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α-Lipoic acid supplementation enhances heat shock protein production and decreases post exercise lactic acid concentrations in exercised standardbred trotters

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ABSTRACT

Heat shock protein (HSP) expression is an adaptive mechanism against the disruption of cell homeostasis during exercise. Several antioxidant supplementation strategies have been used to enhance tissue protection. In this study, we examined the effects of a redox modulator, α-lipoic acid (LA) on HSP responses in six standardbred trotters following intense aerobic exercise. DL–LA supplementation (25 mg kg^{-1} d^{-1}) for five weeks increased the resting levels of HSP90 (1.02 ± 0.155 in control and 1.26 ± 0.090 after supplementation in arbitrary units) and the recovery levels of inducible HSP70 (0.89 ± 0.056 in control and 1.05 ± 0.089 after supplementation in arbitrary units) in skeletal muscle. Furthermore, LA increased skeletal muscle citrate synthase activity at rest and lowered the blood lactate concentration during exercise without any changes in the heart rate. LA had no effect on concentrations of HSP60, HSP25 or GRP75 in skeletal muscle. LA decreased the exercise-induced increases in plasma aspartate aminotransferase and creatine kinase concentrations during recovery. Our results suggest that LA supplementation may enhance tissue protection and increase oxidative capacity of the muscle in horse.

1. Introduction

Heat shock proteins (HSPs) play a critical role in cells assisting in protein folding and preventing improper protein aggregation. In response to environmental stress, the major function of HSPs is to defend cells against damage by binding to partially denatured proteins, dissociating protein aggregates, regulating the correct folding and co-ordinating the transport of newly synthesised polypeptides (Fehrenbach and Northoff, 2001). Endurance training induces HSP response (Thompson et al., 2002; Atalay et al., 2004; Moran et al., 2004) in an intensity-dependent manner (Milne and Noble, 2002). The horse is an excellent animal model to study the exercise-related oxidative insults due to its high maximal oxygen uptake (VO_{2max}) and natural ability for exercise. There is little information regarding the induction and the protective actions of HSPs during physical exercise, especially in horses (Pösö et al., 2002; Kinnunen et al., 2005).

Dietary antioxidant supplementation affords protection against exercise-induced oxidative stress and muscle damage (Atalay et al., 2006). α-Lipoic acid (LA) is present in bound form in all animal cells. Free LA may function as a metabolic antioxidant by not only preventing oxidative stress but also by supporting cellular metabolic processes (Sen and Packer, 2000). LA acts synergistically with other antioxidants, and is capable of regenerating and recycling both water- and lipid-soluble antioxidants from their oxidised forms (Packer et al., 1997). On the other hand, in cells LA itself is continuously reduced to dihydrolipoic acid, DHLA (Handelman et al., 1994; Haramaki et al., 1997). Consequently many biological effects of LA supplementation can be attributed to the antioxidant properties of LA and DHLA. In addition, LA enhances glucose intake by its insulin-mimetic action, which also appears to be a major consequence of LA treatment in most cells (Sen and Packer, 2000).

The widespread belief that more of a good nutrient results in improved performance is not completely true with the antioxidant nutrients (Atalay et al., 2006). Excess supplementation of antioxidants combined with exercise may result in increased oxidative stress (Childs et al., 2001) and may attenuate the exercise-induced adaptations, including blunting of HSP induction (Khassaf et al., 2003; Fischer et al., 2006). The antioxidant properties of LA may not solely explain its wide spread cytoprotective effects and ability to stimulate the synthesis of protective proteins. The effects of LA supplementation on HSP synthesis and its supportive actions in

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tissue protection in relation to acute exercise are not clear. Therefore, in this study we sought to test whether LA may also up-regulate the synthesis of HSPs in muscle and support tissue protection and adaptation in standardbred trotters.

2. Materials and methods

2.1. Animals, exercise protocols and supplementation

The experimental protocol was approved by the Ethics Committee of the MTT Agrifood Research Finland. Six clinically healthy standardbred trotters, 5–13 years of age and 400–508 kg in weight, were examined in this study. Two of the horses were mares and four geldings. All horses had been in regular training for several years. The horses were housed in box stalls and fed hay silage (ad libitum) and oats (2.2 ± 0.24 kg) to meet the recommended nutrient requirements (Meyer, 1996) and to maintain a moderate body condition score (Henneke et al., 1983).

Before starting this series of tests the administration of additional vitamins was discontinued for five weeks (control period), to rule out a previous antioxidant supplementation effect. The performance tests were carried out before and after LA supplementation. Prior to each performance test, the individual treadmill speed (V_{La4}) resulting in a blood lactate level of 4 mmol/l was determined for each horse with the standardised exercise test (SET). The SET consisted of a 10-min warm-up period at 1.7 m/s, followed by 4 exercise intervals, 2 min each, at speeds of 7, 8, 9 and 10 m/s on a high-speed treadmill with a 2.5° incline. Blood samples for lactate analysis were collected before the test and during the last 10 s of each exercise speed. Exercise speed causing a blood lactate level of 4 mmol/l (V_{La4}) was calculated from the velocity of the treadmill and blood lactate concentration in the SET (Persson, 1983).

In the subsequent performance test the treadmill speed was kept under the anaerobic threshold, i.e. under the individual V_{La4} to make sure that lactic acid will not accumulate in the skeletal muscles. The performance test protocol is presented in the Table 1. The results of the first performance test prior to the LA supplementation are further considered as control.

After five-week control period, DL–LA (Changshu Fushilai Medicine & Chemical Co. Ltd., China) was supplemented to the horses at 25 mg kg^{-1} d^{-1} mixed in molasses, for five consecutive weeks. The purity of LA was confirmed by comparing with reagent grade LA using HPLC methods (Sen et al., 1999).

2.2. Samples

Blood samples were drawn from jugular vein at rest and immediately after standard exercise tests, and at 2, 6, 24 and 48 h of recovery after control and LA supplementation periods. The samples were collected in lithium–heparin tubes and centrifuged immediately to separate plasma for biochemical analysis. Plasma samples were aliquoted and snap-frozen in liquid nitrogen.

Tissue samples from the middle gluteal muscle were obtained at rest and after 6, 24 and 48 h of recovery. Biopsy specimens were obtained under local anaesthesia as described previously (Lindholm and Piehl, 1974). The samples were first rinsed quickly with ice-cold saline solution and blotted onto filter paper, and then snap-frozen in liquid nitrogen.

For assays of stress proteins the frozen muscle tissues were handled as described previously (Kinnunen et al., 2005). Unless otherwise stated, all chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

Table 1
The procedure for the performance tests and sampling times.

Treadmill speed (m^{-1} s^{-1})	Time (min)	Gait
Blood and muscle samples (rest)		
1.7	15	Walk (warming up)
6.2–6.8	10	Trot
1.7	10	Walk
6.2–6.8	10	Trot
1.7	10	Walk
6.2–6.8	10	Trot
Blood samples (post-ex)		
1.7	10	Walk
Active cooling down (10 min)		
Blood samples (after 2 h recovery)		
Blood and muscle samples (after 6 h recovery)		
Blood and muscle samples (after 24 h recovery)		
Blood and muscle samples (after 48 h recovery)		

2.3. Analyses

HSP expression was determined using Western Blot as described earlier (Kinnunen et al., 2005). Citrate synthase (CS) activity was measured as described previously (Shepherd and Garland, 1969) and total protein concentration using a BCA protein assay kit (Pierce, Rockford, IL, USA). Lactate concentrations were measured from blood with an enzymatic lactate analyser (YSI 2300 STAT, Yellow Springs Instrument Co., Yellow Springs, OH, USA) using lactate oxidase as a catalyst. Analyses of plasma creatine kinase (CK) and plasma aspartate aminotransferase (AST) were carried out in the Laboratory of Equine Hospital (Ypäjä, Finland) using a clinical chemistry analyzer Kone-Pro (Konelab, Thermo Clinical Labsystems Oy, Finland) according to IFCC reference procedures (Schumann et al., 2002a,b, respectively). Heart rates were measured using the Polar S810 heart rate meter (Polar Electro Oy, Kempele, Finland).

2.4. Statistical analyses

Data were analyzed using SPSS for Windows version 14.0. A multivariate linear mixed model was used to assess whether duration of exercise and use of lipoate (on/off) have an effect on physical quantities, as it takes into account the correlation structure of the data due to repetitions. In addition to main effects of duration of exercise and use of LA, each model includes an interaction term for duration of exercise and use of LA as an explanatory variable. The interaction term was also included because the effect of LA did not seem to be uniform at each time point. Post hoc tests were then used to make pair-wise comparisons to assess whether the use of LA has an effect at separate time points and also whether the later time points differ from the starting point, again because the effect of LA seemed vary at different time points. The paired samples *t*-test was used to assess whether the LA supplementation had an effect on blood lactate before and after the exercise and heart rate during the exercise. Spearman's correlation coefficient was used to assess the correlation between variables. *P*-values less than 0.05 were treated as statistically significant.

3. Results

Intense aerobic exercise had no significant effect on HSP levels in the horses. However, in horses subjected to five-week LA supplementation the abundance of inducible HSP70 increased 19% and was significantly higher after 24-h recovery compared with non-supplemented horses ($p < 0.05$, Fig. 1). LA supplementation increased the basal level of HSP90 by 24% ($p < 0.05$, Fig. 1) and the overall level of constitutive HSC70, even though there were no statistically significant difference in pair-wise comparisons of HSC70 (Fig. 1). There were no significant changes in muscle HSP60,

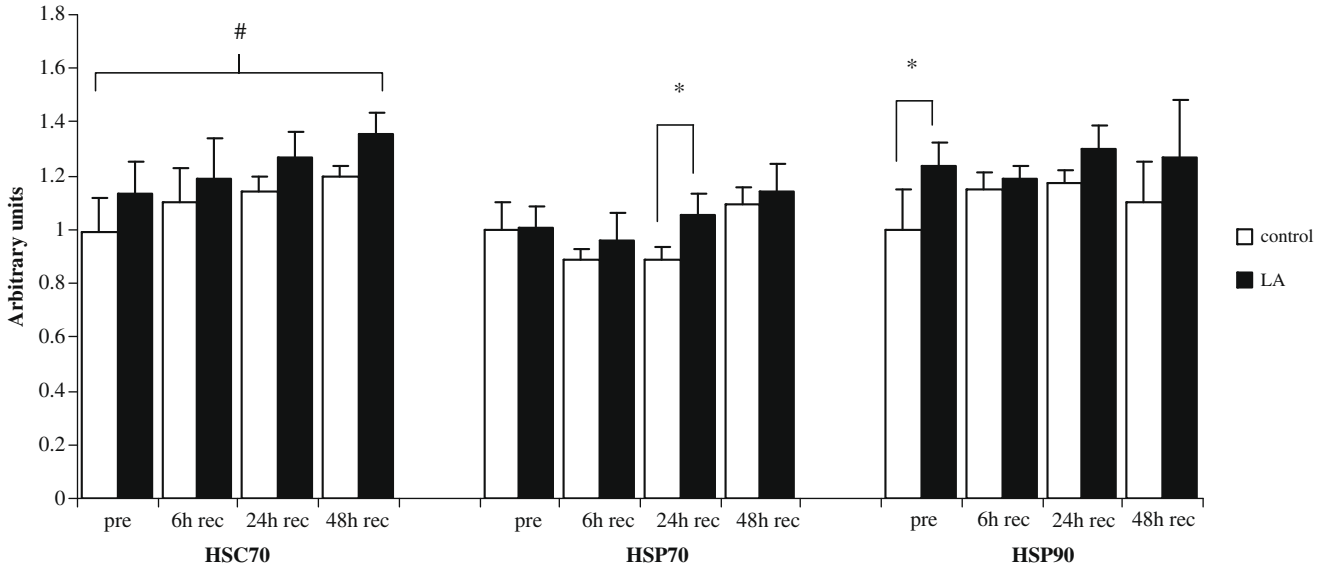

Fig. 1. Effect of exercise and LA supplementation on the levels of constitutive HSC70, HSP70 and HSP90 in the middle gluteal muscle of the horse. Values are means SEM, values marked with * differ significantly ($p < 0.05$) from their counterpart according to pair-wise comparisons in linear mixed model analysis, # overall effect of LA supplementation without pair-wise comparisons.

Table 2
Effects of LA on HSP responses in equine middle gluteal muscle following intense aerobic exercise.

		At rest	During recovery		
			6 h	24 h	48 h
HSP60 (arbitrary units)	Control	1.00 ± 0.012	0.94 ± 0.028	0.96 ± 0.104	1.09 ± 0.082
	Lipoate	1.07 ± 0.065	0.98 ± 0.099	1.07 ± 0.124	1.05 ± 0.203
HSP25 (arbitrary units)	Control	1.00 ± 0.145	0.89 ± 0.058	0.94 ± 0.128	1.09 ± 0.092
	Lipoate	0.96 ± 0.127	0.92 ± 0.065	1.23 ± 0.195	1.01 ± 0.208
GRP75 (arbitrary units)	Control	1.00 ± 0.099	1.01 ± 0.078	1.08 ± 0.086	0.95 ± 0.109
	Lipoate	0.95 ± 0.097	1.13 ± 0.077	0.96 ± 0.108	1.01 ± 0.177

Values are means ± SEM.

HSP25 or GRP75 responses due to LA supplementation or exercise either (Table 2).

LA supplementation increased CS activity in equine muscle at rest ($p < 0.05$, Fig. 2). Furthermore, in non-supplemented animals, muscle CS activity increased in response to exercise ($p < 0.05$, Fig. 2). LA supplementation reduced the post-exercise concentration of blood lactate ($p < 0.05$, Fig. 2) without significant changes in heart rates (Fig. 2).

Plasma CK (Fig. 3) concentration increased significantly in response to exercise both before and after LA supplementation ($p < 0.001$ and 0.05, respectively). However, before supplementation (control) the CK concentration remained elevated still after the 24-h recovery compared with resting levels ($p < 0.05$). In response to LA supplementation, the increase in plasma CK concentration after 6-h recovery was only 35%, compared with a 97% increase before supplementation, although this difference was not statistically significant. There were no statistically significant changes in plasma AST due to exercise or LA supplementation.

Prior to the LA supplementation the plasma AST level after 24-h recovery correlated negatively with the resting levels of muscle HSP25 and HSP60 ($r = -0.829$, $p < 0.05$ in both). Consistent with this, the plasma AST level correlated negatively with muscle HSP25 level after the recovery period of 24-h ($r = -0.943$, $p < 0.01$) and with muscle HSP90 level after the recovery period of 48 h ($r = -0.886$, $p < 0.05$). After LA supplementation the plasma CK level correlated negatively with muscle HSP25 level after the recovery period of 24 h ($r = -1.000$, $p < 0.001$) and further with muscle HSC70 after 48 h of recovery ($r = -0.886$, $p < 0.05$).

4. Discussion

In the present study, LA supplementation enhanced the skeletal muscle HSP response and CS activity, a marker of oxidative metabolism, in treadmill-exercised horses. Oxidative stress is one of the physiological inducers of HSP expression. In contrast to these results, vitamin E and vitamin C supplementation have been shown to decrease exercise-induced HSP expression in human skeletal muscle (Khassaf et al., 2003; Fischer et al., 2006). The mechanism by which LA increases the basal levels of HSP90 and the levels of HSP70 during recovery remains to be elucidated. It has been suggested that LA might activate certain, yet unidentified, signalling intermediates by inducing intramolecular disulphide bond formation, a signal for oxidant exposure and function as an HSP inducer (McCarty, 2001, 2006). The role of LA as an enhancer of HSP induction is supported by our previous studies in which we demonstrated that LA supplementation up-regulated the mRNA for HSP90, but had no effect at the protein level in rat kidney tissue (Oksala et al., 2007) and that LA supplementation up-regulated heat shock factor-1 (HSF-1) at both mRNA and protein levels (Oksala et al., 2007).

Our results show that acute exercise itself did not significantly influence the levels of HSPs in the muscle of regularly trained trotters. This is consistent with our previous study (Kinnunen et al., 2005), where one bout of exercise at moderate intensity had no effect on muscle HSP levels. In horses, increased HSP72 mRNA levels have been reported following exercise, but as the authors emphasised, that it is not certain if the increase in HSP72 mRNA levels led

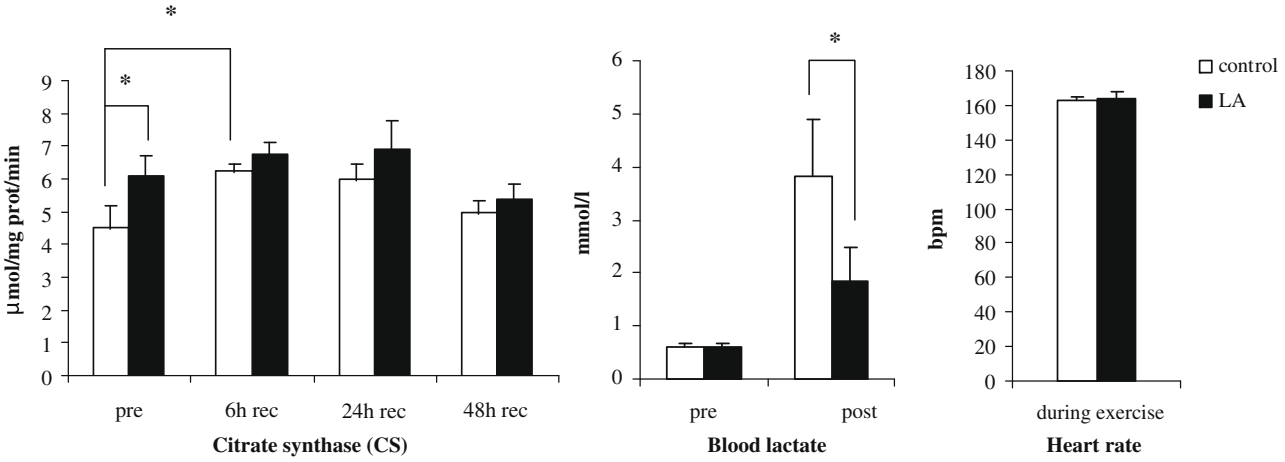

Fig. 2. Effect of exercise and LA supplementation on muscle CS activity, concentration of blood lactate and heart rate during exercise. Values are means SEM, values marked with * differ significantly ($p < 0.05$) from their counterpart.

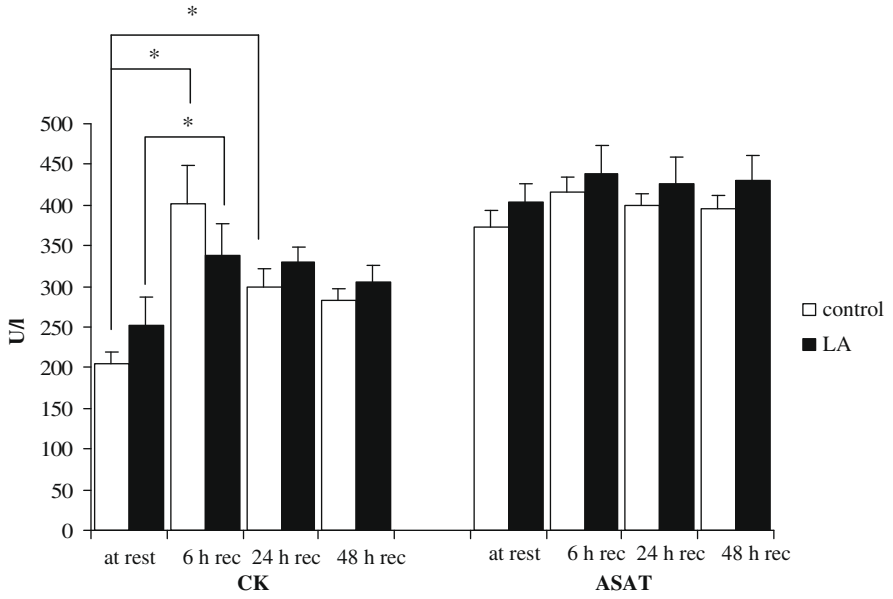

Fig. 3. Effect of exercise and LA supplementation on plasma levels of CK and AST. Values are means SEM, values marked with * differ significantly ($p < 0.05$) from their counterpart.

to an increase in protein levels (Pösö et al., 2002). However, in agreement with our results, in a previous study (Smolka et al., 2000) there was no induction of HSP72 in muscles of trained rats after 2-h recovery following acute exercise, even though there was a significant increase in HSP72 content in the group of sedentary rats exercised to exhaustion.

In non-supplemented horses the negative correlation between the resting levels of muscle HSP60 and HSP25 and the recovery level of plasma AST supports the cytoprotective role of HSPs in skeletal muscle. This is in line with the negative correlations between the levels of plasma AST and HSP25 and HSP90 during recovery (24 and 48 h, respectively). The tendency towards an increase in plasma CK levels following LA supplementation is supported by the earlier study with vitamin E supplementation and eccentric exercise in older men (Sacheck et al., 2003). However, there are also other findings inconsistent with these papers (Williams et al., 2004a), demonstrating lower CK levels with LA-supplementation following endurance exercise. Nonetheless, the duration of exercise (Child et al., 1998), age, gender and exercise training (Williams et al., 2004b) may also influence the release of CK during exercise. Because LA supplementation decreased the rate of CK increase at recovery after constant exercise duration, we suggest that LA might decrease acute, exercise-induced muscle damage. This is, in turn, supported by the negative correlation of plasma CK level with muscle HSP25 and HSC70 during recovery (24 and 48 h, respectively). Antioxidant supplementation has been earlier reported to correlate negatively with CK and to improve the delicate balance between oxidants and antioxidants during endurance type exercise (Hargreaves et al., 2002).

Our findings, that LA supplementation increased skeletal muscle CS activity, could be explained by the role of LA on the oxidative metabolism in mitochondria (Sen and Packer, 2000). According to this hypothesis, LA enhances the activities of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase possibly by improving their co-factor availability (Hagen et al., 2002). This may increase oxidative capacity of the skeletal muscle and lead to improved performance in endurance events. However, our group has reported that intragastrically supplemented LA did not increase the levels

of lipoyl lysine in skeletal muscle (Khanna et al., 1998). Lipoyl lysine is the bound form of LA and a co-factor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase in skeletal muscle (Khanna et al., 1998). On the other hand, LA enhances the glucose intake by the muscle cells by increasing glucose transporters at the plasma membrane (Packer et al., 1997). Enhanced glucose metabolism, in turn, may increase pyruvate availability for TCA cycle and result in increased oxidative capacity of the muscles (Savitha et al., 2005).

However, it has been reported that LA supplementation has no effect on CS activity in the muscle (Jacob et al., 1996) or brain (Seaton et al., 1996) of the rat, which may indicate that the effects are species dependent. Although we did not observe any significant correlation between the CS activity and either HSP70 or HSP90 at the protein level, it has been reported earlier that HSP72 mRNA is markedly associated with muscle oxidative capacity (Bruce et al., 2003). It has been well demonstrated that endurance trained animals have higher levels of HSP72 (Ecochard et al., 2000). It is unclear whether the increase in HSP70 content during recovery is a direct LA effect or a consequence of increased oxidative capacity of the skeletal muscle following LA administration. However, given the small increase in blood lactate compared to what has been reported after racing, and the significantly better buffer capacity of equine muscle than human muscle, in this experiment lactate was likely to have only a minor detrimental effect on muscle (Lindholm and Saltin, 1974; Marlin et al., 1989).

In conclusion, LA-induced increase in the HSP levels before the exercise and during recovery may further minimize disruption of muscle homeostasis during acute exercise. Furthermore, the LA-induced increase in CS activity may suggest enhancement of the oxidative capacity of skeletal muscles which may increase performance in endurance events. Moderate LA administration may, therefore, be useful for trotters in training in order to boost oxidative metabolism and HSP expression in the skeletal muscle. On the other hand, the continuous reduction of LA to DHLA may stress the energy generation capacity and reducing power of cells particularly at high doses of LA. Furthermore, prolonged megadose supplementation of micronutrients may be associated with several adverse health effects. Therefore, in spite of the promising actions of LA based on the present and other studies, caution should be exercised when considering the use of antioxidants at high doses.

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