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Research article

EFFECTS OF PROLONGED EXERCISE ON OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE IN ENDURANCE HORSE

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ABSTRACT

Increased oxidative stress during prolonged endurance exercise may end up with muscle damage, fatigue and decreased physical performance. We have recently shown that acute exercise at moderate intensity induced lipid peroxidation, protein oxidation and oxygen radical absorbance capacity (ORAC) in trained trotters. The aim of this study was to measure the changes in oxidative stress and antioxidant defense following an 80-km ride in the blood of endurance horses. Blood samples were collected before and immediately after the ride. Unlike to our previous studies performed on trotters, in endurance horses there were no measurable changes in antioxidants or oxidative stress marker lipid hydroperoxides (LPO) after prolonged exercise. ORAC, vitamin E and lipid hydroperoxide (LPO) concentration or glutathione related enzyme activities were not altered due to the 80-km ride. However, the base line levels of oxidative stress marker were higher in endurance horses compared to trotters. A positive correlation between the pre-ride LPO concentration and erythrocyte glutathione peroxidase (GPx) activity after the ride was observed, which may indicate a protective response of glutathione peroxidase against exercise-induced oxidative stress. Our results suggest that endurance horses have higher oxidative stress levels compared to trotters and a single 80-km ride probably did not suffice to induce oxidative stress and to activate antioxidant defense mechanisms.

KEY WORDS: Horse, endurance, oxidative stress, antioxidants, ORAC.

INTRODUCTION

During physical exercise, oxygen flux to active skeletal muscles increases, which leads to enhanced production of reactive oxygen species (ROS) and free radicals. Strenuous physical exercise may induce oxidative stress. Oxidative stress has been defined as an imbalance of the pro-oxidant/antioxidant equilibrium in favour of the pro-oxidants (Sen and Packer, 2000). Although ROS function as messengers in signal transduction and regulate a variety of cellular functions, increased oxidative stress plays an important role in diverse disease processes and aging (Sen et al., 2000; Atalay and Laaksonen, 2002). The maximal oxygen uptake (VO_{2max}) of horses is over 160 ml O_2 kg^{-1}·min^{-1}, exposing horse to the oxidative stress (Derman and Noaks, 1994).

In biological systems, cells respond to mild oxidative stress by inducing their antioxidant

defenses and other protective systems (Sen et al., 1994a; Sen et al., 2000; Atalay and Laaksonen, 2002; Atalay et al., 2004). The antioxidant capacities of tissues are well matched to the rates of oxygen consumption and radical production (Powers et al., 1999). A variety of endo- and exogenous antioxidants act in concert to protect tissues against oxidative damage and related chronic diseases. The balance between not only oxidants and antioxidants, but also between various antioxidants, may be of major importance in the protection against ROS-mediated injury.

While regular physical exercise has beneficial effects on health, acute exhaustive exercise may attenuate these benefits via the induction of oxidative stress. Our group and others have shown that glutathione-dependent antioxidant protection in the skeletal muscle is influenced by endurance training, although the training effects are highly tissue specific (Laaksonen et al., 1999; Atalay et al., 2000; Sen and Packer, 2000).

Fatigue to some degree, in which ROS play a role, is expected after a long race also in horses (Foreman, 1998; Assenza et al., 2004). Different pathological alterations during strenuous exercise, including increased ROS formation, may end up with muscle damage, fatigue and decreased physical performance. We have recently reported increased lipid hydroperoxide levels without any induction of the antioxidant responses following single bout treadmill exercise at moderate intensity in trained trotters (Kinnunen et al., 2005a). We also demonstrated that higher antioxidant capacity prior to exercise was associated with lower degree of plasma lipid peroxidation at 4 h post-exercise (Kinnunen et al., 2005a). Exercise-induced oxidative stress has been little studied in endurance horses (Mills et al., 1996; Hargreaves et al., 2002; Williams et al., 2004), however these results are controversial. The main purpose of this study was to examine the association between antioxidant defense mechanisms and oxidative stress after prolonged sub-maximal exercise and to elucidate mechanisms of antioxidant protection in endurance horses. Understanding the magnitude of the endurance ride-induced oxidative insult may help to manage health and welfare of the horses during prolonged exercise.

METHODS

Three clinically healthy Arabian thoroughbreds (one mare, two stallions) and one warmblooded crossbred gelding, 8 to 15 years old, were taken to the 80-km ride at speeds varying from 9-11.7 $km \cdot h^{-1}$ (average of 10.15 $km \cdot h^{-1}$) on 20-km route consisting mainly of farm tracks and roads with minimal changes in altitude. Environmental conditions during the ride ranged from 15 to 18 °C with occasional showers. Animal care and experimental procedure were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Samples

The first blood samples were collected following the first veterinary check before the ride (pre); the other samples (post) were collected when the horses had crossed the finish line. Blood samples were collected from the jugular vein. The blood was collected into serum tubes and lithium-heparin tubes which were centrifuged immediately after collection for the separation of the red blood cells (RBC). RBC were washed with ice-cold saline solution and divided into aliquots. RBC and plasma aliquots were frozen in liquid nitrogen and stored at -80 °C until analyzed.

Analyses

Oxygen radical absorbing capacity (ORAC) assays were performed using a multi-well plate reader according to the methods previously published (Kinnunen et al., 2005b). Briefly, the antioxidant capacity of the samples was measured by the inhibition of the decrease of the fluorescence. For this purpose fluorescein (FL) was used as a target of free radical attack, with 2,2'-azobis(2-amidinopropane) dihydrochloride as a peroxyl radical generator.

Lipid hydroperoxides (ROOHs) in whole plasma were determined as described by Arab and Steghens (Arab and Steghens, 2004) based on oxidation of Fe II to Fe III by lipid hydroperoxides under acidic conditions, followed by complexation of Fe III by xylenol orange. Plasma vitamin E (α-tocopherol) was determined by high-performance liquid chromatography according to the method of De Leenher et al., (De Leenheer et al., 1979). Glutathione peroxidase (GPx), glutathione reductase (GRD) and glutathione-S-transferase (GST) activities were determined from red blood cells spectrophotometrically as described before (Sen et al., 1992; Atalay et al., 2000). Plasma creatine kinase (CK) and aspartate amino transferase (ASAT) activities were analyzed using a standard spectrophotometer (Kone PRO, Finland).

Statistical analysis

The data were analyzed by SPSS for Windows 11.0. Means and standard errors (s.e.) were calculated. Paired samples t-test was used to analyze the difference between the means of pre and post exercise values, independent-samples t-test was used to examine the difference between endurance horses

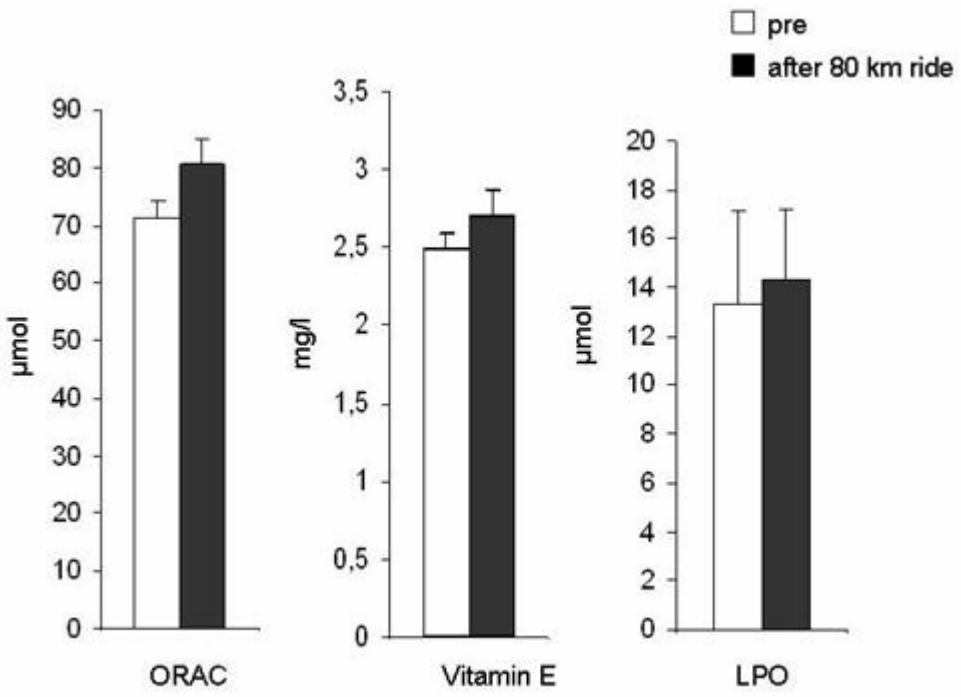

Figure 1. Effect of the 80-km ride on plasma ORAC, lipid hydroperoxide (LPO) and vitamin E concentrations in four endurance horses. Values are means ± SEM. The level of significance is set at $p < 0.05$, paired samples t-test.

and trotters. Pearson's correlation coefficient was used to test the correlation between samples. The level of significance was set at $p < 0.05$.

RESULTS

In endurance horses, total plasma antioxidant capacity measured as ORAC (oxygen radical absorbance capacity) did not change by acute exercise (Figure 1). Plasma vitamin E concentration tended to increase slightly during the 80-km ride, but the change was not statistically significant ($p > 0.05$, Figure 1). The pre-exercise ORAC was 92% higher in endurance horses than in trotters (71.21 and 37.05 µmol respectively, $p < 0.000$, Kinnunen et al., 2005b). The difference after exercise was 88% (80.7 and 43.0 µmol respectively, $p < 0.000$, Kinnunen et al., 2005b). In plasma vitamin E levels the difference compared to trotters was even more obvious, endurance horses having 110% higher pre-exercise concentration of vitamin E (2.49 and 1.18 $mg \cdot L^{-1}$ respectively, $p < 0.000$, (Kinnunen et al., 2005b). After exercise the difference was 140% and statistically significant (2.70 and 1.16 $mg \cdot L^{-1}$ respectively, $p < 0.000$).

There were no statistically significant changes in the activities of glutathione related enzyme systems in erythrocytes during the ride (Table 1). At the same time, there was a positive correlation between glutathione-S-transferase (GST) and glutathione reductase (GRD) activities ($r = 0.995$, $p < 0.005$) in red blood cells before the ride (Table 1). After the ride the glutathione-S-transferase activity correlated positively with glutathione peroxidase (GPx) activity ($r = 0.976$, $p < 0.05$) in red blood cells. There were significant differences in RBC glutathione related enzyme activities between two groups of horses. Glutathione-S-transferase activity was 360% higher in endurance horses than in trotters at pre- (0.023 and 0.005 $\mu mol \cdot mg^{-1}$ $Hb^{-1} \cdot min^{-1}$ respectively, $p < 0.001$) and post-exercise (0.022 and 0.005 $\mu mol \cdot mg^{-1}$ $Hb^{-1} \cdot min^{-1}$ respectively, $p < 0.001$, Kinnunen et al., 2005a; Kinnunen et al., 2005b). Glutathione reductase (GRD) was 70% higher in endurance horses than in trotters pre-exercise (1.84 and 1.08 $nmol \cdot mg^{-1}$ $Hb^{-1} \cdot min^{-1}$ respectively, $p < 0.01$, Kinnunen et al., 2005a; Kinnunen et al., 2005b) and 66% higher post-exercise (1.66 and 1.00 $nmol \cdot mg^{-1}$ $Hb^{-1} \cdot min^{-1}$ respectively, $p < 0.05$,). RBC's glutathione peroxidase (GPx) activity was also higher in endurance horses. Pre-exercise GPx activity was 76% higher and post-exercise 100% higher compared to trotters (0.43 and 0.25 $\mu mol \cdot mg^{-1}$ $Hb^{-1} \cdot min^{-1}$ and 0.53 and 0.26 $\mu mol \cdot mg^{-1}$ $Hb^{-1} \cdot min^{-1}$ respectively, $p < 0.05$ and $p < 0.01$ respectively (Kinnunen et al., 2005b).

Lipid hydroperoxide (LPO) concentration in plasma did not change by the ride. However, pre-ride LPO concentration correlated positively with RBC GPx activity after the ride ($r = 0.970$, $p < 0.05$, Figure 2). There were no significant changes in plasma creatine kinase (CK) and aspartate aminotransferase (ASAT) activities due to the ride

Table 1. The activities of glutathione related enzyme systems in red blood cells and plasma levels of muscle originated enzymes before and after the 80-km ride in four endurance horses. Values are means (± SEM). The level of significance is set at $p < 0.05$, paired samples t-test.

	Pre	After 80 km ride
Glutathione peroxidase ($\mu mol \cdot mg^{-1}\ Hb^{-1} \cdot min^{-1}$)	.43 (.06)	.53 (.03)
Glutathione reductase ($nmol \cdot mg^{-1}\ Hb^{-1} \cdot min^{-1}$)	1.84 (.15)	1.66 (.13)
Glutathione-S-transferase ($\mu mol \cdot mg^{-1}\ Hb^{-1} \cdot min^{-1}$)	.020 (.002)	.020 (.002)
Creatine kinase ($U \cdot l^{-1}$)	275 (43)	568 (137)
Aspartate aminotransferase ($U \cdot l^{-1}$)	387 (13)	423 (35)

(Table 1). Plasma LPO concentration was 456% higher in endurance horses before exercise, however the difference was not statistically significant due to small sample size and high variation between individuals (mean 13.3 and 2.39 µmol respectively, $p = 0.06$, Kinnunen et al., 2005b). After exercise the LPO concentration in endurance horses was only 174% higher and differed significantly from the LPO concentration in plasma of the trotters (14.3 and 5.21 µmol respectively, $p < 0.042$).

DISCUSSION

In this study, there was no detectable change either in lipid peroxidation or oxidative stress markers in endurance horses after an 80-km ride. We did not observe any changes in ORAC or LPO concentrations following 80-km ride. However, there are previous reports indicating increases in plasma LPO or TBARS levels after different types of physical exercise in horses (McMeniman and Hintz 1992; Mills et al., 1996; Chiaradia et al., 1998; Hargreaves et al., 2002; Marlin et al., 2002 and Williams et al., 2004). Similarly in our previous study (Kinnunen et al., 2005a), LPO remained high for several hours after exercise (Mills et al., 1996; Chiaradia et al., 1998; Williams et al., 2004). Plasma LPO started to increase during the last phase of the 80-km ride (Williams et al., 2004), suggesting that in our study the 80-km was ridden at lower intensity and/or in environmentally less challenging conditions. This is supported by the differences in muscle derived enzyme activities in plasma in our study compared with the earlier reports. We did not observe any statistically significant change in plasma CK or ASAT activities, the indexes of cell membrane leakage. However, Williams et al., (2004) as well as Hargreaves et al., (2002) have reported significant increase in both enzyme activities following 80-km ride.

There was no change in plasma vitamin E concentration after 80 km ride either. The unchanged level of plasma vitamin E during prolonged exercise is consistent with the previous reports (Hargreaves et al., 2002; Marlin et al., 2002; Williams et al., 2004). The slight increase in vitamin

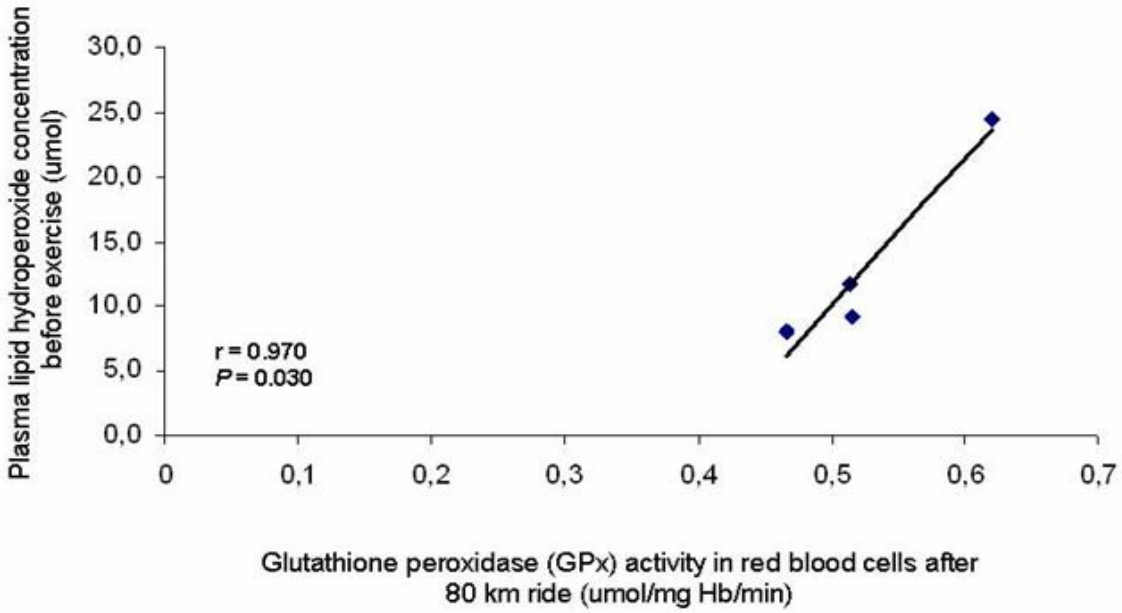

Figure 2. Plasma lipid hydroperoxide (LPO) concentration before exercise vs. glutathione peroxidase (GPx) activity in red blood cells following the 80km ride. The level of significance is set at $p < 0.05$, r = Pearsons correlation coefficient.

E concentration in this study was probably due to hemoconcentration (Sürmen-Gür et al., 1999). Furthermore, in our study, overall plasma α-tocopherol concentrations were lower compared to those reported previously (Hargreaves et al., 2002; Williams et al., 2004).

GPx activity is a key component of the glutathione homeostasis. The positive correlation between the pre-ride LPO concentration and red blood cells' GPx activity after the ride may indicate a protective role of GPx in exercise-induced oxidative stress. The 23% increase following 80-km ride in RBC GPx activity in this study is supported by a previous finding (Williams et al., 2004). However, Hargreaves et al., (2002) have also reported no change in RBC GPx activity following 80-km ride in less challenging environmental conditions.

It has been well demonstrated that endurance exercise training results in an increase in the antioxidant capacity (Miyazaki et al., 2001; Fatouros et al., 2004; Ficicilar et al., 2005). Prolonged exercise results in an increased production of oxidants in skeletal muscle and regular exercise training up-regulates muscle antioxidant enzyme activities (Sen et al., 1992; Powers et al., 1999; Powers and Sen, 2000). The previous studies indicate that regular endurance exercise training results in increased GPx activity in active skeletal muscle and the magnitude of this effect is impacted by both the intensity and daily duration of exercise (Powers et al., 1999). Similarly in our study, endurancetrained horses had significantly higher post-exercise GPx activity in red blood cells compared to trotters (Kinnunen et al., 2005b).

Glutathione reductase (GRD) is not considered as a primary detoxificant of ROS, however GRD recycles oxidised glutathione to its reduced form and has a central role in the glutathione dependent antioxidant protection (Powers and Hamilton, 1999; Atalay et al., 2000; Sen and Packer, 2000). Our results are consistent with the previous findings where endurance training resulted in little or no change in GRD activity in skeletal muscle (Sen et al., 1992).

Glutathione and its related enzymes are well implicated in the circumvention of cellular oxidative stress and maintenance of intracellular thiol redox status (Sen et al., 1994a; Halliwell and Cutteridge, 1999). This is supported by the positive correlations between erythrocyte GST and GRD activities before and GST and GPx activities after 80-km ride, even if there was no change in the GST activity itself.

Horses are subjected to a considerable level of lipid peroxidation at rest as indicated by resting concentrations of plasma TBARS (McMeniman and Hintz, 1992). The pre-exercise plasma LPO concentration in endurance horses was significantly higher compared with trotters (Kinnunen et al., 2005b). However, 80-km ride did not induce lipid peroxidation in endurance horses. According to these findings, it can be suggested that low intensity exercise, even if taking several hours, does not induce oxidative stress in the endurance horses.

In this study we observed that horses trained for endurance ride had higher basal status of oxidative stress compared with more intensively trained trotters reported earlier (Kinnunen et al., 2005b). Depending on the intensity and duration, endurance training is known to enhance the antioxidant capacity (Sen et al., 1992; Powers et al., 1999). However, an 80-km ride of this intensity is not enough to induce antioxidant defense mechanisms. Furthermore, it has been reported, that a single bout of repeated submaximal exercise does not alter vitamin E status in the plasma of the horse (McMeniman and Hintz, 1992; White et al., 2001). This is consistent with our previous studies too, where a single bout of moderate intensity treadmill exercise did not change the plasma vitamin E concentration in trotters (Kinnunen et al., 2005b).

CONCLUSIONS

Prolonged exercise in endurance horses caused neither measurable changes in oxidative stress nor plasma antioxidants following 80-km ride in contrast to a shorter exercise in trotters. Endurance horses, however, had higher oxidative stress at rest compared to trotters (Kinnunen et al., 2005a; Kinnunen et al., 2005b). In summary, endurance horses have basal oxidative stress compared to trotters and an 80-km ride at moderate speed may not suffice to induce oxidative stress or to activate antioxidant defense mechanisms.

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KEY POINTS

- Reactive oxygen species (ROS) at lower concentrations have physiological role in the signal transduction and in the regulation of cellular functions. However, the overproduction of ROS results in oxidative stress, an imbalance favoring pro-oxidants over antioxidants.
- Increased oxidative stress which occurred during prolonged and strenuous physical exercise may end up with muscle damage, fatigue and decreased performance.
- Prolonged exercise at moderate intensity does not induce oxidative stress in endurance horses.
- Endurance horses have higher oxidative stress at rest compared to trotters which were trained for short bouts of exercise.

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