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Original Contribution

EFFECT OF LONG-TERM COLD EXPOSURE ON ANTIOXIDANT ENZYME ACTIVITIES IN A SMALL MAMMAL

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Abstract—Aerobic organisms continually face exposure to reactive oxygen species (ROS) and many have evolved sophisticated antioxidant systems to effectively remove them. Any increase in ROS production or weakening in this defense system may ultimately lead to oxidative stress and cellular damage. We investigated whether long-term cold exposure, which is known to lead to an elevation in metabolic rate, increased the activities of the ROS-scavenging enzymes, catalase (CAT), selenium-dependent glutathione peroxidase (GPx), and total superoxide dismutase (Total-SOD) in liver, cardiac muscle, kidney, skeletal muscle (*vastus lateralis*), and duodenum of short-tailed field voles (*Microtus agrestis*), born and maintained at either 8 ± 3°C or 22 ± 3°C. CAT, GPx, and Total-SOD activities were determined at age 61 ± 1.9 days. An increase in CAT activity in voles maintained at 8 ± 3°C was observed in skeletal muscle (71%) and kidney (20%), with both CAT and GPx activities significantly elevated (by 40 and 43%, respectively) in cardiac muscle, when compared to voles at 22 ± 3°C. Total-SOD activity and protein content did not differ significantly between groups in any tissue. We suggest that the compensatory increases in CAT (skeletal muscle, cardiac muscle, kidney) and GPx (cardiac muscle), but not Total-SOD activities, resulting from long-term cold exposure may reflect the elevated metabolic rate, and possibly also increased ROS production, at this time. © 2000 Elsevier Science Inc.

Keywords—Cold acclimation, Free radicals, Catalase, Glutathione peroxidase, Superoxide dismutase, Short-tailed field vole, Microtus agrestis

INTRODUCTION

The continuous production of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$), are an inevitable consequence of utilizing inspired molecular oxygen for oxidative phosphorylation [1]. It has been widely hypothesized that the production of ROS, localized particularly around mitochondria (the major site of oxygen consumption and ROS production), results in lipid peroxidation, protein oxidation, and nucleic acid damage [1–4]. The potential of ROS to damage macromolecules has led to their production being linked to senescence and the degenerative diseases associated with aging, e.g., heart disease, Parkinson's disease, diabetes mellitus, and mitochondrial diseases [5–8]. This linkage between ROS production and senescence forms the basis of Harman's free radical theory of aging [9].

Since ROS are produced continuously during aerobic respiration, organisms utilize a sophisticated suite of antioxidants (vitamins C and E, glutathione, ubiquinol, and β-carotene) and have evolved antioxidant-scavenging enzymes (catalase, glutathione peroxidase, and superoxide dismutase) to convert ROS into less reactive species [1,10]. While the nature of these antioxidants differ among species, their presence appears universal in aerobic organisms [1]. The antioxidant system, however, is not completely effective, with around 2–5% of the ROS produced escaping conversion [10], and it is this small percentage that appears to be the causative factor in the cellular physiological attrition, which accompanies senescence [3,11].

It has been recognized since the early twentieth century [12] that within particular groups of animals, species with higher metabolic rates per unit mass tend to have shorter maximum life spans [13], although this general

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linkage breaks down when comparisons are made across groups. For example, birds combine high metabolic rates with long lives [14] and marsupials combine low metabolic rates with short lives [15]. Within species, a direct link appears to exist between an individual's energy expenditure and probability of mortality, with an experimentally elevated energy expenditure being linked with a reduction in lifespan in kestrels *Falco tinnunculus* [16], zebra finches *Taeniopygia guttata* [17], honeybees *Apis mellifera* [18], and houseflies *Musca domestica* [19] when compared to controls. In addition, long-lived *Caenorhabditis elegans* mutants have reduced metabolic rates compared to control strains [20]. This inverse relationship between metabolic rate and lifespan is commonly termed Pearl's rate of living theory [21] and is consistent with the free radical theory of aging [9], because it suggests increases in metabolic rate are accompanied by increased ROS production [4]. It appears, however, that in addition to lower mass-specific metabolic rates, species with long maximum lifespans also have lower levels of in vivo ROS production and higher cellular resistance to oxidative stress. They do not sustain elevated cellular concentrations of antioxidant enzymes [22–24], suggesting the link of metabolic rate to longevity is not straightforward.

Almost all studies examining the relationship of metabolic rate, ROS production, and antioxidant enzyme activity have experimentally elevated metabolic rate through physical activity [4,25,26]. It is now well established that strenuous or exhaustive exercise increases the rate of ROS production [25,26], leading to an increase in lipid peroxidation in skeletal muscle [25]. However, the effects of exercise on the antioxidant enzyme system are controversial [10], with changes in antioxidant enzymes during exercise appearing to counteract increased ROS production in some tissues, particularly skeletal muscle, but changes are less prominent in other tissues such as the liver and heart [26].

Long-term cold exposure elevates metabolic rate in mammals and leads to increased hypertrophy of metabolically active tissues [27] such as brown adipose tissue (BAT), liver, kidney, small intestine, and heart [28,29]. Increases in mitochondrial volume density, capillary diameter, aerobic enzyme activity (e.g., succinate and malic dehydrogenase, cytochrome dehydrogenase), and tissue oxygen consumption, particularly in skeletal muscle and liver, are also associated with long-term cold exposure [30–33]. Several studies have investigated whether exposure to low temperatures results in compensatory changes taking place in the antioxidant defense system. Acute cold stress, through immersion in cold water, in two mouse strains significantly decreased blood glutathione (GSH) levels and induced perturbation of GSH metabolism in several visceral organs [34]. Similarly, plasma and liver GSH decreased in cold-adapted rats, although GSH in BAT GSH increased significantly [35]. Voluntary short-term cold exposure by ice-bathing in humans elicited elevated activities of erythrocytic superoxide dismutase (SOD), catalase (CAT), and baseline concentrations of GSH when compared to controls [36]. Increased levels of SOD, CAT, Total and Se-dependent glutathione peroxidase (GPx), and glutathione reductase levels were observed in BAT of cold-acclimated rats *Rattus norvegicus* [37] when compared to controls. However, cold acclimation increased BAT protein content in these rats and the differences between cold-acclimated rats and controls were not significant when enzyme activities were expressed per unit of protein. A significantly increased erythrocyte SOD and an increased GPx activity has also been observed in cold-acclimated rats; however, short-term cold acclimation only resulted in a slight rise in erythrocyte SOD activity but a significant decrease in GPx activity [38]. Additional studies using rat models [39,40] have further indicated that the effects of cold exposure on the antioxidant defense system appear to be both tissue specific and dependent on the duration of the cold exposure experienced.

The aims of this study were to examine whether long-term cold exposure, leading to an increase in the metabolic rate of a small mammal, could induce compensatory changes in the ROS-scavenging enzymes CAT, GPx, or SOD measured in various metabolically active lean tissues. Because antioxidant enzymes scavenge ROS, any alteration in their activity may indirectly indicate changes in ROS generation. The short-tailed field vole, *Microtus agrestis*, was an ideal species to use for this study because previous work in our laboratory has shown that long-term cold exposure leads to significant increases in oxygen consumption when measured at controlled temperatures of 25°C, 20°C, and 10°C (by over 50%), body mass, food intake, and in the dry weight of a variety of morphological parameters when compared to controls not exposed to low temperatures [41]. Short-tailed field voles also regulate their body temperature continuously, i.e., they do not exhibit torpor or hibernate, at low ambient temperatures [42].

MATERIALS AND METHODS

Animals

Two breeding populations of short-tailed field voles *Microtus agrestis* were kept in captivity at Aberdeen, UK at either 8 ± 3°C (cold group) or 22 ± 3°C (warm group), which lies slightly below the lower critical temperature (25–30°C) of this species [27]. Fourteen pups (7 males and 7 females) born and maintained at either 8 ±

Table 1. Mean Percentage Change in Antioxidant Enzyme Activities and Protein Content in Cold-Acclimated Voles (8 ± 3°C), as Percentage of Baseline Activities Measured in Warm-Acclimated Individuals (22 ± 3°C), and Mean Absolute Values (WARM ± SEM–COLD ± SEM)

Tissue	CAT (U)	GPx (U)	Total-SOD (U)	Protein content
	+71%	+25%	+1%	−3%
Skeletal muscle	281 (± 43)–481 (± 78)	1.1 (± 0.1)–1.3 (± 0.1)	160 (± 8)–162 (± 14)	3.5 (± 0.1)–3.4 (± 0.1)
	+40%	+43%	+22%	−10%
Heart	911 (± 114)–1273 (± 86)	3.6 (± 0.4)–5.1 (± 0.5)	175 (± 15)–202 (± 19)	3.2 (± 0.2)–2.9 (± 0.1)
	+20%	+3%	−0.3%	+1%
Kidney	1245 (± 67)–1491 (± 73)	8.9 (± 0.5)–9.2 (± 0.9)	218 (± 18)–217 (± 13)	3.3 (± 0.1)–3.3 (± 0.1)
	+6%	+4%	+3%	−0.2%
Liver	928 (± 56)–980 (± 74)	11.8 (± 0.7)–12.3 (± 0.9)	202 (± 13)–207 (± 18)	3.8 (± 0.1)–3.7 (± 0.1)
	+10%	+2%	+0.5%	+3%
Duodenum	572 (± 48)–629 (± 70)	0.89 (± 0.1)–0.90 (± 0.1)	266 (± 30)–268 (± 35)	2.5 (± 0.1)–2.5 (± 0.1)

+ indicates increase in activity; − indicates decrease in activity; CAT = catalase; GPx = selenium-dependent glutathione peroxidase; Total-SOD = total-superoxide dismutase; U = units of enzyme; protein content = mg protein ml^{-1}

3°C (cold group) or 22 ± 3°C (warm group) were used during this study. Individuals were weaned at day 18, individually housed in shoebox cages containing sawdust, and provided, ad libitum, with water and a pelleted rodent diet (rat and mouse number 1 maintenance diet; Special Diets Services, BP Nutrition, UK). Photoperiod was maintained at 16L: 8D.

Materials

All biochemical reagents (analytical grade) were purchased from Sigma Chemical Co. (Poole, UK).

Antioxidant enzyme assays

Voles were sacrificed by cervical dislocation, the appropriate tissues dissected, weighed, immersed in liquid N_2 generally within 60 s of death, and kept frozen at −70°C. Prior to enzyme determinations, thawed tissue samples were homogenized in 20 volumes of ice cold 50mM phosphate buffer (pH 7.4), centrifuged at 3200 × g for 20 min at 5°C. The supernatant fraction was used for antioxidant enzyme determinations.

Catalase activity of 1% Triton X-100–treated supernatant was determined at 20°C by the disappearance of H_2O_2 at 240 nm [43,44]. One unit of catalase represents the decrease of 1μmol of H_2O_2 per minute. The Se-dependent isozyme of glutathione peroxidase (Se-GPx) was assayed at 25°C [44,45], where NADPH oxidation was followed spectrophotometrically at 340 nm in the presence of reduced glutathione (GSH) and H_2O_2 [45]. To correct for spontaneous reactions in the absence of enzyme, blanks were run without sample and then subtracted from the assay values [44]. One unit of GPx is defined as the amount of enzyme that oxidizes 1μmol of NADPH per minute. Total superoxide dismutase (Total-SOD) activity was assayed by the inhibition, at 25°C, of pyrogallol autoxidation by SOD (with and without sample) and was followed kinetically at 420 nm [46]. One unit of SOD is defined as the amount of enzyme that causes 50% inhibition of pyrogallol autoxidation. Changes in absorbance were read on a SPECTRAmax Plus microplate spectrophotometer (Molecular Devices Corp. Sunnyvale, CA, USA) and analyzed using SOFTmax Pro software (Molecular Devices Corp.). All enzyme activities are expressed per mg protein. Protein content for separate tissue samples was determined using the method of Lowry et al. [47].

Statistical analyses

All values reported are mean ± SEM, except where indicated. Data were analyzed using Student's *t*-test, using SPSS (Version 8) statistical software. Significance was considered at p values $< .05$.

RESULTS

Antioxidant enzymes

We determined whether cold acclimation elevated specific activities of catalase (CAT), selenium-dependent glutathione peroxidase (GPx), and total superoxide dismutase (Total-SOD). Antioxidant enzyme levels were determined in liver, cardiac muscle, kidney, skeletal muscle (*vastus lateralis*), and duodenum.

Catalase activity

Levels of CAT in cold-acclimated voles were elevated in all tissues (Table 1) when compared to the warm-acclimated individuals (Fig. 1). Significant increases in CAT activity of 71%, 40%, and 20% compared to warm-acclimated (control) levels were observed in the skeletal muscle ($t_{26} = 2.26$, $p = .03$), cardiac muscle ($t_{26} = 2.56$, $p = .02$), and kidney ($t_{26} = 2.48$, $p = .02$), respectively.

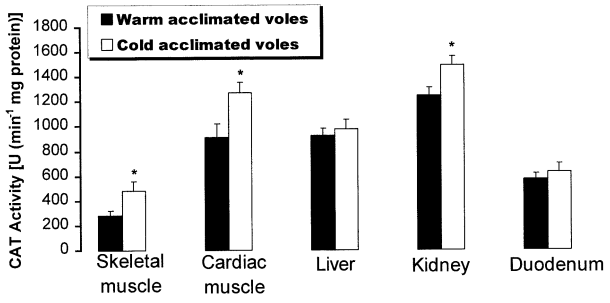

Fig. 1. Catalase activities in skeletal muscle (*vastus lateralis*), heart, liver kidney, and duodenum of cold- and warm-acclimated short-tailed field voles, *Microtus agrestis*. All results are expressed in international Bergmeyer units (U) and are mean ± SEM ($n = 14$ in each group). One unit of catalase represents the decrease of 1 μmol of H_2O_2 per minute at 240 nm [43]. Asterisks denote significant differences in activity between cold-acclimated compared to warm-acclimated voles. *$p <$ .05.

Selenium-dependent glutathione peroxidase activity

As with CAT, GPx activities were higher in all tissues of cold-acclimated voles when compared to controls (Table 1 and Fig. 2). However, a significant increase (43%) in GPx activity was measured only in the cardiac muscle ($t_{26} = 2.31$, $p = .03$).

Total superoxide dismutase activity

Total-SOD activity of cold-acclimated voles showed only very small increases in activity when compared to controls (Table 1 and Fig. 3). Cardiac muscle had the highest increase in Total-SOD activity (22%) and this approached significance ($t_{24} = 1.86$, $p = .076$), but the changes in other tissues were not significant.

Tissue protein content

The differences seen in CAT and GPx activities in the tissues of cold- and warm-acclimated voles were not explained by differences occurring in tissue protein content (Fig. 4). Protein content did not differ between groups in either skeletal muscle ($t_{26} = 0.85$, $p = .40$), cardiac muscle ($t_{26} = 1.76$, $p = .09$), kidney ($t_{26} = 0.20$, $p = .84$), liver ($t_{26} = 0.07$, $p = .95$), or duodenum ($t_{26} = 0.65$, $p = .52$).

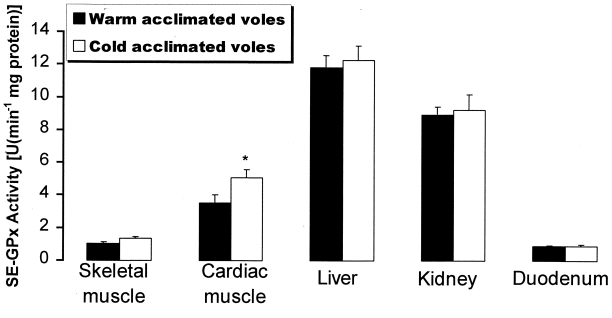

Fig. 2. Selenium-dependent glutathione peroxidase (GPx) activities in skeletal muscle (*vastus lateralis*), heart, liver, kidney, and duodenum of cold- and warm-acclimated short-tailed field voles, *Microtus agrestis*. Mean ± SEM ($n = 14$ in each group). One unit of GPx is defined as the amount of enzyme that oxidizes 1 μmol of NADPH per minute at 340 nm in the presence of reduced glutathione [45]. *$p <$ .05.

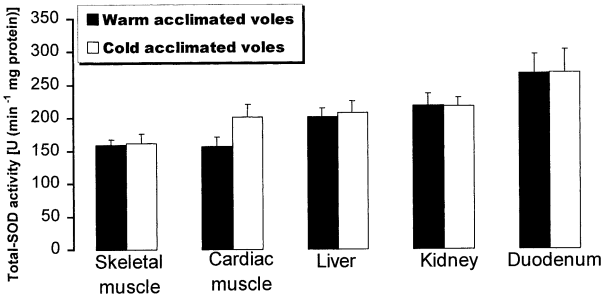

Fig. 3. Total superoxide dismutase (Total-SOD) activity in cold- and warm-acclimated voles. One unit of SOD is defined as the amount of enzyme that causes 50% inhibition of pyrogallol autoxidation at 420 nm [46]. No significant differences were determined in Total-SOD activity in any of the tissues studied.

DISCUSSION

This study was conducted to investigate whether long-term cold acclimation, resulting in an increased metabolic rate, could elicit changes in the antioxidant enzyme status of the short-tailed field vole. Cold exposure leads to an elevation in metabolic rate by over 50% when compared to control individuals [28]. Long-term exposure to cold temperatures is also known to significantly increase both aerobic enzyme activities and tissue oxygen consumption in various rodent models [32,33], although its effect on the antioxidant defense system appears less straightforward [34,35,37–40]. In this study, an enhancement of skeletal muscle, cardiac muscle and kidney CAT, and cardiac muscle GPx, but not Total-SOD, activities in cold-acclimated voles was observed when compared to control (warm-acclimated) individuals.

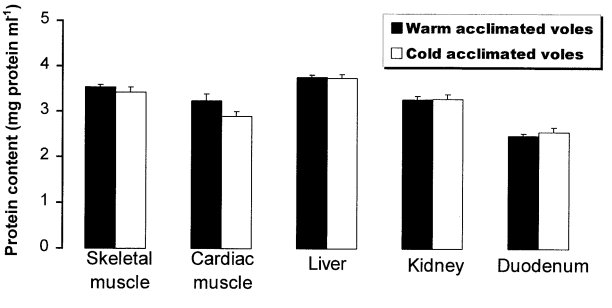

Fig. 4. Effect of cold acclimation on protein content of skeletal muscle (*vastus lateralis*), heart, liver, kidney, and duodenum. Values are mean ± SEM ($n = 14$ in each group).

Total-SOD activity showed relatively little intertissue differences in our study, which is consistent with other published data [10], despite such tissues varying considerably in mass-specific metabolic rate [27]. While heart, skeletal muscle, and erythrocyte SOD activity increases after acute bouts of exhaustive exercise, albeit in some but not all studies [48–51], little effect on basal SOD levels, except in Mn-SOD, have been observed after activity of moderate intensity or short duration [26]. The suggested reason for this is that the basal levels of SOD are sufficient to reduce the superoxide ion to H_2O_2 during moderate oxidative stress [10,26]. Therefore, during long-term cold acclimation, Total-SOD basal levels in the tissues studied, with the possible exception of cardiac muscle, may have been sufficient to cope with any increase in ROS generation. Generally, however, mammalian cardiac muscle exhibits relatively low basal SOD activity, despite a relatively high specific metabolic rate [27] and potential for ROS generation, thus making it highly susceptible to ROS damage [51]. This may be why an increase, approaching significance, in Total-SOD was observed only in cardiac muscle of cold-acclimated voles.

The effect of exercise on antioxidant enzyme levels appears to depend on both basal tissue antioxidant capacity and on the specific locations where ROS are produced [52]. As with cardiac muscle, skeletal muscles generally exhibit relatively low antioxidant enzyme activities, but also contain postmitotic cells that accumulate ROS damage over time [49] and account for, in mammals, a large share of total organismal oxygen consumption [51]. This may result also in skeletal muscles being particularly prone to oxidative damage. It has been postulated that while basal levels of SOD in most tissues appear sufficient to cope with moderate oxidative stress [10], the factor that determines the rate of tissue damage by ROS is the successful removal of peroxides such as H_2O_2 [26]. This may be why, during cold acclimation in this study, increases specifically in CAT and to a lesser extent GPx activities were observed. Very little is known about the mechanisms involved in upregulation of the antioxidant enzyme system, although regulation may occur in response to the intramitochondrial environment [26], with induced oxidative stress through H_2O_2 exposure upregulating CAT and other peroxidases via an oxyR gene [53].

The increases seen in skeletal muscle CAT and GPx activity were unlikely to be in response to shivering thermogenesis, as shivering appears important only as a short-term response to cold exposure [33,54], although we cannot discount that previous shivering episodes in these animals did not lead to antioxidant enzyme induction. However, unlike other studies [37,39,40], the animals in our experiment were born at and maintained in the cold, therefore may not have been so reliant on shivering thermogenesis, which accompanies acute cold exposure [33], to maintain body temperature. Previously, however, our laboratory [55] has shown that cold exposure in short-tailed field voles increases voluntary activity on running wheels by 2–4 times, when compared to controls. Therefore, we cannot discount that the elevations in CAT and GPx during cold exposure, in skeletal and cardiac muscle, do not reflect increased locomotory at this time. However, the voles in the current experiment did not have access to wheels and as a result their activity may have been lower.

Various studies have examined the relationship between cold exposure and the antioxidant defense system [34–40]. What is apparent from this body of work is that the effect of cold exposure on this antioxidant system is both tissue specific [35] and appears highly dependent on the extent of the cold exposure regime encountered [35, 39]. This study is the first to examine this relationship using animals bred at and then maintained at both a warm (control) and a cold environmental temperature. The results of the study indicate that long-term cold exposure in short-tailed field voles resulted in an upregulation in CAT and GPx activity, in various tissues, thereby possibly offering greater protection from ROS tissue damage. This suggests a dynamic equilibrium exists between oxygen consumption and ROS production, with different antioxidant enzymes and tissues reacting to the long-term oxidative stress.

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ABBREVIATIONS

BAT—brown adipose tissue
CAT—catalase
GSH—glutathione
ROS—reactive oxygen species
Se-GPx—selenium-dependent glutathione peroxidase
Total-SOD—total superoxide dismutase