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ORIGINAL ARTICLE

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Exercise-induced oxidative stress and muscle stress protein responses in trotters

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Abstract Acute exercise induces oxidative stress and heat shock protein (HSP) expression. Information on the protection of stress proteins against oxidant insult and muscle damage during moderate exercise is scanty. We aimed to show how a single bout of moderate exercise affects the markers of oxidative stress and heat shock factor-1 (HSF1; the transcriptional regulator of HSP synthesis), and HSP70, HSP90 and glucose-regulated protein (GRP75) expression in horses. Eight clinically normal and regularly trained standardbred trotters were treadmill-exercised for 45 min at moderate intensity. Blood samples were collected prior to and immediately after exercise and at 4 and 24 h of recovery. Muscle biopsy samples from the middle gluteal muscle were taken before exercise and after 4 h of recovery. Acute exercise did not activate HSF1 or induce expression of HSP70, HSP90 or GRP75 in skeletal muscle. One bout of acute exercise increased protein oxidation, which was measured by protein carbonyls in plasma and muscle, but it did not effect 4-hydroxynonenal protein adducts, which are markers of lipid peroxidation. Furthermore, mild muscle damage was observed 4 h after exercise. Our results showed that horses are susceptible to oxidative stress. One bout of exercise at moderate intensity and duration did not induce HSP responses despite the increased protein oxidation and tissue inflammation in equine muscle.

Keywords Exercise · Horse · Muscle damage · Oxidative stress · Stress protein response

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Introduction

Increased oxygen metabolism results in induced formation of reactive oxygen species (ROS). Even moderate-intensity exercise may lead to oxidative stress, a state where increased generation of ROS overwhelms body antioxidant protection and subsequently induces lipid, protein and DNA damage (Sen et al. 2000). Oxidative damage of proteins may result in protein denaturation, aggregation, and loss of essential biological function. Intensive acute exercise, especially the eccentric type, may result in inflammation and muscle tissue damage. Studies examining racehorses in training have shown that the most common reason for wastage is musculoskeletal injury (Rose et al. 1983). Furthermore, exercise-related oxidative insults are implicated in muscle damage and decreased muscle performance (Reid et al. 1992; Sen et al. 2000). In other species, the relationship between exercise and oxidative tissue damage has been well demonstrated. Although the horse presents an excellent model for studies on oxygen consumption and ROS, information on exercise-induced oxidative stress in horses is very limited (Chiaradia et al. 1998).

Organisms cope with oxidative insults via a wide variety of defence systems. In addition to antioxidant protection, heat shock protein (HSP) expression has recently been considered as an adaptive mechanism and a marker of certain cellular insults, including exercise-induced oxidative stress (Powers et al. 2001). HSPs are a family of proteins that are induced by various types of stresses and are involved in the cytoprotection and protein restoration. Although it has been reported that physical exercise induces HSP expression (Fehrenbach et al. 2000; Liu et al. 2000), prolonged and exhaustive exercise protocols have been used in these studies. The information on HSP induction patterns in horses is limited, and the relationship between exercise-induced oxidative insult, muscle damage and HSP expression is unclear. Understanding of the role and expression pattern of stress proteins and their association with oxidative insults may provide valuable information on tissue protection mechanisms, and may help to reduce the deleterious effects of physical exercise.

In the present study we aimed to test the effects of one moderate bout of exercise on stress protein expression. Unlike the previous studies, we used moderate intensity and duration of exercise, which represent the basic daily training of horses. Furthermore, we compared stress protein expression with the level of markers of oxidative stress and muscle damage.

Methods

Animals and training

The Ethics Committee for Animal Experiments of the Agricultural Research Center of Ypäjä, Finland approved the experimental protocol. Eight clinically normal standardbred trotters, 6–9 years of age, were examined in this study. Three of the horses were mares and five were stallions. All horses had been in regular training for several years. The exercise protocol used was a modification from the standard exercise test for horses. The animals were exercised on the treadmill at the uphill 3° slope. The detailed protocol is shown in Table 1.

Table 1 Exercise protocol modified from a standard exercise test and effect of the exercise on lactate accumulation in the blood of trotters. Values are means (SEM)

Speed ($m\ s^{-1}$), slope 3°	Gait	Time (min)	Lactate ($mmol\ l^{-1}$)
Pre-stage	Rest	–	0.62 (0.04)
1.7	Walk	10	Not measured
4.2	Slow trot	15	Not measured
6	Fast trot	2	1.66 (0.17)*
7	Fast trot	2	2.91 (0.3)*
8	Fast trot	2	4.97 (0.6)*
9	Fast trot	2	8.4 (0.98)*
1.7	Walk	5	8.17 (1.43)*
1.7	Walk	10	5.67 (1.21)*
1.7	Walk	15	3.78 (0.93)*
1.7	Walk	20	2.37 (0.72)*

*Difference due to acute exercise, $P < 0.05$, paired samples t-test

Samples

Blood samples were drawn prior to and immediately after exercise, and at 4 and 24 h of recovery. The samples were collected into lithium–heparin tubes and centrifuged immediately to separate plasma for biochemical analysis. Plasma samples were divided into aliquots and frozen in liquid nitrogen.

Biopsy specimens from the middle gluteal muscle were obtained under local anaesthesia using the method of Lindholm and Piehl (1974). Removal of repeated biopsy samples using this technique was previously reported to have no effect on tissue findings (Lindholm and Piehl 1974). Tissue samples were collected before and 4 h after exercise. The samples were first rinsed quickly with ice-cold saline solution, blotted on to a filter paper, and finally submerged in liquid nitrogen.

For assays of stress proteins, the frozen muscle tissues were ground in liquid nitrogen and homogenised in 0.1 M phosphate buffer, pH 7.4, containing protease inhibitors: 5 mM phenylmethylsulfonyl fluoride, 4 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin, 10 mg ml^{-1} aprotinin. Muscle homogenates were stored at −80°C until analysed. Unless otherwise stated, all other chemicals and reagents were obtained from Sigma (St. Louis, Miss.) and were of analytical grade or the highest grade available.

Analyses

Protein carbonyls and 4-hydroxynonenal (4-HNE) protein adducts were measured using Western blot techniques. Briefly, using 20 μg of protein extract per lane, samples were electrophoresed on SDS-PAGE (sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis) as previously reported (Gordillo et al. 2002). For protein carbonyl measurements, samples were derivitised with 2,4-dinitrophenyl hydrazine, as previously described (Gordillo et al. 2002).

For HSP analysis, protein extracts (30 μg protein per well) with molecular weight markers were electrophoresed on SDS/PAGE, transferred to a nitrocellulose membrane (Millipore, Bedford, Mass.), and stained with Ponceau S solution to check and normalise the equal loading and transfer. Membranes were blocked with 5% fat-free milk solution at 37°C for 1 h and treated with monoclonal antibodies (StressGen, Victoria, Canada). As primary antibodies, we used antibodies that recognise the inducible forms of heat shock protein 70 (HSP70), heat shock protein 90 (HSP90) and glucose-regulated protein 75 (GRP75). A polyclonal primary antibody (Alexis, San Diego, Calif.) was used for the detection of heat shock factor-1 (HSF1). Horseradish peroxidase-conjugated anti-mouse, anti-rat and

anti-rabbit immunoglobulins (StressGen and Zymed, San Francisco, Calif.) were used as secondary antibodies. The membranes were developed with an enhanced chemiluminescence method (NEN, Life Science Products, Boston, Mass.) and quantified using image analysis software (NIH-Image, USA).

A gel mobility shift assay was performed as previously described (Kaarniranta et al. 2002; Oksala et al. 2002). The protein extracts were prepared similarly to those in the Western blot and mixed with isotope-labelled probes corresponding to the two overlapping heat shock elements. Protein–DNA complexes were resolved on a nondenaturing polyacrylamide gel. Gels were dried and the radioactivity on the gel was detected by autoradiography.

Muscle β-glucuronidase activity was measured using the method of Barrett (1972), and total protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, Ill.). Citrate synthase activity was measured according to the method of Shepherd and Garland (1969). Analyses of lactate and aspartate aminotransferase (ASAT) were carried out in the Laboratory of Equine Hospital (Ypäjä, Finland) and Säveri Laboratories (Kuopio, Finland), respectively, using standard methods.

For light microscopy, sections were cut in a cryostat at a thickness of 10 µm (Leica, CM 1900, USA) and stained by using standard histological and enzyme–histochemical methods. Staining was performed using haematoxylin and eosin (HE). Muscle fibres were classified into slow twitch (type I) and fast twitch (type II) fibres by pre-incubation at pH 4.3 with ATPase. In each sample, 150–200 fibres were counted. All pictures were taken using Olympus BX microscope with a JVC colour video camera.

Statistical analysis

The data were analysed using SPSS for Windows version 10.0. Means and standard errors (SEM) were calculated, and a paired samples *t*-test was used to compare the difference between means. Analysis of variance for repeated measures was used for multiple comparisons. Correlations were calculated using the Pearson's correlation coefficient. The level of significance was set at $P < 0.05$.

Results

The amount of protein carbonyls in the gluteus medius muscle was significantly increased at 4 h after exercise ($P = 0.006$, Figs. 1 and 2). Plasma protein carbonyls increased immediately after exercise ($P = 0.024$) and were further elevated after 4 h of recovery ($P = 0.018$), but returned to pre-exercise level at 24 h of recovery ($P = 0.149$) as shown in Figs. 1 and 2. In the middle gluteal muscle, the level of 4-HNE-modified proteins, which are markers of lipid peroxidation, was, however, at the pre-exercise level after 4 h of recovery (Table 2).

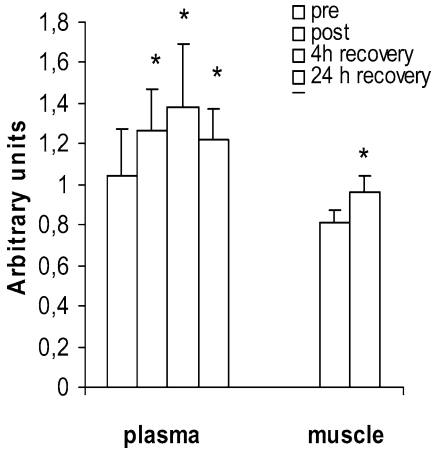


Fig. 1 Effect of an exercise bout on protein carbonyl levels measured from (**a**) plasma and (**b**) gluteus medius muscle in trotters. Values are means (SEM). *Difference due to acute exercise and recovery, $P < 0.05$, by paired samples *t*-test

Acute exercise did not induce HSF1 DNA binding activity examined by the gel mobility shift assay and HSF1 protein expression was not affected by acute exercise (Fig. 3).

A single bout of exercise at this intensity did not induce HSP70, HSP90 or GRP75 expression in the middle gluteal muscle of the exercising horse (Table 2). Citrate synthase activity, a marker of muscle oxidative capacity, showed the tendency to decrease at 4 h of recovery ($P = 0.058$). Plasma ASAT activity increased significantly immediately after and at 4 h after exercise ($P < 0.05$), but returned to pre-exercise level at 24 h of recovery. However, the activity of β-glucuronidase was not affected by the exercise (Table 2, $P = 0.275$).

The concentrations of blood lactate during exercise are presented in Table 1. Lactate concentration began to

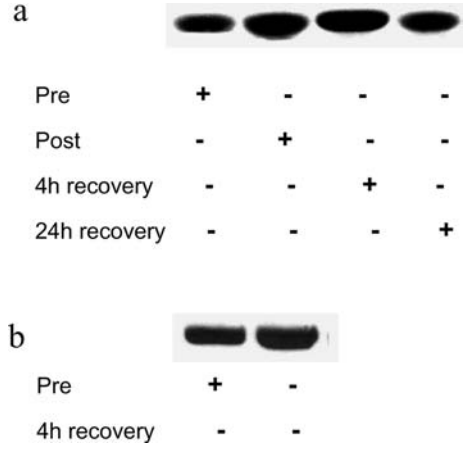


Fig. 2 Western blots using anti-dinitrophenyl antibody, demonstrating protein oxidation in (**a**) plasma and (**b**) gluteus medius muscle of trotters

Table 2 Effects of exercise on stress protein expression (*HSF1* heat shock factor-1; *HSP* heat shock protein; *GRP* glucose-regulated protein), activities of citrate synthase and β-glucuronidase, and the amount of 4-hydroxynonenal (*4-HNE*)-modified proteins in gluteus medius biopsy samples, and plasma aspartate aminotransferase (*ASAT*) activity. Values are means (SEM)

Muscle measurements	Pre-exercise		After 4 h recovery	
Citrate synthase [μmol (mg prot)$^{-1}$ min^{-1}]	0.396 (0.028)		0.333 (0.024)	
β-Glucuronidase [μmol (mg prot)$^{-1}$ min^{-1}]	0.012 (0.005)		0.010 (0.004)	
4-HNE protein adducts (arbitrary units)	1.03 (0.16)		1.02 (0.14)	
HSF1 (arbitrary units)	1.48 (0.33)		1.44 (0.19)	
HSP70 (arbitrary units)	1.60 (0.06)		1.63 (0.05)	
HSP90 (arbitrary units)	1.06 (0.08)		0.99 (0.09)	
GRP (arbitrary units)	2.04 (0.31)		2.29 (0.42)	
Plasma measurements	Pre-exercise	Post-exercise	4 h recovery	24 h recovery
ASAT (U l^{-1})	340 (18)	357 (17)*	381 (22)*	356 (19)

*Difference due to acute exercise and recovery, $P < 0.05$, by paired samples *t*-test

increase when the treadmill speed reached 6 m s^{-1} ($P < 0.001$). The blood lactate concentration started to decrease after the trotting, but it was still significantly higher ($P = 0.012$) after 20 min of recovery compared to the pre-exercise level.

Light microscopy sections stained by HE showed a mild swelling and inflammation of muscle fibres (Fig. 4). Classification of muscle fibres by ATPase at pH 4.3 showed that 18 (2)% of fibres were type I and 82 (2)% were type II fibres. No correlation was detected between any variables measured following exercise.

Discussion

Our study showed that despite increased oxidative stress detected by elevated plasma and muscle protein carbonyl levels, a single bout of exercise at moderate intensity did not induce stress protein response in equine muscle.

Exercise-induced protein carbonylation, an indicator of protein oxidation, has not previously been described in horses. Protein carbonyl groups are the most widely studied markers of protein oxidation and are frequently used as reliable markers of oxidative stress, and they can be assayed as stable hydrazone derivatives formed with 2,4-dinitrophenyl hydrazine (Gordillo et al. 2002). Oxidative modification of proteins is also known to inactivate catalytic functions of the proteins and induce the proteolytic system, which may also lead to a leakage of the plasma membrane and impair muscle function (Radak et al. 2000). Despite the fact that increased protein oxidation is well characterised in active cells and correlates with aging (Stadtman 2001), published information on the exercise-induced protein carbonylation is conflicting. It has been recently shown that acute exercise did not increase protein carbonyls in the soleus or rectus femoris in rats, although chronic exercise induced protein oxidation moderately in the oxidative soleus muscle (Liu et al. 2000). We did not observe any increase in 4-HNE protein adducts after exercise, nor any correlation between protein carbonyls and 4-HNE protein adducts. Similarly, in our previous experiments we have observed no correlation between lipid peroxidation and protein oxidation levels for the detection of exercise-induced oxidative stress in rat tissues (Sen et al. 1997). This discrepancy between protein oxidation and 4-HNE protein adducts could be attributed to the different peak times of these two oxidative products. A previous study compared the kinetics of iron- and ascorbate-induced protein oxidation and reactive aldehydes derived from lipid peroxidation in isolated rat liver mitochondria (Reinheckel et al. 1998). In that study, although oxidative protein modification could be detected immediately

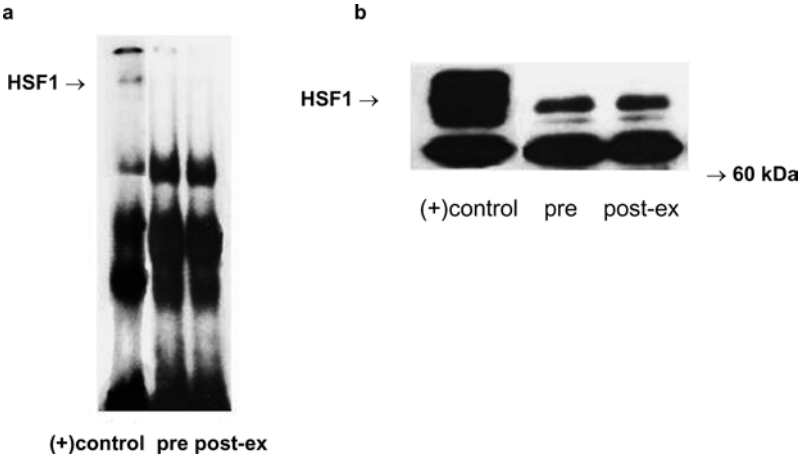
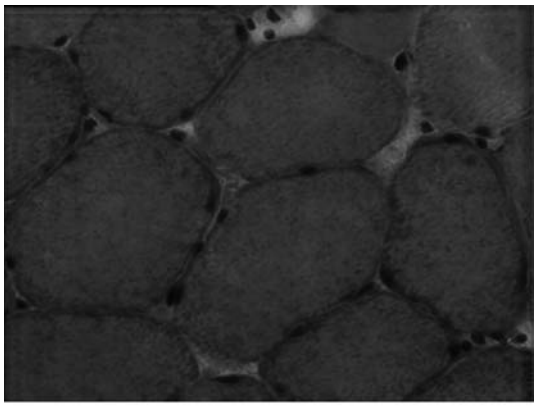


Fig. 3a Heat shock factor-1 (*HSF1*) DNA-binding activity as analysed by mobility shift assay. **b** HSF1 protein expression detected by Western blot assay before and 4 h after exercise in gluteus medius muscle of trotters



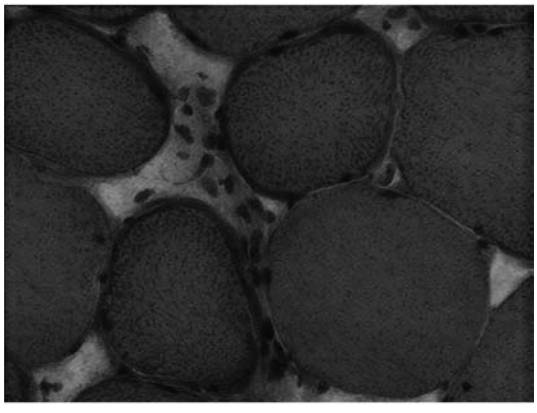


Fig. 4 Light microscopy of the gluteus medius muscle biopsy samples (**a**) before and (**b**) 4 h after exercise. Specimens were stained by haematoxylin and eosin, ×20 magnification

after the oxidative insult, reactive aldehydes and 4-HNE protein adducts showed a lag phase prior to the increase after the oxidative insult (Reinheckel et al. 1998).

Although it has been demonstrated that both acute and chronic exercise can increase HSP expression, we did not observe any HSP induction 4 h after the exercise. Furthermore, acute exercise did not induce either HSF1 DNA binding activity in mobility shift assays or the expression of HSF1 protein (the transcription factor responsible for HSP expression). Consistent with our results, Smolka et al. (2000) showed that in the soleus muscle of trained rats acute exercise did not influence HSP72 expression despite the increase in protein carbonyl content. However, HSP expression was reported to vary widely among different species (Liu and Steinacker 2001), and information on HSP induction patterns in horses is very limited (Pösö et al. 2002). In the studies reporting acute exercise-induced HSP expression in other species, prolonged and exhaustive exercise protocols were used (Salo et al. 1991). The dependence of the HSP70 induction on exercise intensity has also been demonstrated in human studies (Liu et al. 2000). We used a relatively mild exercise protocol, since our aim was to demonstrate HSP, oxidative stress responses and muscle damage 4 h after a bout of exercise of moderate intensity, which is typical during the daily activity of horses. Furthermore, the horses used in our study had all been in regular training for several years, and they were adapted to chronic training at many levels. Studies on chronic-exercise models clearly showed that endurance training induces skeletal muscle and myocardial HSP expression (Fehrenbach et al. 2000; Powers et al. 2001). However, in trained animals, the effect of a single bout of acute exercise on HSP induction was smaller compared to the untrained animals (Thompson et al. 2002).

Excessive contraction of skeletal muscles leads to tissue damage in which cellular contents escape into the blood. Muscle hypoxia during exercise is also known to increase muscle-cell-membrane permeability and leakage of the cellular enzymes. Therefore, plasma ASAT and muscle β-glucuronidase were measured to elucidate possible muscle damage. We observed no increase ofβ-glucuronidase activity. A previous study in standardbred trotters suggested that plasma activities of the lysosomal enzymes were poor indicators of exercise-induced muscle damage, and increased lysosomal enzymes in plasma after exercise mainly originated from the accumulation of neutrophils in the muscle (Raulo et al. 1996). Although no change in β-glucuronidase activity was detected after exercise, we did, however, observe an increased plasma ASAT activity, a marker of impaired membrane integrity. Furthermore, the direct evidence we obtained in histological examination pointed out a minor muscle injury evident by the invasion of inflammatory cells into the extra cellular space and swelling of muscle fibres.

In conclusion, we showed for the first time that a moderate intensity and duration of exercise increased oxidative stress and caused minor muscle damage, without inducing the HSP defence system in horses. Our results suggest that prior to designing a training session, determination of the susceptibility of the horse to oxidative stress might be useful to minimise muscle damage.

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