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Original Contribution

"Cold training" affects rat liver responses to continuous cold exposure

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ARTICLE INFO

ABSTRACT

Continuous exposure of homeothermic animals to low environmental temperatures elicits physiological adaptations necessary for animal survival, which are associated to higher generation of pro-oxidants in thermogenic tissues. It is not known whether intermittent cold exposure (cold training) is able to affect tissue responses to continuous cold exposure. Therefore, we investigated whether rat liver responses to continuous cold exposure of 2 days are modified by cold training (1 h daily for 5 days per week for 3 consecutive weeks). Continuous cold increased liver oxidative metabolism by increasing tissue content of mitochondrial proteins and mitochondrial aerobic capacity. Cold training did not affect such parameters, but attenuated or prevented the changes elicited by continuous cold exposure. Two-day cold exposure increased lipid hydroperoxide and protein-bound carbonyl levels in homogenates and mitochondria, whereas cold training decreased such effects although it decreased only homogenate protein damage in control rats. The activities of the antioxidant enzymes GPX and GR and H_2O_2 production were increased by continuous cold exposure. Despite the increase in GPX and GR activities, livers from cold-exposed rats showed increased susceptibility to in vitro oxidative challenge. Such cold effects were decreased by cold training, which in control rats reduced only H_2O_2 production and susceptibility to stress. The changes of PGC-1, NRF-1, and NRF-2 expression levels were consistent with those induced by cold exposure and cold training in mitochondrial protein content and antioxidant enzyme activities. However, the mechanisms by which cold training attenuates the effects of the continuous cold exposure remain to be elucidated.

1. Introduction

It is long known that homeothermic animals continuously exposed to low environmental temperature undergo physiological adaptations necessary for their survival. Indeed, they increase heat production to offset the increased heat loss, thus maintaining a relatively constant body temperature. During the sustained phase of cold acclimation, additive heat is produced by nonshivering thermogenesis (NST) [1], a process involving an array of changes in the whole metabolic activity of the organism. In fact, metabolic changes occurring mostly in brown adipose tissue [2,3] have also been found in other tissues including liver, skeletal muscle, and kidney [4].

It is also well known that the major components of the adaptive response to cold are increased in the number of mitochondria and in the activity of their electron transport system [5].

Several studies have shown the pivotal role of a transcriptional coactivator, the peroxisomal proliferator-activated receptor-γ

coactivator (PGC-1) [6], which activates the expression of regulatory proteins including nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) [7]. In turn, these factors activate the expression of the nuclear genes for components of the oxidative phosphorylation apparatus [7]. Since mitochondria are the main producers of reactive oxygen species (ROS) [8], cells that induce PGC1 in response to various physiological stimuli must adapt to an elevated generation of ROS. However, it seems that the deleterious consequences of the increased oxidative metabolism can be attenuated by the PGC1 ability to affect positively the expression of numerous ROS-detoxifying enzymes [9].

In a previous work, we found that the increase in PGC-1 expression elicited by continuous cold exposure in rat liver was associated to mitochondrial proliferation [10]. Despite the increase in the activity of antioxidant enzymes, such as GPX and GR, liver from cold exposed rats displayed a decrease in the whole antioxidant capacity and an increase in the oxidative damage to lipids and proteins [11].

It is known that regular physical activity (training), which has several healthy effects [12], induces changes in rat liver similar to those elicited by continuous cold exposure. Indeed, it increases liver PGC-1 expression, oxidative metabolism, mitochondrial proteins, and antioxidant enzyme activities [13]. However, differently

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from cold training, it increases the whole antioxidant capacity [14] and decreases the tissue oxidative damage [13,14].

The protocols of physical training and intermittent cold exposure exhibit the same limited duration of the daily treatments. Differently from the continuous cold exposure, the intermittent cold exposure has been scarcely studied. Such studies are interesting because humans experience such a condition when they go out of their warm house during the winter. This consideration has prompted us to investigate the possibility that intermittent cold exposure can act as a cold training, affecting liver oxidative metabolism and oxidative damage in rats kept at room temperature or continuously exposed to low environmental temperature. The tissue choice was due to observation that various environmental changes represent challenges for hepatic tissue due to its central role in the maintenance of body homeostasis and its function as energy supplier for the metabolic active tissues. In particular, 2 days of cold exposure induce similar increases in liver and muscle oxidative capacity, but higher oxidative damage in liver despite its higher antioxidant capacity [15], suggesting a strong impact of cold stress on hepatic tissue.

2. Materials and methods

2.1. Animals

The experiments were performed on thirty two male Wistar rats supplied by Nossan (Correzzana, Italy) at day 40 of age. All rats were subjected to the same conditions (one per cage, constant artificial circadian cycle of 12 h of light and 12 h of darkness, 24 °C and $50 \pm 10\%$ relative humidity), were fed the same control diet, a commercial rat chow purchased from Nossan, and received water on an *ad libitum* basis.

After 5 days, rats were randomly divided into two groups of sixteen animals each: control (C) group, and cold trained (T) group. The rats of C group were kept at 24 °C, whereas rats of the T group, during the light phase of the L:D cycle, were kept into a temperature-controlled chamber and exposed to 4 °C for 1 h and then returned to 24 °C. The procedure was repeated for 5 days in a week for 3 consecutive weeks. From day 70, half of C and T rats were continuously exposed for 2 days period to cold environment (4 °C). Thus, there were four groups of rats: control (C), continuous cold exposed (CE), cold trained (T), and cold trained and cold exposed (T+CE) rats.

Treatment, housing, and killing of animals met the guidelines set forth by the University's Italian Ministry. All experimental procedures involving animals were approved by the "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University "Federico II" of Naples.

2.2. Liver homogenate preparation

After the end of the period of cold exposure, all animals were sacrificed and livers were rapidly excised and placed into ice-cold homogenization medium (HM) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, 10 mM Tris, pH 7.4). Tissues, freed from connective tissue, were weighed, finely minced, and washed with HM. Finally, tissue fragments were gently homogenized (20% w/v) in the same solution using a glass Potter–Elvehjem homogenizer set at a standard velocity (500 rpm) for 1 min. Aliquots of homogenates were used for analytical procedures and preparation of mitochondrial fractions.

2.3. Preparation of mitochondria

The homogenates, diluted 1:1 with HM, were freed of debris

and nuclei by centrifugation at 500g for 10 min at 4 °C. The resulting supernatants were centrifuged at 10,000g for 10 min. The mitochondrial pellets were re-suspended in washing buffer (WB) (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4) and centrifuged at the same sedimentation velocity. Mitochondrial preparations were washed in this manner twice before final suspension in WB. Mitochondrial protein was measured by the biuret method [16].

2.4. Cytochrome oxidase activity

Cytochrome oxidase (COX) activity was determined at 30 °C by the procedure of Barré et al. [17] using liver homogenates and mitochondrial suspensions diluted with modified Chappel–Perry medium so that the preparations contained per ml either 100 mg of tissue or 0.2 mg of mitochondrial proteins.

2.5. Oxygen consumption

Oxygen consumption of homogenates and mitochondria was monitored at 30 °C by a Hansatech respirometer in 1 ml of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 50 µl of homogenate or 0.25 mg of mitochondrial protein per ml and 10 mM succinate plus 5 µM rotenone (Rot), or 10 mM pyruvate plus 2.5 mM malate as substrates, in the absence (State 4) and in the presence (State 3) of 500 µM ADP. State 4 oxygen consumption of homogenates was determined in the presence of oligomycin (2 µg/ml).

2.6. Oxidative damage

The extent of the peroxidative processes in liver preparations was determined by measuring the level of lipid hydroperoxides (HPs) according to Heath and Tappel [18]. Determination of protein oxidative damage was performed measuring protein-bound carbonyl levels by the procedure of Reznick and Packer [19] for homogenates and by the modified procedure of Schild et al. [20] for mitochondria.

2.7. Antioxidant enzyme activities

Glutathione peroxidase (GPX) activity was assayed at 37 °C according to Flohé and Günzler [21] with H_2O_2 as substrate. Glutathione reductase (GR) activity was measured at 30 °C according to Carlberg and Mannervik [22].

The rate of mitochondrial H_2O_2 release was measured at 30 °C following the increase in fluorescence (excitation at 320 nm, emission at 400 nm) due to oxidation of p-hydroxyphenylacetate (PHPA) by H_2O_2 in the presence of horseradish peroxidase (HRP) [23] in a computer-controlled Jasko fluorometer equipped with a thermostatically controlled cell-holder. The reaction mixture consisted of 0.1 mg ml^{-1} mitochondrial proteins, 6 U ml^{-1} HRP, 200 µg ml^{-1} PHPA, and 10 mM succinate, plus 5 µM rotenone, or 10 mM pyruvate plus 2.5 mM malate added at the end to start the reaction in a medium containing 145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, and 0.1 mM EGTA at pH 7.4. Measurements with different substrates in the presence of 500 µM ADP were also performed. Furthermore, the effects of two respiratory inhibitors were investigated: rotenone (Rot), which blocks the transfer of electrons from Complex I to ubiquinone [24], and antimycin A (AA), which interrupts electron transfer within the ubiquinone-cytochrome b site of Complex III [25]. Inhibitor concentrations (5 µM Rot, 10 µM AA) which do not interfere with the detection PHPA-HRP system were used [26].

2.8. Susceptibility to oxidative challenge

The susceptibility of liver homogenates to *in vitro* oxidative challenge was evaluated by the change in hydroperoxide levels induced by treatment of 10% tissue homogenate with Fe and ascorbate (Fe/As), at a concentration of 100/1000 μM, for 10 min at room temperature. The reaction was terminated by the addition of 0.2% 2,6-di-t-butyl-p-cresol (BHT) and the hydroperoxide levels were evaluated as previously described.

2.9. Western blot analysis

The levels of expression of PGC-1, NRF-1, NRF-2, and cytochrome c were determined by Western blot analysis. Liver fragments were gently homogenized (1:10, w/v) in 500 mM NaCl, 0.5% nonidet P-40, 6 mM EDTA, 6 mM EGTA, 1 mM dithiotreitol, 40 mM Tris–HCl, pH 8.0, in the presence of antiprotease mixture including 40 μg/ml PMSF, 5 μg/ml leupeptin, 5 g/ml aprotinin, 7 g/ml pepstatin. Homogenates were centrifuged at 1000g for 10 min at 4 °C and the resulting supernatants were used for sample preparation. Samples were prepared by diluting 10 μl of supernatant containing 1.5 mg/ml of proteins with 5 μl of 3% SDS, 30% glycerol, 15% β-mercaptoethanol, 0.1% bromophenol blue, 0.187 M Tris base, pH 6.8, and were boiled for 5 min before loading on the gel. Then, samples were electrophoresed through 6% stacking and 12% running SDS-PAGE gel according to Laemmli [27]. Gel was run in the mini protean equipment (Bio-Rad) for about 1 h at constant voltage (25 V). Separated hepatic proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with a 1:1000 dilution of antibodies to PGC-1 (SC-13067), NRF-1 (SC-33771), NRF-2 (SC-22810), and cytochrome c (SC-7159) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2.5% non-fat dry milk, 10% Tween 20. Rabbit polyclonal antibodies raised against amino acids 1-300 mapping near the N-terminus of PGC-1, 204-503 mapping at the C-terminus of NRF-1, 1-180 mapping near the N-terminus of NRF-2α, and 1-104 of cytochrome c were used. Antibody binding was detected by carrying out secondary antibody incubations using peroxidase-conjugated anti first IgG antibodies (Santa Cruz Biotechnology) diluted 1:4000. Secondary antibody was detected using the ECL system according to the manufacturer's recommendation (Santa Cruz Biotechnology). The blots were stripped by treating them for 10 min with 0.2 M NaOH followed by 5-min wash with H_2O and two 5-min washes with 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.1% Tween 20. The blots were again blocked for 30 min with 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2.5% non-fat dry milk, 10% Tween 20, washed as above, and incubated for 2 h with a 1:2000 dilution of anti-actin antibody (Santa Cruz Biotechnology) in blocking solution. Remaining procedures, as described for other antibodies, were followed. The actin was used for loading standardization. To compare protein expression levels among groups, a standard control sample was run on each gel and all group values were then compared with such a sample that was assigned a value of 1.

2.10. Statistical analysis

The data obtained in eight different experiments were expressed as means ± standard error. Data were analyzed with a two-way analysis of variance method. After a significant treatment effect (continuous cold exposure, cold training, or interaction) was found the Student–Newman–Keuls multiple range test was used to determine the statistical significance between means. The level of significance was chosen as $P < 0.05$. The values of P for significant effects of treatments or their interaction are reported in figures and table legend. All analyses were performed using GraphPad Prisma 6.5 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Cytochrome oxidase activities and mitochondrial protein content

COX activity in liver homogenates was significantly increased by cold exposure and was not modified by cold training, which attenuated the response to cold exposure so that, in T+CE group, COX activity was lower than in CE group and higher than in C and T groups (Fig. 1, upper panel). COX mitochondrial activity was significantly increased by cold exposure, but not by cold training, which prevented the cold exposure effect so that in T+CE group enzyme activity was lower than in CE group and not significantly different from those found in C and T groups (Fig. 1, upper panel). The ratio between COX activities in homogenates and mitochondria provided a rough estimate of tissue content of mitochondrial proteins. Such content was significantly increased by cold exposure and not modified by cold training, which reduced cold exposure effect so that, in T+CE group, protein content was lower than in CE group but higher than in C and T groups (Fig. 1, bottom panel).

3.2. Cytochrome c content

Cytochrome c content in liver homogenates was increased by cold exposure, but not by cold training, which attenuated cold
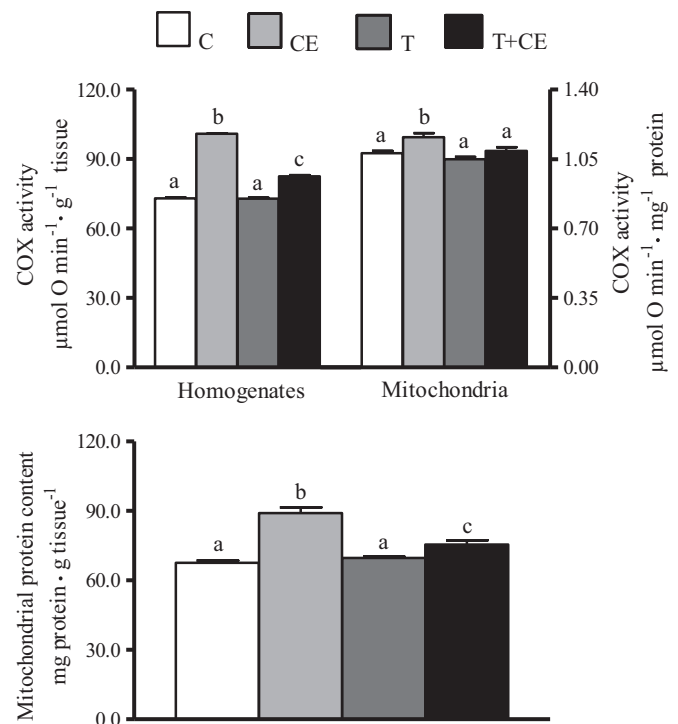

Fig. 1. Cytochrome oxidase activities (upper panel) and mitochondrial protein content (bottom panel) in liver from control (C), cold exposed (CE), cold trained (T), cold trained and cold exposed (T+CE) rats. Values are means ± S.E.M. of eight different experiments. Cytochrome oxidase activity (COX) is expressed in μmol O min^{-1} per milligram mitochondrial protein or g tissue. Mitochondrial protein is expressed as mg protein per g tissue. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0001$) on homogenate COX. Significant effect of cold exposure ($P=0.007$), and cold training ($P=0.037$) on mitochondrial COX. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.002$) and interaction between treatments ($P=0.0001$) on mitochondrial proteins.
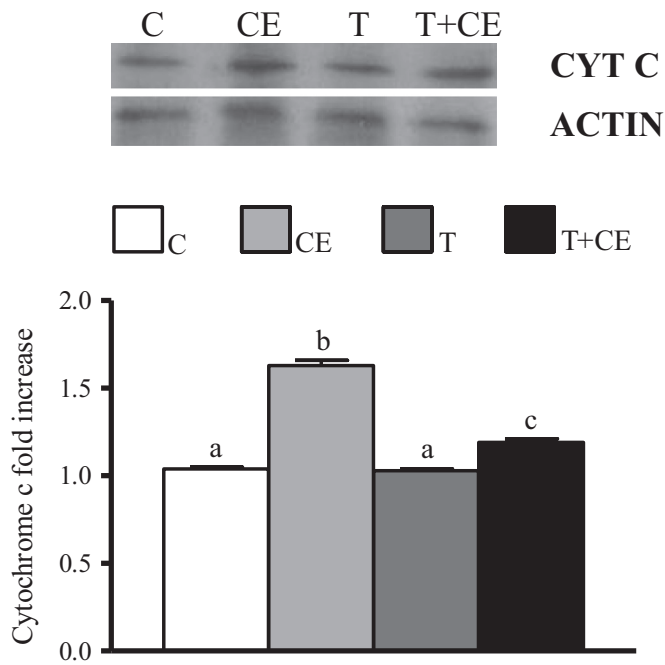
Fig. 2. Cytochrome c content in liver homogenates from control (C), cold exposed (CE), cold trained (T), cold trained and cold exposed (T+CE) rats. Liver total proteins were isolated and analyzed using Western blot analysis. Representative blot cytochrome c and actin protein expressions are shown (above). Bar graphs (below) correspond to the respective densitometric quantification (means ± S.E.M. of three independent experiments). Actin was used for loading standardization. Ratios of band intensities to the actin band intensities were compared with a standard control sample that was assigned a value of 1. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0001$).

exposure effect so that, in T+CE group, cytochrome c content was lower than in CE group but higher than in C and T groups (Fig. 2).

3.3. O_2 consumption

The rates of O_2 consumption by liver preparation are reported in Fig. 3. In homogenates (upper panel), the rates of succinate- and pyruvate/malate-supported O_2 consumption during State 4 were not affected by treatments, whereas during State 3 were increased by cold exposure, but not by cold training, which prevented the cold exposure effect so that, in T+CE group, the rates were lower than in CE group and not significantly different from those found in C and T groups. RCR values, which were 4.9 ± 0.2, 5.5 ± 0.2, 4.7 ± 0.1, and 5.0 ± 0.3 in the presence of succinate and 3.1 ± 0.2, 2.9 ± 0.3, 2.9 ± 0.3, and 2.9 ± 0.2 in the presence of pyruvate/malate for C, CE, T, and T+CE, respectively, were not significantly affected by cold exposure and cold training.

In mitochondria (bottom panel), the rates of succinate-supported O_2 consumption during State 4 were not affected by treatments, whereas during State 3 they were increased by cold exposure, and not modified by cold training, which prevented CE effect so that in T+CE group the rates were lower than in CE group and not significantly different from those found in T groups. RCR values, which were 4.9 ± 0.6, 4.9 ± 0.5, 4.7 ± 0.3, and 5.1 ± 0.4 for C, CE, T, and T+CE, respectively, were not significantly affected by treatments.

The rates of pyruvate/malate-supported O_2 consumption during State 4 were increased by cold exposure and were not affected by cold training, which did not modify the response to cold exposure, so that, in T+CE group, the rate was higher than in T group, but it was not significantly different from that found in both
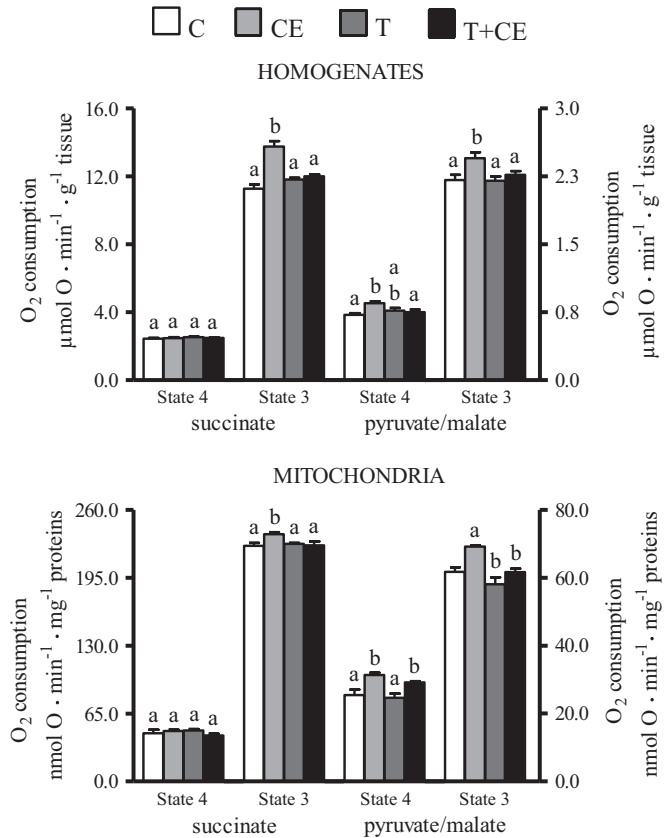

Fig. 3. Rates of O_2 consumption rat liver homogenates (upper panel) and mitochondria (bottom panel) from control (C), cold exposed (CE), cold trained (T), cold trained and cold exposed (T+CE) rats. Values are means ± S.E.M. of eight different experiments. Rates of O_2 consumption were measured in the absence (State 4) and in the presence (State 3) of ADP with Complex II (succinate) and Complex I (pyruvate/malate) linked substrates. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.011$), cold training ($P=0.0117$), and interaction between treatments ($P=0.0001$) on succinate-sustained State 3 homogenate respiration. Significant effect of cold exposure ($P=0.0397$), and interaction between factors ($P=0.0065$) on pyruvate/malate-sustained State 4 homogenate respiration. Significant effect of cold training ($P=0.01$) for pyruvate/malate-sustained State 3 homogenate respiration. Significant effect of interaction between treatments ($P=0.0261$) on succinate-sustained State 3 mitochondrial respiration. Significant effect of cold exposure ($P=0.0001$) on pyruvate/malate-sustained State 4 mitochondrial respiration. Significant effect of cold exposure ($P=0.0003$), and cold training ($P=0.0003$) on pyruvate/malate-sustained State 3 mitochondrial respiration.

CE and C groups. During the State 3, the rates were increased by cold exposure, and were not modified by cold training, which prevented the cold exposure effect so that, in the T+CE group, the rate was lower than in CE group, and not significantly different than that found in C and T groups. RCR values, which were 2.3 ± 0.4, 2.2 ± 0.3, 2.4 ± 0.5, and 2.1 ± 0.4 for C, CE, T, and T+CE, respectively, were not significantly affected by cold exposure and cold training.

3.4. Oxidative damage

The levels of indicators of lipid and protein damage are reported in Fig. 4. In liver homogenates, the levels of lipid hydroperoxides were significantly increased by cold exposure, and were not modified by cold training, which attenuated the cold exposure effect so that, in T+CE group, HP value was lower than in CE group and higher than in C and T groups. The levels of the protein-bound carbonyls were increased by cold exposure, and were slightly reduced by cold training, which also reduced the cold exposure effect so that, in T+CE group, protein damage was lower than in CE
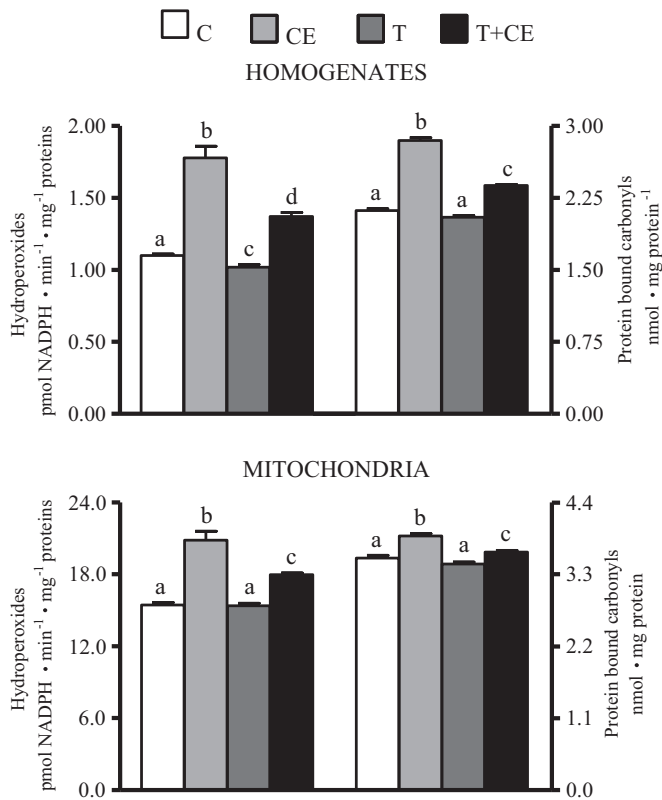
Fig. 4. Oxidative damage in liver homogenates (upper panel) and mitochondria (bottom panel) from control (C), cold exposed (CE), cold trained (T), cold trained and cold exposed (T+CE) rats. Values are means ± S.E.M. of eight different experiments. Hydroperoxides (HPs) are expressed in nmol NADP min^{-1} g^{-1} tissue and pmol NADP min^{-1} mg^{-1} mitochondrial protein. Protein-bound carbonyls (CO) are expressed in nmol mg^{-1} protein. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.008$) on homogenate hydroperoxides. Significant effect of continuous cold exposure ($P=0.0001$), cold training ($P < 0.0001$), and interaction between treatments ($P=0.0001$) on homogenate carbonyls. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0014$), and interaction between treatments ($P=0.0019$) on mitochondrial hydroperoxides. Significant effect of continuous cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0213$) on mitochondrial carbonyls.

Fig. 5. Antioxidants enzyme activity in liver homogenates (upper panel) and mitochondria (bottom panel) from C, control; CE, cold exposed; T, cold trained; T+CE, cold trained and cold exposed rats. Values are means ± S.E.M. For each value eight rats were used. Glutathione peroxidase activity (GPX) is expressed in μmol NADPH min^{-1} per g tissue. Glutathione reductase (GR) is expressed in μmol NADPH min^{-1} per g tissue. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0236$), and interaction between factors ($P=0.0025$) on homogenate GPX. Significant effect of cold exposure ($P=0.0002$) on homogenate GR. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between factors ($P=0.0001$) on mitochondrial GPX. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0069$), and interaction between treatments ($P=0.0003$) on mitochondrial GR.

group and higher than in C and T groups.

In liver mitochondria, the levels of lipid hydroperoxides were increased by cold exposure but not by cold training, which reduced cold exposure effect so that, in T+CE group, HP level was lower than in CE group and higher than in C and T groups. The levels of the protein-bound carbonyls were increased by cold exposure, and were not affected by cold training, which reduced the cold exposure effect so that, in T+CE group, protein damage was lower than in CE group, higher than in T group and not significantly different from that found in C group.

3.5. Antioxidant enzyme activities

The activities of antioxidant enzymes in the liver homogenates and mitochondria are reported in Fig. 5. In liver homogenates (upper panel), GPX activity was increased by cold exposure, and was not affected by cold training, which prevented the effect of cold exposure, so that, in T+CE group, GPX activity was lower than in CE group and not significantly different from that found in C and T groups. GR activity was increased by cold exposure, and was not affected by cold training, which did not modify the cold exposure effect so that, in T+CE group, GR activity was higher than in C and T groups, but not significantly different from that found in CE group.
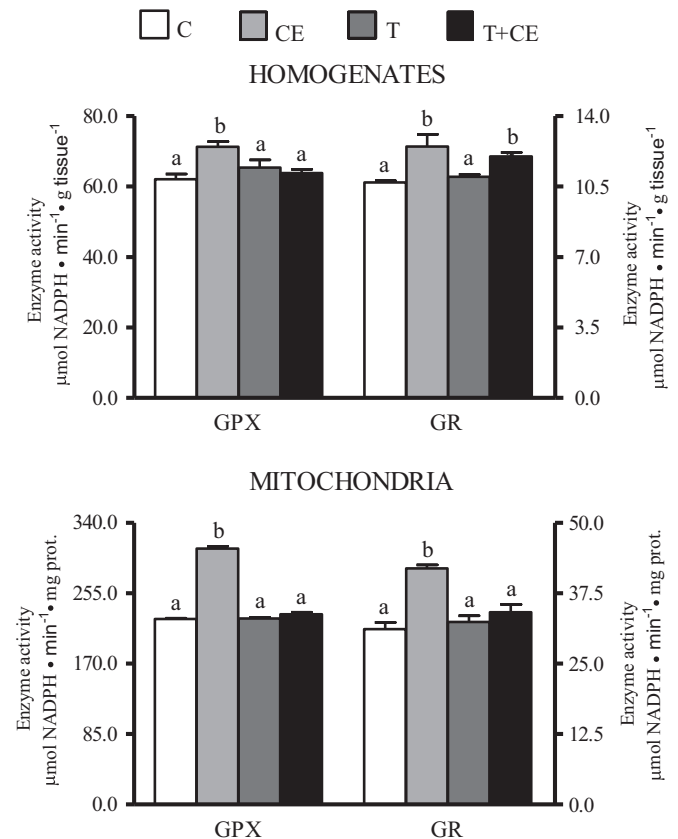
In liver mitochondria (lower panel), GPX and GR activities were increased in CE rats and were not modified in T rats. However, cold training prevented cold exposure effect so that both enzymatic activities in T+CE were lower than in CE group and not significantly different from those found in C group.

3.6. Mitochondrial H$_2$O$_2$ release

The rates of H$_2$O$_2$ mitochondrial release are reported in Table 1. The rate of succinate-supported H$_2$O$_2$ release was increased by cold exposure, and was decreased by cold training, which prevented cold exposure effect so that, in T+CE group, the rate was lower than in CE group, higher than in T group, and not significantly different from that found in C group. In the presence of rotenone, the rate was increased by cold exposure, and was decreased by cold training, which also reduced cold exposure effect so that, in T+CE group, the rate was lower than in CE group, and higher than in T and C groups. In the presence of ADP, the rates did not exhibit significant differences among group. In the presence of antimycin A, the rate was increased by cold exposure, and was decreased by cold training, which prevented cold exposure effect so that, in T+CE group, the rate was lower than in CE group, higher than in T group, and not significantly different from that found in C group.

Table 1
Rates of H_2O_2 release by rat liver mitochondria.

Substrate	Group			
	C	CE	T	T+CE
Succinate (Succ)	158.8 ± 0.7^a	165.6 ± 0.3^b	155.9 ± 0.5^c	159.9 ± 0.3^a
Succ+Rot	108.0 ± 0.7^a	118.1 ± 0.8^b	104.9 ± 0.3^c	111.9 ± 0.8^d
Succ+Rot+ADP	60.6 ± 0.6^a	62.0 ± 0.4^a	60.4 ± 0.3^a	60.4 ± 0.6^a
Succ+Rot+AA	819.9 ± 0.8^a	827.1 ± 2.4^b	814.2 ± 0.5^c	821.1 ± 0.7^a
Pyruvate/malate (Pyr/Mal)	240.4 ± 0.6^a	250.9 ± 1.8^b	234.9 ± 0.3^c	240.2 ± 0.7^a
Pyr/Mal+ADP	171.4 ± 0.7^a	172.8 ± 0.9^a	170.9 ± 0.6^a	172.0 ± 0.3^a
Pyr/Mal+AA	930.1 ± 2.3^a	948.5 ± 4.1^b	921.0 ± 1.9^c	921.1 ± 0.7^c
Pyr/Mal+Rot	269.5 ± 0.9^a	268.4 ± 1.3^a	267.1 ± 0.5^a	267.9 ± 0.7^a

Values are means $\pm$ S.E.M. For each value eight rats were used. Mitochondrial H_2O_2 release rate is expressed in pmol min^{-1} mg^{-1} protein. C, control; CE, cold exposed; T, cold trained; T+CE, cold trained and cold exposed rats. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0069$) in the presence of succinate. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0309$) in the presence of succinate, and rotenone. Significant effect of cold exposure ($P=0.0001$), and cold training ($P=0.0002$) in the presence of succinate, rotenone, and antimycin. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0168$) in the presence of pyruvate/malate. Significant effect of cold exposure ($P=0.0012$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0013$) in the presence of pyruvate/malate, and antimycin.

The rate of pyruvate/malate supported H_2O_2 release was increased by cold exposure, and was decreased by cold training, which prevented the cold exposure effect so that, in T+CE group, the rate was lower than in CE group, higher than in T group, and not significantly different from that found in C group. In the presence of ADP, the rates did not exhibit significant differences among groups. In the presence of antimycin A, the rate was increased by cold exposure, and was decreased by cold training, which also prevented the cold exposure effect so that, in T+CE group, the rate was lower than in CE group and C groups, and not significantly different from that found in T group. In the presence of rotenone, the rates did not exhibit significant differences among group.

3.7. Susceptibility to oxidative challenge

The susceptibility to *in vitro* oxidative challenge was increased by cold exposure, and was decreased by cold training, which reduced the cold exposure effect so that, in T+CE group, the susceptibility was lower than in CE group but higher than in C and T groups (Fig. 6).

3.8. PGC-1, NRF-1, and NRF-2 expression

The expressions of PGC-1, NRF-1, and NRF-2 were increased by cold exposure, and were not affected by cold training, which reduced the cold exposure effect so that, in T+CE group, the protein expression was lower than in CE group but higher than in C and T groups (Fig. 7).

4. Discussion

The increased tissue heat production characterizing metabolic response to cold stress arises from an increase in oxidative metabolism. COX is the final acceptor of electrons in the electron transport chain, and its *in vitro* activity is positively correlated to the maximal oxygen consumption [28] so that such an activity can be used as a measure of the tissue capacity for oxidative
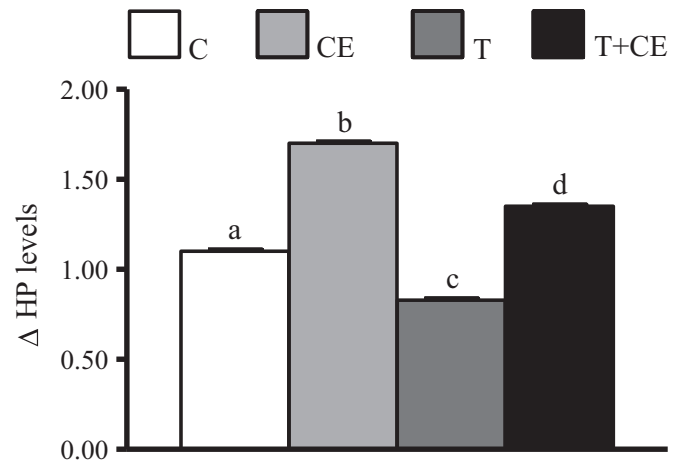

Fig. 6. Susceptibility to oxidative attack of liver homogenates from C, control; CE, cold exposed; T, cold trained; T+CE, cold trained and cold exposed rats. Values are means $\pm$ S.E.M. Liver susceptibility to oxidative stress was evaluated by change in Hp levels (pmol NADPH min^{-1} per g of tissue) after Fe/As treatment. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between treatments ($P=0.0004$).

metabolism. Increased COX activities were previously found in rat hepatic tissue after prolonged cold exposure (6 months) [29] and subsequently after shorter periods (2 and 10 days) [15].

The present results confirm the above findings as well as the observation that the small enhancement in tissue respiration elicited by 2 day cold exposure is due to increase in the mitochondrial respiratory capacity and tissue content of mitochondrial proteins [30]. Further support to observation that cold exposure induces mitochondrial biogenesis has been obtained by determining cytochrome c tissue expression, whose changes have been considered to supply information on tissue content of mitochondrial proteins [31].

The moderate effect of cold exposure on mitochondrial COX activity agrees with the previous finding that, cold exposure modifies mitochondrial population decreasing the percentage content of the heavy fraction provided with high oxidative capacity and increasing that of the light fraction provided with lower respiratory capacity [32]. The less increase generally found in ADP-stimulated (State 3) respiration of mitochondria and homogenates could be due to a fall in the activity of other respiratory complexes which should reduce the electron throughput. Support to such an idea is supplied by the observation that, in rat liver, mitochondrial content of cytochromes c+c1 and b decreases after 48 h of cold stress [33].

Differently from the continuous cold exposure, the intermittent one does not induce significant changes in COX activity and O_2 consumption but it attenuates or prevents the response to the subsequent continuous cold exposure. These cold training effects agreed with the results concerning tissue content of mitochondrial protein and cytochrome c. The effects of intermittent cold exposure have been scarcely studied. However, it was reported that rats subjected to such a procedure showed a certain degree of cold acclimation as manifested by better survival in extreme cold [34] and elevated adrenaline-induced thermogenesis [35].

It was also reported that intermittent (5 °C, 6 h/day, 4 weeks) and continuous cold exposure (5 °C, 4 weeks) resulted in the same cold adaptability as assessed by an enhanced cold tolerance (less drop of colonic temperature at -5 °C) and greater noradrenaline-induced heat production [36]. Since both procedures increase the weights of interscapular and dorsocervical brown adipose tissue (BAT) [36], it is conceivable that intermittent cold exposure, as well as continuous exposure [37], recruits nonshivering
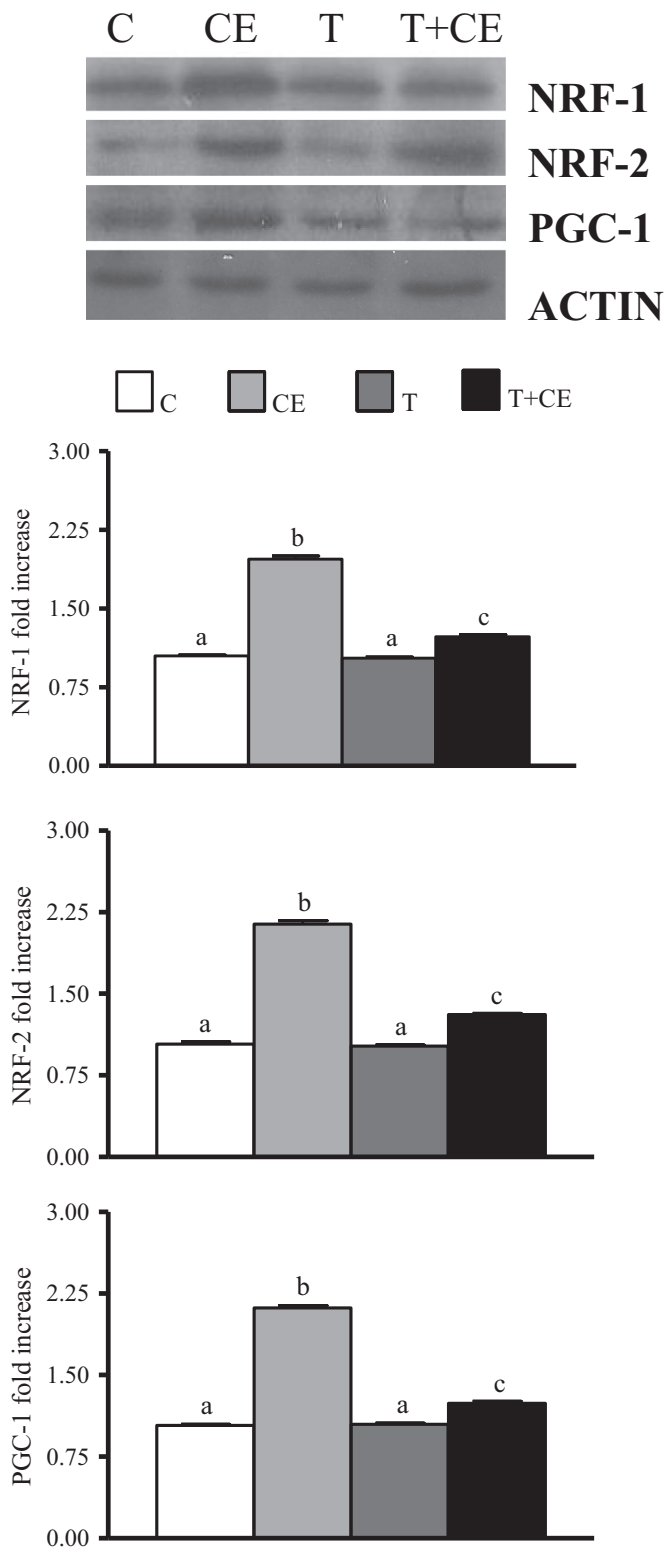
Fig. 7. Levels of NRF-1, NRF-2, and PGC-1 in rat liver. Liver total proteins from control (C), cold exposed (CE), cold trained (T) and cold trained and cold exposed (T+CE) rats, were isolated and analyzed using Western blot analysis. Representative blots of PGC-1, NRF-1, NRF-2, and actin protein expressions are shown (above). Bar graphs (below) correspond to the respective densitometric quantification (means ± S.E.M. of three independent experiments). Actin was used for loading standardization. Ratios of band intensities to the β-actin band intensities were compared with those of a standard control sample that was assigned a value of 1. Different letters indicate significant differences ($P < 0.05$) between groups. Significant effect of cold exposure ($P=0.0001$), cold training ($P=0.0001$), and interaction between factors ($P=0.0001$) on PGC-1, NRF-1, and NRF-2 levels.

thermogenesis in BAT. On the other hand, our observation that, in the liver, cold training does not increase the number of mitochondria and their respiratory capacity, and also attenuates those elicited by subsequent continuous cold exposure suggests that NST recruitment in BAT decreases the need for additional heat production by hepatic tissue. The idea that BAT recruitment can decrease the response of other tissues to cold exposure agrees with the finding that cold-induced oxidative stress in skeletal muscle is lower in wild-type than inUCP1-knockout mice [38].

On the other hand, continuous cold exposure also induces harmful responses that could be attenuated by intermittent cold exposure. In fact, it is known that the increased respiratory capacity displayed from rat liver following cold exposure is associated with oxidative stress [39]. This effect has been confirmed by the present results which show that 2-day continuous cold exposure increases the levels of indicators of lipid and protein oxidative damage. Such results have also shown that the intermittent cold exposure, which has scant effect on oxidative damage, is able to significantly reduce or prevent its increase due to subsequent exposure to continuous cold. This finding raises the question whether previous exposure to intermittent cold is convenient for animals exposed to continuous cold, since the intermittent cold attenuates oxidative damage of the liver but also its respiratory capacity. It is apparent that an affirmative answer to this question can be given only if after cold training additional heat produced by liver is really not indispensable for thermoregulation.

Another important issue concerns the mechanisms behind the protective effect of cold training against oxidative damage. Oxidative stress results from a disturbance of the normal cell balance between production of ROS and the capacity to neutralize their action. In previous works, we demonstrated that, in rat liver, cold exposure increases the rates of mitochondrial H_2O_2 release in the presence of Complex I and Complex II-linked substrates [40,41], and microsomal NADPH-dependent H_2O_2 production [41]. Moreover, we also found that cold exposure decreases whole antioxidant capacity and increases the susceptibility to oxidative challenge of the hepatic tissue [29], whereas it does not modify in coherent fashion the levels of single scavengers and the activities of the antioxidant enzymes we measured [29,41]. The results reported in this paper show that 2 day cold exposure increases the rates of mitochondrial H_2O_2 release during State 4 respiration, whereas intermittent cold exposure induces their little but significant decrease and attenuates their increase due to continuous cold.

Analyzing the effects of respiratory inhibitors on H_2O_2 release, which in their presence depends only on concentration of ROS generators, we found that the continuous and intermittent cold exposure induce opposite changes in the concentration of Complex III generator. Indeed, in the presence of antimycin, the changes in H_2O_2 release rates were irrespective of substrate, although they depend on Complex III generator in the presence of succinate+rotenone and on Complex I and III in the presence of pyruvate/malate. Moreover, the treatments did not modify the rates of H_2O_2 release when in the presence of pyruvate/malate and rotenone they were dependent only on Complex I generator.

The results concerning the activities of GPX and GR in homogenates and mitochondria show that intermittent cold reduces the increases elicited by continuous cold exposure even though it has no effect when applied to the control animals. GPX and GR play a pivotal role in redox homeostasis, but the measurement of their activities does not provide full information on whole effectiveness of the antioxidant defense system. Therefore, our results do not indicate that the cold training effect on oxidative damage is only due to the decrease in H_2O_2 release because the levels of other components of the antioxidant system could be increased following cold training. On the other hand, our finding that cold

training decreases tissue susceptibility to *in vitro* oxidative challenge does not allow concluding that this effect is due to an increase in the effectiveness of the antioxidant defense system. Indeed, the method we used is based on hydroperoxide measurement and other cold-induced changes in biochemical characteristics of the hepatic tissue, such as the unsaturation degree of membrane polyunsaturated fatty acids [42], can determine its susceptibility to peroxidative processes. A 2-day cold exposure increases lipid unsaturation degree in hepatic tissue [29], but it is not known whether cold training causes similar increase, which would allow hypothesizing that such procedure also increases liver antioxidant capacity.

To learn more on the possible relationship between cold training and liver antioxidant defenses, we determined the expression levels of PGC-1, which is involved in induction of the synthesis of antioxidant enzymes [43]. Furthermore, because PGC-1 has also emerged as a master regulator of mitochondrial biogenesis, which is actuated *via* regulation of the expression of transcription factors such as nuclear respiratory factors 1 and 2 (NRF-1 and NFR-2) [7], we also determined the expression of such factors. Such determinations revealed changes in PGC-1, NRF-1, and NRF-2 expression which were consistent with the effects of continuous and intermittent cold exposure on both mitochondrial protein content and antioxidant enzyme activity. However, they did not throw light on the mechanisms underlying the decrease, induced by cold training, in liver susceptibility to oxidative challenge. With regards to this, it is necessary to show that the activity and expression of PGC-1α respond to a variety of positive and negative signaling pathways [44–46]. Moreover, there is evidence that PGC-1α is not exclusively required for the expression of respiratory chain and antioxidant proteins [47], although it is required for normal basal expression levels. Because of the complexity of the signaling pathways involved in the regulation of the mitochondrial biogenesis as well as antioxidant defense system, other studies are necessary to establish the mechanisms underlying the liver response to our protocol of cold training.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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