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Research Article

Functional and biochemical characteristics of mitochondrial fractions from rat liver in cold-induced oxidative stress

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Abstract. We determined characteristics of rat liver mitochondrial fractions, resolved at 1000 (M_1), 3000 (M_3), and 10,000 g (M_{10}) after 2 and 10 days cold exposure. In all groups, the M_1 fraction exhibited the highest oxidative capacity, oxidative damage, H_2O_2 production rate, and susceptibility to stress conditions, and the lowest antioxidant levels. Cold exposure increased cytochrome oxidase activity in all fractions and succinate-supported O_2 consumption in the M_1 and M_{10} fractions during state 3 and state 4 respiration, respectively. With succinate, the H_2O_2 release rate increased in all fractions during state 4 and state 3 respiration, whereas with pyruvate/malate, it increased only during state 4 respiration. Increases in tissue mitochondrial proteins caused a faster H_2O_2 flow from the mitochondrial to cytosolic compartment, which was limited by the reduction in the M_1 fraction. Despite increased liposoluble antioxidant levels, cold also caused enhanced oxidative damage and susceptibility to oxidative challenge and Ca^{2+}-induced swelling in all fractions. These changes leading to elimination of H_2O_2-overproducing mitochondria avoided excessive tissue damage. We propose that triiodothyronine, whose levels increase in the cold environment, brings about the biochemical changes producing oxidative damage and those limiting its extent.

Key words. Cold exposure; thyroid hormone; oxidative damage; mitochondrial fractions; antioxidans; hydrogen peroxide release.

The mitochondrial population can be resolved by differential centrifugation into fractions with definable properties [1, 2], which can be altered according to the thyroid state [3, 4]. Early studies suggested that light mitochondrial fractions represent transitional forms in the process of development into heavy mitochondrial structures [4]. Subsequent investigation suggested that this process is controlled by thyroid hormone in both triiodothyronine (T_3)-treated and cold-exposed rats [5]. More recently, rat liver mitochondria have been resolved at 1000 (M_1), 3000 (M_3), and 10,000 (M_{10}) g, with the heaviest fraction containing mitochondria that are the largest and characterized by the greatest respiratory activity [6]. This fraction has also been found to exhibit the lowest antioxidant capacity, and the greatest capacity to produce reactive oxygen species (ROS) and the greatest susceptibility to stress conditions [7, 8]. Previous studies have also suggested that the lightest fraction, beside comprising light mitochondria with low respiratory activity and capacity to produce ROS, also contains a moderate amount of degenerating mitochondria, which increases following T_3 treatment [9].

T_3 administration induces rat liver oxidative stress [10], apparently as the result of decreased tissue antioxidant capacity [11] and enhanced mitochondrial capacity to produce ROS [12, 13]. Mitochondria also exhibit oxidative damage and oxidant susceptibility increases, which are due to corresponding increases in all mitochondrial fractions which compensate for the decrease in the relative amount of the heaviest fraction [9].

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Even a cold-induced increase in serum T_3 levels is associated with liver oxidative damage due to the reduced antioxidant capacity of the tissue [14] and increased flux of ROS from mitochondria to the cytosolic compartment [15]. Moreover, mitochondria from cold-exposed rats also share the high oxidative damage and susceptibility to oxidants of mitochondria from T_3-treated rats [15].

The effects of cold exposure on mitochondrial fractions have not been investigated. Knowledge about them would provide further information on mechanisms underlying the response of liver mitochondria to cold stress and the possible role played by thyroid hormone. Because serum T_3 levels, which peak after about 2 days [16], produce effects on mitochondrial protein mass and function within 10 days [16,17], here we examined the effects of 2 and 10 days cold exposure on the relative amount and functional and biochemical characteristics of rat liver mitochondrial fractions.

Materials and methods

Animals

The experiments were carried out on 60-day-old male Wistar rats, supplied by Nossan (Correzzana, Italy) at day 45 of age. Animals were randomly assigned to one of three groups: control (C), kept at a room temperature of 24 ± 1 °C, cold-exposed (CE_2 and CE_{10}), kept at 4 ± 1 °C for 2 or 10 days, respectively. All rats were subjected to the same conditions (one per cage, constant artificial circadian cycle of 12 h light and 12 h of darkness), and fed the same diet, a commercial rat chow purchased from Nossan, and water on an ad libitum basis.

Experimental procedure

Soon after a 12-h overnight fast, the animals were subjected to the measurement of resting metabolic rate (RMR) by an open-circuit indirect calorimetric system (Columbus Instruments International, Columbus, Ohio). The animals were killed by decapitation under ether anesthesia. Blood samples were collected and plasma levels of total (TT_3) and free (FT_3) triiodothyronine were determined using commercial RIA kits (DiaSorin, Salluggia, Italy). The livers were rapidly removed and placed in small beakers on ice. They were subsequently finely minced, weighed, and washed with ice-cold isolation medium (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 20 mM Tris, pH 7.4) containing 0.1% fatty acid-free albumin. Tissue fragments were gently homogenized in the same solution (1:10 w/v) using a Potter-Elvejem homogenizer set at a standard velocity (500 rpm) for 2 min. Aliquots of the homogenates were used for determination of cytochrome oxidase (COX) activity.

Preparation of mitochondrial fractions

Homogenates were freed of debris and nuclei by centrifugation at 500 g for 10 min at 4 °C. The resulting supernatants were centrifuged at 10,000 g for 10 min. The pellets designated as M_w (whole mitochondrial fractions) were washed twice with 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4. Aliquots were resuspended in the same solution and used either for determination of COX or subjected to a series of sequential centrifugation steps lasting 10 min at 1000, 3000, and 10,000 g. The pellets were designed M_1, M_3, and M_{10}, respectively. Aliquots of all mitochondrial fractions were resuspended in 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4, and used for biochemical determinations. Other aliquots were washed with 220 mM mannitol, 70 mM sucrose, 20 mM Tris, pH 7.4, resuspended in the same solution, and used for mitochondrial swelling.

In agreement with previous reports [7, 9], preliminary determinations of activities of marker enzymes showed that fractions M_1 and M_3 were virtually uncontaminated by other cellular organelles, whereas the M_{10} fraction was contaminated by microsomes.

The protein content in the mitochondrial preparations was determined, upon solubilization in 0.5% deoxycholate, by the biuret method [18] with bovine serum albumin as standard.

COX activity

COX activity was determined polarographically at 30 °C, using a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio), by the procedure of Barré et al. [19]. In brief, liver homogenates and mitochondrial suspensions, diluted with modified Chappel-Perry medium (1 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, 50 mM Hepes buffer, pH 7.4), were incubated at 0 °C for 30 min with Lubrol PX (0.5 mg/g tissue or 0.225 mg/mg of mitochondrial proteins) (Sigma, Milan, Italy) to unmask enzyme activity. Then, preparations were added to 1.5 ml of 30 µM cytochrome c, 4 µM rotenone, 0.5 mM dinitrophenol, 10 mM Na-malonate, 75 mM Hepes buffer, pH 7.4, so that the reaction medium contained 100 mg/ml tissue or 0.2 mg/ml mitochondrial proteins. COX activity was measured as the difference between the O_2 consumption, observed after the addition of substrate (4 mM Na-ascorbate with 0.3 mM N,N,N′,N′-tetramethyl-p-phenylene-diamine), and the rate of O_2 consumption, observed after the addition of substrate alone, in order to take the auto-oxidation of ascorbate into account.

In vitro COX activity is positively correlated with the maximal oxygen consumption, so that such an activity can be used as a measure of the aerobic metabolic capacity of biological preparations.

Oxygen consumption

Mitochondrial respiration was monitored at 30 °C by a Gilson respirometer in 1.6 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 0.25 mg mitochondrial protein per milliliter and succinate (10 mM) (plus rotenone 5 µM) or pyruvate/malate (10/2.5 mM) as substrates, in the absence (state 4) and presence (state 3) of 500 µM ADP.

H_2O_2 release

The rate of mitochondrial H_2O_2 release was measured at 30 °C following the linear 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) [20] in a computer-controlled Jasko fluorometer equipped with a thermostatically controlled cell holder. The reaction mixture consisted of 0.1 mg/ml mitochondrial proteins, 6 U/ml HRP, 200 µg/ml PHPA, and 10 mM succinate (plus rotenone 5 µM) or 10 mM pyruvate/2.5 mM malate added at the end to start the reaction in the same incubation buffer as used for measurements of O_2 consumption. Measurements with the different substrates in the presence of 500 µM ADP or 10 µM antimycin A were also performed. Known concentrations of H_2O_2 were used to establish the standard concentration curve. In preliminary experiments, the effect of catalase addition on the measured rates of H_2O_2 production was studied. Such experiments showed a dose-dependent drop in fluorescence in the presence of the enzyme.

The effects of two respiratory inhibitors were also investigated: rotenone (Rot), which blocks the transfer of electrons from complex I to ubiquinone [21], and antimycin A (AA), which interrupts electron transfer within the ubiquinone-cytochrome b site of complex III [22]. Inhibitor concentrations (5 µM Rot, 10 µM AA), which do not interfere with the detection PHPA-HRP system [13], were used.

Capacity to remove H_2O_2

Capacity to remove H_2O_2 (CR) was determined by comparing the ability of mitochondrial samples to reduce H_2O_2-linked fluorescent emission with that of desferrioxamine solutions [23]. H_2O_2 was generated by glucose oxidation catalyzed by glucose oxidase (GOX). The nonfluorescent substrate PHPA was oxidized to the stable fluorescent product 2,2′-dihydroxy-biphenyl-5,5′-diacetate $(PHPA)_2$ [20] by the enzymatic reduction of H_2O_2 catalyzed by HRP. Fluorescence was monitored on the Jasko fluorometer (excitation wavelength 320 nm, emission wavelength 400 nm). Assays were performed in quartz fluorometer cuvettes containing a magnetic stirrer and maintained at 30 °C. The reaction was started by adding 10 µl of 80 µg/ml GOX to a mixture containing 0.2 mg/ml PHPA, 6 U/ml HRP, 5 mM glucose in 145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4. After 100 s, 10 µl of desferrioxamine solutions (containing from 1 to 12 nmol), or mitochondrial samples (containing from 0.1 to 1 mg of mitochondrial proteins) were added to 2.0 ml final volume. The additions were made to the cuvettes via externally mounted syringes. The values of fluorescence change for unit of time obtained after addition of desferrioxamine or mitochondria were converted to a relative percentage of the values obtained before the addition. The values for desferrioxamine were used to fit standard curves by the Fig. P program (Biosoft, Cambridge, Mass.). The values for samples served to obtain, from equations describing the standard curves, evaluations of their capacity to remove H_2O_2, expressed as equivalent desferrioxamine concentration.

Oxidative damage to lipid and proteins

The extent of the peroxidative processes in mitochondrial fractions was determined by measuring the level of lipid hydroperoxides (HPs) according to Heath and Tappel [24].

Protein-bound carbonyls were determined by the procedure of Schild et al. [25]. For each determination, three trichloroacetic acid (TCA)-precipitated samples containing 1 mg mitochondrial protein were dissolved in 300 µl of 0.1 M NaOH for 5 min. Two samples were treated with 3 ml of 10 mM dinitro- phenylhydrazine in 2.5 M HCl for 1 h at room temperature. The third sample (the blank) was incubated with 2.5 M HCl. The reaction was stopped by adding 3.3 ml of 20% TCA. The pellets were washed twice with 3 ml of 10% TCA. The protein pellets were washed twice with absolute ethanol/ethylacetate (1:1) and once with 3 ml of 10% TCA. The protein pellets were finally dissolved in 6 M guanidine hydrochloride and the absorption at 370 nm (dinitrophenylhydrazine minus sample blank) was determined. Protein recovery was estimated for each sample. The carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22,000 M^{-1} cm^{-1} and expressed as nmol carbonyl/mg of protein.

Antioxidant levels

Ubiquinols ($CoQH_2$) were oxidized to ubiquinones (CoQs) with ferric chloride as the oxidation reagent. $CoQH_2$ were oxidized from 0.5 ml mitochondrial suspension with 0.5 ml of 2% $FeCl_3$ and 2.0 ml ethanol. CoQs were extracted by 5.0 ml n-hexane, which was then removed by evaporation under N_2 at 40 °C. The residue was dissolved in ethanol and subjected to the mobile phase in HPLC (machine: SpectraSeries P100 isocratic pump; Thermo Separation Products, San Jose, Calif.; column: Ultremex 5 250 × 4.6 mm, 5-µm particle size; Phenomenex, Torrance, Calif.). The eluant was a mixture of metha-

nol/ethanol 3/7 (v/v) containing 20 mM lithium perborate, and the flow rate was 1 ml/min [26]. The total content of CoQs ($CoQH_2$ + CoQ) was then determined. The eluted CoQs were determined separately using a SpectraSeries UV100 detector (Thermo Separation Products, San Jose, Calif.) (275 nm). Quantitation was obtained by using external standards.

For vitamin E determination, mitochondrial fractions were deproteinized with methanol and extracted with n-hexane. The extracts were evaporated under N_2 at 40 °C and the residues were dissolved in ethanol. Vitamin E content was determined using the HPLC procedure of Lang et al. [26]. Quantification was obtained using an external standard.

For reduced glutathione (GSH) determination, mitochondrial suspensions were diluted (1:1) with 0.5 N $HClO_4$, centrifuged at 2000 g for 10 min at 4 °C, and the supernatants neutralized with NaOH and diluted 1:7 with 0.1 M sodium phoshate buffer, pH 7.4. GSH concentration was measured as described by Griffith [27].

Susceptibility to oxidative stress

Response to oxidative stress was determined as previously described [28]. Briefly, several dilutions of the mitochondrial suspensions in the protein concentrations range 20–0.005 mg/ml were prepared with 15 mM Tris (pH 8.5). The assays were performed in microtiter plates. Enhanced chemiluminescence reactions were initiated by addition of 250 µl of the reaction mixture to 25 µl of the samples. The reaction mixture was obtained by dissolving a tablet containing substrate in excess (sodium perborate) and signal-generating reagents (sodium benzoate, indophenol, and luminol) (Amerlite Signal Reagent Tablets) in buffer at pH 8.6 (Amerlite Signal Reagent Buffer). The plates were incubated at 37 °C for 30 s under continuous shaking and then transferred to a luminescence analyzer (Amerlite Analyzer). The emission values were fitted to dose-response curves using the statistical facilities of the Fig.P graphic program (Biosoft).

Mitochondrial swelling and transmembrane electrical potential

Mitochondrial swelling was spectrophotometrically measured by determining the apparent absorbance at 540 nm in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 2 mM succinate, 4 µM Rot, 0.3 mg mitochondrial protein/ml, 100 µM Ca^{2+}, and 1 mM EGTA or 1 µM cyclosporin A (CSA) where indicated.

The mitochondrial membrane potential ($\Delta\Psi$) was estimated through fluorescence changes of safranine (8 µM), recorded on the Jasko fluorometer (excitation wavelength 495 nm, emission wavelength 586 nm) in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 2 mM succinate, 6 µM rotenone, 0.3 mg mitochondrial protein/ml reaction mixture, 100 µM Ca^{2+}. $\Delta\Psi$ was calculated according to Åkerman and Wikström [29] using a calibration curve obtained by incubating mitochondria in a medium containing 200 mM sucrose, 10 mM Hepes, pH 7.2, 6 µM rotenone, 0.38 mM EDTA, 8 µM safranine, 38.5 ng/ml valinomycin, and KCl at concentrations from 0 to 0.96 mM.

Statistical analysis

The data obtained in eight different experiments were expressed as mean ± SE. Statistical analysis was performed using one-way or two-way analysis of variance as appropriate. When a significant F ratio was found, the Student-Newman-Keuls multiple-range test was used to determine the statistical significance of differences between individual means. Probability values ($p < 0.05$) were considered significant.

Results

Thyroid state assessment

The thyroid state was documented by modifications in (i) the heart weight/body weight (HW/BW) ratio, (ii) the resting metabolic rate, and (iii) the plasma levels of FT_3 and TT_3 (table 1). While body weight was not significantly affected by cold exposure, the heart weight increased so that CE_{10} rats exhibited a HW/BW ratio increased in comparison to the controls. The RMR and plasma levels of FT_3 and TT_3, were significantly increased after 2 days of cold exposure and did not undergo further increases after a further 8 days of such exposure.

COX activity

COX activities in homogenates and the whole mitochondrial population were significantly increased by 2 days of cold exposure. Further increases were induced by 10 days of cold exposure (table 2).

In all groups, the enzyme activity was higher in the M_1 and lower in the M_{10} fraction. Furthermore, COX activity increased in the M_1 and M_3 mitochondria after 2 days and in M_{10} mitochondria after 10 days of cold exposure.

Mitochondrial protein content

The ratio between the COX activities in liver homogenates and the whole mitochondrial fraction supplied a rough estimate of the hepatic content of mitochondrial proteins. The 2-day cold exposure was associated with an increase in such a content, whereas the 10-day exposure did not induce any further significant increase (fig. 1, upper panel).

The percentage content of the M_{10} fraction proteins in the mitochondrial population, which was the least abundant, was not significantly different in the various groups. Conversely, the content of the M_1 proteins, which was the highest in controls animals, decreased significantly dur-

Table 1. Indicators of thyroid state in control and cold-exposed rats.

Parameters	Groups		
	C	CE_2	CE_{10}
HW/BW (mg/g)	2.45±0.04	2.85±0.06[a]	3.14±0.08[a,b]
RMR (l O_2/min per kilogram BW)	1.49±0.05	2.24±0.10[a]	2.36±0.06[a]
TT_3 (ng/dl)	57 ±6	140 ±12[a]	128 ±7[a]
FT_3 (pg/dl)	308 ±30	550 ±39[a]	525 ±26[a]

Values are the mean ± SE of eight different experiments. HW/BW, heart weight/body weight; RMR, resting metabolic rate; TT_3 and FT_3, serum level of total and free triiodothyronine, respectively. [a] Significant vs C rats; [b] significant vs CE_2 rats. The level of significance was chosen as $p < 0.05$.

Table 2. Effect of cold exposure on COX activity of mitochondrial fractions from rat liver.

Preparation	Groups		
	C	CE_2	CE_{10}
Homogenate	73.7 ±1.6	114.5 ±6.2[a]	134.1 ±6.90[a,b]
M_w	1.02±0.05	1.22±0.04[a]	1.32±0.02[a,b]
M_1	1.23±0.08	1.48±0.06[a]	1.57±0.07[a]
M_3	1.01±0.06[c]	1.19±0.11[a,c]	1.28±0.01[a,c]
M_{10}	0.41±0.04[c,d]	0.46±0.02[c,d]	0.69±0.07[a,b,c,d]

Data represent the mean ± SE of eight experiments. COX activity is expressed as µmol O/min per gram for liver and µmol O/min per milligram protein for mitochondria. [a] Significant vs C rats; [b] significant vs CE_2 rats; [c] significant vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

ing cold exposure and became lower than the content of the M_3 fraction (fig. 1, lower panel).

Lipid peroxidation and protein-bound carbonyls

In all mitochondrial fractions from 10-day cold-exposed rats, hydroperoxide levels were higher than control values. Similar results were obtained for protein carbonyls. However, in M_1 and M_{10} fractions, protein carbonyl levels were already significantly higher after 2 days cold exposure (fig. 2).

The extent of the oxidative reaction of lipids and proteins was generally greater in the M_1 than in the M_3 and M_{10} fractions. However, not all differences were significant (fig. 2).

Oxygen consumption

With succinate as the substrate, in the control group, the M_{10} fraction exhibited the lowest rate of state 4 O_2 consumption, whereas in the other groups, this rate was not significantly different in the three fractions. Moreover, the respiratory rate of the M_{10} fraction was the only one which underwent a gradual increase during cold exposure. In all groups, the state 3 respiration rate was the highest and lowest in the M_1 and M_{10} fractions, respectively, and increased after 10 days cold exposure only in the M_1 fraction (table 3).

With pyruvate/malate as substrates, there were no differences in state 4 respiratory rates among groups, whereas there was a difference between the M_1 and M_{10} fraction in the control group. The state 3 respiratory rates, which, in all groups, exhibited the highest values in the M_1 fraction and the lowest values in the M_{10} fraction, were not affected by cold exposure (table 3).

H_2O_2 release

In the presence of succinate, in both C and CE_{10} groups, the rates of H_2O_2 release during state 4 and state 3 respiration were significantly higher in M_1 than in other fractions, whereas in the CE_2 group, differences were not found between M_1 and M_3 fractions in state 3. Cold exposure increased the above rates during basal and stimulated respiration, after 10 days, in M_1, and, after 2 days, in M_3 fractions. Moreover, in the M_{10} fraction, cold exposure increased release rates, after 2 days, during basal respiration, and, after 10 days, during stimulated respiration (table 4).

An almost similar pattern was found when pyruvate and malate were used as the substrates, but cold exposure did not affect H_2O_2 release rates by any fraction during stimulated respiration (table 4).

Effect of inhibitors on H_2O_2 release

As shown in table 5, in all groups and fractions the succinate-supported H_2O_2 release rates were lowered by Rot and increased sometimes by further addition of AA. Moreover, inhibitor presence sometimes affected both

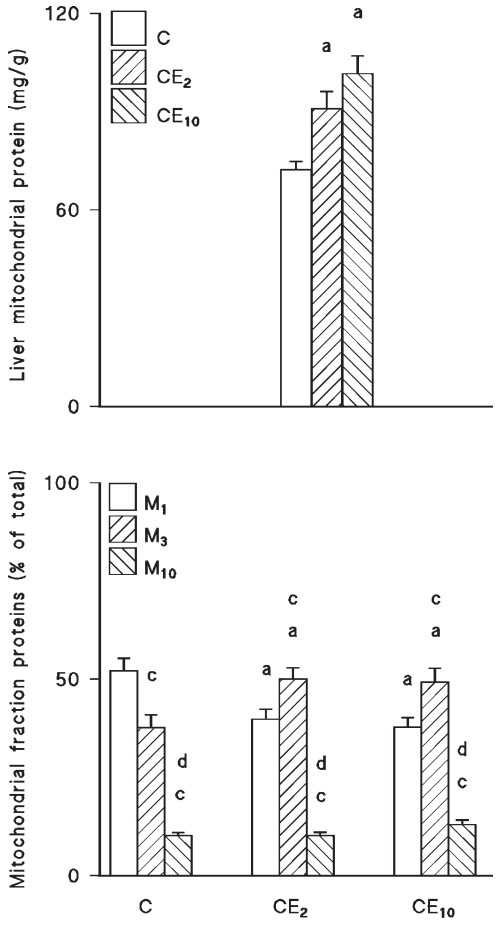

Figure 1. Effect of cold exposure on mitochondrial protein content (upper panel) and protein distribution among mitochondrial fractions (lower panel) in rat liver. Preparations from control (C), 2-day cold-exposed (CE_2), and 10-day cold-exposed (CE_{10}) rats. Data represent the mean ± SE of eight experiments. [a] Significant vs C rats; [b] significant vs CE_2 rats; [c] significan vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

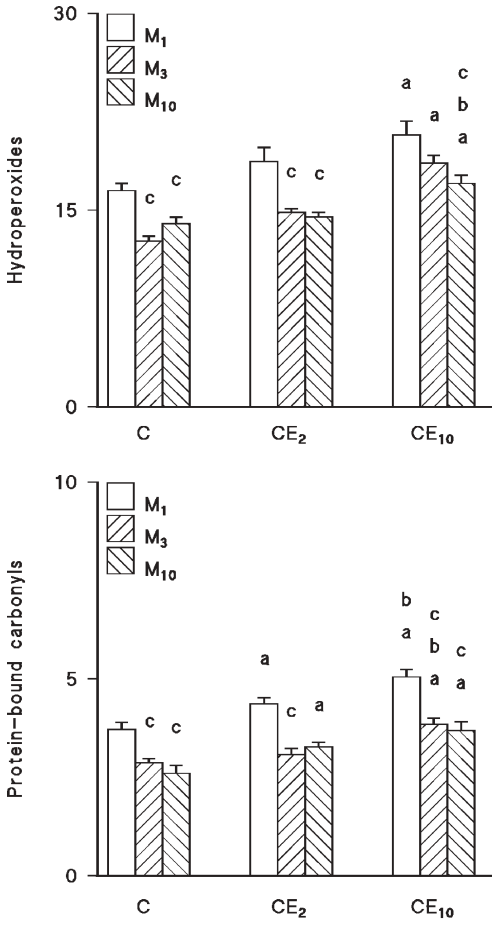

Figure 2. Effect of cold exposure on oxidative damage of mitochondrial fractions. Hydroperoxides (upper panel) are expressed as pmol NADPH/min per milligram protein. Protein-bound carbonyls (lower panel) are expressed as nmol/mg protein. Preparations from control (C), 2-day cold-exposed (CE_2), and 10-day cold-exposed (CE_{10}) rats. Data represent the mean ± SE of eight experiments. [a] Significant vs C rats; [b] significant vs CE_2 rats; [c] significant vs M_1 fraction. The level of significance was chosen as $p < 0.05$.

Table 3. Effect of cold exposure on oxygen consumption of mitochondrial fractions from rat liver.

Group	Fraction	Succinate			Pyruvate/malate		
		state 4	state 3	RCR	state 4	state 3	RCR
C	M_1	34.8±1.3	246.7±13.0	7.5±0.7	10.3±0.7	32.2±1.9	3.2±0.3
	M_3	31.7±2.0	196.4±4.5[c]	6.3±0.4	9.4±0.6	26.1±0.9	3.3±0.3
	M_{10}	20.4±1.5[c,d]	71.1±6.5[c,d]	2.7±0.1	7.0±0.5[c]	15.5±0.7[c,d]	1.9±0.1
CE_2	M_1	35.1±2.4	256.2±10.7	7.5±0.6	9.1±1.2	34.3±1.6	3.0±0.3
	M_3	29.1±2.1	196.0±10.6[c]	6.3±0.5	9.4±0.8	29.2±1.1	2.9±0.3
	M_{10}	28.2±1.9[a]	70.7±2.6[c,d]	2.1±0.2	8.4±1.1	16.3±1.3[c,d]	1.8±0.1
CE_{10}	M_1	37.7±1.5	276.1±11.1[a]	8.0±0.5	10.8±0.8	36.7±2.5	3.0±0.1
	M_3	31.9±1.8	196.9±4.5[c]	6.7±0.3	10.4±0.4	28.7±1.5[c]	3.0±0.2
	M_{10}	38.3±3.2[a,b]	68.2±6.3[c,d]	1.7±0.2	9.3±0.8	14.2±1.4[c,d]	1.8±0.2

Data represent the mean ± SE of eight experiments. Oxygen consumption rates are expressed in nmol O/min per milligram protein.
[a] Significant vs C rats; [b] significant vs CE_2 rats. [c] significant vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

Table 4. Effect of cold exposure on H_2O_2 release by succinate and pyruvate/malate-supplemented mitochondrial fractions from rat liver.

Fraction	Substrate and additions	Group		
		C	CE_2	CE_{10}
M_1	succinate	119.6±4.3	121.1±1.6	134.7±5.3[a,b]
	succinate + ADP	68.6±4.1	69.1±2.2	78.2±2.2[a,b]
	pyruvate/malate	259.2±1.5	264.3±3.8	283.0±4.4[a,b]
	pyruvate/malate + ADP	172.5±1.3	177.5±1.1	181.1±7.3
M_3	succinate	81.1±1.1[c]	109.1±1.7[a,c]	120.1±1.1[a,b,c]
	succinate + ADP	53.2±0.9[c]	63.3±4.1[a]	66.4±1.7[a,c]
	pyruvate/malate	239.9±4.4[c]	257.3±1.9[a]	263.0±1.8[a,c]
	pyruvate/malate + ADP	164.7±1.8	172.6±2.5	168.3±3.5[c]
M_{10}	succinate	60.7±1.7[c,d]	102.0±1.0[a,c]	113.0±1.7[a,b,c]
	succinate + ADP	37.0±1.1[c,d]	41.5±2.4[c,d]	52.0±2.1[a,b,c,d]
	pyruvate/malate	193.3±4.7[c,d]	205.8±1.7[a,c,d]	210.4±7.4[a,c,d]
	pyruvate/malate + ADP	154.3±2.5	157.1±1.2[c,d]	161.3±2.7[c]

Data represent the mean ± SE of eight experiments. H_2O_2 release rates are expressed in pmol/min per milligram protein. [a] Significant vs C rats; [b] significant vs CE_2 rats; [c] significant vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

Table 5. Effect of inhibitors on H_2O_2 release by liver mitochondrial fractions from control and cold exposed rats.

Fraction	Substrate and additions	Group		
		C	CE_2	CE_{10}
M_1	succinate (succ)	170.3±2.4	164.1±1.7	185.4±1.7[a,b]
	succ + Rot	116.2±4.5 *	118.5±0.8 *	133.1±3.8[a,b,] *
	succ + Rot +AA	875.9±4.7 *	919.0±12.5 *	934.7±29.3 *
	pyruvate/malate (pyr/mal)	258.7±0.7	265.3±3.1	284.9±2.7[a,b]
	pyr/mal + AA	949.3±6.0 *	973.1±2.8 *	973.9±9.2 *
	pyruvate/malate	258.8±1.1	263.2±3.0	282.0±5.8[a,b]
	pyr/mal + Rot	304.1±2.3 *	308.9±12.5 *	308.1±4.6 *
M_3	succinate	154.8±2.8	154.7±1.7	154.0±3.2[c]
	succ + Rot	82.9±2.2[c,] *	108.8±1.9[a,c,] *	119.2±1.8[a,b,c] *
	succ +Rot + AA	854.2±14.6 *	884.2±24.3 *	894.3±15.4 *
	pyruvate/malate	235.8±4.3[c]	259.0±1.5[a]	267.4±2.4[a,c,]
	pyr/mal + AA	921.9±21.0 *	926.0±7.0 *	938.0±18.0 *
	pyruvate/malate	238.2±6.2[c]	258.8±1.7[a]	265.7±2.4[a,c]
	pyr/mal + Rot	269.9±2.9[c,] *	283.2±1.8[c,] *	280.2±5.0[c,] *
M_{10}	succinate	115.4±0.9[c,d]	128.1±2.5[a,c,d]	159.5±11.6[a,b,c]
	succ + Rot	60.6±1.7[c,d,] *	102.2±1.0[a,c,] *	112.5±1.2[a,b,c] *
	succ + Rot + AA	777.5±19.8[c,d,] *	874.4±14.7[a,] *	886.1±11.7[a,] *
	pyruvate/malate	192.8±3.8[c,d]	207.3±1.3[a,c,d]	209.4±4.6[a,c,d]
	pyr/mal + AA	821.8±9.1[c,d,] *	865.7±10.9[a,c,d,] *	892.7±16.1[a,c,] *
	pyruvate/malate	190.5±4.5[c,d]	202.1±0.8[a,c,d]	210.3±4.1[a,c,d]
	pyr/mal + Rot	221.1±10.2[c,d,] *	236.8±0.8[c,d,] *	235.7±5.7[c,d,] *

Data represent the mean ± SE of eight experiments. H_2O_2 release rates are expressed in pmol/min per milligram protein. [a] Significant vs C rats; [b] significant vs CE_2 rats; [c] significant vs M_1 fraction; [d] significant vs M_3 fraction. * Significant effect of the last inhibitor added vs mitochondria under the same conditions without that inhibitor. The level of significance was chosen as $P < 0.05$.

fraction and treatment-linked differences. Thus, in the presence of Rot, in C and CE_2 groups, the rates of H_2O_2 release became significantly different in M_1 and M_3 fractions, whereas there was no difference in the M_3 and M_{10} fractions from the CE_2 group. Cold exposure increased such rates in the M_3 fraction already after 2 days. In the presence of AA, for both the CE_{10} and the CE_2 group, there were no differences between the three mitochondrial fractions.

Pyruvate/malate-supported H_2O_2 release rates were strongly increased by addition of AA whereas they were slightly increased by Rot addition. Even in this case, the cold and fraction-linked effects on the release rates in the presence of the inhibitors were sometimes different from those found in the presence of the substrate alone. Thus, in the presence of AA, the rates of H_2O_2 release were not different in the M_1 and M_3 fractions from the C and CE_{10} groups and in the M_3 and M_{10} fractions from the CE_{10}

group. Cold exposure did not increase such rates in the M_1 and M_3 fractions. In the presence of Rot, differences were found between the release rates in M_1 and M_3 fractions from the CE_2 group, and cold exposure did not increase the rates in any fraction.

Capacity to remove H_2O_2

The capacity of the three fractions to remove H_2O_2 was not affected by cold exposure, whereas, in all treatment groups, it was not significantly different in the M_1 and M_3 fractions and was lower in the M_{10} fraction (fig. 3).

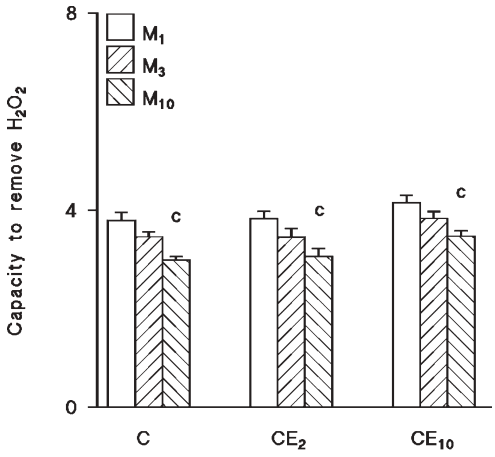

Figure 3. Effect of cold exposure on the capacity of mitochondrial fractions to remove H_2O_2. The capacity to remove H_2O_2 is expressed as equivalent concentration of desferrioxamine (nmol/mg protein). Preparations from control (C), 2-day cold-exposed (CE_2), and 10-day cold-exposed (CE_{10}) rats. Data represent the mean ± SE of eight experiments. [c] Significant vs M_1 fraction. The level of significance was chosen as $p < 0.05$.

Antioxidants

In all groups, the vitamin E content was lower in the M_1 fraction. Moreover, the vitamin E content was increased in all fractions after 2 days and underwent a further increase in M_1 and M_3 fractions after 10 days of cold exposure. Coenzyme Q9 levels reached a higher value already after 2 days cold exposure in M_1, whereas they increased progressively in the M_3 fraction and were high only after 10 days in the M_{10} fraction. Moreover, coenzyme Q9 levels, which were not significantly different in the fractions from C rats, were lower in the M_{10} fraction after 2 days and in M_1 and M_{10} fractions after 10 days of cold exposure. The coenzyme Q10 levels increased after 10 days cold exposure in all fractions, reaching the highest value in the M_{10} fraction. The levels of GSH, which were lower in the M_{10} fraction, were decreased in all fractions by 2 days and restored to control values by 10 days cold exposure (table 6).

Response to oxidative stress

The relationship between light emission (E) and protein concentration (C) of mitochondria stressed by sodium perborate (fig. 4) was described by the same equation $[E = a\,C/\exp(b\,C)]$ used in previous studies [8, 28]. In this equation, the *a* value depends on the concentration of substances, such as the cytochromes, able to react with H_2O_2 to produce the $HO^{\bullet}$ radicals inducing the luminescent reaction. The *b* value depends on the concentration of substances able to prevent the formation or interacting with $HO^{\bullet}$ radicals, thus reducing the levels of light emission. The emission maximum ($E_{max} = a/e\,b$) can indicate the susceptibility of the preparations to oxidative challenge [28]. The curves in figure 4 show that the susceptibilities of the mitochondrial fractions from C rats were the lowest and cold-induced increases in such susceptibilities depended on the period of cold exposure. These qualitative evaluations are confirmed by the E_{max} values reported in table 7. Examination of the parameters characterizing light emission shows that differences in the

Table 6. Effect of cold exposure on antioxidant levels of mitochondrial fractions from rat liver.

Fraction	Parameters	Groups		
		C	CE_2	CE_{10}
M_1	Vit E	0.24±0.01	0.31±0.02[a]	0.39±0.02[a,b]
	CoQ9	1.41±0.05	1.92±0.04[a]	2.18±0.12[a]
	CoQ10	0.15±0.01	0.18±0.02	0.24±0.01[a]
	GSH	15.2 ±0.4	12.5 ±0.7[a]	16.7 ±0.4[b]
M_3	Vit E	0.34±0.02[c]	0.42±0.02[a,c]	0.48±0.02[a,b,c]
	CoQ9	1.73±0.06	2.29±0.10[a]	2.61±0.15[a,b,c]
	CoQ10	0.19±0.01	0.26±0.02	0.31±0.03[a,c]
	GSH	16.6 ±0.6	13.9 ±0.6[a]	18.3 ±1.2[b]
M_{10}	Vit E	0.34±0.02[c]	0.49±0.03[a,c,d]	0.51±0.02[a,c]
	CoQ9	1.51±0.10	1.54±0.06[c,d]	1.83±0.13[a,b,d]
	CoQ10	0.23±0.02[c]	0.25±0.02	0.37±0.03[a,b,c,d]
	GSH	10.8 ±0.6[c,d]	8.8 ±0.6[a,c,d]	12.5 ±0.7[b,c,d]

Data represent the mean ± SE of eight experiments. Vitamin E (Vit E), coenzyme Q10 (CoQ10), coenzyme Q9 (CoQ9), and GSH levels are expressed in nmol/mg protein. [a] Significant vs C rats; [b] significant vs CE_2 rats;[c] significant vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

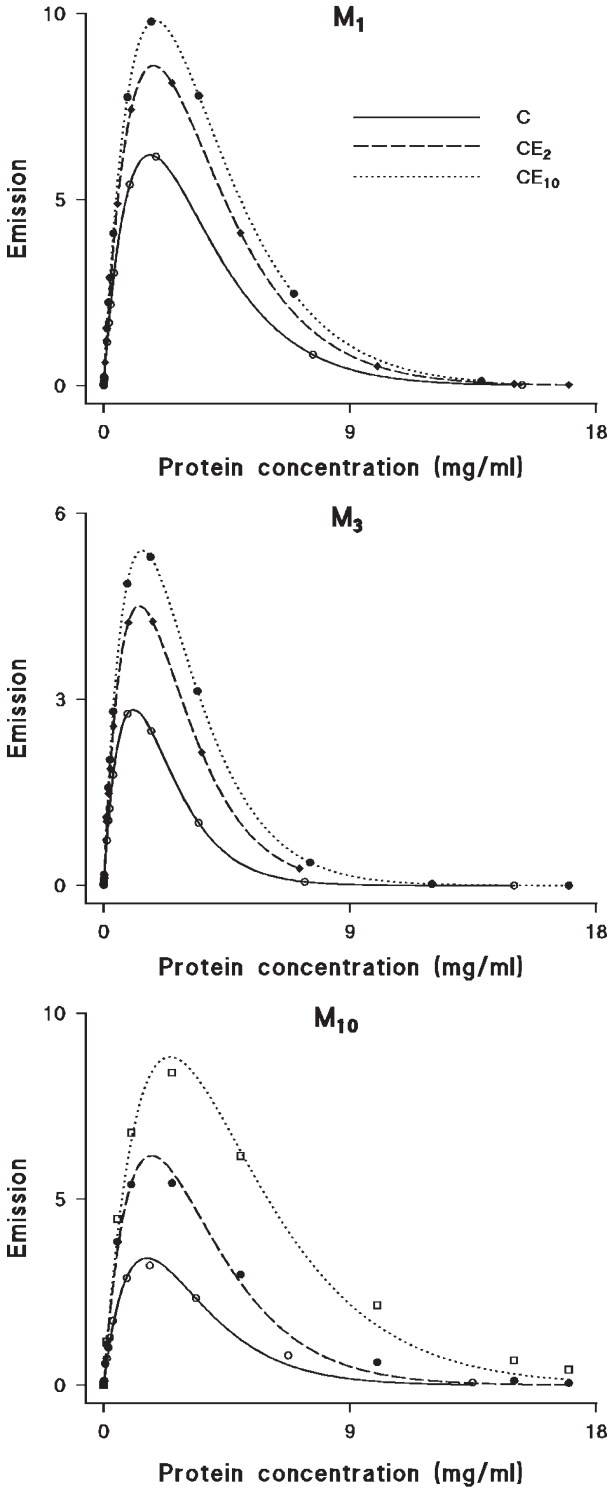

Figure 4. Response to oxidative stress in vitro of mitochondrial fractions from rat liver. The susceptibility to stress was evaluated by determining the variations with concentrations of light emission from a luminescent reaction. Emission values are given as percentage of an arbitrary standard (44 ng/ml peroxidase). The curves are computed from experimental data using the equation: E = *a* C/exp (*b* C). Preparations from control (C) (solid lines), 2-day cold-exposed (CE_2) (dashed lines), and 10-day cold-exposed (CE_{10}) (dotted lines) rats. One representative experiment of eight similar experiments is shown.

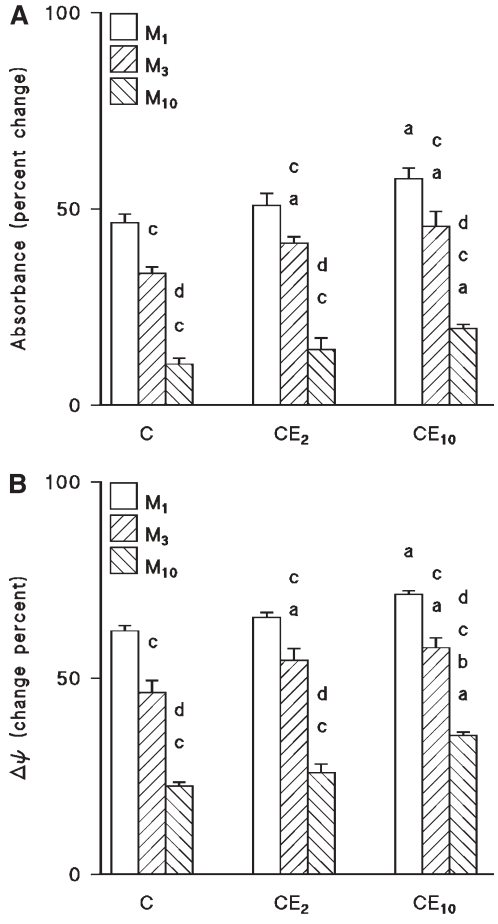

Figure 5. Effect of cold exposure on Ca^{2+}-induced swelling (A) and membrane potential dissipation (B) of mitochondrial fractions from control (C), 2-day cold-exposed (CE_2) and 10-day cold-exposed (CE_{10}) rats. Swelling of mitochondrial preparations (0.3 mg/ml) was monitored as decrease of the absorbance at 540 nm in a standard medium containing 100 µM Ca^{2+} and was expressed as percent of the initial value before Ca^{2+} addition. Membrane potential ($\Delta\Psi$) of mitochondrial preparations (0.3 mg/ml) was estimated through fluorescence changes of safranin (8 µM) (excitation wavelength 495 nm, emission wavelength 586 nm) in a standard medium containing 100 µM Ca^{2+}. $\Delta\Psi$ was calculated using a suitable calibration curve. The decrease of $\Delta\Psi$ for each preparation was expressed as percent of the initial value before Ca^{2+} addition. The initial values of absorbance of preparations from control (C) rats were 0.92 ± 0.04, 0.84 ± 0.02, and 0.54 ± 0.04 for M_1, M_3, and M_{10} fractions, respectively, those of preparations from 2-day cold-exposed (CE_2) rats were 0.87 ± 0.03, 0.85 ± 0.02 , and 0.46 ± 0.04 for M_1, M_3, and M_{10} fractions, respectively, those of preparations from 10-day cold-exposed (CE_{10}) rats were 0.85 ± 0.04, 0.80 ± 0.02, and 0.35 ± 0.03 for M_1, M_3, and M_{10} fractions, respectively. Initial values of $\Delta\Psi$ of mitochondrial fractions from control (C) rats were 188.4 ± 10.5 mV, 171.5 ± 10.6 mV, and 76.8 ± 6.3 mV for M_1, M_3, and M_{10} fractions, respectively, those of preparations from CE_2 rats were 174.2 ± 9.4 mV, 164.0 ± 10.1 mV, and 65.4 ± 6.2 mV for M_1, M_3, and M_{10} fractions, respectively, those of preparations from CE_{10} rats were 170.9 ± 9.3 mV, 151.1 ± 9.9 mV, and 61.9 ± 5.8 mV, for M_1, M_3, and M_{10} fractions, respectively. Data represent the mean ± SE of eight experiments. [a] Significant vs C preparations; [b] significant vs CE_2 preparations; [c] significant vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

Table 7. Effects of cold exposure on parameters characterizing the response to oxidative stress of mitochondrial fractions from rat liver.

Fraction	Parameter	Group		
		C	CE_2	CE_{10}
M_1	*a*	9.93±0.44	10.47±0.54	13.64±0.43[a]
	b	0.66±0.02	0.57±0.03	0.51±0.03
	E_{max}	5.5 ±0.4	6.8 ±0.6	9.8 ±0.6[a,b]
M_3	*a*	7.41±0.44	8.21±1.11[c]	9.79±0.86[c]
	b	0.96±0.07[c]	0.75±0.06[a]	0.71±0.04[a]
	E_{max}	2.8 ±0.2[c]	4.0 ±0.3[c]	5.1 ±0.4[a,c]
M_{10}	*a*	7.36±0.36	11.32±0.91[a,c,d]	11.83±1.00[a]
	b	0.79±0.11[d]	0.68±0.07	0.51±0.01[a]
	E_{max}	3.0 ±0.3[c]	6.1 ±0.7[a,d]	8.5 ±0.8[a,b,d]

Data are the mean ± SE of eight experiments. For explanation of symbols see text. The relation between light emission and protein concentration of mitochondria is described by the equation: $E = a\ C/\exp(b\ C)$. [a] Significant vs C rats; [b] significant vs CE_2 rats; [c] significant vs M_1 fraction; [d] significant vs M_3 fraction. The level of significance was chosen as $p < 0.05$.

emission curves and in the emission peak are imputable to increases in *a* values and decreases in *b* values. The increases in *a* values were generally consistent with the increases in COX activities, whereas there was no similar concordance between the changes in *b* values and those in antioxidant levels. This is due to both the lack of correlation among the mitochondrial levels of the individual antioxidants and the *b* value dependence on the concentration of substances lacking in conventional antioxidant activity [30].

Mitochondrial swelling and membrane potential dissipation

As shown by the absorbance changes in figure 5, in all groups, the extent of Ca^{2+}-induced swelling was higher in the M_1 and lower in the M_{10} fractions. Moreover, the swelling was significantly increased only in the M_3 fraction after 2 days and in all fractions after 10 days of cold exposure. Mitochondrial swelling was drastically reduced by CSA or EGTA (unreported results), pointing to the role played by the permeability transition pore. Furthermore, Ca^{2+}-induced swelling was preceded by a rapid decline in the membrane potential ($\Delta\Psi$) whose dependence on fraction and treatment was similar to that of the swelling (fig. 5).

Discussion

Although the liver contributes to increased oxygen uptake in cold-exposed rats [31, 32], the increase in tissue oxygen consumption is not associated with similar changes in mitochondrial respiration [15, 33, 34]. Such results are consistent with our present finding that cold-induced increases in state 4 and state 3 respiration rate occur only in the M_{10} and M_1 fraction, respectively.

However, other findings of the present work show that the changes in both functional and biochemical characteristics of the three mitochondrial fractions constitute an adequate response to stress imposed on the liver by cold exposure.

First, we found that the percentage content of the M_1 fraction, provided with the highest oxidative capacity, is decreased by cold exposure in apparent contrast with the observation that the specific oxidative capacities of both the overall mitochondrial population and the tissue increase. In fact, the higher oxidative capacity of the mitochondrial population is due to the cold-induced enhancement in oxidative capacity in all fractions, whereas that of the tissue is also due to enhancement of the mitochondrial protein content. This suggests that the enhanced metabolic capacity of the liver is mainly due to a proliferation of mitochondria which, despite their increased oxidative capacity, do not exhibit a higher oxygen consumption. The above proliferation also compensates the reduction in the percentage content of the M_1 fraction provided with the highest respiratory activity. Increased metabolic activity of the liver could also be obtained through an increase in the amount of the M_1 fraction, without resorting to two changes energetically expensive for hepatic cells, such as acceleration of the synthesis of mitochondrial proteins and the turnover of the metabolically more active fraction. It is, therefore, conceivable that the liver response to cold exposure offers other advantages characterized by greater adaptive value.

Within mitochondria, H_2O_2 rises by dismutation of the superoxide anion radical, which is generated by one-electron autoxidation of electron carriers at a Fe-S centre of complex I [35] or at the segment between NADH dehydrogenase and ubiquinone/cytochrome b of complex III [36]. H_2O_2 can be released in the cytoplasm and converted to $^{\bullet}OH$ radicals, which play a major role in determining the extent of tissue oxidative damage.

Both previous [8] and the present results show that M_1 mitochondria release H_2O_2 at the highest rate and, therefore, in control rats they supply a major contribution to

oxidative damage of hepatocytes. Because cold exposure produces some increase in H_2O_2 release rate, which remains higher in the M_1 fraction, the reduction in the relative amount of such a fraction limits liver oxidative damage in cold-exposed rats.

The mechanisms underlying the cold exposure-induced changes in rates of H_2O_2 release by M_1 and M_3 mitochondria seem to differ from those operative in M_{10} mitochondria. Because cold exposure does not modify the capacity of mitochondrial fractions to remove H_2O_2, cold-linked increases in H_2O_2 release rates reflect increases in H_2O_2 production rates. In intact mitochondria, such rates are related to the concentration of autoxidizable electron carriers in the reduced form [37]. Cold exposure increases COX activity and coenzyme Q content in all mitochondrial fractions, but it also produces different and temporally oscillatory changes in cytochromes aa3, c+c1, and b from rat liver mitochondria [38]. This indicates that the above increases cannot be shared by other components of the respiratory chain, including autoxidizable electron carriers. On the other hand, possible cold-induced decreases in the relative content of other electron carriers should reduce electron flow through the respiratory chain. If so, the lack of changes in O_2 consumption by M_1 and M_3 mitochondrial fractions characterized by higher activity of complex IV could be explained. Moreover, the reduced electron flow should produce an increase in the reduction degree of autoxidizable carriers even for want of increases in their concentrations. Blocking the respiratory chain with an inhibitor is known to increase both the reduction state of electron carriers on the substrate side of the inhibitor and H_2O_2 production if the ROS generator is located on that side. Thus, a partial block of the electron flow through the respiratory chain, resulting from a decrease in electron carrier level, should have effects similar to those produced by in vitro of treatment mitochondrial with small amounts of an inhibitor able to produce increased ROS production. Support for this idea is obtained by analyzing the effects of respiratory inhibitors on H_2O_2 release. Addition of antimycin increases both succinate- plus Rot- and pyruvate/malate-supported H_2O_2 production, because the electron carriers located between the substrate side and cytochrome b-560 become completely reduced. Accordingly, antimycin stimulation of H_2O_2 production occurring at complexes I and III (with pyruvate/malate) is higher than that occurring only at complex III (with succinate + Rot). On the other hand, with any substrate, in the presence of antymicin, and with pyruvate/malate, in the presence of Rot, the cold-linked stimulation of H_2O_2 release, which is found in the M_{10} fraction, is lacking in the M_1 and M_3 fractions. This indicates that cold exposure produces significant enhancement in rates of H_2O_2 production by the lightest fraction increasing its levels of autoxidizable electron carriers. Conversely, the increases in rates of H_2O_2 generation by the heavier fractions are due to an increased reduction degree of such carriers. If so, the cytochromes $c+c_1$, which are located on the oxygen side of the autoxidizable carriers and exhibit lower levels in liver mitochondria after 2 and 10 days of cold exposure [38], could be responsible for the block of the respiratory chain. However, one cannot exclude the possibility that a further block of respiration at the complex I level could also explain the unchanged rate of pyruvate/malate-supported respiration in cold-exposed rats.

Analysis of characteristics affecting susceptibility to oxidative damage of mitochondrial fractions allows us to propose a mechanism for the cold-induced decrease in the percentage content of the heavy mitochondrial fraction. Mitochondria are both the main site of production and the main target of ROS, so that changes in ROS production result in changes in the damage to mitochondrial components. Accordingly, in cold-exposed rats, lipid and protein oxidative damage undergoes a generalized increase, remaining greater in the M_1 fraction which exhibits a higher H_2O_2 generation rate than the other fractions. The above increase is not associated with a decrease in low-molecular-weight scavenger levels, except for a transitory fall in GSH levels, which could be due to an early incapacity of the mitochondrial transport system for GSH to compensate its consumption.

Of course, measurement of selected antioxidants provides limited information regarding the antioxidant status of biological preparations. Moreover, other biochemical characteristics of the mitochondria, such as polyunsaturated fatty acids (PUFAs) and iron ligand content, determine their susceptibility to oxygen radicals. The levels of PUFAs in membrane phospholipids affect the extent of oxidative damage because of the high content of weakly bound allylic hydrogens. Accordingly, recent results suggest that enhancement of the synthesis of lipids highly susceptible to oxidative attack contributes to increased lipid peroxidation in tissues from cold-exposed rats [14].

The mitochondrial content of Fe^{2+} complexes affects oxidative damage of membrane lipids [39, 40] through conversion of H_2O_2 into $HO^{\bullet}$ radicals via the Fenton reaction. The H_2O_2 breakdown on iron chelated by proteins can also lead to oxidative modification of such macromulecules [41], because $HO^{\bullet}$ radicals, formed at the point of metal binding, can interact with the ligand, giving rise to site-specific damage [42].

A convenient approach to show possible cold-induced changes in the mitochondrial content of iron Fe^{2+} complexes is to challenge mitochondrial preparations with hydrogen peroxide, using light emission, resulting from the interaction between $HO^{\bullet}$ radicals and detector molecules, as an index of oxidative damage.

Our results show that susceptibility of mitochondrial fractions to oxidative challenge is higher in cold-exposed

than in control rats. The changes, which are mainly due to increased Fe^{2+} complex content, are particularly remarkable in the M_{10} fraction. This suggests that after cold exposure, the M_{10} fraction contains a greater amount of damaged, uncoupled mitochondria coming from the degradation of M_1 mitochondria. This idea is supported by the increase in succinate-supported state 4 respiration, COX activity, and liposoluble antioxidant content of the M_{10} fraction. Further support is supplied by the results obtained challenging mitochondrial preparation with Ca^{2+} loads in vitro.

In the presence of Ca^{2+}, oxidative alterations of mitochondrial inner membrane protein thiols results in CSA-inhibitable mitochondrial swelling and a fall in the mitochondrial membrane potential, mediated by an inner membrane permeabilization referred to as the MPT [43]. In all groups, Ca^{2+}-loaded mitochondria of the M_1 and M_{10} fractions exhibit the highest and the lowest swelling, respectively. Moreover, the value of the absorbance of the M_{10} fraction before the addition of Ca^{2+} is 58.7% of the value of the M_1 fraction in control rats, and 41.2% in CE_{10} rats. This suggests, that, beside light mitochondria endowed with a low susceptibility to permeabilization, the M_{10} fraction contains disrupted mitochondria coming from the M_1 fraction, which have reached a high degree of swelling and low membrane potential, and the amounts of such mitochondria in the light fraction increase after cold exposure.

Of course, mechanical damage, during tissue homogenization, may have led to transfer of more fragile M_1 mitochondria to the light fraction. However, generation of light mitochondria during homogenization should explain only a part of the mitochondrial subpopulation redistribution, because rat liver mitochondria appear swollen in situ after cold exposure [44]. Moreover, swelling in situ of liver mitochondria is found in another condition leading to oxidative stress, i.e., exercise [44], which also decreases the amount of heavy mitochondria and increases that of damaged mitochondria [45].

Thus, taken together our results support the idea that the characteristics of the M_1 mitochondria, i.e., a high capacity to produce ROS and a high susceptibility to oxidants, not only determine the extent of oxidative damage from which they suffer, but also make them more susceptible to modifications that result, finally, in their degradation.

A last problem concerns the possible factors responsible for the modulations of the characteristics of the mitochondrial population induced by rat exposure to low environmental temperature. Experimental hyperthyroidism, elicited by T_3 treatment, induces oxidative stress in the liver [11], accelerated mitochondrial ROS generation [12, 13], and predisposes the tissue to free radical-mediated oxidative damage, decreasing its antioxidant capacity [11] and increasing the Fe^{2+} complex content [9, 11] and degree of lipid unsaturation [46]. Such modifications are similar to those found in the tissue from rats made functionally hyperthyroid by 10 days of cold exposure [14, 15]. The observation that the cold-induced changes in characteristics of the mitochondrial population are apparent or greater after 10 day cold exposure [15] agrees with the idea that modifications in mitochondrial protein mass and function are late effects of the thyroid state alteration [16, 17]. Therefore, we proposed that T_3 brought about the biochemical changes underlying tissue oxidative damage found after 10 days of either cold exposure or T_3 treatment.

This idea is supported by the present results, which show that the characteristics of the mitochondrial fractions are modified by cold exposure in a time-dependent way through mechanisms similar to those operative following T_3 treatment. In fact, mitochondria in the livers of hyperthyroid rats appear swollen in situ [47] and, when isolated, they exhibit a high susceptibility to Ca^{2+}-induced permeabilization of the inner membrane [48, 49] and contain relatively low amounts of heavy mitochondria and high amounts of damaged mitochondria [9]. In light of these similar effects of cold exposure and T_3 treatment on mitochondria, T_3 is conceivably also responsible for ROS-induced MTP-mediated mitopsosis, which, purifying the mitochondrial population of ROS-overproducing mitochondria, limits liver oxidative damage in cold-exposed rats. However, other biofactors whose levels can be modified by cold may also be involved. An intriguing possibility, currently under study in our laboratory, is that thyroxine (T_4) contributes to cold-induced changes in the mitochondrial population. In fact, unlike T_3 administration, cold exposure increases serum levels of T_4, which seem to exert intrinsic biological activity in cold-exposed rats [50].

1 Gear A. R. L. (1965) Some features of mitochondria and fluffy layer regenerating rat liver. Biochem. J. **95:** 118–137
2 Kuff E. I. and Schneider W. C. (1954) Intracellular distribution of enzymes. XII. Biochemical heterogeneity of mitochondria. J. Biol. Chem. **206:** 677–685
3 Katyare S. S., Fatterpaker P. and Sreenivasan A. (1970) Heterogeneity of rat liver mitochondrial fractions and the effect of tri-iodothyronine on their protein turnover. Biochem. J. **118:** 111–121
4 Satav J. G., Rajwade M. S., Katyare S. S., Netrawall M. S., Fatterpaker P. and Sreenivasan A. (1973) The significance of promitochondrial structures in rat liver for mitochondrial biogenesis. Biochem. J. **134:** 687–695
5 Goglia F., Liverini G., Lanni A., Iossa, S. and Barletta A. (1986) Tri-iodothyronine enhances the formation of light mitochondria during cold-exposure. Comp. Biochem. Physiol. **85B:** 869–873
6 Lanni A., Moreno M., Lombardi A. and Goglia F. (1996) Biochemical and functional differences in rat liver mitochondrial subpopulations obtained at different gravitational forces. Int. J. Biochem. Cell. Biol. **28:** 337–343
7 Venditti P., Di Meo, S. and De Leo T. (1996) Effect of thyroid state on characteristics determining the susceptibility to oxidative stress of mitochondrial fractions from rat liver. Cell. Physiol. Biochem. **6:** 283–295

8 Venditti P., Costagliola I. R. and Di Meo S. (2002) H_2O_2 production and response to stress conditions by mitochondrial fractions from rat liver. J. Bioenerg. Biomembr. **34:** 115–125
9 Venditti, P. Daniele M. C., Masullo P. and Di Meo S. (1999) Antioxidant-sensitive triiodothyronine effects on characteristics of rat liver mitochondrial population. Cell. Physiol. Biochem. **9:** 38–52
10 Videla L. A. (2000) Energy metabolism, thyroid calorigenesis, and oxidative stress: functional and cytotoxic consequences. Redox Rep. **5:** 265–275
11 Venditti P., Balestrieri M., Di Meo S. and De Leo T. (1997) Effect of thyroid state on lipid peroxidation, antioxidant defences, and susceptibility to oxidative stress in rat tissues. J. Endocrinol. **155:** 151–157
12 Fernández V. and Videla L. A. (1993) Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat liver submitochondrial particles. Free Radic. Res. Commun. **18:** 329–335
13 Venditti P., De Rosa R. and Di Meo S. (2003) Effect of thyroid state on H_2O_2 production by rat liver mitochondria. Mol. Cell. Endocrinol. **205:** 185–192
14 Venditti P., De Rosa R., Portero-Otin M., Pamplona R. and Di Meo S. (2004) Cold-induced hyperthyroidism produces oxidative damage in rat tissues and increases susceptibility to oxidants. Int. J. Biochem. Cell. Biol. **36:** 1319–1331
15 Venditti P., De Rosa R. and Di Meo S. (2004) Effect of cold-induced hyperthyroidism on H_2O_2 production and susceptibility to stress conditions of rat liver mitochondria. Free Radic. Biol. Med. **36:** 348–358
16 Goglia F., Liverini G., De Leo T. and Barletta A. (1983) Thyroid state and mitochondrial population during cold exposure. Pflügers Arch. **396:** 49–53
17 Liverini G., Goglia F., Lanni A., Iossa S. and Barletta A. (1990) Elevated hepatic mitochondrial oxidative capacities in cold exposed rats. Comp. Biochem. Physiol. **97B:** 327–331
18 Gornall A. G., Bardawill C. J. and David M. M. (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:** 751–766
19 Barrè H., Bailly L. and Rouanet J. L. (1987) Increased oxidative capacity in skeletal muscles from acclimated ducklings: a comparison with rats. Comp. Biochem. Biophys. **88B:** 519–522
20 Hyslop P. A. and Sklar L. A. (1984) A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes. Anal. Biochem. **141:** 280–286
21 Palmer G., Horgan D. J., Tisdale H., Singer T. P. and Beinert H. (1968) Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. XIV. Location of the sites of inhibition of rotenone, barbiturates, and piericidin by means of electron paramagnetic resonance spectroscopy. J. Biol. Chem. **243:** 844–847
22 Turrens J. F., Alexandre A. and Lehninger A. L. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch. Biochem. Biophys. **237:** 408–414
23 Venditti P., Masullo P. and Di Meo S. (2001) Hemoproteins affects H_2O_2 removal from rat tissues. Int. J. Biochem. Cell Biol. **33:** 293–301
24 Heath R. L. and Tappel A. L. (1976) A new sensitive assay for the measurement of hydroperoxides. Anal. Biochem. **76:** 184–191
25 Schild L., Reinheckel T., Wiswedel I. and Augustin W. (1997) Short-term impairment of energy production in isolated rat liver mitochondria by hypoxia/reoxygenation: involvement of oxidative protein modification. Biochem. J. **328:** 205–210
26 Lang J. K., Gohil K. and Packer L. (1986) Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Anal. Biochem. **157:** 106–116
27 Griffith O. W. (1980) Determination of glutathione disulphide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. **106:** 207–212
28 Venditti P., De Leo T. and Di Meo S. (1999) Determination of tissue susceptibility to oxidative stress by enhanced luminescence technique. Methods Enzymol. **300:** 245–252
29 Åkerman K. E. O. and Wikström M. K. F. (1976) Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. **68:** 191–197
30 Di Meo S., Venditti P. and De Leo T. (1996) Tissue protection against oxidative stress. Experientia **52:** 786–794
31 Stoner H. B. (1973) The role of the liver in nonshivering thermogenesis in the rat. J. Physiol. **232:** 285–296
32 Guernsey D. L. and Stevens E. D. (1977) The cell membrane sodium pump as a mechanism for increasing thermogenesis during cold acclimation in rats. Science **196:** 908–910.
33 Iossa S., Barletta A. and Liverini G. (1991) The effect of thyroid state and cold exposure on rat liver oxidative phosphorylation. Mol. Cell. Endocrinol. **75:** 15–18
34 Iossa S., Liverini G and Barletta A. (1991) Effect of thyroid state and cold exposure on rat liver mitochondrial protein mass and function. J. Endocrinol. **131:** 67–73
35 Turrens J. F. and Boveris A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem. J. **191:** 421–427
36 Loschen G., Flohé L. and Chance B. (1971) Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. FEBS Lett. **18:** 261–264
37 Boveris A. and Chance B. (1973) The mitochondrial generation of hydrogen peroxide. Biochem. J. **134:** 707–716
38 Bravo C., Vargas-Suárez M., Rodriguez-Enriquez S., Loza-Tavera H. and Moreno-Sánchez R. (2001) Metabolic changes induced by cold stress in rat liver mitochondria. J. Bioenerg. Biomembr. **33:** 289–301
39 Halliwell B. and Gutteridge J. M. C. (1990) Role of free radicals and catalytic metal ions in human disease. Methods Enzymol. **186:** 1–85
40 Kowaltowski A. J. and Vercesi A. E. (1999) Mitochondrial damage induced by conditions of oxidative stress. Free Radic. Biol. Med. **26:** 463–471
41 Halliwell B. and Gutteridge J. M. C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. **219:** 1–14
42 Gutteridge J. M. C. (1987) Ferrous-salt-promoted damage to deoxyribose and benzoate: the increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. Biochem. J. **243:** 709–714
43 Vercesi A. E., Kowaltowski A. J., Grijalba M. T., Meinicke A. R. and Castilho R. F. (1997) The role of reactive oxygen species in mitochondrial permeability transition. Biosci. Rep. **17:** 43–52
44 Venditti P., Masullo P. and Di Meo S. (1999) Effect of exercise duration on characteristics of mitochondrial population from rat liver. Arch. Biochem. Biophys. **368:** 112–120
45 Salas M., Tuchweber B. and Kourounakis P. (1980) Liver ultrastructure during acute stress. Pathol. Res. Pract. **167:** 217-233
46 Hoch F.L. (1988) Lipids and thyroid hormones. Prog. Lipid Res. **27:** 199–270
47 Paget G. E. and Thorp J. M. (1963) The effect of thyroxin on the fine structure of rat liver cell. Nature **199:** 1307–1308
48 Kalderon B., Hermesh O. and Bar-Tana J. (1995) Mitochondrial permeability transition is induced by in vivo thyroid hormone treatment. Endocrinology **136**: 3552–3556
49 Venditti P., De Rosa R. and Di Meo S. (2003) Effect of thyroid state on susceptibility to oxidants and swelling of mitochondria from rat tissues. Free Radic. Biol. Med. **35:** 485–494
50 Du Breuil A. and Galton V. A. (1978) Thyroxine: studies concerning its intrinsic physiological activity. Acta Endocrinol. **88:** 87–93