Adaptation of signal transduction and muscle proteome in trained horses

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Adaptatie van signaaltransductie en spier-proteoom in getrainde paarden
(Met een samenvatting in het Nederlands)

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‘So eine Arbeit wird eigentlich nie fertig,
man muß sie für fertig erklären,
wenn man nach Zeit und Umständen
das mögliche getan hat.’

J.W. Goethe, Italienische Reise, 16 maart 1787

Aan mijn ouders en Wim

Contents

Chapter 1

General Introduction

For centuries the horse has been recognized for its athletic capacity. This capacity is a remnant of the past in which all horses lived in the wild and were prey animals. When sensing danger, the horse's instinctive reaction is flight rather than fight. During its domestication this, for a companion animal, less favorable characteristic was outweighted by the appreciation of its athletic capacity. In history, horses have been considered important companions for various reasons. Egyptians and Persians used horses in warfare, for riding and pulling war chariots. The use of horses in sports events dates back to the classic Olympic Games, where chariot and bareback races were organized and successful horses were honored for the rest of their lives.

Men have always been trying to increase the horses' athletic capacity in many different ways. For instance, Arabic nomads starved their horses and have them run to a well or to their "stables", whereas Romans increased the intensity by putting increasing amounts of sand in the carriage. In addition, Romans introduced breeding strategies to select specific athletic properties, and this has strongly influenced breeding in Europe.

To date, equine training practice focuses on technical skills, rather than conditional exercises. This is remarkable, because scientists in the field of human and equine exercise physiology agree that basic training principles, which are based on physical conditioning exercises, apply to humans *and* horses. From a physiological point of view physical conditioning exercises form the basis of athletics and, at least in humans, also increases health. Despite the beneficial effects on health, training is also associated with health problems in humans and horses. Especially when an elite athlete is training too vigorously during successive periods of time, health problems may occur, indicating a state of chronic overload. These practical observations have led to an increased interest by the human and horse oriented scientific community, in unraveling the mechanisms behind chronic overload, also known as overreaching or overtraining.

As an introduction to the experimental chapters in this thesis, in this chapter differences between the equine and human athlete are addressed, as well as basic principles of training and overtraining in horses and humans. Furthermore, a brief survey is given of the present knowledge regarding muscle structure and contraction, and short-term and long-term exercise-induced muscle adaptation with emphasis on metabolic signaling.

The overall aim of the thesis is to study adaptive responses in muscle upon exercise and training with the underlying goal to find early markers that play a role in the development of overtraining and that can be applied in horses and men. In the final section of this chapter the outline of the thesis is presented.

The horse versus the human athlete

Despite the fact that, from a genetic point of view, there is a large homogeneity between mammal species, it is likely that adaptations to physical training are different between humans and horses. This could be related to the genetic differences between physiological parameters important during exercise and training (Table 1.1).[1-3] These differences can, in part, be explained by 1) differences in propulsion

and posture, which might explain the differences in fiber composition of muscles, and 2) differences in metabolism. For example, the aerobic capacity of horses is approximately twice that of a human male elite athlete. This is, among other factors, caused by the fact that the horse is capable of increasing hematocrit values upon increased oxygen demand. Besides an increased aerobic capacity, horses have a higher anaerobic capacity as well. Although explanations for such differences have not yet been fully elucidated, the higher percentage of type 2 fibers in equine skeletal muscle could be one of the causes for such higher capacity.[4,5] In addition, during strenuous exercise, horses produce higher amounts of lactate in comparison to human athletes. This poses no problem to the organism since the high red blood cell volume generates a buffering capacity that is 60% higher than that of men. Another important difference, that still is under investigation, is the fact that the main energy source (max. 70%) of the horse comprises of short-chain fatty acids that are derived from the hindgut fermentation of fibers.[6] Altogether, the physiological differences between men and horses, and the fact that research of athletic training related to muscle adaptive signaling processes in horses is less well developed in comparison to that in men, give lead to the research presented in this thesis that hopefully will increase our understanding of muscle adaptive responses during equine training.

Basic principles of training and overtraining

Physical training in horses and humans, in general, aims at the following five goals: 1) delaying onset of fatigue, 2) maintenance of maximum performance, 3) improvement of skills, 4) minimizing the incidence of injuries, and 5) maintaining willingness and enthusiasm for exercise.[3,7] In athletes, the most important goal is to achieve increments in performance. To reach this goal, episodes of exercise and rest have to be well balanced. Only then, exercise results in a positive training stimulus resulting in long-term adaptations. The basic principle of training is that a single exercise session leads to

Table 1.1 Differences between human and equine athletes

	Human		Horse*	
	rest	maximal exercise	rest	maximal exercise
VO2 (ml/kg/min)	2.5	80	2.5	160
lactic acid (mM)	0.5-1.7	15	0.7-1.8	30
Hematocrit (%)	42	46	35	65
HR (beats/min)	50	195	32	240
Cardiac Output (l/min)	5	35	25	300
muscle mitochondrial density (%)	2-6		6-8.5	

** Thoroughbred racehorse*

fatigue, which in turn, results in short-term adaptive responses (Fig 1.1). When exercise is performed regularly, and training volume and training loads are increased gradually, the slight overcompensation that occurs during the recovery of a single training session leads to an overall improvement of performance (Fig 1.2). These responses result in, among others, cardiovascular, respiratory, neuromuscular, structural, endocrine and metabolic muscle adaptations that lead to an increase in performance.

When training is too vigorous, performance is reduced due to an imbalance between training volume (load) and recovery. This condition is known as chronic overload. Various stages can be distinguished, e.g., mechanical overload, metabolic overloading or over-reaching and overtraining syndrome or staleness. In mechanical overload, muscle (connective) tissue, cartilage, tendon and bone are unable to cope with the increased demands of exercise. This often results in repetitive strain injuries (orthopedic problems in horses). In contrast to mechanical overloading, which is caused by biomechanical factors, other causes of decrements in performance are related to endogenous muscle and liver glycogen stores that are of major importance for muscle contraction[8]. Intensive training predominantly relies on carbohydrates to supply energy for the working muscles. When these stores are not completely replenished during the recovery phase, metabolic overtraining may occur. This is coupled to overreaching or short-term overtraining. When the imbalance between training and rest is sustained, the total amount of physiological stress results in a dysfunction of central systems (neuro-endocrine, central nervous system) with possibly changes in behavior. This is referred to as the full blown overtraining syndrome or staleness. This syndrome is hardly ever caused by training alone, and factors like too many competitions, too much training, infectious diseases, allergic reactions, mental stress, and nutritional deficiencies contribute to the development of the full blown overtraining syndrome.[9]

Muscle tissue is, because of its total amount in the body (40-50% of the total body weight is skeletal muscle) always involved in the underlying adaptive processes during training. Muscle adaptation to exercise and training involves structural and molecular cellular signaling processes. The current

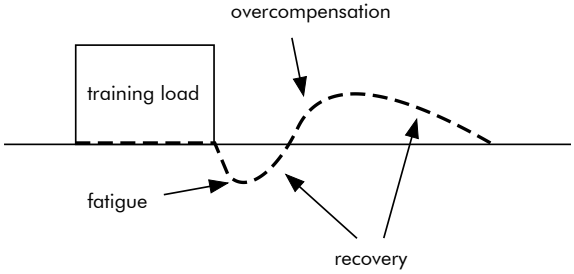

Figure 1.1
Principle of training: a single session
The performance curve (dashed line) of a single training session is shown in comparison with basic performance level. During training (training load), performance is higher but declines when the horse starts to get tired (fatigue). When the training session ends and the horse gets rest, the horse recovers from the training load resulting in a performance capacity that at first increases to the baseline level but increases further with rest (overcompensation). If the rest period is extended further the horse adapts again, and performance capacity is reduced to baseline.

knowledge of these processes will be discussed in the next section.

Skeletal muscle structure and contraction

In the embryonic phase, myoblasts fuse into multinucleated myofibers and eventually form skeletal muscle tissue. The core of the myofiber is filled with organelles, of which the myofibrils are the most abundant. Myofibrils form a bundle that extends along the muscle fiber. The main structural compounds in myofibrils are myosin, actin, tropomyosin, and troponin. These proteins are organized into sarcomeres that give the striated appearance of a transverse section of skeletal muscle (Fig 1.3).

For muscle contraction to occur, the muscle is innervated by hundreds of separate motor nerves, each of which diverges in hundreds of branches to single muscle fibers. One motor nerve and the muscle fibers it innervates, form a motor unit. The type of exercise determines how many and which motor units are activated. For muscle contraction to occur at the cellular level, the myosin head must bind to actin to form actomyosin. This complex interaction is, however, inhibited by the troponin-tropomyosin complex. During depolarization of the muscle fiber membrane, this inhibition is neutralized when the complex falls apart due to the release of calcium ions from the sarcoplasmic reticulum into the cytosol. This process initiates contraction. When the calcium ions are transported back into the sarcoplasmic reticulum, which is an ATP-dependent process, the actin and myosin binding is detached, which allows relaxation of the muscle.

Different motor units are suited for different exercise intensities and durations. The conduction velocity of the motor nerve and the contractile and biochemical properties of the muscle fibers, such as the expression pattern of myosin heavy chains (MHC), myosin light chains and ATPase enzymes, determine motor unit properties. Expression of these proteins is already determined in the first year of life, and neuromuscular activity is an important determinant in this process.[10, 11] The different properties of fibers have lead to various classifications.

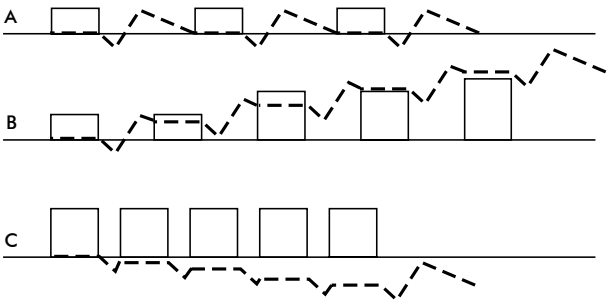

Figure 1.2
Principle of training: training strategies
The performance curve (dashed line) during different training strategies.
A. regular training sessions with the same load and relatively long rest periods do not increase performance
B. regular training sessions with increasing training loads with sufficient rest periods
C. regular training sessions with increasing training loads without sufficient rest periods decreases performance

The two main classes are slow-twitch, or type 1 fibers, and fast-twitch, or type 2 fibers. In general, type 1 fibers have a higher oxidative capacity, and are therefore more fatigue resistant whereas type 2 fibers are less fatigue resistant but can generate more power.

Type 2 fibers can be divided in 2a, 2b and 2x fibers, of which the 2a fibers have a higher oxidative capacity than 2b fibers that have a higher oxidative capacity than 2x fibers. Numerous studies have shown that some fibers have intermediate properties. For example, it has been shown that type 2b fibers can be transformed into hybrid 2a/b and type 2a fibers with chronic endurance exercise,[12-14] whereas inactivity leads to a shift in the opposite direction.[15] The majority of the available research suggests that

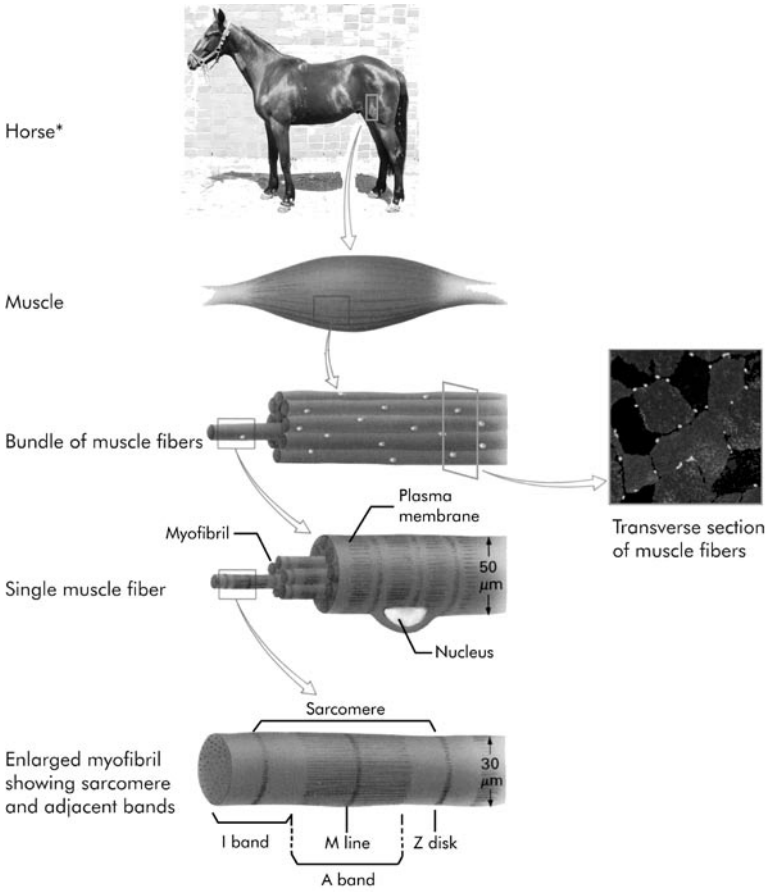

Figure 1.3
General muscle structure
*Adapted from Lodish, H. et al. Molecular cell biology, 3rd ed, 1998, pg 1023. * Morris, one of our test animals*

type 2a fibers do not transit into type 1 fibers due to training. These adaptive processes in fibers often relate to metabolic changes.

Metabolic molecular signaling upon exercise

The release of free calcium in muscle contraction itself plays an important role as a feed-forward mechanism. Calcium release, in a concerted action with the energy and redox state, initiates an important series of metabolic molecular cellular signaling events.

Energy required for muscle contraction is supplied in the form of ATP by either aerobic or anaerobic processes. Glucose, fatty acids, and to a lesser extent amino acids form the substrates for anaerobic energy supply, whereas anaerobic generation of ATP is formed by the release of energy from ATP, phosphocreatine and/or by glucose-coupled ATP formation in the glycolytic pathway (Fig 1.4). Glucose is the main carbohydrate energy source. It can be derived from extracellular stores or from glycogen stores in the muscle cell. When muscle cells require glucose from extracellular stores, for the replenishment of their intracellular glycogen or

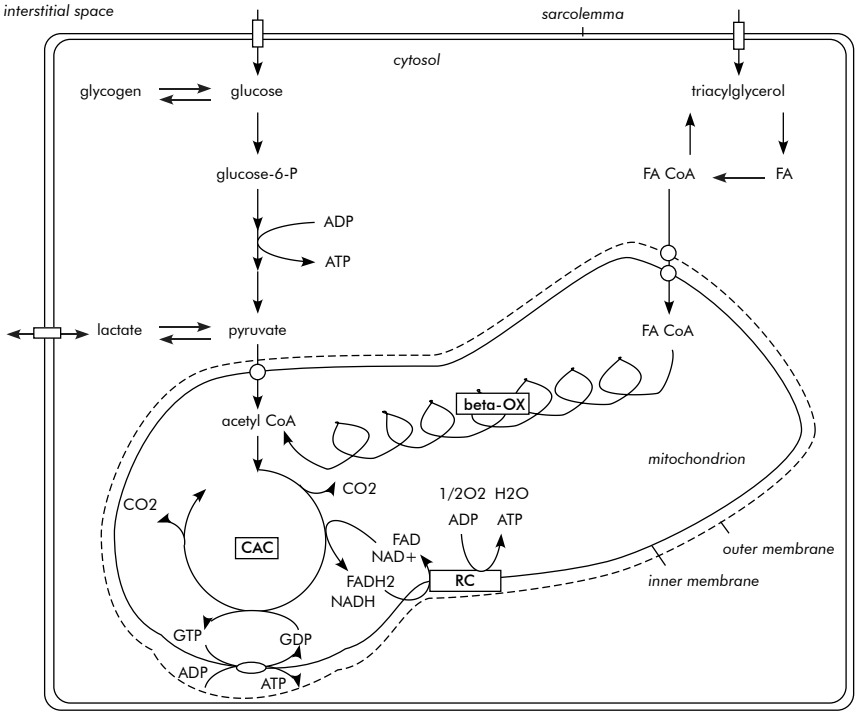

Figure 1.4
Muscular energy converting pathways
CAC, citric acid cycle; beta-OX, beta-oxidation; RC, respiratory chain; FA, fatty acid.

directly for generating energy, glucose has to be taken up from the blood stream and transported over the muscle cell membrane. Specialized transporter proteins facilitate glucose transport across the cellular membrane. More than twelve different transporter proteins have been found, but GLUT4 is to the best of the present knowledge the most important glucose transporter in muscle cells.[16] Its expression and location is regulated by insulin, exercise and training. Under resting conditions, GLUT4 resides in intracellular stores, whereas upon muscle contraction or insulin stimulation, GLUT4 translocates to the sarcolemma,[17] where it passively facilitates glucose transport resulting in glucose uptake. When glucose has been transported over the membrane, it is immediately converted to glucose-6-phosphate by the enzyme hexokinase. This enzymatic reaction prevents the formed glucose-6-phosphate from being transported back into the extracellular space. Under resting conditions, e.g., when carbohydrate stores have to be replenished and insulin levels are relatively high, most glucose-6-phosphate is converted to glycogen, by glycogen synthase. During muscle exercise, when there is a need to generate energy, glucose-6-phosphate enters the glycolytic pathway to form pyruvate, which is then metabolized in the mitochondria to form ATP. In addition, glycogen can be converted by glycogen phosphorylase into glucose-1-phosphate and also enter the glycolytic pathway.

As mentioned above, GLUT4 translocation is stimulated both by insulin and muscle contraction. The signaling cascade preceding the actual translocation process by insulin and contraction are essentially independent of each other (Fig 1.5). Several candidates have been suggested to mediate

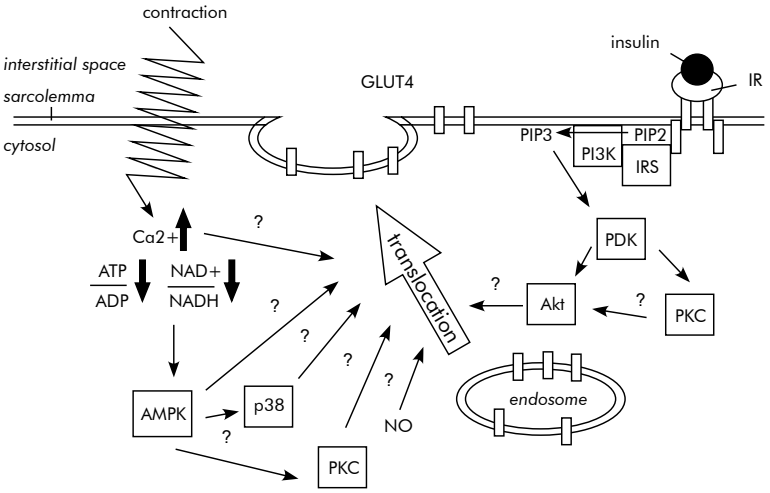

Figure 1.5
GLUT-4 translation
AMPK, AMP-activated protein kinase; PDK; PKC, protein kinase C; IR, insulin receptor; NO, nitric oxide; PI3K, phosphoinositol-3-kinase; p38 MAPK, p38 mitogen-activated protein kinase
Adapted from Ryder et al. 44

contraction-induced GLUT4 translocation. For example, the enzyme AMP-activated protein kinase (AMPK), which functions as a metabolic sensor and is activated when ATP/ADP or CrP/Cr ratios are low (and therefore AMP is elevated), has been put forward as a key factor in this process. Activated AMPK acutely switches on catabolism by phosphorylation of acetyl coenzyme A carboxylase (ACC). However, the mechanism by which AMPK could actually mediate GLUT4 translocation is still unclear. In addition, glycogen levels,[18,19] nitric oxide (NO),[20] and protein kinase C (PKC)[21-24] are from a theoretical point of view attractive candidates. In all cases, calcium plays a central role. For example, it has been shown that conventional and novel PKC isoforms are calcium-sensitive. It is known that multiple pathways are simultaneously involved in contraction-mediated GLUT4 translocation and that this may depend on muscle fiber type.

Insulin-dependent GLUT4 translocation is a receptor-mediated process that activates a cascade of enzymatic reactions involving different intermediates. The most important intermediates are insulin receptor substrate 1 (IRS-1), phosphatidyl inositol-3-kinase (PI3-K) and 3-phosphoinositide-dependent protein kinase-1 (PDK1). Activation of this cascade simultaneously liberates calcium from intracellular stores, thereby activating some calcium-sensitive PKC isoforms. The activation of the classical isoforms α and β is thought to serve as a negative feedback system to the insulin receptor.[25,26] Other PKC isoforms might be activated by PDK1 or in conjunction with PDK1 to induce GLUT4 translocation. PKC activation is however not essential for GLUT-4 translocation and is more likely to play a regulatory role.

The optimal function of metabolic processes is crucial for performance. The beneficial effects of training can in part be explained by the optimization of these pathways. For example, GLUT4 expression and translocation efficiency are increased upon training.[27, 28] Also, neuronal NO synthase expression is higher in trained horses.[29] Finally, insulin sensitivity is increased upon exercise and upon training.[28, 30] These effects will be explained further in more detail in the next section.

Muscle adaptation and molecular signaling

The signaling cascades through which GLUT-4 translocation is induced, also diverge into signals aimed at long-term adaptation. These affect cellular differentiation and proliferation through transcriptional regulation via activation of different mitogen-activated protein kinases (MAPKs) (Fig 1.6). MAPKs comprise a family of three types of kinases that repond to different extracellar signals. In general, these kinases are activated in the cytosol, and subsequently translocate to the nucleus or activate other proteins that translocate to the nucleus. There they activate specific transcription factors, hence affecting protein transcription. This process occurs in essentially all different cell types in the body.

The first class of MAPKs are extracellular signal-related kinases 1 and 2 (ERK1/2). ERK1/2 were reported to be activated in muscle tissue upon exercise in men,[31-33] and several studies suggest that contraction *per se* is sufficient for this activation.[31,34-36] Also downstream targets of ERK1/2, p90rsk and MSK1 and MSK2, were activated upon exercise.[37,

[38] Contraction is likely to activate mechanosensitive receptors like integrins, that are coupled to phospholipase C and results in activation ERK1/2 through a cascade of proteins including Ras, PI3K and Raf. Hormones like insulin, growth hormone (GH), insulin-like growth factor type 1 (IGF-1) and mechanogrowth factor (MGF), activate ERK1/2 through receptor tyrosine kinases (RTKs) that share the Ras-Raf cascade. In a manner similar to insulin, IGF-1 can activate ERK1/2 through the IGF-1 receptor and Ras-Raf signaling. In contrast to insulin and GH which are produced not in the vicinity of muscle cells, IGF-1 and MGF are locally secreted by the contracting muscle and hence could effect muscle adaptation in a paracrine and autocrine fashion.[39]
Another MAPK, p38 MAPK, which shares some kinase substrates with ERK1/2 was also found to be activated upon exercise in men.[31,38,40] p38 MAPK responds to cellular stress, like metabolic and oxidative stress. And, therefore, this kinase is likely to play a role in contraction-induced activation of AMPK. Although different p38 MAPK isoforms exist, little is known about isoform-specific biological responses.
Finally, the last MAPK pathway that is activated upon exercise is that of c-Jun NH2-terminal kinase (JNK).[31,41] JNK activation is most prominent in injury-promoting exercise.[42]
Differences in MAPK activation upon different modes of exercise and contraction [31,33,35,42,43] suggest regulation of specific genes and gene patterns.[28] More research is necessary to address the specific role of exercise and training on the molecular signaling pathways.

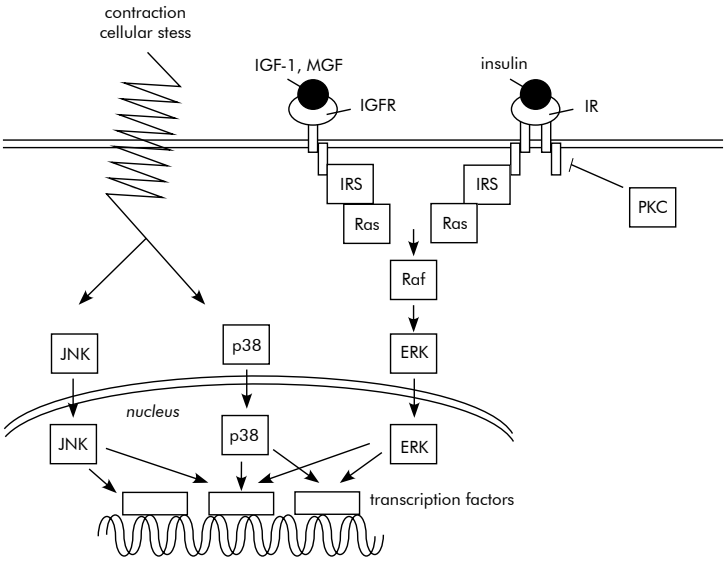

Figure 1.6
Signaling pathways of exercise and insulin
IR, insulin receptor; IRS, insulin receptor substrate; IGFR, insulin-like growth factor receptor; IGF-1, insulin-like growth factor 1; MGF, mechanogrowth factor; ERK, extracellular regulated kinase; JNK, c-Jun NH2-terminal kinase; p38, p38 mitogen-activated protein kinase; PKC, protein kinase C

Outline of the thesis

Most of the knowledge on signaling is based on muscle research in humans and laboratory animals. This has provided us with a more detailed picture of adaptive processes that goes along with physical training in human athletes. The physiological differences between (trained) horses and humans, however, might indicate that molecular adaptive responses, whether positive or negative, are not entirely analogous between these species. Although our understanding of equine exercise physiology has greatly increased over the last 20 to 30 years, molecular signaling adaptations in equine muscle have not been thoroughly investigated yet. Such research endeavors could give us additional information regarding equine exercise physiology. Therefore, the major aim of the present thesis was to investigate the effects of normal training and periods of intensified training on muscle adaptive (signaling) processes that could lead to the identification of early muscle-derived markers for overtraining.

Chapter 2 describes and discusses the original research design of the whole training study. The study comprised of periods of normal and intensified training and the use of standardized exercise tests to monitor athletic performance. Biopsy samples taken during this study enabled the investigation of signaling enzymes and proteins in equine skeletal muscle at rest and upon training and intensified training.

Because PKC isoforms are important regulators of cell signaling, their localization in equine muscle fiber at rest was investigated in **chapter 3**. Certain PKC isoforms are known to influence MAPK signaling. Hence, it was hypothesized that MAPK intermediates in muscle, known to be involved muscle adaptive processes in humans, are activated upon acute exercise in horses as well. MAPK signaling pathways were, therefore, studied at rest and upon acute exercise (**chapter 4**). In addition, in **chapter 5** the question whether or not these MAPK pathways are adapted upon training and intensified training was addressed. The finding of muscle and fiber specific properties regarding signaling proteins, led to the presumption that these differences could affect overall muscle function. It was postulated that training might induce muscle fiber transitions. This was investigated by measuring the portion of myosin heavy chain isoforms in muscle upon training (**chapter 6**).

In **chapter 7** the use of proteomic technology was explored, which is new to equine science and medicine, by comparing healthy horses and horses suffering acute tying-up. These techniques were subsequently applied to biopsy specimens of the training study, to investigate the assumption that alterations in global muscle protein expression are induced upon training and/or intensified training, independent of fiber distribution (**chapter 8**). Intensified training potentially reverses or opposes these normal adaptive processes upon training. This could reveal potential early markers for overtraining.

Finally, in **chapter 9** the results of the thesis are integrated and discussed in the light of the present literature regarding exercise, training and overtraining, and with respect to muscle-derived early markers of overtraining.

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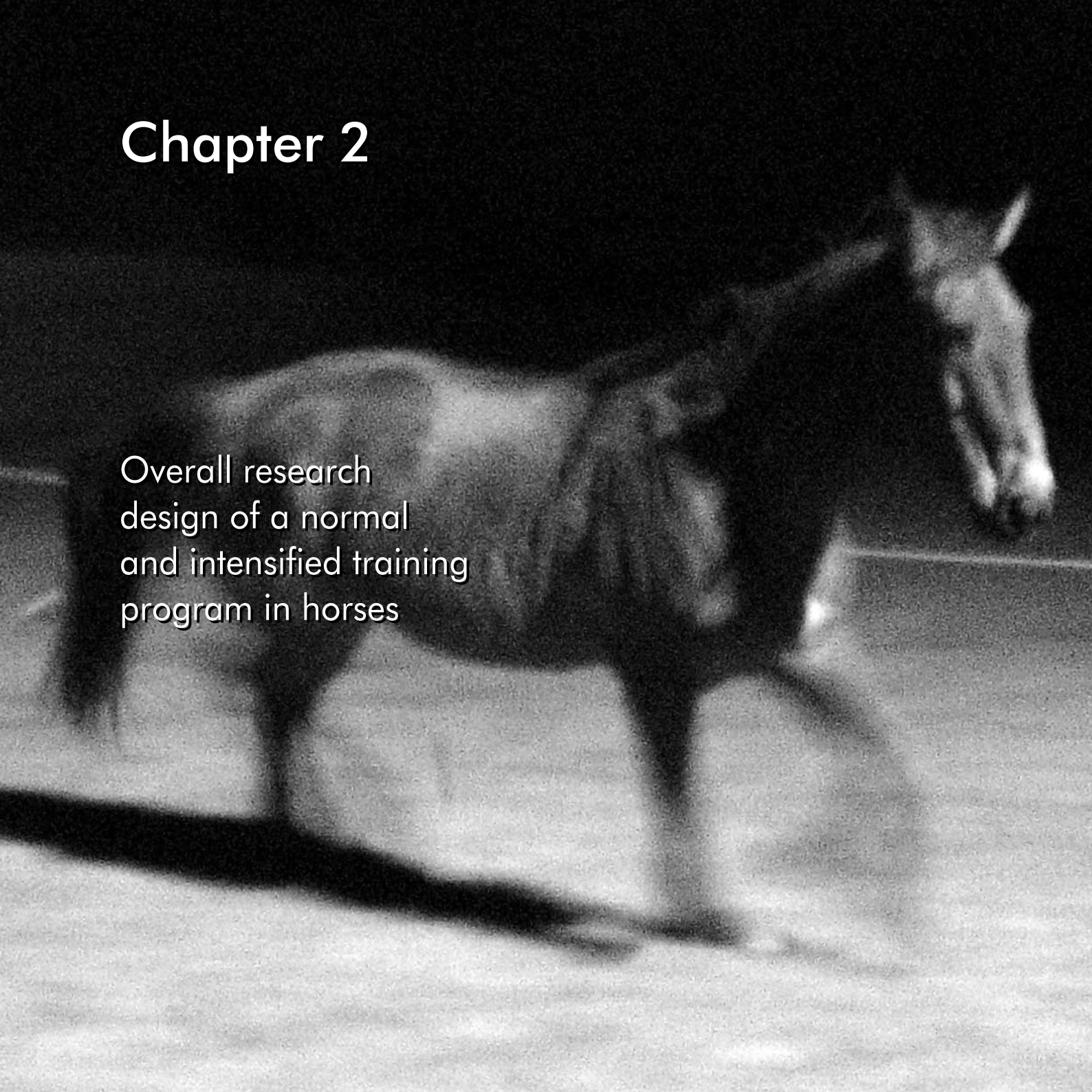
Chapter 2



Overall research design of a normal and intensified training program in horses

The research design in this thesis is based on the findings in human studies, and is specifically designed to investigate a wide range of physiological parameters, that are under influence of normal and intensified physical training. Although other aspects during physical training, like behavioral changes, changes in the growth hormone axis, and metabolic changes and specific skeletal muscle alterations were studied by different research teams, it is compulsory to compare findings in skeletal muscle with the outcomes of the general training parameters. Therefore, this chapter also provides more insight in the used training methods.

Original Research Design

Horses

A total of twelve healthy Standardbred geldings were included in the study. Horses had an average age of 19 ± 2 (SD) months and an initial mean body weight of 386 ± 45 kg at the start of the initiation phase (*see* below). The horses were healthy and had no known history of health problems. The horses were individually housed in boxes, and were fed according to their individual requirements. Water and salt blocks were available *ad libitum*. The study was approved by the Committee on Animal Welfare at the Faculty of Veterinary Medicine, Utrecht University.

Training

The training period, of which a schematic representation is shown in Fig 2.1 and Table 2.1, comprised of a total of 32 weeks. This period was divided into four parts e.g.: (1) an initiation phase of 4 weeks in which animals were acclimatized to the treadmill (Kagra, Graber HG, Switzerland) by performing light exercise for 20-45 min a day, 3-4 days a week; (2) a training phase of 18 weeks, consisting of endurance training and high intensity training (HIT) for 4 days a week; (3) an intensified training phase of 6 weeks, in which training intensity and training volume were further increased and resting days were eliminated during the last three weeks; (4) a recovery phase of 4 weeks in which training was performed at lower intensity.
Horses were divided in pairs according to their age. Per pair, one horse was randomly assigned to the increased intensity protocol in phase 3 whereas the other horse continued the "normal" training load of phase 2. Because of organization purposes, pairs of horses were trained in two groups in two consecutive years (year 1, three pairs; year 2, three pairs).
During the training period (phase 2), days of endurance training were alternated by days of high intensity training. Each training session started with 30 min of walking in a horse-walker and an 8-min warming-up on a treadmill (4 min at 1.6 m/s and 4 min at 4.5 m/s). An endurance training session consisted of continuous level running for 20-24 min at 60% $HF_{est\text{-}max}$ (*see* section 'Adaptations of the original design' for explanation) or 16-18 min at 75% $HF_{est\text{-}max}$. Each high intensity training session consisted of three 3-min bouts or four 2-min bouts at 80% interspersed with 3-min or 2-min bouts at 60% $HF_{est\text{-}max}$. In phase 3, horses assigned to the intensified program performed endurance training at 75% $HF_{est\text{-}max}$ and high intensity training at 75% and 80% $HF_{est\text{-}max}$. In phase 4, horses performed endurance training for 20 min at 60% and 70% $HF_{est\text{-}max}$ for 4 days a

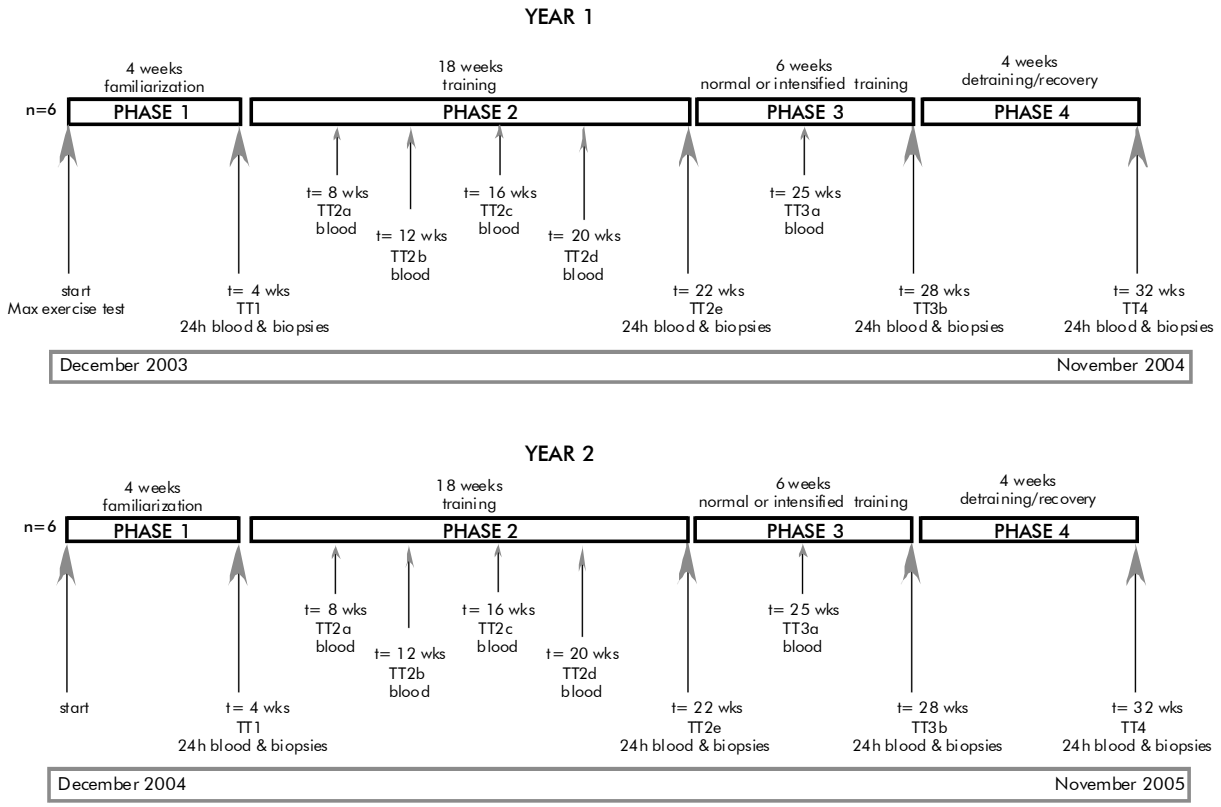


Figure 2.1
Timeline of training and tests
Three pairs of horses were trained in year 1 and three pairs of horses were trainined in year 2. One horse of each couple was admitted to the intensified training program in phase 3. Pairs entered the study with an interval of approximately two weeks because of organizational purposes. Time-trials (TT) were performed regularly to monitor performance level.

week.
On rest days horses walked in a horse-walker for 60 min. Body weight was measured once every week during phase 1 and 2, and twice a week during phase 3 and 4.

Incremental exercise test

At the start of the training period in the first year, an incremental maximal exercise test was performed in 6 horses to obtain the individual maximal heart rate. After 30 minutes walk in the horse-walker the horses trotted on a treadmill for 2 minutes at 5 m/s and 2 minutes 6 m/s. Intensity was subsequently increased (1 m/s) every 2 minutes until the horses started to gallop. From then on speed remained the same but inclination was increased (1%) every 2 minutes until they reached fatigue. Fatigue was defined as the point at which the horse could not keep up with the treadmill despite human encouragement. Heart rate was monitored using a Polar S610, which was

Table 2.1 Training loads

	PHASE 1				PHASE 2
	week 1	week 2	week 3	week 4	wks 5-22
ET					
intensity (% of HFest-max)	30%	30%	40%	50%	60%/75%
duration (min)	20-30	25-45	30-45	35-45	20-24/16-18
frequency/wk	3	4	4	4	2
HIT					
intensity (% of HFest-max)					80%
duration (min)					3x3'/4x2'
frequency/wk					2

	PHASE 3			PHASE 4
	NORMAL	INTENSIFIED		
	wks 23-29	wks 23-25	wks 26 - 28	wks 29-32
ET				
intensity (% of HFest-max)	60%/75%	75%	75%	60%(3x)/70%(1x)
duration (min)	20-24/16-18	16-18	16-20	20
frequency/wk	2	3	3	4
HIT				
intensity (% of HFest-max)	80%	80%	80%	
duration (min)	3x3'/4x2'	3x3'	4x3'	
frequency/wk	2	3	4	

ET, endurance training session; HIT, high intensity training session.

set to store heart rates every 5 seconds. The training intensity (velocity and inclination) was adjusted according to the measured heart rates on a weekly basis.

Time-trial procedures

A schematic representation of the time-trial procedures is shown in Fig 2.2.
Every four weeks a time-trial (TT) was performed to determine the level of performance. Horses walked prior to the time-trial for 30 minutes in a horse-walker. Next, treadmill warming-up consisted of walking (1.6 m/s) and slow trotting (4.5 m/s) on a treadmill for 5-10 min. This was immediately followed by the actual time-trial consisting of trotting at speeds between 6 and 8.5 m/s and inclinations between 0 and 4%, corresponding to 80% $HF_{est\text{-}max}$. Cooling-down consisted of walking on the treadmill for 5 min and 30 min of walking in a horse-walker.
Heart rate was constantly monitored to adjust the time-trial speed and inclination to reach 80% of $HF_{est\text{-}max}$, using a Polar S610, which was set to store mean heart rates every 5 s.

Venous blood was drawn before, every five minutes during and 0, 5, 10, 15 min and 1, 3, 6 and 24 h after the time-trials from a catheter in the jugular vein. Blood was stored on ice, and later lactate and pH were routinely analyzed in whole blood samples from samples taken at the start, during and immediately after the time-trial. All other parameters were performed at a later stage from samples of plasma and serum that was stored at -20°C.
Muscle biopsy specimens were taken before and after each time-trial at the end of the four training phases (TT1, 2e, 3b, 4; *see* Fig 2.1). Biopsies were taken with a 7 mm diameter modified Bergström needle before and after each time-trial. Biopsies were taken from the left and the right vastus lateralis and pectoralis descendens muscle. Blood was carefully removed and each biopsy was divided in two parts. One part was immediately frozen in liquid nitrogen and used for biochemical analysis. The other part was frozen in isopentane cooled to its melting point in liquid nitrogen and used for immunohistochemical studies. All samples were subsequently stored at -80°C until further processing.

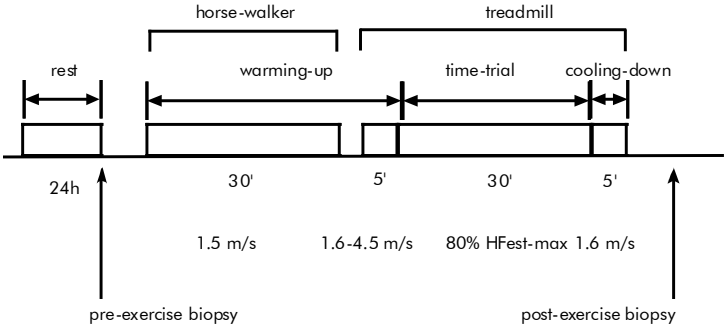


Figure 2.2
Schematic representation of the time-trial procedure

Adaptations of the original design

During the training program observations were encountered that required adaptation of the original research design. These adaptations are explained below.

Use of heart rate to guide intensity of training sessions and time-trials

In the original research design it was planned to use the individual maximal heart rate in guiding training intensity, e.g., velocities and inclinations, of training sessions and time-trials.
In the first year of the training study, 6 horses performed an incremental exercise test to obtain the maximal heart rate and an average value of 221 ± 17 bpm was found. However, a maximum heart rate, as determined by a plateau in heart rate despite an increase in exercise load, was not reached in all horses. Therefore the found value can only be considered as average peak heart rate.

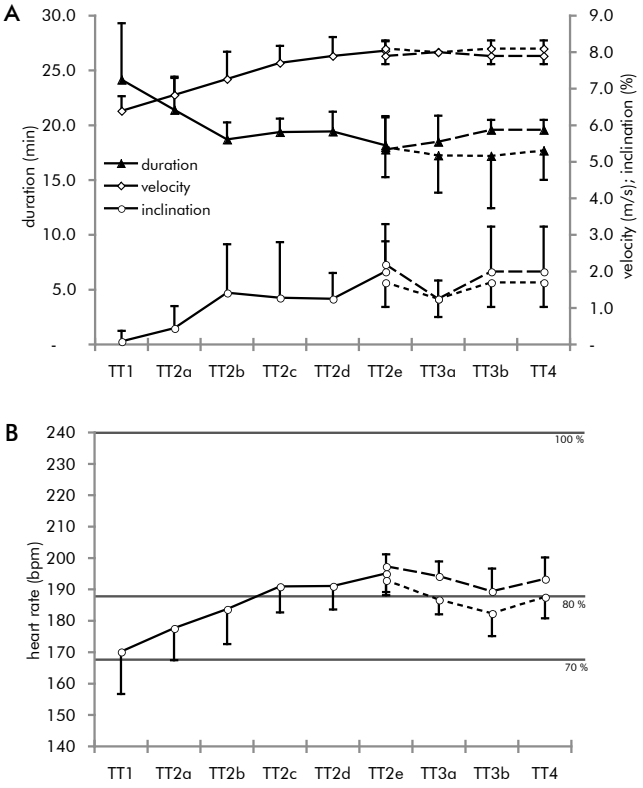


Figure 2.3
Velocity, slope duration and heart rates during time-trials
Velocity, inclination and duration [A] and heart rate [B] of consecutive time-trials are presented as mean ± SD; Continuous lines represent phase 1 and 2 (n=12); Dashed lines represent normal training group in phase 3 and 4 (n=5); Dotted lines represent intensified training group in phase 3 and 4 (n=5). Horizontal lines indicate % HFest-max in panel B.

Since the incremental exercise test posed a high risk of injury in these untrained horses, it was decided not to perform additional maximal exercise tests to obtain individual maximal heart rates. Based on the maximal heart rate of horses that indeed reached a plateau heart frequency during the maximal exercise test, and based on measurements of maximal heart rate in 2-year old Standardbred stallions in a previous training study,[1] an estimated maximal heart rate ($HF_{est\text{-}max}$) of 240 bpm was predicted. This $HF_{est\text{-}max}$ was used for all horses in both years of the study to guide training and time-trials.

Adaptation of time-trial procedures

Adaptations were especially needed in the time-trial procedure. In time-trial 1 in the first year of the training study it was observed that the horses were unable to trot at speeds and inclinations corresponding to 80% $HF_{est\text{-}max}$. They already started to gallop before this heart frequency was reached. Therefore, it was decided to perform the time-trials at the highest possible heart rate at which the horses were still able to trot. The average velocities, slopes, duration and heart rates are depicted in Fig 2.3.
At the end of phase two, horses indeed did reach 80% $HF_{est\text{-}max}$ and consecutive individual time-trials (TT3a/3b/4) were conducted at the same speed and inclination to monitor performance.
During the first time-trials of the first year it was observed that the planned duration of the time-trial of 30 minutes was too long for the animals under study and therefore, we reduced the time-trials to 20 minutes. This explains the variation in durations of these time-trials as shown in Fig 2.3.

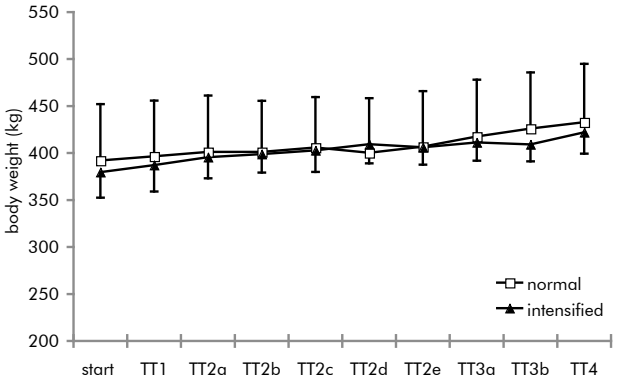


Figure 2.4
Body weight during the training program
Body weight is presented as mean ± SD of horses assigned to the normal training group and to the intensified training group during the entire training program; TT1-2e (n=6), TT3a-4 (n=5).

Observations and evaluation

Preliminary evaluation revealed that performance of horses increased upon training because horses were able to complete the time-trial at higher speeds and inclination at the end of phase 2 (TT2e) compared to phase 1 (TT1).

The aim of the research presented in this thesis was to find early markers for overtraining. Overtraining is associated with deterioration in performance, behavioral changes and possibly loss of body weight. Therefore, these parameters were monitored during the training study.
One couple suffered from serious orthopedic injuries at the end of phase 2 in the second year, and was taken out of the study during phase 3. This might indicate that these horses suffered mechanical overloading.
In phase 3 we compared the effect of normal and intensified training on level of performance. Preliminary results suggest that mean physical performance in TT3a/3b/4 was not significantly lower in the intensified training group compared to the normal training group although the mean duration of the time-trials of the intensified training group was decreased.
Other signs that possibly reflect overtraining were the change in gate at the planned speeds during training and time-trials. Some horses were unable to continue trotting and started galloping. In addition changes in behavior, both during exercise and during rest periods were found (E. Roelfsema, M. van Dierendonck, personal communications).
Animals significantly gained weight during the training period (Fig 2.4). This increase in body weight could be caused both by training, suggesting an increase in muscle mass, and by growth, because young horses were used that were not fully-grown. All horses showed a small temporary decrease in weight during some time points in the training phase (7 ± 3 (SD) kg). But there was no difference between the normal and intensified training group, which suggest that overtraining did not result in loss of body weight.

Final remarks

Although it was not easy to find clear signs of overtraining, it is likely to assume that adaptations in skeletal muscle signaling and structure could, in part, be responsible for the observed changes in performance upon training and intensified training. Furthermore, peripheral changes in skeletal muscle metabolic signaling processes might precede central changes that possibly have some involvement in the development of the full-blown overtraining syndrome. Therefore, in the next chapters we will focus on muscle metabolic process during exercise and (intensified) training.

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Chapter 3

Immunohistochemical identification and fiber type specific localization of protein kinase C isoforms in equine skeletal muscle

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Abstract

Objective—To investigate whether protein kinase C (PKC) isoforms are expressed in equine skeletal muscle and determine their distribution in different types of fibers by use of immunofluorescence microscopy.

Animals—5 healthy adult Dutch Warmblood horses.

Procedure—In each horse, 2 biopsy specimens were obtained from the vastus lateralis muscle. Cryosections of equine muscle were stained with PKC isoform (α, β1,β2,δ,ε, or ζ)-specific polyclonal antibodies and examined by use of a fluorescence microscope. Homogenized muscle samples were evaluated via western blot analysis.

Results—The PKC α, β1, β2, δ, ε and ζ isoforms were localized within the fibers of equine skeletal muscle. In addition, PKC α and β2 were detected near or in the plasma membrane of muscle cells. For some PKC isoforms, distribution was specific for fiber type. Staining of cell membranes for PKC α was observed predominantly in fibers that reacted positively with myosin heavy chain (MHC)-IIa; PKC δ and ε staining were more pronounced in MHC-I positive fibers. In contrast, MHC-I negative fibers contained more PKC ζ than MHC-I positive fibers. Distribution of PKC β1 was equal among the different fiber types.

Conclusions and Clinical Relevance—Results indicated that PKC isoforms are expressed in equine skeletal muscle in a fiber type-specific manner. Therefore, the involvement of PKC isoforms in signal transduction in equine skeletal muscle might be dependent on fiber type.

Introduction

Protein kinase C (PKC) plays a major role in cell signaling.[1] In skeletal muscle, different atypical PKC isoforms have been identified and linked to carbohydrate metabolism. Although the involvement of PKC isoforms in carbohydrate metabolism has been a matter of discussion.[2] Results of recent studies indicate their involvement in insulin signaling and thus indirectly in cellular glucose uptake and metabolism.[3,4]

On the basis of the manner of activation, the PKC family can be categorized into 3 distinct classes, each consisting of different isoforms. The classical isoforms of PKC are α, $\beta 1$, $\beta 2$, and γ that are calcium- and diacylglycerol-dependent for their activation and use phosphatidyl serine as a cofactor. Other PKC isoforms δ, ε, η, and θ are calcium-independent and only require diacylglycerol for their activation and use phosphatidyl serine as a cofactor. In addition, PKC ζ and λ (also known as ι) belong to the class of atypical PKC isoforms because these only require phosphatidyl serine as a cofactor. The PKC family has a wide variety of substrates in numerous cell types including receptors, G proteins, enzymes, cytoskeletal and nuclear proteins, and proto-oncogene products.[1] Identification of which isoform is linked to which substrate is only partly elucidated.

In skeletal muscle, PKC isoforms are linked to glucose transporter protein-4 (GLUT-4) translocation and the regulation by insulin signaling.[5-8] In addition, PKC has been associated with diabetes mellitus and insulin resistance[9-13] and could play a role in excessive glycogen accumulation in equine muscle such as that associated with polysaccharide storage myopathy in horses.[14] Interestingly, Valberg et al.[15] found that type 2 muscle fibers were primarily affected in polysaccharide storage myopathy in horses.

To the authors' knowledge, PKC isoforms have been identified biochemically in skeletal muscle,[16,17] but their exact localization within muscle fibers has not been described in detail. This is surprising because the subcellular localization of PKC isoforms can provide information concerning their possible function in the physiologic functions of muscle. The purpose of the study reported here was to investigate whether PKC isoforms are expressed in equine skeletal muscle and determine their distribution in different types of fibers by use of immunofluorescence microscopy. We hypothesized that PKC isoforms are localized to specific types of muscle fibers.

Materials and Methods

Animals

Five healthy Dutch Warmblood horses owned by Utrecht University were used in this study. The mean age ± SD of the horses was 6.8 ± 1.8 years. There were 2 mares and 3 geldings with a mean weight of 633 ± 48 kg. The study was approved by the Committee on Animal Welfare of the Faculty of Veterinary Medicine, Utrecht University.

Biopsy specimens

Two percutaneous needle biopsy specimens were taken under local anesthesia with lidocaine hydrochloride plus adrenalin (Alfacaine 2% plus adrenalin (100 ml), Alfasan, Woerden, The Netherlands) using a modified Bergström needle

with a diameter of 7 mm. The biopsy specimens were obtained from the vastus lateralis muscle at a point located 15 cm ventral from the center of the tuber coxa and 10 cm caudal from the cranial border of the vastus lateralis muscle; the specimens were obtained from 1 incision at a depth of 5 cm. The vastus lateralis muscle was selected for this study because training has shown to induce glycogen disposal and changes in metabolic enzyme activities in this muscle.[18] In addition, the triceps brachii and the pectoralis descendens muscles were evaluated in the same way (data not shown). For these muscles biopsy procedures were identical as those for the vastus lateralis muscle. Biopsy specimens from the triceps brachii were obtained at a point located 5 cm above the olecranon and 3 cm cranial of the caudal border of the triceps brachii muscle; the specimens were obtained from 1 incision at a depth of 4 cm. Biopsy specimens from the pectoralis descendens muscle were obtained at a point 20 cm caudal of the imaginary line through the shoulder joints in the middle of the muscle; the specimens were obtained from 1 incision at a depth of 4 cm. For immunohistochemical studies, 1 biopsy sample was frozen in isopentane that was cooled to melting point in liquid nitrogen. The other sample was frozen immediately in liquid nitrogen and used for biochemical analysis.

Homogenization of muscle specimens

Frozen muscle was homogenized in ice-cold homogenization buffer (20 mM Tris-HCl, 10 mM EDTA, 1% Triton-X100, Complete protease inhibitor cocktail (Roche, Mannheim, Germany), pH 7.4) by use of a tissue homogenizer and centrifuged (11,000 x g for 30 minutes at 4°C). The supernatant was diluted with an equal volume of sample buffer, boiled for 4 minutes and centrifuged (10,000 x g for 5 minutes at 20°C). Processed samples were stored at -20°C until electrophoresis was performed.

Evaluation via western blot analysis

Homogenized muscle samples were separated via 10% polyacrylamide gel electrophoresis as described by Laemmli[19] and transferred to nitrocellulose for 1 hour (100 V, 4°C) in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nitrocellulose sheets were blocked with 2% non-fat dry milk and 0.05% Tween-20 in PBS solution for 30 minutes. Incubation of the nitrocellulose sheets with anti-PKC specific antibodies (sc-208/sc-209/sc-210/sc-937/sc-214/sc-216, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) (diluted 1:500 in 0.2% non-fat dry milk in PBS) was performed for 16 hours at 20°C with gentle shaking. Blots were washed 3 times (10 min/wash) in PBS solution containing 0.05% Tween-20 and incubated for 2 hours with horseradish peroxidase-conjugated swine antibodies raised against rabbit IgG or horseradish peroxidase-conjugated goat antibodies raised against goat IgG (DAKO, Glostrup, Denmark) diluted 1:5000 in 2% non-fat dry milk in PBS solution containing 0.05% Tween-20. Blots were washed 6 times (15 min/wash) in PBS with 0.05% Tween-20 and for 10 minutes with PBS. Chemiluminescence was performed with Super Signal West Dura Extended kit (Pierce, Rockford, Ill., USA) and visualized with a FluorS Imager and analyzed by use of QuantityOne software program (Bio-Rad Laboratories, Hercules, Calif., USA).

Immunocytochemical evaluation of frozen muscle tissue sections

Muscle biopsy specimens were cryosectioned (5 μm), thaw-mounted on glass slides, and air-dried at 20°C for 3 hours and stored at -80°C. Prior to staining, sections were thawed and dried for 30 minutes. Sections were treated with PBS solution containing 0.5% Triton X-100 for 5 minutes, washed with PBS solution for 5 minutes, and probed for 2 hours with rabbit polyclonal PKC, mouse myosin heavy chain (MHC) and mouse laminin specific antibodies (A4.840 and N2.261 (developed by H.M. Blau) and 2E8 (developed by E. Engvall) Developmental Studies Hydridoma Bank, Iowa City, Iowa, USA) diluted 1:25 in PBS solution containing 1% bovine serum albumin. After washing 3 times (5 min/wash) with PBS solution, the sections were incubated with goat polyclonal Alexa-conjugated antibodies (Molecular Probes, Leiden, The Netherlands) raised against rabbit IgG, mouse IgM, and mouse IgG diluted 1:100 (Alexa350) or 1:200 (Alexa488 and Alexa568) in PBS solution for 45 minutes. Finally, sections were washed for 3 times with PBS solution and mounted with Mowiol (Calbiochem, San Diego, Calif., USA).

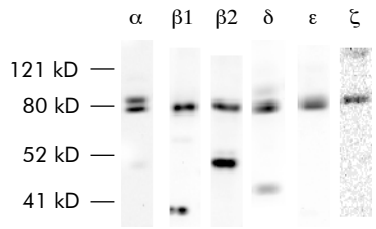

Figure 3.1
Results of western blot analysis of a homogenate of a specimen of skeletal muscle from a horse.
Staining was performed with isoform-specific polyclonal protein kinase C (PKC) antibodies and peroxidase-conjugated secondary antibodies. Equal amounts of protein were applied. The position of the molecular weight marker in kilodaltons is indicated on the left.

Image analysis

Images from all sections were captured by use of a Nikon Eclipse SE800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) that was coupled to a digital CCD camera and processed with Lucia image software (Laboratory Imaging Ltd., Prague, Slovakia). Exposure time of the fluorescent sections was constant for each antibody stain evaluated. Images were corrected for autofluorescence and background staining by use of data obtained from images of stained serial sections in which the primary antibody was omitted. From each biopsy specimen, 100 fibers were evaluated. The presence of cytosol or membrane staining in fibers was scored subjectively via visual assessment of the abundance of PKC isoforms. Biopsy procedures, histochemical techniques and first scoring, and second scoring were performed by different researchers to exclude bias. Subsequently, fields of serial sections were matched via visual assessment to determine fiber types on the basis of MHC composition. Laminin staining was used to identify individual fibers and membranes.

Results

Results of specific immunofluorescence staining of equine muscle sections with anti-human PKC antibodies illustrated the general application of

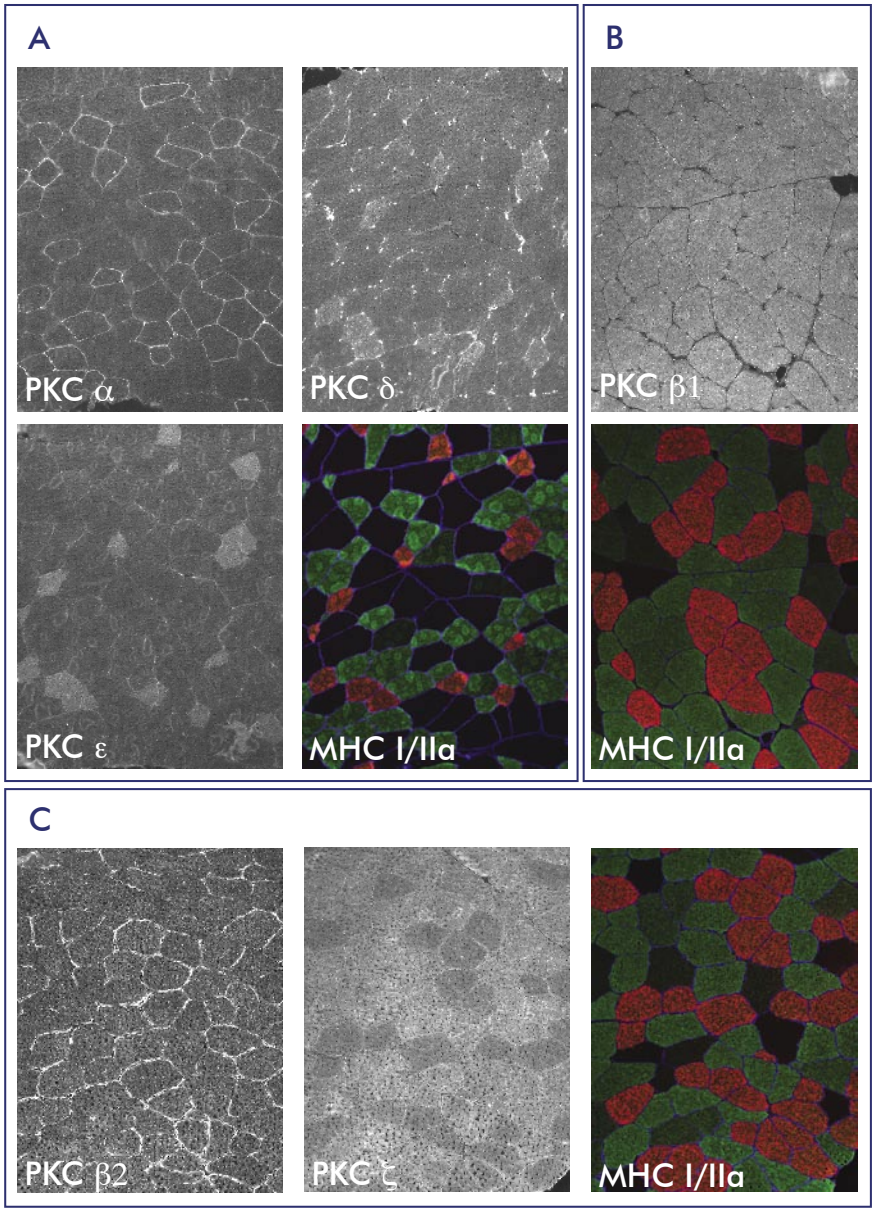

Figure 3.2 Immunofluorescence photomicrographs of serial sections of vastus lateralis muscle stained with polyclonal PKC antibodies and fluorescence-labeled secondary antibodies illustrating PKC isoform distribution in skeletal muscle of horses

Notice that PKC α expression is high near the plasma membrane of type 2a fibers (i.e., green MHC-IIA stained fibers) [A]. Expression of PKC δ and ε is higher in type 1 fibers (i.e., red MHC-I stained fibers) than in type 2a and 2b fibers (i.e., black, unstained fibers) [A]. PKC β1 is distributed equally over all fiber types [B]. PKC β2 is present near the plasma membrane of all fiber types and PKC ζ is predominant in both type 2a and 2b fibers [C]. In the images of MHC-I/IIA, laminin staining is blue. The scale bar in the upper left corner of panel C indicates a length of 100 μm and is representative for all images.

these antibodies and the possible conservation of PKC among different species. Assessment of equine muscle homogenates via western blot analysis confirmed specific binding of PKC antibodies (Fig 3.1). Native PKC isoforms were detected as bands of approximately 80 kd, whereas posttranslational modified PKC isoforms appeared as bands between approximately 36 and 49 kd.[20,21] The lower bands of PKC isoforms β1, β2 and δ are comprised of the phosphorylated constitutively active catalytic domain of PKC at approximately 49 kd and a smaller dephosphorylated inactive catalytic domain.
Five hundred fibers were analyzed; of these, 26% were identified as MHC-I-positive, 58% were MHC-IIa-positive, and 16% yielded negative results for MHC-I and MHC-IIa. Immunofluorescence staining of equine muscle sections with anti-PKC α revealed membrane fluorescence illumination in a subset of fibers (Fig 3.2). Triple-staining of serial sections with anti-MHC-I, anti-MHC-IIA and anti-laminin antibodies indicated that membrane staining of PKC α was restricted to approximately 80% of the MHC-IIA-positive fibers. Compared with the MHC-I-positive fibers, the MHC-I-negative fibers had greater anti-PKC ζ antibody staining. Immunofluorescence staining against PKC δ and ε was more intense in MHC-I-positive fibers than it was in MHC-I-negative fibers. Protein kinase C β1 and β2 were detected in all fibers. Interestingly, PKC β2 was predominantly found in the membrane area of the muscle fibers. The specific distribution of PKC isoforms did not appear to be specific for fibers of the vastus lateralis muscle because fibers of the triceps brachii and pectoralis descendens muscles had similar staining patterns (data not shown).

Discussion

Results of the study of this report indicated the presence and localization of different PKC isoforms in equine skeletal muscle. Protein kinase C plays a major role in signaling events in a wide variety of cells. In skeletal muscle cells, different PKC isoforms have been linked to glucose metabolism.[22] Furthermore, it has been shown that PKC isoforms play a role in the insulin signaling pathway.[3,4] Our data indicated the presence and cellular localization of PKC α, β1, β2, δ, ε and ζ in different skeletal muscle fibers of horses. To identify different fiber types we applied the MHC staining technique. Although ATPase staining has been used extensively in equine muscle research Rivero et al.[23] recently presented an well-designed study in which the correlation between ATPase staining and MHC staining in equine muscle has been established. Findings of this study confirmed data obtained from similar studies in other species.[24]
In approximately 80% of the MHC-IIa-positive fibers (classified as type 2a fibers), there was intense membrane-localized staining for PKC α. This localization of PKC α is consistent with findings of other immunohistochemical studies in humans[25] and rabbits[26] and denotes an important role for PKC α in receptor-mediated signaling. In addition, Chen et al.[27] reported that PKC α reduced the insulin-stimulated increase in antiphosphotyrosine-precipitable phosphatidyl inositol-3 kinase activity in Chinese hamster ovary cells.
Although PKC β2 was also localized in the vicinity of the plasma membrane, PKC β1, δ, ε and ζ were distributed more homogenously within skeletal muscle

fibers. In addition, PKC δ and ε were detected in MHC-I-positive fibers (classified as type 1 fibers). Furthermore, MHC-I-negative fibers (type 2a and 2b fibers) contained more PKC ζ MHC-I-positive fibers. This might suggest different roles for PKC isoforms in signaling events related to glucose uptake and insulin signaling.[28,29] One of the major limitations of the above mentioned published studies,[3,4,22,27] is the evaluation of whole muscle homogenates and muscle fractions only. Contamination among membrane and subcellular organelle specific fractions may well have influenced the results of those studies with regard to PKC isoform distribution.

Nevertheless, it is noteworthy that differential expression of other proteins (i.e., uncoupling protein 3[30] and GLUT-4[24]) that are involved in muscle glucose metabolism has been identified in different muscle fibers as well. Thus, it is reasonable to assume that a link between glucose uptake and metabolism and PKC isoform expression is fiber type-specific. Whether the expression of different PKC isoforms in muscle is fiber type-specific is a matter of further investigation.

In conclusion, our data indicated that PKC isoforms are expressed differently in type 1 and type 2 skeletal muscle fibers in horses and suggested a possible role of these isoforms in glucose and or insulin signaling events. The role of PKC isoforms in signaling pathways and pathologic processes in muscles of horses remains to be elucidated.

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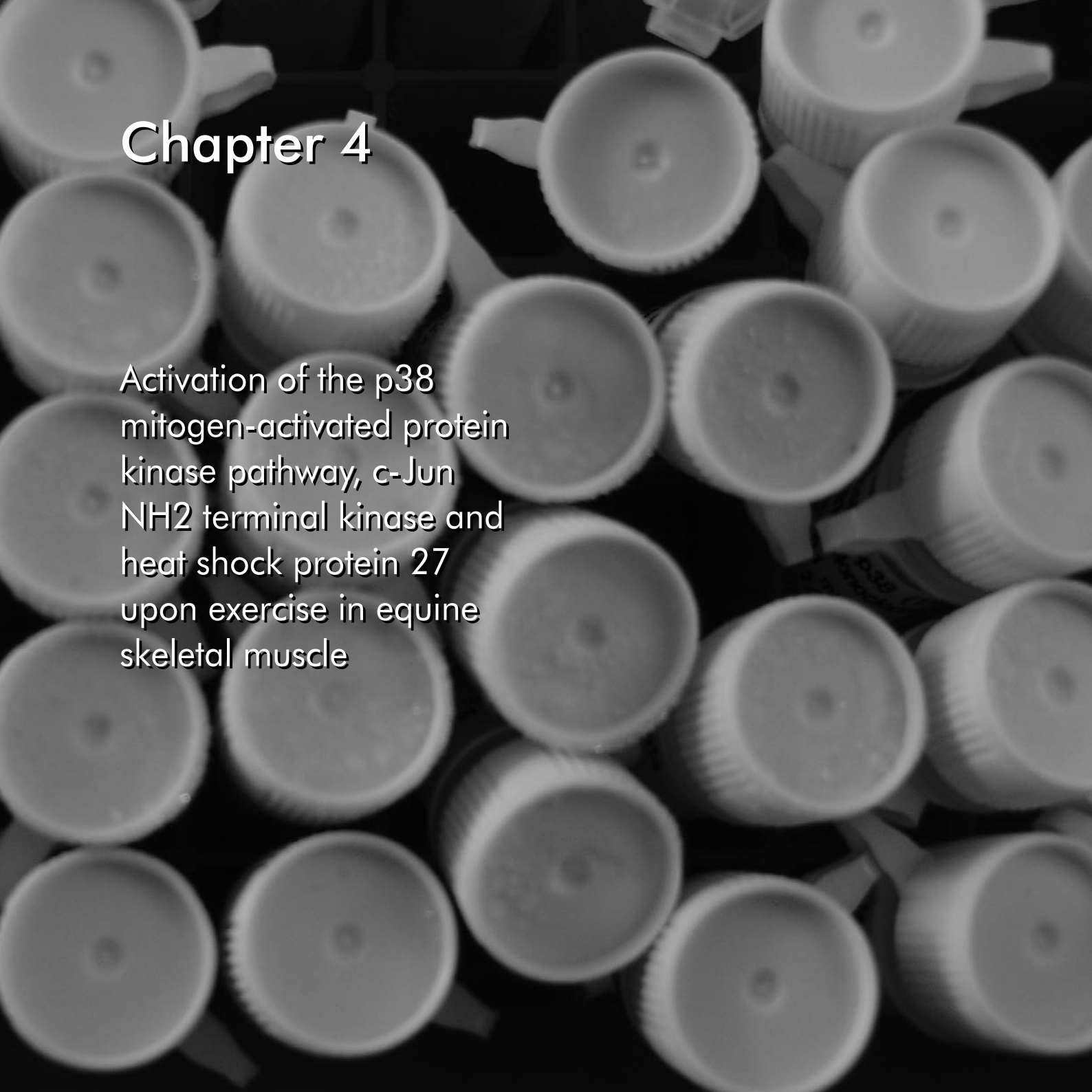

Chapter 4

Activation of the p38 mitogen-activated protein kinase pathway, c-Jun NH2 terminal kinase and heat shock protein 27 upon exercise in equine skeletal muscle

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Abstract

Objective—To investigate the effects of exercise on activation of mitogen-activated protein kinase (MAPK) signaling proteins in horses.
Animals—6 young trained Standardbred geldings.
Procedure—Horses performed a 20-minute bout of exercise on a treadmill at 80% of maximal heart rate. Muscle biopsy specimens were obtained from the vastus lateralis and pectoralis descendens before and after exercise. Amount of expression and intracellular location of phosphospecific MAPK pathway intermediates were determined by use of western blotting and immunofluorescence staining.
Results—Exercise resulted in a significant increase in phosphorylation of p38 pathway intermediates, c-Jun NH2 terminal kinase (JNK), and heat shock protein 27 (HSP27) in the vastus lateralis muscle, whereas no significant changes were found in phosphorylation of extracellular regulated kinase. In the pectoralis descendens muscle, phosphorylation of p38 and HSP27 was significantly increased after exercise. Immunohistochemical analysis revealed fiber-type specific locations of phosphorylated JNK in type 2a/b intermediate and 2b fibers and phosphorylated-p38 in type 1 fibers. Phosphorylated-HSP27 was strongly increased after exercise in type 1 and 2a fibers.
Conclusions and Clinical Relevance—The p38 pathway and JNK are activated in the vastus lateralis muscle after a single 20-minute bout of submaximal exercise in trained horses. Phosphorylation of HSP27 as detected in the study reported here is most likely induced through the p38 signaling pathway.

Introduction

To survive, skeletal muscle fibers must adapt to various physiologic and mechanical stimuli. In particular, skeletal muscles are repeatedly exposed to exercise stress that involves high-force contractions and increased metabolic demands. Depending on the type of exercise (eg, resistance or endurance exercise), training will eventually lead to muscle hypertrophy and strength or increased oxidative capacity. These effects have been studied extensively in humans and rats. However, the initial signaling processes leading to these long-term adaptations are still largely unclear.

Because MAPKs (mitogen-activated protein kinases) have been implicated in proliferation and differentiation of muscle cells, [1,2] their pathways may be involved in exercise-induced adaptations in skeletal muscle. The MAPK comprises a family of 3 distinct subdivisions: Extracellular regulated kinases 1 and 2 (ERK1/2), p38, and (c-Jun NH2 terminal kinase JNK). These kinases elicit signal transduction through phosphorylation of protein on threonine, serine, and tyrosine residues and appear to have a distinct effect on function of muscle cells through exercise.[3] The ERK1/2 plays a role in early exercise-induced changes in gene expression and protein synthesis. This system is activated by numerous extracellular cues, such as growth factors and hormones typically transduced via receptors. Furthermore, p38 is a stress-activated kinase that responds to cellular stress, such as mechanical forces and metabolic stress. It shares some downstream signaling intermediates, such as MAPK-activated protein kinase 2 (MAPKAPK2), mitogen- and stress-activated kinases 1 and 2, and the cAMP response element binder with the ERK1/2 pathway. Similar to p38, JNK is a stress-activated MAPK cascade. It responds to stimuli, such as oxidative stress, inflammatory cytokines, and growth factors. Cumulative acute responses to single bouts of exercise are responsible for chronic adaptations to training. Evidence for this concept came from a study[4] in which investigators found that total ERK1/2 protein was increased by 190% in trained subjects, compared with the content in sedentary subjects, whereas p38 expression was 32% lower in the trained group. Therefore, to study early events during training, the effect of a single bout of exercise on MAPK signaling intermediates is an important research target. There is a vast amount of evidence for the involvement of MAPK cascades during exercise or muscle contraction. Exercise resulted in increased phosphorylation of ERK and p38 and activation of JNK in humans[5-7] and rats.[8-11] Interestingly, electrical stimulation of slow-twitch and fast-twitch skeletal muscles in rats resulted in discrepancies in phosphorylation of p38.[12] It has been found in multiple studies[13-15] that expression of proteins is dependent on type of muscle fiber, which suggests that these functional differences of various types of muscle (e.g., white vs. red or fast-twitch vs. slow-twitch) could be explained in part by the differential expression pattern of proteins in the muscle fibers. However, we are not aware of any studies that examined MAPK activation for single muscle fibers.

Other stress-induced proteins that have an important role in maintaining cellular homeostasis during physiologic stress belong to the family of heat shock proteins (HSPs). This suggests that HSPs could be

involved in responses to exercise-induced stress.[16] Interestingly, HSP27 can be activated through ERK1-2 and p38 MAPK cascades via their common downstream target MAPKAPK2.[17] In a study[18] in which trotters that exercised in accordance with a regularly scheduled training regimen performed a 45-minute bout of exercise at moderate intensity, no effect was found on skeletal muscle content of heat shock factor-1, HSP70, or HSP90 immediately or 4 hours after exercise. In contrast in another study,[19] submaximal exercise for 60 minutes in Finnhorses tended to cause a transient increase in HSP72 mRNA. Knowledge about the effects of exercise on HSP27 is limited to studies[16,20-22] conducted in men in which HSP27 protein was induced in response to eccentric exercise. Therefore, more research is warranted to address the effect of exercise on HSP27.

To our knowledge, there is no information regarding MAPK activation and HSP27 in horses. In the study reported here, our objective was to investigate the effect of exercise on activation of various MAPK pathways and HSP27 in equine skeletal muscle. In addition, we wanted to examine activation of these pathways in single muscle fiber types by performing immunohistochemical analysis on selected muscle sections.

Materials and Methods

Animals

Six young trained Standardbred geldings were included in the study. Mean ± SD age was 26 ± 1 months, and mean body weight was 429 ± 43 kg. Horses were housed separately in stables. This study was approved by the Committee on Animal Welfare of the Faculty of Veterinary Medicine of Utrecht University.

Exercise protocol

Horses were accustomed to trotting on a treadmill. The horses were allowed 24 hours of rest before performing the exercise test. On the test day, muscle biopsy specimens were obtained before horses exercised. Then, horses warmed up by walking for 30 minutes in a horse-walker. The exercise test consisted of trotting on a treadmill for 4 minutes at low intensity (4.5 m/s), followed by trotting for 20 min at a speed (8 m/s in all horses) and incline of the treadmill (1% for 3 horses and 2% for the other 3 horses) corresponding to 80% of maximal heart rate followed by a cooling-down period of 5 minutes of walking at 1.5 m/s. Muscle biopsy specimens were obtained within 10 minutes after each horse completed the exercise test.

Muscle biopsy procedure

Percutaneous needle biopsy specimens were collected form the vastus lateralis and pectoralis descendens muscles. The vastus lateralis muscle was selected for the study because it is important for forward movement in horses and because the vastus lateralis has typically been used in human studies. The pectoralis descendens muscle was selected because this muscle is important for propulsion and for posture.

Local anesthesia was achieved by administration of a solution of lidocaine hydrochloride plus adrenalin (Alfacaine 2% plus adrenalin, Alfasan, Woerden, The Netherlands), and biopsy specimens were obtained

by use of suction applied to a modified Bergström needle with a diameter of 7 mm (Maastricht Instruments, Maastricht, The Netherlands). Biopsy specimens were obtained before exercise from the vastus lateralis muscle at a point 15 cm ventral to the center of the tuber coxa and 7 cm caudal to the cranial border of the vastus lateralis muscle; specimens were obtained at a depth of 5 cm. Biopsy specimens were obtained before exercise from the pectoralis descendens muscle at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle; specimens were obtained at a depth of 4 cm. Biopsy specimens from each muscle were obtained after exercise by use of a new incision made 3 cm caudal to the point for the biopsy specimens obtained before exercise.
A portion of each biopsy specimen was frozen immediately in liquid nitrogen and used for biochemical analysis. The remaining portion of each biopsy specimen was frozen in isopentane cooled to the melting point in liquid nitrogen and used for immunohistochemical analysis.

Homogenization of muscle biopsy specimens

Frozen muscle (100 mg) was homogenized (1:10) in ice-cold homogenization buffer (50 mM HEPES, 10% glycerol, 2 mM EDTA, 1% Triton-X100, 10 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, and Complete protease inhibitor cocktail (Roche, Mannheim, Germany); pH, 7.5) by use of a tissue homogenizer (2 times for 10 s/homogenization). Samples were subsequently rotated end over end for 30 minutes at 4°C and then centrifuged (10,000 x g for 30 minutes at 4°C). Bio-Rad protein assay kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) was used on the supernatant to determine protein concentration. Supernatants were diluted with purified water and concentrated (6X) sample buffer to achieve a final concentration of 5 mg/mL; samples were boiled for 5 minutes and then centrifuged (10,000 x g for 5 minutes at 20°C). Processed samples were stored at –80°C until electrophoresis was performed.

Determination of signaling intermediates

Homogenized muscle (35 μg) was separated via PAGE (12% gel) and transferred to nitrocellulose sheets (100 V for 1 hour at 4°C) by use of blotting buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Equal loading and blotting was confirmed by staining with PonceauS (Sigma Chemical Co, Steinheim, Germany). Nitrocellulose sheets were blocked by the addition of 5% non-fat dry milk and 0.05% Tween-20 in PBS solution and incubation for 1 hour. Antibodies (Cell Signaling Technology, Westburg BV, Leusden, The Netherlands) raised against phosphospecific ERK1/2, p38, MKK3/6, MAPKAPK2, and HSP27 were diluted 1:1000 in 5% bovine serum albumin and 0.05% Tween-20 in PBS solution, added to nitrocellulose sheets, and incubated for 16 hours at 20°C with gentle shaking. Blots were washed 3 times (5 min/wash) in PBS solution containing 0.05% Tween-20 and incubated for 1 hour with horseradish peroxidase–conjugated swine antibodies raised against rabbit IgG (DAKO, Glostrup, Denmark) (diluted 1:5000 in blocking solution); blots were then washed 3 times (5 min/wash) in PBS solution with 0.05% Tween-20 and rinsed with PBS solution. Chemiluminescence was evoked by use of Super Signal West Dura extended kit (Pierce, Rockford, Ill.,

USA) and detected using Clear blue X-ray film (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Films were scanned with a GS800 high-resolution scanner and analyzed by use of Quantity One densitometry analysis program (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Homogenized human muscle biopsy specimens were used as positive control specimens.

The amount of phosphorylation was calculated by dividing the measured optical density by the optical density for the positive control specimens that was equal for all western blots. These data were analyzed by use of paired *t* tests to determine significant effects of acute exercise and differences between the vastus lateralis and pectoralis descendens muscles. Effects were considered significant at values of $P < 0.05$.

Determination of phosphorylated JNK

Homogenized muscle (70 μg) was separated via PAGE (12% gel) as described previously. Nitrocellulose sheets were blocked by incubation with 50% Odyssey blocking buffer (LI-COR Biosciences, Westburg BV, Leusden, The Netherlands) and 0.05% Tween-20 in PBS solution for 45 minutes. Mouse monoclonal antibodies raised against phosphospecific JNK were diluted 1:2000 in 50% blocking buffer and 0.05% Tween-20 in PBS solution, added to nitrocellulose sheets, and incubated for 16 hours at 20°C with gentle shaking. Blots were washed 3 times (5 min/wash) in PBS solution containing 0.05% Tween-20 and incubated for 1 hour with Alexa680-conjugated antibodies (Molecular Probes, Leiden, The Netherlands.) raised against mouse IgG (diluted 1:5000 in 50% blocking buffer and 0.05% Tween-20 in PBS solution); blots were then washed 3 times (5 min/wash) in PBS solution with 0.05% Tween-20 and rinsed with PBS solution. Fluorescence of blots was detected with a Odyssey high-resolution infrared scanner (LI-COR Biosciences, Westburg BV, Leusden, The Netherlands) and analyzed by use of Quantity One, as described previously.

Immunohistochemical evaluation of frozen sections of muscle tissue

Biopsy specimens of the vastus lateralis muscle were cryosectioned at a thickness of 5 mm, thaw-mounted on glass slides, and air-dried for 3 hours at 20°C. Serial sections were washed in PBS solution for 5 minutes, incubated with PBS solution containing 0.5% Triton X-100 for 5 minutes, washed with PBS solution for 5 minutes, and probed with phosphorylated-p38, phosphorylated HSP27, phosphorylated JNK, or a combination of specific antibodies against myosin heavy chain (MHC) type I and type IIA (A4.840 and N2.261 [developed by H. M. Blau], Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA), diluted 1:25 in PBS solution, by incubation overnight at 4°C. After washing 3 times (5 min/wash) with PBS solution, the sections were incubated for 45 minutes with Alexa-conjugated antibodies (Molecular Probes, Leiden, The Netherlands) diluted 1:200 (Alexa488) or 1:500 (Alexa555) in PBS solution. Finally, sections were washed 3 times (5 min/wash) with PBS solution and mounted with Mowiol (Calbiochem, San Diego, Calif., USA) containing 4′-6′-Diamino-2-phenylindol (DAPI) nuclear stain.

To address effects of fiber type, serial sections were stained with specific antibodies against MHC I and MHC IIA, such that the fibers containing MHC IIB or IIX (referred to in the study reported here as type 2b fibers) remained unstained. Fibers with low intensity

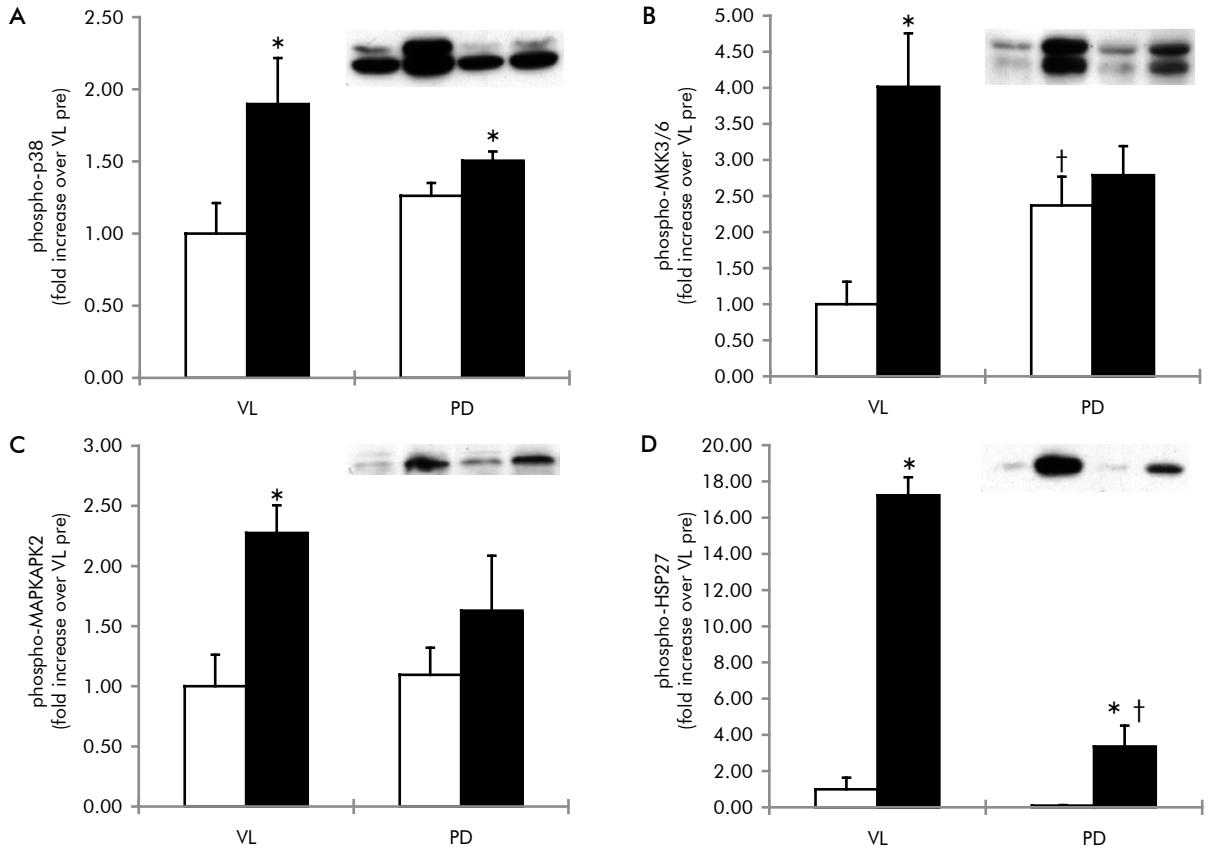

Figure 4.1

Mean ± SEM phosphorylation of the 38 MAPK pathway as indicated by results for p38 [A], MKK3-6 [B], MAPKAPK2 [C], and HSP27 [D] in the vastus lateralis (VL) and pectoralis descendens (PD) muscles of 6 horses before (white bars) and after (black bars) a 20-minute bout of exercise.

*Results are reported as the fold increase, compared with results for the positive control sample. Notice that the scale on the y-axis differs among portions of the figure. Insets are radiographs of a western blot for a representative horse (panel A, p38 isoforms γ [top band]) and α [bottom band]; panel B, phosphorylated MKK6 [top band] and phosphorylated MKK3 [bottom band]; panel C, phosphorylated MAPKAPK2; and panel D, phosphorylated HSP27. For p38 and MKK3-6, total density of both bands was used for statistical analysis. * within a muscle, value differs significantly ($P < 0.05$) from the value before exercise; † within an exercise period, value differs significantly ($P < 0.05$) from the value for the VL muscle.*

for only MHC IIA were termed type 2a/b intermediate fibers, and fibers with high intensity were termed type 2a fibers. Fibers that had positive results when stained with MHC I were termed type 1 fibers.

Image analysis and quantification

Images from all sections were captured by use of a fluorescence microscope that was coupled to a Bassler A113C progressive scan color CCD camera (Ahlersburg, Germany). Images were processed by use of Lucia image analysis software (Laboratory Imaging Ltd, Prague, Slovakia). Exposure time of the fluorescent sections was constant for each antibody stain evaluated. Images were corrected for autofluorescence and background staining by use of data obtained from images of stained serial sections in which the primary antibody was omitted. All sections obtained before and after exercise sections were stained together on 1 slide for each antibody to limit staining variability. For each biopsy specimen, 50 fibers were evaluated. Fields of serial sections were matched via visual assessment to determine fiber types on the basis of MHC composition. The DAPI stain was used to identify nuclei.

For semiquantitative purposes, staining intensity for phosphorylated p38 and phosphorylated HSP27 was determined in the cytosol of each fiber by use of the image analysis software. The mean grayscale (range, 0 [no stain] to 255 [maximal stain]) for each fiber was calculated to yield a mean grayscale per fiber type before and after exercise. Mean background for a specific section was subtracted from the mean grayscale per fiber to yield the corrected mean staining intensity. In addition, staining intensity of phosphorylated p38 was determined in and near the nuclei by use of values in the regions in which there was overlap of DAPI stain and phosphorylated p38 stain.

Results

Activation of various MAPK pathways was investigated in horses. Therefore, MAPK phosphorylation was assessed in equine skeletal muscle. Acute exercise for 20 minutes resulted in a significant ($P = 0.02$) 1.9-fold increase in p38 phosphorylation at threonine 180 and tyrosine 182 in the vastus lateralis muscle. In addition, phosphorylation increased significantly for upstream MKK3/6 at serine 189 and 208 (4.0-fold increase; $P = 0.02$) and downstream MAPKAPK2 at threonine 334 (2.3-fold increase; $P = 0.01$) and HSP27 at serine 82 (17.2-fold increase; $P < 0.001$; Fig 4.1). In the pectoralis descendens muscle, phosphorylation of p38 and HSP27 were also increased significantly, but the fold increases were lower than for the vastus lateralis muscle.

To investigate whether other MAPK pathways were activated concurrently with p38, phosphorylation of ERK1-2 and JNK was assessed in a comparable manner. No significant increase in phosphorylation of ERK1-2 was detected, whereas a significant ($P = 0.009$) 3.4–fold increase in phosphorylation of JNK at threonine 183 and tyrosine 185 were found in the vastus lateralis muscle (Fig 4.2).

To further assess phosphorylation of p38, localization of signaling intermediates was determined for the vastus lateralis muscle. Immunofluorescence staining of phosphorylated p38 and phosphorylated HSP27 revealed differences in the amount of expression

among fibers. Therefore, fiber typing was assessed to address potential expression of these proteins dependent on fiber type. Sections stained with phosphorylated p38 revealed localization on the basis of specific fiber types (Fig 4.3). In general, phosphorylated p38 was found in type 1 fibers. In addition, staining for phosphorylated p38 found in and near nuclei. This finding was supported by quantification of the staining intensity for single fibers. The corrected mean staining intensity was 61, 31, 33, and 27 grayscales before and 50, 35, 27, and 28 grayscales after exercise for type 1, 2a, 2a/b, and 2b

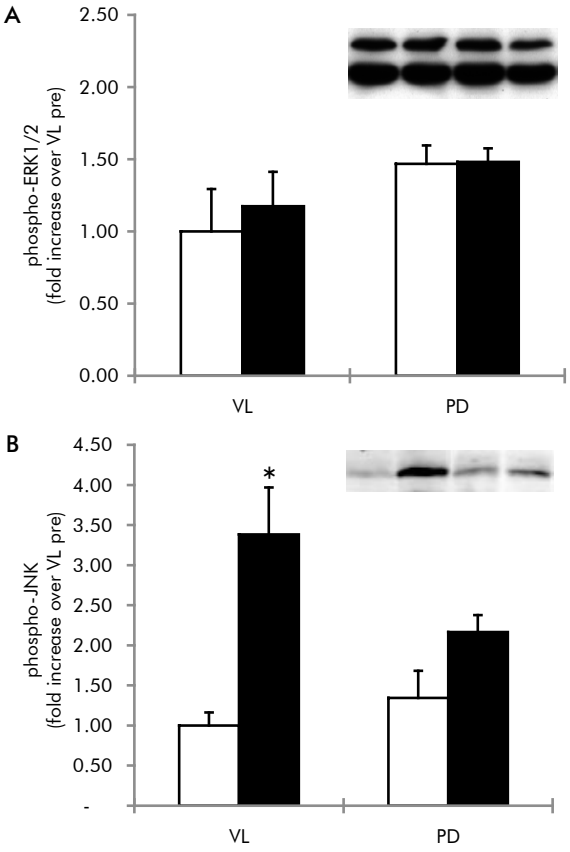

Figure 4.2
Mean ± SEM phosphorylation of ERK1-2 [A] and JNK [B] in VL and PD muscles of 6 horses before (white bars) and after (black bars) a 20-minute bout of exercise.
Results are reported as the fold increase, compared with results for the positive control sample. Notice that the scale on the y-axis differs between portions of the figure. Insets are radiographs of a western blot for a representative horse (panel A, ERK isoforms; 44-kd band [top band] and 42-kd band [bottom band]; and panel B, phosphorylated JNK). Total density of both ERK bands was used for statistical analysis. See Fig 4.1 for remainder of key.

fibers, respectively. The corrected mean intensity in and near nuclei was 101 grayscales before exercise and 105 grayscales after exercise; these values did not differ significantly.

The most striking difference was found when sections were stained with phosphorylated HSP27. Before exercise, the phosphorylated HSP27 signal was almost as low as background values, whereas a clear increase in staining was found after exercise (Fig 4.4). Phosphorylated HSP27 was not distributed equally over all muscle fibers. Typically, the highest concentrations were found in type 1 and 2a fibers

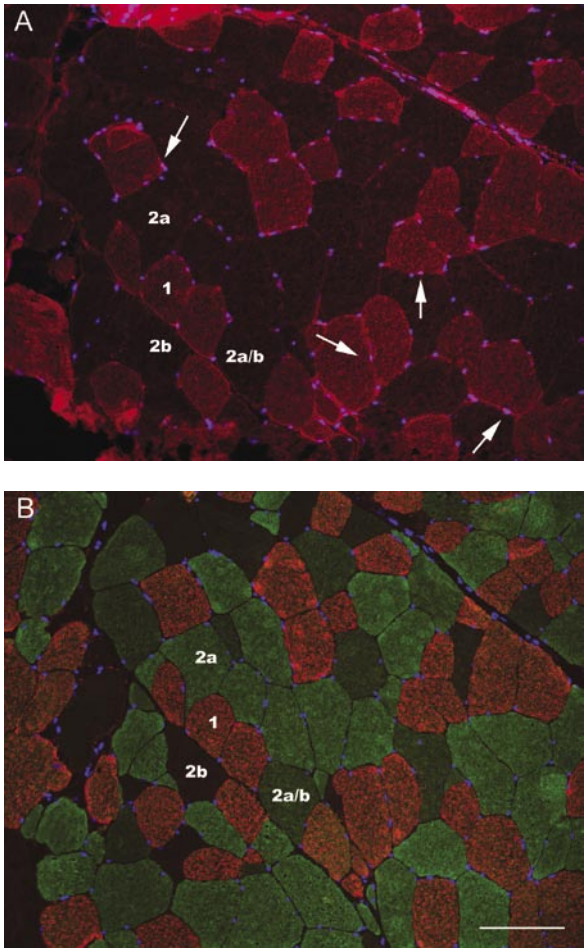

Figure 4.3
Photomicrographs depicting the location of phosphorylated p38 [A] and MHC composotion [B] in the VL muscle of a representative horse obtained after a 20-minute bout of exercise.
Cryosections were stained with a rabbit IgG phospho-p38 antibody and a goat anti rabbit-IgG Alexa555-conjugated antibody, which resulted in red staining specific for phosphorylated p38. Sections were embedded with DAPI stain, which resulted in blue staining of nuclei (arrows). Panel A reveals strong p38 staining in type 1 fibers, whereas panel B represents a serial section of the muscle biopsy specimen from panel A stained with specific antibodies against MHC I and MHC IIa, which reveals type 1 (red), 2a (dark green), 2a/b intermediate (light green), and 2b (black) fibers. Bar = 100 µm.

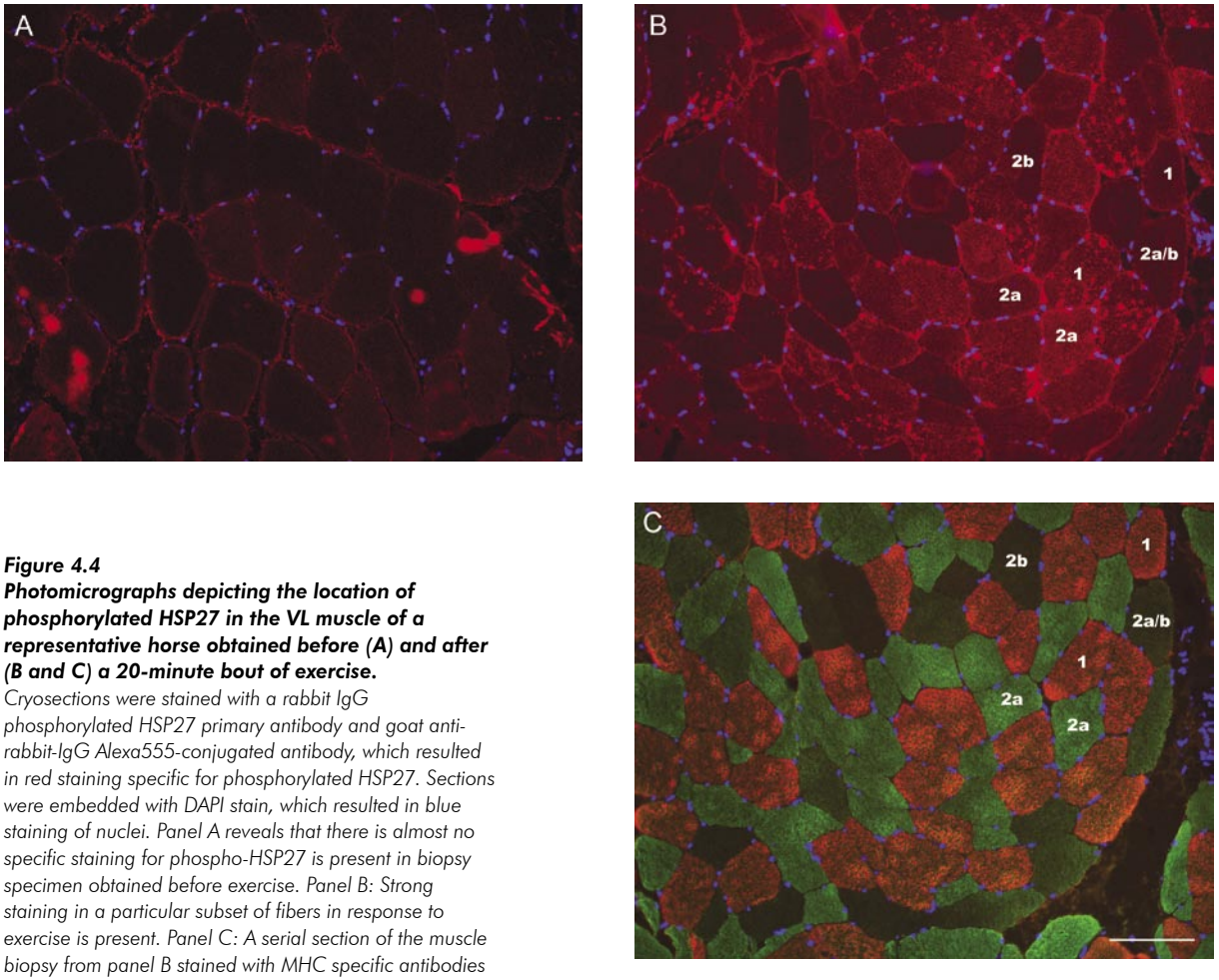

Figure 4.4
Photomicrographs depicting the location of phosphorylated HSP27 in the VL muscle of a representative horse obtained before (A) and after (B and C) a 20-minute bout of exercise.
Cryosections were stained with a rabbit IgG phosphorylated HSP27 primary antibody and goat anti-rabbit-IgG Alexa555-conjugated antibody, which resulted in red staining specific for phosphorylated HSP27. Sections were embedded with DAPI stain, which resulted in blue staining of nuclei. Panel A reveals that there is almost no specific staining for phospho-HSP27 is present in biopsy specimen obtained before exercise. Panel B: Strong staining in a particular subset of fibers in response to exercise is present. Panel C: A serial section of the muscle biopsy from panel B stained with MHC specific antibodies against MHC I and MHC IIa, showing type 1 (red), 2a (strong green) 2a/b intermediate (light green) and 2b (black) fibers. Panel B reveals strong staining in a particular subset of fibers in response to exercise, and panel C represents a serial section of the muscle biopsy specimen from panel B stained with MHC specific antibodies against MHC I and MHC IIa, showing type 1 (red), 2a (strong green) 2a/b intermediate (light green) and 2b (black) fibers. Bar = 100 µm. See Fig 4.3 for remainder of key.

and the lowest concentrations in type 2a/b and 2b fibers. Staining was equally distributed over the intracellular space in some fibers, whereas staining was concentrated in a punctuate pattern in other fibers. These qualitative findings were supported by quantification of the staining intensity on single fibers. The corrected mean staining intensity before exercise was 11, 8, 9, and 10 grayscales before exercise and 48, 47, 20, and 21 grayscales after exercise for type 1, 2a, 2a/b, and 2b fibers, respectively. Changes in phosphorylated HSP27 in fiber types 1 and 2a differed significantly. The distribution of fibers when analyzed for phosphorylated and p38 and phosphorylated HSP27 was 41% type 1, 42% type 2a, 8% type 2a/b, and 9% type 2b fibers, with no significant difference before and after exercise. Staining of sections with phosphorylated JNK yielded a low staining intensity in type 2a/b intermediate and type 2b fibers (data not shown).

Discussion

Analysis of results of the study reported here indicates that a single bout of submaximal exercise in horses activates the p38 MAPK pathway but not ERK1/2 or JNK. Increases were found in phosphorylation of p38, the upstream element MKK3/6 (which phosphorylates p38 MAPK), and the downstream element MAPKAPK2. In addition, the MAPKAPK2-target HSP27 was also phosphorylated in response to exercise. Our results for p38 are in line with results from other studies[5,23] in which investigators reported activation of p38 and MAPKAPK2 in men after 60 minutes of 1-legged cycling exercise at 70% of maximal oxygen consumption. In contrast to our findings, however, those investigators also reported transient phosphorylation of ERK1/2. An explanation for this discrepancy is not easy to provide, but we suggest that the time frame at which we obtained the biopsy specimen after exercise in our study (approx 10 min after cessation of exercise) may be responsible for the observed differences. Evidence for this assumption is provided in another study[24] in which investigators reported that phosphorylation of ERK is rapidly decreased during recovery after exercise. Another plausible explanation could be that the exercise was too mild or of too short a duration. It has been reported[24] that ERK1/2 is activated in an intensity-dependent manner, and the exercise bout performed by the horses in the study reported here was relatively short and caused glycogen concentrations to decrease only mildly (20% to 25%).

Interestingly, our results differed between the vastus lateralis and pectoralis descendens muscles. The pectoralis descendens muscle had a significant increase in phosphorylation of p38, but phosphorylation of upstream MKK3/6 and downstream MAPKAPK2 did not increase significantly. A feasible explanation could be that the load to the vastus lateralis muscle is greater than to the pectoralis descendens muscle because of the exercise-specific movements. The vastus lateralis muscle is subject to higher mechanical forces than the pectoralis descendens muscle because of its function in propulsion.[25]

Several mechanisms may have been responsible for the activation of the p38 pathway. Those possibilities include increased blood flow to muscles leading to increased amounts of hormonal factors and

activation of receptor-mediated signaling, release of autocrine and paracrine factors (eg, interleukin 6) that stimulate surface receptors on cells and activate signaling cascades,[26] and muscle contraction *per se*.[27] Differential expression of proteins in fiber types may explain functional discrepancies observed in various types of skeletal muscle. For example, in studies[28,29] conducted by our laboratory group, we determined that protein kinase C isoforms, an important family of protein kinases involved in modulation of glucose uptake and insulin signaling, are expressed in a fiber-type-specific manner in skeletal muscle.[13] Other investigators[30] reported that incremental static stretch *in vitro* in isolated soleus muscle obtained from rats (i.e., a muscle with predominantly type 1 fibers) resulted in higher amounts of phosphorylation of p38, compared with the amount of phosphorylation of p38 for the extensor digitorum longus muscle (a muscle primarily consisting of type 2 fibers).

Studies have provided conflicting results regarding effects of exercise on JNK activation. Although electrical stimulation of skeletal muscle of rats increases JNK activity[31] and phosphorylation of JNK,[32] it is still unclear whether JNK is activated by aerobic exercise,[33,34] resistance exercise, or only eccentric exercise.[35] Activation of JNK may be related to age,[36] duration of exercise,[37] exercise-induced muscle damage,[30] and contraction-induced tension.[38] On the basis of results for the study reported here, we conclude that exercise of the vastus lateralis muscle of young horses activates JNK, although the amount of phosphorylated JNK appears to be lower in the vastus lateralis and pectoralis descendens muscles, compared with the amount of phosphorylated p38 in those muscles, because more protein and a highly sensitive detection system had to be used to measure the amount of phosphorylated JNK.

To address potential distribution among specific fiber types and effects of exercise on the MAPK pathways, we performed immunohistochemical analysis of the muscle biopsy specimens. Phosphospecific p38 was mainly found in type I fibers and in and near nuclei. Nuclear staining intensity was slightly increased after exercise. This corresponds with results published elsewhere.[34] In addition, specific phosphorylated JNK staining was found in type 2b and 2a/b intermediate fibers but not in type 1 and 2a fibers. Because type 2b and 2a/b fibers only represent a small percentage of the biopsy specimens of the vastus lateralis muscle, this may also be the reason for the lower detection by use of western blotting.

A robust increase in phosphorylation of HSP27 after exercise was found in the study reported here. This supports results of other studies[16,39] in which investigators detected activation and increased expression of HSPs in response to exercise. To our knowledge, the study reported here is the first in which investigators detected an effect of short-term exercise on phosphorylation of HSP27. This finding was supported by immunohistochemical analysis in which staining for phosphorylated HSP27 was strongly increased after exercise.

The role of HSP27 in skeletal muscle is largely unknown. We are aware of only 2 studies[16,22] in men in which HSP27 protein was induced after 48 hour in response to eccentric exercise. On the basis of results of the investigation of HSP27 in other tissues and cell types, it can be concluded that HSP27 plays a role in the regulation of actin dynamics during stress.[40] This implies that it is likely that HSP27 plays a role in

preventing skeletal muscle damage and activating repair mechanisms following exercise. The increase in phosphorylation of HSP27 in the study reported here may be an early event in the exercise-induced modulation of HSP27.

In the study reported here, we found that a single bout of 20 minutes of exercise at submaximal intensity results in activation of the p38 pathway and phosphorylation of HSP27 and JNK in equine skeletal muscle. Analysis of our findings and those of other researchers indicates that MAPK pathways and HSPs are activated by exercise and that the MAPK family actually involved may be dependent on type, intensity, and duration of exercise. Therefore, the activation of multiple MAPK pathways and HSPs is likely and may be indicative of the importance of the time course of the signaling pathway response during exercise and recovery after exercise.

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Chapter 5

Expression and phosphorylation of p38 MAPK, JNK and HSP27 upon normal and intensified training in equine skeletal muscle

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Abstract

Objective—Previously, we have shown activation of JNK, p38 MAPK and HSP27 upon exercise in equine skeletal muscle. The aim of the present study was to investigate the modulation of these proteins during a period of training and intensified training of horses.
Animals—Sixteen young Standardbred geldings were used. A group of six untrained horses were trained for 18 weeks, and two groups of five trained horses respectively entered a 6-week normal or intensified training protocol.
Procedure—Training of untrained horses consisted of endurance training and interval training on a treadmill. Horses trained four days a week and training load increased gradually during the training period. Trained horses from the normal training group followed the same training procedure for six weeks and horses in the intensified training group trained with higher loads during the first three weeks whereas rest days were eliminated during the last three weeks of training. Muscle biopsies of the vastus lateralis were obtained before and after the training periods immediately before and after an acute bout of exercise. Expression and phosphorylation levels of p38 MAPK, JNK and HSP27 were determined via Western blotting.
Results—Normal or intensified training did not result in an altered expression level of p38 MAPK, JNK or HSP27 protein. In all horses, irrespective of training status, a significant increase in phosphorylation of HSP27 was found upon exercise. However, p38 MAPK and JNK phosphorylation was not increased by exercise in untrained horses. This could be related to the higher basal phosphorylation levels for p38, JNK and HSP27 in these horses.
Conclusions and Clinical Relevance—We conclude that a period of normal or intensified training has no effect on p38 MAPK, JNK or HSP27 protein expression. or exercise-induced HSP27 phosphorylation. Basal p38 phosphorylation is decreased upon training. The latter finding might be indicative for the beneficial effects of training on basal stress levels in equine skeletal muscle.

Introduction

Activation of mitogen activating protein kinase (MAPK) signaling pathways is among the initial molecular processes that lead to long-term adaptations in muscle in response to physical training.[1] The activation of MAPK pathways in response to acute exercise and muscle contraction has extensively been studied and documented in men[2-6] and rat.[7-10] It has previously been shown that the type, intensity and/or duration of the exercise regimen determine the type of MAPK that is actually activated. For example, *in situ* concentric muscle contractions in rat induced a marked increase in ERK1/2 phosphorylation, with little effect on p38 phosphorylation, whereas eccentric contractions resulted in a dramatic increase in p38 phosphorylation, and little effect on ERK1/2 phosphorylation.[7] In addition, Widegren et al. showed that ERK1/2 phosphorylation level rises with increased exercise intensity.[11] Recently, we have also shown that p38 MAPK and JNK are also activated upon exercise in trained horses.[12]

Interestingly, Yu and coworkers showed that MAPKs are not only activated upon exercise, but are also differentially regulated upon training as well. More specifically, these authors found that in trained subjects total ERK1/2 protein was increased by approximately 190% in comparison to sedentary subjects, whereas p38 protein expression was 32% lower in the trained group.[13] These results have led to the hypothesis that depending on intensity and type of exercise, MAPK pathways are differentially activated and thus may play a role in specific adaptations induced by different training programs. This idea is strengthened by the results of Lee et al., who showed in a training study in rats that low-intensity training (~40-45% of VO2max) and moderate-to-high-intensity training (~75% of VO2max) results in selective post-exercise activation of p38 MAPK and ERK1/2.[10]

Other proteins implicated in the response to exercise stress are the family of heat shock proteins (HSPs). Evidence for the modulation of HSPs by training is limited to studies that have focused on large size HSPs. For example, an increase in HSP60 and HSP72 has been found in rat muscle after endurance training.[14,15] Furthermore, other training studies in rats also showed an increase in HSP72.[16,17] The magnitude of the increase was dependent on muscle type, being higher in fast (type 2) muscles (plantaris and white gastrocnemius) and lower in slow twitch (type 1) muscles (soleus and red gastrocnemius). Interestingly, an increase in HSP72 mRNA has also been found in the gluteus medius muscle in Finn horses during training,[18] which suggests an increased protein expression. Finally, in humans it has been found that the increase in HSP70 to training is dependent on training volume and intensity.[19,20]

In the framework of MAPK signaling, the small heat shock protein HSP27 is of particular interest, because it can be activated through ERK1/2 and p38 MAPK cascades via their common downstream target MAPKAPK2.[21] Recently, we have shown that both p38 MAPK and HSP27 are phosphorylated upon exercise in young trained horses. Therefore, the aim of the present study was to investigate the modulation and exercise-induces phosphorylation of MAPKs and HSP27 protein during a period of normal and intensified training in equine skeletal muscle.

Materials and methods

Animals

A total of 16 young Standardbred geldings were used in the study. Six untrained horses were included in a training protocol. Their mean age was 18 ± 0.4 (SD) months and their mean body weight was 367 ± 24 kg. Two groups of trained horses (n=5) were randomly admitted to a normal training protocol (aged 25 ± 2 months; weight 416 ± 61 kg) or an intensified training protocol (aged 26 ± 1 months; weight 410 ± 17 kg), respectively. Horses were individually housed and fed according to their individual needs. Water was available *ad libitum*. This study was approved by the Committee on Animal Welfare of the Faculty of Veterinary Medicine of the Utrecht University.

Training protocol

Horses were familiarized with trotting on a treadmill for four weeks by trotting at speeds corresponding to 30-50% of estimated maximal heart rate ($HF_{est\text{-}max}$, 240 bpm; *see* chapter 2 for explanation) for 20-35 minutes for 3-4 days a week. After this period, the first exercise test was performed. Horses then continued training for 18 weeks. Horses trained four days a week in which sessions of endurance training (ET) at 60% and 75% $HF_{est\text{-}max}$ and high intensity training (HIT) at 80% $HF_{est\text{-}max}$ were alternated and training load (speed, inclination and duration) were gradually increased over time to reach $HF_{est\text{-}max}$. After this period, horses performed a second exercise test.

Normal and intensified training protocol

No familiarization period was necessary since horses were used to exercising on a treadmill. Hence the first exercise test was performed immediately before the training period. The normal training procedure consisted of training at a frequency and load as described in the previous section. The intensified training consisted of longer ET en HIT sessions in the first three weeks. The frequency of training was further increased from 5 to 7 days a week during the last three weeks of training. After this period, horses performed a second exercise test.

Exercise test

The exercise tests were performed as described earlier.[12] After taking pre-exercise muscle biopsies horses walked in a horse-walker for 30 min. Then horses warmed up on the treadmill for 4 min at low intensity (4.5 m/s), which was then immediately followed by 30 min (untrained horses) or 20 min (trained horses) at a speed (6 m/s in untrained horses; 8 m/s in trained horses) and inclination (0-1% in untrained horses; 1-2% in trained horses) corresponding to 70% (untrained) or 80% (trained) $HF_{est\text{-}max}$. The exercise test ended with a 5-minute cooling-down (1.5 m/s). Post-exercise biopsies were taken within 10 min after ending the test.

Muscle biopsy procedure

Percutaneous needle biopsy specimens were taken under local anesthesia with a modified Bergström needle as described before.[12] The pre-exercise biopsy specimens were obtained from the vastus lateralis muscle at a point located 15 cm ventral from the center of the tuber coxa and 7 cm caudal from the cranial border of the muscle; the specimens were obtained at a depth of 5 cm. The post-exercise biopsy

specimens were taken from a new incision 3 cm caudally from the pre-exercise biopsy specimens. The biopsy samples were frozen immediately in liquid nitrogen and stored at -80°C.

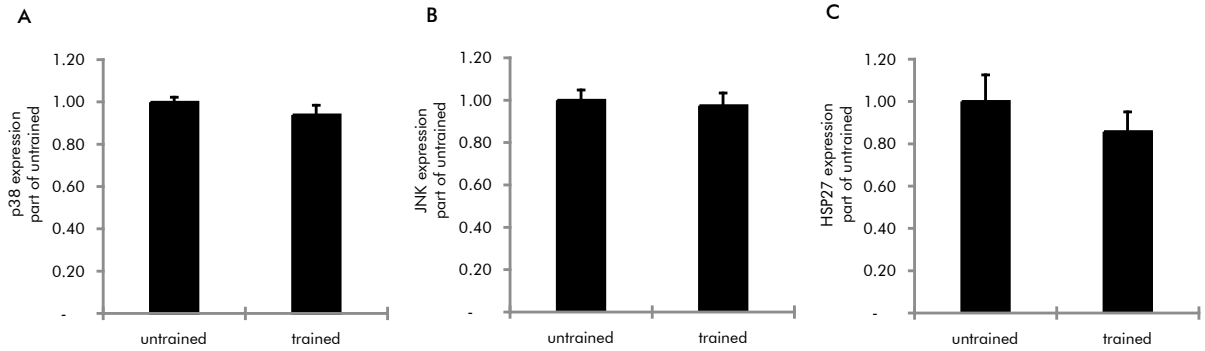
Homogenization of muscle biopsy specimens

Frozen muscle (100 mg) was homogenized (1:10) in ice-cold homogenization buffer (50 mM HEPES, 10% glycerol, 2 mM EDTA, 1% Triton-X100, 10 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, Complete protease inhibitor cocktail, pH 7.5) by use of a tissue homogenizer for 2 times 10 s. The samples were subsequently rotated end over end for 30 min at 4°C and centrifuged (10,000 x g for 30 minutes at 4°C). Bio-Rad protein assay kit was used to determine protein concentration in the supernatant. Supernatants were diluted with purified water and 6 times concentrated sample buffer to a final concentration of 5 mg/ml, boiled for 5 minutes and centrifuged (10,000 x g for 5 minutes at 20°C). Processed samples were stored at -80°C until electrophoresis was performed.

Determination of signaling intermediates

Homogenized muscle (35-70 μg) was separated via 12% polyacrylamide gel electrophoresis and transferred to nitrocellulose for 1 h (100 V, 4°C) in

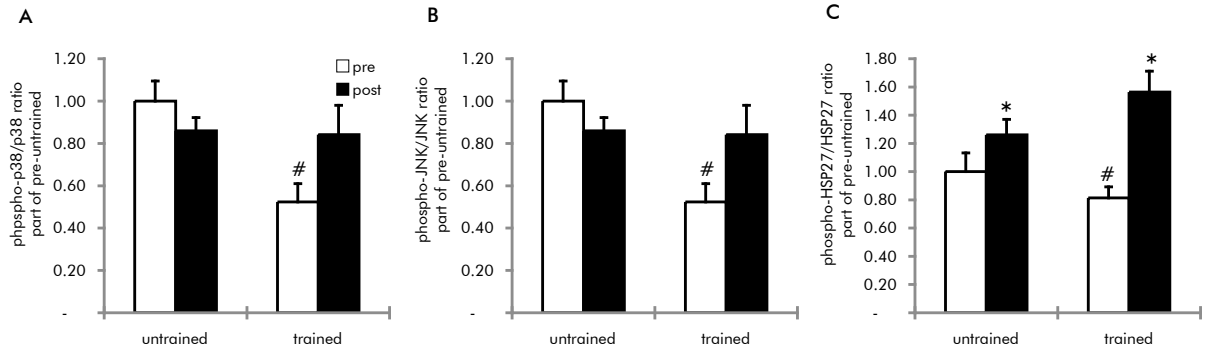
Figure 5.1
p38 MAPK [A], JNK [B] and HSP27 [C] protein expression upon training in horses.
Expressed as part of control (untrained) ± SEM (n=6).



blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nitrocellulose sheets were blocked with 50% Odyssey Blocking Buffer and 0.05% Tween-20 in PBS solution for 45 min. Incubation of the nitrocellulose sheets with specific antibodies (Cell Signaling Technology, Westburg B.V., Leusden, The Netherlands) raised against normal and phosphospecific proteins p38 MAPK, JNK and HSP27 (diluted 1:1000 or 1:2000 in 5% BSA and 0.05% Tween-20 in PBS) was performed for 16 h at 20°C with gentle shaking. Blots were washed 3 times (5 min/wash) in PBS containing 0.05% Tween-20 and incubated for 1 h with Alexa680-conjugated (Molecular Probes, Leiden, The Netherlands) or IRdye800-conjugated antibodies (LI-COR Biosciences, Westburg B.V., Leusden, The Netherlands) diluted 1:5000 in 50% Odyssey Blocking Buffer and 0.05% Tween-20 in PBS, were washed 3 times (5 min/wash) in PBS with 0.05% Tween-20 and rinsed with PBS. Fluorescence of blots was detected with an Odyssey high-resolution infrared scanner (LI-COR Biosciences, Westburg B.V., Leusden, The Netherlands) and analyzed by use of a Quantity One densitometry analysis program (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).

Statistical analysis

Figure 5.2
Changes in basal and exercise-induced changes in phosphorylated protein/protein ratio upon training in horses.
*P38 phosphorylation [A]; JNK phosphorylation [B]; HSP27 phosphorylation [C]. Expressed as part of control (pre-exercise untrained) ± SEM (n=6). Pre-exercise (white), post-exercise (black). * significantly different from control (P<0.05); # significantly different between normal and intensified training (P<0.05).*



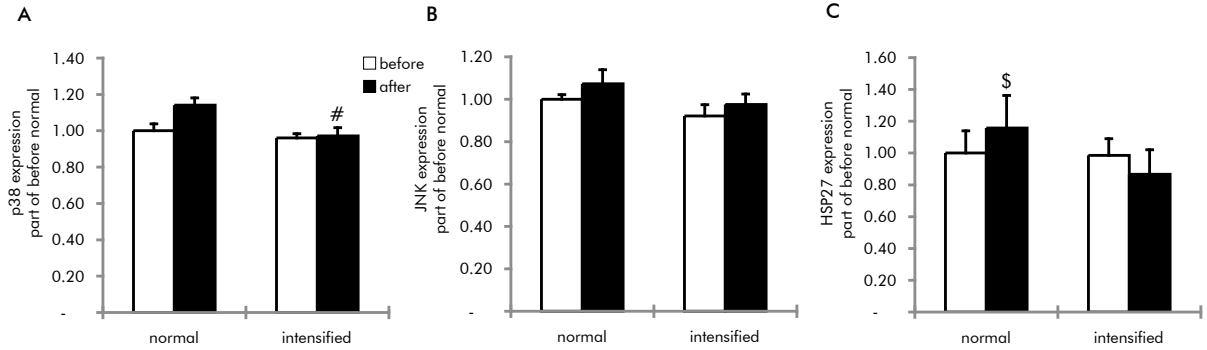
The amount of total protein or phosphorylated protein was calculated by dividing the measured fluorescence by that of a positive control that was equal for all Western blots. The ratio of phosphorylated protein over total protein was subsequently calculated. Because data were not normally distributed non-parametric statistical analysis was performed. Data were analyzed using Mann-Whitney test to determine significant effects of exercise and training, and Wilcoxon rank-sum test to determine significant differences between normal and intensified training. An effect was considered significant at $P<0.05$.

Results

Training

Training did not result in changes in expression level of p38 MAPK, JNK or HSP27 protein (Fig 5.1). Relative basal phosphorylation levels of p38 and HSP27 were significantly higher in untrained horses compared to these horses after training (Fig 5.2). In addition, exercise-induced increase in p38 and JNK phosphorylation were statistically not significant in untrained horses. Exercise-induced phosphorylation HSP27 was nevertheless significantly increased in all training conditions.

Figure 5.3
Changes in p38 MAPK [A], JNK [B] and HSP27 [C] protein expression upon normal or intensified training in trained horses.
Expressed as part of control (before normal training) ± SEM (n=5).
 # significant different between normal training and intensified training group;
$ significant different effect after normal or intensified training (P<0.05).



Intensified training

HSP27 protein expression was significantly increased upon normal training, but not upon intensified training of trained horses (Fig 5.3). JNK and p38 expression was not changed upon normal or intensified training, although the expression level of p38 MAPK was significantly higher in the group after intensified training in comparison with that of the group after normal training. Significant exercise-induced increases in phosphorylation were found for HSP27 and JNK in all groups, except in the group after normal training, where a decrease in JNK phosphorylation was found (Fig 5.4). No significant increase or change in exercise-induced p38 MAPK phosphorylation was found in any condition.

Discussion

In the present study we hypothesized that a period of normal training in previous untrained horses and intensified training in trained animals results in changes of MAPK signaling pathways. We investigated whether or not basal protein levels changed upon our interventions. We found no changes in the expression of p38 MAPK, JNK or HSP27 upon normal or intensified training. In addition, in already trained horses, normal training significantly increased HSP27 expression.

Only a limited number of studies have examined the training-induced effects on basal HSP expression levels. In a human study significant increases in HSP70 levels were found in high intensity training but not in low-intensity endurance training.[19] Furthermore it has been reported that a bout of eccentric exercise that was repeated after 1-6 weeks after the first one, resulted in lower basal HSP27 and HSP70 expression.[22] In addition, an increase in HSP72 mRNA in the gluteus medius muscle was found upon training in Finnhorses.[18]

The absence of a change in HSP27 expression upon normal or intensified training in our study could be related to the fact that training sessions were of a moderate intensity [*see* chapter 2, in combination with the absence of considerable skeletal muscle damage related to low plasma creatine kinase activity (data not shown)]. In addition, it is also known that the expression of HSPs is, in general, induced very rapid (within hours after the application of a stressor). Therefore, it is likely that HSP27 expression is induced within hours after an exercise session in horses as well. This has already clearly been shown in rat and human studies.[4,22,23] The results of studies in horses, however, are conflicting. In one study, no induction of HSP70 and HSP90 was found 4 hours after a period (45 min) of moderate intensity exercise (walking and trotting) in 6-9 year old trained Standardbred trotters,[24] whereas in another study a transient induction of HSP72 mRNA was found upon a 60-min submaximal exercise test in trained Finnhorses.[18] From these findings one might speculate whether or not HSP27 protein is induced upon exercise, and if this is different between normal and/or intensified trained horses in comparison to untrained horses. This has, to the best of our knowledge, not been investigated before.

Only a limited number of studies were published regarding the effects of training on basal MAPK expression. For instance, an increase in basal ERK in parallel with a decrease in p38 MAPK has been

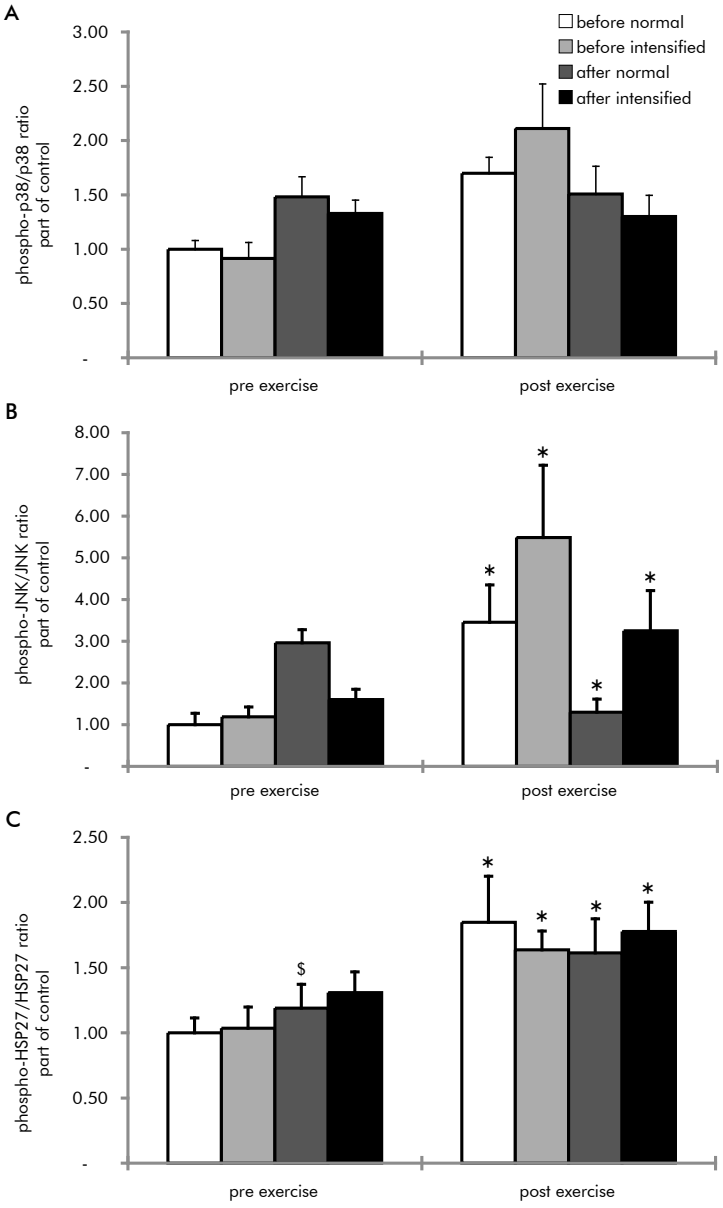


Figure 5.4
Changes in basal and exercise-induced changes in phosphorylation/protein ratio upon normal or intensified training in trained horses.
*p38 phosphorylation [A]; JNK phosphorylation [B]; HSP27 phosphorylation [C]. Expressed as part of control (pre-exercise before normal training) ± SEM (n=5). * significantly different upon exercise (P<0.05); # significantly different upon normal or intensified training (P<0.05).*

found in trained humans and rats in comparison with sedentary controls.[10,13] These studies used muscle biopsies from different subjects before and after training. Differences in experimental design could explain the differences between these latter findings and our results.

Besides the training-induced changes in p38 MAPK, JNK and HSP27 protein expression, we have also analyzed the activation of these proteins by measuring relative phosphorylation levels upon training and acute exercise. As has been shown previously,[12] acute exercise resulted in a significant increase in phosphorylation of HSP27. The biological significance of phosphorylation of HSP27 is still unknown but it has been suggested that HSP27 interacts with the cytoskeleton through the modulation of actin microfilaments.[25, 26] This might indicate a regulatory role of HSP27 in translocation processes in skeletal muscle through focal adhesion complexes.[27] In untrained horses, acute exercise did not increase phosphorylation of p38 MAPK or JNK, whereas after training a statistically non-significant exercise-induced increase in phosphorylation of p38 MAPK and JNK was found. These results are in contrast with our previous findings and with the results of the intensified training study in which trained horses showed a significant increase in exercise-induced JNK phosphorylation. Although an explanation for these differences is not easy to provide, we suggest that activation of p38 MAPK in these horses must at least have occurred transiently, because of the significant increase in phosphorylation of HSP27 upon exercise in all training conditions.[21] The fact that we are not able to measure a significant effect on p38 MAPK phosphorylation might indicate that p38 MAPK phosphorylation has already returned to basal levels. A most likely cause could be related to the timing of post-exercise biopsies[11] and/or the small sample size (5-6 horses per group).

Remarkably, JNK phosphorylation was reduced upon exercise after continuation of normal training (control animals of the intensified trained animals). In addition, in the same group we found a higher HSP27 protein level. At this moment we have no explanation for these findings.

In conclusion, we found no changes in expression of p38 MAPK, JNK or HSP27 in skeletal muscle upon normal or intensified training in horses. Changes in exercise-induced phosphorylation levels were not induced by training or intensified training, although basal phosphorylation levels appeared higher in untrained horses. Therefore, we suggest that normal training or intensified training does not result in major changes of MAPK signaling or basal HSP27 expression in equine muscle.

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Chapter 6

Effect of normal training and intensified training on myosin heavy chain content in equine skeletal muscle

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Abstract

Fiber type distribution plays a major role in muscle plasticity. To address specific differences between various muscles and changes upon training we investigated fiber type distribution by measuring differences and variation in myosin heavy chain (MHC) isoform content of skeletal muscles. Six Standardbred horses were trained for 18 weeks. Before and after training biopsy specimens were taken from the vastus lateralis muscle before and after an exercise test. As a reference, biopsies were taken from six trained horses from vastus lateralis muscle and pectoralis descendens muscle. Subsequent isolation of MHC-enriched fractions from muscle biopsies and electrophoretic separation yielded distinct bands for MHC I, IIa and IIx, Individual pre- and post-exercise biopsies showed variation in MHC isoform distribution, which was not statistically significant at group level. Mean MHC distribution before and after training was not significantly different. The comparison of vastus lateralis and pectoralis densendens muscles did however show a significant difference in MHC content. Expression of MHC IIx was higher and expression of MHC I was lower in pectoralis desendens than in vastus lateralis muscle. We conclude that electrophoretic separation of MHC proteins is capable of identifying differences in fiber type distribution despite individual variations between multiple biopsies from the same muscle. Exercise training did not result in a significant change in MHC distribution.

Introduction

Skeletal muscle can adapt in response to variations in contractile activity, substrate availability and environmental factors. This is important for maintaining cellular and physiological integrity during periods of mechanical and metabolic stress, such as exercise and training. To meet altered functional demands, changes in the phenotype of muscle fibers is a key factor.[1-3] The major fiber constituent that determines fiber phenotype is the myosin heavy chain. This protein exists in slow and fast isoforms coded by different genes.[4] One slow isoform, MHC I, and three fast isoforms, MHC IIa, IIb and IIx exist, in adult mammals. MHC IIb and IIx have comparable functional properties, but with increasing bodysize of species the expression profile of these isoforms changes.[5] The IIb isoform is expressed in small mammals, whereas MHC IIx is also expressed in larger mammals, and exclusively in large species like humans[4] and horses.[6] Different isoforms can be expressed in a single fiber but the most abundant isoform determines overall physiological and contractile properties of a fiber.[4] A significant alteration in the expression of myosin heavy chain (MHC) isoforms therefore contributes to adaptation in response to altered functional demands.
Endurance training induces increases in functional capacity of muscle by adapting for example capillary density, mitochondrial density and increasing activity of key metabolic enzymes. Since these changes are specifically related to different fiber types, it is no surprise that fast-to-slow fiber transitions were observed in response to exercise training. This was reflected by an increase in MHC I and/or IIa expression, and a decrease in MHC IIb and/or IIx expression. In accordance with this, unloading, inactivity and denervation of muscle, for example as a result of certain pathological conditions, results in the opposite effect, e.g., slow-to-fast fiber transition. For example, muscles of horses suffering equine motor neuron disease have a very high percentage of type 2b fibers.[7]
In the present study, the major aim was to investigate if a physical training program comprising sessions of endurance training and high intensity training, resulted in muscle adaptation at the level of fiber type. Therefore, MHC composition was determined before and after a medium-term training program.

Methods

Horses

A total of six untrained Standardbred geldings aged 18 ± 0.4 (SD) months and weighing 367 ± 24 kg and 10 trained Standardbred geldings aged 26 ± 2 months and weighing 430 ± 44 kg were used in the study. The horses had no history of health problems and received no medication. The horses were housed individually in stables. This study was approved by the Committee on Animal Welfare of the Faculty of Veterinary Medicine of the Utrecht University.

Training protocol and muscle biopsy collection

Untrained horses were familiarized with trotting on a treadmill by performing a light exercise session consisting of level trotting at 30-50% estimated maximal heart rate ($HF_{est\text{-}max}$; *see* chapter 2 for explanation) for 30-45 min for 3 to 4 days a week

during four weeks. The next 18-week training period consisted of endurance training (ET) and high intensity training (HIT) sessions. ET and HIT were alternated every training day. Horses trained 4 days a week. Horses also walked in a horse-walker for 60 min, every day of the week, whether training or rest day.

An ET session consisted continuous level running at 60 and 75% $HF_{est\text{-}max}$. Each HIT session consisted of four 2-min or three 3-min bouts at 80% $HF_{est\text{-}max}$ and interspersed with 2-min or 3-min periods at 60% $HF_{est\text{-}max}$. Intensity and volume of training sessions were gradually increased to meet these criteria. Body weight was measured weekly.

Exercise tests were performed after familiarization and after training as described previously. Biopsies specimens from the vastus lateralis muscle were taken before and after the exercise test as described previously. The post-exercise biopsy specimens were taken at the same depth but from a new incision 3 cm caudally from the pre-exercise biopsy specimens. Biopsy specimens were taken from the left muscle before training and from the right muscle after training. Biopsy specimens were immediately frozen in liquid nitrogen and stored at -80°C.

As a reference and to address muscle specific differences in MHC isoform expression, biopsy specimens of trained horses were taken from the vastus lateralis and pectoralis descendens muscle.

Sample preparation

Muscle tissue (50 mg) was homogenized as described previously.[8] Muscle homogenate was centrifuged (10,000 x g, 30 min, 4°C). Supernatant was stored at -80°C. MHC-enriched fractions were prepared from the remaining insoluble pellet. To each pellet 300 µl of fresh extraction buffer (100 mM Na-pyrophosphate, 5 mM EDTA, 1m M DTT, pH 8.5) was added. After resuspending vigorously, samples were incubated on ice for 30 min. After centrifugation (10,000 x g, 30 min, 4°C), supernatant was collected and diluted with electrophoresis sample buffer to a final concentration of 0.2 µg/µl. These MHC-enriched fractions were boiled for 2 min and centrifuged quickly before applying to gel.

SDS-PAGE

Electrophoretic separation of equine muscle MHC was performed essentially according to Rivero and coworkers[9] with minor modifications. In brief, 5 µl of MHC-enriched fractions were run on a single 1mm thick, 18 cm long, 8% acrylamide/bis (50:1) running gel with 4% stacking gel containing 30% glycerol and 4 mM EDTA for 27 hours at a constant current of 10 mA and a maximal voltage of 300V using a cooling unit and a Styrofoam box filled with ice to ensure a temperature below 10°C. The upper running buffer consisted of 100 mM Tris, 150 mM glycine and 0.1% SDS and the lower running buffer consisted of 50 mM Tris, 75 mM glycine and 0.05% SDS. Gels were subsequently stained with Coomassie Brilliant Blue (CBB) for 1 h at 60°C and destained overnight using multiple changes of destaining solution.

Gels were scanned using a calibrated densitometer (GS800, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) and percentages of MHC were calculated with densitometry analysis software (Quantity One, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Percentages of MHC content were determined in pre- and post-exercise

biopsies specimens.
To address variation between biopsy specimens, differences in MHC distribution between pre- and post-exercise biopsies was calculated. Subsequently, the average of these two specimens was calculated and used as the actual distribution in an individual horse. Results were compared before and after training, and between muscle types. Data were analyzed using a paired Student's t-test. Data were considered statistically significant at $P<0.05$.

Results

Electrophoretic separation of MHC-enriched fractions of equine muscle resulted in three distinctive bands representing MHC I, IIa and IIx isoforms (Fig 6.1A).

A variation between biopsies taken from the same muscle from 2 separate incisions with an in-between distance of 3 cm was found. This was reflected by differences in the distribution between pre- and post-exercise biopsies specimens (Fig 6.1B). A standard error of 4 ± 4 % , 3 ± 2 %, and 4 ± 4 % was found for MHC I, IIa and IIx respectively (n=24).
Training did not result in a significant change in composition of MHC content. Biopsy specimens of six untrained horses contained 56 ± 8 % of MHC I, 26 ± 3 % of MHC IIa and 18 ± 7 % MHC IIx, and after training this respectively was 57 ± 6 %, 26 ± 3 %, 17 ± 7 % (Fig 6.2).
In another group of six trained horses the measurement of MHC distribution yielded 53 ± 8 % MHC I, 29 ± 5 % MHC IIa, 18 ± 4 % MHC IIb for vastus lateralis muscle and 20 ± 3 %, 29 ± 4 %, 51

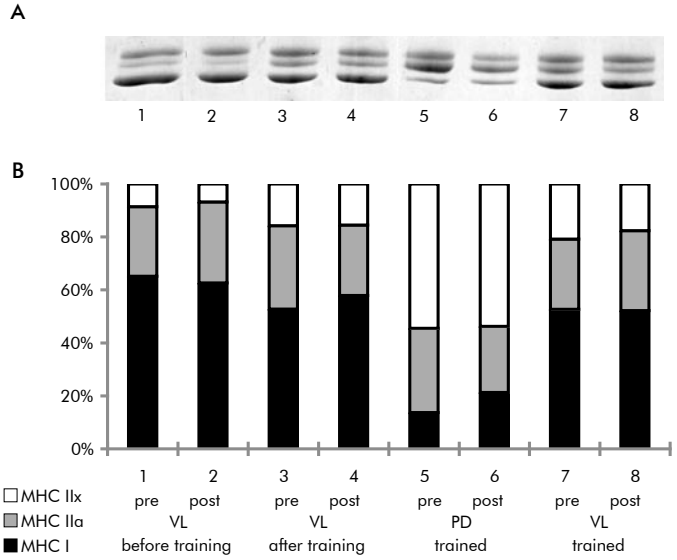

Figure 6.1
Typical individual examples of MHC isoforms separated on gel [A] and MHC composition [B]
Lanes 1-4 and lanes 5-8 each represent an individual horse. VL, vastus lateralis; PD, pectoralis descendens.

± 6 % for pectoralis descendens muscle (Fig 6.3). Mean differences in percentage MHC composition were statistically significant for MHC I ($P<0.0006$) and MHC IIb ($P< 0.0003$).

Discussion

Different muscles in the body serve different motoric functions. For example, vastus lateralis muscle is especially involved in locomotion whereas the pectoralis descendens muscle also is important for posture. This determines the forces that each muscle has to resist and therefore muscle fiber properties are adapted to muscle function already early in life. Since differences in muscle fiber properties are correlated with fiber distribution, histological techniques are routinely used to address this. Since it was previously shown that the electrophoretic separation of MHCs correlates well with immunohistological techniques for determining fiber type distribution using MHC specific monoclonal antibodies,[9] it is likely that alterations in MHC distribution reflect differences in fiber type distribution. In the present study we used the electrophoretic technique since it is far less time-consuming than the histological approach and is less sensitive for investigator interpretation errors. We found that vastus lateralis muscle contains 33% less MHC IIx in favor of MHC I. This suggests that oxidative capacity of vastus lateralis muscle is greater than pectoralis descendens muscle as a result of the presence of more type 1and less type 2b fibers. Adaptation of muscle is the main factor involved in the increased performance levels upon exercise training. In the present study we found that an 18-week treadmill-training program with sessions of endurance and high intensity training did not result in a transition in MHC isoform composition. This is in contrast with previous studies in horses. For example, in an 8-month endurance training study in 4-year old Andalusian stallions, a decrease in MHC IIx was found in gluteus medius.[10] This was accompanied

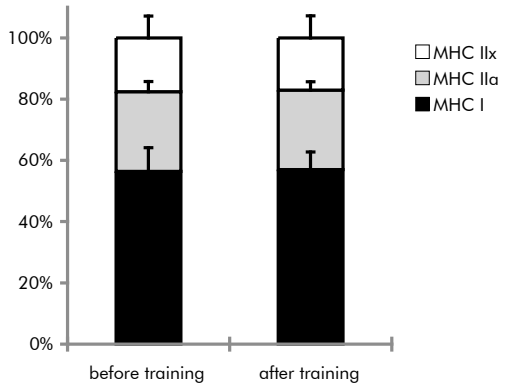

Figure 6.2
Composition of MHC content before and after training.
Mean ± SD of MHC composition of VL muscle are given of 6 horses that were subjected to an 18-week training program. No significant differences were found.

by an increase in MHC IIa after the first 3 months and an increase in MHC I after 8 months. In another study in 4-year old Andalusian mares from the same research group, involving weight pulling (75 kg) at walking and trotting speeds (2.3-3.5 m/s), MHC IIx decreased and MHC I increased upon training.[11] Additionally, in a study in which endurance-trained Arabian horses were compared with sedentary horses, muscle tissue contained less MHC IIx in favor of MHC IIa.[12] The fact that the gluteus medius muscle was used in these studies and not vastus lateralis muscle could explain these discrepancies. Although differences were found in fiber distribution between vastus lateralis muscle of sedentary and trained men, the differences in species-specific demands of vastus lateralis muscle between humans and horses might explain our findings. In addition, the total training frequency and/or duration of training sessions were lower in the present study compared to other equine training studies. Although this was partly imposed by the use of younger animals, it could well be that the relative training load was lower, resulting in adaptations other than a shift in fiber type distribution. For example, it is likely that in the present study muscle mass was increased since body weight was significantly increased upon training from 367 ± 24 kg to 388 ± 22 ($P<0.009$).

In addition to differences in training program and muscle investigated, technical shortcomings could in part explain, especially when using a relatively small number of animals. Although comparing different muscle type showed a statistically significant difference in MHC distribution, the comparison of pre- and post-exercise biopsies, showed a discrepancy in MHC distribution, that in individuals cases reached values of 17%. Although it has been shown previously that differences and adaptations in MHC distribution are a function of biopsy depth,[13] the procedures used in the present study such as the depth at which biopsy specimens were taken, is expected to reach the part of the muscle where adaptation processes are most likely to have taken

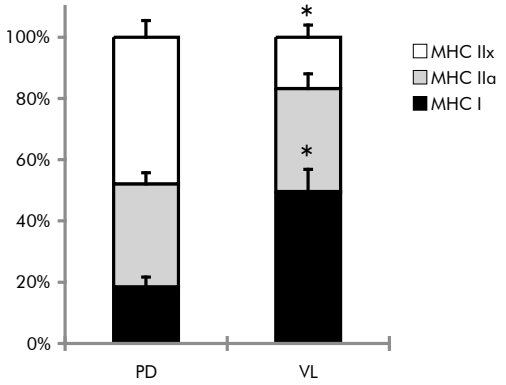

Figure 6.3
Composition of MHC content in pectoralis denscendens and vastus lateralis muscle.
Mean ± SD of MHC composition are given of 6 trained horses.
** differences in MHC I and IIx were statisically significant ($P<0.05$).*

place. Since small variations in fiber distribution already have huge effects on whole muscle capacity, the discrepancies between multiple biopsies of the same muscle are likely to hamper the detection of small training-induced transitions.
In summary, it can be concluded that the investigation of MHC content using electrophoretic separation in muscle biopsy specimens is useful in detecting differences between different muscles. And, although it cannot be excluded that technical shortcomings in combination with a small number of horses obscure the presence of potential small training-induced transitions in fiber types, in the present study no change was found in MHC distribution upon this form of training.

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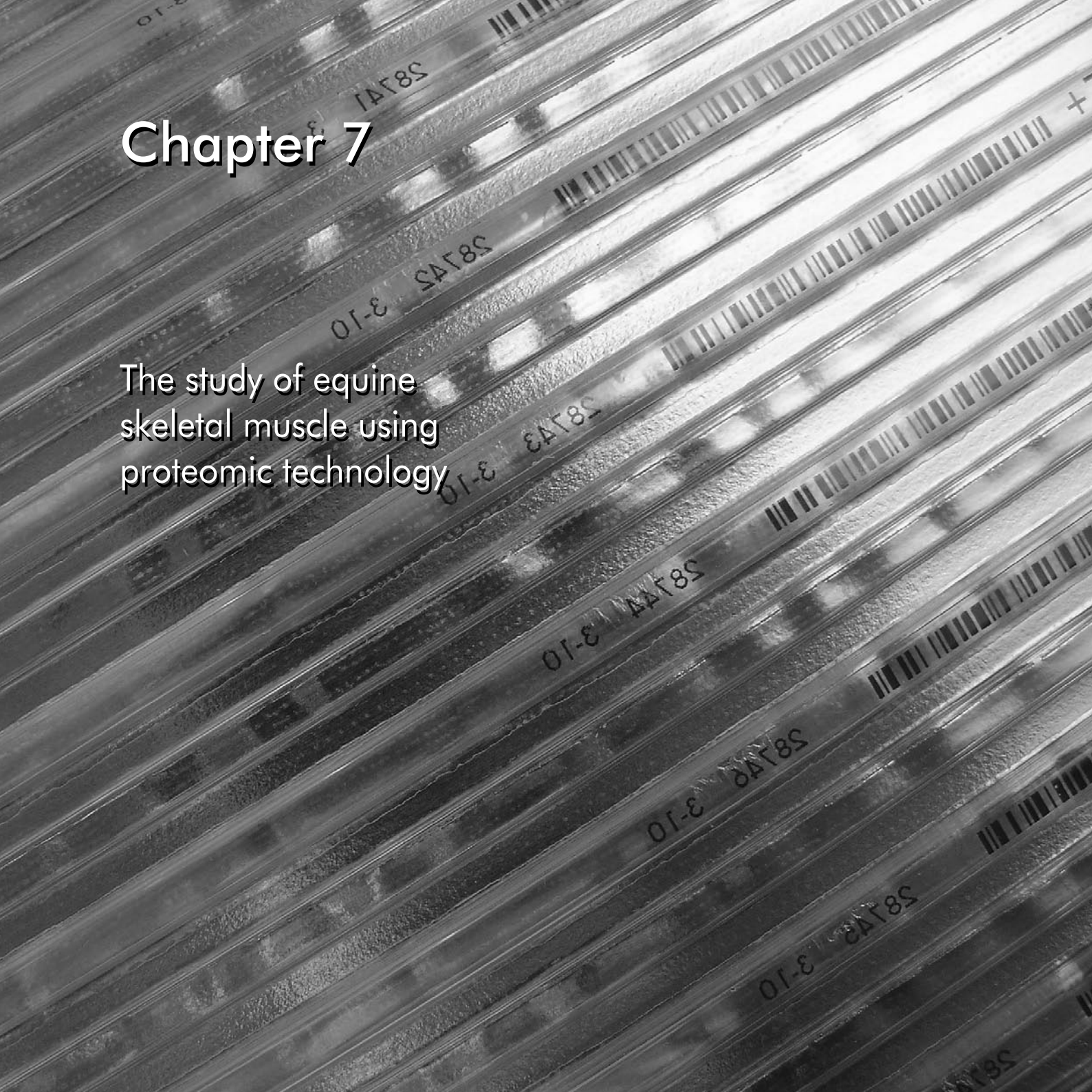

Chapter 7

The study of equine skeletal muscle using proteomic technology

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Submitted

Abstract

Hypothesis—Proteomic technology is a useful tool in studying equine muscle pathology.
Animals—Biopsy specimens of 3 horses suffering from acute tying-up and of 3 healthy horses. Mouse C2C12 myotubes and myoblasts were used in a positive control experiment.
Methods—We performed two-dimensional gel electrophoresis, using an immobilized pH gradient combined with gel electrophoresis, and MALDI-TOF mass spectrometry for identification of proteins that are differentially expressed in tying-up.
Results—2D-gel electrophoresis of skeletal muscle sequential extracts yielded more than 350 protein spots on each gel, of which 14 were differentially expressed more than two times ($p<0.05$). Of this group, 1 spot was unique to tying-up. In addition, 9 spots were of increased abundance ($\geq$ 2x) and 4 spots were decreased ($\leq$ 2x) in tying-up. In-gel digestion followed by peptide mass fingerprinting enabled identification of alpha actin, tropomyosin alpha chain and creatine kinase M chain (CKM). These proteins were significantly increased in the proteome, which in the case of CKM may be due to posttranslational modification.
Conclusions and Clinical Importance—We show that in tying-up, several proteins are differentially expressed. Since changes in the rates of synthesis and degradation of proteins are likely to lead to pathological conditions, identification of differentially expressed proteins in tying-up might result in new hypotheses for the mechanisms that underlie this condition. This paper demonstrates the potential value of proteomic technologies for skeletal muscle research.

Introduction

Proteomic technology can play an important role in the discovery of novel biological markers underlying health and disease. Proteomics comprises a wide range of techniques enabling the identification of proteins and of differential expression of proteins between healthy and diseased states.[1,2] In one of the most popular approaches, extracted proteins are separated by isoelectric point and molecular weight using two-dimensional gel electrophoresis (2DGE), followed by protein staining. Subsequently, proteins of interest can be identified using mass spectrometry (MS).

The technique has already been successfully applied in a mouse model for Duchenne muscular dystrophy,[3] bovine dilated cardiomyopathy,[4] and skeletal muscle of type 2 diabetic patients,[5] proteomics has already proven its use and effectiveness.

Most published studies have focused on the complete muscle of rat origin only.[6-9] Only two studies applied proteomics on small muscles biopsy samples.[5,10] This latter approach could provide important knowledge in veterinary (patho)physiology.

For instance, tying-up is a serious condition that eventually can lead to death. In general, two categories of tying-up can be distinguished: 1) sporadic tying-up, typically caused by an imbalance between load and loading capacity of working muscles, or too much eccentric exercise; and 2) recurrent exertional rhabdomyolysis (RER), which commonly affects performance horses of a specific breed (Arabians, Standardbred, Thoroughbred). Tying-up is probably caused by an underlying muscle disorder. For instance, a hereditary form of RER, polysaccharide storage disease (PSSM), has mainly been found in Quarter horses[11] and results in excessive glycogen storage and abnormal polysaccharide accumulation, which probably reflect an increased synthesis rather than decreased utilization of glycogen.[12] Furthermore, RER in Thoroughbreds has been found to be associated with increased post-exercise myoplasmic calcium levels.[13] In all cases, horses suffering from RER seem to benefit from a low carbohydrate and high fat diet, as suggested by the reported reduced post-exercise plasma creatine kinase (CK) activities found.[14] To date, increased plasma CK activities and histological evaluation of muscle biopsies are the only clinical objective measures for diagnosis of tying-up.[14-16]

The search for new markers of tying-up could be of major importance from both a clinical as well as a scientific point of view because it will result in a better understanding of the mechanisms underlying this condition and, in turn result in a more successful treatment strategy.

Therefore, the major aims of this study were 1) to demonstrate the potential use and importance of proteomic technology for (equine) muscle research and 2) to find clues for the underlying mechanisms of acute tying-up at the protein level.

Materials and methods

Horses and muscle biopsy specimens

Three horses suffering from tying-up were used. Horses were selected from clinical cases that were presented after referral. We selected only those horses that had plasma CK levels beyond 100,000 IU/liter

(Table 7.1). Biopsies were taken post mortem. As a control group biopsies from three non-tying-up diagnosed horses were used. From two horses biopsies were taken post mortem and one by needle biopsy. These horses had no history of myopathy. The percutaneous needle biopsy was taken after application of local anesthesia by use of a modified Bergström needle as described previously.[17] All needle and post mortem biopsy specimens were taken from the vastus lateralis muscle from one incision at a depth of 5 cm on the crossing of a line 15 cm ventrally from the center of the tuber coxa and 10 cm caudally from the cranial border of the vastus lateralis muscle.
All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

Cell culture and induction of differentiation

Mouse C2C12 myoblasts (ATCC# CRL1772, American Type Culture Collection, Manassas, VA, USA) were cultured on 60-mm dishes coated with Matrigel (Becton Dickinson Labware, Bedford, MA, USA) in low-glucose Dulbecco modified Eagle medium (DMEM) containing antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and 9% fetal bovine serum (FBS) (Life Technologies, Rockville, MD, USA). After reaching 80% confluency, cells were further cultured in 0.5% heat-inactivated FBS for 72 h to induce myogenic differentiation.[18]

Homogenization and sample preparation for 2DGE

Cells were trypsinized and collected with a rubber policeman. Cells were centrifuged for 5 min 500 x g at 4 °C. The cell pellet was taken up in rehydration buffer (8 M urea, 2% CHAPS, 1% DTT and 0.5% IEF buffer [IPG buffer pH 3-10, Amersham Biosciences, Roosendaal, The Netherlands]) and sonicated for 2x 5s on ice. The homogenate was rotated end over end for 30 min at 4°C and subsequently centrifuged (30 min, 11,000 x g, 4°C).
Frozen muscle was crushed to a fine powder in liquid nitrogen. The tissue powder was taken up in rehydration buffer and homogenized using a disperser (Yelloline disperser, IKA Works Inc, Wilmington, NC, USA) at 30,000 rpm for 2 times 10 s. The homogenate was rotated end over end for 30 min at 4°C and subsequently centrifuged (30 min, 11,000 x g, 4°C).
For sequential extraction of muscle, 50 mg muscle tissue was crushed to a fine powder and taken up in 40 mM Tris with a Complete protease inhibitor cocktail (Roche, Mannheim, Germany) and rotated for 30 min at 4°C. After centrifugation, the supernatant (E1) was collected. The remaining pellet was washed twice and taken up in 40 mM Tris, 8M urea, 2% CHAPS, 0.5% DTT and 0.5% IEF buffer. After rotation and centrifugation the supernatant (E2) was collected.
Protein concentration in the samples was measured using Biorad protein assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The supernatant was aliquoted and stored at -80°C until further processing.

2DGE: IEF and SDS-PAGE

Solubilized cell samples were resolved on 24 cm IEF strips with a linear pH range of 3 to 10 (Amersham Biosciences, Roosendaal, The Netherlands). Gel

strips were run simultaneously in an IPG-PHOR electrophoresis unit (Amersham Biosciences, Roosendaal, The Netherlands) using a progressive increasing voltage protocol comprising: 14 h 30 V, 1 h 500 V, 1 h 1000 V, 2h 1000-8000 V gradient step, and 8000 V until a total of 74,000 Vh was reached. Strips were loaded with 100 (muscle biopsy) or 125 μg (cells) protein.
IEF strips were first incubated in equilibration buffer (6 M urea, 30% glycerol, 2% CHAPS, 50 mM Tris, pH 8.8) supplemented with 1% DTT for 20 min. Strips were then incubated in equilibration buffer supplemented with 2.5% iodoacetamide for 20 min. After rinsing thoroughly in electrophoresis running buffer (25 mM Tris, 192 mM glycine, 10% SDS), strips were applied to 12% polyacrylamide gels, sealed with 0.5% agarose and run for 5 h at 200 V and 18°C in a multi gel electrophoresis unit (DODECA, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).
In the case of C2C12 cells, 10 individual cultures in each group of myoblasts and myotubes were pooled to normalize dish-to-dish variability. Homogenates were made from these pooled cells and IEF was performed in triplicate. The IEF strips and polyacrylamide gels were run simultaneously to reduce variability.
Horse biopsies were treated individually. Each biopsy was separately homogenized and applied to a single IEF strip. All individual horse samples were run simultaneously.

Staining

The silver staining protocol was adapted from Shevchenko et al.[19] It involved fixation for 1 h in 50% methanol and 5% acetic acid, overnight incubation in 50% methanol; rinsing in deionized water for three times 10 min, 3 min 0.02% sodium thiosulphate, three 5 min washes in deionized water, 1h 0.1% silvernitrate, rinsing two times 1 min in deionized water, and developing with 2% sodium carbonate and 0.04% formaldehyde. After sufficient staining (5-10 min) the gel was transferred to 5% acetic acid for 5 min and finally washed in deionized water for 30 min.

Image analysis and statistics

The stained gels were digitized using a GS800 calibrated densitometer and processed using PD-Quest v. 7.0.3 software (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). A number of spots on each 2D gel were matched manually. Subsequently, the software program matched the remaining spots on the basis of the manually matched spots. The automated matches were checked manually. Spot data were normalized to the total intensity of the gel (C2C12 cell line) or to total intensity of valid spots (horse study), in which case saturated spots were excluded. Spot data of gels belonging to a specific group were then averaged. Averaged spot data of the different groups were compared to detect quantitative and qualitative differences. Differences were checked for their significance ($p<0.05$) with a Student's t-test.

In-gel digestion

Protein spots were manually excised from the gel on a glass plate and processed on a Massprep digestion robot (Waters, Manchester, United Kingdom). A solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulphate was used for destaining. Cysteines were reduced with 10 mM DTT in 100

mM ammonium bicarbonate for 30 min followed by alkylation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 min. Spots were washed with 100 mM ammonium bicarbonate to remove excess reagents and were subsequently dehydrated with 100% acetonitrile (ACN). Trypsin (6 ng/mL) in 50 mM ammonium bicarbonate was added to the gel plug and incubation was preformed at 37°C for 5 h. The peptides were extracted with 1% v/v formic acid/ 2% v/v ACN.[19]

MALDI-TOF MS

Matrix assisted time of flight (MALDI-TOF) MS was performed as described previously.[20] 1.5 mL of each peptide mixture and 0.5 mL matrix solution (10 mg/mL CHCA in 50% ACN/ 0.1% TFA) was spotted automatically onto a 96 well-format target plate. The spots were allowed to air-dry for homogeneous crystallization. Spectra were obtained using a MALDI-TOF mass spectrometer (M@LDI-LR, Waters, Manchester, United Kingdom). The instrument was operated in positive reflector mode. The acquisition mass range was 900–3000 Da. The instrument was calibrated on 8-10 reference masses from a tryptic digest of alcohol dehydrogenase. In addition, a near point lockmass correction for each sample spot was performed using adrenocorticotropic hormone fragment 18–39 (MH1 2465.199) to achieve maximum mass accuracy. Typically 100 shots were combined and the background was subtracted. A peptide mass list was generated for the subsequent database search.

Database search

The obtained protein fingerprints were searched with ProteinLynx Global Server v. 2.0 (Waters, Manchester, United Kingdom) or the Mascot search engine (http://www.matrixscience.com) against the Swiss-

Table 7.1 Horse characteristics

breed	sex	age (yr)	plasma CK activity (IU/l)	biopsy	diagnosis	group
Friesian	mare	0.5	n.d.	post mortem		control
Pony	gelding	28	n.d.	post mortem		control
Standardbred	mare	4	n.d.	needle biopsy		control
Iceland pony	gelding	6	114 000	post mortem	rhabdomyolysis following castration	tying-up
Trakehner	mare	1.7	180 000	post mortem	atypical rhabdomyolysis	tying-up
Groninger	gelding	7	179 000	post mortem	SCAD	tying-up

SCAD, Short-chain Acyl-CoA dehydrogenase deficiency; n.d., not determined.

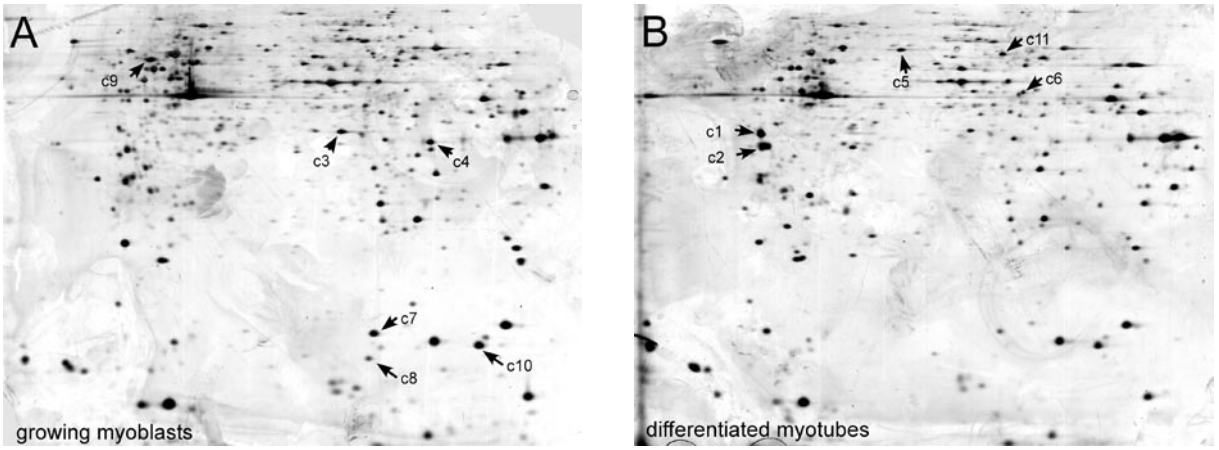
Prot database (http://expasy.ch/sprot) for protein identification. One miss-cleavage was tolerated; carbamidomethylation was set as a fixed modification and oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as optional modifications. The peptide mass tolerance was set to 100 ppm. No restrictions were made on the protein Mr and the pI. A protein was regarded as identified when it had a significant ProteinLynx or MASCOT probability score ($p<0.05$) and at least five peptide mass hits or sequence coverage of at least 25% of the complete protein sequence.

Results

Myogenic differentiation

More than 350 protein spots were detected on each group of three gels containing the extracts of growing myoblasts or differentiated myotubes (Fig 7.1). Of these proteins 118 were differentially expressed ($dOD \geq 2x$, $p < 0.05$). A number of 60 were excised and characterized. Of this group, 7 spots were unique to growing myoblasts, 12 spots were unique to differentiated myocytes. In addition, 20 spots were of increased abundance ($\geq 2x$) after differentiation

Figure 7.1
2DGE images of growing [A] and differentiated [B] C2C12 cell proteomes.
Numbered spots were excised from the gel using an automated spot cutter and analyzed and identified using MALDI-TOF MS.



and 21 spots were of increased abundance (≥ 2x) during growth. In-gel digestion followed by peptide mass fingerprinting enabled identification of 11 of these (Table 7.2).

2DGE of equine skeletal muscle

Solubilized proteins of three healthy horses were separated with 2DGE and silver stained. Large spot clusters (A-G, Fig 7.2, Table 7.3) could be identified by comparing the 2DGE patterns of equine vastus lateralis muscle with that of mouse gastrocnemius muscle (www.expasy.org).[21] A number of 16 spots was randomly excised and analyzed further with MALDI-TOF MS (Fig 7.2). Of these spots 8 were matched to a known protein (Table 7.3).

Acute tying-up

A comparison was made between the sequential protein extracts, E1 and E2, of healthy and tying-up animals. A number 14 of protein spots were found to be differentially expressed (dOD≥ 2x, $p<0.05$) compared to healthy horses (Fig 7.3). A number of 9 were increased in tying-up, and a number of 4 were decreased in tying-up. One spot was unique to horses suffering from tying-up. These spots were excised from the gel and processed for further analysis. This yielded 3 spectra that were matched to a known protein (spots 20, 22 and 29; Table 7.4). In addition, nine protein spots that were not differentially expressed were identified as well using MALDI-TOF MS (spots 31-39; Table 7.4).

Table 7.2 Proteins identified by MALDI-TOF MS in C2C12 differentiation

spot nr	protein	accession nr	MW (Da)	pI	matched peptides	coverage (%)	regulation
c1	Tropomyosin beta chain	P58774	32933	4.66	14	39	↑
c2	Tropomyosin 1 alpha chain, splice variant 2	P58771	32746	4.71	23	53	↑
c3	Annexin A1	P10107	38869	7.15	18	57	↓
c4	Annexin A2	P07356	38806	7.53	9	34	↓
c5	Protein disulfide-isomerase A3 precursor (EC 5.3.4.1)	P27773	57050	5.98	21	35	↑
c6	Pyruvate dehydrogenase E1 component alpha subunit, somatic form, mitochondrial [Precursor]	P35486	43888	8.49	12	26	↑
c7	Nucleoside diphosphate kinase B	Q01768	17466	6.97	6	34	↓
c8	Nucleoside diphosphate kinase A	P15532	17311	6.84	8	47	↓
c9	Tubulin beta 5 chain	P99024	50095	4.78	12	17	↓
c10	Peptidyl propyl cis trans isomerase A	P17742	18000	7.88	8	41	↓
c11	Dihydrolipoyl dehydrogenase	O08749	54748	7.97	12	29	↑

Spot nr corresponds to the number in Fig 7.1. The accession nr is the identification code for that specific protein in SwissProt and the pI and Mr are the theoretical values given by SwissProt. The matched peptides and coverage are indicative for the amount of protein fragments that corresponded to the theoretical peptides. The upward (downward) arrow under regulation indicates that the expression of that protein was significantly (≥2, $p<0.05$) increased (decreased) upon differentiation of C2C12 cells.

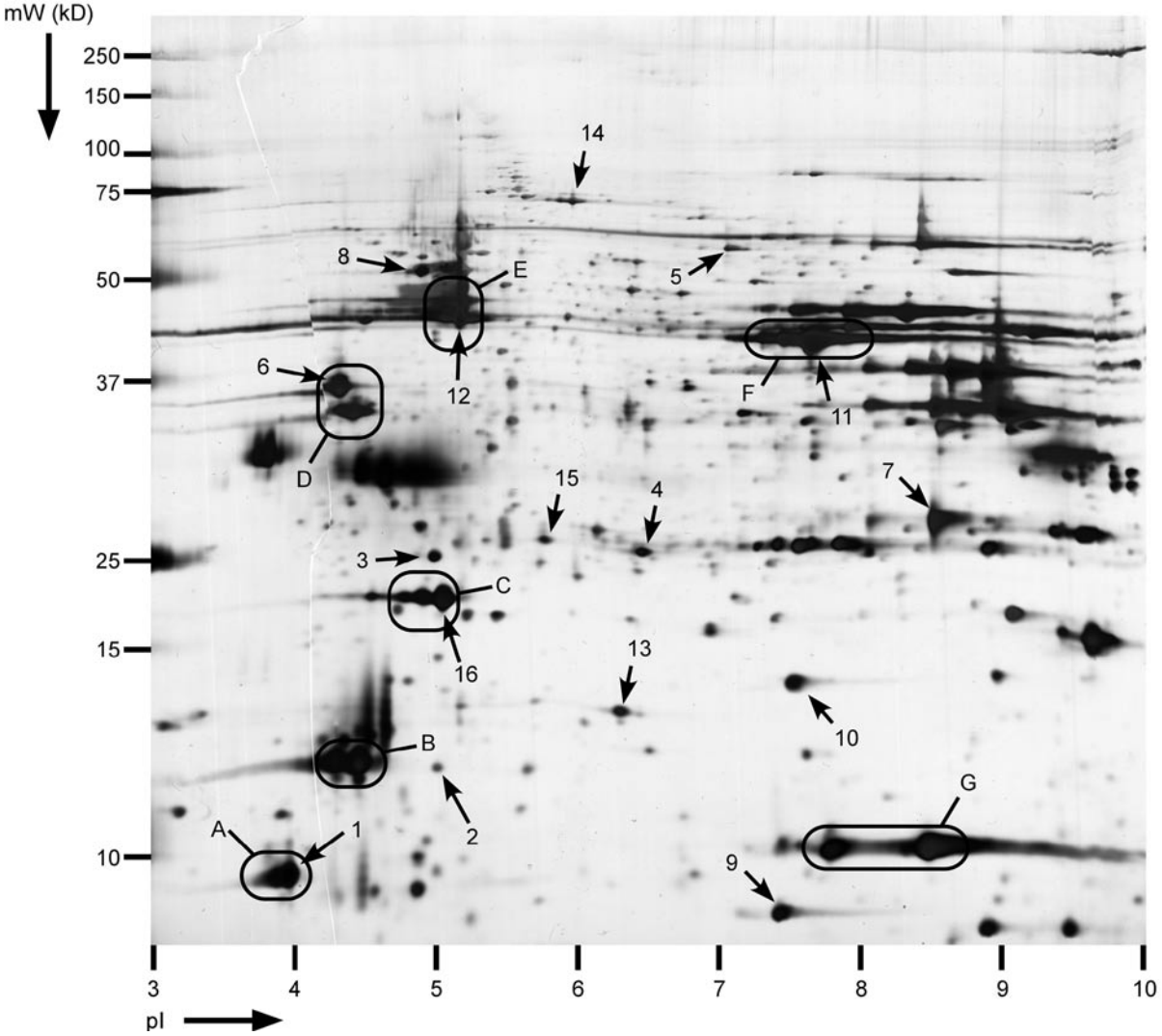


Figure 7.2
2DGE image of total equine skeletal muscle homogenate.
Encircled spots regions were visually matched to known spots in mouse skeletal muscle (see: www.expasy.org/cgi-bin/map2/def?MUSCLE_MOUSE)
Numbered spots were manually excised from the gel and analyzed using MALDI-TOF MS.

Discussion

We demonstrated that proteomic technology is useful in veterinary muscle research. Furthermore, we found 14 protein spots that were differentially expressed (dOD $\geq$ 2x, $p<0.05$) in tying-up compared to healthy horses. Since therapy to cure tying-up is limited, detailed studies to unravel the mechanisms of the underlying disorder are warranted. The identification of differentially expressed proteins could potentially reveal a protein or a cluster of proteins that is involved in the clinical symptoms of tying-up.
In order to validate 2DGE on muscle, we used the mouse derived C2C12 cell line. The cell line has a low variability because of the homogeneity of cells in culture in comparison to mixed cells from a muscle biopsy. Furthermore, the genetic map of the mouse has now been completely unraveled and almost no restrictions for identifying proteins are present. Finally, over 1500 genes have been reported to change their RNA expression profiles during myocyte differentiation[22] and therefore applying the approach of the differentiating cell line provides us with good positive control tools.[23-25] We were able to detect differential expression of proteins and identify some in the C2C12 cell line. For example, expression of

Table 7.3 ***Proteins identified by MALDI-TOF MS in equine skeletal muscle***

spot nr	protein (homologue)	accession nr	MW (Da)	pI	matched peptides	coverage (%)
3	Myosin light chain 1, slow twitch muscle B (human)	P08590	21926	5.03	7	35
6	Tropomyosin beta chain (human)	P07951	32945	4.66	10	28
7	Carbonic anhydrase III, horse	P07450	29647	7.84	12	56
8	ATP synthase beta chain, mitochondrial precursor	P06576	56525	5.26	12	27
9	Hemoglobin beta chain, horse	P02062	16055	6.52	11	86
11	Creatine kinase M chain (human)	P06732	43302	3.77	11	30
14	Serum albumin precursor, horse	P35747	70550	5.95	11	17
16	Myosin light chain 1, slow twitch muscle B (human)	P08590	21787	5.07	11	48
A	myosin light chain 3	P05978				
B	myosin regulatory light chain 2	P97457				
C	myosin light chain 1	P05977				
D	tropomyosin 1 alpha chain	P58771				
E	actin	P99041				
F	creatine kinase M chain	P07310				
G	hemoglobin beta 1 chain	P02088				

Spot nr corresponds to the number in Fig 7.2. The accession nr is the identification code for that specific protein in SwissProt and the pI and Mr are the theoretical values given by SwissProt. The matched peptides and coverage are indicative for the amount of protein fragments that corresponded to the theoretical peptides. If a protein homologue was found of another species, the species is given between parentheses. Spots in regions A-G were annonated by their similar patterns in comparison with published data of mouse gastrocnemius muscle. Since these regions comprise several spots, pI and MW cannot be given.

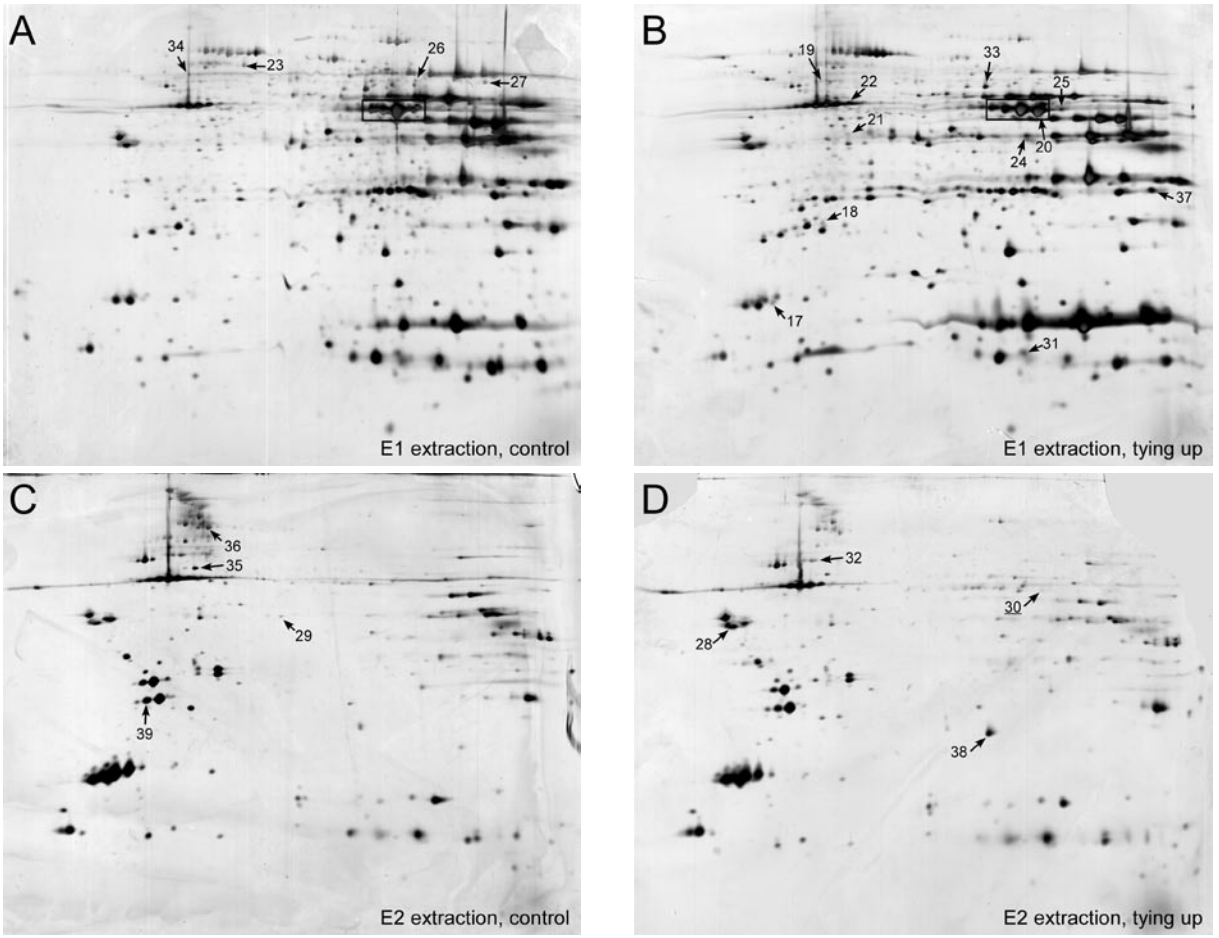


Figure 7.3
2DGE images of sequential extracts of equine skeletal muscle from healthy horses and horses suffering from tying-up.
Numbered spots were excised from the gel using an automated spot cutter and analyzed using MALDI-TOF MS. Spots 17-30: a spot number depicted on a certain gel means that the spot is most abundant or only present (underlined, spot 30) in the group the gel represents. Spots 31-39 were excised and identified using MALDI-TOF MS; they were however not differentially expressed. The box indicates the region of CK protein spots that is enlarged in Fig 7.4

tropomyosin beta chain and tropomyosin 1 alpha chain, both cytoskeletal proteins, were increased in differentiated myotubes. This is in line with previous studies[23-25] and reflects formation of myofibrils upon differentiation of myocytes. Thus, the validation of proteomic technology by 2DGE in C2C12 cells seems to be successful.

For a global survey of the equine muscle proteome we performed a simple mapping of extracts of muscle biopsy specimens. Large protein spots, like actin, creatin kinase, etc. could be annotated by comparing the silver stained 2DGE patterns of equine vastus lateralis muscle with that of mouse gastrocnemius muscle (see: www.expasy.org).[21] Some spots were excised from the gels and analyzed using MALDI-TOF MS. In most cases horse proteins could be identified based on the mass fingerprint of a homologue from another species, e.g. human, rat, mouse or bovine. The fact that few spectra of equine proteins could be matched with predicted spectra of horse proteins in databases on the Internet clearly indicates that there still is a large gap between mouse, rat and human research and equine research. Fortunately most proteins are largely homologous between species, but the limited resources regarding the equine genome can in some cases hamper identification. It would be of major importance if more of the equine genome would be unraveled in the near future.

To demonstrate the potential of proteomics in the veterinary field, we analyzed biopsies of healthy horses and horses suffering from tying-up. Because of the complexity of the muscle homogenate, and

Table 7.4 ***Proteins identified by MALDI-TOF MS in sequential extracts of equine skeletal muscle and differentially regulated proteins in tying-up***

spot nr	Protein (homologue)	accession nr	MW (Da)	pI	matched peptides	coverage (%)	regulation
20	Creatine kinase M chain (human)	P07310	43018	7	12	33	↑
22	Actin alpha 1 (human)	P68133	42024	5.32	11	33.5	↑
29	Tropomyosin alpha chain (rat)	P04692	32533	4.72	25	60	↑
31	Hemoglobin, horse	P02062	15998	6.94	10	85	=
32	Desmin (bovine)	O62654	53368	5.27	16	35	=
33	Aldehyde dehydrogenase, cytosolic, horse	P15437	54709	6.94	14	27.4	=
34	Actin alpha 1 (human)	P68133	42024	5.32	11	33.5	=
35	ubiquinol cyt c reductase complex core protein I, mitochondrial precursor (bovine)	P31800	53444	5.94	11	22	=
36	HSP71 (human)	P08107	70294	5.48	20	30	=
37	Glutathione S-transferase Mu 5 (human)	P46439	25716	7.3	10	26	=
38	Alpha crystallin B chain (mouse)	P23927	20056	6.76	7	34	=
39	Myosin light chain 1, slow twitch (human)	P08590	21787	5.07	11	48	=

Spot nr corresponds to the number in Fig 7.3. The accession nr is the identification code for that specific protein in SwissProt and the pI and Mr are the theoretical values given by SwissProt. The matched peptides and coverage are indicative for the amount of protein fragments that corresponded to the theoretical peptides. The upward arrow under regulation indicates that the expression of that protein was significantly increased (≥2, $p<0.05$) in horses suffering from tying-up. Proteins that were identified in sequential extracts but were not differentially expressed (=) are also given. If a protein homologue was found of another species, the species is given between parentheses.

the relatively huge amount of some typical skeletal muscle proteins, we decided to perform a sequential extraction in the study of tying-up horses. This technique uses differences in solubility between proteins and divides the proteome in different sub-proteomes. The patterns of E1 and E2 extracts indicate that more basic and metabolic enzyme proteins were extracted during the first step, whereas more acidic and structural proteins were extracted in the second step. Using this strategy we were able to identify three proteins that appeared to be differentially expressed in horses suffering from tying-up (Table 7.4). In addition we were able to identify several other proteins in these extracts.

Actin alpha 1, tropomyosin alpha chain and creatin kinase M chain were significantly increased in tying-up. Since these proteins can be found in multiple spots in 2DGE (Fig 7.2),[21] the altered expression of a single protein spot in the entire protein cluster could mean that that protein is modified in some way. Interestingly, over 60 mutations are known in the gene for actin alpha 1 (ACTA1). For example, ACTA1 mutations are related to congenital muscle disorders and are associated with changes in size and distribution of muscle fiber types.[26] Although it may be secondary to the pathology, the increase in a specific actin alpha 1 protein spot may also be the reflection of a genetic predisposition for tying-up. Mutations in the gene that codes for tropomyosin alpha chain (TPM3) are also related to congenital myopathies.[27]

Another striking finding was that the staining patterns of spots containing the CK protein of tying-up horses were significantly different from that of healthy horses. In horses with tying-up a CK protein variant with a higher isoelectric point and a slightly smaller mass was present that was almost absent in healthy horses (Fig 7.4). Our results indicate that this CK protein spot is more positively charged, and therefore more basic. An alteration in protein charge in general means an altered balance of non-covalent bonds resulting in a conformational change and therefore an altered 3D-structure. The conformational change of a protein might in turn result in activation or inactivation of its biological function or affect its binding to sub-units. In case of the CK protein, the positive charge and the smaller mass are likely to be

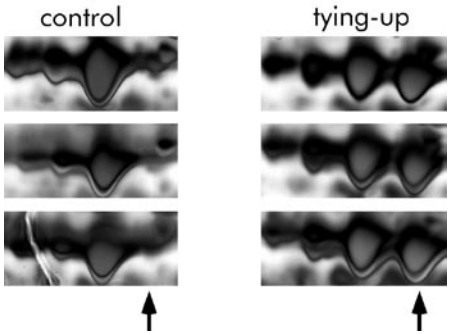


Figure 7.4
Close-up of the 2DGE region containing Creatine Kinase M chain protein spots.
The arrow indicates the CK protein spot that is increased in skeletal muscle of horses suffering from tying- up.

the result of a posttranslational modification like, for example, a dephosphorylation. Because of the lack of the exact amino acid sequence of equine CK protein we were not able to identify a possible modification site. Although the possibility that the observed modification is related to small amounts of blood that were present in the muscle biopsies, as indicated by the presence of hemoglobin, and therefore represent muscle-derived plasma CK, cannot be excluded, the modification might well be related to the sensitiveness for muscle damage in the tying-up horses.

In conclusion, the present study demonstrates that using proteomic technology, skeletal muscle proteins can be identified that could serve as markers for specific myopathies associated with tying-up. This study demonstrates that proteomics can be of value in veterinary skeletal muscle research. To the best of our knowledge this is the first study using proteomic technology on equine muscle biopsies with a direct clinical link. It is clear however, that more effort has to be put in proteomics and genomics studies of equine muscle biopsies to come to the full potential and clinical relevance of this promising technique.

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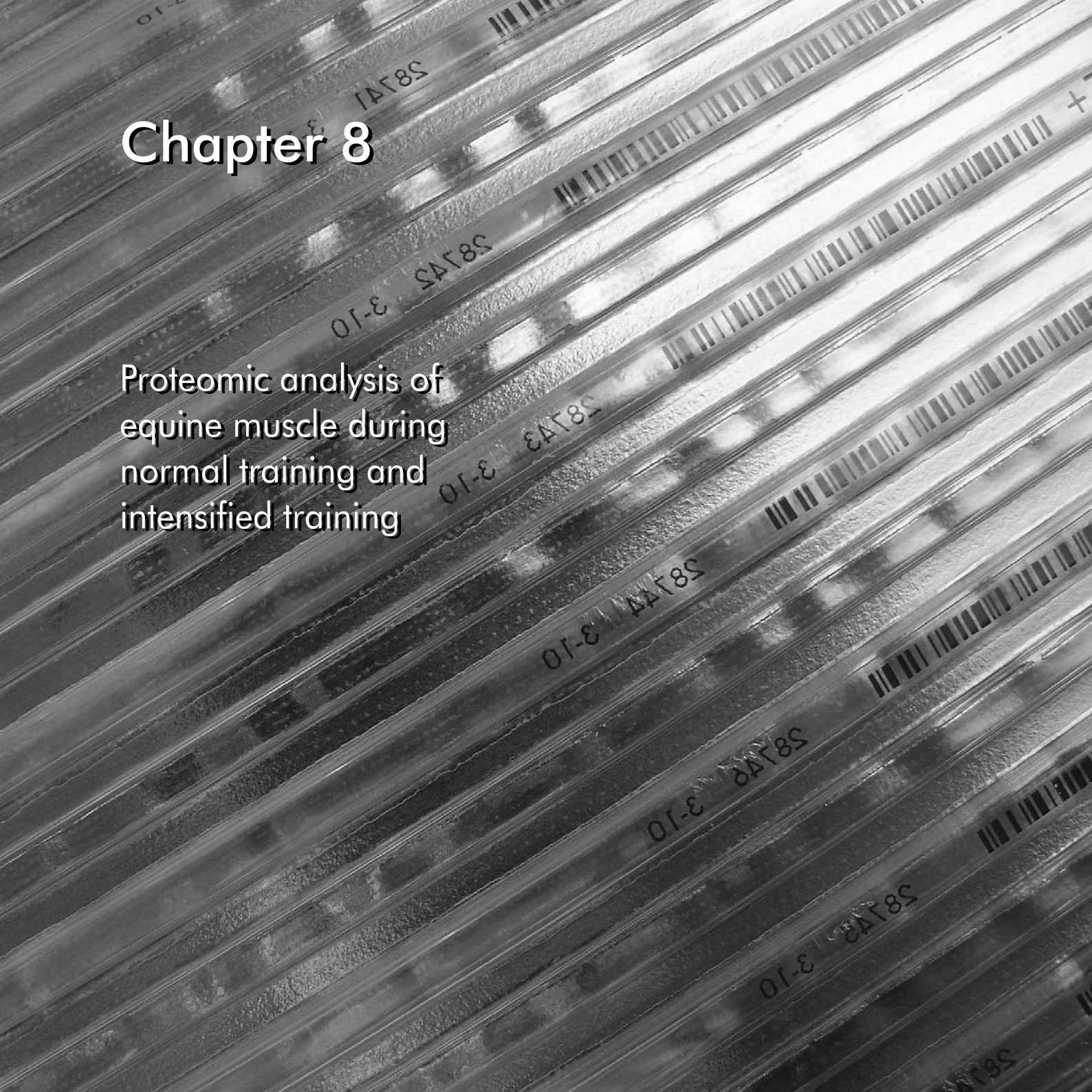

Chapter 8

Proteomic analysis of equine muscle during normal training and intensified training

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Abstract

Hypothesis—Specific proteins are differentially expressed upon normal and/or intensified training. Discovery of such proteins could serve as early markers for training-induced adaptive processes and/or for overtraining.

Animals—A total of 16 young Standardbred geldings were use in this study. Six untrained horses were trained for 18 weeks, and two groups of five trained horses respectively entered a 6-week normal or intensified training protocol.

Methods—Two-dimensional gel electrophoresis (2DGE) was performed on skeletal muscle biopsy specimens, using an immobilized pH gradient combined with gel electrophoresis. Gels were stained, digitized and analyzed using specific software.

Results—2DGE of skeletal muscle sequential extracts yielded 62 protein spots that were differentially (2 times more or less, $P<0.05$) expressed upon training, of which 23 protein spots were decreased and 39 protein spots were increased. In the intensified trained horses a group of 7 protein spots was significantly increased ($\geq 2x$, $P<0.05$) upon training compared to control groups.

Conclusions and Clinical Importance—We show that in normal and intensified training, several proteins are differentially expressed in skeletal muscle tissue. Their exact identity and biological relevance has to be established in future studies.

Introduction

The major aim of physical training in athletes is to improve overall performance. Long-term adaptative responses in skeletal muscle are an essential element in this process. For instance, an increase in muscle mass and an increased activity of specific metabolic enzymes and in concert with changes in the expression of structural muscle proteins are known to contribute to these functional changes in muscle.[1-3] Many of these proteins have been identified over the past decades using conventional techniques. These proteins belong to the highly abundant present proteins in skeletal muscle. Other proteins, most of them expressed to a lesser extent, might play an even important role in the adaptive processes upon training and could, therefore, contribute to increased performance level as well.

In contrast to normal adaptation to training, overly training (staleness) in combination with an imbalance between training frequency and (active) rest, can result in a long-term deterioration in performance.[4] Although overtraining research has mainly focused on behavioral aspects and systemic variations of biochemical, hormonal and immunological markers, measured in blood and urine samples of healthy and overtrained elite athletes, at present, no universal biological marker has yet emerged from the scientific literature.[5] An early phase of the full-blown overtraining syndrome is caused by metabolic derangements in skeletal muscle.[6] Although speculative in nature, it is not unlikely that specific changes in skeletal muscle function precede later stages of overtraining. Although overtraining studies in horses are limited,[3,7-12] a few studies have addressed the role of skeletal muscle during experimental induced harsh training. For instance, in one study, overload training in Standardbred geldings did not result in changes in fiber area, number of capillaries per fiber or mitochondrial density.[8] Furthermore, lower basal muscle glycogen levels were found in, according to the definition of the authors, overtrained Standardbred horses compared to normal trained horses.[3]

During the last decade, proteomic technology has shown to be useful in identifying new proteins that are differentially expressed in muscle following denervation,[13] immobilization,[14] hindlimb suspension-induced atrophy and hypertrophy,[15] in red and white porcine muscle,[16] and in pathological conditions such as bovine dilated cardiomyopathy,[17] and Duchenne muscular dystrophy.[18] It should be noticed that the majority of these studies were performed in laboratory animals, using intact post-mortem skeletal or heart muscles, in which robust experimental conditions were artificially introduced. In contrast, the use of small muscle biopsies to study (patho)physiological differences in combination with proteomics, is a technique that has only recently been explored successfully by several research groups.[19,20] Numerous proteins that play a role in the functional changes upon training have been identified over the past decades using conventional techniques. The advantage, however, of proteomic technology would be the ability to investigate a wide range of proteins simultaneously and discover proteins that have not yet been identified.

Therefore, the major aim of the present study was to study the equine proteome at different stages of training and intensified training.

Materials and methods

Horses

A total of sixteen Standardbred gelding were used in the study. Six untrained horses (aged 18 ± 0.4 (SD); weight 367 ± 24 kg) followed a training protocol. Five trained horses (aged 25 ± 2 months; weight 416 ± 61 kg) were admitted to the normal training group and another five trained horses (aged 25 ± 2 months; weight 410 ± 17 kg) were admitted to the intensified training group. Horses were housed individually in stables, and were fed according to their individual requirements. Water and salt blocks were available *ad libitum*.

All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

Training protocol

Horses were familiarized with trotting on a treadmill during four weeks of training by trotting at speeds corresponding to 30-50% estimated maximal heart rate ($HF_{est\text{-}max}$; see chapter 2 for explanation) for 20-35 min for 3-4 days a week. Pre-training biopsies were taken after this period. Horses then continued training for 18 weeks. Horses trained 4 days a week in which sessions of endurance training at 60% and 75% $HF_{est\text{-}max}$ and high intensity training at 85% $HF_{est\text{-}max}$ were alternated and training load (speed, inclination and duration) were gradually increased over time. After this period, the second biopsy specimen was taken.

Normal and intensified training protocol

The normal training procedure consisted of six weeks of training at a frequency and load as described in the previous section, which aimed at retaining performance level. The intensified training consisted of longer training sessions in the first three weeks, and the frequency was increased from 5 to 7 days a week in the last three weeks of training.

Muscle biopsy specimens

Percutaneous needle biopsy samples were taken from the vastus lateralis muscle before and after training using a modified Bergström needle under local anesthesia as described before.[21] Biopsy specimens were obtained from the vastus lateralis muscle at a point located 15 cm ventral from the center of the tuber coxa and 7 cm caudal from the cranial border of the muscle; the specimens were obtained at a depth of 5 cm. Biopsies were taken from the left muscle before training and the right muscle after training. Blood was carefully removed and was immediately frozen in liquid nitrogen. Biopsy specimens were subsequently stored at -80°C until further processing.

Sequential extraction of muscle

Muscle tissue (50 mg) was crushed to a fine powder and taken up in 40 mM Tris containing Complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mM EDTA, and rotated for 30 min at 4°C. After centrifugation, the supernatant (E1) was collected. The remaining pellet was washed twice and taken up in rehydration buffer (8M urea, 2%

CHAPS, 0.5% DTT and 0.5% IEF buffer [IPG buffer pH 3-10, Amersham Biosciences, Roosendaal, The Netherlands]). After rotation and centrifugation the supernatant (E2) was collected.
Protein concentration in the samples was measured using computed densitometry analysis (Quantity One, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) of Coomassie Brilliant Blue stained 1D SDS-PAGE gels. The supernatant was aliquoted and stored at -80°C until further processing.

2DGE: IEF and SDS-PAGE

Individual E1 and E2 muscle extracts from horses before and after training, and pools of five E1 extracts of horses before and after normal or intensified training, on 24 cm IEF strips with a linear pH range of 3 to 10. Gel strips were run in an IPG-PHOR electrophoresis unit (Amersham Biosciences, Roosendaal, The Netherlands) using a progressive increasing voltage protocol comprising: 14 h 30 V, 1 h 500 V, 1 h 1000 V, 2h 1000-8000 V gradient step, and 8000 V until a total of 74,000 Vh was reached. Strips were loaded with 100 μg (E1) or 150 μg (E2) protein.
IEF strips were first incubated in equilibration buffer (6 M urea, 30% glycerol, 2% CHAPS, 50 mM Tris, pH 8.8) supplemented with 1% DTT for 20 min. Strips were then incubated in equilibration buffer supplemented with 2.5% iodoacetamide for 20 min. After rinsing thoroughly in electrophoresis running buffer (25 mM Tris, 192 mM glycine, 10% SDS), strips were applied to 12% polyacrylamide gels, sealed with 0.5% agarose and run for 5 h at 200 V and 18°C in a multi gel electrophoresis unit[f]. Each sample of an individual extract (training) was separated on two gels in two different experiments. The gel with the best resolution was analyzed further. Each sample containing pools of five individuals (intensified training) were separated on three gels within one experiment. These three gels were all analyzed further.

Staining

The silver staining protocol was adapted from Shevchenko et al..[22] It involved fixating for 1 h in 50% methanol and 5% acetic acid, overnight incubation in 50% methanol; rinsing in deionized water for three times 10 min, 3 min 0.02% sodium thiosulphate, three 5 min washes in deionized water, 1h 0.1% silvernitrate, rinsing two times 1 min in deionized water, and developing with 2% sodium carbonate and 0.04% formaldehyde. After sufficient staining (5-10 min) the gel was transferred to 5% acetic acid for 5 min and finally washed in deionized water for 30 min.

Image analysis and statistics

Stained gels were digitized using a GS800 calibrated densitometer and processed using PD-Quest v. 7.0.3 software (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). A number of spots on each 2D gel were matched manually. Subsequently, the software program matched the remaining spots on the basis of the manually matched spots. The automated matches were checked manually. Normalization of spot data consisted of calculating the ratio of spot density and the total density of valid spots. Saturated spots were excluded in both cases. In the training experiment, gels of the same individual were paired, and differences between the untrained and trained

group were tested using a paired Student's t-test. In the intensified training experiment, spot data of the different groups were compared to detect differences with a Student's t-test. Differences were regarded statistically significant at $P<0.05$. The molecular weight and iso-electric point of differentially expressed protein spots were estimated by use of a molecular weight marker, which was used on all gels, and the range of the used IEF gel strips (pH 3-10, linear range).

Results

Training

A total of 653 protein spots were matched in gels containing E1 sequential extracts of individual skeletal muscle biopsies from 6 horses before and after training (Fig 8.1). A number of 20 protein spots were significantly differentially expressed upon training, of which 5 protein spots were decreased (≤2x, $P<0.05$) and 15 protein spots were increased (≥2x, $P<0.05$) in staining intensity (Table 8.1). In addition, a total of 511 protein spots were matched in gels containing E2 sequential extracts (Fig 8.2). A number 42 protein spots was significantly differentially expressed upon training, of which 18 protein spots decreased (≤2x, $P<0.05$) and 24 protein spots increased (≥2x, $P<0.05$) (Table 8.2).

Normal versus intensified training

A total of 219 protein spots were matched in gels containing pooled (n=5) E1 sequential skeletal muscle extracts of horses before and after normal or intensified training (Fig 8.3). In the intensified trained horses a group of 7 protein spots was significantly increased (≥2x, $P<0.05$) upon training compared to the normal training group after training and both groups before training (Table 8.3).

Discussion

Biological findings

In the present study we demonstrated that normal and intensified training in horses resulted in significant changes in the skeletal muscle proteome. Upon training, expression level of 39 proteins increased, whereas in 23 proteins a significantly decreased level in expression was found.

Because two different groups of horses were used to study the effect of intensified training, individual extracts were pooled. This allowed us to use, in one single experiment, triplicate gels of each group before and after training. Hence, inter-experimental variation was reduced, possibly at the expense of a higher risk of false positive results. We assumed that only the intensified training program would potentially induce (early) overtraining and therefore only differences between the group after intensified training and all other groups would be considered biologically relevant. For the same reason, only proteins were selected that showed a difference of at least two magnitudes in expression level. This margin may be considered reasonable since the number of horses in the present study was limited, and the individual differences could potentially affect the end results. Employing such an approach we found that 7 proteins increased upon intensified training. Remarkably, one of the spots (Fig 8.3/Table 8.3,

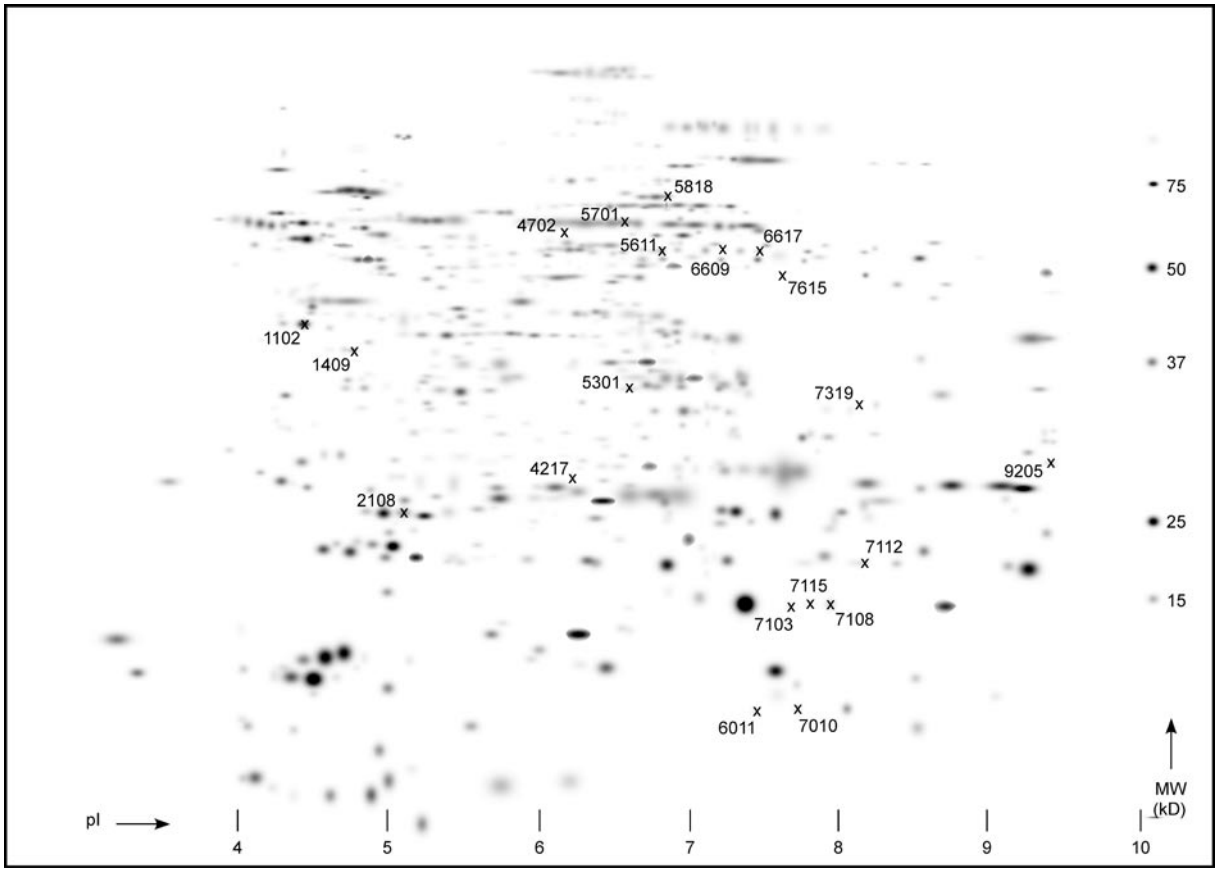

Figure 8.1
Image of master gel with matched protein spots of E1 sequential extracts of equine skeletal muscle before and after training
Protein spot numbers that showed differential expression upon normal training of previous untrained horses are depicted. Estimated pI and MW and regulation of these protein spots are described in Table 8.1. MW, molecular weight marker; pI, iso-electric point.

spot 1407) that increased in expression level upon intensified training shows comparable characteristics (location on gel, estimated molecular weight and iso-electric point) as a protein spot that was decreased upon normal training (Fig 8.1/Table 8.1, spot 1409). This suggests differential regulation of this protein upon normal versus intensified training.

Technical aspects

Compared to other proteomics studies using muscle tissue, we applied a relatively high amount of protein in combination with silver staining. This resulted in overstaining of a considerable number of protein spots. For this reason, these spots were excluded in the analysis. This in turn, also explains the relatively

Table 8.1 ***Differentially expressed proteins in E1 sequential muscle extracts upon training***

spot nr	MW (kD)	pI	untrained (PPM)	trained (PPM)	Ratio to untrained	effect
1402	42	4.4	5494	2703.2	0.49	-
1409	38	4.8	969	361.9	0.37	-
2108	26	5.1	3150	1379.5	0.44	-
4217	28	6.2	71.5	180.5	2.52	+
4702	60	6.2	60.6	167.6	2.76	+
5301	35	6.6	550.3	1158.5	2.11	+
5611	54	6.8	461.7	1172.6	2.54	+
5701	62	6.6	2708.3	941.7	0.35	-
5818	71	6.9	870.8	2214.3	2.54	+
6011	12	7.4	261.4	536.7	2.05	+
6609	55	7.2	292.1	830.3	2.84	+
6617	54	7.5	439.6	1205.1	2.74	+
7010	12	7.7	432.9	881.8	2.04	+
7103	15	7.7	341.8	1127.9	3.3	+
7108	15	7.9	625.4	1731.5	2.77	+
7112	19	8.2	646.7	1587.5	2.45	+
7115	15	7.8	224.4	712.1	3.17	+
7319	33	8.1	178.7	363.8	2.04	+
7615	49	7.6	78.4	174.2	2.22	+
9205	29	9.4	1825.4	240.8	0.13	-

Effect: - decreased, + increased expression upon training

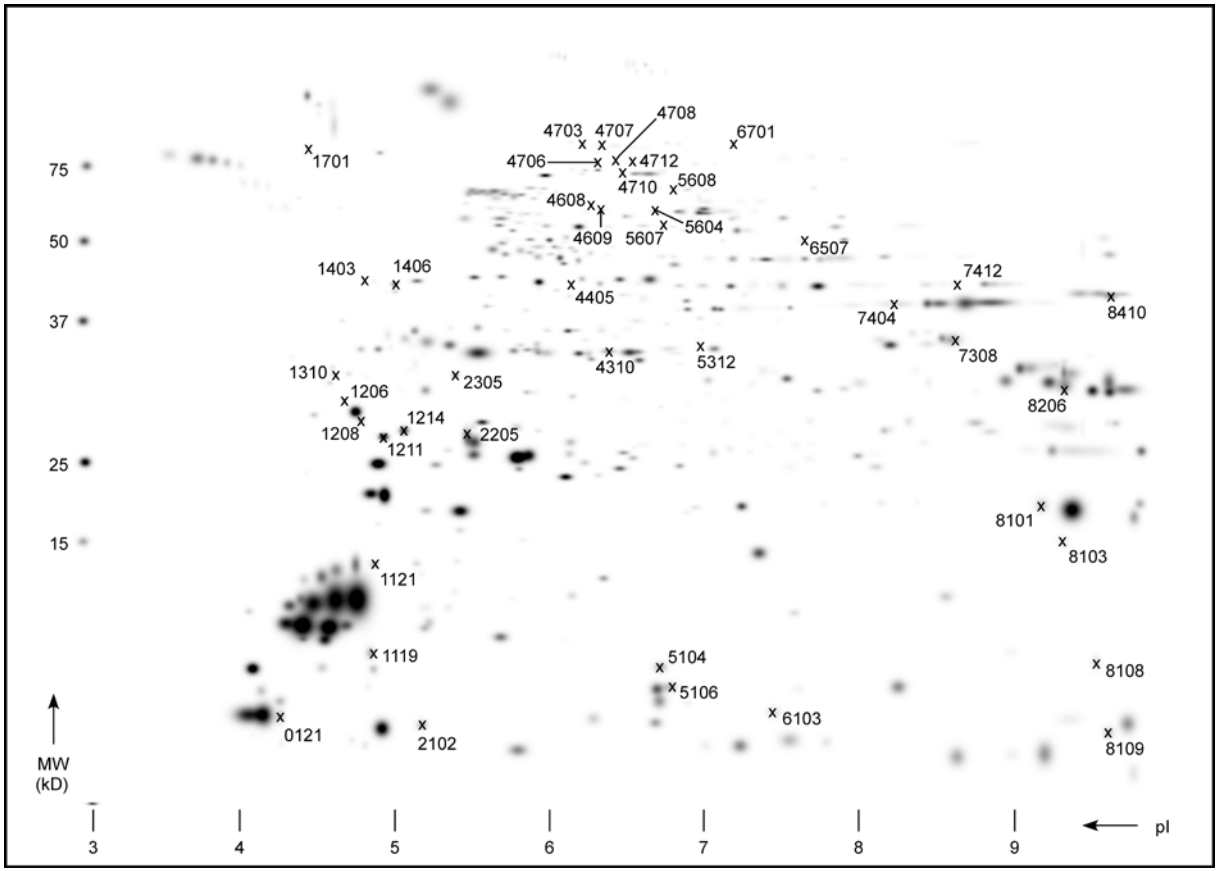

Figure 8.2
Image of master gel with matched protein spots of E2 sequential extracts of equine skeletal muscle before and after training
Protein spot numbers that showed differential expression upon normal training of previous untrained horses are depicted. Estimated pI and MW and regulation of these protein spots are described in Table 8.2.

Table 8.2 ***Differentially expressed proteins in E2 sequential muscle extracts upon training***

spot nr	MW (kD)	pI	untrained (PPM)	trained (PPM)	Ratio to untrained	effect
121	11	4.3	1194.2	448.4	0.38	-
1119	13	4.8	636.2	80.7	0.13	-
1121	14	4.9	625.4	285.1	0.46	-
1206	30	4.7	142.4	22.2	0.16	-
1208	28	4.8	280.7	58.2	0.21	-
1211	27	4.9	1897.3	911.1	0.48	-
1214	27	5.1	922	331.5	0.36	-
1310	32	4.6	99.5	30.8	0.31	-
1403	43	4.8	82.4	33.2	0.4	-
1406	42	5.0	291.1	98.3	0.34	-
1701	83	4.4	172.1	65.7	0.38	-
2102	11	5.2	980.2	47.9	0.05	-
2205	27	5.5	1541.5	662.1	0.43	-
2305	32	5.4	449.4	131.4	0.29	-
4310	34	6.4	224	588.7	2.63	+
4405	43	6.1	43.9	18	0.41	-
4608	61	6.3	30.2	64.9	2.15	+
4609	59	6.3	44.3	135.6	3.06	+
4703	85	6.2	45.9	119.4	2.6	+
4706	77	6.3	24	108.9	4.53	+
4707	83	6.3	82.5	243.7	2.95	+
4708	77	6.4	23.2	96	4.14	+
4710	72	6.5	39.6	105.5	2.66	+
4712	77	6.5	35.2	136.1	3.87	+
5104	12	6.7	886.7	434.4	0.49	-
5106	12	6.8	561.9	139.2	0.25	-
5312	35	7.0	59.2	137	2.31	+
5604	59	6.7	73.1	147.8	2.02	+
5607	55	6.8	40	110.4	2.76	+
5608	66	6.8	42	98.5	2.34	+
6103	12	7.5	568.8	190.7	0.34	-
6507	50	7.7	70.6	183.4	2.6	+
6701	84	7.2	27.7	64.9	2.35	+
7308	35	8.6	1137.8	2612.6	2.3	+
7404	39	8.3	599.9	1294.4	2.16	+
7412	42	8.6	76.9	155.6	2.02	+
8101	19	9.2	631.3	1383.4	2.19	+
8103	15	9.3	412.8	1035.3	2.51	+
8108	12	9.6	390.5	922.8	2.36	+
8109	11	9.6	858.1	2048.4	2.39	+
8206	31	9.4	205.7	562.2	2.73	+
8410	41	9.7	655.2	1384	2.11	+

Effect: - decreased, + increased expression upon training

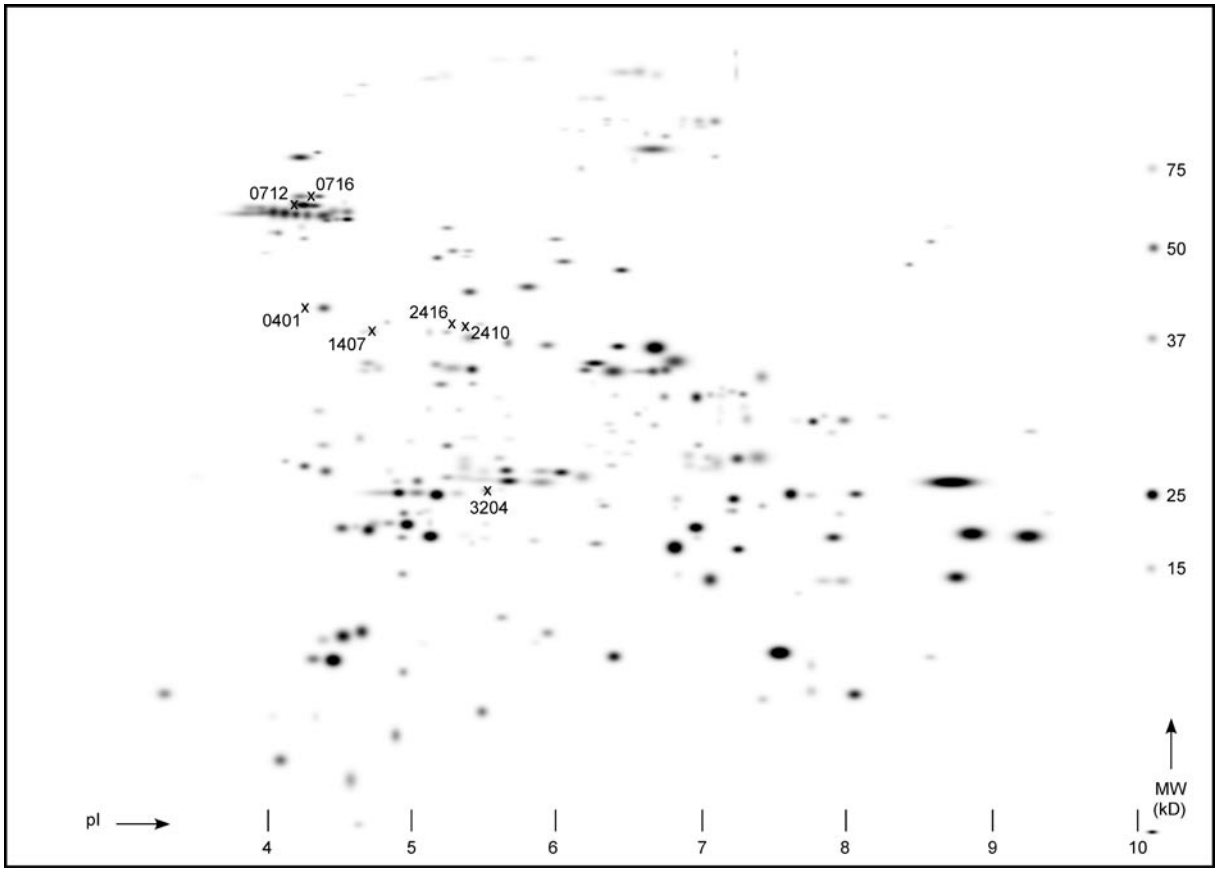

Figure 8.3
Image of master gel with matched protein spots of E1 sequential extracts of equine skeletal muscle before and after normal or intensified training
Protein spot numbers that showed differential expression upon intensified training of horses compared to trained horses are depicted. Estimated pI and MW and regulation of these protein spots are described in Table 8.3.

low amount of matched protein spots on the master gels (Fig8.1-8.3). Overstaining was, however, a thoughtful considered choice, since we focused on low-copy proteins. To date, silver staining can be regarded as the most sensitive staining method to visualize low-copy proteins.
Although this approach was, in part, successful, future studies should focus on further decreasing sample complexity, by using subcellular fractionation techniques. This, in combination with proteomic analysis of specific cellular muscle compartments like mitochondria and nuclei could serve as basis for muscle proteomic research. Although, such an approach might be difficult when frozen muscle tissue is to be used, it encompasses an interesting strategy that could potentially result in identifying differentially expressed proteins with a clear biological role. Furthermore, the use of a sensitive staining method with a high dynamic range, like Sypro Ruby,[23] is, because of the broad difference between low and high copy proteins in muscles, recommended in future studies.

Overall conclusion

Previously we demonstrated that proteomic technology is useful in equine muscle research (chapter 7). In the present study this technology was successfully repeated to investigate training-induced differential expression of the equine muscle proteins. We conclude that training in horses results in changes in the muscle proteome and that a period of intensified training resulted in differential expression of some unknown proteins. Identification of these proteins, which is currently underway, might shed more light on the biological relevance of these proteins in muscle adaptive processes upon normal training and intensified training.

Table 8.3 ***Differentially expressed proteins in E1 upon intensified training***

spot nr	MW (kD)	pI	control (PPM)	intensified (PPM)	Ratio to control	effect
401	41	4.3	305.8	1129.3	3.69	+
712	63	4.2	3508.2	10872.7	3.1	+
716	65	4.3	747.8	1882.6	2.52	+
1407	38	4.7	573.8	1550.5	2.7	+
2410	38	5.4	613.8	1285.5	2.09	+
2416	39	5.3	231.7	782.3	3.38	+
3204	25	5.5	1008.9	2485.9	2.46	+

Effect: + increased expression upon intensified training

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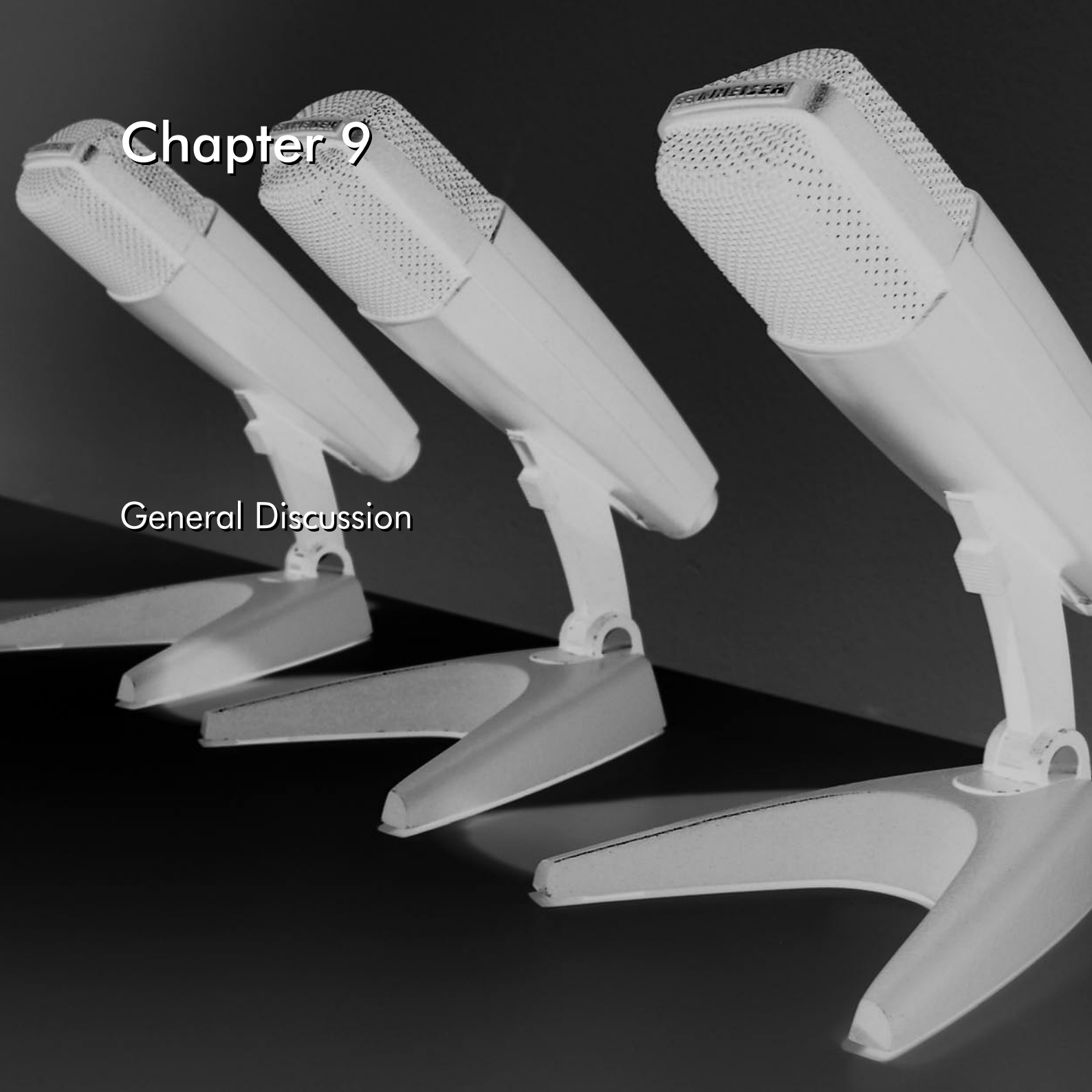
Chapter 9

General Discussion



In the present thesis the localization and activation of signaling proteins, known from human studies, in equine muscle were investigated under conditions of rest, after an acute bout of exercise and before and after a period of (intensified) training. Proteins of interest (PKC, ERK, p38 MAPK, JNK and HSP27) were studied using histological and biochemical techniques. In order to investigate training-induced fiber transitions, MHC content was determined as well. In addition, for the first time in horses, proteomic technology was used in rhabdomyolysis and used to elucidate (intensified) training-induced muscle adaptation at the protein level.

Subcellular and fiber-type specific localization of PKC isoforms

The protein kinase C (PKC) family consists of 12 isoforms and the expression level of these isoforms varies among different tissues. The regulation mechanism of the tissue type-dependent expression of PKC isoforms is largely unclear. It has been reported that the level of expression varies within different stages of (muscle) development.[1-6]
In this thesis it has been found that PKC α, β1,β2, δ, ε and ζ are expressed in adult warmblood equine skeletal muscle (chapter 3). Some isoforms were differentially expressed in muscle fiber types and the subcellular localization at rest was different among PKC isoforms. More specifically, PKC α and β2 were predominantly localized at the sarcolemmal membrane, whereas PKC β1, δ, ε and ζ were found within the cytosol of muscle fibers. In addition, PKC α was mainly expressed in a subset of type 2a fibers. PKC δ and ε were found in type 1 fibers and PKC ζ was found in type 2 fibers. Distribution of PKC β1 was equal among type 1 and 2 fibers.
Previous studies have reported that PKC α, β1,β2, δ, ε and ζ are ubiquitous proteins expressed in most tissues.[7, 8] At present, few rat and human studies reported on subcellular localization of PKC isoforms in skeletal muscle using histological techniques under resting conditions.[6,9] In these studies, expression of PKC α has been localized at the surface membrane. These results are in line with the present study.
In addition, PKC β1 and β2 are expressed in the cytosol of human skeletal muscle fibers. It is likely that the differential localization of PKC isoforms is related to the localization of target proteins of PKC. It is known from studies in cell lines that the insulin receptor tyrosine kinase activity is inhibited by PKC β1 and β2,[10] and PKC α was found to stimulate insulin receptor degradation via interaction with insulin receptor substrate-1.[11] The localization of PKC α and β2 in the present study suggests that these proteins are likely candidates for this negative feed back mechanism for insulin signaling in equine muscle. Although we have not looked into the exercise-induced activation of PKC isoforms, it is interesting to mention that various studies in mice and humans suggest that PKC isoforms are activated upon exercise.[12-14] Most PKC isoforms are diacylglycerol-dependent and use phosphatidyl serine as a cofactor or require other lipoidal activators. Hence, translocation of PKC to an intracellular or sarcolemmal membrane is probably required for its full activation.[15] These observations confirm the importance of the subcellular localization of PKC isoforms in equine muscle as well.

Activation of MAPK signaling upon acute exercise and a period of (intensified) training

Mitogen-activated protein kinases (MAPKs) comprises three enzymes, ERK1/2, p38 MAPK and JNK, that are activated upon extracellular signals and cellular stress.[16] MAPKs have been implicated in proliferation and differentiation of muscle cells.[17,18] Hence, their pathways may be involved in exercise- and training-induced adaptations in skeletal muscle.

In this study, the exercise-induced activation of p38 MAPK, JNK and ERK1/2 was studied in trained horses (chapter 4). This revealed that only p38 MAPK and JNK were significantly phosphorylated upon exercise. It is remarkable that p38 MAPK was predominantly found in the cytosol and nuclei of type 1 muscle fibers of vastus lateralis muscle. The overall phosphorylation effects were stronger in vastus lateralis muscle compared to pectoralis decendens muscle, which is likely due to differences in fiber type distribution.

p38 MAPK and JNK were studied further with respect to training-induced adaptation of basal protein expression and phosphorylation in vastus lateralis (chapter 5). It was found that the expression level of these proteins at rest was not influenced by a period of training or intensified training. However, phosphorylation levels of these proteins were higher in untrained horses compared to these horses after a period of training.

In humans and rat studies it was also found that exercise induces activation of MAPK pathways.[19-22] Furthermore, other studies suggest that the extent of activation of these pathways is dependent on the duration, intensity and type of exercise.[23,24]

Exercise did indeed induce phosphorylation of these proteins after training or intensified training in this study. This was not statistically significant in all conditions. Individual variations in duration and intensity of exercise might have influenced the extent and time-frame of activation of MAPK cascades in the present study. This might be an explanation for the absence of a significant exercise-induced activation of p38 MAPK and JNK in all conditions. Another explanation for this discrepancy can be found in a previous study in which it was shown that MAPK phosphorylation rapidly declines within 15 min during recovery.[25] Phosphorylation levels might, in part, have returned to basal levels, because post-exercise biopsies were not taken immediately after exercise but 10 to 15 minutes later. Taking a series of biopsies immediately after exercise is recommended in future studies.

It has previously been reported that p38 MAPK expression is lower in trained compared to sedentary humans[26] and rats.[27] In addition, it has been shown that low-intensity training and moderate-to-high-intensity training result in selective post-exercise activation of p38 MAPK and ERK1/2 in rat soleus muscle.[27] Results of the present study are not in line with these findings. An explanation for the discrepancies might be species-specific differences in adaptation to (intensified) training and the use of (different) muscles with different fiber properties, as established by the type 1 fiber specific localization of p38 MAPK. We did find, however, higher basal phosphorylation levels of p38 MAPK and JNK in untrained compared to trained horses. It is possible that higher basal phosphorylation levels of p38 MAPK and JNK reflect higher stress levels early in the training phase in the horses in this study. Higher

stress levels might be the result of psychological stress, due to frequent handling, or due to a lower level of fitness.

p38 MAPK-induced HSP27 phosphorylation upon acute exercise and a period of (intensified) training

Heat shock proteins (HSPs) are known for their important role in maintaining cellular homeostasis during physiological stress, such as exercise.[28-30] HSP27 is phosphorylated by the MAPKAPK2, a downstream kinase of p38 MAPK.[31]
In this thesis we investigated the phosphorylation of p38 pathway intermediates upon exercise (chapter 4) and the p38 pathway target HSP27 (chapter 4 and 5). Results revealed that upstream intermediates MKK3/6 and the downstream intermediate MAPKAPK2 were phosphorylated upon exercise (Fig 9.1). In addition, significant exercise-induced phosphorylation of HSP27 was found in all conditions, without any differences before and after (intensified) training. This suggests that the p38 MAPK pathway was at least transiently activated upon exercise in all conditions. Furthermore, HSP27 phosphorylation was induced strongly in type 1 fibers, which was in line with localization of p38 MAPK, but also in type 2a fibers. Although in general, expression of heat shock proteins is increased upon stressful events such as exercise, the specific knowledge of HSP27 in exercise is limited to few studies in men in which HSP27 protein was induced in response to eccentric exercise.[28-30,32]
To the best of our knowledge this is the first time that HSP27 phosphorylation upon an acute bout of exercise is published. Although the biological significance of phosphorylation of HSP27 upon exercise is not known, some studies suggest that HSP27 interacts with the cytoskeleton through the modulation of actin microfilaments.[33, 34] This, in turn, might reflect a regulatory role of HSP27 in translocation processes in skeletal muscle through focal adhesion complexes,[35] like GLUT-4 translocation. HSPs function as molecular chaperones that protect proteins form unfolding, aggregation and subsequent degradation and, therefore, might have a protective effect against muscle damage upon exercise. Hence, the phosphorylation of HSP27 might be the first step in increasing the ability of HSP27 to function as a molecular chaperone, and might precede a potential exercise-induced induction of HSP27 protein. The specific localization of the exercise-induced phosphorylation response in type 1 and 2a fibers might reflect the higher metabolic stress level in these fiber types, and is in line with the fact that type 1 and 2a have a higher oxidative capacity and, therefore, endure more oxidative stress.

Differences in muscle MHC distribution upon a period of training

The major muscle fiber constituent that determines fiber phenotype is myosin heavy chain (MHC). This protein exists in slow (MHC I) and fast (MHC IIa, IIb, IIx) isoforms coded by different genes.[36] Although specific muscle fiber type distribution is mainly determined at birth and during the first year of life,[37] various studies indicate that fiber distribution

is subject to change upon specific pathological conditions,[38] aging,[39-42] and training.[43-48] In addition, it has also been shown that fiber type distribution between sedentary and trained subjects is not identical. This has led to the hypothesis that physical training can, to a certain level, influence fiber distribution.

The present study indicates that 18 weeks of medium-term training in young Standardbred geldings does not induce changes in specific MHC isoform contents in muscle after training compared to the pre-training contents in vastus lateralis muscle (chapter 6). Preference was given to vastus lateralis muscle and pectoralis descendens muscle because these muscles revealed, in contrast to gluteus medius muscle, a clear EMG signal in conscious horses at rest.

The MHC findings suggest no conversion of fiber types upon training. Since immature horses were used, these findings also suggest that potential effects of growth during the training period have not influenced fiber type distribution. Furthermore, results indicate that fiber distribution in horses is muscle-type dependent, because differences were found in the MHC distribution between vastus lateralis muscle and pectoralis descendens muscle. For instance, pectoralis descendens muscle contains more MHC IIx and less

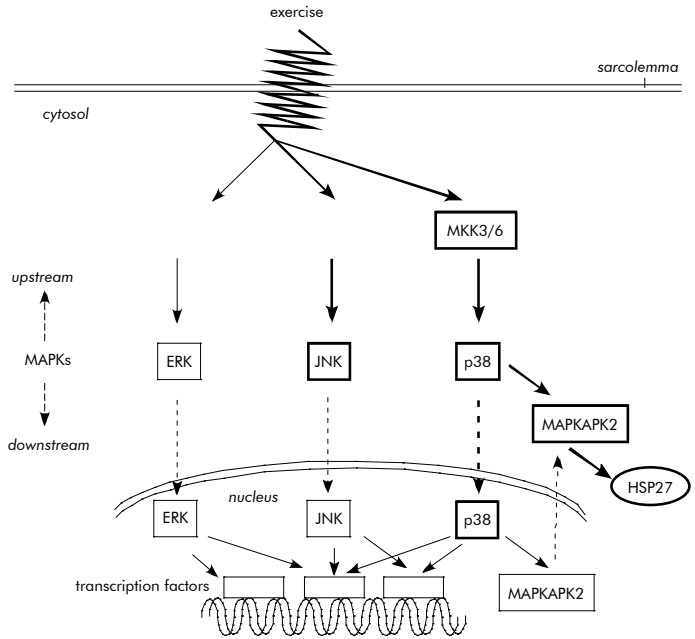


Figure 9.1
Overview of signaling intermediates studied in this thesis.
Thick lines and boxes represent the intermediates that were phosphorylated upon exercise in horses.
MAPKAKP2, MAPK activating protein 2; MKK3/6, MAPK kinase 3 and 6.

MHC I compared to vastus lateralis muscle. Comparison of MHC content in the equine gluteus medius[49] with that of the studied muscles in this thesis suggests that gluteus medius contains more MHC IIa than pectoralis descendens and vastus lateralis, less MHC I than vastus lateralis and more than pectoralis descendens, and more MHC IIx than vastus lateralis and less than pectoralis descendens. Nevertheless, these findings must be interpreted with care because of the well known differences in specific muscle fiber distribution between different breeds.[50] Our results do support the notion that fiber distribution is dependent on the functional requirements of a specific muscle. Previous findings in horses also suggest that training does not induce fiber transitions in gluteus medius muscle.[51-53] Nevertheless, the absence of signs of fiber transitions might also be explained by the slow and gradual increase in training load and by the fact that horses were only allowed to trot. Such a gait results in little vertical displacement during exercise and, therefore, changes in exercise-specific muscle forces are likely to be lower than, for example, upon galloping.[54] This potentially has resulted in more subtle adaptations. From our findings it cannot be excluded yet that fiber characteristics like fiber diameter were unchanged upon training, but it is reasonable to assume that major transitions are not easily induced upon training in horses. It is nonetheless likely that, despite absence of changes in MHC distribution, training-induced changes in other key proteins have taken place. For this reason, proteomic technology was used as a strategy to find differences in expression levels of known and unknown proteins, without a preconceived idea.

Use of proteomic technology in equine muscle

Proteomics comprises a wide range of techniques enabling the identification of proteins and of differential expression of proteins between different conditions. In one of the most popular approaches, extracted proteins are separated by isoelectric point and molecular weight using two-dimensional gel electrophoresis (2DGE), followed by protein staining. Subsequently, proteins of interest can be identified using mass spectrometry.
Although the use proteomic technology to investigate adaptations in skeletal muscle was not new.[55-59] the endeavor to use small muscle biopsy specimens of equine origin was original.
The technique was successfully used in: i) a differentiating mouse muscle cell line, ii) equine muscle biopsies, and iii) pathological cases of a muscle disorder (tying-up) (chapter 7). Differentially expressed proteins were found in growing and differentiated myoblasts. In addition, the protein pattern of equine muscle, as was established by performing 2DGE, was comparable with that of mouse muscle. Various mouse and equine proteins were subsequently identified using mass spectrometry. Furthermore, an important technical advance was the use of a sequential extraction method that yielded two muscle extracts thereby reducing muscle complexity. In addition, this enabled the loading of higher amounts of protein onto the gels, leading to visualization of more low-copy proteins. This technique has been used to study differences between the proteomes of horses suffering acute tying-up and that of clinical healthy horses. Horses suffering from a episode of severe tying-up expressed a creatine

kinase M chain protein variant with a smaller mass and a higher iso-electric point. This indicates that this creatine kinase protein is more positively charged, which is indicative of an altered protein 3D-structure. Such a conformational change of a protein might, in turn, result in activation or inactivation of its biological function or affect its binding to relevant sub-units. In case of the creatine kinase protein, the positive charge and the smaller mass are likely to be the result of a posttranslational modification such as, for example, a dephosphorylation. These modifications might well be related to a predisposing factor for muscle damage in the tying-up horses.
We also investigated differential expression of muscle proteins upon training (chapter 8). Comparing the proteomes of individual horses before and after a period of training 39 proteins had an increased expression level and 23 proteins showed a decreased expression level. In addition a group of 7 proteins were increased in expression upon intensified training.
Although the lack of an equine genomic database makes the identification of muscle proteins of equine origin not easy, identification of the differentially expressed proteins in (intensified) training is a challenge. Identification of these proteins will, hopefully, shed more light on the biological relevance in the process of training and overtraining.

The grey zone between adaptation and pathophysiology upon intensified training

It has been recognized that successive periods of intensive training might lead to the full-blown overtraining syndrome in human and equine athletes. To date, in apparent clinically healthy human athletes that show a decrement in athletic performance, diagnosis of overreaching (first stage) and/or overtraining (end stage) is made by the use of the profile of the mood (POMS) questionnaire.[60] The tentative diagnosis of overreaching and/or overtraining in horses still remains a major challenge because the use of questionnaires is not possible in horses.
At present behavioral markers in concert with decrements in performance without any clinical adverse signs are the only options available, yet. More objective parameters would contribute positively to the diagnosis and adequate treatment of overreaching and prevent horses from falling into the full-blown overtraining syndrome. In addition, such findings could increase the welfare of the equine athlete. Various markers have been postulated in human overtraining research. These include non-invasive, invasive, biological, biochemical, hormonal and immunological markers.[61,62] No standard marker for humans has yet emerged from such studies. Furthermore, most of the markers have not yet been investigated in horses. In addition, many markers have been found by comparing healthy elite athletes with those already diagnosed as overtrained.
In the present thesis, it was hypothesized that changes in muscle protein expression and/or activation upon intensified training might be suggestive of early markers of overtraining.
The most remarkable findings are the changes in the skeletal muscle proteome upon intensified training (chapter 8). Analysis of the differential expression of muscle proteins in horses before and after normal or

intensified training yielded a group of 7 proteins that showed an increase in expression after intensified training. These proteins require additional study with reference to potential early markers for overtraining. The design of the present study enabled the comparison of potential markers at several time points upon training and/or intensified training and, therefore, discriminates between early and late markers for normal and potential adverse adaptive processes. The intensity of the training program was guided by use of the average peak heart rate. The use of this parameter is limited and, therefore, training intensities in the present study might have varied between individual horses. As a consequence, this might implicate that fitness levels of horses at the end of the training phase were different. This might have influenced the effects of intensified training. An objective measure for the effect of training in general was the performance level during the exercise tests (chapter 2). From these results it can be concluded that exercise time was reduced during and after intensified training. It cannot be excluded that this originated from a physiological training stimulus (fatigue). Training intensity and volume in the intensified training phase was gradually increased and rest days were omitted during the last 3 weeks. Hence, the physical implications of the intensified training program in the present study were not unambiguous. For a better understanding of the use and implications of intensified training for the study of overtraining, the use of the individual maximal heart rate to guide training and the use of a more sensitive exercise test for determining performance level are recommended.

Final remarks

From the overall findings of this thesis we hypothesize that, like in human muscle, PKC isoforms, p38 MAPK, JNK and HSP27 play a major role in equine muscle cell signaling. Since these proteins are involved in the insulin signaling cascade, the findings emphasize the importance of the equine muscle in glucose homeostasis as well. The similarities between human and equine muscle cell signaling make the horse an excellent model to study biological markers for guiding training and preventing overtraining in humans and horses. Moreover, the application of such markers could increase the welfare of the equine athlete in clinical practice. The unraveling of the equine genome would facilitate the identification of new markers using proteomic technology.
Despite the similarities between the human and equine athlete and the way they adapt to training, it remains to be elucidated whether or not other aspects (mental, neuro-endocrine, metabolic) act analogous in the process of developing overtraining.

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Appendix

Nederlandse Samenvatting (voor leken)

Paarden worden al eeuwen gewaardeerd om hun atletisch vermogen. Dit vermogen komt voort uit het feit dat paarden in het wild als prooidier moesten overleven. Wanneer het paard precies is gedomesticeerd is niet bekend, maar waarschijnlijk is dit gebeurd door stammen die in Noordoost-Europa, Noordoost-Azië of in Centraal Azië woonden. Dit denkt men omdat daar grote kuddes paarden rondzwierven. De eerste gedomesticeerde paarden waren waarschijnlijk in de eerste plaats bedoeld als lastdier en niet als rijdier. De geschiedenis leert ons verder dat de Grieken een van de eersten waren die de rijkunst hebben ontwikkeld. In 365 v.C. schreef een Griekse cavalerie-officier, Xenophon, boeken over de rijkunst en het africhten van paarden voor oorlogvoering. Het gebruik van paarden in de sport dateert van de eerste Olympische Spelen, waarin het onderdeel wagenrennen werd georganiseerd.
De mens heeft altijd geprobeerd de atletische capaciteit van paarden te verbeteren. Zo lieten Arabische nomaden de paarden hongeren om ze vervolgens naar een waterput of hun 'stal' te laten rennen, terwijl Romeinen hun paarden zwaardere lasten lieten trekken door de kar met toenemende hoeveelheden zand te beladen.
Vandaag de dag concentreert het trainen van paarden, in de praktijk, zich voornamelijk op technische vaardigheden. Dit is opmerkelijk aangezien we uit training bij mensen hebben geleerd dat kracht en conditionele training van belang is om prestaties te verbeteren en blessures te voorkomen. Op basis van het erfelijk materiaal vertonen paard en mens grote overeenkomsten. Echter, de verschillen tussen paard en mens, in voortbeweging, de verwerking en het gebruik van voedingstoffen (metabolisme), en de grotere aërobe en anaërobe capaciteit (de capaciteit om zuurstof en voedingsstoffen te transporteren en om te zetten in energie) van paarden, wijzen mogelijk op verschillen die van invloed kunnen zijn in de aanpassing aan training.
Training bij zowel het paard als de mens heeft tot doel: 1) het uitstellen van vermoeidheid, 2) het behouden van de maximale capaciteit, 3) het verbeteren van vaardigheden, 4) het verminderen van de kans op blessures en 5) het behoud van het enthousiasme en de wil om te trainen. Bij paarden dient ook het welzijn gewaarborgd te zijn. Om deze doelen te bereiken is het van belang periodes van training af te wisselen met periodes van rust. Alleen dan kan training leiden tot lange termijn aanpassingen en verbetering van prestatie.
Hoewel bewegen, althans bij de mens, in het algemeen een gunstig effect op de gezondheid lijkt te hebben, kan overmatige training leiden tot gezondheidsproblemen. Als tijdens periodes van zware training onvoldoende rust wordt ingebouwd kan dit leiden tot chronische overbelasting. Deze overbelasting komt in verschillende gradaties voor. De ernstigste vorm is het overtrainings-syndroom. Dit syndroom komt zowel bij mensen als paarden voor, hoewel de precieze mechanismen nog onbekend zijn.
Het basisprincipe van training is dat een enkele trainingssessie leidt tot vermoeidheid. Door deze prikkel worden fysiologische aanpassingen opgewekt. Door trainingssessies vervolgens langer en zwaarder te maken zullen deze korte termijn aanpassingen leiden tot lange termijn aanpassingen

met als gevolg een verbetering van de atletische capaciteit. Aanpassingen vinden o.a. plaats in hart en bloedvaten, longen en spieren.
Specifieke aanpassingen in spier door training zijn het onderwerp van dit proefschrift. Verder zouden specifieke veranderingen in de spier als gevolg van intensieve training mogelijk kunnen dienen als voorspellers (markers) voor overtraining.

Spieren bestaan uit bundels van spiervezels. In deze vezels zitten de zogenaamde contractiele eiwitten, die zorgen voor spiercontractie. Er bestaan verschillende typen spiervezels elk met verschillende eigenschappen. Dit maakt dat de spieren meer of minder geschikt zijn voor bepaalde soorten spiercontracties, zoals kracht of duur.
Voor spiercontractie is energie nodig. Deze energie, in de vorm van ATP, wordt verkregen uit glucose en vetten uit de voeding. Glucose wordt door de spier uit het bloed gehaald via speciale transporteiwitten in de wand van de spiervezels.
Aanpassing van spieren door training is het gevolg van een toegenomen energiebehoefte, verhoogde rek- en trekkrachten in de spieren, en veranderingen in hormoonhuishouding. Deze veranderingen worden 'vertaald' via de activatie van specifieke signaaleiwitten in de spiervezels (signaaltransductie). Deze signaaleiwitten zorgen er voor dat het eiwitspectrum (proteoom) van de spiervezel zich aanpast en waardoor de spier beter kan functioneren onder de veranderde behoeften. Deze kennis is voornamelijk opgedaan uit onderzoek bij mensen en proefdieren, zoals ratten en muizen. Hoewel vermoedelijk ook bij paarden deze processen een belangrijke rol spelen, is er nog weinig onderzoek op dit terrein verricht. Om een beter inzicht te krijgen in de aanpassingen van spieren door training bij paarden is het onderzoek zoals beschreven in dit proefschrift uitgevoerd.

Het onderzoek beschreven in dit proefschrift gaat dieper in op de plaats en activatie van signaaleiwitten die we kennen uit onderzoek in menselijk spierweefsel. In de studies hebben wij spierweefsel van paarden in rust, na acute inspanning en na periodes van normale en intensieve training bestudeerd. Bepaalde signaaleiwitten werden onderzocht door middel van microscopische en biochemische technieken.
Uit het onderzoek blijkt dat signaaleiwitten die we kennen uit onderzoek bij de mens, ook aanwezig zijn in spieren van paarden. Deze signaaleiwitten bevinden zich op verschillende plaatsen in de spiervezel. Ook de hoeveelheid van deze signaaleiwitten lijkt te variëren tussen verschillende typen spiervezels. Deze verschillen liggen mogelijk ten grondslag aan de verschillen in functionele eigenschappen van de diverse spiervezeltypes.
Enkele van de onderzochte signaaleiwitten werden geactiveerd door acute inspanning. Dit wijst tevens op een mogelijke rol van deze eiwitten voor de aanpassingen in spier op lange termijn. Langdurige training (18 weken) gevolgd door normale of intensieve training (6 weken) resulteerde niet in veranderingen in de verdeling van vezeltypen maar wel in veranderingen in het totale eiwitspectrum. Deze veranderingen in eiwitspectrum werden gevonden met behulp van een nieuwe techniek, te weten proteomics. Deze techniek biedt de mogelijkheid om alle eiwitten in een mengsel, en verschillen tussen

mengsels, te onderzoeken op hoeveelheid zonder dat deze eiwitten bekend hoeven zijn. De techniek werd geoptimaliseerd en getest op spieren van gezonde paarden en van ernstig zieke paarden met acute spierverkramping (tying-up), hetgeen leidt tot veel spierschade.

Samenvattend kan worden gesteld dat net zoals in spieren van mensen, bepaalde signaaleiwitten een belangrijke rol spelen in signaaloverdracht in spiercellen van paarden. Omdat deze signaaleiwitten ook een rol spelen in de signaaloverdracht door insuline, benadrukken onze bevindingen dat de spieren in het paard ook van belang zijn in de regulatie van de glucosehuishouding. De overeenkomsten tussen signaaloverdracht tussen spiercellen van menselijke oorsprong en die van paarden, maken het paard een uitstekend model om biologische markers te vinden om training te sturen en om chronische overbelasting en/of het overtrainings-syndroom te voorkomen. Deze markers zouden mogelijk ook kunnen leiden tot een verbetering van het welbevinden van het paard als atleet. De verdere ontrafeling van het erfelijk materiaal van het paard zal in de toekomst de identificatie van nieuwe markers mogelijk vergemakkelijken.
Ondanks de overeenkomsten tussen de mens en het paard als atleet, en de vergelijkbare manier waarop ze zich aanpassen aan training, blijft de vraag bestaan of andere (mentale, hormonale, metabole) aspecten al dan niet overeenkomsten vertonen in het ontwikkelen van overtraining.

Curriculum Vitae

Mireille van Ginneken werd geboren op 16 maart 1973 te Etten-Leur. Zij behaalde daar in 1991 het VWO diploma aan de Katholieke Scholengemeenschap Etten-Leur. Aansluitend werd de studierichting Medische Biologie aan de Universiteit Utrecht (UU) gevolgd. Tijdens die studie werd een 8-maands stage doorgebracht bij de afdeling Haematologie, Academisch Ziekenhuis Utrecht, en werd de localisatie van heterotrimere eiwitten in bloedplaatjes bestudeerd. Een 11-maands stage werd gelopen bij de afdeling Fysiologische Chemie en betrof een moleculair biologisch onderzoek betreffendet de expressie van het B-50-GFP fusie-eiwit in neuronale cellen. In april 1996 behaalde zij het doctoraaldiploma. Na haar afstuderen was zij enige tijd vrijwilligster bij het Landelijk Informatiecentrum Hepatitis. Van april 1997 tot januari 2002 werkte zij bij de afdeling Interne Geneeskunde, sectie Endocrinologie, Universiteit Maastricht (UM), onder leiding van Dr. B.H.R. Wolffenbuttel (thans hoogleraar aan de Rijksuniversiteit Groningen), aan een celmodel voor endotheeldysfunctie bij diabetes. In maart 2000 was zij 4 weken te gast op het laboratorium van Prof. Dr. G.L. King, Harvard Medical School, Joslin Diabetes Institute, Boston, Verenigde Staten. In september 2002 gestart met het onderzoek beschreven in dit proefschrift onder leiding van Prof. Dr. A. Barneveld, Dr. J.H. van der Kolk (UU) en Dr E. van Breda (UM). Hiervoor werd zij vanuit de Hoofdafdeling Gezondheidszorg Paard (UU), gedetacheerd bij het Nutrition & Toxicology Research Institute Maastricht, binnen de capgroep Bewegingswetenschappen (UM).

Publications

Full papers

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W Engels, PJMR Lemmens, MME van Ginneken and BHR Wolffenbuttel
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De aanloop was lang en de eindsprint kort en hevig. Zonder de nodige 'trainers' en 'coaches' had ik deze 'wedstrijd' uiteraard niet kunnen winnen.
Alle collega's en oud-collega's wil ik bij deze dan ook van harte bedanken voor hun inzet in de vorm van praktische en mentale ondersteuning in de afgelopen jaren. Ik heb van jullie alle aspecten van het vak mogen leren, en dit proefschrift is het resultaat. Ook wil ik familie en vrienden bedanken voor hun onvoorwaardelijke vertrouwen. Jullie steun was onontbeerlijk om de eindstreep te halen.

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