CMLS, Cell. Mol. Life Sci. 61 (2004) 2244–2252
1420-682X/04/172244-09
DOI 10.1007/s00018-004-4125-9

CMLS Cellular and Molecular Life Sciences

Research Article

Role of nitric oxide in the functional response to ischemia-reperfusion of heart mitochondria from hyperthyroid rats

P. Venditti, R. De Rosa, L. Cigliano, C. Agnisola and S. Di Meo*

Dipartimento di Fisiologia Generale ed Ambientale, Università di Napoli, V. Mezzocannone 8, 80134 Napoli (Italy), Fax: +39 81 2535090, e-mail: dimeo@biol.dgbm.unina.it

Received 24 March 2004; received after revision 9 June 2004; accepted 5 July 2004

Abstract. We investigated the role of nitric oxide (NO) in the mitochondrial derangement associated with the functional response to ischemia-reperfusion of hyperthyroid rat hearts. Mitochondria were isolated at 3000 g from hearts subjected to ischemia-reperfusion, with or without N^{ω}-nitro-L-arginine (L-NNA, an NO synthase inhibitor). During reperfusion, hyperthyroid hearts displayed tachycardia and low functional recovery. Their mitochondria exhibited O_2 consumption similar to euthyroid controls, while H_2O_2 production, hydroperoxide, protein-bound carbonyl and nitrotyrosine levels, and susceptibility to swelling were higher. L-NNA blocked the reperfusion tachycardic response and increased inotropic recovery in hyperthyroid hearts. L-NNA decreased mitochondrial H_2O_2 production and oxidative damage, and increased respiration and tolerance to swelling. Such effects were higher in hyperthyroid preparations. These results confirm the role of mitochondria in ischemia-reperfusion damage, and strongly suggest that NO overproduction is involved in the high mitochondrial dysfunction and the low recovery of hyperthyroid hearts from ischemia-reperfusion. L-NNA also decreased protein content and cytochrome oxidase activity of a mitochondrial fraction isolated at 8000 g. This and previous results suggest that the above fraction contains, together with light mitochondria, damaged mitochondria coming from the heaviest fraction, which has the highest cytochrome oxidase activity and capacity to produce H_2O_2. Therefore, we propose that the high mitochondrial susceptibility to swelling, favoring mitochondrial population purification from H_2O_2-overproducing mitochondria, limits hyperthyroid heart oxidative stress.

Key words. Ischemia-reperfusion; hydrogen peroxide release; mitochondrial function; nitric oxide; hyperthyroidism.

Introduction

Although restoration of blood flow is necessary to rescue the ischemic myocardium, oxidative stress occurs during reoxygenation, contributing to so-called ischemia-reperfusion injury [1]. The mechanisms of the cellular and subcellular disruption associated with this phenomenon are not well established. Mitochondria play an essential role in cell viability, and are both a source of reactive oxygen species (ROS) and a primary target for oxidative stress. Their derangement under oxidative stress may account for the reperfusion-induced tissue damage. Experimental evidence indicates that, during reperfusion of ischemic myocardium, the respiratory chain is a major site of ROS production [2, 3]. ROS in turn produce mitochondrial dysfunction [4], which is inversely correlated to the functional recovery during reperfusion of myocardial tissue [5].

Asayama and Kato [6] suggested that hyperthyroidism-induced heart dysfunction is a result of oxidative damage due to an increased production of ROS. Indeed, hyperthyroidism increases H_2O_2 production by heart mitochondria [7, 8]. However, the increased oxidative damage observed in the hyperthyroid heart [9, 10] seems also to depend on its low effectiveness in preventing oxidative alterations [10].

* Corresponding author.

In previous work, we tested this possibility using ischemia-reperfusion as a model of oxidative myocardial injury related to ROS production [11]. During reperfusion, hyperthyroid hearts displayed significant tachycardia and a low recovery of left ventricular developed pressure (LVDP) and maximal rate of developing left ventricular pressure dP/dt_{max}, associated with extensive peroxidative processes. Vitamin E prevented these functionally and biochemically altered responses to reperfusion suggesting that they were associated with the reduced capability of the hyperthyroid heart to face oxidative stress [11]. The high susceptibility to ischemia-reperfusion of hyperthyroid hearts was associated with mitochondrial dysfunction, H_2O_2 production, and oxidative damage higher than those shown in mitochondria from euthyroid hearts [12].

The mechanisms by which ROS mediate the decline in mitochondrial function are not entirely clear. Nitric oxide (NO) and its potent oxidative derivative peroxynitrite are putative species responsible for altered mitochondrial function in myocardial ischemia-reperfusion. Nitric oxide synthase (NOS) stimulation upon ischemia-reperfusion [13] and inhibition of mitochondrial function by both NO [14] and peroxynitrite [15] have been reported. Moreover, we found that the perfusion of hearts with the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NNA) prevented the tachycardic response to ischemia-reperfusion of hyperthyroid heart and the consequent contractile dysfunction [16].

In the present study we tested the possibility that the reperfusion-induced mitochondrial dysfunction seen in hyperthyroid hearts is due to overproduction of NO, comparing the change in mitochondrial function following ischemia-reperfusion of hearts from euthyroid and hyperthyroid animals in the presence and absence of L-NNA.

Materials and methods

Animals, preparation set up and equipment

The animals (60-day old male Wistar rats from Nossan, Correzzana, Italy) were housed in separate cages at 24 ± 1 °C, with an artificial lighting cycle (LD 8–20 h) and ad libitum water and commercial rat chow (Nossan) provision. From day 50, animals were randomly assigned to one of two groups: E, euthyroid control rats (n = 16, mean weight ± SEM 303 ± 9 g), and H, rats made hyperthyroid with 10 daily intraperitoneal injections of triiodothyronine (T_3) (10 µg/100 g body weight) (n = 16, mean weight 262 ± 6 g). Hearts from each group were randomly assigned to two subgroups, according to their in vitro perfusion with basal saline or with L-NNA, as described below, giving four heart groups: E_c (controls, euthyroid hearts, n = 8), H_c (controls hyperthyroid hearts, n = 8), $E_{L\text{-}NNA}$ (L-NNA- treated euthyroid hearts, n = 8) and $H_{L\text{-}NNA}$ (L-NNA- treated hyperthyroid hearts, n = 8). Animals were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/100 g body weight) combined with ether. After electrocardiographic recording and heparinization, a rapid thoracotomy was performed and the aorta cannulated retrogradely. The hearts were excised and flushed to get rid of blood for 1 min with Krebs-Henseleit (KH) buffer containing (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA 0.5, glucose 11, pH 7.4, and gassed with 5% CO_2 in O_2. The same buffer was used to perfuse the isolated hearts at 37 °C and a head pressure of 70 mm Hg according to Langendorff [17]. After stabilization (20 min) and 15-min perfusion, hearts were subjected to global normothermic ischemia for 20 min and then reperfused for 25 min with KH oxygenated buffer. $E_{L\text{-}NNA}$ and $H_{L\text{-}NNA}$ heart perfusion was shifted from KH to KH containing 0.2 mM L-NNA 5 min before ischemia. Functional performance was determined immediately before ischemia and at the end of reperfusion as previously described [11]. The investigation conformed to the guidelines for care and use of laboratory animals of the Italian Health Ministry.

Preparation of mitochondria

At the end of the perfusion experiments, large heart vessels, valves, and atria were trimmed away and the ventricles were cut open, rinsed free of liquid, and weighed. Then the ventricles were finely minced and washed with ice-cold isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 20 mM Tris, pH 7.4) containing 0.1 mg/ml nagarse and 0.1% fatty acid-free albumin. Tissue fragments were gently homogenized in the same solution (10% w/v) using a glass Potter-Elvehjem homogenizer set at a standard velocity for 1 min. The homogenates were freed from debris and nuclei by centrifugation at 500 g for 10 min at 4 °C and the resulting supernatants were centrifuged at 3000 g for 10 min. The mitochondrial pellets were washed twice with 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4, before final suspension in the same solution. Basically, this procedure was similar to that described by Tyler and Gonze [18]; however, we slightly modified the procedure, by reducing both the speed used to free homogenates from debris and nuclei (500 g instead of 700 g) and that used to obtain the mitochondrial pellet (3000 g instead of 8000 g). These changes reduced both the contaminations by cytoplasmic and microsomal material and the amount of damaged mitochondria [12]. We defined this mitochondrial fraction, on which most of the present study was performed as M_3. We also isolated a second mitochondrial fraction (M_8) by a subsequent centrifugation step at 8000 g, in order to investigate whether hyperthyroidism or L-NNA treatment modify the characteristics of the M_3 fraction, by affecting the dynamics of the mitochondrial population.

For both fractions, the protein content and the activities of cytochrome oxidase (COX) and oligomycin-insensitive

adenosine triphosphatase (ATPase) were determined. COX activity was determined polarographically at 30 °C, using a Gilson glass respirometer (Yellow Springs Instruments, Ohio, USA), by the procedure of Barré et al. [19]. ATPase activity was determined according to Krieger et al. [20]. Mitochondrial protein content was determined, upon dissolving in 0.5% deoxycholate, by the biuret method [21] with bovine serum albumin (BSA) as standard.

M_3 preparations were used for the biochemical assays described below.

Analytical procedures

Mitochondrial respiration was monitored at 30 °C by a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instrument) in 1.6 ml incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO4, 3 mM $MgCl_2$, 0.1 mM EGTA, rotenone 5 µM, pH 7.4) with 0.5 mg mitochondrial protein per milliliter. Succinate (10 mM) was used as substrate, in the absence (state 4) and presence (state 3) of 500 µM ADP.

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 by H_2O_2 in the presence of horseradish peroxidase [22] in a computer-controlled Jasko fluorometer. Succinate (10 mM) was used as substrate. Measurements 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.

The 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]. Briefly, p-hydroxyphenylacetate (PHPA) oxidation to the stable fluorescent product 2,2′-dihydroxy-biphenyl-5,5′-diacetate $(PHPA)_2$ [22] by the H_2O_2 generated from glucose oxidase (GOX) was monitored with a Jasko fluorometer (excitation wavelength 320 nm, emission wavelength 400 nm) at 30 °C. The reaction was performed in the presence of desferrioxamine (1 – 12 nmoles), or mitochondrial samples (0.1 – 1.0 mg of mitochondrial proteins). 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 desferrioxamine values were used to fit standard curves by the Fig. P program (Biosoft, Cambridge, Mass.). The sample values were plotted on the standard curves to obtain their capacity to remove H_2O_2, expressed as equivalent desferrioxamine concentration.

The extent of peroxidative reactions was determined by measuring the hydroperoxides (HPs) according to Heath and Tappel [24].

Quantification of protein-bound carbonyls was performed by the procedure of Schild et al. [25]. Protein recovery was estimated for each sample. 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.

Nitrotyrosine (N-Tyr) content was evaluated by ELISA titration according to a slightly modified propcedure of Tanaka et al. [26]. Samples were diluted (1:2000, 1:5000, 1:15,000, 1:30,000, 1:45,000, 1:60,000, and 1:90,000), coated into the wells of a microtiter plate and incubated for 2 h at room temperature. Nitrated BSA was used as a standard. For N-Tyr titration, sheep antiN-Tyr antibody and donkey anti-sheep horseradish peroxidase-conjugated antibody, as primary and secondary antibody, respectively, were used. N-Tyr content was expressed as nmol nitrotyrosine per milligram of protein.

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) when required.

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 [27] 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 EDTA, 8 µM safranine, 38.5 ng/ml valinomycin, and KCl at concentrations from 0 to 0.96 mM.

All chemicals used (Sigma, Milan, Italy) were of the highest grades available.

Statistical analysis

The data obtained from each heart group were expressed as mean values ± SE and were analyzed with two-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Differences between M_3 and M_8 fractions in the same subgroup were statistically analyzed with an unpaired Student's t test. The level of significance was chosen as $p < 0.05$. Statistics and graphics were performed with GraphPad Prism (v. 4.00; GraphPad Software, San Diego, Calif.).

Results

The hyperthyroid state of T_3-treated animals was reflected in their higher heart/body weight ratio (E = 2.36 ± 0.07 mg/g; H = 3.36 ± 0.07 mg/g, $p < 0.05$, unpaired Stu-

dent's t test) and in vivo heart rate (E = 440 ± 7 beats/min; H = 542 ± 8 beats/min, $p < 0.05$).

Basal heart performance and functional recovery from ischemia-reperfusion

Unlike basal preischemic coronary flow, preischemic ventricular performance was significantly affected by T_3 treatment of animals. Hyperthyroid rats displayed a higher intrinsic heart rate than euthyroid animals (table 1). L-NNA treatment of Langendorff preparations significantly increased cardiac inotropism [(LVDP) and $(dP)/dt_{max}$] in the euthyroid animals and not in the hyperthyroid animals, so that LVDP and dP/dt_{max} values were higher in euthyroid than in hyperthyroid animals.

Functional recovery values (= the percent ratio between the value at the end of the reperfusion period and the value of the same parameter just before the onset of the ischemic period) of the Langendorff preparations after ischemia-reperfusion are reported in Figure 1.

The main effect of T_3 treatment was a strong tachycardia during reperfusion, with a heart rate at 25-min reperfusion twice as much as the preischemic value. This tachycardia was accompanied by a recovery of ventricular contractility (LVDP and dP/dt_{max}) significantly lower than that of euthyroid hearts. Hyperthyroid hearts displayed slightly lower recovery of coronary flow with respect to the other groups.

Heart perfusion with L-NNA did not affect functional recovery in the euthyroid hearts. The tachycardic response of hyperthyroid hearts was abolished by L-NNA. As a consequence, LVDP and dP/dt_{max} in such hearts were close to those of euthyroid hearts. The perfusion with L-NNA also increased coronary flow recovery of hyperthyroid hearts up to the euthyroid levels.

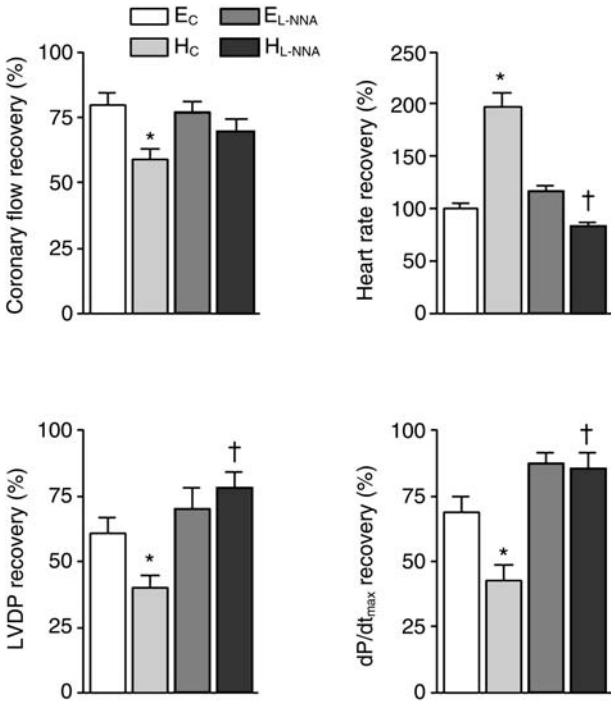

Figure 1. Reperfusion recovery of coronary flow, heart rate, LVDP and dP/dt_{max} following 20-min ischemia in Langendorff preparations from euthyroid and hyperthyroid rats. From each group of animals, half of the preparation was perfused with 0.2 mM L-NNA 5 min before the onset of ischemia, and during the reperfusion. Data are means ± SE of eight preparations. * Significant effect of T_3 treatment. † Significant effect of L-NNA (two-way ANOVA followed by Bonferroni post hoc test, $p < 0.05$).

Characteristics of mitochondrial subpopulations

As shown in table 2, in the M_3 fraction, COX activity was not affected by L-NNA treatment, but was increased by hyperthyroidism in L-NNA-treated hearts. ATPase activity and protein content of the above fraction were not significantly affected by T_3 treatment or L-NNA perfusion. In the M_8 fraction, COX activity and protein content were increased by T_3 administration in untreated hearts and decreased by L-NNA perfusion in hyperthyroid hearts. M_8 ATPase activity was not modified by T_3 treatment or L-NNA perfusion.

In all groups, the protein amount was lower in the M_8 than in the M_3 fraction. Conversely, oligomycin-insensitive ATPase activity was higher in the M_8 fraction, indicating, as expected, that such a fraction was more contaminated by other cellular components. In E_c, $E_{L\text{-}NNA}$, and H_c hearts, COX activity of the M_8 fraction was higher than that of the M_3 fraction, whereas in $H_{L\text{-}NNA}$ hearts it was lower.

Table 1. Preischemic coronary flow and left ventricle performance (basal performance) of Langendorff preparations from euthyroid and hyperthyroid rats.

Group	Coronary flow (ml/min)	Heart rate (beats/min)	LVDP (torr)	DP/dt_{max} (torr/s)
E_C	12.72 ± 0.95	220.91 ± 17.5	60.79 ± 5.20	861.83 ± 83.4
$E_{L\text{-}NNA}$	11.73 ± 1.01	211.14 ± 19.88	84.91 ± 5.01†	1183.63 ± 85.10†
H	12.84 ± 1.07	276.38 ± 18.85*	53.61 ± 7.15	628.29 ± 78.36
$H_{L\text{-}NNA}$	11.83 ± 1.91	279.13 ± 18.9*	54.28 ± 7.17*	667.15 ± 83.49*

Values are means ± SE of eight experiments.

* Significant difference for hyperthyroid (H_C or $H_{L\text{-}NNA}$) vs euthyroid (E_C or $E_{L\text{-}NNA}$) animals ($p < 0.05$).

† Significant difference between L-NNA-treated ($E_{L\text{-}NNA}$ or $H_{L\text{-}NNA}$) and L-NNA-untreated (E_c or H_c) preparations ($p < 0.05$).

Table 2. Cytochrome oxidase and oligomycin-insensitive ATPase activities in mitochondrial fractions from euthyroid and hyperthyroid rat hearts subjected to ischemia-reperfusion.

Parameter	Fraction	Group			
		E_c	$E_{L\text{-}NNA}$	H_c	$H_{L\text{-}NNA}$
COX	M_3	1.93 ± 0.11	1.98 ± 0.11	2.33 ± 0.08	2.54 ± 0.16*
	M_8	2.42 ± 0.12‡	2.28 ± 0.04‡	2.86 ± 0.06*,‡	2.38 ± 0.07†
ATPase	M_3	5.8 ± 0.4	5.9 ± 0.2	6.5 ± 0.6	5.8 ± 0.6
	M_8	14.1 ± 1.1‡	15.3 ± 1.0‡	15.0 ± 1.0‡	14.6 ± 1.1‡
Protein (%)	M_3	84.2 ± 3.1	89.9 ± 3.9	76.6 ± 3.8	89.1 ± 4.4
	M_8	15.8 ± 1.7‡	10.1 ± 0.8‡	23.4 ± 2.8*,‡	10.9 ± 0.4†,‡

Values are means ± SE of eight experiments. M_3 and M_8, mitochondrial fractions isolated at 3000 and 8000 g, respectively. COX activity is expressed in µmol O/min per milligram mitochondrial protein; oligomycin-insensitive adenosine-triphosphatase (ATPase) activity, expressed as percent of total ATPase activity, is used as a measure of contamination of mitochondrial preparations. Protein (%) represents the percent distribution of mitochondrial proteins in the two fractions.

* Significant difference for hyperthyroid animals vs respective euthyroid controls ($p < 0.05$).

† Significant for L-NNA-treated hearts vs respective untreated controls ($p < 0.05$).

‡ Significant for M_8 fraction vs M_3 fraction ($p < 0.05$).

Mitochondrial oxygen consumption

The rate of state 4 oxygen consumption was not dependent on either L-NNA perfusion or T_3 treatment (table 3). Conversely, the rate of state 3 oxygen consumption, which was not different in mitochondria from untreated euthyroid and hyperthyroid hearts, was strongly increased by L-NNA perfusion. This increase was higher in hyperthyroid preparations, so that the mitochondrial respiration rate was significantly higher in the $H_{L\text{-}NNA}$ than in the $E_{L\text{-}NNA}$ group.

Mitochondrial H_2O_2 release and capacity to remove H_2O_2

The rate of succinate-supported H_2O_2 release, in the presence and absence of ADP, and the rate of antimycin A-stimulated H_2O_2 release were significantly higher in mitochondrial preparations from hyperthyroid hearts than in those from the corresponding euthyroid controls (table 4). L-NNA treatment decreased the rate of H_2O_2 release during state 3 and state 4 respiration in both euthyroid and hyperthyroid preparations, but did not significantly modify H_2O_2 release in all antimycin A-treated preparations (table 4).

The capacities to remove H_2O_2 were not significantly different in preparations from untreated hearts ($E_C = 2.47 \pm 0.04$; $H_C = 2.68 \pm 0.10$, $p > 0.05$). L-NNA perfusion significantly increased the mitochondrial capacity to remove H_2O_2 of hyperthyroid hearts, but not of euthyroid hearts. Thus, the capacity of mitochondria to remove H_2O_2 was higher in $H_{L\text{-}NNA}$ than in the $E_{L\text{-}NNA}$ group ($E_{L\text{-}NNA} = 2.87 \pm 0.08$; $H_{L\text{-}NNA} = 3.52 \pm 0.10$, $p < 0.05$). This indicates that the mitochondria from $H_{L\text{-}NNA}$ hearts are endowed with a greater level of substances which remove H_2O_2 by producing H_2O (H_2O_2-metabolizing enzymes) or $^{\bullet}OH$ radical (iron ligands).

Oxidative damage of mitochondria

Oxidative damage in heart mitochondria was increased by T_3 and decreased by L-NNA treatment. In fact, the hydroperoxide level and protein-bound carbonyl content were both higher in hyperthyroid preparations than in the respective euthyroid controls and decreased significantly after L-NNA treatment in both euthyroid and hyperthyroid preparations (table 5).

Table 3. Effects of ischemia-reperfusion on O_2 consumption of heart mitochondria from euthyroid and hyperthyroid rats.

Group	O_2 consumption (nmol/min per milligram protein)		
	state 4	state 3	RCR
E_c	78.7 ± 1.7	220.3 ± 9.1	3.0 ± 0.2
$E_{L\text{-}NNA}$	74.8 ± 3.0	255.2 ± 8.3†	3.2 ± 0.2
H_c	86.1 ± 4.5	238.3 ± 8.7	2.8 ± 0.2
$H_{L\text{-}NNA}$	83.4 ± 3.1	315.1 ± 12.8*,†	3.6 ± 0.3

Values are means ± SE of eight different experiments RCR, respiratory control ratio.

* Significant difference for hyperthyroid animals vs respective euthyroid controls ($p < 0.05$).

† Significant for L-NNA-treated hearts vs respective untreated controls ($p < 0.05$).

Table 4. Effects of ischemia-reperfusion on H_2O_2 release by heart mitochondria from euthyroid and hyperthyroid rats.

Group	H_2O_2 release (pmoles/min per milligram protein)		
	succinate	succinate + ADP	succinate + AA
E_c	135.3 ± 2.4	89.9 ± 2.7	823.2 ± 7.2
$E_{L\text{-}NNA}$	113.7 ± 1.4†	76.8 ± 1.3†	824.1 ± 12.9
H_c	172.9 ± 1.2*	109.1 ± 3.6*	896.7 ± 4.1*
$H_{L\text{-}NNA}$	153.2 ± 1.0*,†	95.1 ± 3.4*,†	913.6 ± 14.8*

Values are means ± SE of eight experiments.

* Significant difference for hyperthyroid animals vs respective euthyroid controls ($p < 0.05$) AA, antimycin A.

† Significant for L-NNA-treated hearts vs respective untreated controls ($p < 0.05$).

Table 5. Effect of ischemia-reperfusion on hydroperoxide, protein-bound carbonyl and nitrotyrosine levels in heart mitochondria from euthyroid and hyperthyroid rats.

Group	Parameter		
	HPs	CO	N-Tyr
E_c	24.8 ± 0.8	2.88 ± 0.12	0.43 ± 0.08
$E_{L\text{-}NNA}$	17.2 ± 0.7†	2.40 ± 0.20†	0.22 ± 0.03†
H_c	36.1 ± 0.6*	3.56 ± 0.06*	1.10 ± 0.06*
$H_{L\text{-}NNA}$	22.5 ± 1.1*,†	2.81 ± 0.11*,†	0.49 ± 0.06*,†

Values are the means ± SE of eight experiments for HPs and CO and six experiments for N-Tyr. Protein-bound CO is expressed as nmol/mg protein. HPs are expressed as pmol NADPH/mg protein. N-Tyr levels are expressed as nitro-BSA equivalents (nmol/mg protein).

* Significant difference for hyperthyroid animals vs respective euthyroid controls ($p < 0.05$).

† Significant for L-NNA-treated hearts vs respective untreated controls ($p < 0.05$).

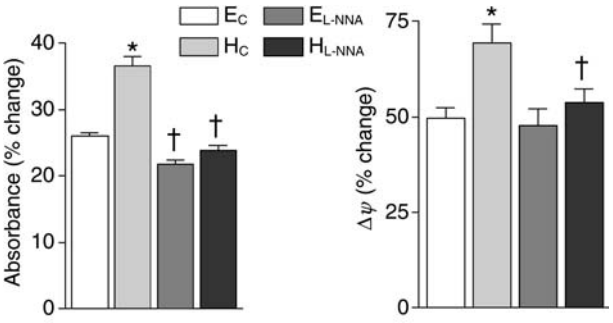

Figure 2. Effect of L-NNA treatment on Ca^{2+}-induced swelling (left panel) and membrane potential dissipation (right panel) of mitochondria from euthyroid and hyperthyroid hearts subjected to ischemia-reperfusion. Swelling of mitochondrial preparations (0.3 mg/ml), monitored as decrease in the absorbance at 540 nm in the presence of Ca^{2+}, is 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 in the presence of Ca^{2+}. $\Delta\Psi$ was calculated using a suitable calibration curve and its decrease is expressed as percent of the initial value before Ca^{2+} addition. The initial absorbance values were 0.78 ± 0.06, 0.76 ± 0.08, 0.78 ± 0.07, and 0.81 ± 0.06 for E_c, $E_{L\text{-}NNA}$, H_c, and $H_{L\text{-}NNA}$ preparations, respectively. Initial values of $\Delta\Psi$ were 149.2 ± 12.1, 169.5 ± 8.9, 89.4 ± 3.6, and 127.6 ± 6.7 mV for E_C, $E_{L\text{-}NNA}$, H_C, and $H_{L\text{-}NNA}$ preparations, respectively. Values are means ± SE of eight experiments. *Significant difference for hyperthyroid animals vs respective euthyroid controls ($p < 0.05$). † Significant for L-NNA-treated hearts vs respective untreated controls.

NO-linked damage of mitochondrial proteins was higher in hyperthyroid untreated hearts than in their euthyroid controls. It was strongly reduced in the hyperthyroid hearts by L-NNA treatment so that it was not significantly different in $H_{L\text{-}NNA}$ and $E_{L\text{-}NNA}$ groups. The extent of tyrosine nitration was also higher in hyperthyroid hearts than in euthyroid ones and was significantly reduced in both euthyroid and hyperthyroid preparations by L-NNA treatment.

Mitochondrial swelling

To verify whether animal treatment with T_3 or heart treatment with L-NNA affected the susceptibility of mitochondria to Ca^{2+}-dependent swelling, succinate-energized mitochondria were incubated in the presence of 100 µM Ca^{2+} and absorbance changes were monitored. Figure 2 (left panel) shows that the Ca^{2+}-loaded mitochondrial suspensions from untreated hearts subjected to ischemia-reperfusion suffer an extensive decrease in absorbance measured at 540 nm, significantly greater than for hyperthyroid preparations. The decreases in absorbance were compatible with a Ca^{2+}-induced mitochondrial permeability transition (MPT). In fact, they were drastically reduced when either Ca^{2+} was eliminated from the reaction medium with the Ca^{2+} chelator EGTA or when a specific inhibitor of MPT, cyclosporin A, was added to the medium (unreported results). Mitochondrial swelling of hyperthyroid preparations was more strongly reduced by L-NNA treatment so that it was not significantly different from that of euthyroid preparations. Figure 2 (right panel) shows that the mitochondria also underwent a decrease in membrane potential ($\Delta\Psi$). The change was more extensive in hyperthyroid than in euthyroid preparations from untreated hearts. The fall in the membrane potential was reduced by L-NNA perfusion only in hyperthyroid preparations so that it was not significantly different in $H_{L\text{-}NNA}$ and $E_{L\text{-}NNA}$ groups.

Discussion

The results reported here confirm our previous observations that (i) during reperfusion, hyperthyroid rat hearts display a strong tachycardia, which is accompanied by a recovery in ventricular contractility significantly lower than that of euthyroid hearts [11, 16], and (ii) L-NNA perfusion protects hyperthyroid hearts against the reperfusion-induced tachycardia, thus increasing their inotropic recovery up to the euthyroid level [16].

Thyroid hormone induces up-regulation of NOS gene expression in rat hypothalamus [28], increases rat liver NOS activity [29], notwithstanding a decrease in the mitochondrial NOS [30], and induces NO overproduction by human vascular endothelium [31] and rat phagocytic cells [32]. If these properties are shared by cardiac tissue, these observations might explain the strong protective effect of L-NNA on the functional response of hyperthyroid hearts to ischemia-reperfusion. However, they do not supply information on the mechanisms and sites of cellular derangement and their contribution to myocardial ischemia-reperfusion injury.

Accumulating evidence points to mitochondria as a primary intracellular target for oxidative stress, which may account for an NO-linked reperfusion-induced tissue damage. During reperfusion of an ischemic heart, mitochondrial derangement is inversely correlated to the functional recovery of the tissue [5]. In particular, the low functional recovery of hyperthyroid hearts after ischemia-reperfusion is associated with both a high impairment of state 3 respiration, which declines to the euthyroid level, and severe oxidative damage of mitochondria [12]. Moreover, NOS stimulation upon ischemia- reperfusion [13] and inhibition of mitochondrial function by both NO [14, 33] and peroxynitrite [15] have been reported, implicating such substances in altered mitochondrial function in myocardial ischemia-reperfusion.

The results of the present study strongly support the idea that L-NNA treatment improves the functional recovery of hyperthyroid hearts by protecting their mitochondria from the oxidative damage linked to reperfusion-induced NO overproduction. In fact, mitochondrial N-Tyr levels, determined at the end of the ischemia-reperfusion protocol, were remarkably higher in hyperthyroid than in euthyroid hearts. The above differences were reduced when the heart perfusion was performed in the presence of the NOS inhibitor. Furthermore, the changes in N-Tyr levels were associated with opposite changes in mitochondrial function: state 3 oxygen consumption rates were higher in mitochondria whose levels of N-Tyr were lower.

Because the mitochondrial respiration rate was evaluated in isolated preparations, we can exclude the possibility that the low value it exhibits in the H_c group is due to an NO-dependent inhibition of COX [34], which is largely competitive with oxygen [35] and reversed by light [36]. The decline in the respiratory capacity is more likely to involve peroxynitrite, which can be formed from the reaction of NO with superoxide anion radical ($O_2^{\bullet -}$) and causes slow but irreversible inhibition of many mitochondrial components [37]. The respiratory chain is a major source of ROS during reperfusion of ischemic myocardium [2, 3]. Under physiologic conditions, single electron transfer by reduced respiratory components to oxygen produces small quantities of $O_2^{\bullet -}$, which is converted to H_2O_2 by the superoxide dismutase [38]. Hydrogen peroxide may either be detoxified by H_2O_2-metabolizing enzymes or permeate mitochondrial membranes and enter the cytoplasm. However, H_2O_2 may also be removed by hemoproteins, which can initially form a ferryl species that then decomposes to release $^{\bullet}OH$ radicals [39]. These are highly reactive and short-lived species, which would be expected to damage mitochondrial components at or near the site of their formation. Information on the rate of H_2O_2 generation by intact mitochondria can be obtained combining the determinations of the rate of mitochondrial H_2O_2 release and the mitochondrial capacity to remove H_2O_2 [23]. Because mitochondrial ROS generation depends on the concentration and reduction degree of autoxidizable electron carriers [40], the higher reduction of the respiratory chain associated with ischemia is currently assumed to promote the transfer of electrons to oxygen to generate superoxide radicals upon resumption of respiration [41].

The higher content of respiratory chain components in hyperthyroid heart mitochondria [42] would lead to increased ROS generation during ischemia-reperfusion. This idea is indirectly supported by our observation that both the rate of mitochondrial H_2O_2 release in the presence of antimycin A and the capacity to remove H_2O_2 are higher in hyperthyroid hearts than in euthyroid controls. In the presence of antimycin A, the respiratory chain components located between the substrate side and cytochrome b-560 become completely reduced and the ROS production rate depends on their concentration only. A similar condition occurs in hearts reperfused after ischemia, leading to increased ROS generation, which should end rapidly as the electron carriers of the respiratory chain are reoxidized. However, results we obtained in a previous study [12] suggest that during reperfusion, the rate of H_2O_2 production does not return to the preischemic level. In fact, we found that the rate of H_2O_2 release by mitochondrial preparations from reperfused euthyroid and hyperthyroid hearts was significantly higher than that from the respective controls subjected to continuous perfusion, and remained higher in mitochondria from hyperthyroid hearts [12]. The results of the present study show that L-NNA treatment decreases the mitochondrial H_2O_2 release rate, which again remains higher in hyperthyroid preparations. Although, even in this case, the release rate was determined by in vitro measurements, the values it assumes depend on processes occurring in vivo during reperfusion.

Indirect information on the dependence of mitochondrial ROS production on thyroid state and inhibitor treatment is also supplied by reperfusion-induced changes in the indices of oxidative damage. The high reactivity of $^{\bullet}OH$ radicals makes mitochondria a putative site of reperfusion-induced oxidative damage whose severity will depend on the capacity of these organelles to produce H_2O_2 and remove it via the Fenton reaction. The finding that the oxidative damage to mitochondrial lipids and proteins is wider in hyperthyroid than in euthyroid hearts and in untreated than in L-NNA-treated hearts is consistent with the hypothesis of a higher increase in H_2O_2 production rate during reperfusion. Thus, the aforementioned failure of the H_2O_2 release rate to recover the preischemic value [12], its lowering due to heart L-NNA treatment, and its enhancement due to animal T_3 treatment should depend on the rates of electron flow across the respiratory chain. These, in turn, should depend on changes in the inhibition degree of respiratory complexes by NO or $ONOO^-$ and

the consequent changes in the reduction degree of autoxidizable electron carriers.

The above considerations suggest that the mechanism which, during reperfusion, causes the tissue oxidative damage and dysfunction is a positive feedback loop. In fact, the concomitance of reflow-mediated perturbations, such as NOS activation and increased ROS production, which have synergistic effects, should enhance oxidative stress and mitochondrial dysfunction. The concurrence of higher ROS generation and NOS activity, together with the reduced efficacy of the mitochondrial antioxidant defence system associated with hyperthyroidism [43], should increase the extent of mitochondrial dysfunction and tissue impairment.

On the other hand, a system able to provide protection against excessive tissue dysfunction appears to be operative. During ischemia-reperfusion, the Ca^{2+} concentration increases in myocardial cells [44]. In the presence of Ca^{2+}, oxidative alterations of protein thiols of the mitochondrial inner membrane promote an MPT [45] that leads to mitochondrial swelling. The dependence of Ca^{2+}-induced MPT on oxidative alterations of the inner membrane represents a link between oxidative challenge of mitochondria and their susceptibility to swelling. This is supported by our finding that mitochondria from untreated hyperthyroid hearts exhibit the highest susceptibility to in vitro Ca^{2+}-induced swelling.

The susceptibility to permeabilization seems to have important implications for the regulation of cellular ROS production. Studies on liver mitochondrial fractions suggest that the light fractions, characterized by low respiratory activity, contain transitional forms in the process of development into the heavy mitochondrial structures with high respiratory activity [46, 47]. The heavy fraction also exhibits the lowest antioxidant level [46–48] and the highest rates of H_2O_2 production and susceptibility to Ca^{2+}-induced swelling [47]. However, biochemical and morphological studies have shown that the lightest fraction contains, together with neoformed mitochondria, disrupted mitochondria which exhibit a very low respiratory capacity [46, 49]. Such mitochondria seem to come from the degradation of heavy mitochondria, because conditions leading to increased ROS production and Ca^{2+} overload, including exercise [50] and hyperthyroidism [46, 48], decrease the amount of heavy mitochondria and increase the amount of the damaged mitochondria in the light fraction from rat liver. Even the mitochondrial population of the heart appears to consist of mitochondria of different ages, which show different susceptibility to ischemia-reperfusion-induced alterations. Previously, we found that, after ischemia-reperfusion-induced oxidative stress, the amount of recovered proteins and the activity of COX increased in the light (M_8) and decreased in the heavy (M_3) mitochondrial fraction from hyperthyroid hearts [12]. The observation reported here that such changes are reversed by L-NNA treatment supports the idea that the M_8 fraction contains membranes coming from degradation of M_3 mitochondria and the amount of such membranes depends on oxidative stress severity. The relationship between extent of mitochondrial degradation and severity of oxidative stress is also supported by the finding that the number of 'open' mitochondria increases when the insult caused by ischemia-reperfusion is greater [44]. Moreover, it is consistent with the view that cells defend themselves against excessive oxidative damage by MPT-mediated removal of mitochondria with high ROS production [51]. If so, the mechanism that, during reperfusion of the hyperthyroid heart, enhances the swelling of Ca^{2+}-loaded mitochondria is a negative feedback loop. In fact, the perturbation itself, represented by the thyroid hormone-mediated enhancement in ROS generation, should accelerate removal of ROS-overproducing mitochondria, thus limiting heart oxidative damage and dysfunction in animals exposed to ischemia-reperfusion.

1 Granger D. N. and Korthuis R. J. (1995) Physiological mechanisms of postischemic tissue injury. Annu. Rev. Physiol. **57:** 311–332

2 Das D. K., George A., Liu X. K. and Rao P. S. (1989) Detection of hydroxyl radical in the mitochondria of ischemic-reperfused myocardium by trapping with salicylate. Biochem. Biophys. Res. Commun. **165:** 1004–1009

3 Ambrosio G., Zweier J. L., Duilio C., Kuppusamy P., Santoro G., Elia P. P. et al. (1993) Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J. Biol. Chem. **268:** 18532–18541

4 Lucas D. T. and Szweda L. I. (1998) Cardiac reperfusion injury: aging, lipid peroxidation, and mitochondrial dysfunction. Proc. Natl. Acad. Sci. USA **95:** 510–514

5 Venditti P., Masullo P. and Di Meo S. (2001) Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress. Cell. Mol. Life Sci. **58:** 1528–1537

6 Asayama K. and Kato K. (1990) Oxidative muscular injury and its relevance to hyperthyroidism. Free Radic. Biol. Med. **8:** 293–303

7 López-Torres M., Romero M. and Barja G. (2000) Effect of thyroid hormones on mitochondrial oxygen free radical production and DNA oxidative damage in the rat heart. Mol. Cell. Endocrinol. **168:** 127–134

8 Venditti P., Puca A. and Di Meo S. (2002) Effects of thyroid state on H_2O_2 production by rat heart mitochondria: sites of production with complex I and complex II-linked substrates. Hormone Metab. Res. **35:** 55–61

9 Asayama K., Dobashi K., Hayashibe H., Megata Y. and Kato K. (1987) Lipid peroxidation and free radical scavengers in thyroid dysfunction in the rat: a possible mechanism of injury to heart and skeletal muscle in hyperthyroidism. Endocrinology **121:** 2112–2118

10 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

11 Venditti P., Masullo P., Agnisola C., and Di Meo S. (2000) Effect of vitamin E on the response to ischemia-reperfusion of Langerdorff heart preparations from hyperthyroid rats. Life Sci. **66:** 697-708

12 Venditti P., Agnisola C. and Di Meo S. (2002) Effect of ischemia-reperfusion on heart mitochondria from hyperthyroid rats. Cardiovasc. Res. **56:** 76–85
13 Liu P., Hock C. E., Nagele R. and Wong P. Y. K. (1997) Formation of nitric oxide, superoxide, and peroxinitrite in myocardial ischemia-reperfusion injury in rats. Am. J. Physiol. **272:** H2327–H2336
14 Borutaite V. and Brown, G. C. (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem. J. **315:** 295–299
15 Radi R., Rodriguez M., Castro L. and Telleri R. (1994) Inhibition of mitochondrial electron transport by peroxinitrite. Arch. Biochem. Biophys. **308:** 89–95
16 Masullo P., Venditti P., Agnisola C. and Di Meo S. (2000) Role of nitric oxide in the reperfusion induced injury in hyperthyroid rat hearts. Free Radic. Res. **32:** 411–421
17 Langendorff O. (1895) Untersuchungen am überlebenden Saugethierherzen. Arch. Ges. Physiol. (Pfluger) **61:** 291–332
18 Tyler D. D. and Gonze J. (1967) The preparation of heart mitochondria from laboratory animals. Methods Enzymol. **10:** 75–77
19 Barré H., Bailly L. and Rouanet J. L. (1987) Increased oxidative capacity in skeletal muscles from cold-acclimated ducklings: a comparison with rats. Comp. Biochem. Physiol. **88B:** 519–522
20 Krieger D. A., Tate C. A., McMillin-Wood J. and Booth F. W. (1980) Populations of rat skeletal muscle mitochondria after exercise and immobilization. J. Appl. Physiol. **48:** 23–28
21 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
22 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
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 Tanaka S., Choe N., Hemenway D. R., Zhu S., Matalon S. and Kagan E. (1998) Asbestos inhalation induces reactive nitrogen species and mitochondria nitrotyrosine formation in the lungs and pleura of the rat. J. Clin. Invest. **102:** 445–454
27 Åkerman K. E. O. and Wikström M. K. F. (1976) Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. **68:** 191–197
28 Ueta Y., Chowdrey H. S. and Lightman S. L. (1995) Hypothalamic nitric oxide synthase gene expression is regulated by thyroid hormones. Endocrinology **136:** 4182–4187
29 Fernandez V., Cornejo P., Tapia G. and Videla L. A. (1997) Influence of hyperthyroidism on the activity of liver nitric oxide synthase in the rat. Nitric Oxide Biol. Chem. **1:** 463–468
30 Carreras M. C., Peralta J. G., Converso D. P., Finocchietto P. V., Rebagliati I., Zaninovich A. A. et al. (2001) Modulation of liver mitochondrial NOS is implicated in thyroid-dependent regulation of O_2 uptake. Am. J. Physiol. **281:** H2282–H2288
31 Napoli R., Biondi B., Guardasole V., Matarazzo M., Pardo F., Angelini V. et al. (2001) Impact of hyperthyroidism and its correction on vascular reactivity in humans. Circulation **104:** 3076–3080
32 Huffman L. J., Judy D. J., Rao K. M., Frazer D. G. and Goldsmith W. T. (2000) Lung responses to hypothyroidism, hyperthyroidism, and lipopolysaccharide challenge in rats. J. Toxicol. Environ. Health A. **61:** 623–639
33 Poderoso J. J., Peralta J. G., Lisdero C. L., Carreras M. C., Radisic M., Schopfer F. et al. (1998) Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. Am. J. Physiol. **274:** C112–C119
34 Cleeter M. W. Y, Cooper J. M., Darley-Usmar V. M., Moncada S. and Shapira A. H. V. (1994) Reversible inhibition of cytochrome oxidase, the terminal enzyme of the mitochondrial respiratory chain by NO. FEBS Lett. **345:** 50–54
35 Brown G. C. and Cooper, C. E. (1994) Nanomolar concentrations of NO reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. **356:** 295–298
36 Borutaite V., Budriunaite A. and Brown G. C. (2000) Reversal of nitric oxide-, peroxynitrite-, and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex Iactivity by light and thiols. Biochim. Biophys. Acta **1459:** 405–412
37 Cassina A. and Radi R. (1996) Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch. Biochem. Biophys. **328:** 309–316
38 Halliwell B. and Gutteridge J. M. C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. **186:** 1–85
39 Loschen G., Azzi A., Richter C. and Flohé L. (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett. **42:** 68–72
40 Chance B., Sies H. and Boveris A. (1979) Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59:** 527–605
41 Kehrer J. P. and Lund L. G. (1994) Cellular reducing equivalents and oxidative stress. Free Radic. Biol. Med. **17:** 65–75
42 Nishiki K., Ericinska M., Wilson D. F. and Cooper S. (1978) Evaluation of oxidative phosphorylation in hearts from euthyroid, hypothyroid, and hyperthyroid rats. Am. J. Physiol. **235:** C212–C219
43 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
44 Halestrap A. P. (1999) The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem. Soc. Symp. **66:** 181–203
45 Zoratti M. and Szabo I. (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta **1241:** 139–176
46 Venditti P., De Leo T. and Di Meo S. (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
47 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
48 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
49 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.
50 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
51 Skulachev V. P. (2002) Programmed death phenomena: from organelle to organism. Ann. N. Y. Acad. Sci. **959:** 214–237