Free Radical Biology and Medicine 65 (2013) 916–924

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

The impact of cold acclimation and hibernation on antioxidant defenses in the ground squirrel (*Spermophilus citellus*): An update

Milica Vucetic [a], Ana Stancic [a], Vesna Otasevic [a], Aleksandra Jankovic [a], Aleksandra Korac [b], Milica Markelic [b], Ksenija Velickovic [b], Igor Golic [b], Biljana Buzadzic [a], Kenneth B. Storey [c], Bato Korac [a,*]

[a] University of Belgrade, Institute for Biological Research "Sinisa Stankovic," Department of Physiology, Bulevar despota Stefana 142, 11060 Belgrade, Serbia
[b] University of Belgrade, Faculty of Biology, Center for Electron Microscopy, Belgrade, Serbia
[c] Carleton University, Department of Biology, Ottawa, Ontario, Canada

ARTICLE INFO

Article history:
Received 4 April 2013
Received in revised form
14 August 2013
Accepted 28 August 2013
Available online 4 September 2013

Keywords:
Hibernation
Cold acclimation
Antioxidant
SOD
CAT
GSH-Px

ABSTRACT

Any alteration in oxidative metabolism is coupled with a corresponding response by an antioxidant defense (AD) in appropriate subcellular compartments. Seasonal hibernators pass through circannual metabolic adaptations that allow them to either maintain euthermy (cold acclimation) or enter winter torpor with body temperature falling to low values. The present study aimed to investigate the corresponding pattern of AD enzyme protein expressions associated with these strategies in the main tissues involved in whole animal energy homeostasis: brown and white adipose tissues (BAT and WAT, respectively), liver, and skeletal muscle. European ground squirrels (*Spermophilus citellus*) were exposed to low temperature (4 ± 1 °C) and then divided into two groups: (1) animals fell into torpor (hibernating group) and (2) animals stayed active and euthermic for 1, 3, 7, 12, or 21 days (cold-exposed group). We examined the effects of cold acclimation and hibernation on the tissue-dependent protein expression of four enzymes which catalyze the two-step detoxification of superoxide to water: superoxide dismutase 1 and 2 (SOD 1 and 2), catalase (CAT), and glutathione peroxidase (GSH-Px). The results showed that hibernation induced an increase of AD enzyme protein expressions in BAT and skeletal muscle. However, AD enzyme contents in liver were largely unaffected during torpor. Under these conditions, different WAT depots responded by elevating the amounts of specific enzymes, as follows: SOD 1 in retroperitoneal WAT, GSH-Px in gonadal WAT, and CAT in subcutaneous WAT. Similar perturbations of AD enzymes contents were seen in all tissues during cold acclimation, often in a time-dependent manner. It can be concluded that BAT and muscle AD capacity undergo the most dramatic changes during both cold acclimation and hibernation, while liver is relatively unaffected by either condition. Additionally, this study provides a basis for further metabolic study that will illuminate the causes of these tissue-specific AD responses, particularly the novel finding of distinct responses by different WAT depots in hibernators.

Introduction

Oxidative metabolism of the cell is necessarily accompanied by production of reactive oxygen species (ROS). Maintenance of cellular homeostasis under conditions of increased ROS production is achieved by a proportional increase in endogenous antioxidant defense (AD) [1–3]. Cellular AD involves a wide range of enzymes and several nonenzymatic components [4]. The first line enzymes involved in AD include superoxide dismutase isoforms 1 and 2 (SODs, EC 1.15.1.1), glutathione peroxidase (GSH-Px, EC 1.11.1.19), and catalase (CAT, EC 1.11.1.6).

Compartmentalization of metabolic processes within cells leads to corresponding subcellular-specific responses by AD components [1,5]. Thus, mitochondria are equipped with the SOD 2 (also known as MnSOD) isoform that removes superoxide anion radicals ($O_2^{\bullet -}$) produced mainly by the respiratory chain or through the β-oxidation pathway [6,7]. The same function in the cytosol is performed by another SOD isoform—SOD 1 (also known as CuZnSOD) [7]. Both SODs catalyze the dismutation of $O_2^{\bullet -}$ by successive oxidation and reduction of a transition metal ion at the active site in a ping-pong-type mechanism with a remarkably high

Abbreviations: AD, antioxidant defense; BAT, brown adipose tissue; WAT, white adipose tissue; rpWAT, retroperitoneal WAT; gnWAT, gonadal WAT; scWAT, subcutaneous WAT; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide anion radical; H_2O_2, hydrogen peroxide

* Corresponding author. Fax: 381 11 2761 433.
E-mail address: koracb@ibiss.bg.ac.rs (B. Korac).

0891-5849/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.08.188

reaction rate [8]. The hydrogen peroxide (H_2O_2) produced is not a radical, but is still highly reactive, and so is promptly removed. This is accomplished, preferentially, by GSH-Px that is ubiquitously present in the mitochondria, cytosol, membrane fraction, and extracellular space [9]. It reduces fatty acid hydroperoxides and H_2O_2 to water, at the expense of glutathione. Besides the mitochondria and cytosol, intensive oxidative metabolism also takes place in peroxisomes [10]. There, fatty acid oxidation occurs by a mechanism that is somewhat distinct from that in mitochondria, and involves generation of H_2O_2 in the first reaction of the pathway. The major enzyme involved in the decomposition of H_2O_2 to H_2O in peroxisomes is CAT [11]. Altered function and subcellular localization of these AD enzymes are frequently associated with diseases and stress conditions [12].

In attempting to correlate AD with different energy requirements (i.e., metabolic modes), hibernating animals present a particularly interesting model. Namely, the metabolic plasticity that these animals show over the euthermia–torpor cycle is amazing [13,14]. It has been shown that seasonal hibernators, like ground squirrels, are characterized by torpor bouts lasting for several days during which body temperature is maintained below 10 °C with rate of oxygen consumption reduced to approximately 5% of resting level [15]. On the other hand, in regard to heat production, all mammals act similarly during cold acclimation [16–18]. This involves increased resting metabolism [19–22], increased respiratory capacity of brown adipose tissue (BAT) [23], and increased expression of the key element of energy dissipation—uncoupling protein 1 [18]. Numerous studies have examined the AD status that follows such metabolic reprogramming during energy-demanding thermogenic processes in BAT and skeletal muscle of nonhibernating rats [24–27]. Besides these two thermoeffector organs, liver and white adipose tissue (WAT) also play important roles in the metabolic aspects of cold acclimation by providing the fuel for heat generation [28,29], and adjusting their metabolism to current conditions and capabilities [29,30]. Our earlier study revealed changes in AD that paralleled metabolic reprogramming of rat WAT during cold acclimation [31]. On the other hand, the mode of metabolic recruitment and reorganization of AD during cold exposure in hibernators, that are believed to be continually primed for thermogenesis [32–34], is quite understudied.

During winter torpor, hibernators are characterized by a sharp decrease in body temperature and metabolic activity [35]. Earlier, we as well as others authors have investigated antioxidant enzyme activity in hibernators during winter torpor; increased activity of AD enzymes and the main regulators of AD enzyme protein expression, in BAT and some other tissues, had been shown [13,15,36–38]. This up-regulation of AD during hibernation was interpreted primarily as preconditioning, i.e., protection of the tissue against oxidative damage to come with the enormous increase in metabolic activity during arousal from torpor. However, contemporary viewpoints are somewhat extended and include cytoprotective effects of AD during hypothermic excursion, as well [14]. Indeed, it has been shown that BAT increases its metabolic activity despite the global suppression of metabolism by the whole animal, probably to meet the need for modification of thermoregulatory responses during deep torpor [15,16]. It is clear that AD status represents an indispensable part of this newly established homeostasis.

Taking all this into account we aimed to investigate changes occurring at the level of protein expression by enzymes involved in the first line of antioxidant defense (SOD 1, SOD 2, GSH-Px, and CAT) during both cold acclimation and hibernation in different ground squirrel tissues that are involved in the maintenance of whole body energy balance: BAT, liver, skeletal muscle, and retroperitoneal, gonadal, and subcutaneous WAT.

Materials and methods

Animals

The experimental protocol was approved by the ethical committee for the treatment of experimental animals of the Institute for Biological Research, Belgrade, Serbia. Adult male European ground squirrels (*Spermophilus citellus*) were trapped during mid-July in the Deliblatska peščara (southeastern part of Vojvodina, Serbia) and transported to the animal facility at the Institute for Biological Research, Belgrade, Serbia. Ground squirrels were housed in individual plastic cages at room temperature and fed rodent chow, fresh carrots, and apples *ad libitum* until early September when one group continued to be maintained under these conditions (control group) and another group was moved to a cold chamber set to an ambient temperature of 4 ± 1 °C, with food and water *ad libitum*. Active, euthermic squirrels that did not enter into hibernation under these low temperature conditions were sampled as the cold-exposed group and were sacrificed after 1, 3, 7, 12, or 21 days. Animals that entered into torpor (hibernation group) were sampled after each individual had been hibernating for 2–5 days (as indicated by continuous rectal temperature reading of ~4 °C). Control animals were sampled on the same day as the hibernating ones. All animals were sacrificed by decapitation between 8 and 10 a.m. to avoid any cyclic daily variation in antioxidant levels. Tissues (interscapular BAT, liver, skeletal muscle (*m. quadriceps*), retroperitoneal, gonadal, and subcutaneous WAT) were removed within 3 min. The liver was perfused with cold saline and minced. Minced tissues were washed thoroughly to remove all traces of blood and were snap-frozen in liquid nitrogen and stored at −80 °C until subsequent Western blotting.

SDS-PAGE and Western blotting

Western blots were conducted as described previously [41,42] using antibodies against SOD 1 (ab13498; 0.2 μg ml^{-1}), SOD 2 (ab13533; 1:5000), CAT (ab1877; 1:1000), GSH-Px 1 (ab16798; 1:2000), and beta actin (ab8226; 1:1000) (all purchased from ABCAM, UK). Quantitative analysis of immunoreactive bands was conducted with ImageQuant software. Band volume was the sum of all the pixel intensities within a band, i.e., 1 pixel=0.007744 mm^2. We averaged the ratio of dots per band for the target protein and beta actin in corresponding samples, from three similar independent experiments, and expressed them relative to the euthermic control, which was standardized as 100%. Data were then statistically analyzed.

Additional assays and statistical analysis

Protein content was estimated using bovine serum albumin as a reference [43]. Analysis of variance (ANOVA) was used to test within-group comparisons. If the F test indicated an overall difference, Tukey's t test was applied to evaluate the significance of the differences. Statistical significance was set at $P < 0.05$.

Results

Fig. 1 shows the protein expression of key AD enzymes in interscapular BAT during cold acclimation and hibernation. Hibernation induced significant increases of about 1.5- to 2-fold in the protein levels of CAT and both SOD isoforms ($P < 0.001$), while it had the opposite effect on the protein expression of GSH-Px that decreased to about 40% of control values ($P < 0.001$). Similarly, low temperature increased the protein content of CAT on Day 1 of cold acclimation ($P < 0.05$), SOD 1 on Days 12 and 21 ($P < 0.01$

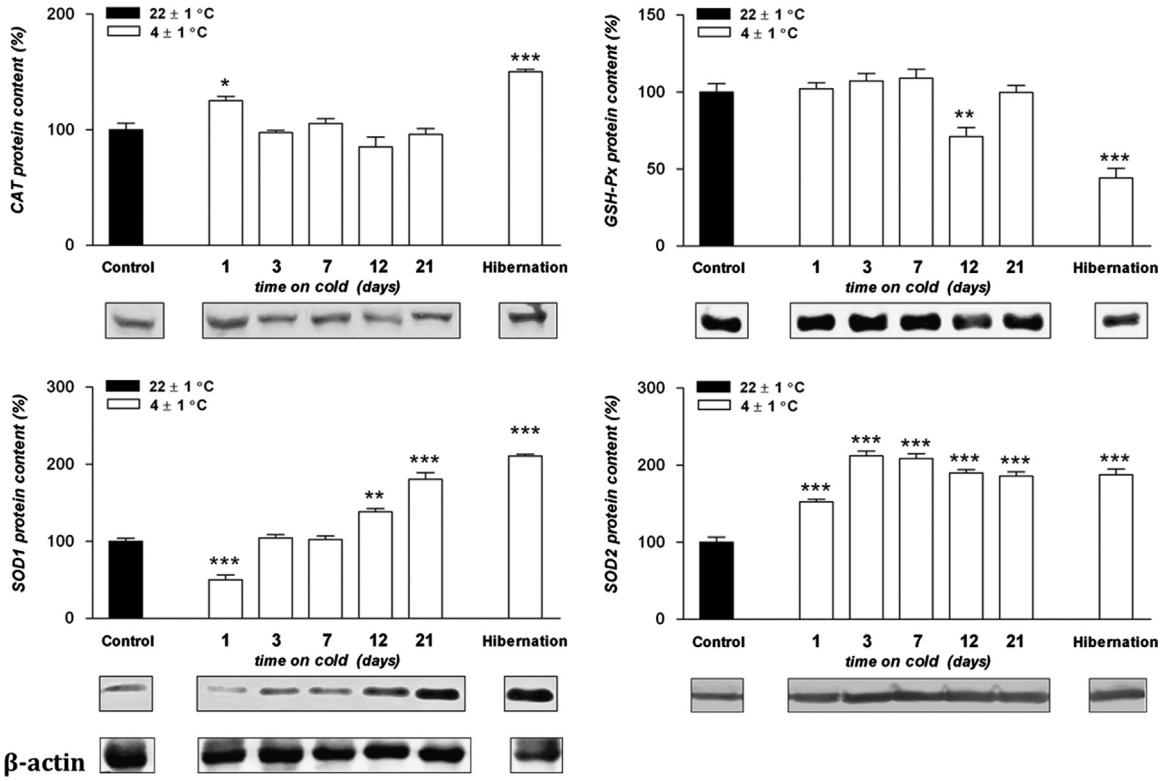

Fig. 1. The effects of different cold exposure (1, 3, 7, 12, or 21 days) or hibernation (2–5 days) on the protein expression of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase 1 and 2 (SOD 1 and 2) in ground squirrel interscapular brown adipose tissue (BAT). Protein content is expressed relative to a euthermic control, which was standardized as 100%. Band images from a representative blot of three trials are shown. Data were quantified as described under Materials and methods. The values represent the mean ± SEM. Asterisks indicate significant differences as compared to control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and $P < 0.001$, respectively), and SOD 2 during the whole acclimation period ($P < 0.001$).

Changes in the protein expression of AD enzymes in quadriceps muscle are depicted in Fig. 2. Opposite to BAT, hibernation decreased the protein content of SOD 2 isoform to about 30% of control values ($P < 0.001$) in skeletal muscle, while increasing about twice the levels of GSH-Px, CAT, and SOD 1 ($P < 0.001$). Acclimation to cold had the same effects on CAT and SOD 1 protein expression as hibernation. Namely, on Day 1 of cold exposure protein contents of these enzymes decreased, but then, after 7 days, levels rose above control levels and remained high throughout the following 2 weeks. On the other hand, contrary to hibernation, cold exposure decreased the protein content of GSH-Px on Days 12 and 21 ($P < 0.01$). SOD 2 was elevated on Day 1 of cold exposure but returned to control values at all subsequent sampling times.

Liver protein contents of AD enzymes are shown in Fig. 3. The results of Western blots clearly showed that neither cold acclimation nor hibernation affected protein levels of GSH-Px or SOD 2. Liver CAT was similarly unaffected by cold exposure, but hibernation induced a slight drop in CAT content ($P < 0.05$). On the contrary, SOD 1 protein expression was decreased for approximately 25% over the whole period of cold acclimation, and was strongly suppressed during hibernation to about 15% of control values ($P < 0.001$).

Fig. 4 shows changes in the protein content of CAT, GSH-Px, SOD 1, and SOD 2 in rpWAT during cold exposure and hibernation. Protein expression of CAT was unchanged during either condition. However, cold exposure increased the protein content of the other AD enzymes, as follows: GSH-Px was elevated over the whole period of cold exposure by about 1.5–2.5 times, SOD 2 increased during early cold exposure by about 1.6 times (Days 3 and 7; $P < 0.001$), and SOD 1 increased during prolonged cold exposure for approximately 1.4-fold (Days 12 and 21; $P < 0.01$). On the other hand, torpor led to decreases in GSH-Px and SOD 2 protein content ($P < 0.01$), but elevated SOD 1 ($P < 0.01$) in this WAT depot.

The protein contents of CAT and the mitochondrial SOD isoform were not affected in gnWAT either during acclimation to low temperature or during torpor (Fig. 5). Hibernation also had no effect on the content of the preferentially cytosolic SOD isoform (SOD 1), but increased GSH-Px protein expression for approximately 1.6-fold ($P < 0.001$). During cold acclimation, however, the protein level of SOD 1 was uniformly decreased ($P < 0.01$ or $P < 0.001$) whereas the content of GSH-Px increased on Days 1, 3, and 21.

Results for AD enzyme protein content in scWAT are presented in Fig. 6. This depot was characterized by decreased protein expression of GSH-Px, SOD 1, and SOD 2 over the whole cold-exposure period (21 days), as well as by an increased content of CAT starting from Day 3 of cold exposure. Similar results were seen during hibernation, except that the cytosolic SOD isoform was unaffected under these conditions.

Discussion

This study depicted patterns of AD enzyme protein expression in response to cold acclimation and hibernation in the main tissues of ground squirrels that are involved in the maintenance of whole body energy homeostasis and thermogenesis: liver, quadriceps muscle, interscapular BAT, scWAT, and retroperitoneal and gonadal depots of visceral WAT. We showed that skeletal muscle and BAT undergo the most dramatic changes in AD protein content during both cold acclimation and hibernation. Also, both conditions affected the AD levels of the three WAT depots differently, suggesting that they have specific and different involvements in overall metabolic homeostasis. On the other hand, liver only slightly changed its AD capacity in

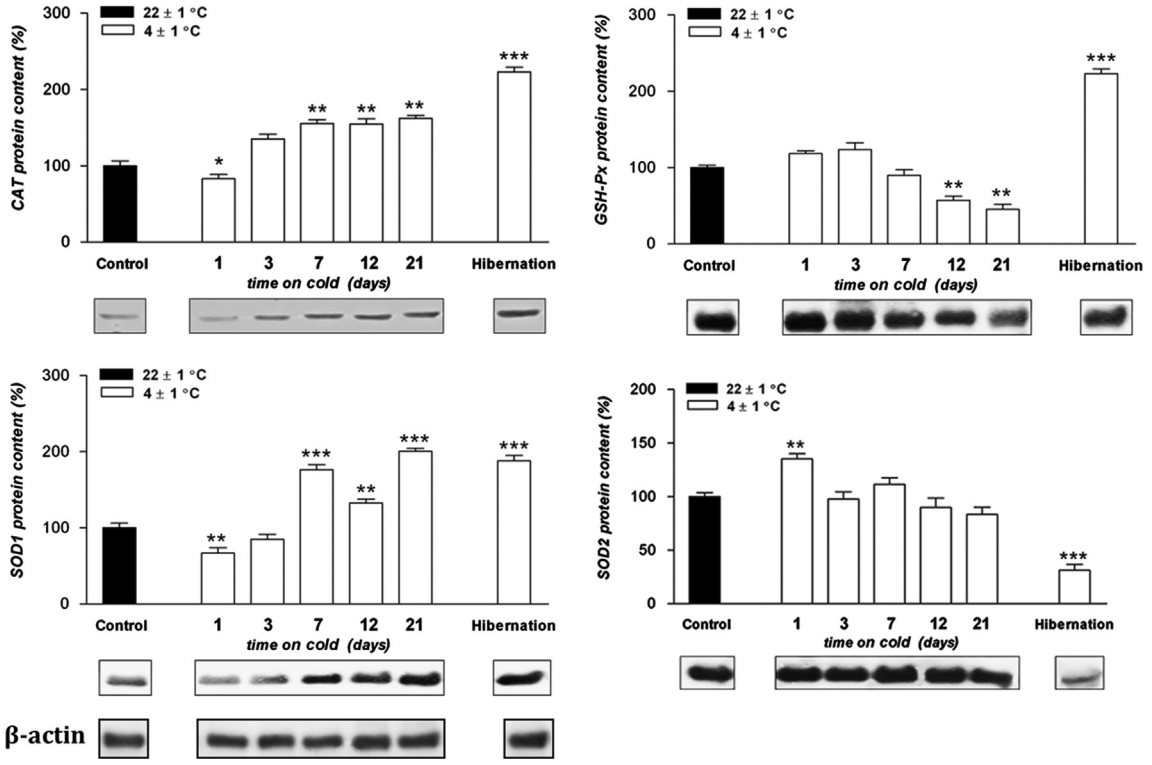

Fig. 2. Changes in the protein expression of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) 1 and 2 in the skeletal muscle, quadriceps, of ground squirrels during cold exposure or hibernation. Asterisks indicate significant differences as compared to control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Other information as in Fig. 1.

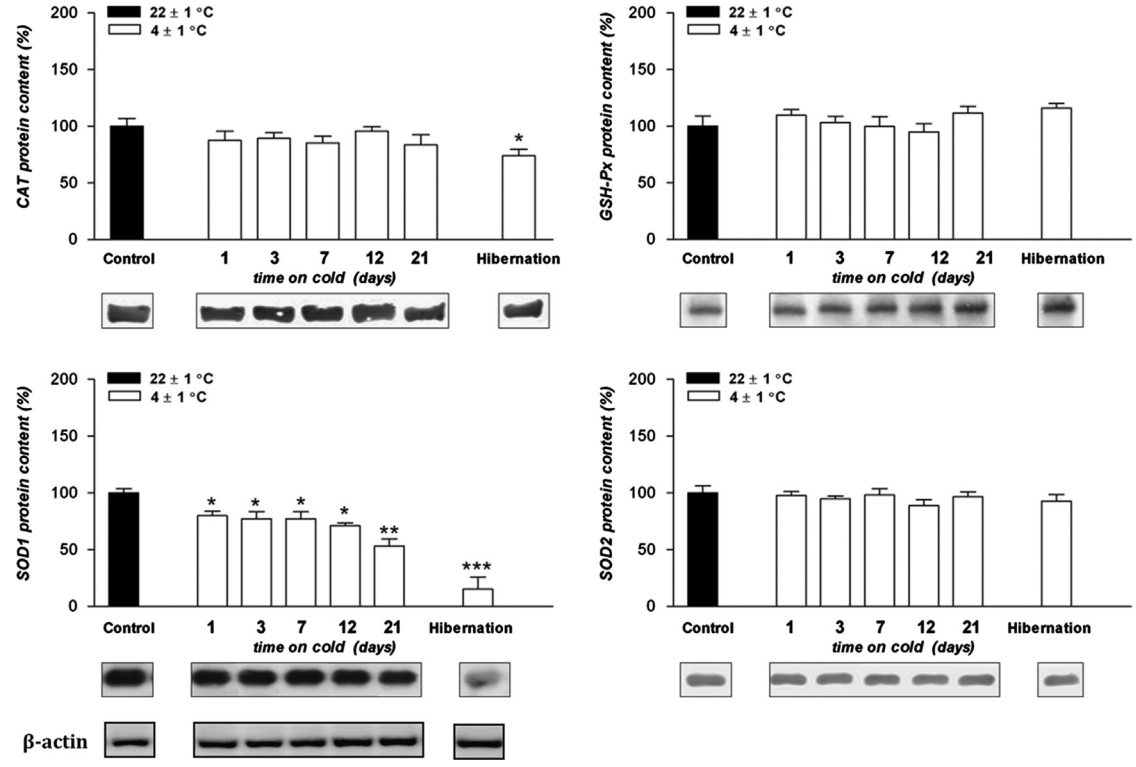

Fig. 3. Effects of hibernation of cold exposure on protein contents of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) 1 and 2 in the liver of ground squirrels. Asterisks indicate significant differences as compared to control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Other information as in Fig. 1.

response to either cold exposure or torpor. The results clearly point toward an orchestrated, tissue-dependent reorganization of AD under circumstances of altered metabolic demands, as discussed in more detail below.

Cold exposure in euthermic mammals (and arousal from hibernation) induces increases in heat production and enhances tolerance to cold through both shivering and nonshivering thermogenesis [44]. Skeletal muscles and BAT are the main effectors of

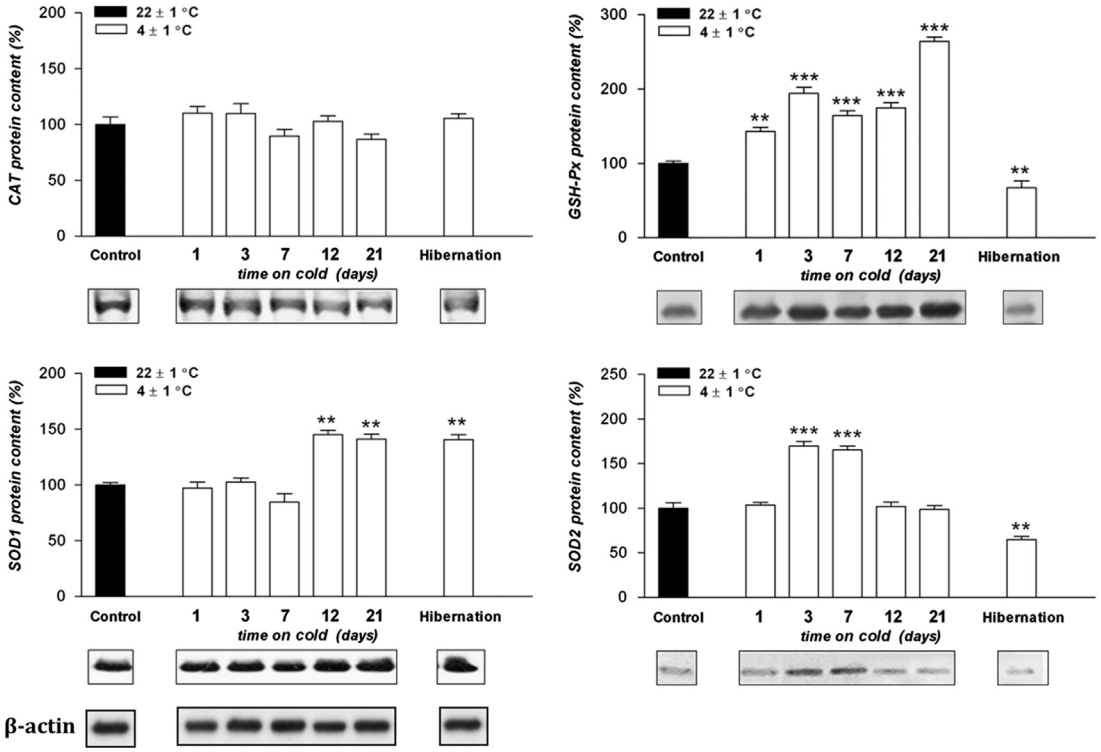

Fig. 4. Protein levels of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) 1 and 2 in retroperitoneal white adipose tissue (rpWAT) of ground squirrels given different lengths of cold exposure or during deep hibernation. Asterisks indicate significant differences as compared to control: ** $P < 0.01$, *** $P < 0.001$. Other information as in Fig. 1.

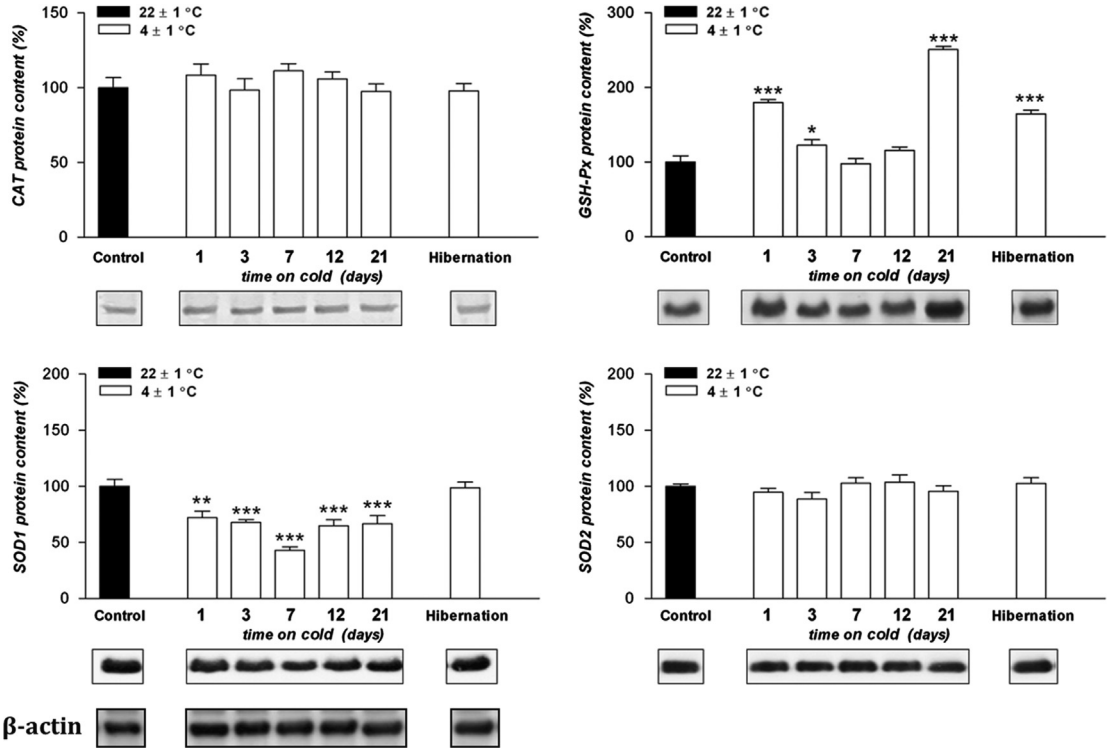

Fig. 5. Gonadal white adipose tissue (gnWAT) contents of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) 1 and 2 in ground squirrels exposed to low temperature for 1, 3, 7, 12, or 21 days or during deep torpor. Asterisks indicate significant differences as compared to control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Other information as in Fig. 1.

these thermoregulatory processes. Considering that heat production is highly energy demanding, it necessarily requires an increased metabolic rate that, in turn, results in elevated generation of ROS. Generally, maintenance of cellular homeostasis under conditions of increased ROS production is achieved by a proportional increase in tissue AD [1,24,45–48]. Thus, although there is little evidence of tissue-specific metabolic reprogramming in euthermic hibernators during cold exposure, the increased content of SOD2 in BAT and CAT

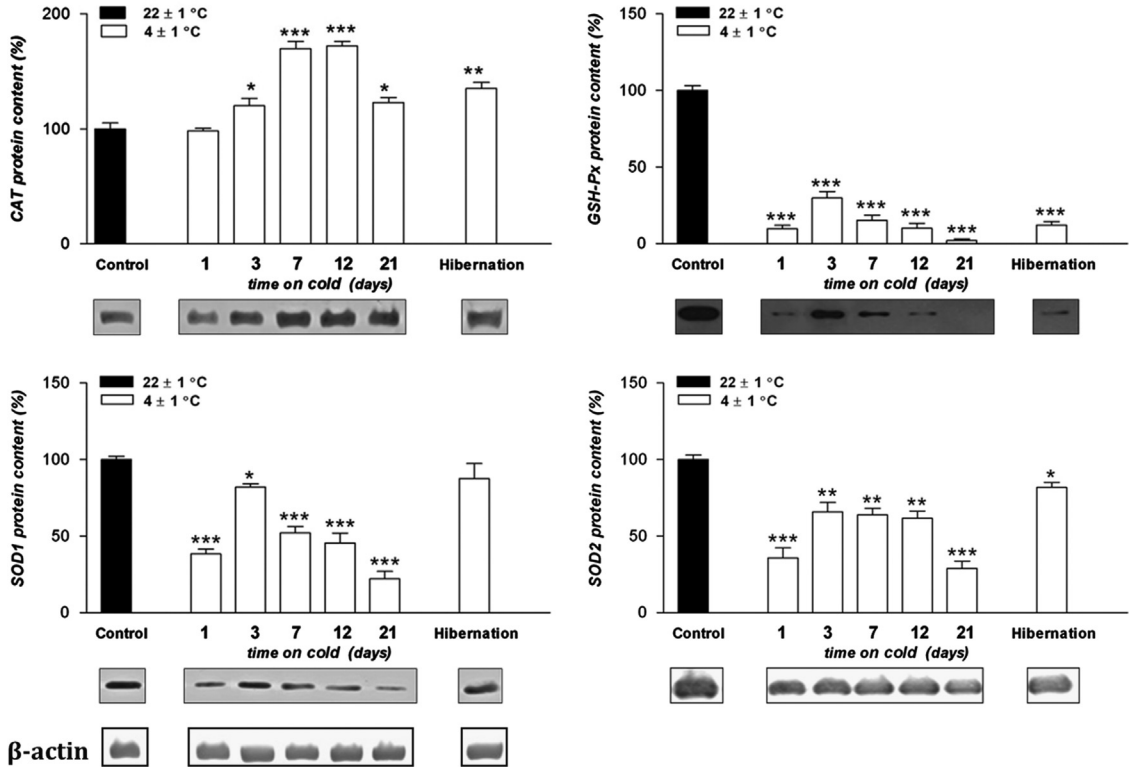

Fig. 6. The effects of different cold exposure time and hibernation on protein levels of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) 1 and 2 in ground squirrel subcutaneous white adipose tissue (scWAT). Asterisks indicate significant differences as compared to control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Other information as in Fig. 1.

in skeletal muscle, observed in the present study in response to cold acclimation, unambiguously points toward an increased need of these tissues for protection from oxidative stress, especially in mitochondria and peroxisomes. This is in accordance with findings that both shivering and nonshivering thermogenic processes rely on intense oxidative metabolism of lipids [49–51], as well as with results that showed increased respiratory capacity of BAT mitochondria from cold-exposed 13-lined ground squirrels [23]. However, increased levels of the preferentially cytosolic SOD 1 isoform in both BAT and skeletal muscle during prolonged cold exposure (7–21 days) indicate that the cytosol may be exposed to increased oxidative pressure as well, at least in this period considering that we did not detect any alteration of its protein expression (this study) nor in its activity [36] after short-term cold exposure. The pattern of AD remodeling in BAT and skeletal muscle of nonhibernating rats exposed to low temperature [24–26,52], that followed specific metabolic reprogramming in these tissues [51], seems quite different from AD remodeling in hibernating species, observed in this study. Consequently, these findings raise questions about low temperature-induced metabolic adjustments in hibernating species.

Contrary to BAT and skeletal muscle, cold exposure had little effect on AD capacity in liver. This is in accordance with our previous study that revealed unchanged GSH-Px and SOD-specific activity in the liver of nonhibernating squirrels kept at low temperatures [36]. Considering that liver has an important role in the adaptive processes that supports thermogenesis [53] and that Liu et al. [23] reported increased respiratory capacity of liver mitochondria from 13-lined ground squirrels, these results are quite surprising. However, earlier results from our laboratory revealed high basal specific activity of SOD in rats and squirrel liver in comparison with other organs and tissues (kidney, BAT, lung, heart, and spleen) [54]. Thus, basal levels of antioxidant enzymes in squirrel liver probably meet the AD needs of this tissue during exposure to low temperatures.

The role of WAT during cold exposure in nonhibernating rats has long been viewed as that of a passive reservoir of lipid fuel, particularly for BAT thermogenesis [28]. However, recent studies have revealed its active role in overall energy homeostasis, especially as it is now recognized to be an important endocrine tissue [55]. Novel reports even indicated that different physiological stimuli, including cold, may increase oxidative metabolism of this tissue in rats [30]. However, the metabolism of WAT in hibernating species during cold acclimation is still highly elusive. The present examination of different WAT depots revealed that, with respect to GSH-Px, the two visceral depots responded to cold stimulus in the same manner, i.e., by increasing GSH-Px protein expression. Similarly, our previous results showed increased activity of this AD component in rat rpWAT during cold acclimation [31]. Contrary to the visceral depots, GSH-Px protein levels in the subcutaneous WAT depot were markedly decreased throughout the cold-exposure period, whereas protein expression of CAT was increased. Considering the different subcellular localization and substrate responsiveness of GSH-Px and CAT [56], these results may suggest a different compartmentalization and intensity of oxidative metabolism within white adipocytes in viscera *versus* under the skin. Also, in rpWAT protein expression of SODs showed a phase dependency. Namely, levels of the mitochondrial SOD isoform rose during acute cold exposure (3–7 days), and then returned to control levels, whereas the cytosolic SOD isoform followed the opposite pattern of protein expression. A different pattern of changes is seen in rpWAT of rats, where activity of the mitochondrial SOD isoform is dominant in the period of 7–21. Day [31], i.e., in a period of increased mitochondrial metabolism [57]. These results suggest different oxidative needs of this visceral WAT depot in hibernating versus nonhibernating species during cold acclimation. Also, in the other visceral depot, gnWAT, phase dependency of SOD protein expression was not seen, indicating differences in metabolic reprograming even between visceral depots.

Hence, the above results indicate that there are tissue-specific differences in the levels and responsiveness of AD enzymes in cold-exposed, euthermic, active squirrels that differ from those seen in nonhibernating rats during exposure to low temperature. In general, cold-induced reorganization of AD status in ground squirrel tissues indicates cold-induced adjustments of oxidative metabolism intensity and subcellular localization, and we intend to investigate this issue in the future.

Opposite to cold acclimation, hibernation induces a completely different metabolic profile in the organism [14]. Our previous work with ground squirrels was the first one to show changes in AD in the various tissues of hibernating animals [36]. Namely, we showed that, compared with autumn active squirrels, hibernating animals had increased both SOD and CAT activity, while slightly decreased activity of GSH-Px in BAT. These results are in great accordance with the results for the protein expression levels of these enzymes obtained in the present study: increased protein levels of both SOD isoforms and CAT, and decreased content of GSH-Px protein. Such same-directional changes of protein expressions and activities (present and previous work) clearly suggest the importance of the translational regulation of investigated AD enzymes during the hibernating period, as well as during cold exposure. Enhanced protein levels and enzymatic activities of AD in brown fat of hibernating squirrels are consistent with sustained or even increased metabolic activity of this tissue during torpor and with a significant modification of thermogenic capacity in this period [39,40]. Accordingly, it has also been shown that cytochrome oxidase activity and transcript levels of other respiratory chain components are higher in BAT of torpid *versus* euthermic little brown bats [58]. Furthermore, increased protein levels of carnitine palmitoyl transferase-1β, the rate-limiting enzyme in β-oxidation [58], as well as increased activities of mitochondrial and peroxisomal β-oxidation enzymes [59] were detected in hibernating species during torpor. All these point to increased oxidative metabolism, and thus increased oxidative stress, in BAT during hibernation. Hence, the decreased protein levels and activity of GSH-Px in BAT detected here and in our previous study seem quite surprising. However, it has been recently shown that a strong increase in peroxiredoxin transcripts and protein expression takes place in BAT of hibernating ground squirrels [37]. This is a group of antioxidants that destroys a wide range of hydroperoxides, typically using thioredoxin as the electron donor [60,61]. Accordingly, it is possible that peroxiredoxins take over the role of GSH-Px, reducing the need for this enzyme during hibernation.

In skeletal muscle we detected a similar enhancement of antioxidant capacity as in brown fat. This is consistent with results that showed activation of upstream regulator of AD enzymes protein expression—NFκB during torpor [62], as well as with suggested improved capacity for oxygen-based fuel catabolism in muscle during hibernation [15,63]. However, unlike in BAT, muscle showed a decreased level of mitochondrial SOD 2 isoform during torpor. This could be related to different compartment-dependent fuel metabolism in these tissues. Indeed, Eddy et al. [58] found that muscle did not show increased levels of carnitine palmitoyl transferase-1β, as was characteristic of BAT under these conditions.

Contrary to BAT and skeletal muscle, which show up-regulation of the expression of some genes during torpor [40], liver shows global suppression of transcriptional machinery during torpor [64]. This is consistent with the lack of change in GSH-Px and SOD 2 or even the decreased levels of CAT and SOD 1 protein expression observed for liver during hibernation in the present study. These changes are in accordance with AD enzyme activities that we detected in the liver of hibernating squirrel compared to autumn active ones: no marked change in total SOD activity and slight decrease of the CAT activity [36]. Decreased energy requirements (e.g., suppression of energy-consuming transcription and translation machinery) and mitochondrial oxidative metabolism (respiration) [15,65] in hibernation suggest a decreased oxidative pressure on the liver, which may account for such response of liver AD.

The main role of WAT during winter torpor is to provide the fatty acids required as the fuel for energy metabolism by hibernating species during this period [35]. In line with this is the highly expressed temperature-stable pancreatic triacylglycerol lipase that allows WAT lipolysis at near-freezing temperature [66]. Furthermore, considering that expression of the key regulator of the metabolic shift toward the combustion of stored fatty acids, pyruvate dehydrogenase kinase 4, rises during hibernation in WAT, it seems that WAT itself also uses fatty acids as its major oxidative fuel during winter torpor [67]. However, this is not consistent with our results for AD changes in WAT during hibernation. Namely, in two WAT depots (scWAT and rpWAT), protein expression of SOD 2 was decreased during hibernation, suggesting decreased oxidative pressure in WAT mitochondria, where β-oxidation of fatty acids mainly takes place. However, in the subcutaneous depot of WAT protein expression of CAT rose significantly. This may suggest enhanced oxidative metabolism in peroxisomes and is in accordance with the study of Buck et al. [67]. On the other hand, protein expression of cytosolic SOD 1 was unchanged (scWAT and gnWAT) or increased (rpWAT) during hibernation, suggesting increased oxidative pressure in this cellular compartment. The different AD profile of visceral and subcutaneous WAT, or even between the two visceral depots, indicates specificity of their metabolic response and their role in overall metabolic homeostasis. All these results suggest that white adipose tissue of hibernating animals should not be viewed as a homogeneous tissue and should be examined separately in each depot type.

In conclusion, this study gave comprehensive insights into the changes of tissue-specific antioxidant capacity that probably enable tissue metabolic plasticity of hibernating animals. In summary, it can be concluded that brown fat and skeletal muscle undergo the greatest changes in AD capacity during both cold exposure and hibernation, while liver AD showed minimal adjustments for either low temperature exposure or winter torpor. Up-regulation of antioxidant enzyme protein expression in BAT and skeletal muscle during hibernation seems to be required for both metabolic function at near-freezing temperatures and to precondition the tissues to deal with an oxidative burst during arousal from hibernation. Thus, our future studies will be focused on the metabolic remodeling and its transcription and translational regulation in these tissues.

Acknowledgment

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant 173055.

References

[1] Halliwell, B.; Gutteridge, J. M. C. The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **280:**1–8; 1990.
[2] Halliwell, B.; Gutteridge, J. M. C.; Cross, C. E. Free radicals, antioxidants and human disease: where are we now? *J. Lab. Clin. Med.* **119:**598–620; 1992.
[3] Johnson, P. Antioxidant enzyme expression in health and disease: effects of exercise and hypertension. *Comp. Biochem. Physiol. C* **133:**493–505; 2002.
[4] Aruoma, I. E. Characterization of drugs as antioxidant prophylactics. *Free Radic. Biol. Med.* **20:**675–705; 1996.
[5] Mates, J. M. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* **153:**83–104; 2000.
[6] Guan, Y.; Hickey, M. J.; Borgstahl, G. E.; Hallewell, R. A.; Lipock, J. R.; O'Connor, D.; Hsieh, Y.; Nick, H. S.; Silverman, D. N.; Tainer, J. A. Crystal structure of Y34F

mutant human mitochondrial manganese superoxide dismutase and the functional role of tyrosine 34. *Biochemistry* **37:**4722–4730; 1998.
[7] Majima, H.; Oberley, T. D.; Farukowa, K.; Mattson, M. P.; Yen, H. C.; Szweda, I. I.; St. Clair, D. K. Prevention of mitochondrial injury by Mn-SOD reveals a primary mechanism for alkaline-induced cell death. *J. Biol. Chem.* **273:** 8217–8224; 1998.
[8] Hsieh, Y.; Guan, Y.; Tu, C.; Bratt, P. J.; Angerhofer, A.; Lepock, J. R.; Hickey, M. J.; Tainer, J. A.; Nick, H. S.; Silverman, D. N. Probing the active site bof human Mn-SOD: the role of glutamine 143. *Biochemistry* **37:**4731–4739; 1998.
[9] Freeman, M. L.; Meredith, M. J. Subcellular localization of glutathione and thermal sensitivity. *Radiat. Res.* **115:**461–471; 1988.
[10] Reddy, J. K.; Hashimoto, T. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu. Rev. Nutr.* **21:**193–230; 2001.
[11] Chance, B.; Sies, H.; Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59:**527–605; 1979.
[12] Kamei, A.; Houdou, S.; Takashima, S.; Suzuki, Y.; Becker, L. E.; Armstrong., D. L. Peroxisomal disorders in children: immunohistochemistry and neuropathology. *J. Pediatr.* **122:**573–579; 1993.
[13] Carey, H. V.; Andrews, M. T.; Martin, S. L. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol. Rev.* **83:**1153–1181; 2003.
[14] Storey, K. B. Out cold: Biochemical regulation of mammalian hibernation—a mini-review. *Gerontology* **56:**220–230; 2010.
[15] Muleme, H. M.; Walpole, A. C.; Staples, J. F. Mitochondrial metabolism in hibernation: metabolic suppression, temperature effects, and substrate preferences. *Physiol. Biochem. Zool.* **79:**474–483; 2006.
[16] Smith, R. E.; Roberts, J. C.; Hittelman, K. J. Nonphosphorylating respiration of mitochondria from brown adipose tissue of rat. *Science* **154:**653–654; 1966.
[17] Lindberg, O.; de Pierre, J.; Rylander, E.; Afzelius, B. A. Studies of the mitochondrial energy-transfer system of brown adipose tissue. *J. Cell Biol.* **34:**293–310; 1967.
[18] Liu, X.; Li, Q.; Lin, Q.; Sun, R. Uncoupling protein1 mRNA, mitochondrial GTP-binding, and T4 5'-deiodinase of brown adipose tissue in euthermic Daurian ground squirrel during cold exposure. *Comp. Biochem. Physiol. A* **128:**827–835; 2001.
[19] Adolph, E. F.; Lawrow, J. W. Acclimation to cold air, hypothermia, and heat production in the golden hamster. *Am. J. Physiol.* **166:**62–74; 1951.
[20] Hart, J. S. Climatic and temperature changes in the energetics of homeotherms. *Rev. Can. Biol* **16:**133–174; 1957.
[21] Farrand Jr R. L.; Folk, G. E. Responses of hamster during the first 2 weeks of cold exposure. *Fed. Proc* **16:**35; 1957.
[22] Farrand, R. L. Cold acclimatization in the golden hamster. *State Univ. Iowa Studies Nat. Hist* **20:**1–29; 1959.
[23] Liu, C. C.; Frehn, J. L.; Laporta, A. D. Liver and brown fat mitochondrial response to cold in hibernators and nonhibernators. *J. Appl. Physiol.* **27:**83–89; 1969.
[24] Petrovic, V.; Buzadzic, B.; Korac, A.; Vasilijevic, A.; Jankovic, A.; Korac, B. Free radical equilibrium in interscapular brown adipose tissue: relationship between metabolic profile and antioxidative defense. *Comp. Biochem. Physiol. C* **142:**60–65; 2006.
[25] Petrovic, V.; Buzadzic, B.; Korac, A.; Vasilijevic, A.; Jankovic, A.; Micunovic, K.; Korac, B. Antioxidative defence alterations in skeletal muscle during prolonged acclimation to cold: role of L-arginine/NO-producing pathway. *J. Exp. Biol.* **211:**114–120; 2008.
[26] Petrovic, V.; Buzadzic, B.; Korac, A.; Korac, B. Antioxidative defense and mitochondrial thermogenic response in brown adipose tissue. *Genes Nutr* **5:**225–235; 2010.
[27] Buzadzic, B.; Korac, A.; Petrovic, V.; Vasilijevic, A.; Jankovic, A.; Korac, B. Adaptive changes in interscapular brown adipose tissue during reacclimation after cold: the role of redox regulation. *J. Therm. Biol.* **32:**261–269; 2007.
[28] Trayhurn, P.; Duncan, J. S.; Rayner, D. V. Acute cold-induced suppression of ob (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system. *Biochem. J.* **311:**729–733; 1995.
[29] Hauton, D.; Richards, S. B.; Egginton, S. The role of the liver in lipid metabolism during cold acclimation in non-hibernator rodents. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **144:**372–381; 2006.
[30] Mulligan, J. D.; Gonzalez, A. A.; Stewart, A. M.; Carey, H. V.; Saupe, K. W. Upregulation of AMPK during cold exposure occurs via distinct mechanisms in brown and white adipose tissue of the mouse. *J. Physiol.* **580:**677–684; 2007.
[31] Jankovic, A.; Buzadzic, B.; Korac, A.; Petrovic, V.; Vasilijevic, A.; Korac, B. Antioxidative defense organization in retroperitoneal white adipose tissue during acclimation to cold—the involvement of L-arginine/NO pathway. *J. Therm. Biol.* **34:**358–365; 2009.
[32] Turner, J. J.; Anderson, B. O.; Bintz, G. L. Whole body lipids and fatty acids synthase activity in Richardson's ground squirrels, *Spermophilus richardsonii*. *Physiol. Zool.* **62:**1383–1397; 1989.
[33] Dicker, A.; Cannon, B.; Nedergaard, J. Cold acclimation-recruited non-shivering thermogenesis: the Syrian hamster is not an exception. *Am. J. Physiol.* **269:**767–774; 1995.
[34] Cannon, B.; Bengtsson, T.; Bronnikov, G.; Svoboda, P.; Zhao, J.; Nedergaard, J. Non-shivering thermogenesis in a hibernator, the golden hamster: mediation exclusively through beta 3-adrenergic receptors which are desensitised due to cold acclimation. In: Geiser, F., Hulbert, A. J., Nicol, S. C., editors. *Adaptation to cold*. Armidale: University of New England Press; 1996. p. 271–279.
[35] Lyman, C. P., Willis, J. S., Malan, A., Wang, L. C. H., editors. *Hibernation and torpor in mammals and birds*. New York: Academic Press; 1982.
[36] Buzadzic, B.; Spasic, M.; Saicic, Z. S.; Radojicic, R.; Petrovic, V. M.; Halliwell, B. Antioxidant defenses in the ground squirrel *Citellus citellus* 2. The effect of hibernation. *Free Radic. Biol. Med.* **9:**407–413; 1990.
[37] Jr Morin, P.; Storey, K. B. Antioxidant defense in hibernation: cloning and expression of peroxiredoxins from hibernating ground squirrel, *Spermophilus tridecemlineatus*. *Arch. Biochem. Biophys.* **461:**59–65; 2007.
[38] Jr Morin, P.; Ni, Z.; McMullen, D. C.; Storey, K. B. Expression of Nrf2 and its downstream gene targets in hibernating 13-lined ground squirrel, *Spermophilus tridecemlineatus*. *Mol. Cell. Biochem.* **312:**121–129; 2008.
[39] Hittel, D. S.; Storey, K. B. The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*). *Arch. Biochem. Biophys.* **401:**244–254; 2002.
[40] Hittel, D. S.; Storey, K. B. Differential expression of mitochondria-encoded genes in a hibernating mammal. *J. Exp. Biol.* **205:**1625–1631; 2002.
[41] Petrovic, V.; Korac, A.; Buzadzic, B.; Korac, B. The effects of L-arginine and L-NAME supplementation on redox-regulation and thermogenesis in interscapular brown adipose tissue. *J. Exp. Biol.* **208:**4263–4271; 2005.
[42] Korac, A.; Buzadzic, B.; Petrovic, V.; Vasilijevic, A.; Jankovic, A.; Micunovic, K.; Korac, B. The role of nitric oxide in remodeling of capillary network in rat interscapular brown adipose tissue after long-term cold acclimation. *Histol. Histopathol.* **23:**441–450; 2008.
[43] Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275; 1951.
[44] Himms-Hagen, J. Cellular thermogenesis. *Annu. Rev. Physiol.* **38:**315–351; 1976.
[45] Buzadzic, B.; Blagojevic, D.; Korac, B.; Saicic, Z. S.; Spasic, B. M.; Petrovic, V. M. Seasonal variation in the antioxidant defense system of the brain of the ground squirrel (*Citellus citellus*) and response to low temperature compared with rat. *Comp. Biochem. Physiol. C* **117:**141–149; 1997.
[46] Buzadzic, B.; Blagojevic, D.; Korac, B.; Saicic, Z. S.; Spasic, B. M.; Petrovic, V. M. Seasonal changes in the activity of antioxidative defense in the kidneys of the euthermic ground squirrel (*Citellus citellus*). *J. Environ. Pathol. Toxicol. Oncol.* **17:**271–276; 1998.
[47] Buzadzic, B.; Korac, B.; Petrovic, V. M. The effect of adaptation to cold and re-adaptation to room temperature on the level of glutathione in rat tissues. *J. Therm. Biol.* **24:**373–377; 1999.
[48] Korac, B.; Buzadzic, B. Doxorubicin toxicity to the skin: possibility of protection with antioxidants enriched yeast. *J. Dermatol. Sci.* **25:**45–52; 2001.
[49] Weber, J. M.; Haman, F. Fuel selection in shivering humans. *Acta Physiol. Scand.* **184:**319–329; 2005.
[50] Vaillancourt, E.; Haman, F.; Weber, J. M. Fuel selection in Wistar rats exposed to cold: shivering thermogenesis diverts fatty acids from re-esterification to oxidation. *J. Physiol* **587:**4349–4359; 2009.
[51] Vucetic, M.; Otasevic, V.; Korac, A.; Stancic, A.; Jankovic, A.; Markelic, M.; Golic, I.; Velickovic, K.; Buzadzic, B.; Korac, B. Interscapular brown adipose tissue metabolic reprogramming during cold acclimation: Interplay of HIF-1α and AMPKα. *Biochim. Biophys. Acta* **1810:**1252–1261; 2010.
[52] Otasevic, V.; Buzadzic, B.; Korac, A.; Stancic, A.; Jankovic, A.; Vucetic, M.; Korac, B. Effects of L-arginine and L-NAME supplementation on mRNA, protein expression and activity of catalase and glutathione peroxidase in brown adipose tissue of rats acclimated to different temperatures. *J. Therm. Biol.* **36:**269–276; 2010.
[53] Jansky, L. Effect of cold and exercise on energy metabolism, in small mammals. In: Tromps, E., editor. *Progress in animal biometeorology*. Amsterdam: Swets and Zeitlinger; 1976. p. 239–258.
[54] Petrovic, V. M.; Saicic, Z.; Milic, B.; Spasic, M.; Radojicic, R. Distribution of superoxide dismutase in the ground squirrel (*Citellus citellus*): effect of the hibernation and arousal. *Comp. Biochem. Physiol.* **75:**699–700; 1983.
[55] Ahima, R. S.; Flier, J. S. Adipose tissue as an endocrine organ. *Trends Endocrinol. Metab.* **11:**327–332; 2000.
[56] Halliwell, B., Gutteridge, J. M. C., editors. *Free radicals in biology and medicine*. New York: Oxford University Press; 2007.
[57] Jankovic, A.; Korac, A.; Buzadzic, B.; Otasevic, V.; Stancic, A.; Vucetic, M.; Markelic, M.; Velickovic, K.; Golic, I.; Korac, B. Endocrine and metabolic signaling in retroperitoneal white adipose tissue remodeling during cold acclimation. *J. Obes.* **2013:**937572; 2013.
[58] Eddy Jr S. F.; Morin, P.; Storey, K. B. Differential expression of selected mitochondrial genes in hibernating little brown bats, *Myotis lucifugus*. *J. Exp. Zool.* **305:**620–630; 2006.
[59] Kabine, M.; Clemencet, M. -C.; Bride, J.; Said El Kebbaj, M.; Latruffe, N.; Cherkaoui-Malki, M. Changes of peroxisomal fatty acid metabolism during cold acclimatization in hibernating jerboa (*Jaculus orientalis*). *Biochimie* **85:**707–714; 2003.
[60] Hofmann, B.; Hecht, H. J.; Flohe, L. Peroxiredoxins. *Biol. Chem.* **383:**347–364; 2002.
[61] Rhee, S. G.; Chae, H. Z.; Kim, K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic. Biol. Med.* **38:**1543–1552; 2005.
[62] Allan, M. E.; Storey, K. B. Expression of NF-kB and downstream antioxidant genes in skeletal muscle of hibernating ground squirrels, *Spermophilus tridecemlineatus*. *Cell Biochem. Funct.* **30:**166–174; 2012.
[63] Postnikova, G. B.; Tselikova, S. V.; Kolaeva, S. G.; Solomonov, N. G. Myoglobin content in skeletal muscles of hibernating ground squirrels rises in autumn and winter. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **124:**35–37; 1999.

[64] Knight, J. E.; Narus, E. N.; Martin, S. L.; Jacobson, A.; Barnes, B. M.; Boyer, B. B. mRNA stability and polysome loss in hibernating Arctic ground squirrels (*Spermophilus parryii*). *Mol. Cell. Biol.* **20:**6374–6379; 2000.

[65] Chung, D.; Lloyd, G. P.; Thomas, R. H.; Guglielmo, C. G.; Staples, J. F. Mitochondrial respiration and succinate dehydrogenase are suppressed early during entrance into a hibernation bout, but membrane remodeling is only transient. *J. Comp. Physiol. B* **181:**699–711; 2011.

[66] Bauer, V. W.; Squire, T. L.; Lowe, M. E.; Andrews, M. T. Expression of a chimeric retroviral lipase mRNA confers enhanced lipolysis in a hibernating mammal. *Am. J. Physiol. Regul. Integr. Comp. Physiol* **281:**1186–1192; 2001.

[67] Buck, M. J.; Squire, T. L.; Andrews, M. Y. Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. *Physiol. Genomics* **8:**5–13; 2002.