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Effect of vitamin E on characteristics of liver mitochondrial fractions from cold-exposed rats

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Abstract In cold exposed rats, it is known that vitamin E induces an increase in the respiration of the whole mitochondrial population isolated from liver. To obtain information on the effects of cold exposure and vitamin E treatment on the dynamics of mitochondrial population, we determined characteristics of rat liver mitochondrial fractions, resolved at 1,000 (M_1), 3,000 (M_3), and 10,000*g* (M_{10}). We found that cold exposure increased the liver content of total mitochondrial proteins irrespective of vitamin E treatment. Conversely, protein distribution among the mitochondrial subpopulations was differentially affected by cold and antioxidant integration. In a cold environment, the M_1 fraction, characterized by the highest O_2 consumption and H_2O_2 production rates, underwent a remarkable protein content reduction, which was attenuated by vitamin E. These changes were dependent on the opposite effects of the two treatments on mitochondrial oxidative damage and susceptibility to swelling. The proteins of the other fractions, in which the above effects were lower, underwent smaller (M_3) or no change (M_{10}) in the treatment groups. The cold also led to an increase in O_2 consumption of the M_1 fraction which was accentuated by vitamin E treatment. This phenomenon and the vitamin-induced recovery of the M_1 proteins supply an explanation of the previously reported increase in the respiration of the whole mitochondrial population induced by vitamin E in the liver from cold exposed rats.

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Introduction

It is long known that the mitochondrial population is heterogeneous and can be resolved, by differential centrifugation, into fractions having different properties (Gear 1965; Kuff and Schneider 1954). Their studies suggested that the rat liver mitochondria undergo a process of development in which light mitochondria, with low respiratory activity, change in heavy mitochondria with high respiratory activity (Satav et al. 1973). Such a process is controlled by the thyroid hormone in both T_3-treated and cold-exposed rats (Goglia et al. 1986). Subsequent investigations on mitochondrial fractions, resolved at 1,000 (M_1), 3,000 (M_3), and 10,000 (M_{10}) *g*, showed that the M_1 fraction, containing the oldest mitochondria, exhibits the lowest levels of antioxidants (Venditti et al. 1996; Venditti et al. 1999; Venditti et al. 2002), and the highest levels of H_2O_2 production and susceptibility to both in vitro oxidative challenge and Ca^{2+}-induced swelling (Venditti et al. 2002). Because both H_2O_2 production and susceptibility to oxidants depend on the mitochondrial content of respiratory chain components, the process of mitochondrial maturation is self-limiting leading to mitochondrial degradation. The T_3 treatment induces rat liver oxidative stress (Venditti et al. 1997), enhancing the production of reactive oxygen (ROS) (Venditti et al. 2003a) and nitrogen (RNS) (Fernández et al. 1997) species. It also increases the oxidative damage and the susceptibility to oxidants and Ca^{2+} load (Venditti et al. 2003b) of the mitochondrial population, which is characterized by a reduced amount of M_1 mitochondria and an increased

amount, in the M_{10} fraction, of degenerating mitochondria, coming from the degradation of M_1 mitochondria (Venditti et al. 1996).

The functional hyperthyroid state, elicited by cold exposure, increases the mitochondrial production of ROS (Venditti et al. 2004a) and RNS (Peralta et al. 2003), resulting in modifications of the liver mitochondrial population similar to those elicited by T_3 treatment (Venditti et al. 2004a, b).

Vitamin E administration is able to reduce the T_3-induced changes in the oxidative damage and distribution of proteins in the mitochondrial fractions, which, however, show an incomplete recovery of their resistance to the oxidants (Venditti et al. 1999).

Information about the vitamin E effects on the mitochondrial fractions from rats exposed to a low environment temperature are lacking. Because such information would contribute to further clarify the mechanisms underlying the response of the liver mitochondria to cold stress, we have examined the characteristics of liver mitochondrial fractions from rats subjected to cold exposure and vitamin E treatment.

Materials and methods

Animals

The experiments were carried out on 60 days old male Wistar rats, supplied at weaning by Nossan (Correzzana, Italy). 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), and were provided with water ad libitum and a commercial rat chow diet (Nossan), containing 105 IU/kg of vitamin E. From day 45, the animals were randomly assigned to one of three groups: control rats (C), kept at room temperature of 24±1 °C; cold-exposed rats (CE), kept at 4±1 °C for 10 days; cold exposed vitamin E-treated rats (CE+VE), kept at 4±1 °C for 10 days and treated for 15 days with daily intramuscular injections of vitamin E (10 mg/100 g body weight). Control and cold exposed rats were injected with vitamin E vehicle for the same period.

Experimental procedure

The animals were killed by decapitation under ether anesthesia. Blood samples were collected and later analysed to determine plasma levels of free thyroxine (FT_4) and triiodothyronine (FT_3) by using commercial RIA kits (DiaSorin, Salluggia, Italy). The livers were rapidly removed and placed in small beakers on ice. Then, they were finely minced, weighed, and washed with ice-cold homogenization medium (HM) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, 20 mM Tris, pH 7.4). Tissue fragments were gently homogenized in the same solution (1:5 w/v) using a Potter-Elvejem homogenizer set at a standard velocity (500 rpm) for 2 min. Aliquots of the homogenates were used to determine the cytochrome oxidase (COX) activity.

Preparation of mitochondrial fractions

The homogenates, diluted 1:1 with HM, 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 suspended in the same solution and used either for COX determination or subjected to a series of sequential centrifugation steps lasting 10 min at 1,000, 3,000, and 10,000*g*. The pellets were designed as M_1, M_3, and M_{10}, respectively. Aliquots of all mitochondrial fractions were suspended 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, suspended in the same solution, and used to determine mitochondrial swelling and membrane potential.

In agreement with previous reports (Venditti et al. 1996; Venditti et al. 1999), preliminary determinations of activities of marker enzymes showed that fractions M_1 and M_3 were scarcely contaminated 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 (Gornall et al. 1949) with bovine serum albumin as standard.

Cytochrome oxidase activity

The COX activity of homogenates and mitochondrial suspensions was determined polarographically at 30 °C, using a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instruments, Ohio, USA), by the procedure of Barrè et al. (1987).

In vitro COX activity is positively correlated to 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 of mitochondrial protein per ml and succinate (10 mM) (plus rotenone 5 μM) or pyruvate/malate (10/2.5 mM) as substrates, in the absence (State 4) and in the presence (State 3) of 500 μM ADP.

H_2O_2 release

The rate of the 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) (Hyslop and Sklar 1984) 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 used for the measurements of O_2 consumption. Measurements with the different substrates in the presence of 500 μM ADP were also performed. Known concentrations of H_2O_2 were used to establish the standard concentration curve. In preliminary experiments the effect of the catalase addition on the rates of H_2O_2 production was studied. Such experiments showed a dose-dependent drop of the fluorescence in the presence of the enzyme.

The effects of respiratory inhibitors were also investigated: rotenone, which blocks the transfer of electrons from the Complex I to the ubiquinone (Palmer et al. 1968), and antimycin A (AA), which interrupts electron transfer within the ubiquinone-cytochrome b site of the Complex III (Turrens et al. 1985). Inhibitor concentrations (5 μM Rot, 10 μM AA), which do not interfere with the detection PHPA-HRP system (Venditti et al. 2003a), were used.

Capacity to remove H_2O_2

The capacity to remove the H_2O_2 (CR) was determined, as previously described (Venditti et al. 2001), by comparing the ability of mitochondrial samples to reduce the H_2O_2-linked fluorescent emission with that of desferrioxamine solutions. Thus, the capacity of mitochondrial samples to remove H_2O_2 was expressed as equivalent desferrioxamine concentration.

Oxidative damage to lipid and proteins

The extent of the peroxidative processes in the mitochondrial fractions was determined by measuring the level of lipid hydroperoxides (HPs) according to Heath and Tappel (1976).

To assess protein-bound carbonyls, the procedure of Schild et al. (1997) for rat liver mitochondria was employed.

Antioxidant levels

Ubiquinols (CoQH2) from 0.5 ml of mitochondrial suspension were oxidized to ubiquinones (CoQs) with 0.5 ml of 2% $FeCl_3$ and 2.0 ml of ethanol. The total content of CoQs (CoQH2 + CoQ) was then determined according to Lang et al. (1986). Vitamin E content was determined using the HPLC procedure of Lang et al. (1986). GSH concentration was measured as described by Griffith (1980).

Mitochondrial swelling and membrane potential dissipation

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 rotenone, 0.3 mg mitochondrial protein/ml, 100 μM Ca^{2+}, and 1 mM EGTA or 1 μM cyclosporin A (CSA) where indicated.

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 (1976) using a calibration curve obtained 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 are expressed as mean ± standard error. 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

Thyroid state was documented by the modifications in heart weight/body weight (HW/BW) ratio and plasma levels of FT_3 and FT_4. Heart weight but not body weight (266±5, 257±5, and 255±8 g for C, CE, and CE + VE rats, respectively) was significantly affected by cold exposure, so that HW/BW ratio was significantly higher in CE (3.25± 0.06 mg/g) and CE + VE (3.17±0.05 mg/g) than in C (2.49± 0.02 mg/g) rats. Plasma FT_3 levels (306±20, 539±38, and 568±50 pg/dl for C, CE, and CE + VE rats, respectively) were significantly increased by the cold exposure irrespective of the vitamin E treatment, whereas FT_4 levels (1.66±

0.08, 1.85±0.18, and 1.89±0.16 ng/dl for C, CE, and CE + VE rats, respectively) were not significantly modified by treatments.

Cytochrome oxidase activity and mitochondrial protein content

COX activities in homogenates and whole mitochondrial populations were significantly increased by 10 days of cold exposure irrespective of vitamin E treatment (Table 1).

In all groups, the highest and lowest enzyme activities were found in the M_1 and the M_{10} fractions, respectively, even though in the CE group the difference between the M_1 and M_3 fraction was not significant. Moreover, COX activities were increased in all mitochondrial fractions by cold exposure, and were restored to control values in the M_{10} fraction by vitamin E treatment.

The protein percentage content of the M_{10} fraction was not significantly modified by the treatments, remaining the least abundant in the mitochondrial population. Conversely, the protein content of the M_1 fraction, which was the highest in control animals, decreased significantly during cold exposure and became lower than the content of the M_3 fraction (Fig. 1, lower panel). When cold exposure and vitamin E treatment were combined the M_1 and M_3 protein contents decreased without reinstating the control values.

The ratio between the homogenate COX activity and the sum of the products of the mitochondrial fractions COX activities and their protein percentage contents provided a rough estimate of the tissue content of mitochondrial proteins. The cold exposure was associated with an increase in such content irrespective of vitamin E treatment (Fig. 1, upper panel).

Another evaluation of the hepatic content of mitochondrial proteins was obtained by the ratio between the COX activities of the homogenates and those of the whole mitochondrial populations. The values so obtained (67.7±5.6, 92.6±2.2, and 93.9±7.1 mg/g for C, CE, and CE + VE rats, respectively) were not substantially different from those obtained by the first method (72.9±6.0, 99.7±3.9, and 104.2±7.0 mg/g for C, CE, and CE+VE rats, respectively).

Lipid peroxidation and protein-bound carbonyls

Hydroperoxide and protein carbonyl levels were generally greater in the M_1 than in the M_3 and M_{10} fractions. However, in the CE + VE group, the differences between the M_1 and M_3 fractions were not significant.

Table 1 Effect of cold exposure and vitamin E treatment on cytochrome oxidase activity of mitochondrial fractions from rat liver

Preparation	Groups		
	C	CE	CE + VE
Homogenate	74.2±0.8	119.8±1.4[a]	116.4±4.2[a]
M_w	0.98±0.04	1.33±0.03[a]	1.24±0.05[a]
M_1	1.15±0.05	1.37±0.03[a]	1.34±0.05[a]
M_3	0.97±0.06[c]	1.24±0.02[a]	1.15±0.04[a,c]
M_{10}	0.44±0.04[c,d]	0.57±0.02[a,c,d]	0.40±0.02[b,c,d]

Data represent the mean ± SEM of eight experiments. Cytochrome oxidase (COX) activity is expressed as μmol O/min/g for liver and μmol O/min/mg protein for mitochondria. Control (C), cold-exposed (CE), and cold-exposed and vitamin E-treated (CE + VE) rats

[a] significant vs. C rats

[b] significant vs. CE rats

[c] significant vs. M_1 fraction

[d] significant vs. M_3 fraction

The level of significance was chosen as $P<0.05$

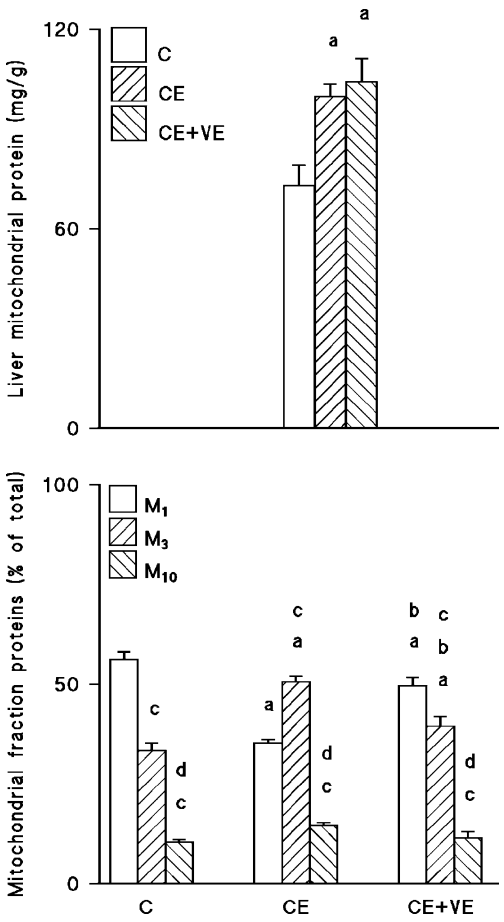

Fig. 1 Effect of cold exposure and vitamin E treatment on content in mitochondrial proteins (upper panel) and protein distribution among mitochondrial fractions (lower panel) in rat liver. Preparations from control (C), cold exposed (CE), and cold exposed, vitamin E treated (CE + VE) rats. Data represent the mean ± SEM of eight experiments. [a] significant vs. C rats; [b] significant vs. CE rats; [c] significant vs. M_1 fraction; [d] significant vs. M_3 fraction. The level of significance was chosen as $P<0.05$

Cold exposure led to an increase in the lipid and protein oxidation in all mitochondrial fractions. Vitamin E treatment significantly reduced lipid oxidation in all fractions and protein oxidation in the M_1 and M_3 fractions (Fig. 2).

Oxygen consumption

The rates of O_2 consumption are reported in Table 2. With succinate as the substrate, in the C group the lowest rate of State 4 respiration was found in the M_{10} fraction. Following cold exposure the above rate increased in all mitochondrial fractions irrespective of the vitamin treatment. However, in the CE group the lowest respiration rates were found in M_3 and M_{10} fractions, whereas in the CE + VE group the highest and the lowest rates were found in the M_1 and M_{10} fractions, respectively.

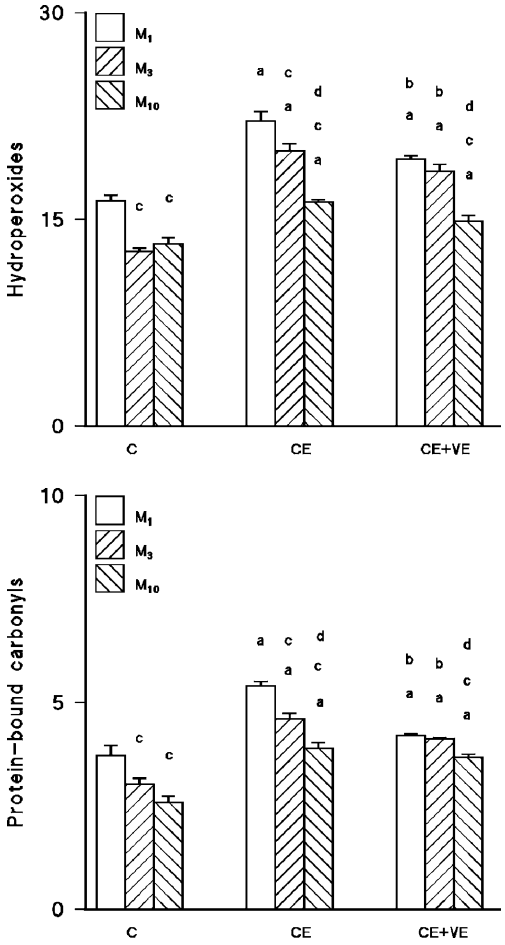

Fig. 2 Effect of cold exposure and vitamin E treatment on oxidative damage of mitochondrial fractions. Hydroperoxides (upper panel) are expressed as pmol NADPH/min/mg protein. Protein-bound carbonyls (lower panel) are expressed as nmol/mg protein. Preparations from control (C), cold exposed (CE), and cold exposed, vitamin E treated (CE + VE) rats. Data represent the mean ± SEM of eight experiments. [a] significant vs. C rats; [b] significant vs. CE rats; [c] significant vs. M_1 fraction; [d] significant vs. M_3 fraction. The level of significance was chosen as $P<0.05$

In all groups, the highest State 3 respiration rates were the highest in the M_1 and the lowest in the M_{10} fractions. Furthermore, in the M_1 fraction respiration rates underwent an increase after cold exposure and further increase following vitamin E treatment, whereas the other fractions were not affected by the treatments.

The RCR value of M_{10} fraction, which was the lowest in all groups, was not affected by the treatments, whereas the RCR values of the M_1 and M_3 fractions were increased by cold exposure irrespective of the vitamin treatment.

With pyruvate/malate as the substrates, the highest and lowest rates of State 4 and State 3 respiration were generally found in the M_1 and M_{10} fractions, respectively. Cold exposure increased the State 4 respiration rates in the M_1 and M_{10} fractions. The concomitant treatment with vitamin E caused a greater increase in the respiration rates so that in all fractions they were significantly higher than those found in the C and CE groups. Conversely, State 3 respiration rates of the M_1 and M_3 fractions were increased by cold exposure and underwent further significant increase when cold exposure was associated with vitamin treatment.

RCR values were generally lower in the M_{10} than in the other fractions and were not affected by the treatments.

H_2O_2 release

In the control group, the highest and lowest rates of H_2O_2 release during State 4 and State 3 respiration were generally found in the M_1 and M_{10} fractions, respectively. In the presence of succinate, cold exposure caused an increase in the release rates during basal and stimulated respiration in the M_1 and M_{10} fractions, whereas the concomitant vitamin treatment prevented such an increase during basal respiration (Table 3). In the presence of pyruvate and malate, cold exposure increased the H_2O_2 release rates in all fractions during basal and stimulated respiration, whereas the concomitant vitamin treatment prevented such an increase in the M_1 fraction during basal and stimulated respiration and in the M_{10} fraction during basal respiration. Moreover, in the M_3 fraction vitamin E treatment reduced the H_2O_2 release rate during stimulated respiration below the control value (Table 3).

Effect of inhibitors on H_2O_2 release

As shown in Table 4, in all groups and fractions the succinate-supported H_2O_2 release rates were lowered by rotenone and increased by further addition of antimycin A. In some cases, the inhibitor presence affected the fraction and treatment-linked differences. Thus, in the presence of AA, in the C and CE + VE groups the rates of H_2O_2 release became no significantly different in the M_1 and M_3 fractions, whereas in the CE + VE group became no different in the M_3 and M_{10} fractions. In the presence of

Table 2 Effect of cold exposure and vitamin E treatment 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	33.2±1.1	219.1±6.6	6.6±0.7	10.4±0.6	30.7±1.3	3.5±0.3
	M_3	30.5±1.4	186.2±4.6[c]	6.1±0.4	9.5±0.5	26.1±0.5[c]	2.7±0.3[c]
	M_{10}	22.7±1.3[c,d]	62.9±3.5[c,d]	2.8±0.2[c,d]	7.3±0.4[c,d]	15.4±0.6[c,d]	2.1±0.1[c]
CE	M_1	50.6±3.3[a]	234.2±5.2[a]	4.9±0.5[a]	13.6±0.8[a]	37.8±1.2[a]	2.8±0.3
	M_3	41.4±2.1[a,c]	193.8±3.0[c]	4.7±0.4[a]	10.7±0.4[c]	30.5±0.9[a,c]	2.9±0.3
	M_{10}	36.1±1.9[a,c]	58.0±3.1[c,d]	1.6±0.2[c,d]	10.0±0.6[a,c]	15.4±0.2[c,d]	1.6±0.1[c,d]
CE + VE	M_1	54.5±1.9[a]	258.4±2.6[a,b]	4.7±0.4[a]	15.3±0.3[a,b]	48.0±0.2[a,b]	3.1±0.1
	M_3	41.7±3.0[a,c]	195.7±3.8[c]	4.7±0.3[a]	12.6±0.1[a,b,c]	34.1±0.3[a,b,c,]	2.9±0.2
	M_{10}	30.7±0.9[a,c,d]	50.8±1.8[c,d]	1.7±0.2[c,d]	12.4±0.2[a,b,c,]	16.7±0.3[c,d]	1.4±0.1[c,d]

Data represent the mean ± SEM of eight experiments. Oxygen consumption rates are expressed in nmol O/min/mg protein. Control (C), cold-exposed (CE), and cold-exposed and vitamin E-treated (CE + VE) rats

[a] significant vs. C rats

[b] significant vs. CE rats

[c] significant vs. M_1 fraction

[d] significant vs. M_3 fraction

The level of significance was chosen as $P<0.05$

rotenone, vitamin E treatment restored the control values of the release rates in the M_{10} fraction, whereas it did not modify the cold exposure effect on the release rate in the same fraction after antimycin addition.

Pyruvate/malate supported H_2O_2 release rates were strongly increased by AA addition and slightly increased by rotenone addition. Even in this case, the cold and fraction-linked effects on the release rates in the presence

Table 3 Effect of cold exposure and vitamin E treatment on H_2O_2 release by succinate and pyruvate/malate-supplemented mitochondrial fractions from rat liver

Fraction	Substrate and additions	Group		
		C	CE	CE + VE
M_1	Succinate	122.2±3.0	142.5±3.9[a]	124.8±0.9[b]
	Succinate + ADP	68.5±3.2	82.3±1.6[a]	80.8±3.0[a]
	Pyruvate/Malate	258.0±0.6	290.1±1.2[a]	257.4±1.4[b]
	Pyruvate/Malate + ADP	171.2±1.4	177.5±0.4[a]	171.2±1.5[b]
M_3	Succinate	116.0±1.7[c]	120.8±0.8[c]	114.7±1.1[c]
	Succinate + ADP	53.5±0.7[c]	70.2±1.2[a,c]	68.0±1.4[a,c]
	Pyruvate/Malate	244.3±4.5[c]	266.0±2.0[a,c]	255.9±1.3[a,b]
	Pyruvate/Malate + ADP	163.0±0.7[c]	171.5±2.2[a,c]	152.1±0.7[a,b,c]
M_{10}	Succinate	104.2±1.4[c,d]	112.3±1.0[a,c,d]	101.4±0.9[b,c,d]
	Succinate + ADP	40.5±0.9[c,d]	61.3±2.7[a,c,d]	63.2±3.0[a,c]
	Pyruvate/Malate	183.8±4.5[c,d]	198.3±3.1[a,c,d]	185.6±1.2[b,c,d]
	Pyruvate/Malate + ADP	54.5±1.6[c,d]	65.3±1.0[a,c,d]	65.0±1.2[a,c,d]

Data represent the mean ± SEM of eight experiments. H_2O_2 release rates are expressed in pmol/min/mg protein. Control (C), cold-exposed (CE), and cold-exposed and vitamin E-treated (CE + VE) rats

[a] significant vs. C rats

[b] significant vs. CE 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 and vitamin E treatment on H_2O_2 release by liver mitochondrial fractions in presence of inhibitors specific for different segments of the respiratory chain

Fraction	Substrate and additions	Group		
		C	CE	CE + VE
M_1	Succinate (Succ)	165.5±2.6	187.8±1.4[a]	160.8±0.7[b]
	Succ + Rot	121.9±3.1 *	142.2±3.7[a] *	125.0±1.0 [b] *
	Succ + Rot + AA	889.3±12.4 *	929.7±5.0[a] *	884.4±8.1[b] *
	Pyruvate/Malate (Pyr/Mal)	257.9±0.6	290.2±1.1[a]	257.2±1.3[b]
	Pyr/Mal + AA	936.6±14.7 *	992.6±5.6[a] *	976.9±9.5[a] *
	Pyruvate/Malate	258.1±0.5	291.0±1.2[a]	257.7±1.1[b]
	Pyr/mal + Rot	293.7±5.4 *	315.2±1.8[a] *	292.6±1.7[b] *
M_3	Succinate	154.2±3.5[c]	153.0±2.3[c]	151.3±0.9[c]
	Succ + Rot	116.1±1.6[c] *	121.7±0.9[c] *	115.1±1.0[c] *
	Succ + Rot + AA	861.3±12.7 *	886.6±5.5[c] *	870.5±3.1 *
	Pyruvate/Malate	244.0±4.0[c]	265.8±2.2[a,c]	256.0±1.3[a,b]
	Pyr/Mal + AA	919.4±16.6 *	974.8±9.8[a] *	968.8±11.0[a] *
	Pyruvate/Malate	243.8±4.1[c]	266.4±1.9[a,c]	255.6±1.2[a,b]
	Pyr/Mal + Rot	274.3±3.7[c] *	290.1±4.8[a,c] *	272.0±2.0[b,c] *
M_{10}	Succinate	115.4±1.0[c,d]	129.9±0.9[a,c,d]	137.0±1.1[a,b,c,d]
	Succ + Rot	103.9±1.3[c,d] *	112.4±1.0[a,c,d] *	100.8±0.9[b,c,d] *
	Succ + Rot + AA	757.6±17.2[c,d] *	835.3±9.1[a,c,d] *	838.0±10.4[a,c,d] *
	Pyruvate/Malate	183.5±4.3[c,d]	198.6±3.2[a,c,d]	185.4±1.3[b,c,d]
	Pyr/Mal + AA	822.0±4.5[c,d] *	868.4±12.9[a,c,d] *	857.8±3.3[a, c,d] *
	Pyruvate/Malate	183.6±4.3[c,d]	198.1±3.0[a,c,d]	185.4±1.1[b,c,d]
	Pyr/mal + Rot	215.5±8.4[c,d] *	253.6±6.3[a,c,d] *	238.3±4.10[a,c,d] *

Data represent the mean ± SEM of eight experiments. H_2O_2 release rates are expressed in pmol/min/mg protein. Control (C), cold-exposed (CE), and cold-exposed and vitamin E-treated (CE + VE) rats

[a] significant vs. C rats

[b] significant vs. CE rats

[c] significant vs. M_1 fraction

[d] significant vs. M_3 fraction

*significant effect of the last inhibitor added vs. mitochondria under same conditions without that inhibitor

The level of significance was chosen as $P<0.05$

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 groups, whereas in the presence of rotenone they became significantly different in the M_3 fractions from the CE + VE groups. Moreover, in the presence of antimycin, vitamin E treatment did not restore the control values of the H_2O_2 release rate in the M_1 and M_{10} fractions and did not reduced the cold induced increase in such a rate in all fractions. In the presence of rotenone, vitamin E treatment was not able to restore the control values of the H_2O_2 release rate in the M_1 fraction, whereas did this in the M_{10} fraction; however, in this fraction it did not reduce the cold effect on the H_2O_2 release rate.

Capacity to remove H_2O_2

Because part of the H_2O_2 produced within mitochondria is removed by H_2O_2 metabolizing enzymes and hemoproteins (Venditti et al. 2001), sometimes changes in the H_2O_2 release do not reflect changes in the H_2O_2 production rates. In all treatment groups, the capacity to remove H_2O_2 of the M_1 and M_3 fractions was not different and was higher than that of the M_{10} fractions. Such a capacity was not affected by cold exposure and vitamin treatment in the M_3 and M_{10}

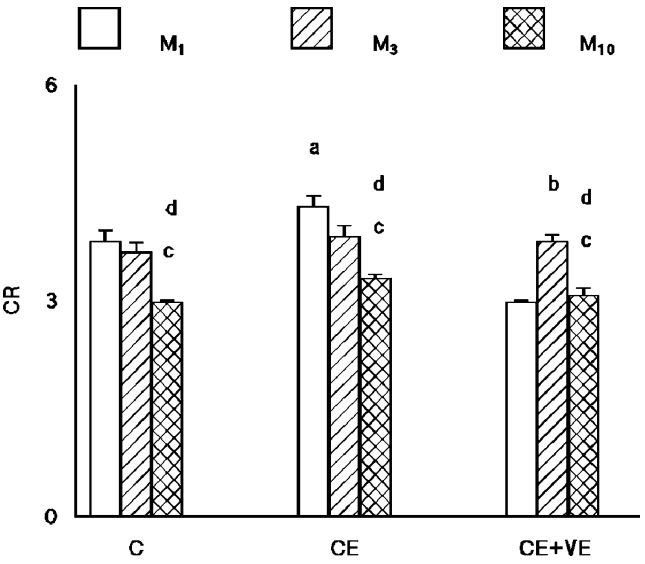

Fig. 3 Effect of cold exposure and vitamin E treatment on capacity of mitochondrial fractions to remove H_2O_2. Capacity to remove H_2O_2 (CR) is expressed as equivalent concentration of desferrioxamine (nmol/mg protein). Preparations from control (C), cold exposed (CE), and cold exposed, vitamin E treated (CE + VE) rats. Data represent the mean ± SEM of eight experiments. [a] significant vs. C rats; [b] significant vs. CE rats; [c] significant vs. M_1 fraction; [d] significant vs. M_3 fraction. The level of significance was chosen as $P<0.05$

fractions and was increased by cold but not by cold and vitamin treatment in the M_1 fraction (Fig. 3).

These results clearly indicate that the changes among fractions and treatment groups in the H_2O_2 release rates correspond to changes in the H_2O_2 production rates.

Antioxidants

The levels of low molecular weight antioxidants are reported in Table 5. In the C and CE + VE groups, the vitamin E content was lower in the M_1, whereas in the CE group the differences among fractions were not significant. Moreover, the vitamin E content was increased in all fractions after cold exposure and underwent further increase following vitamin E treatment. Significant differences among fractions in the coenzyme Q9 content were found only in the M_3 and M_{10} from the C group. In all fractions, cold exposure caused an increase in coenzyme Q9, which was prevented by the concomitant vitamin E treatment in the M_3 and M_{10} fractions only. In all groups, there were no differences between the M_1 and M_3 fractions in the coenzyme Q10 levels, which, in the M_{10} fraction were higher in the C and CE groups and lower in the CE + VE group. The coenzyme Q10 levels were increased by cold exposure in all groups, whereas, following vitamin E treatment, they were not further modified in the M_1 fraction and were reduced below the control values in the M_{10} fraction. The GSH level, which was the lowest in the M_{10} fraction, was increased in this fraction by both cold and vitamin E treatment, whereas was not affected by the treatments in the M_1 and M_3 fractions.

Mitochondrial swelling and membrane potential dissipation

As shown by the absorbance changes in Fig. 4a, in all groups the extent of Ca^{2+}-induced swelling was the highest in the M_1 fraction and the lowest in the M_{10} fraction. Moreover, the swelling was significantly increased by cold exposure in all fractions and reduced to the control values following vitamin E treatment. Mitochondrial swelling was drastically reduced by CSA or EGTA (results not shown), pointing to the role played by the permeability transition pore. Furthermore, in all fractions the cold-linked increase in mitochondrial swelling was preceded by a faster decline of the membrane potential ($\Delta\Psi$), which was reinstated to the control values by vitamin E treatment in the M_1 and M_{10} fractions (Fig. 4b).

Discussion

The metabolic response of the rat liver to a low environmental temperature is associated with the tissue oxidative damage

Table 5 Effect of cold exposure and vitamin E treatment on antioxidant levels of mitochondrial fractions from rat liver

Fraction	Parameters	Groups		
		C	CE	CE + VE
M_1	Vit E	0.25±0.01	0.39±0.02[a]	0.64±0.02[a,b]
	CoQ9	1.41±0.07	2.11±0.12[a]	1.82±0.13[a]
	CoQ10	0.16±0.01	0.24±0.01[a]	0.23±0.02[a]
	GSH	15.32±0.44	16.22±0.42	15.97±0.27
M_3	Vit E	0.34±0.02[c]	0.46±0.02[a]	0.77±0.03[a,b,c]
	CoQ9	1.65±0.05	2.27±0.11[a]	1.56±0.07[b]
	CoQ10	0.19±0.01	0.25±0.02[a]	0.20±0.02
	GSH	16.39±0.49	17.22±0.66	17.43±0.11
M_{10}	Vit E	0.37±0.02[c]	0.51±0.02[a]	0.74±0.02[a,b,c]
	CoQ9	1.33±0.06[d]	1.98±0.11[a]	1.55±0.08[b]
	CoQ10	0.23±0.02[c]	0.31±0.02[a,c,d]	0.17±0.01[a,b,c]
	GSH	11.13±0.64[c,d]	11.76±0.91[c,d]	13.65±0.27[a,b,c,d]

Data represent the mean ± SEM of eight experiments. Vitamin E (Vit E), Coenzyme Q10 (CoQ10), coenzyme Q9 (CoQ9), and GSH levels are expressed in nmol/mg proteins. Control (C), cold-exposed (CE), and cold-exposed and vitamin E-treated (CE + VE) rats

[a] significant vs. C rats

[b] significant vs. CE rats

[c] significant vs. M_1 fraction

[d] significant vs. M_3 fraction

The level of significance was chosen as $P<0.05$

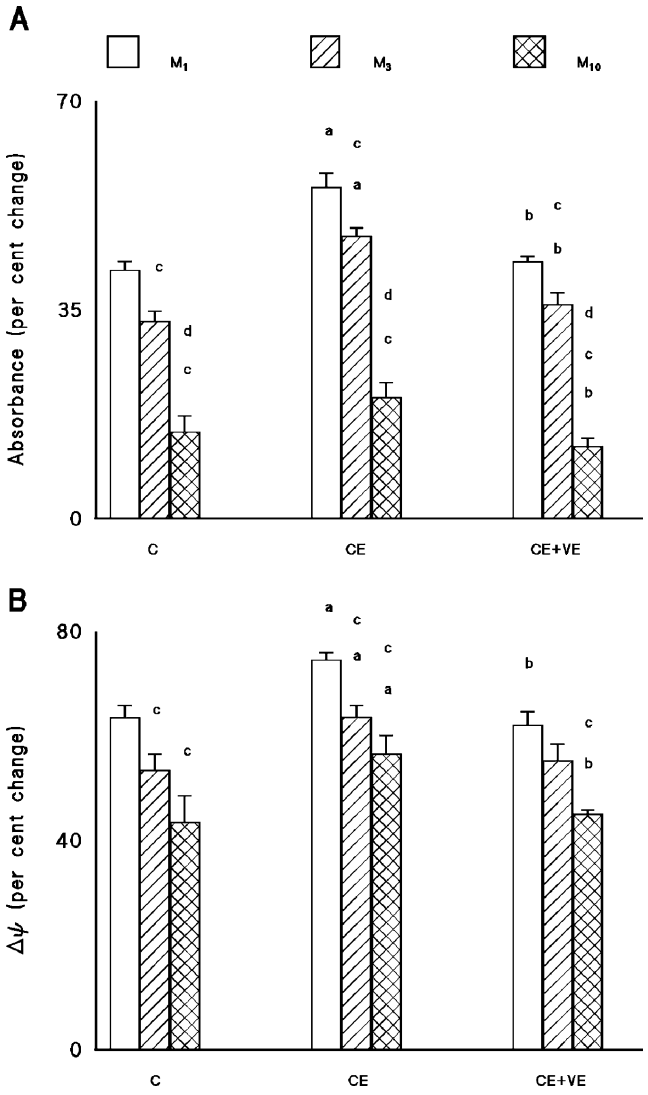

Fig. 4 Effect of cold exposure and vitamin E treatment on Ca^{2+}-induced swelling (**A**) and membrane potential dissipation (**B**) of mitochondrial fractions from control (C), cold exposed (CE) and cold exposed and vitamin E treated (CE + VE) rats. Swelling of mitochondrial preparations (0.3 mg/ml) 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 (ΔΨ) 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+}. ΔΨ was calculated using a suitable calibration curve. The decrease of ΔΨ 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 cold exposed (CE) 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, those of preparations from cold exposed and vitamin E treated (CE + VE) rats were 0.88±0.03, 0.86±0.02, and 0.46±0.04 for M_1, M_3, and M_{10} fractions, respectively. Initial values of ΔΨ of mitochondrial fractions from control (C) rats were 187.3±10.5 mV, 170.5±9.6 mV, and 77.0±6.4 mV for M_1, M_3, and M_{10} fractions, respectively, those of preparations from cold exposed rats (CE) were 170.7±9.0 mV, 150.1±9.6 mV, and 60.9±5.7 mV for M_1, M_3, and M_{10} fractions, respectively, those of preparations from cold exposed and vitamin E treated (CE + VE) rats were 174.3±9.1 mV, 165.0±10.0 mV, and 65.7±6.0 mV for M_1, M_3, and M_{10} fractions, respectively. Data represent the mean±SEM of eight experiments. [a] significant vs. C preparations; [b] significant vs. CE preparations; [c] significant vs. M_1 fraction; [d] significant vs. M_3 fraction. The level of significance was chosen as $P<0.05$

(Venditti et al. 2010). The increases in respiration and oxidative damage depend on increases in the mitochondrial O_2 consumption and H_2O_2 production, respectively. However, they depend also on a remarkable mitochondrial proliferation (Venditti et al. 2004a). The vitamin E treatment attenuates the liver oxidative damage reducing the H_2O_2 mitochondrial release without modifying the aerobic capacity and mitochondrial protein content of the tissue (Venditti et al. 2007).

The study of the characteristics of the rat liver mitochondrial subpopulations has supplied further information on the mechanisms underlying the vitamin E effects on the mitochondria from cold liver.

Our results show that, despite a reduction of the M_1 fraction, provided with the highest oxidative capacity, cold exposure increases the oxidative capacities of liver homogenates and mitochondria, increasing both the oxidative capacities of the mitochondrial fractions and tissue content of mitochondrial proteins.

The vitamin E administration does not modify the cold-induced changes in the oxidative capacities of liver homogenates, whole mitochondrial populations, and heavy mitochondrial fractions. Moreover, it does not reduce the cold-linked increase in the hepatic content of mitochondrial proteins, showing that the vitamin E does not affect the mitochondrial protein synthesis although it is able to regulate gene expression (Azzi and Stocker 2000). Conversely, vitamin E causes an inversion of the cold-linked distribution of the mitochondrial proteins in the M_1 and M_3 fractions and reduces the oxidative damage extent of the mitochondrial fractions, phenomena which also happen when the vitamin is administered to T_3-treated rats (Venditti et al. 1999). These results suggest that the decrease in the liver content of the M_1 fraction is related to the extent of the oxidative damage suffered by this fraction. This idea is supported by some observations. The oxidative damage of mitochondrial components increases in conditions in which the ROS production is rising (Boveris et al. 1999). Thus, then days of cold exposure (Venditti et al. 2007; Venditti et al. 2006) increase the mitochondrial H_2O_2 generation and oxidative damage, whereas vitamin E treatment of the cold-exposed rats attenuates such increases (Venditti et al. 2007). The present results show that the H_2O_2 release rate and oxidative damage are higher in the M_1 than in the M_3 and M_{10} fractions from C rats and remain higher in the M_1

mitochondria from CE rats, despite the generalized increase caused by cold exposure. The high level of oxidative damage reached in the M_1 mitochondria following the cold exposure leads to their degradation and consequent decrease in their hepatic content, a phenomenon which is limited by the oxidative damage reduction due to vitamin E administration.

In the intact mitochondria the H_2O_2 release rates depend on the concentration of autoxidizable electron carriers and on their reduction degree. Mitochondria supplemented with respiratory inhibitors exhibit complete reduction of the electron carriers on the substrate side of the inhibitors so that the H_2O_2 release rates depend only on concentration of autoxidizable carriers located on such a side. Thus, the effects of the respiratory inhibitors here reported suggest that the cold-induced changes in the H_2O_2 release are due to increased autoxidizable carrier concentration at Complex I and Complex III, in the M_1 and M_{10} fractions, and at Complex I in the M_3 fraction.

It could appear surprising that in the cold liver the oxidative damage of the M_3 fraction is similar to that of the other fractions despite the lack of cold-induced increases in both autoxidizable carrier content at Complex III and succinate-supported H_2O_2 release rate. This apparent discrepancy can be explained by the observation that a mitochondrion can intercept and be damaged by ROS produced by extra-mitochondrial sources or released by other mitochondria (Zoccarato et al. 2004).

Another apparently surprising observation is that the COX activity and the coenzyme Q content increase in the M_3 and M_{10} fractions, which do not show changes of the State 3 O_2 consumption. However, the above increases are not necessarily shared by other components of the respiratory chain, as suggested by the differential changes induced by cold in the cytochrome content of rat liver mitochondria (Bravo et al. 2001). Furthermore, components of the respiratory chain are likely damaged by hydroxyl radicals and peroxynitrite, a powerful oxidant generated by the reaction between superoxide and nitric oxide. Such cold effects should result in a slower electron flow through the respiratory chain leading to a reduced O_2 consumption and an enhanced ROS production.

The vitamin E administration does not reinstate the control levels of oxidative damage in the mitochondrial fractions. It is likely that this depends on vitamin inability to affect all the factors determining the mitochondrial susceptibility to oxidative stress.

A factor is the concentration of autoxidizable carriers in the reduced state, which determines the H_2O_2 production rate. The effects of respiratory inhibitors on the H_2O_2 release suggest that the vitamin E treatment scarcely affects the autoxidizable carrier concentration. Indeed, in the presence of the inhibitors, only in some cases the H_2O_2 release rates are restored to the control values and generally their percentage decreases are comparable to or smaller than those found in the presence of the substrate alone. Conversely, in the light of the inverse relationship between rate of electron flow and reduction degree of electron carriers (Barja 1999), the O_2 consumption changes suggest that the reduction degree of both autoxidizable carriers in the M_1 mitochondria and that of the carrier at Complex III in the M_3 mitochondria are lowered by vitamin E treatment. Indeed, the M_1 fraction exhibits an increased O_2 consumption and a decreased H_2O_2 release in the presence of Complex I and Complex II-linked substrates whereas the M_3 fraction exhibits the same changes only in the presence of Complex I-linked substrates. In the M_{10} fraction there are not changes of the O_2 consumption so that the decrease in the H_2O_2 release should be due to a different mechanism. It is possible that it involves the capacity of the α-tocopherol (Gotoh and Niki 1992) and the α-tocopheryl radical (Cadenas et al. 1989) to scavenge the superoxide radical, which could also be involved in the down-regulation of mitochondrial H_2O_2 production in the M_1 and M_3 fractions.

Another factor is the concentration of the hemoproteins, which contribute to the conversion of H_2O_2 in $^{\bullet}OH$ radicals (Turrens et al. 1985), and the oxidative damage that mitochondria undergo at a fixed rate of H_2O_2 production. Indeed, because of the scant influence of the vitamin E on the hemoprotein levels it is reasonable to think that the antioxidant manipulation does not affect the probability that $^{\bullet}OH$ radicals are generated.

Interestingly, the vitamin E treatment restores the protein distribution pattern found in the mitochondrial fractions from the control animals, in which the M_1 and M_{10} fractions display the highest and the lowest protein content, respectively. However, with respect to the controls, the percentages of the M_1 and M_3 proteins remain lower and higher, respectively. This can be explained by the observation that the characteristics of the mitochondrial fractions determine both the extent of the oxidative damage they undergo and their susceptibility to stressful conditions (Venditti et al. 1996; Venditti et al. 1999). Mitochondria exposed to a Ca^{2+} load, undergo an inner membrane permeabilization named mitochondrial permeability transition (MPT) (Vercesi et al. 1997), which requires a potential membrane decrease (Bernardi et al. 1992) and leads to mitochondrial degradation. Because MPT can be promoted by ROS-induced oxidation of protein thiols located in the inner membrane and unmasked by matrix Ca^{2+} (Vercesi et al. 1997), mitochondria which fail to maintain low the ROS level and, hence, keep the pores open, are discarded, whereas the low ROS producing organelles survive (Skulachev 1996). This idea is consistent with the finding that the M_1 fraction, which is provided with the highest capacity to produce ROS and the lowest antioxidant capacity (Venditti et al. 2002), shows a susceptibility to Ca^{2+}-induced MPT higher than the

other fractions. Furthermore, it explains why the cold-induced increase in the H_2O_2 production by the M_1 mitochondria is associated with an increase in their susceptibility to the permeability transition and a decrease in the hepatic content of their proteins.

Previously, we found that the State 3 O_2 consumption by liver mitochondria from cold-exposed rats is increased by vitamin E (Venditti et al. 2007). We also hypothesized that, following cold exposure, the mitochondria consume O_2 at a rate lower than that their equipment of electron carriers would allow and vitamin E preserves the mitochondrial function protecting the respiratory chain components against the oxidative alterations. The results here reported support the above hypothesis showing, however, that the vitamin E increases the O_2 consumption by the whole mitochondrial population also restoring a low susceptibility to the swelling and effectively preventing the loss of the M_1 mitochondria.

In conclusion, these and other observations reported in this paper confirm the timelines to investigate the modifications of mitochondrial subpopulations to obtain information on the mechanisms underlying the response of liver mitochondria to stressful conditions and antioxidant integration.

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