$).

Fig.5 (A) Normalised external work during the first session of the triamcinolone (●), nutritionally deprived (+), prednisolone (Δ) and free-fed (○) groups ± SEM. (B) Normalised external work during the second session of the triamcinolone (●), nutritionally deprived (+), prednisolone (Δ) and free-fed (○) groups ± SEM.

Information of the recovery of the dorsiflexor muscles was obtained by comparing initial external work of the second session with end external work of the first session. This showed a full recovery of the TR and PR rats, whereas the recoveries of the ND and FF rats were not adequate.

Table 2. External work of dorsiflexors

	Triamcinolone	Nutritional deprived	Prednisolone	Free-fed
nor init ext work 1	29.5 ± 0.9 †	27.7 ± 1.1	25.1 ± 1.6	25.9 ± 1.3
nor init ext work 2	29.2 ± 0.6 • ‡ §	22.7 ± 1.3	21.2 ± 1.7	19.6 ± 1.9
nor end ext work 1	18.5 ± 0.5 • ¶ §	9.4 ± 0.6 †	6.6 ± 0.8	8.0 ± 1.3
nor end ext work 2	15.1 ± 0.4 • ¶ §	6.9 ± 0.6	4.8 ± 0.7	4.9 ± 1.2
fatigue 1	37 ± 2 • ¶ §	66 ± 2 †	74 ± 2	70 ± 4
fatigue 2	48 ± 1 • ¶ §	69 ± 3 ‡	78 ± 2	77 ± 5

All values are means ± SEM. nor init ext work, normalised initial external work (in mJ/gram exercising muscle); fatigue, decline in external work as percentage of initial external work; 1, during first session of stimuli; 2, during second session of stimuli; •, $p < 0.001$ compared to ND; †, ‡, ¶, $p < 0.05$, $p < 0.01$ and $p < 0.001$ compared to PR; §, $p < 0.001$ compared to FF.

4.4. Discussion

The results of this study clearly demonstrate a corticosteroid type specific effect on *in situ* muscle work performance. Although both triamcinolone and prednisolone treatment resulted in an increased muscle glycogen store, muscle performance was not comparable. In contrast to prednisolone in an equipotent dose, triamcinolone treatment showed an increase in external work when normalised on skeletal muscle mass. Moreover, dorsiflexor muscles of triamcinolone treated animals were less prone to fatigue than prednisolone treated rats. The observed hyperglycaemia after triamcinolone treatment could play a role in this. Prednisolone treatment showed no changes compared to control. Contrary to *in vitro* contractility, where triamcinolone treatment and chronic undernutrition showed similar alterations (8), in this *in situ* set-up, changes in external work of the TR rats were also significantly different from ND and could therefore not be exclusively attributed to the muscle wasting *per se*.

Balance between energy intake and body weight change showed a clear discrepancy after triamcinolone treatment, implying that weight loss is a consequence of increased energy expenditure not adequately restored by dietary intake. This phenomenon was not observed after prednisolone treatment, and was triamcinolone-specific. No quantitative analysis of total body composition was performed. However, muscle mass was significantly decreased in comparison to body mass reduction after triamcinolone treatment, implying relatively more muscle degradation. Undernutrition however, showed a relatively sparing of muscle mass.

In vitro contractility studies demonstrated that triamcinolone treatment in a higher dose and nutritional deprivation with a comparable body mass reduction, resulted in similar changes in respiratory muscle function, such as a reduced fatigability and an increased half-relaxation time (7, 8). In the present *in situ* study, triamcinolone treatment and nutritional deprivation both resulted in a decrease of absolute initial external work of the dorsiflexor hindlimb muscles. However, when external work was corrected for the exercising muscle mass, triamcinolone treated rats showed an increased external work, whereas nutritional deprivation showed no alterations compared to control rats. How can this discrepancy between these two different contractility studies be explained? The decrease in *in vitro* fatigability after triamcinolone treatment as well as after nutritional deprivation is suggested to be related to a relative increase in cross-sectional area of oxidative muscle fibres (7, 8). In earlier studies, triamcinolone treatment has demonstrated to induce no differences in fibre type distributions, but predominantly type IIx/b fibre atrophy of both the diaphragm (7, 8, 21) and the gastrocnemius muscle (7). Corticosteroid treatment has shown in the EDL to cause both type IIa and IIb atrophy (12). Both the EDL and anterior digitorum longus muscles are stimulated in the rat dynamometer in the present study. The dorsiflexor muscles consist mainly of type IIa and IIb fibres. It is to be expected that triamcinolone treatment will cause type IIx/b atrophy of the dorsiflexor muscles. Prolonged treatment of low-dose methylprednisolone has demonstrated to cause a decrease in distribution of type IIb fibres and atrophy of both type I, IIa and IIx fibres,

resulting in a relatively increase of type IIx fibres and a decrease of type IIb fibres of the diaphragm muscle. In the present study, no structural changes are to be expected after prednisolone treatment, at least not resulting in any changes in muscle performance. Undernutrition has demonstrated to cause atrophy of all fibre types, but proportionally of mostly type IIx/b fibres (7, 8, 22). In contrast to corticosteroid treatment, Kelsen and colleagues found that undernutrition lead to alterations in muscle fibre composition of the diaphragm muscle, resulting in relatively more oxidative fibres (22). We assume that this will not be found in the present study, since nutritionally deprivation did not show an improved fatigability. The results of the present study however indicate that in *in situ* conditions not only the relative fibre contribution determines muscle fatigue but other factors, like intermediary metabolism also may contribute. Most likely the observed hyperglycaemia, which was also demonstrated by others after fluorinated corticosteroids (23-25), will play a significant role. We would like to hypothesise that the combination of exercise-induced increase in skeletal muscle glucose transport capacity (26) and hyperglycaemia in TR rats, most likely results in an elevated supply of glucose during contraction compared to rats with normal plasma glucose levels, and subsequently in a decreased fatigability of the muscles. Obviously, the increased muscle glycogen concentration does not contribute to an increased muscle performance, since the muscle performance in the prednisolone rats was not altered.

Opposite to muscle wasting observed after triamcinolone treatment, prednisolone treatment did not lead to alterations in body and muscle mass compared to FF, and even in a small but significant increase in muscle mass relative to body weight. This implies even a relative conservation of muscle mass after prednisolone treatment in contrast to the muscle wasting observed after an equipotent triamcinolone dose. In line with the absence of a catabolic response, prednisolone treatment in the present study was not associated with alterations in *in situ* muscle function when compared to free-fed animals. The full recovery of both the triamcinolone and prednisolone rats may imply that in corticosteroid-treated rats energy stores are not fully exhausted after the first session or that energy stores are better restored. Muscle glycogen concentrations were indeed found to be increased after either corticosteroid treatment.

Corticosteroids are commonly used in low doses as maintenance therapy or in high doses during disease exacerbations. In clinical practice, triamcinolone is not used extensively, in contrast to other fluorinated corticosteroids. The rationale for studying this corticosteroid is the clear body wasting, not only caused by a decreased food intake (16). The dose of triamcinolone in the present study is within the range of those given to patients with chronic diseases (27, 28). Prednisolone however, is a frequently used corticosteroid in clinical practice. In the present study, the dose of prednisolone was within the range of those given to patients (29), but somewhat higher than maintenance therapy. The results of this study point out that triamcinolone, in contrast to prednisolone treatment in a comparable anti-inflammatory potency, causes muscle wasting. Despite the muscle wasting effect of triamcinolone, the functional properties of the remaining muscle tissue are superior to that in the other groups: less fatigable and almost complete recovery within 5 minutes after a strenuous exercise bout. The differences in systemic effects between different glucocorticoid treatment may be important to consider in the choice of corticosteroids type as medical therapy.

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5

Enzyme activity of rat tibialis anterior muscle differs between triamcinolone and prednisolone treatment and nutritional deprivation.

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European Journal of Applied Physiology 79: 274-279 (1999)

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Abstract

The maximal activity of a selection of enzymes involved in muscle carbohydrate handling, citric acid cycle and fatty acyl β -oxidation respectively were studied after treatment with the fluorinated corticosteroid triamcinolone and compared to a similar treatment of the non-fluorinated corticosteroid prednisolone in an equipotent anti-inflammatory dose. Furthermore, because triamcinolone causes loss of mass and muscle wasting, the effects of triamcinolone were investigated relative to a control group, with the same loss of mass, due to nutritional deprivation. The study was performed in male Wistar rats in the following treatment groups; TR, triamcinolone treatment (0.25 mg/kg/day for 2 weeks), which resulted in a reduction of body mass (24%); ND, nutritional deprivation (30% of normal daily food intake for 2 weeks) resulting in a similar (24%) decrease of body mass as TR; PR, prednisolone treatment (0.31 mg/kg/day for 2 weeks), with a 10% increase in body mass; FF, free-fed control group, with a 12% increase in body mass in 2 weeks. Compared to FF, TR induced an increase in phosphofructokinase (PFK) activity ($p < 0.01$), glycogen synthase [GS(i+d)] activity ($p < 0.05$) and glycogen content ($p < 0.01$) in the tibialis anterior muscle. The PR and ND caused no alterations in PFK or citrate synthase (CS) activity compared to FF. Compared to PR, TR induced an increase in PFK ($p < 0.01$), CS ($p < 0.05$) and GS(i+d) activity ($p < 0.01$). Both TR and PR caused an increased muscle glycogen concentration, being more pronounced in TR ($p < 0.05$). Compared to ND, TR induced an increased CS ($p < 0.05$) and GS(i+d) activity ($p < 0.01$) and glycogen content ($p < 0.01$). The ND resulted in a decreased glycogen content compared to FF ($p < 0.05$). None of the treatments affected the activity of glycogen phosphorylase, β -hydroxyacyl CoA dehydrogenase and lactate dehydrogenase. It was concluded that corticosteroids led to an increased muscle glycogen content. However, the changes in the enzymes of carbohydrate metabolism were corticosteroid type specific and did not relate to undernutrition, which accompanied the triamcinolone treatment.

5.1. Introduction

Glucocorticoids have been commonly used as anti-inflammatory agents in the treatment of chronic obstructive pulmonary disease (COPD)(1). It has been found that patients with COPD often suffer from respiratory and peripheral skeletal muscle weakness (2-4). The cause of this muscle weakness in COPD is not yet clear, but among others, muscle wasting related to nutritional depletion (5) and corticosteroids (6) have been suggested as contributing factors. Corticosteroids can be fluorinated to increase their anti-inflammatory potency. However, it has been shown that this results in more severe systemic effects than non-fluorinated corticosteroids (7).

It has been demonstrated in studies on experimental animal, that the fluorinated corticosteroid triamcinolone causes muscle wasting and predominantly type IIx/b atrophy (8-10). In addition to histochemical changes, triamcinolone exerts many alterations in muscle energy metabolism, which can also be associated with alterations in muscle function. It has been noticed that triamcinolone caused an increase in glycogen concentration in muscle but the exact mechanism of action of triamcinolone on muscle carbohydrate handling is still a matter of debate. Evidence for increased glycogenesis after triamcinolone treatment has been found, associated with increased lactate concentrations (11). In addition, glutamine release of the rat hindquarter has been observed to be increased (12, 13), which implies protein catabolism.

Furthermore, triamcinolone has even been shown to cause *in vitro* uncoupling of oxidative phosphorylation and respiration (14), which will probably be reflected in changed muscle enzyme capacities.

The question that remains to be addressed is whether changes in muscle metabolism after triamcinolone treatment are induced by 1) muscle wasting, related to loss of mass, or induced by 2) corticosteroid treatment *per se*, as studied in a prednisolone group. We hypothesized that triamcinolone-induced muscle atrophy and increased glycogen content is associated with alterations in the activity of muscle enzymes, involved in carbohydrate and β -oxidation. In an earlier study we have demonstrated that a similar administration of the non-fluorinated corticosteroid prednisolone in an equipotent glucocorticoid dose (15) did not affect muscle mass (16). Since triamcinolone has been shown to cause type IIx/b atrophy, a muscle with predominantly type II fibres was studied: the tibialis anterior muscle.

5.2. Methods

Animals

Forty male Wistar rats were treated during two weeks. The initial body mass (BM) of these animals, aged 12 weeks, ranged from 314 to 345 g. The animals were randomly allotted to four groups, a triamcinolone treated group (TR: 0.25 mg/kg/day triamcinolone acetate i.m., which had an *ad libitum* food intake, $n = 10$) and 3 reference groups. Because in previous studies, the amount of muscle wasting after triamcinolone treatment could not be explained by the diminished food intake (17) but also by an increased energy expenditure (9), we did not introduce a pair-fed group, but introduced a nutritionally deprived group (ND: 0.05 ml*day⁻¹ saline i.m.), receiving a food intake of 30% of normal, resulting in a calculated comparable body mass reduction ($n = 10$). A prednisolone treated group was included which received an equipotent anti-inflammatory dose to triamcinolone (PR: 0.31 mg*kg⁻¹*day⁻¹ prednisolone i.m.) ($n = 10$), with an *ad libitum* food intake. Thirdly, a free fed group (FF: 0.05 ml*day⁻¹ saline i.m.) was included which had an *ad libitum* food intake ($n = 10$). The chosen corticosteroids dose is comparable to what is used in patients with COPD as maintenance therapy or during acute exacerbations. The diet provided to all animals was SRM-A 1210 pellets (Hopefarms, Woerden, NL). Water was available *ad libitum* to all animals. The animals were housed in metabolic cages at a temperature of 23°C with a 12 hour dark light cycle. Food intake and body mass of all animals were registered daily. Veterinary oversight, including regular observation of the health of the animals was performed according to the Dutch National Guidelines of Animal Care. The experimental protocol was approved by the Ethical Committee of Animal Research of the Maastricht University.

Muscle preparation and enzyme determination

Rats were anaesthetized with Nembutal (6 mg/100g body mass, intraperitoneally) after short term (<20 s) CO₂ sedation. Tibialis anterior muscle was dissected, immediately weighed and freeze clamped in liquid nitrogen and stored at -80°C until analysis. Muscle was homogenized (10% w/v) in SET-buffer by use of an Ultra-Turrax T25 tissue homogenizer (Janke & Kunkel, GmbH & CO KG) for four 5 s intervals. At 0°C, the homogenization process was completed by treatment with ultrasonic sound (Soniprep 150, MSE) for four 15 s intervals. Total protein was assayed from this homogenate by means of bicinchoninic acid as described earlier (41,42). For enzyme determination, the homogenate was centrifuged (4°C) for 10 min at 2100 x g (3500rpm) and supernatant was assayed for lactate dehydrogenase (LDH; EC 1.1.1.27) (18), phosphofructokinase (PFK; EC 2.7.1.11) (19), citrate synthase (CS; EC 4.1.3.7) (20) and 3-hydroxyacyl CoA dehydrogenase (HAD; EC 1.1.1.35) (21) activities by use of the Cobas Bio centrifugal analyzer (La Roche, Basle, Switzerland). Glycogen synthase (GS) activity was assessed on a centrifugal analyzer (Cobas Fara, Hoffman La-Roche, Basle, Switzerland) in the muscle homogenate at 30°C. To

discriminate between the active GS(i) part of GS and total GS activity GS(i+d), GSi activity was assayed in absence of glucose-6-phosphate (G6P) while G6P (10mM) was added to the homogenate to assay total GS activity.

UDP-glucose was used as starting reagents and formation of UDP was measured according to Danforth (22). Glycogen phosphorylase (GP) activity was assayed according to Schreiber (23) on a centrifugal analyzer and expressed as $\mu\text{mol}/\text{min}/\text{g ww}$. Both the total GP(a+b) and active form GP(a) of glycogen phosphorylase were assessed. Part of the muscle was dissolved for 1h in 1M NaOH (37°), precipitating glycogen with 96% ethanol (10 min at 80°C followed by overnight precipitation at 4°C). Subsequently the pellet was hydrolyzed using 1M HCL at 100°C for 3h after which HCL was neutralized with a KCl saturated KOH/Tris (2.1 M/ 0.12 M) buffer. Glycogen derived glycosyl units were determined using a glucose kit (hexokinase method, Roche) for a centrifugal analyzer. Glycogen was analyzed on a centrifugal analyzer (Cobas Fara) and expressed as μmol glycosyl units per g wet weight.

Statistical analysis

Differences between all groups were first detected with the Kruskal Wallis test and then assessed using the Mann-Whitney U test, because of the non-normal distribution. Level of significance was determined as $p<0.05$. Analysis were performed using the SPSS/PC+ program. Data are presented as group mean $\pm$ standard error of the mean (SEM).

5.3. Results

Animal food intake, body and muscle mass

Food intake of all rats was comparable before the experimental period (± 21 g/day). During treatment, food intake of the FF and PR rats remained stable and was approximately 100 kJ/100 g body mass.

Fig. 1.(A) Mean body mass of the groups as a result of triamcinolone (●) or prednisolone (Δ) treatment or nutritional depletion (+) or ad libitum food intake (○). (B) Mean energy intake per 100 g body mass $\pm$ SEM of the triamcinolone (●), nutritionally deprived (+), prednisolone (Δ) and free-fed (○) groups during experimental period. Significant difference between TR and FF: *, $p<0.05$; **, $p<0.01$.

Food intake of the TR rats decreased after the first injection to 9.7 ± 0.5 g at day 3 and then slowly increased until it was not significantly different from that of the FF rats at day 12 (TR: 19.8 ± 1.0 g, FF: 21.5 ± 0.5 g). Food intake of the ND rats remained stable at approximately 7 g per day. As shown in Figure 1A, body mass of the FF and PR group increased, while body mass of the TR and ND groups decreased. After two weeks treatment body mass of the TR and ND rats were significantly lower than those of the FF and PR rats (Table 1, $p<0.001$). Body mass of the TR rats was slightly lower than of the ND rats ($p<0.05$). Figure 1B depicts the energy intake per 100 g body mass. There was no significant change in energy intake per body mass during treatment period for the FF, PR and ND rats. Energy intake per body mass of the TR rats was decreased during the first days and was even increased compared to all other groups after 9 days on ($p<0.05$).

Table 1. Body and muscle weights

	Triamcinolone		Prednisolone		Nutritional deprived		Free-fed
Body mass (g)	244	± 2	357	± 4	254	± 3	365 ± 2
Tibialis anterior (mg)	438	± 12	748	± 18	561	± 11	708 ± 12
% Muscle mass	1.8	± 0.03	2.1	± 0.06	2.2	± 0.03	1.9 ± 0.03

Values are mean $\pm$ SEM. % muscle mass, muscle mass of tibialis anterior/body mass $\times 100$; ¶, $p<0.001$ compared to PR; ◊, •, $p<0.05$, $p<0.001$ compared to ND; *, §, $p<0.05$, $p<0.001$ compared to FF.

Muscle mass of the tibialis anterior was significantly decreased in the TR and ND group compared to FF and PR (Table 1, $p<0.001$). Muscle mass was significantly lower in the TR group compared to the ND group ($p<0.001$). Muscle mass percentage of body mass was significantly decreased in the TR group compared to PR and ND ($p<0.001$), increased in the PR group compared to FF ($p<0.05$) and even further elevated in the ND group compared to FF ($p<0.001$).

Fig. 2. Mean glycogen content $\pm$ SEM in the tibialis anterior of the triamcinolone (TR), prednisolone (PR), nutritional deprived (ND) and control (FF) group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Muscle glycogen stores and enzyme activities in tibialis anterior

Protein contents per gram muscle wet weight were not significantly different between the groups. Glycogen levels in TR and PR were higher than in FF and ND (Figure 2, $p<0.05$), while glycogen content in TR was significantly higher than in PR ($p<0.05$). Glycogen content in the ND group was significantly lower than in all other groups ($p<0.05$). Total glycogen synthase activity [GS(i+d)] was significantly increased in the TR group compared to all other groups (Table 2, $p<0.05$). Maximal activity of glycogen synthase (d) was calculated and significantly increased in TR compared to PR and ND ($p<0.001$) and FF ($p<0.01$). Glycogen phosphorylase

activity was not different between the groups. Phosphofructokinase (PFK) activity was significantly increased in the tibialis anterior of the TR rats compared to the FF and PR rats (Table 2, $p < 0.01$). Lactate dehydrogenase (LDH) and 3-hydroxyacyl CoA dehydrogenase (HAD) activities were not significantly different between the groups (Table 2). Activity of citrate synthase (CS) was significantly increased in the TR rats compared to the PR and ND rats (Table 2, $p < 0.05$). The ratio of CS over PFK activity was significantly decreased in the TR group (0.6 ± 0.1) compared to FF (3.3 ± 0.9) ($p < 0.01$) and PR (1.6 ± 0.5) ($p < 0.05$).

Table 2. Muscle enzyme activities in tibialis anterior

	Triamcinolone	Prednisolone	Nutritional deprived	Free-fed
GP(a+b)	263 $\pm$ 12	269 $\pm$ 12	240 $\pm$ 14	258 $\pm$ 16
GP(a)	94 $\pm$ 8	96 $\pm$ 7	78 $\pm$ 8	83 $\pm$ 6
GS(i+d)	38 $\pm$ 3 † 0 *	30 $\pm$ 1 °	25 $\pm$ 2	28 $\pm$ 3
GS(i)	17 $\pm$ 1	17 $\pm$ 1	15 $\pm$ 1	16 $\pm$ 1
PFK	507 $\pm$ 127 † #	155 $\pm$ 40	217 $\pm$ 44	117 $\pm$ 40
LDH	10.0 $\pm$ 0.8	12.5 $\pm$ 1.5	10.2 $\pm$ 1.0	10.3 $\pm$ 0.8
HAD	118 $\pm$ 15	109 $\pm$ 15	87 $\pm$ 8	89 $\pm$ 12
CS	225 $\pm$ 29 † °	167 $\pm$ 31	168 $\pm$ 14	194 $\pm$ 29

All values are means $\pm$ SEM; GP, glycogen phosphorylase in U/mg protein, (a) is the active form and (a+b) the total glycogen phosphorylase; GS, glycogen synthase in U/mg protein, (i) is the active form and (i+d) the total glycogen synthase; PFK, phosphofructokinase in U/g protein; LDH, lactate dehydrogenase in U/mg protein; HAD, 3-hydroxyacyl CoA dehydrogenase in U/g protein; CS, citrate synthase in U/g protein; †, ‡, ¶, $p < 0.05$, $p < 0.01$, $p < 0.001$ compared to PR; °, 0, • $p < 0.05$, $p < 0.01$, $p < 0.001$ compared to ND; *, #, $p < 0.05$, $p < 0.01$ compared to FF.

5.4. Discussion

This study demonstrated that the triamcinolone-induced effects on muscle enzyme capacity were not related to the reduction in muscle mass *per se*, or corticosteroid treatment *per se*, but to underlying metabolic alterations different from nutritional deprivation or prednisolone treatment.

Triamcinolone treatment caused a relative increase in energy intake after 9 days, considering the food intake per 100 g body mass. However muscle mass reduction was even more pronounced in TR rats than in ND rats. This would imply an increased energy expenditure and an increased net protein degradation in the TR rats. The ND caused a reduction in body mass, but the reduction in muscle mass was relatively small, which could be expected in view of the metabolic adaptations to starvation, that is increase fat oxidation instead of net protein degradation. In contrast to triamcinolone, prednisolone caused no alterations in energy intake or muscle mass. Tibialis anterior muscle mass relative to body mass was even increased similar to nutritional deprivation. These findings were in line with earlier studies, in which it has been shown that in contrast to oral treatment or subcutaneous administration of prednisolone (24-26), intramuscular treatment of prednisolone has appeared to cause no catabolism (27, 28).

The observed increase in muscle glycogen content would seem to be induced by corticosteroids *per se*, since enhanced glycogen levels were observed in the tibialis anterior muscle of both TR and PR treated animals. However, the increase was significantly higher after treatment with the fluorinated corticosteroid triamcinolone than after prednisolone administration. The substantially increased glycogen content after triamcinolone treatment was accompanied by an increased glycogen synthase (i+d) activity, which was caused by an increase of the inactive form GS(d). When this enzyme is dephosphorylated, it becomes active (GS(i)). After triamcinolone treatment, the supply of phosphorylated GS is increased, which may be surplus. The observation of increased GS activity corroborates earlier findings of Peters and colleagues (29), who have not distinguished between the active and inactive forms.

In contrast, the increased glycogen content after prednisolone treatment was not accompanied by alterations in the maximal activity of GP or GS. These findings would suggest that glycogen synthesis is regulated by factors other than the content (i.e. maximal activity) of GS or GP, which supports the allosteric activity of GS by glucose. Nutritional deprivation resulted in an expected decrease of glycogen content in the tibialis anterior muscle (30-33), without alterations in the maximal activity of glycogen handling enzymes.

Both PFK and CS enzyme capacities were increased in tibialis anterior muscle after triamcinolone treatment, implying an increase in both glycolytic and oxidative enzyme capacity. This could also have been an indirect result of muscle wasting with a marked loss of contractile proteins and a relative preservation of metabolic proteins, but in that case, all muscle enzyme capacities would have had to be increased, which was not observed in this study. The fact that the increase of PFK was more pronounced than that of CS would indicate that the glycolytic pathway was more sensitive to triamcinolone treatment than the citric acid cycle. In the present study, oxidative phosphorylation was not studied, but earlier studies have shown that oxidative phosphorylation and respiration in isolated mitochondria obtained from muscle tissue were less tightly coupled after triamcinolone treatment (14, 34). The consequence of relative uncoupling is most likely upregulation of enzyme activities involved in the rate determining steps of glycolysis and citric acid cycle activity as observed in the present study, in order to obtain sufficient ATP.

In contrast to the increase of PFK activity after triamcinolone treatment, we observed after prednisolone treatment no alterations in PFK activity in the muscle, while Lieu and coworkers have even found a decrease of PFK activity (26). The results of these studies are difficult to compare because of the differences in treatment and types of muscles under investigation. Lieu and colleagues (26) have injected higher doses of prednisolone than in the present study and injected it subcutaneously, which induced catabolism. Nutritional deprivation in the present study caused no alteration in PFK, while Russell and colleagues (33) fed rats 25% of their normal diet over 3 weeks after which they observed a decrease in PFK capacity in the gastrocnemius muscle. Despite a decreased glycogen level and wasting of the tibialis anterior after nutritional deprivation, no alterations in the maximal activity of enzymes involved in carbohydrate and β -oxidation were observed in the present study.

It has been suggested that several factors may contribute to muscle wasting and muscle weakness in patients with COPD, including nutritional depletion (5), corticosteroid medication (6), acute respiratory failure (35) and inactivity (36). In this study we found that corticosteroids led to increased muscle glycogen contents and may have induced a type specific upregulation of enzymes of carbohydrate metabolism. Undernutrition was associated with glycogen depletion but not to major changes in these enzymes. Both increased muscle glycogen levels (37) and decreased glycogen contents (38) have previously been reported in patients with COPD. Altered PFK and CS activities have also been demonstrated in muscles of COPD patients (36, 39), but the pattern of changes has not been consistent among the studies. Furthermore, a very recent study showed in skeletal muscle of COPD patients an upregulation of the activity and gene expression of cytochrome oxidase (40). Further studies are needed to determine whether biochemical analysis of peripheral muscle biopsies could be useful in differentiating between the specific contributions of nutritional depletion and corticosteroids in patients with COPD, aimed at targeted intervention strategies.

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6

Effects of corticosteroids and undernutrition on glutamine metabolism in rat skeletal muscle.

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Abstract

Corticosteroid treatment affects muscle glutamine metabolism. We have investigated to what extent undernutrition contributes to these alterations and whether acute and chronic corticosteroid treatment exert comparable metabolic alterations. The study was performed in Wistar rats in the following treatment groups: TR, triamcinolone treatment (0.25 mg/kg/day i.m.) for 3 days (n=8) or for 14 days (n=10); PF, pair fed (similar food intake as TR) for 3 days (n=8) or for 14 days (n=10); PW, pair weight (comparable body weight reduction as TR) for 14 days (n=10); FF, free fed for 3 days (n=8) or 14 days (n=10).

In contrast to undernutrition, acute corticosteroid treatment increased intramuscular glutamine concentration and glutamine shunting and decreased intracellular disposal and production of glutamine in the muscle. Chronic corticosteroid treatment however, was accompanied by an increased muscle glutamine efflux, an increased glutamine *de novo* production and increased glutamine membrane transport rates, which were also observed (although less pronounced) after chronic undernutrition. Glutamate is likely to be the precursor for glutamine production after chronic triamcinolone treatment, but after chronic undernutrition influx of possible precursors for glutamine production, like glutamate or branched chain amino acids was not altered.

In conclusion, chronic weight loss either induced by triamcinolone or by undernutrition is associated with similar alterations in muscle glutamine metabolism.

6.1. Introduction

Corticosteroids are commonly used as anti-inflammatory agents in the treatment of various diseases including chronic obstructive pulmonary disease (COPD) (1). Muscle weakness and wasting are frequently reported after a prolonged treatment with corticosteroids (2, 3). It is not clear to what extent this is related to the metabolic effects of corticosteroids or to the associated undernutrition. Muscle wasting is the result of a disturbed protein balance. Protein metabolism was indeed found to be disturbed in COPD patients (4, 5). Of the metabolic alterations, particularly glutamine metabolism was found to be changed. Alterations in glutamine efflux (4) and intramuscular glutamine concentrations were found (5, 6). The results are however inconsistent, which could be related to differences in patient characteristics, muscles studied and corticosteroid use.

Glutamine is an important nitrogen and ammonia carrier in interorgan metabolism (7). It is a non-essential amino acid and can be synthesised from glutamate and ammonia with the means of the enzyme glutamine synthase (GS).

During metabolic stress, glutamine is considered to be a conditionally essential amino acid, as metabolic fuel for the gut and other rapidly dividing cells e.g. the immune system. Intramuscular concentrations were observed to be decreased after chronic starvation (8) or corticosteroid treatment (9). In addition, it has been observed that the activity of glutamine synthetase is increased in muscle and lung in response to various catabolic disease states (10, 11) and after corticosteroid treatment (12).

To discriminate between the effects of corticosteroid treatment *per se* and those of undernutrition on muscle glutamine metabolism, a rat study was conducted. The fluorinated corticosteroid triamcinolone was used, since this corticosteroid induces besides a hypermetabolic state, also an abrupt drop in food intake.

First aim of the study was to compare the effects on muscle glutamine metabolism after corticosteroid treatment or undernutrition. Second aim was to study possible differences in glutamine metabolism between acute and chronic effects of corticosteroid treatment, when dietary intake is normalised.

6.2. Methods

Animals

Fifty four male Wistar rats ($\pm$ 350g, Harlan-Winkelmann, Borcheln, Germany) were studied. The diet provided to all animals was standard laboratory rat chow (SRM-A 1210, Hopefarms, Woerden, The Netherlands) and water *ad libitum*. They were subjected to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.), whereas room temperature was maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (13) and approved by the Ethical Committee of Animal Research of the Maastricht University.

Experimental groups

Rats were randomly divided into four groups. The first group (TR) received a daily injection of triamcinolone (1.25 mg/ml) of 0.25 mg/kg body weight (approximately 0.06 ml) intramuscular (i.m.) for 3 days (n=8) and 2 weeks (n=10) and *ad libitum* food intake. The second group (pair-fed; PF) had a similar food intake as TR, and received daily 0.05 ml 150 mM NaCl i.m. for 3 days (n=8) and 2 weeks (n=10). The third group (pair-weight; PW) had a comparable body weight as TR by reducing daily food intake and received daily 0.05 ml 150 mM NaCl i.m. for 2 weeks (n=10). The fourth group was free fed (FF) for 3 days (n=8) and for 2 weeks (n=10), and received daily 0.05 ml 150 mM NaCl i.m. All injections were given in the hamstrings of the left leg.

Study and sampling procedure

Rats were randomly assigned to metabolic cages, taking an adaptation period of 4 days prior to the experiment into account. Food intake and body weight (BW) of all animals were registered daily at 8:00 A.M., before the injections. All infusion and sampling experiments were performed as described previously (14, 15). In brief, under anaesthesia a laparotomy was performed. The inferior caval vein, the right vena renalis, the aorta and the left carotid artery were cannulated. To study amino acid membrane transport kinetics, a primed (1.1 μ Ci/100 g BW) continuous infusion (1 μ Ci/100 g BW/h) of L-[3,4-3H]-Glutamine (GLN) and L-[2,6-3H]-Phenylalanine (PHE) was given into the right renal vein. For flow measurements, the indicator dilution method with para-aminohippuric acid (PAH) was used (pH 7.4, iso-osmolar, Sigma A 1422, St Louis, MO, USA). A primed (0.15 ml/100 g BW, 50 mM) continuous infusion (0.75 ml/100 g BW/h, 5 mM) of PAH was infused in the aorta.

After 30 minutes, blood (1.2 ml per catheter) was sampled from the caval vein and carotid artery at a maximum rate of approximately 500 μ l/min. All blood was collected in heparinized cups (Lithium-Heparin Micro-sample container CB100, Sarstedt, Germany) on ice. Hereafter, the right gastrocnemius muscle was dissected freely, directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis. Gastrocnemius muscle from the left leg was dissected freely and weighed.

For PAH determinations, 50 μ l heparinized blood was added to 500 μ l Trichloroacetic acid GR 10% (w/v) (TCA 10%) (Merck 807, Darmstadt, Germany), vortexed and centrifuged at 8,900 g at 4°C. The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube (Modulohm 88302, Herlev, Denmark) was filled with heparinized blood and centrifuged at 10,000 g at room temperature. Plasma was obtained by whole blood centrifugation at 8,900 g at 4°C for 5 minutes. For arterial and venous glucose determinations, 200 μ l plasma was vortexed with 20 μ l of 50% TCA, put into liquid nitrogen and stored at -80°C. For plasma amino acid determinations 200 μ l plasma was added to 8 mg 5-Sulfosalicylic acid (SSA 5%) (Brunschwig, 10346, Amsterdam, The Netherlands) for deproteinization, vortexed, frozen in liquid nitrogen and stored at -80°C.

For the determination of tissue free amino acid concentrations and specific activity, frozen muscle tissue was pulverised using a mortar and pestle precooled in liquid nitrogen and homogenised and deproteinized using a Mini-beater (Biospec products, Bartlesville) Approximately 100 mg tissue was added to 500 μ l SSA (5%) plus glass beads (1mm). This was put in the mini-beater for 30 seconds. The homogenate was frozen in liquid nitrogen and stored at -80°C until further determinations of the supernatant.

To determine the tissue dry weight approximately 200 mg pulverised tissue was freeze dried for 24 hours in a Speedvac (type SC200, Savant Instruments Inc., Farmingdale, NY, USA) connected with a refrigerated condensation trap (type RT 400, Savant Instruments Inc., Farmingdale, NY, USA).

Biochemical analysis

Plasma glucose and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (15, 16). Amino acids and specific activity were determined by fully automated high performance liquid chromatography (HPLC) using 2-3 μ M spherisorb ODS II packing material and pre-column derivatization with ortho-phthalaldehyde as described previously (17).

Calculations

Plasma flow across the hindquarter using PAH in the indicator dilution method is calculated as follows:

$$\text{Plasma flow} = \text{IPAH} / \{[\text{PAH}]_{\text{ven}} - [\text{PAH}]_{\text{art}}\}$$

in (ml/100 g BW/min), IPAH is the infusion rate of PAH (μ mol/100 g BW/min) and [PAH]_{ven} or art the venous or arterial plasma PAH concentration (μ M).

Hindquarter substrate fluxes are calculated by multiplying the inferior caval vein-arterial concentrations differences with the mean hindquarter plasma flow of the group and are expressed in nmol/100 g BW/min. A positive flux indicates net release, a negative flux reflects a net uptake. Tissue concentrations of amino acids are expressed in mmol/L and derived by the multiplication of tissue homogenate amino acid concentration (mmol/kg wet weight) and the percentage tissue water content. Branched-chain amino acids were calculated as the sum of valine, leucine and isoleucine.

Glutamine turnover

Three compartment modelling was used to calculate glutamine turnover (18, 19). The model divides an organ in two vascular compartments and a tissue compartment. Plasma-tissue membrane transport rates are calculated as the glutamine flux in vivo from the arterial pool to the intracellular muscle pool ($F_{m,a}$) and from the intracellular pool to the venous pool ($F_{v,m}$). Metabolic shunting can be derived from the flux entering the venous pool from the arterial pool bypassing the intracellular free glutamine pool ($F_{v,a}$). Whole body glutamine and phenylalanine rate of appearance (R_a) were calculated from the arterial dilution of the tracer amino acid at steady state (20). Glutamine can be incorporated in muscle protein (intracellular disposal). Production of glutamine can be obtained by protein breakdown (intracellular production) and by the transamidation of glutamate. Knowing the relative amino acid concentration of phenylalanine and glutamine in protein (21) enables us to calculate glutamine R_a derived from protein breakdown and non-protein derived glutamine R_a (*de novo* production).

Statistics

All data are presented as group means $\pm$ SEM. Kruskal-Wallis Oneway ANOVA was used to test significant differences. Differences between all groups were assessed using a non-parametric test (Mann-Whitney U). A significance level of $p \leq 0.05$ was selected.

6.3. Results

Animal food intake, body and muscle weights

The daily food intake of the different groups treated during 2 weeks, is depicted in figure 1A. The TR rats showed a reduced intake during the first days of the experiment. After this acute response, food intake gradually returned to normal levels. Initial body weights of the groups (Fig.1B) were not significantly different.

Fig. 1. (A) Food intake of the groups as a result of triamcinolone treatment (●) or pair-weight (▲), pair-fed (Δ) or ad libitum food intake (○). (B) Weight change during triamcinolone treatment (●) or pair-weight (▲), pair-fed (Δ) or ad libitum food intake (○).

After 3 days, body weight of the TR group was significantly decreased compared to the FF group, while body weight of the PF group was not significantly different from FF (Table 1). During the 2 weeks treatment, the weights of the TR and PW rats decreased by 20% and 22%, while body weight of the PF and FF rats increased: values at the end of treatment period were not significantly different (Figure 1B). As shown in table 1, gastrocnemius weight decreased after triamcinolone treatment, but also after 2 weeks severe undernutrition (PW). Total gastrocnemius weight expressed as percentage of body weight was significantly decreased after 14 days in the TR group compared to all other groups (Table 1). No difference in relative muscle weight was observed between the PW, PF and FF groups.

Table 1. Body and muscle weights.

		FF	PF	PW	TR
Final BW	3d	354 ± 9	337 ± 9		315 ± 8 a
(g)	14d	343 ± 7	334 ± 4	266 ± 4 c	251 ± 5 c f g
Weight gastro	3d	1.90 ± 0.06	1.85 ± 0.07		1.67 ± 0.06 a
(g)	14d	1.89 ± 0.05	1.88 ± 0.03	1.55 ± 0.02 c	1.26 ± 0.05 c f i
Gastro/BW	3d	0.54 ± 0.01	0.55 ± 0.01		0.53 ± 0.01
	14d	0.56 ± 0.01	0.56 ± 0.01	0.58 ± 0.01	0.50 ± 0.01 a e i

Values are mean ± SEM. BW, body weight; Gastro, gastrocnemius. a, b, c, $p < 0.05$, $p < 0.01$, $p < 0.001$ vs FF; d, e, f, $p < 0.05$, $p < 0.01$, $p < 0.001$ vs PF; g, h, i, $p < 0.05$, $p < 0.01$, $p < 0.001$ vs PW.

Intramuscular and arterial concentrations and hindquarter fluxes after 3 days

Hematocrits and muscle wet/dry weights were not significantly different between the groups. When we compare the TR with the FF group, an increased arterial glucose concentration was observed in the TR rats (42 ± 2 mM) compared to FF rats (12 ± 1 mM, $p < 0.01$). Comparing TR with PF, the TR rats had higher arterial glucose levels than the PF rats (PF: 11 ± 1 mM, $p < 0.001$). As shown in Table 2, intramuscular glutamine (GLN) concentration was increased in the TR rats ($p < 0.05$) and glutamate (GLU), phenylalanine (PHE) and branched chain amino acid (BCAA) concentrations were unchanged. Efflux of PHE and BCAA were increased in the TR rats (Table 3, $p < 0.05$), while GLU influx and GLN efflux were unchanged.

Table 2. Intramuscular substrate concentrations in the gastrocnemius.

		FF	PF	PW	TR
GLU	3d	448 ± 33	431 ± 22		354 ± 43
	14d	685 ± 35	700 ± 70	743 ± 69	721 ± 63
GLN	3d	2113 ± 78	2188 ± 92		2517 ± 153 a
	14d	2414 ± 51	2517 ± 99	3143 ± 99 c	2423 ± 190 h
PHE	3d	108 ± 3	99 ± 8		116 ± 5
	14d	68 ± 7	52 ± 3	54 ± 3	42 ± 3 b e g
BCAA	3d	386 ± 19	430 ± 29		430 ± 33
	14d	349 ± 18	317 ± 26	321 ± 21	251 ± 12 b d g

Values are mean ± SEM. Concentrations are in $\mu\text{mol/kg}$ wet weight. GLU, glutamate; GLN, glutamine; PHE, phenylalanine; BCAA, branched chain amino acids (the sum of valine, leucine and isoleucine). a, b, c, $p < 0.05$, $p < 0.01$, $p < 0.001$ vs FF; d, e, $p < 0.05$, $p < 0.01$ vs PF; g, h, $p < 0.05$, $p < 0.01$ vs PW.

Table 3. Hindquarter substrate fluxes

		FF	PF	PW	TR
Flow	3d	3.7 ± 1.0	2.7 ± 0.9		3.9 ± 1.1
	14d	3.1 ± 0.5	3.9 ± 1.2	3.0 ± 0.9	4.0 ± 0.8
Glucose	3d	-479 ± 969	-2052 ± 564		-2993 ± 2280
	14d	-1638 ± 1298	-18 ± 1446	-1315 ± 709	-6993 ± 2085 a d g
GLU	3d	-28 ± 20	-34 ± 20		-16 ± 8
	14d	-19 ± 4	-23 ± 8	-20 ± 3	-85 ± 18 b e h
GLN	3d	151 ± 44	116 ± 23		127 ± 48
	14d	160 ± 39	204 ± 53	324 ± 68 a	494 ± 65 c e g
PHE	3d	-4 ± 3	1 ± 4		14 ± 6 a
	14d	5 ± 4	9 ± 3	14 ± 3	19 ± 2 b d
BCAA	3d	-61 ± 33	-47 ± 32		27 ± 24 a
	14d	-72 ± 13	-59 ± 21	-71 ± 11	-13 ± 14 a h

Values are mean ± SEM. Flow in $\text{ml}/100 \text{ g BW}/\text{min}$. Fluxes in $\text{nmol}/100 \text{ g body weight}/\text{min}$. BCAA is the sum of valine, leucine and isoleucine. a, b, c, $p < 0.05$, $p < 0.01$, $p < 0.001$ vs FF; d, e, $p < 0.05$, $p < 0.01$ vs PF; g, h, $p < 0.05$, $p < 0.01$ vs PW.

Intramuscular and arterial concentrations and hindquarter fluxes after 2 weeks

Comparing the TR with the FF group, an increased arterial glucose concentration was observed in the TR rats (TR: 20 ± 3 mM, FF: 12 ± 1 mM, $p < 0.01$). Arterial glucose of the PW and PF groups were unchanged (11 ± 1 mM and 10 ± 1 mM resp.) and were therefore significantly lower than the TR group ($p < 0.01$). Intramuscular concentrations of PHE and BCAA were decreased in the TR rats (Table 2). The PW group showed an increased concentration of GLN in the gastrocnemius and GLN, PHE and BCAA concentrations in the gastrocnemius were significantly higher than in the TR group (Table 2). Intramuscular concentrations of PHE and BCAA were also higher in the PF rats than in the TR rats (Table 2). Glucose and GLU influx and PHE and GLN efflux were increased in the TR group (Table 3), while BCAA influx was decreased. The PW group showed an increased GLN efflux from the hindquarter (Table 3). Influx of glucose and GLU was higher in the TR group than in the PW group (Table 3), while influx of BCAA was lower. Efflux of GLN was also higher in the TR group than in the PW group. Influx of glucose and GLU and efflux of GLN and PHE were higher in the TR rats than in the PF rats (Table 3).

Table 4. Muscle membrane transport rates of glutamine.

		FF	PF	PW	TR
Fm,a	3d	730 $\pm$ 117	593 $\pm$ 79		728 $\pm$ 145
	14d	393 $\pm$ 79	818 $\pm$ 219	628 $\pm$ 75 a	1099 $\pm$ 92 c h
Fv,m	3d	788 $\pm$ 85	683 $\pm$ 99		822 $\pm$ 143
	14d	524 $\pm$ 71	1011 $\pm$ 270	952 $\pm$ 97 b	1616 $\pm$ 166 c h
Fv,a	3d	1768 $\pm$ 90	1190 $\pm$ 68 b		2020 $\pm$ 239 d
	14d	1332 $\pm$ 105	1641 $\pm$ 271	1319 $\pm$ 113	1184 $\pm$ 100

Values are mean $\pm$ SEM. In nmol/100 g body weight/min; Fm,a, plasma membrane rate from the arterial to the free amino acid muscle pool; Fv,m, plasma membrane rate from the free amino acid muscle pool to the venous pool; Fv,a, plasma membrane rate from the arterial to the venous pool; 3 d, 3 days; 14 d, 14 days. a, b, c, $p < 0.05$, $p < 0.01$, $p < 0.001$ vs FF; d, $p < 0.05$ vs PF; h, $p < 0.01$ vs PW.

Fig. 2. Intracellular glutamine disposal and production from the free amino acid pool in the muscle after 3 and 14 days. FF, free fed; PF, pair fed; PW, pair weight; TR, triamcinolone treated; +, $p < 0.05$; ++, $p < 0.01$ vs PF.

Glutamine turnover over the hindquarter

Acute triamcinolone treatment resulted in no significant changes in glutamine turnover compared to the FF group, but glutamine shunting ($F_{v,a}$) was increased compared to the PF group (Table 4, $p < 0.05$). Furthermore, both intracellular disposal and production of glutamine were decreased in the TR group compared to the PF group (Fig.2). After 2 weeks, membrane transport rates from the arterial to the intracellular free amino acid pool ($F_{m,a}$) and from the intracellular pool to the venous pool ($F_{v,m}$) were increased in TR compared to FF ($p < 0.001$).

Fig. 3. De novo glutamine production in the hindquarter of the rat after 3 and 14 days. FF, free fed; PF, pair fed; PW, pair weight; TR, triamcinolone treated; #, $p < 0.01$; *, $p < 0.001$ vs FF.

Furthermore *de novo* glutamine production was increased (Fig.3, $p < 0.01$). After 2 weeks, the PW group showed both an increased $F_{m,a}$ and $F_{v,m}$ and also an increased glutamine *de novo* production. The increases in $F_{m,a}$ and $F_{v,m}$ were significantly higher in the TR group than in the PW group ($p < 0.01$).

6.4. Discussion

In contrast to undernutrition, the present study indicates that acute triamcinolone treatment caused hyperglycaemia and an increased intramuscular glutamine concentration associated with an increased glutamine shunting in the muscle and decreased glutamine disposal and production. Chronic triamcinolone treatment on the other hand showed similar alterations in muscle glutamine metabolism as undernutrition (PW), like an increased glutamine *de novo* production, an increased glutamine efflux and increased glutamine membrane transport rates into and from the free amino acid pool in the muscle. These alterations were nevertheless more pronounced in the TR group, as reflected in a higher reduction in muscle mass relative to the body mass reduction. Glutamate is a likely precursor for *de novo* glutamine production induced by TR, but after undernutrition precursors of glutamine production are not clear.

The effects of triamcinolone treatment on muscle glutamine metabolism were compared with those of undernutrition after 3 days treatment, since triamcinolone treatment caused after 3 days the most dramatic decrease in food intake (22). In the present study, this resulted only in the triamcinolone group in a decreased body weight and muscle mass, which probably was the result of an increased muscle protein degradation, as indicated by the increased phenylalanine efflux. Otherwise, data of the pair fed group showed that after this short period of time undernutrition *per se* did not result in a significantly decreased body weight or muscle mass, and revealed unchanged net protein degradation of the hindquarter muscles.

Three days triamcinolone treatment caused an intramuscular increase of glutamine concentration despite unaltered fluxes. This is in contrast to the majority of earlier reports, in which intramuscular glutamine concentrations were found to be decreased after approximately 1 week of treatment with corticosteroids (9, 23-25) or unchanged after 3 days treatment in old rats (25). Otherwise, two days hydrocortisone acetate has also shown to cause an increased intramuscular glutamine concentration in the diaphragm (26). Glutamine efflux from the muscle was consistently found to be increased after 1 to 2 weeks treatment with corticosteroids (9, 23, 27), while in the present study glutamine efflux was not altered after 3 days triamcinolone treatment. In general, an increased muscle glutamine concentration could be caused by an increased *de novo* glutamine production (induction of glutamine synthase), by an increased net muscle protein degradation, or by both. The tracer data in the present study showed acutely no *de novo* glutamine production; increased muscle protein degradation on the opposite seems more likely in line with the increased phenylalanine efflux. A recent study of Minet-Quinard and colleagues has demonstrated that glutamine synthase activity was increased in the extensor digitorum longus (EDL) after 3 days dexamethasone treatment, but in the gastrocnemius this increase was only shown after 5 days dexamethasone treatment (25). Therefore, the differences in glutamine metabolism of the present study with earlier studies could be due to different muscles studied and different treatment periods.

A short period of food restriction in the present study had no significant effect on glutamine concentration or efflux from the muscle. Earlier reports showed decreased intramuscular glutamine levels in the gastrocnemius (28) and increased intramuscular glutamine levels in the diaphragm and EDL (26) or in the gastrocnemius (14) after starvation. However, in the present study, food intake of the PF rats was not totally reduced to zero as in these earlier studies. Therefore, these PF rats should be compared with semistarved rats. Seven days of undernutrition showed an unchanged glutamine efflux from the hindquarter and an increased glutamine concentration in the gastrocnemius (29). Remarkably, the changes in glutamine shunting over the hindquarter in the pair fed group were opposite to those of the TR group, possibly related to an expected adaptive decreased metabolism during undernutrition.

The observed initial decrease in daily food intake in the TR group was restored completely after 2 weeks, while energy intake per gram body weight was even increased compared to the free fed group. This phenomenon and the changes in body mass indicates that the TR group was still hypermetabolic after 2 weeks. After 2 weeks, the pair fed group was not catabolic, since energy intake per gram body weight was not decreased and body weight was increasing. This implies that the PW group was the only nutritionally deprived group after 2 weeks, resulting in a similar body weight reduction as the TR group. However, the comparison between the TR and the PW group is also somewhat limited since muscle mass reduction was not comparable due to the fact that muscle wasting relative to body wasting was more pronounced in the TR group.

Chronic triamcinolone treatment did not alter intramuscular glutamine concentration in the gastrocnemius. As mentioned above, earlier animal studies have found decreased intramuscular glutamine concentrations after 3-9 days treatment with corticosteroids. However, in patients, chronically treated with corticosteroids, increased intramuscular glutamine levels were reported. It is very difficult to compare several studies, because of the

different corticosteroids used, the different treatment periods, the different muscles studied and the different ages of the animals, which are also important to consider (25).

In the present study, an increased muscle glutamine efflux was shown, in line with earlier studies after nine or eight days treatment with dexamethasone (9, 23). The increased glutamate influx of the present study implies that glutamate could be the precursor for this glutamine production. This is confirmed by the observed increased *de novo* glutamine production. Earlier studies have indeed demonstrated an increased glutamine synthase mRNA expression and activity after corticosteroid treatment (12, 25, 30). Undernutrition also showed an increased muscle glutamine efflux, which was earlier reported after 4 days starvation (14). Interestingly, undernutrition showed also an increased *de novo* production, but here the precursors for glutamine are not clear. It has been demonstrated earlier that starvation could increase the maximum activity of glutamine synthase in muscle (31) or in the lung (11), which is in line with the observed increased *de novo* glutamine production in the present study. Despite striking similarities in muscle glutamine metabolism, the remaining amino acids (glutamate, phenylalanine and branched chain amino acids) and other substrates showed a clearly different response. After TR treatment an increased muscle influx of glucose and glutamate and efflux of phenylalanine was found while undernutrition did not induce any of these changes. This discrepancy between the two treatment groups is in line with an earlier study, where glycolytic and oxidative muscle enzyme capacities were found to be increased in the peripheral skeletal muscle of rats treated with triamcinolone, while undernourished rats did not show any of these alterations (22).

It is important to consider the similar effects of triamcinolone treatment and severe undernutrition (PW) on muscle glutamine metabolism. It is possible that both treatments could enhance these effects on glutamine metabolism. Therefore, it is important to prevent undernutrition in patients chronically treated with corticosteroids. The present experimental rat model could be an interesting model to discriminate between the acute and chronic effects of glutamine suppletion on corticosteroid treatment and to investigate the reversibility of the effects of metabolic stress relative to undernutrition on glutamine metabolism. This may provide new perspective to the treatment of cachexia associated with chronic corticosteroid dependent disorders.

Acknowledgements

The authors wish to thank Mrs GAM Ten Have for biotechnical assistance and Mr HMM van Eijk and Mr DR Rooyackers for analytical help.

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7

α and β Glucocorticoid receptor mRNA expression in skeletal muscle.

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Journal of Muscle Research and Cell Motility 19: 757-765 (1998)

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This study was supported in part by ASTRA Pharmaceutica, the Netherlands.

Abstract

The aim of the present study was to investigate the occurrence and autoregulation of both glucocorticoid receptor mRNAs in rat gastrocnemius muscle. The expression of both receptor forms was studied at 1, 4 or 12 hours after intra-tracheal instillation of a high dose (100 μ g) of budesonide; muscular expression was compared with glucocorticoid receptor expression in lung tissue. After Northern blot analysis, hybridization was performed with glucocorticoid receptor, glyceraldehyde-3-phosphate dehydrogenase and glutamine synthetase probes. In the gastrocnemius muscle, both the α and β glucocorticoid receptor mRNA forms were detected and found to be downregulated four hours after the budesonide instillation. α/β glucocorticoid receptor ratios were lower in the gastrocnemius (1.1 ± 0.2) than in the lungs (2.6 ± 0.6). In the lungs, at all time points, the average α glucocorticoid receptor mRNA levels did not differ from controls, although glutamine synthetase mRNA levels were upregulated. The β glucocorticoid receptor mRNA was slightly reduced at 1 and 4 hours. In conclusion, after intra-tracheal instillation of budesonide, both α and β glucocorticoid receptor forms were downregulated in muscle tissue. The difference in α/β glucocorticoid receptor mRNA ratios and concentrations between lung and gastrocnemius muscle supports the hypothesis of differential gene regulation by glucocorticoids in different cell types.

7.1. Introduction

The use of glucocorticoids, the mainstay of treatment in a wide variety of chronic inflammatory diseases, can have severe systemic side-effects, although the severity and frequency are reduced when glucocorticoids are applied topically (Barnes, 1995; Hanania et al., 1995; Kamada et al., 1996). One of the reported side-effects that may occur after glucocorticoid use is skeletal muscle atrophy (Khaleeli et al., 1983; Seene, 1994). The fast-twitch muscles in particular are sensitive to the catabolic action of glucocorticoids (Kelly et al., 1986; Picado et al., 1990; Burtea et al., 1992). A central role in the action of glucocorticoids is played by the glucocorticoid receptor (GR), and GR levels have been investigated in slow and fast-twitch skeletal muscles. Higher concentrations of GR have been demonstrated in the slow-twitch muscles (Almon & Dubois, 1990; Dubois & Almon, 1984; Saartok, 1984; Sauerwein et al., 1991). Therefore, a higher response to glucocorticoids may be expected in slow-twitch muscles, but in contrast, fast-twitch muscles are affected to a greater extent by glucocorticoids. At present, no studies have been performed regarding the response of the GR after exposure to glucocorticoids in fast-twitch skeletal muscles like the gastrocnemius.

The GR is present in the cytoplasm of cells as an inactive protein bound to a complex of different heat shock proteins (Pratt et al., 1992). After binding of the ligand, the receptor becomes activated by dissociation of the heat shock proteins. By binding as a dimer to glucocorticoid responsive elements (GRE) in the DNA, the GR regulates the transcription of genes. The activated GR can also modulate gene transcription by binding to other transcription factors, such as activator protein-1 (AP-1) (Yang-Yen et al., 1990), nuclear factor κ B (NF κ B) (Brostjan et al., 1996), cAMP-responsive element binding protein (CREB) (Peters et al., 1995) and signal transducer and activator of transcription 5 (Stat5) (Stocklin et al., 1996). Glucocorticoid hormone receptors contain a ligand binding C-terminal end, a central DNA binding domain, and a transactivating N-terminal end. Owing to alternative splicing, two functionally distinct receptors α and β are formed, differing at their carboxyterminal end (Hollenberg et al., 1985). The presence of both GR mRNAs has been demonstrated in humans in several tissues (Bamberger et al., 1995; Oakley et al., 1996), but

on a protein level no clear separation between the α and β form has been made. Recently an antibody recognizing the α form has become commercially available; however, this has not yet been the case for the β GR (Lee et al., 1996). In a recent study by Oakley and colleagues (Oakley et al., 1996), three GR mRNA isoforms were described. With Northern blot analysis, an $\alpha 1$ of 7 kb, an $\alpha 2$ of 5.5 kb, and a β GR mRNA form of 4.3 kb was demonstrated. Translation of the $\alpha 1$ and $\alpha 2$ forms results in the same protein, which has ligand-binding capacity. In contrast, the β GR has no steroid-binding domain and is considered as a dominant negative inhibitor of the α GR, influencing its gene regulatory function after activation by glucocorticoids (Bamberger et al., 1995; Oakley et al., 1996). In view of this inhibitory function it is of interest to study β GR form, its relationship to the α GR form and transcriptional regulation by different modulators.

Because of the direct correlation between glucocorticoid receptor number and cellular responsiveness to glucocorticoids (Pui et al., 1984; Iida et al., 1985; Vanderbilt et al., 1987) and because of the inhibitory effect of the β GR on the α GR, it is important to understand thoroughly the mechanisms responsible for maintaining α and β receptor levels and ratios. Several studies (Dong et al., 1988; Kalinyak et al., 1989; Silva et al., 1994; Swezey et al., 1995; Meyer & Schmidt, 1994; Knutsson et al., 1995; Schlecte et al., 1982) have shown that the α GR is downregulated after exposure to glucocorticosteroids. Thus far, little information is available on the autoregulation of the β form. In a recent study (Korn et al., 1997) we demonstrated a downregulation of the β GR mRNA after exposure to budesonide in human bronchial epithelial cells and alveolar macrophages. In rats, no information is available about the existence of the β GR in skeletal muscles. Also no information is available about the regulation of α and β GR forms by glucocorticoids.

The aim of this study was to investigate the presence of both α and β GR mRNAs and their autoregulation by budesonide in rat skeletal muscle (gastrocnemius). Also, α/β GR mRNA ratios were determined. Because of the known effects of glucocorticoids on the GR mRNA levels in the lung, this tissue served as a positive control. Furthermore, glutamine synthetase, upregulated by glucocorticoids, was investigated as a positive control.

7.2. Methods

Animals and tissues

Sixty-five male Wistar rats weighing 200-250 g (Harlan-Winkelmann, Borcheln, Germany) were anaesthetised by subcutaneous injection of 0.2 ml of 100 mg per ml ketamin (Nimatek, AUV Cuijk, the Netherlands) and 0.2 ml of 5 times diluted 23 mg per ml Sedamun (AUV Cuijk, the Netherlands) in the neck. Five animals were used to optimise the instillation technique by aerosolization of black ink. The other 60 rats were included in the study. After intubation the animals were ventilated mechanically with O_2 and N_2O at a frequency of 40 breaths per minute. Under visual inspection the tube ending was localised a few millimetres above the carina. Owing to the increasing use of local application of glucocorticoids in chronic inflammatory airway diseases, intra-tracheal exposure to glucocorticoids was chosen. To this end, a small tube was inserted within the ventilation tube, through which the budesonide solution was administered. 10 mg Budesonide was dissolved in 250 μ l of 70% alcohol and diluted in 50 ml phosphate buffered saline (PBS) to a final concentration of 200 μ g per ml. Either 0.5 ml budesonide solution or 0.5 ml dilutant (control) was injected in aliquots of 50 μ l, administered during the inspiratory phase. Since the animals were similar in weight, the budesonide dosage was equal in all rats: 100 μ g of budesonide intra-tracheally (400 μ g/kg). Animals regained consciousness and were sacrificed 1, 4, or 12 h later with 1.5 ml 60 mg/ml pentobarbital administered intraperitoneally. The right gastrocnemius muscle and both lungs of each animal were sampled. The gastrocnemius muscle was put into 4 ml 4 M guanidinium isothiocyanate (GTC) and frozen immediately to -70° C without homogenisation. The lungs were embedded in tissue-tek (Miles Inc., Elkhart, IN, USA) and

immediately frozen in liquid nitrogen. The whole animal experiment was performed in two days, and control and budesonide exposed animals were handled simultaneously (10 per group).

Histology

From the frozen lung specimens 6 μ M sections were cut and mounted on gelatine-chromium(III)-potassium sulfate coated slides. Sections were stained with hematoxylin-eosin. Morphologic examination on sections from right and left lung was performed to investigate possible traumatic or inflammatory changes in all animals.

RNA-isolation

For part of the samples the method of total RNA extraction was similar as described before (Korn et al., 1997). In brief, tissues were homogenized in GTC, and after CsCl-ultracentrifugation total RNA was recovered. The second, faster approach was based on the method described by Chomczynski et al. (Chomczynski & Sacchi, 1987), using an RNA isolation kit, TRIzol Reagent (Life Technologies, Breda, the Netherlands). Tissue samples were homogenized in 1 ml TRIzol Reagent per 100 mg tissue. After adding 0.2 ml chloroform, the tubes were vigorously shaken, incubated at room temperature for 3 minutes and centrifuged at 4 °C and 12,000 g for 15 minutes. The aqueous phase was mixed with 0.5 ml 2-propanol per 1 ml TRIzol and RNA was recovered by centrifugation at 12,000 g and 4 °C for 10 minutes. The pellet was washed with 75% ethanol and recovered again by centrifugation for 5 minutes at 7,500 g and 4 °C. This wash step was repeated once. RNA pellets were dissolved in 10 μ l distilled water. Total RNA concentration was determined visually, by comparison to RNA samples with known concentrations on a 1% agarose gel.

Northern blot analysis

Northern blot analysis was performed as described before (Korn et al., 1997) with slight modifications. Total RNA (20 μ g) was dissolved in sample buffer (1.3xMOPS, 0.5 M formaldehyde (pH 6.5) and 16.3 M formamide) and fractionated by electrophoresis on an 1.2% agarose gel (1.2 mg/ml agarose in 1xMOPS and 1 M formaldehyde (pH 6.5) with a 1xMOPS running buffer) at 110 V at room temperature for 2 h. The gel was rinsed 3 $\times$ 15 minutes in RNase free water, colored with ethidium bromide, washed in RNase free water 3 $\times$ 15 minutes in RNase free water, 20 minutes in 0.05 M NaOH, rinsed shortly in water and finally washed 30 minutes in 1 M ammonium acetate. The RNA was transferred onto a Nytran membrane (Schleicher and Schuell, Dassel, Germany) by overnight (17 h) capillary blotting with 1 M ammonium acetate. After blotting the RNA was fixed to the membrane by UV-crosslinking and 1 h baking at 80 °C. Since a maximum of 14 samples could be loaded onto the gel, the 20 animals used for each time point had to be separated over 2 gels. 10 Samples/gel were loaded, 5 budesonide exposed animals and their simultaneously handled controls. All 6 gels necessary for this experiment were poured from the same agarose stock, loaded and run at the same time.

Hybridization

Northern blots were hybridized simultaneously with the rat GR, human glyceraldehyde-3-phosphate dehydrogenase and rat glutamine synthetase (a generous gift from Prof. Dr. W.H. Lamers, AMC, Amsterdam, the Netherlands) probes in similar order as described before (Korn et al., 1997). Since glutamine synthetase is upregulated by glucocorticoids (Feng et al., 1990; Abcouwer et al., 1995), this probe (2800 bp) was used as a positive control. The GR probe was 2800 bp long and detected both α and β GR forms (Ko & Takano, 1989). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was 1007 bp long. The probes were labelled with the random primed labelling method using [α - 32 P]-dCTP (Amersham, Buckinghamshire, England). All 6 Northern blots were hybridized simultaneously at 65 °C in 0.5 M phosphate, 1 mM EDTA, 70 mg/ml SDS and 5 mg/ml BSA at the same probe specific

activities. The blots were washed at room temperature with 2xSSC, 3.5 mM SDS and 0.5xSSC, 3.5 mM SDS and at 65 °C with 0.1xSSC, 3.5 mM SDS for 15 minutes each. The sample signals were analysed visually as well as semiquantitatively with a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA, USA). GR and glutamine synthetase mRNA expression levels were determined relative to the control gene GAPDH to correct for the amounts of total RNA loaded onto the gel. These relative expression levels were compared to the relative expression levels of the control animals (100%) receiving only dilutant, resulting in 10 outcomes per timepoint. So the percentages were the result of the comparison of 1 exposed animal with the mean expression of the control animals.

Statistics

For all studies mean $\pm$ standard deviation (SD) was calculated and the Mann-Whitney U test performed. A difference of $p < 0.05$ was considered statistically significant.

7.3. Results

Aerosol instillation and lung morphology

In a pilot study (5 rats), optimisation of glucocorticoid distribution was carried out by aerosoling black ink into the lungs. By staining 6 μ M sections of frozen lung specimens, the distribution of the ink was visualised and shown to be present throughout lungs, until into the alveoli. With morphologic examination of the treated and control rats no signs of bleeding or inflammation were found in the lungs. During the experiment 2 rats died after being anaesthetised, one in the budesonide exposed group (12 h), and one in the control group (1 h), which reduced the total number from 60 to 58.

Northern blot hybridization

The GR mRNA expression levels in the gastrocnemius were 5-8 times lower than in the lungs. Therefore, GR mRNA signals from the gastrocnemius of 17 rats, evenly distributed over the treated and control animals, were too weak for a reliable quantification. Only the $\alpha 1$ and β GR mRNA bands were observed. No $\alpha 2$ band was demonstrated in the lung or gastrocnemius muscle.

Fig. 1. GR mRNA expression in rat gastrocnemius after intra-tracheal instillation of budesonide. After four hours a downregulation was observed for both α and β GR mRNAs. The mRNA levels are expressed relatively to GAPDH within the same sample and subsequently calculated to the mean of the control values within one gel, set at 100%. *Significantly different from control values.

Fig. 2. GR mRNA expression in rat lung after intra-tracheal instillation of budesonide. Rats were sacrificed after 1, 4, and 12 hours. Quantification of α and β GR mRNAs is described in Fig. 1. *Significantly different from control values.

The GR mRNA expression in the gastrocnemius showed a clear downregulation ($p < 0.05$) of both $\alpha 1$ and β GR types 4 hours after the addition of budesonide, to 64% and 67%, respectively (figure 1). Within the first hour no change in $\alpha 1$ and β GR mRNA expression took place (95% and 101%, respectively). After 12 h the gene expression no longer differed from the control animals. The mRNA expression for the $\alpha 1$ GR was $133 \pm 66\%$ ($p = 0.9$) and for the β GR $125 \pm 61\%$ ($p = 0.9$). In the lungs no difference in $\alpha 1$ GR gene expression was observed after 1, 4 or 12 hours (figure 2). The average $\alpha 1$ GR mRNA levels at 1, 4, and 12 hours were 104%, 95% and 113%, respectively. For the β GR mRNA a slight downregulation was observed at 1 and 4 h to 84% and 91%, respectively. At 12 h no change was seen compared to the control (110%).

Fig. 3. Glutamine synthetase (GS) mRNA expression in the rat lung. Both glutamine synthetase forms are displayed relatively to the GAPDH-value. At all times an upregulated expression is observed. Calculation of the mRNA expression in percentages is performed as described in Fig. 1. *Significantly different from control values.

Fig. 4. Northern blot analysis of the GR mRNA from rat lung and gastrocnemius. Both α and β GR are shown. The positions of the α and β GR mRNA bands are determined by the 28S and 18S ribosomal bands. Lanes 1 and 2 represent RNA from the gastrocnemius, while lanes 3 and 4 represent RNA from the lungs. In lanes 1 and 3 a control animal is shown, four hours after instillation. Lanes 2 and 4 represent an exposed animal after four hours. Note the GR mRNA downregulation in lane 2 compared with lane 1, and the clear difference in α/β GR mRNA ratio between the lung and gastrocnemius.

To demonstrate the pharmacological activity of budesonide in the lung, glutamine synthetase mRNA expression was studied as a control gene. The effect of budesonide on the glutamine synthetase gene expression in the lungs is shown in figure 3. Both glutamine synthetase mRNA forms were at all time points upregulated by budesonide. The 2.8 kb mRNA expression was upregulated to 165%, 164%, and 112% at 1, 4, and 12 h, respectively ($p < 0.05$). The 1.4 kb form was also significantly ($p < 0.05$) upregulated at 1 (116%), 4 (138%), and 12 (126%) h, but to a lesser degree.

A difference in α/β GR ratio was observed between the gastrocnemius and lungs, resulting in a lower ratio in the muscle (1.1 ± 0.2), compared to a ratio of 2.6 ± 0.6 in the lungs ($p < 0.05$). In figure 4 an example is shown of a northern blot hybridization with the GR probe. In addition, lower α 1 and β GR mRNA levels are detected in the gastrocnemius muscle compared to the lungs. For the α 1 GR mRNA, the amount in the gastrocnemius muscle is 12% of that in the lungs. For the β GR mRNA the expression is slightly higher, namely 20% of that in the lungs.

7.4. Discussion

The purpose of this study was to investigate the occurrence and regulation of the α and β GR mRNA levels in gastrocnemius muscle in comparison with lung tissue of rats. In both tissues the α 1 and β GR mRNAs were present. Concerning the α 1/ β GR mRNA ratio, a lower ratio was observed in the gastrocnemius muscle compared with the lung. The gastrocnemius showed a reversible downregulation of both α 1 and β GR mRNAs four hours after instillation of budesonide. No downregulation of either receptor form was observed in the lung after exposure to budesonide, except for the β GR mRNA after 1 and 4 hours. As demonstrated by the upregulation of the control gene glutamine synthetase in lung tissue, the steroid was adequately delivered and pharmacologically active.

It has long been known that the response of cells to glucocorticoids is positively correlated with the availability of the ligand and the amount of receptor present in cells, and that both vary strongly between different tissues (Pui et al., 1984; Iida et al., 1985; Vanderbilt et al., 1987). Because of the known inhibitory function of the β on the α GR form (Bamberger et al., 1995; Oakley et al., 1996), it is also important to take α/β GR ratios into consideration in studying these responses. In the present study, 5 to 8 times lower α and β GR mRNA levels were demonstrated in the gastrocnemius compared with the lung. Also, a tissue dependent variation in α/β GR ratio was observed, resulting in a ratio of 2.6 in the lungs compared with a ratio of 1.1 in the gastrocnemius. The α/β GR mRNA ratio in the lungs of rats is similar to that observed in human bronchial epithelial cells and alveolar macrophages (Korn et al., 1997). In theory, because of the lower GR mRNA levels and α/β ratio in the gastrocnemius, a smaller effect of glucocorticoids on gene transcription might be expected in the gastrocnemius compared with the lungs. Nevertheless, we observed a smaller change in gene transcription of the GR. This is similar to the results obtained previously in bronchial epithelial cells and alveolar macrophages (Korn et al., 1997) and in agreement with the *in vitro* model system where α/β ratios equivalent to or lower than 0.5 were clearly inhibiting.

The systemic side-effects that occur after oral glucocorticoid use are well documented. Controversy surrounds the possible systemic side-effects that may take place after a long period inhalation of glucocorticoids (Hanania et al., 1995). A clear relationship, though, is observed with the size of the administered dose, the pharmacokinetics of the steroid, and the method for inhaled drug delivery (Kamada et al., 1996). Among the many side-effects are myopathy and atrophy of fast-twitch skeletal muscles. Type 2b fibres are considered particularly susceptible to the catabolic action of glucocorticoids (Kelly et al., 1986; Almon & Dubois, 1990; Dubois & Almon, 1984). By demonstrating a downregulation of both GR mRNA levels in the gastrocnemius muscle four hours after instillation of 100 μ g budesonide, it can be supposed that the amount of budesonide entering the systemic circulation was high enough ($^{10^{-6}}$ M, see (Korn et al., 1997)) to create a similar change in both α and β GR mRNA patterns to those seen before in bronchial epithelial cells and alveolar macrophages (Korn et al., 1997). No change in gastrocnemius muscle GR mRNA was demonstrated 1 or 12 hours after instillation.

Surprisingly, despite the higher GR amounts and α/β GR ratios, and the broader availability of glucocorticoids in the lungs, no change in GR gene expression was observed in this tissue. In a previous study (Korn et al., 1997) a clear downregulation of the α and β GR mRNA was observed in human bronchial epithelial cells. Since it is not possible to obtain enough bronchial epithelial cells of such high purity from rats, whole lung tissue was investigated in this study. This tissue therefore contained a mixture of different cells such as epithelial cells, alveolar macrophages, mast cells, endothelial cells, smooth muscle cells, fibroblasts, and lymphocytes. A clear downregulation of the α GR by glucocorticoids has been demonstrated before in rat liver, rat lung epithelial cells, cervix carcinoma cells, human lymphocytes, human bronchial epithelial cells, and human alveolar macrophages (Dong et al., 1988; Silva et al., 1994; Swezey et al., 1995; Knutsson et al., 1995; Schlechte et al., 1982; Korn et al., 1997). However, in other reports studying human peripheral blood mononuclear cells, Kaposi's sarcoma cells, rat pulmonary fibroblasts, and human leukaemic T-cells, an upregulation of the GR mRNA expression was found (Adcock et al., 1995; Cole et al., 1996; Swezey et al., 1995; Eisen et al., 1988). Other investigations observe a biphasic expression pattern of the GR mRNA after exposure of rat hepatoma tumour cells and human lung parenchyma cells to glucocorticoids, resulting at first in an upregulation followed by a downregulation of the GR mRNA expression (Okret et al., 1991, 1986; Adcock et al., 1996). This diversity of transcriptional responses to glucocorticoids in different cells is probably caused by the concentrations of various transcription factors and their interactions in the cell, clearly varying from one cell type to another. Opposite responses in GR mRNA expression to glucocorticoids between different cell types can result in absence of an overall

measurable effect, as seen in this study. One of the options to study the GR mRNA expression solely in bronchial cells would be the use of mRNA *in situ* hybridization. However, to be able to determine reliably the differences in α and β GR mRNA expression between control and exposed animals with *in situ* hybridization, these differences must exceed a factor of 2. Since in our studies the changes in α and β GR mRNA levels are less than 50% after exposure to budesonide, it is probably not possible to detect these differences using mRNA *in situ* hybridization. An imperfect distribution of budesonide through the lungs could be another explanation for the unchanged GR mRNA expression found in this study. From the histological examination in the pilot study, however, it can be concluded that the distribution of the glucocorticoid in the lungs was good. Also, the observed upregulated glutamine synthetase mRNA expression indicates proper steroid function in the lungs (Feng et al., 1990; Abcouwer et al., 1995).

Recently, a downregulation of the GR mRNA after topical glucocorticoid application has been shown *in vivo* in peripheral lymphocytes (Knutsson et al., 1995), bronchial epithelial cells and alveolar macrophages (Korn et al., 1997) of humans. In the publication by Knutsson and coworkers (1995), a downregulated α GR mRNA expression was observed after intranasal inhalation of budesonide and fluticasone propionate. In the present study the amount of steroid per kg of body weight instilled in the rats was approximately 40 times more than the amount inhaled by the human volunteers in the study by Korn et al. (Korn et al., 1997). The size of the administered dose could be an explanation for the observed downregulation of both α and β GR mRNA expression at four hours.

In a recent study of Oakley and coworkers (Oakley et al., 1996), the β GR was investigated. As described before in a study by Bamberger and colleagues (1995), the β GR had a dominant negative inhibitory effect on the activity of the α form. Oakley and coworkers made the striking observation that, using Northern blot analysis, three GR mRNA forms were present. Instead of the routinely demonstrated α (7 kb) and β (5 kb) GR mRNA (Hollenberg et al., 1985; Rosewicz et al., 1988; Brönnegård et al., 1991; Zeiner & Gehring, 1993), an $\alpha 1$ band of 7 kb, an $\alpha 2$ band of 5.5 kb and a β band of 4.3 kb were demonstrated (Oakley et al., 1996). Similar results can be observed in the study of Brönnegård and Okret (1991). Also, in a previous study by Korn and colleagues (1997), the third isoform was occasionally seen in patients expressing high levels of GR mRNA and in a bronchial epithelial cell line. In the present study we investigated both $\alpha 1$ and β GR mRNA forms, and the identity of these isoforms was determined by the location of the 28 and 18S ribosomal bands. However, in rats, no sign of the third $\alpha 2$ band was observed.

In conclusion, in rats both α and β GR mRNA forms are present and modulated by budesonide in the gastrocnemius muscle. Systemic effects of intra-tracheally instilled budesonide on the GR mRNA levels in the gastrocnemius muscle were observed. However, no GR mRNA downregulation of intra-tracheally instilled budesonide in rat lung was observed, probably owing to the diversity of the cell population. The $\alpha 1/\beta$ GR mRNA ratios and concentrations were lower in the gastrocnemius muscle compared with the lungs, which suggests a possible difference in gene regulation by glucocorticoids between these tissues.

Acknowledgments

The assistance of Peter Kelderman with the animal study is greatly appreciated.

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8

Summary and general discussion

8.1. Introduction

The studies described in this thesis were designed to discriminate between the effects of corticosteroids and undernutrition on muscle functioning, as determined by muscle mass, morphology and muscle metabolism. Our aim was to investigate whether the metabolic pathways and the functional and structural consequences of muscle wasting are comparable between these two situations. The current literature on this particular subject reveals many unclarities and omissions. Firstly the results of our studies will be framed in perspective of the literature; thereafter the results regarding the differentiation between corticosteroids and undernutrition will be discussed.

8.2. Effects of undernutrition on muscle mass, morphology, contractility and metabolism

In all the studies reported in the present thesis, it was shown that undernutrition resulted in muscle mass reduction of both peripheral skeletal and respiratory muscles. However in contrast to diaphragm muscle wasting, wasting of peripheral skeletal muscles was less pronounced than body wasting. As mentioned in chapter 1, muscle wasting of the diaphragm was accompanied by an overall fibre type atrophy pattern (1-5), which was confirmed by our results in chapter 2. However in chapter 3, atrophy was limited to type IIx/b fibres under conditions of less severe and shorter undernutrition.

As earlier observed in *in vitro* diaphragm bundles after nutritional deprivation, existence of a decreased fatigability (1-4), an increased half-relaxation time (1) and a leftward shift of the force-frequency curve was confirmed in chapter 2 (1, 2). In addition, an increased time to peak tension was observed, which was not reported earlier. Considering the fact that the cross-sectional area of type I fibres is relatively increased in the diaphragm (chapter 2)(1), it is not surprising that *in vitro* diaphragm fatigability was decreased and that half-relaxation time and time to peak tension were increased. However, *in situ* muscle contractility of dorsiflexor muscles showed only a reduced external work as a consequence of the diminished muscle mass, while normalised for muscle mass, no changes were observed (chapter 4). This could imply different muscle atrophy patterns between the diaphragm muscle and the studied peripheral skeletal muscle. This is however not likely, since other studies have found similar effects of undernutrition on the diaphragm and peripheral skeletal muscles, like a generalised muscle fibre atrophy (1, 3). Therefore, other alterations have to be considered, as muscle metabolism and also motor unit characteristics.

In our studies muscle metabolism of both the diaphragm and peripheral skeletal muscles after chronic undernutrition was investigated. In chapter 4, it was confirmed that glycogen levels in the peripheral skeletal muscle after undernutrition are decreased (6). However, other studies also showed alterations in muscle enzyme activities involved in carbohydrate and β -oxidation (7, 8). In chapter 5 unchanged PFK, LDH, CS and HAD activities were found in the peripheral skeletal muscle. No muscle enzyme activities were measured in the diaphragm. Lactate concentrations were found to be unchanged in the diaphragm after undernutrition, while in the peripheral skeletal muscle an increased lactate level was observed (data not published), which was also reported by others (8, 9).

In addition, muscle energy status was studied. Earlier studies observed decreased PCr levels associated with maintained ATP levels in the peripheral skeletal muscle after undernutrition (8-10). In chapter 3 as well, a decreased energy metabolism in the diaphragm was found as shown by decreased PCr levels and total adenine nucleotides. However, a decreased diaphragmatic ATP concentration could also be demonstrated. This has not been reported before in the diaphragm. In addition, total creatine pool was maintained, suggesting the establishment of a new metabolic equilibrium, with lower ATP and PCr levels. This was accompanied by an increased creatine concentration. A similar tendency was also observed earlier in the gastrocnemius muscle (10). This increase could be caused by an increased Cr

and ATP production from PCr in order to satisfy the need for ATP. In future research it would be interesting to compare muscle energy metabolism after undernutrition between the peripheral skeletal muscle and the diaphragm, since in contrast to the findings in the diaphragm muscle in chapter 2, ATP levels were never found to be decreased (8-10) and lactate levels were increased in peripheral skeletal muscles (8, 9).

In order to obtain more insight in muscle amino acid metabolism, muscle glutamine metabolism was studied in the peripheral skeletal muscle. This conditionally essential amino acid is an important nitrogen and ammonia carrier in interorgan metabolism (11). Little information is available on muscle glutamine metabolism after chronic undernutrition. However, an increased glutamine concentration in the gastrocnemius muscle was demonstrated after 2 days of undernutrition (12), and confirmed in chapter 6 of this thesis. Additionally, glutamine efflux from the hindquarter was studied in chapter 6 and found to be increased, as well as glutamine *de novo* production and membrane transport rates. However in the diaphragm, unchanged intramuscular glutamine levels were found after undernutrition and basal intramuscular glutamine levels were twice as high as in the gastrocnemius (data not published). This suggests different muscle glutamine metabolism between the diaphragm and the peripheral skeletal muscle. Further research on this phenomenon is needed.

It can be concluded from these studies that muscle wasting induced by undernutrition differs between the peripheral skeletal muscle and the diaphragm. More information could be obtained in future research, studying simultaneously diaphragm and peripheral skeletal muscle energy metabolism. However, it should be realised that amino acid and substrate fluxes cannot (yet) be studied across the diaphragm muscle.

8.3. Effects of corticosteroids on muscle mass, morphology, contractility and metabolism

Intramuscular *prednisolone* treatment in the studies of the present thesis did not result in a decrease in body and muscle mass (chapter 4). After intramuscular or intraperitoneal *prednisolone* administration no body or muscle mass reduction was reported earlier (13, 14). Reductions in body and muscle mass were however observed after subcutaneous *prednisolone* administration (15-17). The observed absence of muscle wasting after i.m. *prednisolone* treatment was accompanied by minor alterations in *in situ* peripheral skeletal muscle contractility, as an increased recovery during a rest period. This implies that muscle wasting is not related to muscle recovery.

More striking effects were found after *triamcinolone* treatment. It was clearly demonstrated that *triamcinolone* treatment resulted in loss of body and muscle mass. Strikingly, wasting of the tibialis anterior and the gastrocnemius muscle was more pronounced than body wasting. This was also found earlier after *triamcinolone* treatment in the gastrocnemius (13, 18). Diaphragm muscle mass was however decreased to a comparable extent than body mass (chapter 2+3) as observed earlier (1, 13, 19). In chapter 2, the effects of muscle mass reduction on muscle fibre atrophy was described. Atrophy of type IIa and IIx/b fibres in the diaphragm was found after 0.5 mg *triamcinolone* per kg body weight during 4 weeks. Other studies have reported only type IIx/b atrophy of the diaphragm after a similar treatment of *triamcinolone* of 6 weeks (1). Even after a shorter *triamcinolone* treatment with a higher dose, both type I and IIx/b fibre atrophy was observed (20). This variation in atrophy pattern may be partly caused by the complications of corticosteroid treatment. During our study and that of Dekhuijzen and colleagues (1), many *triamcinolone*-treated rats died, while Petrof and coworkers (20) prevented corticosteroid-related pneumonia and reported no deaths. It is likely that high dose *triamcinolone* treatment induced also infections (like pneumonia), which may also affect the muscle atrophy pattern. Anyway, it is clear that at least fibre IIx/b atrophy occurred after *triamcinolone* treatment. In chapter 3, we have found only type IIx/b atrophy in the diaphragm after *triamcinolone* treatment with a lower dose and of shorter duration than in chapter 2. This was also supported by earlier studies (1, 13, 21).

The observed *triamcinolone*-induced atrophy of both type IIa and IIx/b fibres resulted in a decreased *in vitro* diaphragm fatigability, an increased half-relaxation time as well as a leftward shift of the force-frequency relationship (chapter 2), in accordance with earlier studies (1, 13, 21). This could be the consequence of a relative increase in cross-sectional area of type I fibres. In addition, *in situ* muscle function of peripheral skeletal muscles was studied. The absolute external work was reduced, caused by muscle mass loss. However, normalised for muscle mass, an increased external work was found. Furthermore, muscle fatigue was diminished, in line with diaphragm *in vitro* contractility and in contrast to hindlimb muscle fatigue after *prednisolone* treatment (17). As discussed earlier, this decreased fatigue could be caused by a relative increase of type I fibre cross-sectional area, since type I fibres are more fatigue resistant. Furthermore, during a 5 minute rest period in between the stimulation sessions, *triamcinolone* rats fully recovered. This phenomenon was also found for the *prednisolone* rats.

Ferguson and coworkers (22) observed only in the diaphragm increased glycogen levels after cortisone treatment. However in this thesis, we found increased glycogen stores in the peripheral skeletal muscle both after *triamcinolone* and *prednisolone* treatment. This increased muscle glycogen concentration did not seem to be related to alterations in muscle function, except for the abovementioned increased muscle recovery. An increased glucose influx was observed in the hindquarter muscles after *triamcinolone* treatment in chapter 6, but data on glucose muscle influx after *prednisolone* treatment are not yet available. This increased glucose muscle influx is very surprisingly, since other studies have demonstrated a decreased glucose uptake by the muscle after dexamethasone treatment (23). However, the increased glucose muscle influx after *triamcinolone* administration could very well contribute to the increased muscle performance of the peripheral skeletal muscle. In addition, Lieu and colleagues (16) observed after *prednisolone* treatment, in the peripheral skeletal muscle a decreased PFK activity. However, we could not confirm changes in PFK activity after *prednisolone* treatment in the peripheral skeletal muscle. After *triamcinolone* treatment however, PFK activity was increased, in line with an increased glycolysis. In addition, oxidative capacity was also found to be increased after *triamcinolone* in contrast to *prednisolone*. We furthermore confirmed for the diaphragm after *triamcinolone* treatment, the earlier observed increased intramuscular pyruvate and lactate levels after corticosteroid treatment (22, 24), suggesting also in the diaphragm an increased glycolysis. The increased glycolysis could be related to the increased glucose influx.

To the best of our knowledge, no data are available in literature on diaphragm muscle energy metabolism after *triamcinolone* treatment. We found decreased levels of ATP, PCr and total adenine nucleotides and creatine pools (chapter 3), suggesting a metabolic impairment. This could be related to the observed increased glycolysis, but further research is needed.

As mentioned earlier, a disproportional loss of peripheral skeletal muscle mass to body mass was observed after *triamcinolone* treatment. Therefore muscle amino acid metabolism was studied in the peripheral skeletal muscle after *triamcinolone* treatment. We demonstrated an increased intramuscular glutamine concentration only after acute *triamcinolone* treatment (chapter 6), in contrast to earlier studies. A decreased intramuscular glutamine concentration was found after corticosteroid treatment in the peripheral skeletal muscle (24-27), except for the soleus (25). Intramuscular amino acid concentrations by themselves however, do not give any information about muscle uptake or release. Therefore, it is necessary to study amino acid fluxes. Increased glutamine and phenylalanine efflux from the hindquarter was observed in this thesis after chronic *triamcinolone* treatment. This observation is in line with previously reported data (24, 27). This indicates net muscle degradation and metabolic stress. These effluxes were accompanied by an increased glutamate influx, as well as a decreased BCAA influx. Glutamate is probably a precursor for glutamine production, since glutamine *de novo* production was found to be increased. The decreased BCAA influx implies no precursor activity of the BCAAs for glutamine production. In addition we

demonstrated increased glutamine membrane transport rates in the muscle after *triamcinolone* treatment. The latter finding as well as the decreased BCAA influx were not reported previously.

The action of corticosteroids is mediated by glucocorticoid receptors. Two functionally distinct receptor forms α and β have been demonstrated in human tissues (28). The β GR form is considered as a dominant negative inhibitor of the α form (28, 29). Several studies have shown that the α GR is downregulated in lung cells and peripheral lymphocytes after exposure to corticosteroids (30-32). However, little is known about the autoregulation of the β form. In rats, no information is available about the existence of the β GR in skeletal muscles. In chapter 7, the presence of both the α and β glucocorticoid receptor mRNA forms in the rat gastrocnemius muscle was described. These two GR forms were found to be downregulated in the muscle even after corticosteroid inhalation, suggesting a systemic effect in the peripheral skeletal muscle after this local corticosteroid administration. It is to be expected that similar effects could be found after systemic administration, but further research is needed to study GR mRNA expression under these conditions.

In conclusion, we observed different effects of *prednisolone* and *triamcinolone* in an equipotent dose and similar treatment, on muscle wasting, function and metabolism. Both corticosteroids were found to be associated with increased muscle glycogen concentrations and an increased recovery of the peripheral skeletal muscle in between two stimulation sessions. In addition, we observed no muscle wasting and alterations in peripheral skeletal muscle performance and metabolism after *prednisolone* treatment. *Triamcinolone* treatment however, was accompanied by muscle wasting of both the diaphragm and peripheral skeletal muscles, associated with type IIx/b atrophy of the diaphragm and a decreased fatigability of the diaphragm and peripheral skeletal muscles. In addition an increased glycolytic and oxidative capacity was found. Furthermore, decreased ATP, PCr and total adenine nucleotides and creatine pools were observed, suggesting metabolic impairment. Muscle protein metabolism was also disturbed, as indicated by an increased glutamine efflux from the muscle and an increased glutamine *de novo* production and membrane transport rates.

8.4. Differentiation between corticosteroids and undernutrition

During our first experiment it was shown that muscle wasting induced by triamcinolone treatment, was a consequence of an increased energy expenditure not adequately restored by dietary intake, which takes us to the basic difference between triamcinolone treatment and nutritional deprivation. Triamcinolone treatment showed acutely a temporarily anorexia accompanied by hypermetabolism, which was also found chronically. Therefore, one control group was matched for the temporarily diminished food intake and one for the diminished body mass. Unfortunately, peripheral skeletal muscle mass reduction was not proportional to body mass reduction and could not be controlled for.

After both triamcinolone treatment and undernutrition, diaphragm muscle mass was reduced proportional to body mass reduction. On the other hand, peripheral skeletal muscle wasting after triamcinolone treatment, was more pronounced than body wasting, indicating a pronounced catabolic response. During nutritional deprivation, relative sparing of the peripheral skeletal muscle was observed. This discrepancy in muscle wasting between these two treatments, is also reflected by different atrophy patterns of the diaphragm. Triamcinolone treatment caused type II fibre atrophy, while undernutrition caused a generalised atrophy of all fibre types. Both treatments resulted in a relative increase of type I fibre cross-sectional area of the diaphragm, which could be responsible for the decreased fatigability of the diaphragm bundle *in vitro*. Other diaphragm contractility characteristics were also comparable between triamcinolone treatment and nutritional deprivation, like an increased half-relaxation time and a leftward shift of the force-frequency relationship.

Contractility of dorsiflexor hindlimb muscles however, was not comparable between these two treatments as studied in chapter 4. Undernutrition showed a decreased fatigability and external work was actually not altered when normalised for muscle mass, while triamcinolone treatment showed an increased fatigue resistance and external work. Probably muscle structure alterations cannot explain the discrepancy in *in situ* muscle performance between these two treatments.

Metabolic differences after triamcinolone treatment and undernutrition have to be considered as well. As above mentioned, triamcinolone rats were hyperglycaemic and an increased glucose influx in the hindquarter muscles was observed. Undernutrition however, showed no alterations in glucose metabolism. It was even demonstrated that triamcinolone treatment was associated with increased intramuscular glycogen levels, while undernutrition was accompanied by decreased intramuscular glycogen concentrations. In chapter 5 it was shown that muscle enzyme activities of both glycolytic and oxidative pathways were increased after triamcinolone treatment in contrast to undernutrition. These alterations in muscle metabolism could very well explain the increased muscle performance per gram muscle after triamcinolone treatment.

The alterations in muscle energy metabolism seem comparable between triamcinolone treatment and undernutrition, as reflected by decreased ATP and PCr levels and total adenine nucleotides. However, the additionally reduced creatine pool and the increased pyruvate and lactate levels in the muscle after triamcinolone treatment in contrast to undernutrition, suggest other mechanisms for the decreased muscle energy status. The alterations in muscle metabolism after triamcinolone treatment could imply metabolic impairment, while those after undernutrition imply a new metabolic equilibrium with lower ATP and PCr levels.

Surprisingly, muscle glutamine metabolism was almost comparable after triamcinolone treatment and undernutrition. Muscle wasting was accompanied by an increased glutamine *de novo* synthesis, membrane transport rates and glutamine efflux. These alterations were however, more pronounced for the triamcinolone-treated rats.

In summary, despite several similarities in muscle structure, function and metabolism after triamcinolone treatment and undernutrition, some alterations in muscle metabolism were triamcinolone specific. The most important differences were the increased glucose influx, increased glycogen levels and increased glycolytic and oxidative capacities after triamcinolone treatment. These observed differences are important to consider in the choice of adequate interventions to prevent muscle wasting induced by triamcinolone or undernutrition.

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Samenvatting

Veel chronisch zieke patiënten zoals die met chronisch hartfalen (CHF), nierfalen of obstructieve longziekten (COPD) zijn erg beperkt in hun dagelijks functioneren. Dit is niet alleen het gevolg van het slecht functioneren van het aangetaste orgaan maar spierzwakte speelt hierbij ook een belangrijke rol. Bovendien krijgen veel van deze patiënten chronisch corticosteroiden toegediend, een medicijn wat ontstekingsremmend werkt. Een van de bij-effecten van corticosteroiden is het optreden van spierzwakte. Verder vertonen veel van deze patiënten gewichtsverlies, wat ook kan bijdragen aan de spierzwakte. Aangezien chronisch corticosteroidgebruik zelf kan leiden tot ondervoeding, is het relevant om deze twee factoren te bestuderen in relatie tot spierfunctie.

Spierfunctie wordt bepaald door de massa van de spier, de opbouw (morfologie) en door de biochemische processen die zich hierin afspelen (metabolisme). Al deze componenten zijn lastig in patiënten te bestuderen en daarom is gebruik gemaakt van een rattenmodel. Hierbij kunnen ook gemakkelijk interventies worden uitgevoerd en het verstorende effect van de ziekte kan worden uitgeschakeld. We hebben gekozen voor het gefluorideerde corticosteroid triamcinolon. In dit corticosteroid is een fluoridegroep ingebouwd, waardoor het een ontsteking beter kan remmen, maar bovendien ook meerdere bij-effecten veroorzaakt zoals spierverslapping. De ratten die met triamcinolon behandeld werden, werden vergeleken met een paarsgewijs-gevoede groep, ter correctie van de verminderde voedselinname. Vervolgens werd een paarsgewijs-gewichtsgroep geïntroduceerd, omdat het lichaamsgewicht van de met triamcinolon behandelde ratten sterker daalt dan alleen door de verminderde voedselinname verklaard kan worden. Tenslotte werd een controlegroep gebruikt met een normale voedselinname, die diende als referentie voor normale onbehandelde ratten. Het diafragma (de middenrifspier) is de belangrijkste ademhalingsspier. De functie van deze spier werd bestudeerd *in vitro* (buiten de rat) zoals beschreven in hoofdstuk 2. Een bundel van het diafragma werd opgehangen in een weefselbadje en gestimuleerd. Hierbij werd de spierfunctie van de verschillende groepen vergeleken. Het viel op dat de paarsgewijs-gewichtsgroep en de met triamcinolon behandelde groep tot dezelfde veranderingen in contractiliteit van het diafragma leidden, zoals een verminderde vermoeibarheid van de spier en een verlenging van de half-relaxatietijd na stimulatie.

Een spierbundel is opgebouwd uit verschillende spiervezels die verschillende eigenschappen bezitten. Type I vezels kunnen bijvoorbeeld maar een kleine kracht opbouwen, maar zijn pas na lange tijd vermoeid. Type IIx/b vezels kunnen veel kracht opbouwen en zijn snel vermoeid, terwijl de eigenschappen van type IIa vezels ertussenin liggen. Wanneer de spiervezels gekleurd en dwars doorgesneden worden, kunnen de verschillende vezeltypen en hun oppervlakten worden bepaald. Dit werd gedaan in het diafragma van de verschillende groepen (hoofdstuk 2) en we vonden dat het oppervlak van type IIa en IIx/b spiervezels afgenomen was (atrofie) in de met triamcinolon behandelde groep vergeleken met de controlegroep. De paarsgewijs-gewichtsgroep vertoonde een vergelijkbaar beeld maar vertoonde ook type I vezel atrofie. Na een behandeling met een lagere dosis triamcinolon vertoonde het diafragma alleen type IIx/b atrofie (hoofdstuk 3). Dit werd ook gevonden, hoewel in mindere mate, na ondervoeding in de paarsgewijs-gewichtsgroep. In geen enkele groep waren de spiervezels op een andere manier aangetast (myopathie) in het diafragma.

Zoals eerder gezegd wordt de spierfunctie ook bepaald door de biochemische kenmerken in de spier. Energierijke fosfaten, zoals adenosine tri- en difosfaat (ATP en ADP) en creatine fosfaat (CrP), leveren energie voor spiercontractie. Na triamcinolonbehandeling en na ondervoeding vinden we een daling van ATP, ATP/ADP ratio, totale adenine nucleotiden,

CrP en CrP/Cr ratio in het diafragma (hoofdstuk 3). De met triamcinolon behandelde ratten hadden daarnaast ook nog een verlaagde totale creatinepool en een verhoogde concentratie van eindsubstraten van de glycolyse (pyruvaat en lactaat). Dit werd niet gevonden in de paarsgewijze-gewichtsgroep, maar deze had een verlaagde creatineconcentratie in het diafragma. Hieruit werd geconcludeerd dat deze twee groepen met behulp van verschillende metabole routes leiden tot vergelijkbare veranderingen op het niveau van energierijke fosfaten. De ondervoede groep leidt tot een verlaagde energiestatus in de spier maar herwint hierbij een nieuw metabool evenwicht, terwijl de met triamcinolon behandelde groep dit evenwicht niet kan vinden als gevolg van een verstoring tussen de glycolytische afbraakroute en het oxidatief metabolisme (de afbraakroute waar zuurstof voor nodig is).

Zoals gezegd is in hoofdstuk 2 de functie van het diafragma beschreven *in vitro*. In dit model is de spierfunctie voornamelijk afhankelijk van de substraten die in de spier (en het weefselbadje) aanwezig zijn. Hierbij speelt de spiervezelsamenstelling een belangrijke rol evenals de diffusie van substraten. *In situ* spierfunctie (in de levende rat) echter is ook nog afhankelijk van de bloedtoevoer en afvoer, die belangrijk is bij de aan- en afvoer van substraten en afvalproducten. Daarom werd de spierfunctie in een rattendynamometer bestudeerd (hoofdstuk 4), wat fysiologisch meer overeenkomt met een normale spierfunctie. Hierin werden de achterpootspieren bestudeerd van ratten behandeld met triamcinolon en met prednisolon in een vergelijkbare ontstekingsremmende dosis. De met triamcinolon behandelde ratten werden daarnaast ook weer met een ondervoede groep vergeleken. Tijdens dit experiment werd een zenuw van de achterpootspieren gestimuleerd tijdens twee sessies van 60 contracties. Tijdens de eerste sessie daalde de externe arbeid van de met triamcinolon behandelde en de ondervoede ratten als gevolg van de daling in spiermassa. Wanneer de externe arbeid uitgedrukt werd per gram gestimuleerde spier, dan was de externe arbeid aan het begin van de sessie hoger voor de met triamcinolon behandelde ratten dan voor de met prednisolon behandelde ratten. Verder daalde de externe arbeid het minst (dus minder vermoeibaarheid werd geconstateerd) in de met triamcinolon behandelde ratten vergeleken met alle ander groepen. Prednisolonbehandeling en ondervoeding hadden geen effect op de externe arbeid (per gram gestimuleerde spier) tijdens de sessies. Het herstel in externe arbeid, tijdens de 5 minuten rust tussen de sessies in, was voor de met triamcinolon en prednisolon behandelde ratten nagenoeg volledig, in tegenstelling tot de ondervoede en controlegroep. Dit zou kunnen samenhangen met het verhoogde spierglycogeengehalte na triamcinolon of prednisolonbehandeling. Vervolgens werden de activiteiten van enkele spierenzymen bestudeerd (hoofdstuk 5). De fosfofructokinase-activiteit (PFK: een glycolytisch enzym) en de glycogeen synthetase (GS) activiteit waren verhoogd in de met triamcinolon behandelde groep, terwijl er geen veranderingen waren in spierenzymactiviteiten in de met prednisolon behandelde en de ondervoede groep. Vergeleken met prednisolonbehandeling en ondervoeding was ook de citraat-synthase-activiteit (CS: enzym uit de citroenzuurcyclus) in de met triamcinolon behandelde groep toegenomen. In geen enkele groep was de activiteit van glycogeen fosforylase (GP), 3-hydroxyacyl CoA dehydrogenase (HAD: enzym uit de vetzuur β -oxidatie) en lactaat dehydrogenase (LDH: vorming van melkzuur) veranderd. Hieruit werd geconcludeerd dat de veranderingen in spierenzymactiviteiten corticosteroïd-type specifiek zijn en onafhankelijk van de samenhangende ondervoedingstoestand.

Tot nu toe hebben we alleen het koolhydratenmetabolisme en de energierijke fosfaten bestudeerd. Een spier bestaat grotendeels uit eiwit, en we verwachten ook veranderingen op eiwitniveau na triamcinolontoediening. Het metabolisme van glutamine, een belangrijk aminozuur tijdens stresssituaties, werd bestudeerd in de achterpootspier (hoofdstuk 6). Hierbij werd kortdurende (3 dagen) triamcinolonbehandeling, waarbij ook een verminderde voedselinname meespeelt, vergeleken met langdurige behandeling (14 dagen). Drie dagen

triamcinolontoediening leidde tot verhoogde glutamineconcentraties in de achterpootspier van de rat, en een verminderde in- en uitbouw van glutamine in spiereiwit. Chronische triamcinolontoediening leidde tot een verhoogde afgifte van glutamine uit de achterpootspier, een verhoogde glutamineproductie en verhoogde snelheden van transport van glutamine in en uit de spier. Chronische ondervoeding resulteerde in dezelfde veranderingen, maar in een mindere mate.

In de voorafgaande studies zijn de corticosteroïden telkens toegediend in de spier. Dit betekent dat ze in de bloedbaan terechtkomen en systemisch effecten kunnen veroorzaken in het hele lichaam. Bij patiënten worden corticosteroïden vaak lokaal toegediend, bijvoorbeeld in de vorm van inhalatiesteroïden bij patiënten met ademhalingsstoornissen. De glucocorticoid receptor (GR) speelt een centrale rol tijdens de werking van corticosteroïden. In verschillende humane weefsels is messenger RNA van twee functioneel verschillende glucocorticoid receptors, α en β gevonden. In de rat is de aanwezigheid van de β GR vorm in skeletspieren nog niet beschreven. Daarom hebben wij in de rat de expressie van de α en de β GR vorm in een perifere skeletspier bestudeerd, ook om na te gaan of er systemische effecten na corticosteroïd inhalatie optraden (hoofdstuk 7). Zowel het mRNA van de α als de β GR vorm werd gevonden in deze spier. Tenslotte worden de belangrijkste resultaten van de studies samengevat en bediscussieerd in hoofdstuk 8.

In het algemeen zijn er redelijk wat overeenkomstige effecten op de spier gevonden na triamcinolonbehandeling en na ondervoeding, waaruit geconcludeerd zou kunnen worden dat ondervoeding deels verantwoordelijk is voor de veranderingen na triamcinolonbehandeling. Er zijn echter aanwijzingen gevonden dat deze effecten door verschillende mechanismen veroorzaakt worden. Na triamcinolonbehandeling is er sprake van een verhoogde glucoseopname door de spier en verhoogde glycogeenconcentraties in de spier. Zowel de glycolytische als de oxidatieve capaciteit van de spier is verhoogd, maar de verhoogde lactaat- en pyruvaatconcentraties duiden op een verstoring tussen deze twee afbraakroutes. Prednisolonbehandeling leidt in tegenstelling tot triamcinolonbehandeling niet tot spiermassaverlies en metabole veranderingen. Om spierzwakte te voorkomen bij patiënten die chronisch behandeld worden met corticosteroïden, is het van belang om de oorzaak van deze spierzwakte te achterhalen en eventueel rekening te houden met het type corticosteroïd.

Nawoord

Diegene die nu even snel dit nawoord leest in de hoop er een smeug verhaal te vinden moet ik helaas teleurstellen. Momenteel ben ik gewoon blij dat dit boekje er eindelijk ligt en wil ik hier alle mensen bedanken die mij in deze tijd hebben geholpen en gesteund.

Allereerst mijn promotor professor Wouters, die mij in de gelegenheid heeft gesteld om mij dit onderzoek te laten doen bij de vakgroep Longziekten. Professor Wouters, hoewel ons contact weinig frequent was, was uw rol in het behouden van de lijn van het onderzoek duidelijk merkbaar. Bedankt voor uw inzicht en wetenschappelijke bijdrage!

Vervolgens mijn co-promotor Annemie Schols, zonder wiens wetenschappelijke en inhoudelijke bijdrage dit onderzoek niet tot z'n recht zou zijn gekomen. Beste Annemie, het was vast een moeilijke aanpassing om dierexperimenteel onderzoek te begeleiden terwijl die ratjes je weinig zeggen. Gelukkig kon jij mij helpen om uit de brij van resultaten juist de interessante bevindingen te benadrukken. Ik heb veel van je geleerd, waarvoor mijn dank.

Verder wil ik alle leden van de beoordelingscommissie, Prof Dr ir WHM Saris, Dr R Dekhuijzen, Prof Dr JP van Hooff, Prof Dr H Kuipers en Prof Dr C Pison bedanken voor de bereidheid dit proefschrift te lezen en te beoordelen, merci beaucoup!

De eerste twee experimenten heb ik in Leuven uitgevoerd, waar ik erg goede herinneringen aan overgehouden heb. Beste professor Decramer, hartelijk dank voor de mogelijkheid die u mij geboden heeft om deze experimenten bij u uit te voeren. Het was een leerzame en rijke ervaring. Ghislaine, hartelijk dank voor alles wat ik van jou (en van Nancy en Anja) heb geleerd! De kelder waar jullie labo in onder was gebracht was erg somber maar met jullie aanwezigheid was het er erg gezellig! Ook erg bedankt voor je wetenschappelijke bijdrage aan de twee artikels, je bent erg kritisch op een motiverende wijze. Nancy, Anja en Heleen, bedankt voor alle praktische hulp tijdens de experimenten, zonder jullie was het nooit gelukt!

Verder heb ik nog een aantal experimenten in samenwerking met Algemene Heelkunde uitgevoerd. Mick, van jou heb ik alles geleerd wat betreft operatietechnieken. Dat ik het in m'n vingers kreeg om efficiënt en nauwkeurig de operaties uit te voeren heb ik aan jou te danken. Het is jammer dat ik niet alsnog chirurg kan worden... Ook de verwerking van de eindeloze spreadsheets had ik niet zonder je hulp gekund. Wetenschappelijk heb ik veel van je geleerd. Je enthousiasme en doelgerichtheid waren erg motiverend. Gabrie, jij ook bedankt voor je hulp bij de ratten en op het lab. Het was ook erg gezellig met je te werken. Ivo, jij ook bedankt voor je rattenhulp en de hulp bij het artikel. Hans, Gabrie en Dennis bedankt voor de aminozuurbepalingen.

De bepalingen voor de energierijke fosfaten zijn uitgevoerd bij Humane Biologie. Ton Wagemakers wil ik bedanken voor deze mogelijkheid. Olav heeft mij erg geholpen met de Cobas waarop de bepalingen gedaan werden. Helaas voor hem sloeg dit apparaat altijd op tilt zodra ik in de buurt kwam (of was het andersom) en heb ik talloze keren een beroep op hem moeten doen. Heel erg bedankt, ook voor al je hulp met het artikel!

Verder wil ik Maarten en Matthijs van Bewegingswetenschappen hartelijk bedanken voor het experiment met de mooie rattendynamometer. Ook veel dank voor jullie wetenschappelijke en praktische bijdragen! Zonder alle praktische hulp van Matthijs en Jolanda had ik niet veel gekund!

Prof van der Vusse wil ik ook hartelijk bedanken voor zijn wetenschappelijke bijdrage aan een deel van het onderzoek en artikels. Uw uitleg was altijd erg duidelijk (met tekening en al) en uw enthousiasme werkte erg motiverend. Jodil bedankt voor de nucleotiden en enzymbepalingen.

Natuurlijk wil ik ook alle andere (oud) AIO's en Francine bedanken voor alle gezelligheid, etentjes en niet te vergeten ook hun praktische hulp bij de ratten experimenten! Erica, Tim, Eva, Mariëlle, Solange, Mieke, Veerle en Jeske. Later ook André, Ramon, Harry, Juanita, Diederick, Mehmed, Karin, Marja en Lotte, en natuurlijk Esther, m'n kamergenoot tijdens de laatste twee jaar. Doordat ik niet-klinisch onderzoek deed had ik ook weinig contact met de

longartsen op de afdeling, wat op zich wel jammer was. Misschien dat een gemeenschappelijke koffiekamer het contact tussen de staf en de AIO's zou bevorderen... Met Erica heb ik veel lief en leed gedeeld omdat wij als eerste twee de AIO-kamer hebben bewoond. Hier kwam Tim op een gegeven moment bij om aan zijn boekje te werken (arme hij, maar het was wel gezellig) voordat Eva en Mariëlle AIO werden.

Ik wil Solange ook nog bedanken voor hoofdstuk 7 van dit boekje. Het was erg leuk om samen met jou een experiment te doen en dan 's avonds laat nog even een Macshake halen...

Mieke en Erica, ik ben jullie ook erg dankbaar voor het feit dat we samen met Annemie over de inleiding konden discussiëren. Bedankt voor al jullie moeite en tijd. Sorry dat er uiteindelijk een heel ander stuk gekomen is, jullie hebben wel erg geholpen in de hele situatie.

Daarnaast ben ik ook nog een aantal mensen van Pathologie mijn dank verschuldigd, waaronder Erik Thunnissen, Cor Beek en John Paulissen en andere mensen van het lab op wie ik een beroep heb moeten doen.

Op het CPV heb ik ook hulp gekregen van een aantal mensen, Frans Weekers, Harry, Peter Kelderman, Brigit en alle anderen, heel erg bedankt!

Alle mensen van het secretariaat Longziekten wil ik ook nog bedanken voor hun secretariële hulp, Manon, France, Resi, Gonda, Anouk, Nicole en eerder ook Marie-José.

Het feit dat ik niet in de buurt van de universiteit woonde deed mij besluiten om te gaan carpoolen. Jeske, Marianne, Annelie bedankt voor al die uren in de auto. Lily, met jou carpoolen was altijd erg gezellig en ik dank je voor al je steun. Francine, al jouw verhalen over het lief en leed van je kroost was altijd erg ontspannend en gezellig. Karla, leuk dat jij ook in Maastricht terechtgekomen bent. Je meelevendheid en heldere blik en je mondigheid uit Amsterdam heb ik erg gewaardeerd.

Voor de laatste twee jaren heb ik naast de twee dagen kinderdagverblijf Casper vaak weg moeten brengen naar de oppas. Lieve Saskia, hartstikke bedankt voor je flexibiliteit en al je zorgen en liefde voor Casper.

Wanneer ik het even niet zag zitten hielpen de mailtjes van Karen, Solange en Inge me weer om alles in perspectief te zien, heel erg bedankt!

Ook alle verdere vrienden en zussen heel erg bedankt voor alle morele steun.

Mam, bedankt voor de mogelijkheid die jullie mij hebben gegeven om te gaan studeren en voor je hulp in moeilijke tijden.

Tenslotte dan de twee mannen in m'n leven. Bert, ik ben blij dat we zo'n sterk team zijn. Met jouw vermogen tot relativeren en jouw goede analyses heb je me enorm geholpen in deze tijd. En Casper, je bent een heerlijk vrolijk onderzoekertje. Door jou leer ik wat belangrijk is in het leven.

Curriculum Vitae

Esther de Lang was born on 8 June 1966 in Eindhoven. She went in 1978 to the Eindhoven's Protestants Lyceum and got her Athenaeum in 1985. She studied medical biology at the Free University in Amsterdam. She studied as a student the antigen-presentation of macrophages from the peritoneal fluid from continuous ambulatory peritoneal dialysis patients (vakgroep histologie en elektronenmicroscopie), the chromosomal *in situ* hybridisation techniques to locate the 11p13 band for the Wilms' tumour gen (vakgroep antropogenetica at the Academisch Medisch Centrum in Amsterdam) and the copulation behaviour of male monkeys (vakgroep vergelijkende fysiologie at the Rijksuniversiteit Utrecht). She married Bert Koerts in 1988 and finished her study in 1991. In 1992 she began as an AIO at the department of Pulmonology at the Maastricht University, which led to this thesis. Her son was born in 1997 and in that year she became a co-ordinator of the animal studies at the department of Pulmonology for two years.

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