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

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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 muscles 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. (Am J Vet Res 2006;67:837–844)

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 have been implicated in proliferation and differentiation of muscle cells, their pathways may be involved in exercise-induced adaptations in skeletal muscle. The MAPK comprises a family of 3 distinct subdivisions: ERK 1-2, p38, and 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. The ERK 1-2 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 MAPKAPK2, mitogen- and stress-activated kinases 1 and 2, and the cAMP response element binder, with the ERK 1-2 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 in which investigators found that total ERK 1-2 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 results in increased phosphorylation of ERK and p38 and activation of JNK in humans and rats. 
Interestingly, electrical stimulation of slow-twitch and fast-twitch skeletal muscles in rats results in discrepancies in phosphorylation of p38. 
It has been found in multiple studies that expression of proteins is dependent on type of muscle fiber, which suggests that these functional differences of various types of muscle (eg, 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 HSPs. This suggests that HSPs could be involved in responses to exercise-induced stress. 
Interestingly, HSP27 can be activated through ERK 1-2 and p38 MAPK cascades via their common downstream target MAPKAPK2. 
In a study 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, 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 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 minutes 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) that elicited 80% of maximal heart rate, which was then followed by a cool-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 from 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 muscle has typically been used in human studies. The pectoralis descendens muscle was selected because this muscle is important for propulsion and posture.

Local anesthesia was achieved by administration of a solution of lidocaine hydrochloride plus adrenaline, and biopsy specimens were obtained by use of suction applied to a modified Bergstrom needle with a diameter of 7 mm. 
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 from the tuber coxa and 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; these portions were used for immunohistochemical analysis.

Homogenization of muscle biopsy specimens—Frozen muscle (100 mg) was homogenized (1:10) in ice-cold homogenization buffer (50mM HEPES, 10% glycerol, 2mM EDTA, 1% Triton-X100, 10mM NaF, 10mM sodium pyrophosphate, 2mM sodium orthovanadate, 1mM phenylmethylsulfonylfluoride) and protease inhibitor cocktail (pH 7, 5) by use of a tissue homogenizer (2 times for 10 seconds/homogenization). Samples were subsequently rotated end over end for 30 minutes at 4°C and then centrifuged (10,000 × g for 30 minutes at 4°C). A protein assay was performed 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 × 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 (25mM Tris, 192mM glycine, and 20% methanol). Equal loading and blotting was confirmed by staining with a temporary protein stain. 
Nitrocellulose sheets were blocked by the addition of 5% nonfat dry milk and 0.05% Tween-20 in PBS solution and incubation for 1 hour. Antibodies raised against phosphospecific ERK1-2, p38, MKK3-6, MAPKAPK2, and HSP27 were diluted 1:1,000 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 (diluted 1:5,000 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. Chemiluminescent analysis was performed by use of a commercially available kit, and results were developed on radiographic film. 
Films were scanned with a high-resolution scanner, and analyzed by use of a densitometry analysis program. 
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

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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 for 45 minutes with 50% blocking buffer and 0.05% Tween-20 in PBS solution. Mouse monoclonal antibodies raised against phosphospecific JNK were diluted 1:2,000 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 fluorophore-conjugated antibodies with emission at 680 nm raised against mouse IgG (diluted 1:5,000 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 high-resolution infrared scanner and analyzed by use of a densitometry analysis program, 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 µm, 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 MHC type 1 and type 2a' (diluted 1:23 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 fluorophore-conjugated antibodies diluted 1:200 (emission at 488 nm) or 1:500 (emission at 555 nm) in PBS solution. Finally, sections were washed 3 times (5 min/wash) with PBS solution and mounted with an antifade reagent containing DAPI nuclear stain.

![Figure 1](image_url)
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 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 digital color camera. Images were processed by use of image analysis software. 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 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]) of 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.

Figure 2—Mean ± SEM phosphorylation of ERK 1-2 (A) and JNK 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 Figure 1 for remainder of key.

Figure 3—Photomicrographs depicting the location of phosphorylated p38 in the VL muscle of a representative horse obtained after a 20-minute bout of exercise (A and B). Cryosections were stained with a rabbit IgG phospho-p38 antibody and a goat anti–rabbit-IgG fluorophore-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 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.
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 M KK3-6 at serine 189/208 (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; $P < 0.001$; Figure 1). In the pectoralis descendens muscle, phosphorylations 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 ERK 1-2 and JNK was assessed in a comparable manner. No significant increase in phosphorylation of ERK 1-2 was detected, whereas a significant ($P = 0.009$) 3.4-fold increase in phosphorylation of JNK at threonine 183 and tyrosine 185 was found in the vastus lateralis muscle (Figure 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 (Figure 3). In general, phosphorylated p38 was found in type 1 fibers. In addition, staining for phosphorylated p38 was 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 greyscales before and 50, 35, 27, and 28 greyscales after exercise for type 1, 2a, 2a/b, and 2b fibers, respectively. The corrected mean intensity in and near nuclei was 101 greyscales before exercise and 105 greyscales 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 (Figure 4). Phosphorylated HSP27 was not distributed equally over all muscle fibers. Typically, the highest concentrations were found in type 1 and 2a fibers 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 inten-
sity 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 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 ERK 1-2 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 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 ERK 1-2. 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 minutes after cessation of exercise) may have been responsible for the observed differences. Evidence for this assumption is provided in another study 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 that ERK 1-2 is activated in an intensity-dependent manner, and the exercise bout performed by the horses in the study reported here is the first in which investigators detected an effect of short-term exercise on single fibers. The corrected mean staining intensity on single fibers 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 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).

Differential expression of proteins in fiber types may explain functional discrepancies observed in various types of skeletal muscle. For example, in studies 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. Other investigators reported that incremental static stretch in vitro in isolated soleus muscle obtained from rats (ie, 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 and phosphorylation of JNK, it is still unclear whether JNK is activated by aerobic exercise, or only eccentric exercise. Activation of JNK may be related to age, duration of exercise, exercise-induced muscle damage, and contraction-induced tension. 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 1 fibers and in and near nuclei. Nuclear staining intensity was slightly increased after exercise. This corresponds with results published elsewhere. 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 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 in humans in which HSP27 protein was induced after 48 hours 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. 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.

References


