

## Antioxidative defence alterations in skeletal muscle during prolonged acclimation to cold: role of L-arginine/NO-producing pathway

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### SUMMARY

Early in cold acclimation (1–7 days), heat is produced by shivering, while late in cold acclimation (12–45 days), skeletal muscle contributes to thermogenesis by tissue metabolism other than contractions. Given that both thermogenic phases augment skeletal muscle aerobic power and reactive species production, we aimed in this study to examine possible changes in skeletal muscle antioxidative defence (AD) during early and late cold acclimation with special emphasis on the influence of the L-arginine/nitric oxide (NO)-producing pathway on the modulation of AD in this tissue. Adult Mill Hill hybrid hooded rat males were divided into two main groups: a control group, which was kept at room temperature (22±1°C), and a group maintained at 4±1°C for 45 days. The cold-acclimated group was divided into three subgroups: untreated, L-arginine treated and N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) treated. The AD parameters were determined in the gastrocnemius muscle on day 1, 3, 7, 12, 21 and 45 of cold acclimation. The results showed an improvement of skeletal muscle AD in both early and late cold acclimation. Clear phase-dependent changes were seen only in copper, zinc superoxide dismutase activity, which was increased in early cold acclimation but returned to the control level in late acclimation. In contrast, there were no phase-dependent changes in manganese superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase, the activities of which were increased during the whole cold exposure, indicating their engagement in both thermogenic phases. L-Arginine in early cold acclimation accelerated the cold-induced AD response, while in the late phase it sustained increases achieved in the early period. L-NAME affected both early and late acclimation through attenuation and a decrease in the AD response. These data strongly suggest the involvement of the L-arginine/NO pathway in the modulation of skeletal muscle AD.

Key words: skeletal muscle, antioxidative defence, nitric oxide, cold.

### INTRODUCTION

Prolonged exposure of mammals to cold induces increases in heat production and enhances tolerance to cold through both shivering and non-shivering thermogenesis (NST) (Himms-Hagen, 1976). Shivering thermogenesis, achieved by increased skeletal muscle activity, occurs early in response to cold exposure (Jansky and Hart, 1968). A later decrease in shivering thermogenesis has been found to be accompanied by an increase in NST (Griggio, 1982). NST is achieved by the uncoupling of oxidative metabolism from ATP production, primarily in brown adipose tissue (Jansky, 1966; Foster and Frydman, 1979). In contrast to the clear role of skeletal muscle in shivering thermogenesis, the extent of NST in this tissue remains controversial, with some reports demonstrating NST in the skeletal muscle of pigeons (Skulachev and Maslov, 1960), ducklings (Barre et al., 1987) and rats (Mollica et al., 2005), whilst others found no NST in this tissue (Golozoubova et al., 2001). However, skeletal muscle, which represents a large percentage of body mass, significantly contributes to the intensification of metabolic activity of the whole organism during thermogenesis (Rolfé and Brand, 1996) due to its huge capacity for  $\beta$ -oxidation and the oxidative capacity of mitochondria (Hoppeler and Fluck, 2003).

Heat production increases metabolic rate and oxygen consumption in metabolically active tissues (Shiota and Masumi,

1988) that is implacably associated with the elevation of reactive oxygen species generation. Cellular homeostasis under conditions of increased reactive oxygen species production is achieved by a proportional increase in tissue antioxidative defence (AD) (Halliwell and Gutteridge, 1990; Buzadžić et al., 1997; Buzadžić et al., 1999; Korać and Buzadžić, 2001; Petrović et al., 2006). AD consists of enzymes – copper, zinc and manganese superoxide dismutase (CuZnSOD and MnSOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione S-transferase (GST, EC 2.5.1.18), thioredoxin reductase (TR, EC 1.6.4.5), glutathione reductase (GR, EC 1.6.4.2) and low molecular mass antioxidants such as vitamins E and C, glutathione (GSH), etc. (Chance et al., 1979; Cadenas et al., 1989; Aruoma, 1996). In recent years, considerable progress has been achieved in the field of redox regulation and the concept of the protective effect of AD was extended to its regulatory role, since AD activity determines reactive species availability (Mugge et al., 1991; Korać and Buzadžić, 2000; Buzadžić et al., 2006).

Reactive species in moderate concentrations, especially superoxide anion radical (O<sub>2</sub><sup>•-</sup>) and nitric oxide (NO), play an important role as regulatory mediators in biological processes (Dröge, 2002). In skeletal muscle, NO is produced by the activity of constitutively expressed endothelial and neuronal NO synthase

(NOS), localized on the surface of the sarcolemmal membrane and the endothelial plasmalemmal caveolae, as well as by inducible NOS, the localization of which varies depending on the state of the cell (Kobzik et al., 1995; Bates et al., 1996). NO regulates many physiological functions of skeletal muscle including glucose uptake and oxidation (Young et al., 1997), mitochondriogenesis (Puigserver et al., 1998), contractile functions (Joneschild et al., 1999; Maréchal and Gailly, 1999), blood flow (Brevetti et al., 2003) and fatty acid oxidation (Jobgen et al., 2006), as well as muscle repair through satellite cell activation and the release of myotrophic factors (Anderson, 2000; Brunelli et al., 2007).

On the other hand, it is known that both endurance training and acclimation to cold increase skeletal muscle oxygen uptake and, consequently, production of reactive oxygen species. So far, most studies have focused on the changes of skeletal muscle AD during exercise (Tiidus et al., 1996; Tonkonogi et al., 2000; Pansarasa et al., 2002). However, knowledge of the changes in AD in skeletal muscle during acclimation to cold is very limited and there are no data concerning the possible role of NO in the modulation of skeletal muscle AD.

Hence, the aim of the present study was to assess changes in AD in skeletal muscle during early and late cold acclimation with a special focus on the possible role of NO in the modulation of AD in this tissue. For this purpose, adult male rats were kept at room temperature, or were exposed to cold for 45 days and received the NO-manipulating agents L-arginine or *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) as drinking liquid, and the effects on AD over time were assessed.

## MATERIALS AND METHODS

Mill Hill hybrid hooded, 4 month old *Rattus norvegicus* (Berkenhout 1769) males were divided into two main groups: a control group, kept at room temperature (22±1°C) for the duration of the experiment, and a second group, maintained at 4±1°C. The cold-acclimated group was divided into three subgroups: (1) untreated, (2) L-arginine treated and (3) L-NAME treated. Drugs were administered as a drinking liquid, as done previously by others (Saha et al., 1996) and by us (Vasiljević et al., 2007) in a dose (2.25% L-arginine HCl and 0.01% L-NAME HCl in tap water) that caused no toxic effects (Petrović et al., 2005). The animals were killed at the same time of the day after 1, 3, 7, 12, 21 or 45 days of cold acclimation. The rats were housed in individual plastic cages with drinking liquid and food *ad libitum*. Each experimental group consisted of six individuals. Body mass, food and fluid intake were recorded daily for each animal. The experimental protocol was approved by the Ethical Committee for the Treatment of Experimental Animals of the Institute for Biological Research, Belgrade.

The rats were killed by decapitation, and the gastrocnemius dissected and rinsed with physiological saline to wash out traces of blood. The tissue was homogenized (Ultra/Turrax homogenizer, Janke und Kunkel Ka/Werke, Staufen, Germany; 0–4°C) in 0.25 mol l<sup>-1</sup> sucrose, 0.1 mmol l<sup>-1</sup> EDTA and 50 mmol l<sup>-1</sup> Tris-HCl buffer, pH 7.4, and the homogenates sonicated (Takada et al., 1982).

### Activity of antioxidative enzymes

Total SOD activity was examined by a modified method of Misra and Fridovich (Misra and Fridovich, 1972). MnSOD activity was determined after preincubation with 4 mmol l<sup>-1</sup> KCN. CuZnSOD activity was calculated as the difference between total SOD and MnSOD activities. Enzymatic activity was expressed in U mg<sup>-1</sup>

protein. SOD units were defined as the amount of the enzyme inhibiting epinephrine (adrenaline) auto-oxidation under appropriate reaction conditions. CAT was assayed as suggested by the supplier (Sigma-Aldrich, St Louis, MO, USA) and the activity expressed in μmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein. GSH-Px was determined with *t*-butylhydroperoxide as a substrate (Paglia and Valentine, 1967) and the activity expressed in nmol of reduced NADPH min<sup>-1</sup> mg<sup>-1</sup> protein. GST was measured by the method of Habig et al. (Habig et al., 1974) and the activity expressed in nmol GSH used min<sup>-1</sup> mg<sup>-1</sup> protein. GR activity was assayed according to Glatzle et al. (Glatzle et al., 1974) and expressed as nmol GSH min<sup>-1</sup> mg<sup>-1</sup> protein.

### Determination of GSH

The content of GSH was examined in the tissue after deproteinization with sulphosalicylic acid. Total GSH was measured by enzyme recycling assay according to Griffith (Griffith, 1980) and expressed in nmol GSH g<sup>-1</sup> tissue.

### Other assays and statistics

Protein content was estimated by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as a reference. Analysis of variance (ANOVA) was used for within-group comparison of the data. If the *F* test showed an overall difference, Tukey's test was applied to identify significant differences. Statistical significance was accepted at *P*<0.05.

## RESULTS

Table 1 shows the changes in body mass during acclimation. Initially, in cold acclimation animals from all examined groups lost body mass (*P*<0.001). These decreases persisted during the whole of the early cold-acclimation period and body mass returned to the control level no sooner than day 45 of late cold acclimation.

During the whole experiment no changes in fluid intake were observed. In contrast, as acclimation to cold started, animals in all examined groups increased food intake by 100% and this increase remained constant to the end of experiment (data not shown).

Generally, the results showed improvement of skeletal muscle AD in both early and late cold acclimation. Clear phase-dependent changes were seen only in CuZnSOD activity (Fig. 1), which was increased in early cold acclimation but returned to the control level in late acclimation. In contrast, there were no phase-dependent changes in MnSOD (Fig. 2) and peroxidative- and GSH-related parts of AD (CAT, GSH-Px, GR and GST; Figs 3–6), the activities of which were increased during the whole period of cold exposure.

Alterations in CuZnSOD activity in gastrocnemius are shown in Fig. 1. In the untreated group, changes in CuZnSOD activity were quite phase dependent, i.e. an increase in its activity was observed

Table 1. Changes in body mass during cold acclimation

Day	Control	Cold		
		Untreated	L-arginine	L-NAME
1	2.0±1.0	-6.8±1.4***	-4.7±1.7***	-1.0±0.2***
3	4.7±2.8	-3.4±1.6**	-2.3±2.9*	-1.8±1.5**
7	8.2±1.7	-11.5±3.5***	-11.3±4.4***	-16.0±5.8***
12	14.4±4.4	-8.7±2.7***	-5.6±12.2***	2.7±3.3***
21	24.5±4.3	8.7±3.2***	9.5±3.3***	4.3±5.1***
45	49.0±16.0	42.8±9.6	39.0±16.9	49.0±4.4

Data for body mass gain (in g) are means ± s.e.m. (*N*=6 in all groups).

\**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 compared with the control acclimated to 22°C for the same time period.

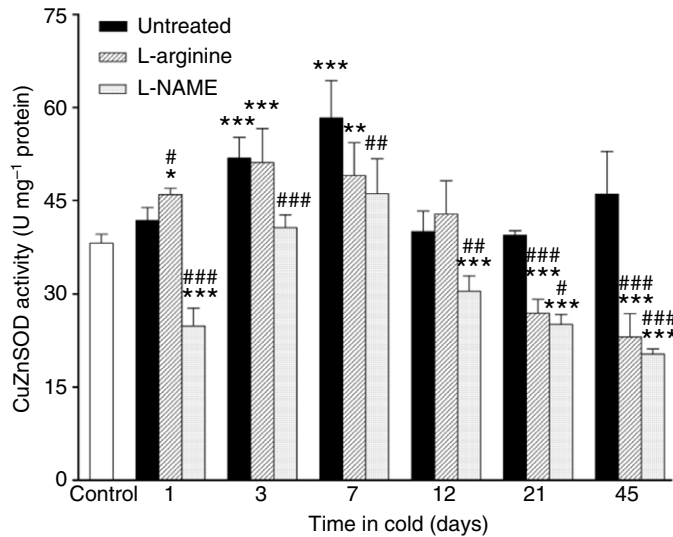


Fig. 1. The effect of cold-exposure ( $4\pm 1^\circ\text{C}$ ) duration on copper, zinc superoxide dismutase (CuZnSOD) activity in the skeletal muscle of untreated, L-arginine-treated and *N*<sup>o</sup>-nitro-L-arginine methyl ester (L-NAME)-treated animals. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  compared with the control kept at room temperature ( $22\pm 1^\circ\text{C}$ ). # $P<0.05$ , ## $P<0.01$ , ### $P<0.001$  compared with the untreated group at the same time of cold acclimation.

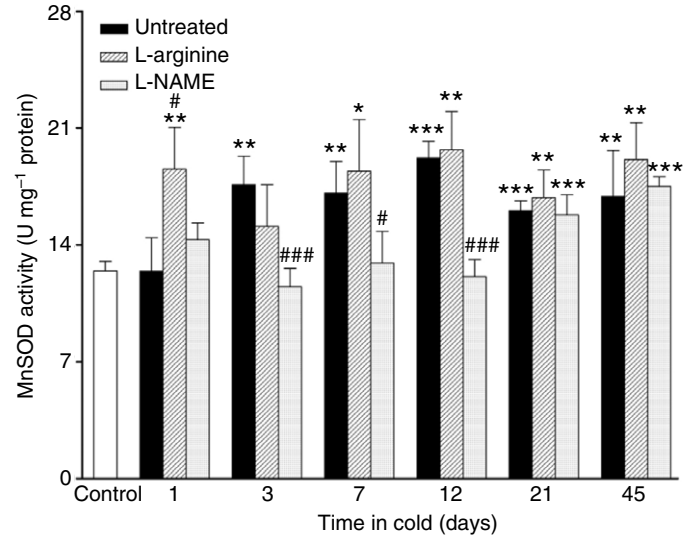


Fig. 2. Time course of changes in manganese superoxide dismutase (MnSOD) activity in skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during early and late acclimation to cold ( $4\pm 1^\circ\text{C}$ ). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  compared with the control maintained at room temperature ( $22\pm 1^\circ\text{C}$ ). # $P<0.05$ , ## $P<0.01$ , ### $P<0.001$  compared with the untreated group at the same time of cold acclimation.

during early cold exposure starting from day 3, which returned to the control level during late cold acclimation. On the other hand, in the L-arginine-treated group the activity of this enzyme increased from day 1 ( $P<0.05$ ) in the early phase, but decreased below the control level after day 21 ( $P<0.001$ ) of the late cold exposure, compared with both the control and the untreated group. In L-NAME-treated animals, CuZnSOD activity was decreased on day 1 of early cold exposure ( $P<0.001$ ) and remained decreased until the end of cold exposure in comparison with the control and untreated group.

Changes in MnSOD activity during cold acclimation are depicted in Fig. 2. As shown, in the untreated group, MnSOD activity increased from day 3 ( $P<0.01$ ), while in L-arginine-treated rats, MnSOD activity was increased on day 1 of early cold exposure compared with the control ( $P<0.01$ ) and untreated group ( $P<0.05$ ). During the late cold acclimation, increase in MnSOD activity achieved early was sustained in the untreated group and was unaffected by L-arginine, related to the untreated group. L-NAME treatment decreased MnSOD activity from day 3 to day 12 compared with the untreated group, and delayed the cold-induced increase in MnSOD activity on day 21 of late cold-acclimation ( $P<0.001$ ).

CAT activity (Fig. 3) progressively increased in all three cold-acclimated groups with the highest increase on day 7 ( $P<0.001$ ). A higher CAT activity was observed on day 3 of the early phase of acclimation in both untreated ( $P<0.05$ ) and L-NAME-treated rats ( $P<0.001$ ), while increased activity of this enzyme was recorded as early as day 1 of exposure to cold in L-arginine-treated animals ( $P<0.05$ ) compared with the control ( $P<0.05$ ) and untreated group ( $P<0.05$ ). CAT activity remained elevated during the whole of the late cold acclimation in both untreated and L-arginine-treated rats, while in the L-NAME-treated group it returned to control values on day 21 and decreased compared with the untreated group at days 21 ( $P<0.01$ ) and 45 ( $P<0.001$ ) of late cold acclimation.

Time-dependent changes of GSH-Px activity are shown in Fig. 4. Similar time-course changes in the activity of this enzyme were observed in all three cold-acclimated groups, during both the early and late phases, with the highest increase seen on day 7 in untreated ( $P<0.001$ ) and L-NAME-treated animals ( $P<0.01$ ). However, in the L-arginine-treated group the maximal increase of GSH-Px activity was recorded on day 3 of early cold exposure and it was higher ( $P<0.05$ ) compared with that observed at the same

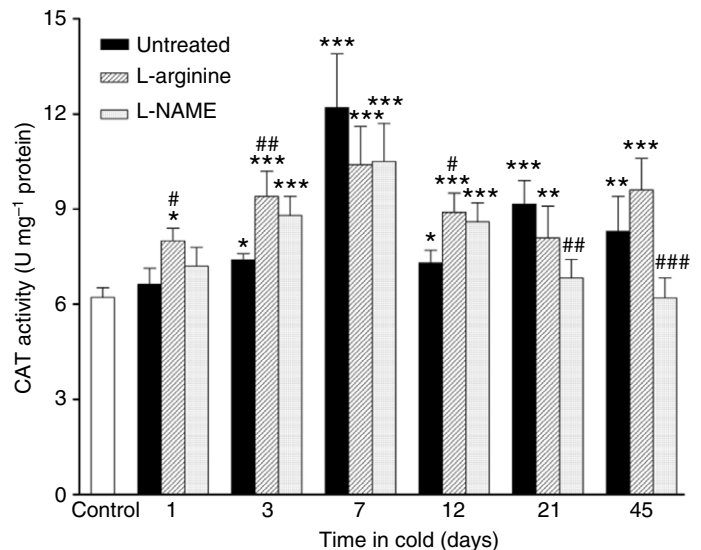


Fig. 3. Time-dependent changes in catalase (CAT) activity in the skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats during acclimation to cold ( $4\pm 1^\circ\text{C}$ ). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  compared with the control acclimated to room temperature ( $22\pm 1^\circ\text{C}$ ). # $P<0.05$ , ## $P<0.01$ , ### $P<0.001$  compared with the untreated group at the same time of cold acclimation.

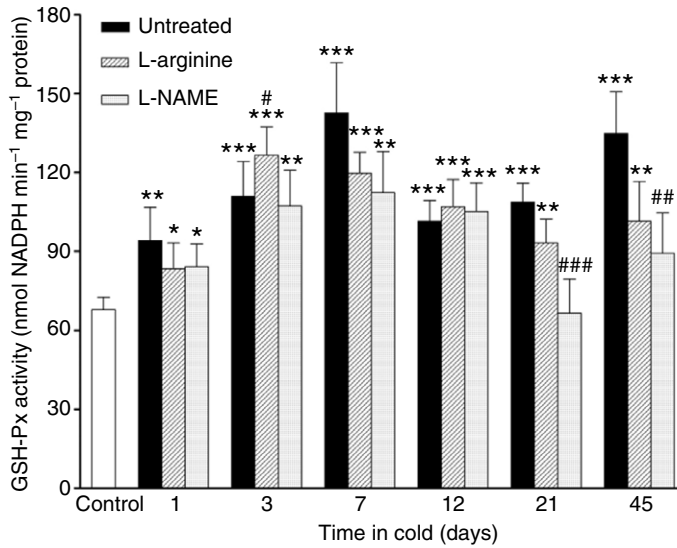


Fig. 4. Time course of changes in glutathione peroxidase (GSH-Px) activity in the skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during exposure to cold ( $4\pm 1^\circ\text{C}$ ).  $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$  compared with the control maintained at room temperature ( $22\pm 1^\circ\text{C}$ ).  $\#P<0.05$ ,  $\#\#P<0.01$ ,  $\#\#\#P<0.001$  compared with the untreated group at the same time of cold acclimation.

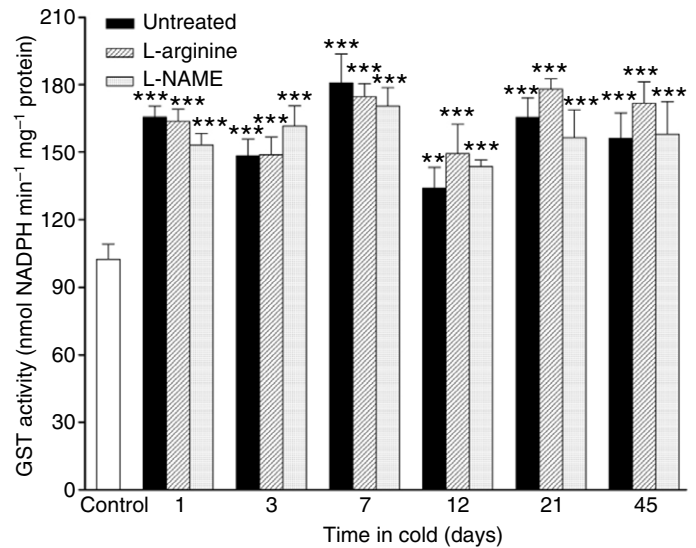


Fig. 5. The effect of short and prolonged cold exposure ( $4\pm 1^\circ\text{C}$ ) on glutathione S-transferase (GST) activity in the skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats.  $**P<0.01$ ,  $***P<0.001$  compared with the control acclimated to room temperature ( $22\pm 1^\circ\text{C}$ ).

time in the untreated group. The increase in GSH-Px activity originated in the early cold acclimation remained during the whole late cold acclimation in untreated and L-arginine-treated groups, while it returned to the control level on day 21 in L-NAME-treated rats and stayed below values observed in the untreated group at days 21 ( $P<0.001$ ) and 45 ( $P<0.01$ ).

As shown, L-arginine in early cold acclimation accelerated the cold-induced increase in MnSOD, CuZnSOD and CAT activity, while in the late phase it sustained increases achieved in the early period. L-NAME affected both early and late acclimation through attenuation and decreases in the AD response. That is, L-NAME treatment postponed the cold-induced increase of MnSOD activity and reversed CAT and GSH-Px activities to the control level on day 21 of late cold acclimation, and decreased CuZnSOD activity during the whole acclimation period. In contrast, neither L-arginine nor L-NAME changed GR and GST activities, which increased from day 1 of cold acclimation and stayed unchanged until the end of the experiment.

From Fig. 5 it can be seen that GST activity was increased in the untreated group on day 1 of early cold exposure ( $P<0.001$ ) and remained elevated for the whole duration of the experiment compared with the control, with the maximum reached on day 7 of cold exposure ( $P<0.001$ ). Both L-arginine and L-NAME failed to affect changes in GST activity seen in the untreated group.

GR activity (Fig. 6) showed time-dependent changes similar to those of GST activity, i.e. it was increased on day 1 of the early phase in all examined groups and remained elevated during the whole period of both early and late cold acclimation, with the maximum increase on day 7 ( $P<0.001$ ).

GSH content in the gastrocnemius (Fig. 7) was decreased from day 1 of early cold exposure in all examined groups, and remained decreased during the whole period of cold acclimation in L-NAME-treated rats in relation to the control. However, in the untreated group the GSH content was restored to the control level on day 45 of late cold acclimation, while in the L-arginine-treated group it

restitution was observed on day 3 of early cold acclimation and remained unchanged until the end of the experiment.

## DISCUSSION

In the present study, clear phase-dependent changes were seen only in CuZnSOD activity, which was increased in early cold acclimation (1–7 days) but returned to control in late acclimation (12–45 days). In contrast, there were no phase-dependent changes in MnSOD activity and the peroxidative- and GSH-related part of AD – CAT, GSH-Px, GR and GST – the activities of which were

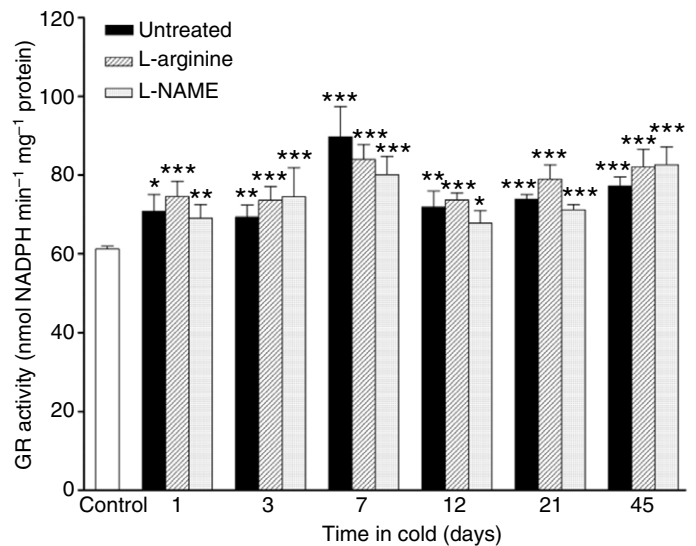


Fig. 6. Changes in glutathione reductase (GR) activity in the skeletal muscle of untreated, L-arginine-treated and L-NAME-treated animals during early and late cold acclimation ( $4\pm 1^\circ\text{C}$ ).  $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$  compared with the control kept at room temperature ( $22\pm 1^\circ\text{C}$ ).

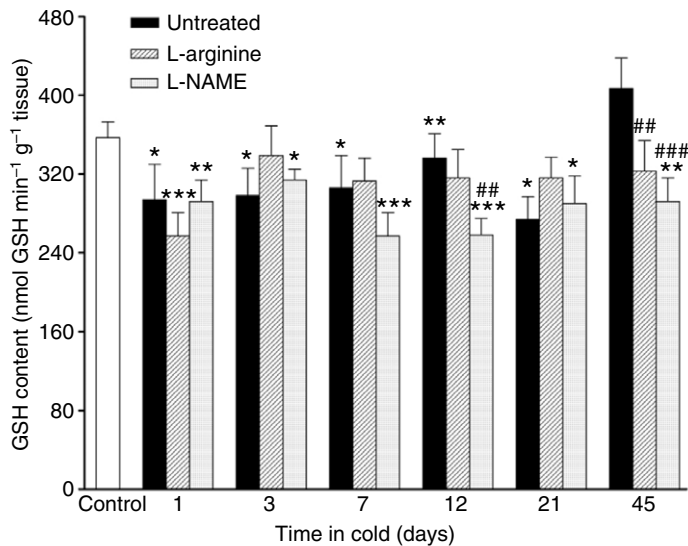


Fig. 7. Changes in GSH content in the skeletal muscle of untreated, L-arginine-treated and L-NAME-treated rats after different time periods of cold exposure ( $4\pm 1^\circ\text{C}$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with the control acclimated to room temperature ( $22\pm 1^\circ\text{C}$ ). ## $P < 0.01$ ; ### $P < 0.001$  compared with the untreated group at the same time of cold acclimation.

increased during the whole cold exposure, owing to their functional engagement in both phases of thermogenesis. L-Arginine treatment exerted effects on early cold acclimation by acceleration of the cold-induced AD response and in the late phase it sustained increases achieved in the early period. In contrast, L-NAME attenuated and decreased the cold-induced skeletal muscle AD response in both early and late acclimation. The effects that these treatments exhibited on AD in both phases of thermogenesis are discussed in more detail below.

In the course of the early period of cold exposure, shivering thermogenesis is activated. During this time, to overcome cold, nerve stimulation induces permanent skeletal muscle contractions and relaxation. This period may therefore be seen as muscular training, which is known to increase the rate of oxygen consumption (Wickler, 1981) and the proportion of the  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  formed from oxygen utilized by mitochondria (Vasilaki et al., 2006). Accordingly, increases in MnSOD and CuZnSOD as well as CAT and GSH-Px activities observed in the untreated group during early cold acclimation could be explained as a response of these enzymes to an increased production of their substrates and are in line with several reports related to exercise-improved AD (Higuchi et al., 1985; Pansarasa et al., 2002). Supplementation with the physiological substrate for NOSs, L-arginine, in the early phase resulted in an acceleration of the increase in cold-induced AD enzymes (MnSOD, CuZnSOD, CAT and GSH-Px) activities, and restoration of GSH level compared with the untreated group. In contrast, L-NAME, the non-selective NOSs inhibitor, postponed the cold-induced increase in MnSOD activity seen in the untreated group in the early phase to day 21 of late cold exposure and decreased CuZnSOD activity during the acclimation to cold. It was found that L-arginine enhanced exercise-induced NOSs activation and NO production, which improves aerobic capacity (Maxwell et al., 2001), mechanical and metabolic muscle capability (Maréchal and Gailly, 1999; Ohta et al., 2007) and increases glucose oxidation (Fu et al., 2005). In contrast, L-NAME, through inhibition of NOSs and a decrease in

NO availability, diminished skeletal muscle contractions, as well as glucose uptake and oxidation (Roberts et al., 1997). Thus, the accelerated increase in MnSOD, CuZnSOD and CAT activities observed here in L-arginine-treated rats during the early acclimation to cold could be connected to improved skeletal muscle contraction and oxidative capacity by NO and, consequently, to  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  production.

However, after a period of 7 (Cannon and Nedergaard, 2004) or 10 days (Peralta et al., 2003) on cold, the impact of shivering decreases and cannot account for thermogenesis (Griggio, 1982), while NST markedly increases. During prolonged exposure to cold, despite decreased shivering, skeletal muscle retains an enhanced capacity for aerobic support of energy metabolism (although, less than in shivering), i.e. working as a supportive tissue to supply brown adipose tissue with oxygen, contributing to the overall acclimation to cold (Wickler, 1981; Wibom et al., 1992). In our study, after 7 days, CuZnSOD activity in the untreated group was restored to the control level, while MnSOD activity remained increased during the whole acclimation period. The different responses of SOD isoforms seen after early cold acclimation could be connected to their different subcellular organization; MnSOD is compartmentalized in the mitochondrial inner membrane, whereas CuZnSOD is found mainly in cytosol. Thus, it might be proposed that in the period when shivering ceases, production of  $\text{O}_2^-$  is such that mitochondrial MnSOD is sufficient to cope with it and to prevent an increase of  $\text{O}_2^-$  in the cytosol and a competent response in CuZnSOD activity.

The observed changes in enzyme activities during cold acclimation could be interpreted in terms of different tissue conditions characterized by different fuel utilization. It is known that maximal utilization of glucose by skeletal muscle is associated with acute cold exposure, while in long-term cold acclimation a marked increase in the utilization of lipid substrates occurs (Vallerand et al., 1990). Accordingly, a conspicuous increase of CAT and GSH-Px activities recorded here from day 7 of cold acclimation could be attributed to increased  $\beta$ -oxidation of fatty acids and, connected to that, increased  $\text{H}_2\text{O}_2$  production. These data are in line with studies on various tissues that reported increases in CAT and GSH-Px activity during cold exposure, as a response to increased peroxidative pressure (Alptekin et al., 1996; Buzadžić et al., 1997; Selman et al., 2000).

L-arginine treatment sustained the shown increases in AD achieved in the early period until the end of cold acclimation. In contrast, L-NAME treatment postponed the cold-induced increase of MnSOD activity and reversed CAT and GSH-Px activities to the control level on day 21 of late cold exposure. These data showing attenuated and delayed cold-induced responses in AD components by L-NAME treatment in late cold acclimation strongly indicate the involvement of the L-arginine/NO pathway in the modulation of skeletal muscle AD. At this stage, considering our data, we cannot say how L-arginine/NO affects skeletal muscle AD. Fu et al. (Fu et al., 2005) showed that L-arginine, by increasing NOSs-derived NO production, enhances expression of the genes responsible for fatty acid oxidation and, thereby,  $\text{H}_2\text{O}_2$  production. In contrast, Nagase et al. (Nagase et al., 1997) showed that incubation of L-arginine under peroxidative conditions leads to the non-enzymatic production of NO-dependent species, which are known to increase production of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  (Navarro et al., 2005). Peralta et al. (Peralta et al., 2003) reported that L-arginine/NO, by mitochondrial NOS (mtNOS) activation, in early cold acclimation increased  $\text{O}_2^-$  uptake but significantly decreased it at day 24 of late cold exposure via inhibition of cytochrome *c* oxidase and an increase in  $\text{O}_2^-$

production. Additionally, these authors emphasized that in skeletal muscle the major NOS isoform that participates in the response to cold acclimation is mtNOS, which in this tissue was described varyingly as post-translationally modified nNOS (Elfering et al., 2002) or as eNOS (Punkt et al., 2006). In the present study, restoration of GSH level was seen in the L-arginine-treated group at day 3 and in the untreated group at day 45, while in the L-NAME-treated group it stayed below the control level during the whole period of cold acclimation. Thus, it could be hypothesized that L-arginine, i.e. NO, acted by restoring the GSH amount to the control level. This hypothesis is based on the results of Moellering et al. (Moellering et al., 1998), who reported induction of GSH synthesis in skeletal muscle as well as in endothelial cells (Moellering et al., 1999) in response to NO via the activation of  $\gamma$ -glutamyl cysteine synthetase. Further studies along these lines are necessary for clarification of the precise mechanisms involved. The data presented here demonstrating that the cold-induced AD response is improved by L-arginine but attenuated by L-NAME clearly showed opposing effects of L-arginine and L-NAME on the modulation of AD in skeletal muscle. However, there are several reports, with different model systems, concerning the same effect of L-NAME treatment as that seen after L-arginine treatment. Conflicting observations in experiments with the NOSs inhibitor L-NAME are explained by non-enzymatic production of NO during incubation with L-NAME in the presence of NADPH, GSH and ascorbate (Moroz et al., 1998). Henningson et al. (Henningson et al., 2000) showed that chronic L-NAME treatment in diabetic rats blocked constitutive NOSs that evoked iNOS-derived NO production to compensate for NO level, while Krippel-Drews et al. (Krippel-Drews et al., 1996) reported that L-NAME can also act directly, not via intracellular messengers. We have recently reported that L-arginine and L-NAME affect pancreatic AD in the same manner (Vasiljević et al., 2007). Thus, tissue-specific effects of these two NO-manipulating agents could be proposed.

The presented results clearly show improvement of skeletal muscle AD in both early and late cold acclimation, indicating intensive oxidative metabolism in this tissue during both shivering and NST. Moreover, L-arginine in early cold acclimation accelerated the cold-induced AD response, while in the late phase it simply sustained increases achieved in the early period. L-NAME exerted effects on both early and late acclimation through attenuation and a decrease in the AD response. These data strongly suggest the involvement of the L-arginine/NO pathway in the modulation, i.e. the improvement of skeletal muscle AD during cold acclimation. However, in order to understand the precise mechanisms, additional studies, primarily related to the determination of the relative contributions of the L-arginine-dependent and NOSs-dependent effects, are needed. Our efforts along these lines are in progress.

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