

Effect of cold acclimation on GSH, antioxidant enzymes and lipid peroxidation in brown adipose tissue

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Cold acclimation increased the activities of superoxide dismutase, catalase, total and selenium (Se)-dependent glutathione peroxidases (GPx) and glutathione reductase by 2–4-fold in the brown adipose tissue (BAT) of cold-acclimated rats. Nevertheless, when expressed per unit protein, the antioxidant enzyme activities were unaltered. Sensitivity to lipid peroxidation and GSH levels both increased by one order of magnitude in the cold on a per weight basis and were still 3–5 times greater in the cold when expressed per mg of protein. We suggest that activation of BAT leads to a large increase in the potential for lipid peroxidation and that the tissue responds to this challenge by increasing practically all of its antioxidant defences. Nevertheless, GSH, and possibly GPx activity, seem to be the principal defences involved in adaptation of the tissue to a higher sensitivity to peroxidative damage after activation.

INTRODUCTION

When small mammals are chronically exposed to cold, achievement of long-term thermoregulation involves an increased generation of heat by non-shivering thermogenesis. It has been shown that the principal component of this extra heat is produced in the brown adipose tissue (BAT) [1,2]. The response of this tissue to cold involves a large increase in oxygen consumption, which is necessary for the oxidation of fatty acids in the mitochondria [3]. It is known that mitochondria are among the principal cellular generators of oxygen free radicals [4] and that the most susceptible macromolecules to free-radical-induced damage are polyunsaturated fatty acids (PUFA). In addition, the higher the mitochondrial rate of oxygen consumption, the greater the rate of oxygen radical generation [5,6].

Thus BAT has a complement of characteristics that probably make it very sensitive to lipid peroxidation during its activation in response to cold exposure. These include: (1) mitochondria are very abundant in BAT; (2) activation of the tissue involves large increases in oxygen consumption and lipid turnover; and (3) increases in phospholipids [7,8] and PUFA, and in the unsaturation index of the fatty acid pattern of phospholipids [7–10], occur during cold acclimation. Increased susceptibility to oxidative stress after activation of BAT would result from the increase in oxygen consumption leading to an increase in oxygen radical generation *in vivo*. Indeed, it has been shown that BAT mitochondria generate H_2O_2 *in vitro* at a higher rate than do liver mitochondria. Furthermore, the rate of H_2O_2 generation in BAT mitochondria (expressed per mg of mitochondrial protein) increases 2.5-fold after cold acclimation [11]. Taking into account the increase in mitochondrial protein that occurs during cold acclimation, the combined effects would result in a 6–9-fold increase in oxygen radical generation per mg of tissue.

Nevertheless, data about levels of oxygen-radical-related parameters in BAT are scarce, except for data on catalase (EC 1.11.1.6) and superoxide dismutase (SOD) [12–14]. These reports have shown that brown fat SOD and catalase are greatly increased in response to cold acclimation. These results, together with the above-mentioned characteristics of BAT, prompted us to perform

a comprehensive study of the changes that occur in the principal oxygen-radical-related parameters after chronic activation of the tissue. Thus the activities of SOD, catalase, selenium (Se)-dependent and non-dependent glutathione peroxidases (GPx) and glutathione reductase (GR), plus oxidized (GSSG) and reduced (GSH) glutathione levels and sensitivity to lipid peroxidation (TBARS) were measured, both in an intense heat-generating BAT, as present in cold-acclimated rats, and in a thermogenically inactive tissue, as present in rats at thermo-neutral temperature. GDP binding was the index used to determine thermogenic activity.

MATERIALS AND METHODS

Animals

Male Wistar rats (227 ± 1 g body weight at the beginning of the experiment), were acclimated to either cold (6 °C) or thermo-neutral conditions (28 °C) for 21 days. Throughout the experiment they were housed in individual cages, with food and water *ad libitum* and a 12 h light/12 h dark schedule. The food provided was a commercial stock diet (Panlab, Barcelona, Spain). After 21 days of exposure, the rats were killed by decapitation. Interscapular BAT (IBAT) was quickly removed and placed in the appropriate medium (for mitochondrial isolation, or for measurement of enzymes, glutathione or peroxidation) and adherent white adipose tissue and muscle were removed.

Samples

A sample was homogenized in 20 vol. of cold 50 mM-potassium phosphate buffer, pH 7.4, and was used for assay of enzyme activities and TBARS. The homogenates were sonicated at 38 W for 30 s and centrifuged at 5 °C and 3200 g for 20 min. Another sample from the same tissue (for glutathione assays) was homogenized in cold 5% trichloroacetic acid containing 0.01 M-HCl. This solution was deoxygenated by bubbling with N_2 . The homogenates were centrifuged at 3200 g for 5 min under a N_2 atmosphere.

Abbreviations used: BAT, brown adipose tissue; IBAT, interscapular brown adipose tissue; SOD, superoxide dismutase (EC 1.15.1.1); GPx, glutathione peroxidase, total and Se-dependent (EC 1.11.1.9); GR, glutathione reductase (EC 1.6.4.2); COX, cytochrome *c* oxidase (EC 1.9.3.1); TBARS, thiobarbituric-acid-reactive substances; Se, selenium; PUFA, polyunsaturated fatty acids.

Enzymes, glutathione and peroxidation

SOD was measured after 24 h of dialysis by monitoring the inhibition by the enzyme of the rate of NADH oxidation in the presence of 5 mM-EDTA, 2.5 mM-MnCl₂ and 0.56 mM-mercaptoethanol [15]. Catalase [16] and both total (cumene hydroperoxide) [17] and Se-dependent GPx [18] were measured by spectrophotometric methods as previously described [19]. GR was assayed by measuring NADPH oxidation at 340 nm in the presence of 4 mM-GSSG and 0.3 mM-NADPH [20]. Glutathione was assayed in trichloroacetic acid extracts by the method of Tietze [21] in the presence of 0.6 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.21 mM-NADPH and 0.5 units of GR/ml of assay mixture. GSSG was assayed by the same method after derivatization of GSH with 2-vinylpyridine [22]. Lipid peroxidation of supernatants from the phosphate buffer homogenates was measured by the TBA test as previously described [19] after incubating supernatant samples with 0.4 mM-ascorbate and 0.05 mM-FeSO₄ for 60 min at 25 °C.

Mitochondrial isolation and GDP binding

The method of Cannon & Lindberg [23], with minor modifications [24], was used to isolate mitochondria. Briefly, 1 g of IBAT was diluted to 5% (w/v) in a medium containing 250 mM-sucrose, 5 mM-Tes and 2 mM-EDTA, pH 7.2, homogenized and submitted to three different centrifugations at 0–4 °C. Cytochrome *c* oxidase (COX) activity and total proteins were measured both in the homogenate and in the final mitochondrial suspension to allow calculation of mitochondrial recovery. COX activities [25] and protein concentrations [26] were measured by current methods.

GDP binding was measured as previously described [27] with some modifications [24]. In short, the mitochondria were incubated in 2 ml of a medium containing [¹⁴C]sucrose and [³H]GDP. The incubation mixture was filtered through a Sartorius cellulose membrane filter. The filters were placed in scintillation vials, and ³H and ¹⁴C were measured in a scintillation counter. The amount of GDP binding was calculated as the excess amount of [³H]GDP found in the filter, correcting for trapped buffer by the use of [¹⁴C]sucrose as an extra-mitochondrial marker.

Statistical analysis

Values were computed as means ± S.E.M. Comparisons between

the two acclimation groups were performed by Student's *t* tests. The *P* < 0.05 level was selected as the point of minimum statistical significance.

RESULTS

Cold exposure for 21 days resulted in changes typical of cold acclimation, namely an increase in protein content (7.6 ± 0.2%, compared with 2.7 ± 0.1% in the control group), mitochondrial proliferation (13.1 ± 1.3 versus 4.6 ± 0.1 mg of mitochondrial protein/IBAT depot) and a higher oxidative capacity, as assessed by COX activity (Table 1). GDP binding was clearly enhanced by cold acclimation (0.83 ± 0.06 versus 0.40 ± 0.02 nmol/mg of mitochondrial protein). This increased thermogenic activity decreased body weight gains (38 ± 4 g versus 104 ± 4 g).

Cold acclimation resulted in very marked increases in all five antioxidant enzymes and COX when the activities were expressed per mg of tissue (Table 1). SOD, catalase and COX reached around 400% of control values in the cold-acclimated group, whereas the glutathione-related enzymes (Se- and non-Se-dependent GPx, and GR) only doubled their activity. Since protein concentration per mg of tissue was around three times higher in cold-acclimated animals than in controls, when the enzymic activities were expressed per mg of protein, none of the increases described above were apparent: SOD, catalase and COX did not differ between the two experimental groups, and total and Se-dependent GPx and GR even showed moderate decreases in the cold-acclimated group.

GSSG also showed significant increases after cold acclimation when expressed per tissue weight, but did not show any change when referred to protein (Table 1). However, a different situation was evident for GSH. The increase in this low-molecular-mass thiol in the cold was one order of magnitude when expressed on a tissue weight basis. When GSH levels were expressed per mg of protein, the cold-acclimated group still showed levels that were almost 300% of control values. Since GSH increased much more acutely than did GSSG, the GSSG/GSH ratio was significantly decreased in cold-acclimated animals.

Analogous results to those obtained for GSH were observed when sensitivity to tissue peroxidation was assayed after 1 h of incubation of the supernatants in the presence of ascorbate and FeSO₄. TBARS values expressed on a tissue weight basis were more than one order of magnitude higher in the cold, and still

Table 1. Antioxidant enzymes, COX, glutathione and peroxidation *in vitro* (ascorbate/Fe²⁺) in BAT after acclimation to thermoneutral (28 °C) or cold (6 °C) temperatures

Values are means ± S.E.M. Numbers of animals are given in parentheses. ****P* < 0.001, ***P* < 0.01, **P* < 0.05 in cold-acclimated versus control animals (28 °C).

	Values/mg of tissue		Values/mg of protein	
	28 °C	6 °C	28 °C	6 °C
SOD (units)	0.25 ± 0.04 (7)	0.97 ± 0.10 (6)***	8.9 ± 1.5 (7)	10.6 ± 0.6 (6)
Catalase (μmol of H ₂ O ₂ /min)	1.59 ± 0.16 (7)	6.08 ± 0.89 (6)***	61 ± 5 (7)	67 ± 8 (6)
Total GPx (nmol of NADPH/min)	4.5 ± 0.4 (7)	10.9 ± 1.1 (6)***	173 ± 17 (7)	121 ± 8 (6)*
Se-dependent GPx (nmol of NADPH/min)	3.4 ± 0.3 (7)	8.0 ± 1.0 (6)***	130 ± 9 (7)	88 ± 6 (6)**
GR (nmol of NADPH/min)	0.76 ± 0.11 (7)	1.69 ± 0.26 (6)***	28 ± 3 (7)	19 ± 2 (6)*
COX (μmol of cytochrome <i>c</i> /min)	0.054 ± 0.003 (5)	0.155 ± 0.014 (6)***	2.0 ± 0.11 (5)	2.0 ± 0.2 (6)
GSH (nmol)	0.08 ± 0.02 (6)	0.80 ± 0.25 (6)*	3.0 ± 0.6 (6)	8.3 ± 2.2 (6)*
GSSG (nmol)	0.02 ± 0.005 (6)	0.05 ± 0.01 (6)*	1.0 ± 0.4 (6)	0.82 ± 0.16 (6)
GSSG/GSH	0.50 ± 0.22 (6)	0.09 ± 0.03 (6)*	—	—
TBARS <i>in vitro</i> (nmol of malondialdehyde)	0.036 ± 0.010 (4)	0.527 ± 0.141 (4)**	1.29 ± 0.36 (4)	5.67 ± 1.52 (4)*

represented 400% of control values when referred to protein mass (Table 1).

DISCUSSION

GDP binding in the BAT of cold-acclimated rats was much higher than that in controls. This elevated thermogenic activity is sustained by an enhanced substrate combustion and a correspondingly higher oxygen consumption [1,28].

Previous studies have shown that catalase and palmitoyl-CoA oxidase, two peroxisomal enzymes, are increased in BAT after cold acclimation. The induction of these enzymes was thought to be secondary to the well-known increase in peroxisomal number in the cold [29]. Decomposition of H_2O_2 is a highly exergonic process, and it has been suggested that it could be involved in non-shivering thermogenesis [30]. Nevertheless, peroxisomes consume only 1% of the oxygen used by BAT and cannot represent an important source of metabolic heat [11–13]. In the absence of a clear role for the increase in peroxisome number in the cold, it has been suggested that these organelles carry out only the first steps of fatty acid oxidation, the remaining acyl-CoA being oxidized in the mitochondria [12]. Alternatively, the peroxisomes could metabolize potentially harmful fatty acids, produce H_2O_2 for regulatory metabolic purposes or produce oxidized substrates needed in BAT [12].

The results obtained in this work clearly show that all of the antioxidant enzymes (SOD, catalase, Se- and non-Se-dependent GPx and GR) and the major non-protein thiol (GSH) are greatly increased (on a per weight basis) in cold-stimulated tissue. The observed SOD levels are similar to those in a recent study [14] in which SOD/mg of tissue also increased in the cold (1.6-fold), whereas SOD/mg of protein was not changed. These increases are expected from the acute increase in sensitivity of the tissue to oxidative stress induced *in vitro* (TBARS) that was observed here. This last change is probably the result of the previously described increases in phospholipids [7,8] and especially in PUFA [7–10], the molecules showing the highest sensitivity to lipid peroxidation, after cold acclimation in BAT.

It is suggested that activation of BAT by chronic cold exposure is not only accompanied by a large increase in the mitochondrial generation of oxygen radicals [11] together with the augmentation of the preferential substrates of lipid peroxidation (PUFA) [7–10], but also involves adaptations that compensate for an increased liability to tissue peroxidation. This is accomplished by increases in all of the antioxidant enzymes and in GSH levels. It is possible that the main objective of increasing the number of peroxisomes in the cold is to increase catalase activity, since this enzyme represents a large proportion of peroxisomal protein, and a higher flux of H_2O_2 can be expected to enter the peroxisome from the cytosol in the activated tissue. This suggestion is fully compatible with a role for peroxisomes, complementary to that of mitochondria, in fatty acid oxidation, as was described above [12]. Nevertheless, it should be pointed out that the increases in the antioxidant enzymes are not apparent when activities are expressed per mg of protein. This is due to the similarly large increase in protein concentration that was observed in the present as well as in previous studies [13,14]. The same applies to COX activity, confirming previous observations [13]. Thus changes in enzyme activities after activation of BAT are unlikely to be detected if the results are expressed exclusively on a protein content basis.

A somewhat different pattern was observed for glutathione. GSH levels per tissue weight were stimulated in the cold by an order of magnitude, and levels of GSH/mg of protein were still around three times higher in cold-acclimated animals than in controls. Thus, of all the parameters studied, GSH seems to be

the principal antioxidant involved in adaptation to the increased peroxidative challenge that BAT encounters after activation. The adaptive character of changes in GSH is further supported by the fact that the increase in GSH was much larger than that in GSSG, resulting in a decreased GSSG/GSH ratio after cold acclimation.

In addition to a direct antioxidant role for GSH, this thiol is involved as a substrate for GPx and is thus needed for the detoxification of lipid peroxides by the enzyme. It should be pointed out that the results given here for the measurement of GPx activity *in vitro* were obtained at saturating concentrations of both the peroxide and GSH (4 mM for the rat enzyme). Thus the absence of changes in GPx activity/mg of protein means that the concentration of active enzyme did not change on a protein basis. Nevertheless, the saturating concentration for GSH mentioned previously (4 mM) is much higher than the GSH levels observed in the tissue, even after cold acclimation (0.8 mM). Thus the large increase in GSH concentration that occurs when the animal acclimates to cold will probably lead to a large increase in flux through GPx *in vivo*, even though the concentration of the enzyme/mg of protein did not change. This increase in GPx activity *in vivo* will be especially important when considering the capacity of the non-Se-dependent enzyme to specifically detoxify fatty acid hydroperoxides, since the large increase in sensitivity to lipid peroxidation (*in vitro* TBARS) can lead to a higher production of lipid hydroperoxides.

In summary, activation of IBAT in response to the cold acutely increases the sensitivity of the tissue to peroxidative damage. Adaptation to this oxidative stress seems to involve principally GSH and the coupled GPx–GR system for the scavenging of lipid hydroperoxides.

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