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H_2O_2 Production and Response to Stress Conditions by Mitochondrial Fractions From Rat Liver

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Rat liver mitochondria, in different steps of the maturation process, were resolved by differential centrifugation at 1000*g* (M_1), 3000*g* (M_3), and 10,000*g* (M_{10}), and their characteristics determining susceptibility to stress conditions were investigated. Some parameters did not show gradual changes in the transition from M_{10} to M_1 fraction because of the contamination of the M_{10} fraction by microsomes and damaged mitochondria with relatively high lipid content. The highest and lowest rates of O_2 consumption and H_2O_2 production were exhibited by M_1 and M_{10} fractions, respectively. Vitamin E and coenzyme Q levels were significantly higher in M_{10} than in M_1 fraction, whereas whole antioxidant capacity was not significantly different. The degree of oxidative damage to lipids and proteins was higher in M_1 and not significantly different in M_3 and M_{10} fractions. The order of susceptibility to both oxidative challenge and Ca^{2+}-induced swelling was $M_1 > M_3 > M_{10}$. It seems that the Ca^{2+}-induced swelling is due to permeabilization of oxidatively altered inner membrane and leads to discard mitochondria with high ROS production. If, as previous reports suggest, mitochondrial damage is initiating stimulus to mitochondrial biogenesis, the susceptibility of the M_1 mitochondria to stressful conditions could be important to regulate cellular ROS production. In fact, it should favor the substitution of the oldest ROS-overproducing mitochondria with neoformed mitochondria endowed with a smaller capacity to produce free radicals.

KEY WORDS: Oxidative capacity; antioxidant capacity; mitochondrial swelling; oxidative stress; mitochondrial turnover; lipid peroxidation; protein oxidation.

INTRODUCTION

It is long known that mitochondrial population is heterogeneous with regard to its sedimentation characteristics, chemical makeup, and enzymatic activities (Gear, 1965; Kuff and Schneider, 1954). Fatterpaker *et al.* (1965) found that heavy, light, and fluffy mitochondrial fractions, obtained by differential centrifugation, exhibited differences in their stability and the activities of some membrane-bound enzymes in a stress condition caused by carbon tetrachloride administration. More recently, the study of three rat liver mitochondrial fractions resolved by differential centrifugation suggested that the light fractions, with low respiratory activity, represent transitional forms in the process of development into the heavy mitochondrial structures with high respiratory activity (Lanni *et al.*, 1996). Subsequently, we found that the susceptibility to oxidative stress, an important feature of mitochondria which is likely involved in their degradation, depends on mitochondrial content of both antioxidants and cytochromes (Di Meo *et al.*, 1996). Accordingly, the study of the three mitochondrial fractions from rat liver showed that the heavy fraction, which was characterized by the greatest cytochrome content and the lowest antioxidant level, also exhibited the lowest capacity to oppose an oxidative challenge (Venditti *et al.*, 1996). Therefore, we suggested that the process of mitochondrial maturation strictly bound to an increase in respiratory components is self-limiting. In fact, it should give rise to a series of events, which should lead after all to degradation of the mitochondria. However, it remains to establish yet whether the differences in antioxidant capacity found in the mitochondrial fractions are associated with differences in the production of reactive

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oxygen species (ROS), which can play a major role in the modulation of the turnover of the mitochondrial proteins and whole mitochondrial population.

In this study, an attempt was made to differentiate mitochondrial fractions with respect to their capacity to produce ROS and oppose them, by determining O_2 consumption, H_2O_2 release, antioxidant level, and extent of oxidative damage. Moreover, the relative stability of the mitochondrial fractions in stress conditions was evaluated by assessing their susceptibility to oxidative challenge and Ca^{2+}-induced swelling.

MATERIALS AND METHODS

Animals

Male Wistar rats (60 days old) were used in the experiments. The animals, purchased at weaning from Nossan (Correzzana, Italy), were housed in separate cages at 24 ± 1°C, with an artificial lighting cycle (LD 8–20 h). All animals were provided with water ad libitum and a commercial rat chow diet (Nossan), containing 105 IU/kg of vitamin E.

Experimental Procedure

The animals were killed by decapitation under ether anesthesia. The livers were rapidly removed and placed in small beakers on ice. Subsequently, the livers were finely minced, weighed, and washed with ice-cold isolation medium (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 20 mM Tris, pH 7.4) containing 0.1% fatty acid-free albumin. Tissue fragments were gently homogenized in the same solution (1:10 w/v) using a Potter–Elvejem homogenizer set at a standard velocity (500 rpm) for 2 min.

Preparation of Mitochondrial Fractions

Mitochondrial fractions were obtained by modifying a recently developed method able to minimize the cytoplasmic contamination of the pellets (Lanni *et al.*, 1996). Accordingly, the homogenates were freed of debris and nuclei by centrifugation at 500*g* for 10 min at 4°C. The resulting supernatants were centrifuged at 10,000*g* for 10 min. The pellets were washed twice with 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4, resuspended in the same solution, and subjected to a series of sequential centrifugation steps lasting 10 min at 1000, 3000, and 10,000*g*. The pellets were designed as M_1, M_3, and M_{10}, respectively. Aliquots of all mitochondrial fractions were resuspended in 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4, and used for biochemical determinations. Other aliquots were washed with 220 mM mannitol, 70 mM sucrose, 20 mM Tris, pH 7.4, resuspended in the same solution, and used for mitochondrial swelling.

In agreement with previous reports (Lanni *et al.*, 1996; Venditti *et al.*, 1996), 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 fractions was determined, upon solubilization in 0.5% deoxycholate, by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

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 mitochondrial H_2O_2 release was measured at 30°C following the linear increase in fluorescence (excitation at 320 nm, emission at 400 nm) because of 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 measurements of O_2 consumption. Measurements with the different substrates in the presence of 500 μM ADP or 10 μM antimycin A were also performed. Known concentrations of H_2O_2 were used to establish the standard concentration curve. In preliminary experiments the effect of catalase addition on the measured rates of H_2O_2 production was studied. Such experiments showed a dose-dependent drop of the fluorescence in the presence of the enzyme.

Capacity to Remove H_2O_2

Capacity to remove H_2O_2 (CR) was determined by comparing the ability of mitochondrial samples to reduce H_2O_2-linked fluorescent emission with that of desferrioxamine solutions (Venditti *et al.*, 2001). H_2O_2 was generated by glucose oxidation catalyzed by glucose oxidase (GOX). The nonfluorescent substrate PHPA was oxidized to the stable fluorescent product 2,2′-dihydroxy-biphenyl-5,5′-diacetate $(PHPA)_2$ (Hyslop and Sklar, 1984) by the enzymatic reduction of H_2O_2 catalyzed by HRP. The fluorescence was monitored on the Jasko fluorometer (excitation wavelength 320 nm, emission wavelength 400 nm). Assays were performed in quartz fluorometer cuvettes containing a magnetic stirrer and maintained at 30°C. Reaction was started by adding 10 μL of 80 μg/mL GOX to a mixture containing 0.2 μg/mL PHPA, 6 U/mL HRP, 5 mM glucose in 145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4. After 100 s, 10 μL of desferrioxamine solutions (containing from 1 to 12 nmoles), or mitochondrial samples (containing from 0.1 to 1 mg of mitochondrial proteins) were added to 2.0 mL final volume. The additions were made to the cuvettes via externally mounted syringes. The values of fluorescence change for unit of time obtained after addition of desferrioxamine or mitochondria were converted to relative percentage of the values obtained before the addition. The values for desferrioxamine were used to fit standard curves by the Fig. P program (Biosoft, Cambridge, MA). The values for samples served to obtain, from equations describing the standard curves, evaluations of their capacity to remove H_2O_2, expressed as equivalent desferrioxamine concentration.

Oxidative Damage to Lipid and Proteins

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

Quantification of protein-bound carbonyls was performed by the procedure of Reznick and Packer (1994) modified by Schild *et al.* (1997). For each determination, three trichloroacetic acid (TCA)-precipitated samples containing 1 mg of mitochondrial protein were dissolved again in 300 μL of 0.1 M NaOH for 5 min. Two of the samples were treated with 3 mL of 10 mM dinitrophenylhydrazine in 2.5 M HCl for 1 h at room temperature. The third tube, used as the blank, was incubated with 2.5 M HCl. The reaction was stopped by addition of 3.3 mL of 20% TCA. The pellets were washed twice with 3 mL of 10% TCA. The protein pellets were washed twice with absolute ethanol/ethylacetate (1:1) and once with 3 mL of 10% TCA. The protein pellets were finally dissolved in 6 M guanidine hydrochloride, and the absorption at 370 nm (dinitrophenylhydrazine minus sample blank) was determined. Protein recovery was estimated for each sample. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22,000 $M^{-1} \cdot cm^{-1}$ and expressed as nmol carbonyl/mg of protein.

Coenzyme Q and Vitamin E

Ubiquinols ($CoQH_2$) were oxidized to ubiquinones (CoQs) with ferric chloride as the oxidation reagent. $CoQH_2$ were oxidized from 0.5 mL of mitochondrial suspension with 0.5 mL of 2% $FeCl_3$ and 2.0 mL of ethanol. CoQs were extracted by 5.0 mL of *n*-hexane, which was then removed by evaporation under N_2 at 40°C. The residue was dissolved in ethanol and subjected to the mobile phase in HPLC (machine: SpectraSeries P100 isocratic pump, Thermo Separation Products, San Jose, CA; column Ultremex 5 250 × 4.6 mm, 5-μm particle size, phenomenex Torrance, CA). Eluant was a mixture of methanol/ethanol 3/7 (v/v) containing 20 mM lithium perborate, and the flow rate was 1 mL/min (Lang *et al.*, 1986). The total content of CoQs ($CoQH_2$ + CoQ) was then determined. The eluted CoQs were determined separately by using a SpectraSeries UV100 detector (Thermo Separation Products, San Jose, CA) (275 nm). Quantitation was obtained by using external standards.

For vitamin E determination, mitochondrial fractions were deproteinized with methanol and extracted with *n*-hexane. The extracts were evaporated under N_2 at 40°C and the residues were dissolved in ethanol. Vitamin E content was determined using the HPLC procedure of Lang *et al.* (1986). Quantification was obtained by using external standard.

Susceptibility to Oxidative Stress and Antioxidant Capacity

Response to oxidative stress was determined as previously described (Venditti *et al.*, 1999a). Briefly, several dilutions of the mitochondrial suspensions in the range of protein concentrations from 20 to 0.005 mg/mL were prepared with 15 mM Tris (pH 8.5). The assays were performed in microtiter plates. Enhanced chemiluminescence reactions were initiated by addition of 250 μL of the reaction mixture to 25 μL of the samples. The reaction mixture

was obtained by dissolving a tablet containing substrate in excess (sodium perborate) and signal generating reagents (sodium benzoate, indophenol, and luminol) (Amerlite Signal Reagent Tablets) in buffer at pH 8.6 (Amerlite Signal Reagent Buffer). The plates were incubated at 37°C for 30 s under continuous shaking and then transferred to a luminescence analyzer (Amerlite Analyzer). The emission values were fitted to dose-response curves using the statistical facilities of the Fig. P graphic program (Biosoft, Cambridge, United Kingdom).

The determination of the overall antioxidant capacity (CA) was performed according to Di Meo *et al.* (1996). Briefly, 250 μL of the above reaction mixture was added to 10 μL of 110 ng/mL peroxidase plus 15 μL of either desferrioxamine, at concentrations ranging from 0.01 to 3 mM, in 15 mM Tris (pH 8.5), or buffer alone. Equal volumes of reaction mixture were also added to both 10 μL of 110 ng/mL peroxidase plus 15 μL of mitochondrial samples (5 mg of protein/mL) (samples) and 10 μL of 15 mM Tris (pH 8.5) plus 15 μL of the same mitochondrial samples (blanks). The emission values obtained from mixture of peroxidase and desferrioxamine were reported against the desferrioxamine concentration on logarithmic coordinates supplying a standard curve. The differences between the emission values obtained from the samples and those obtained from the relative blanks were referred to those of the standard curve and allowed the mitochondrial antioxidant capacity to be expressed as equivalent desferrioxamine concentration.

Mitochondrial Swelling and Transmembrane Electrical Potential

Mitochondrial swelling was spectrophotometrically measured by determining the apparent absorbance at 540 nm in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 2 mM succinate, 4 μM 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 Ackerman 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 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

Oxygen Consumption

The results concerning respiratory characteristics of the mitochondrial fractions show that, using succinate as the substrate, both in State 4 and State 3, M_1 and M_{10} fractions exhibit the highest and lowest rates of oxygen consumption, respectively (Table I). Furthermore, the addition of ADP stimulates oxygen consumption

Table I. Oxygen Consumption in Mitochondrial Fractions From Rat Liver

	Oxygen consumption rate (ng atoms/min/mg protein)					
	Succinate			Pyruvate/malate		
Fraction	State 4	State 3	RCR	State 4	State 3	RCR
M_1	53.4 ± 2.9	328.8 ± 10.3	5.9 ± 0.4	9.1 ± 0.4	45.8 ± 3.4	5.2 ± 0.4
M_3	37.2 ± 3.2^a	218.9 ± 6.1^a	6.5 ± 0.6	7.1 ± 0.6^a	29.4 ± 1.2^a	5.6 ± 0.5
M_{10}	$25.3 \pm 3.3^{a,b}$	$64.4 \pm 7.4^{a,b}$	$2.4 \pm 0.2^{a,b}$	$5.3 \pm 0.6^{a,b}$	$14.5 \pm 1.4^{a,b}$	$1.7 \pm 0.1^{a,b}$

Note. Given values are the mean ± SE of eight different experiments.
[a] Significant vs. M_1 fraction.
[b] Significant vs. M_3 fraction. The level of significance was chosen as $P < 0.05$.

Table II. H_2O_2 Release by Succinate and Pyruvate/Malate-Supplemented Mitochondrial Fractions From Rat Liver

Parameter	Fraction		
	M_1	M_3	M_{10}
H_2O_2 release (pmol/min/mg protein)			
Succinate	120.4 ± 5.1	76.8 ± 2.0^a	$48.2 \pm 1.4^{a,b}$
Succinate + ADP	70.0 ± 3.1	54.5 ± 1.6^a	$31.5 \pm 1.0^{a,b}$
Succinate + AA	1135 ± 17	920 ± 9^a	$622 \pm 22^{a,b}$
Pyruvate/malate	252.8 ± 2.6	221.8 ± 5.3^a	$192.6 \pm 3.6^{a,b}$
Pyruvate/malate + ADP	190.0 ± 3.0	168.4 ± 3.6^a	$147.0 \pm 3.3^{a,b}$
Pyruvate/malate + AA	1191 ± 62	986 ± 31^a	$815 \pm 21^{a,b}$

Note. Given values are the mean ± SE of eight different experiments.
[a] Significant vs. M_1 fraction.
[b] Significant vs. M_3 fraction. The level of significance was chosen as $P < 0.05$.

markedly in M_1 and M_3 fractions and scarcely in M_{10} fraction. Therefore, the respiratory control ratio (RCR) values were not significantly different in M_1 and M_3 fractions and lower in M_{10} fraction. Similar pattern was found when pyruvate and malate were used as the substrates.

H_2O_2 Release

With both succinate or pyruvate/malate the H_2O_2 release rates were significantly higher in the M_1 fraction than in other fractions (Table II). The ADP addition decreased the H_2O_2 release rates in different extent in the three fractions, but such rates remained higher in the M_1 fraction and lower in the M_{10} fraction. The antimycin A-stimulated rates also were higher in the M_1 fraction and lower in the M_{10} fraction. The release of H_2O_2 and O_2 consumption were measured in the same buffer, using the same concentrations of substrates and ADP, and were assayed at the same temperature. This allowed the calculation of the percentage of O_2 released as H_2O_2 by mitochondria. Figure 1 shows that under State 3 condition such a percentage was higher for M_{10} fraction when both succinate and pyruvate/malate were used as substrates. Under State 4 condition, using both succinate and pyruvate/malate as substrates, significant differences were not found among fractions.

Capacity to Remove H_2O_2

Table III reports the capacity to remove H_2O_2 of the mitochondrial fractions from rat liver. The order of CR ($M_1 > M_3 > M_{10}$) indicates that the heavy mitochondria are endowed with greater levels of substances able to remove H_2O_2 preventing the •OH production (H_2O_2-metabolyzing enzymes) or/and substances (iron-ligands) able to remove H_2O_2 converting it into more reactive radicals via Fenton reaction.

Antioxidant Levels

The M_1 fraction exhibited a whole antioxidant capacity lower than that of the M_3 fraction and no significantly different from that of the M_{10} fraction (Table III). In contrast, the levels of lipid-soluble antioxidants, vitamin E and coenzyme Q9 and Q10, in the M_1 fraction were lower than those in M_3 and M_{10} fractions (Table III).

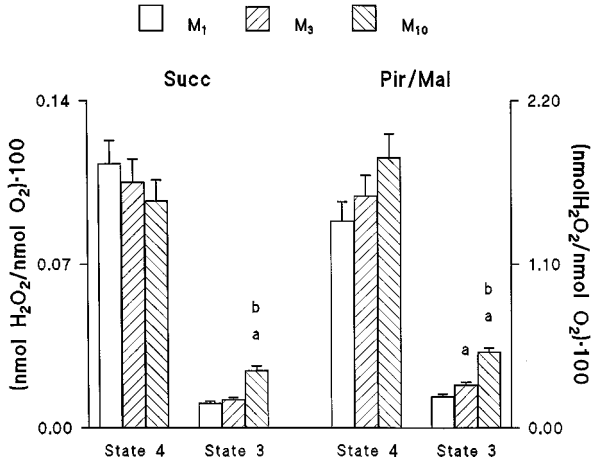

Fig. 1. Percentage of the total oxygen released as H_2O_2 by mitochondrial fractions supplemented with Complex I- or Complex II-linked substrates. Succ = succinate; Pyr/mal = pyruvate/malate, ADP (500 μM). Values are means ± SEM of eight experiments. a: significant vs. M_1 fraction; b: significant vs. M_3 fraction. The level of significance was chosen as $P < 0.05$.

Table III. Capacity to Remove H_2O_2 and Levels of Antioxidants in Mitochondrial Fractions From Rat Liver

	Parameter				
Fraction	CR	CA	Vit E	CoQ9	CoQ10
M_1	4.97 ± 0.20	0.23 ± 0.01	0.80 ± 0.14	1.65 ± 0.07	0.53 ± 0.03
M_3	3.88 ± 0.23^a	0.43 ± 0.05^a	1.65 ± 0.15^a	2.04 ± 0.11^a	0.74 ± 0.05^a
M_{10}	$2.94 \pm 0.17^{a,b}$	0.28 ± 0.02^b	1.96 ± 0.29^a	2.16 ± 0.09^a	0.86 ± 0.08^a

Note. Given values are the mean ± SE of eight different experiments. Capacity to remove H_2O_2 (CR) is expressed as equivalent level of desferrioxamine (nmol/mg protein). Whole antioxidant capacity (CA) is expressed as equivalent concentration of desferrioxamine (nmol/L). Vitamin E (Vit E), coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) are expressed as nmol/mg protein.
[a] Significant vs. M_1 fraction.
[b] Significant vs. M_3 fraction. The level of significance was chosen as $P < 0.05$.

Oxidative Damage

The examination of HP levels in mitochondrial fractions indicates greater extent of peroxidative reactions in the M_1 than in the M_3 and M_{10} fractions (Table IV).

The results which document that degree of carbonyl formation is different for the three mitochondrial fractions are reported in Table IV. Such results indicate that the extent of oxidative attack to proteins is greater in the M_1 than in M_3 and M_{10} fractions.

Susceptibility to Oxidative Stress

The relationship between light emission (E) and protein concentration (C) of mitochondria stressed with sodium perborate was described by the equation $[E = a \cdot C/\exp(b \cdot C)]$ (Venditti *et al.*, 1999a), in which the *a* value depends on the cytochrome content, the *b* value on the antioxidant level, while the emission maximum ($E_{max} = a/e \cdot b$) is an index of the susceptibility of the preparations to oxidative challenge. The curves in Fig. 2 show that the susceptibility to oxidative stress of the M_1 mitochondria is higher than those of M_3 and M_{10} fractions. The examination of the parameters characterizing light emission (Table V) shows that the higher emission by the M_1 fraction is due to both higher *a* value and lower *b* value.

Table IV. Hydroperoxide Levels and Protein-Bound Carbonyl Content in Mitochondrial Fractions From Rat Liver

	Parameter	
Fraction	HPs	CO
M_1	22.92 ± 0.60	3.53 ± 0.15
M_3	16.33 ± 0.45^a	2.57 ± 0.09^a
M_{10}	$19.62 \pm 0.83^{a,b}$	2.46 ± 0.29^a

Note. Given values are the mean ± SE of eight different experiments. Hydroperoxides (HPs) are expressed as pmol NADP/min/mg mitochondrial proteins. Content of protein-bound carbonyls (CO) is expressed as nmol/mg protein.
[a] Significant vs. M_1 fraction.
[b] Significant vs. M_3 fraction. The level of significance was chosen as $P < 0.05$.

Mitochondrial Swelling

To verify whether mitochondrial fractions are differently susceptible to Ca^{2+}-dependent swelling,

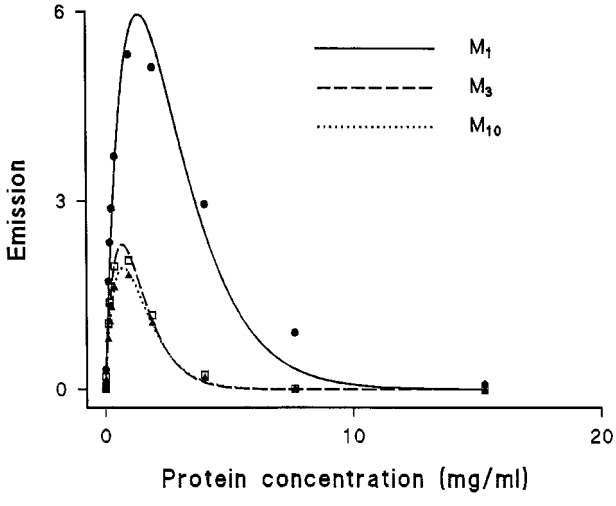

Fig. 2. Response to oxidative stress in vitro of mitochondrial fractions from rat liver. The susceptibility to stress was evaluated by determining the variations with concentrations of light emission from a luminescent reaction. Emission values are given as percentage of an arbitrary standard (44 ng/mL peroxidase). The curves are computed from experimental data using the equation: $E = a \cdot C/\exp(b \cdot C)$. Preparations from M_1 (solid lines), M_3 (dashed lines), and M_{10} (dotted lines) fractions.

Table V. Parameters Characterizing Response to Oxidative Stress of Mitochondrial Fractions From Rat Liver

	Parameter		
Preparation	*a*	*b*	E_{max}
M_1	11.9 ± 0.7	0.76 ± 0.04	5.76 ± 0.26
M_3	8.9 ± 1.1^a	1.45 ± 0.09^a	2.25 ± 0.11^a
M_{10}	7.3 ± 0.5^a	1.34 ± 0.1^a	2.00 ± 0.07^a

Note. Given values are the mean ± SE of eight different experiments. The relation between light emission and protein concentration of mitochondria is described by the equations: $E = a \cdot C/\exp(b \cdot C)$. $E_{max} = a/e \cdot b$ (emission maximum).
The level of significance was chosen as $P < 0.05$.
[a]Significant vs. M_1 fraction.

succinate-energized mitochondria were incubated in the presence of 100 μM Ca^{2+} and mitochondrial membrane integrity was evaluated. Figure 3(A) shows that Ca^{2+}-loaded suspensions of the M_1 fraction suffers an extensive decrease in absorbance measured at 540 nm. The figure also shows that, compared to swelling of the M_1 fraction, that of M_3 fraction was less extensive, while that of the M_{10} fraction was rather poor. The decreases in absorbance are 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 a specific inhibitor of MPT, the immune suppressor cyclosporin A, was added to such a medium (Fig. 3(B)). Furthermore, Fig. 4 shows that mitochondrial swelling induced by Ca^{2+} is preceded by a rapid decrease in membrane potential ($\Delta\Psi$) which is higher for the M_1 fraction and lower for the M_{10} fraction.

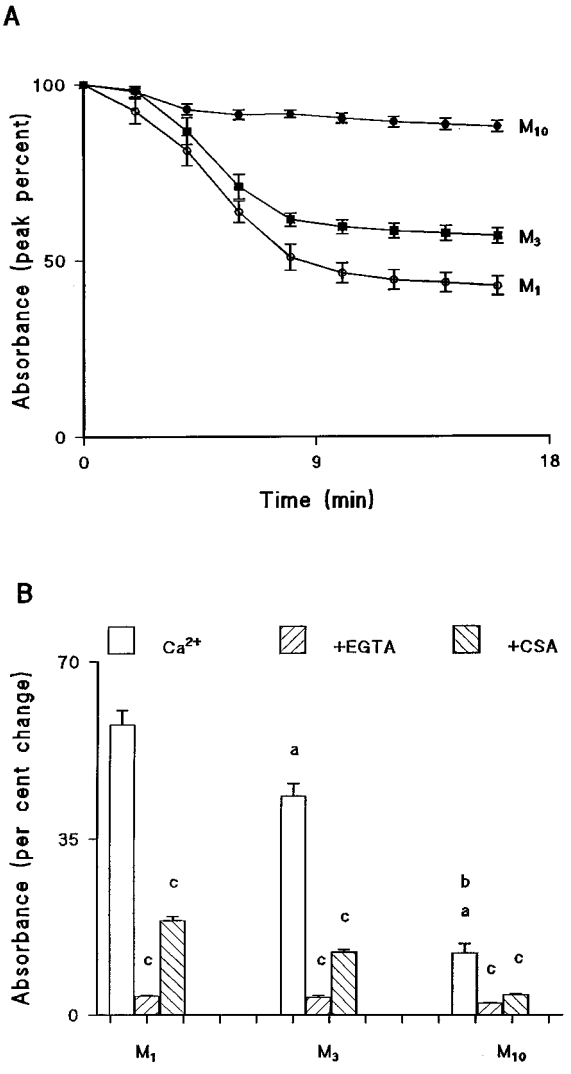

Fig. 3. Mitochondrial swelling induced by Ca^{2+}. Mitochondrial fractions from rat liver (0.3 mg/mL) were incubated in standard medium containing 100 μM Ca^{2+}. (A) Swelling of mitochondrial fractions. The swelling of the mitochondrial fractions was monitored as decrease of the absorbance at 540 nm, and expressed, for each fraction, as percent of the initial value before Ca^{2+} addition. The initial values of absorbance were 0.76 ± 0.09, 0.73 ± 0.06, and 0.49 ± 0.06 for M_1, M_3, and M_{10} fractions, respectively. Time course of the absorbance for each fraction was significantly different from that of the other fractions (two-way ANOVA, $P < 0.0001$). (B) Extent of mitochondrial fraction swelling in the presence of 100 μM Ca^{2+}, 100 μM Ca^{2+} plus 1 mM EGTA, or 100 μM Ca^{2+} plus 1 μM cyclosporin A (CSA). The effect of addition of EGTA or CSA on extent of swelling induced by Ca^{2+} was determined by comparing the percent changes of absorbance at 540 nm obtained during a 16-min period. a: significant vs. M_1 fraction; b: significant vs. M_3 fraction; c: significant vs. the same preparation in the presence of the only Ca^{2+}. The level of significance was chosen as $P < 0.05$.

DISCUSSION

The results presented in this paper widen our knowledge on biochemical and functional characteristics of three mitochondrial fractions resolved by differential centrifugation from rat liver. In particular, more light is thrown upon the characteristics determining the susceptibility of the fractions to stress conditions and the dynamics of the mitochondrial population.

Firstly, by using a method which supplies an evaluation of the antioxidant capacity of the mitochondria (Di Meo *et al.*, 1996), we have confirmed previous results indicating that the antioxidant level in M_1 mitochondria is lower than that in M_3 ones (Venditti *et al.*, 1996). However, the sensitivity of the above method has been found to

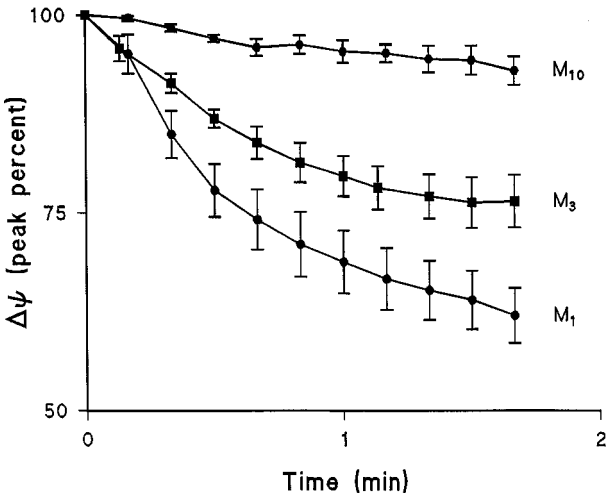

Fig. 4. Membrane potential dissipation induced by Ca^{2+}. Membrane potential ($\triangle\Psi$) of mitochondrial fractions (0.3 mg/mL) was estimated through fluorescence changes of safranin (8 μM) (excitation wavelength 495 nm, emission wavelength 586 nm) in a standard medium containing 100 μM Ca^{2+}. $\triangle\Psi$ was calculated using a suitable calibration curve. The decrease of $\triangle\Psi$ for each fraction was expressed as percent of the initial value before Ca^{2+} addition. Initial values of $\triangle\Psi$ were 169.5 ± 8.9 mV, 149.2 ± 12.1 mV, and 79.4 ± 3.6 mV for M_1, M_3, and M_{10} fractions, respectively. Time course of $\triangle\Psi$ for each fraction was significantly different from that of the other fractions (Two-way ANOVA, $P < 0.0001$).

be low for antioxidants such as vitamin E (Venditti *et al.*, 1997), which appears to have a weak preventive effect for the oxidation of substances in the aqueous phase (Chen *et al.*, 1993). For this reason we have determined the level of lipid-soluble antioxidants, such as vitamin E (Burton *et al.*, 1983) and ubiquinol (Forsmark *et al.*, 1991), which are able to protect polyunsaturated fatty acids within phospholipids of biological membranes. Thus, we have found that also the levels of lipid-soluble antioxidants are lower in M_1 than in M_3 fraction suggesting that the membranes of the heavy mitochondria are less able to oppose peroxidative processes. On the other hand, while overall antioxidant capacity is lower in M_{10} than in M_3 fraction, the levels of lipid-soluble antioxidants are similar in the two fractions. This discrepancy can be explained by the composition of M_{10} fraction, which, besides light mitochondria, contains discrete amounts of both microsomal and mitochondrial membranes (Lanni *et al.*, 1996) with high content of vitamin E and coenzyme Q.

Secondly, by determining the levels of hydroperoxides, we have confirmed previous results (Venditti *et al.*, 1996, 1999b) indicating a greater extent of peroxidative processes in the M_1 than in M_3 fraction. However, in this investigation we have also found that protein oxidation is greater in heavy fraction, a previously unreported finding.

In the whole, the data concerning the levels of the antioxidants seem to be well-correlated with the extent of oxidative damage shown in the three fractions. However, it is well known that oxidative stress, which triggers the unspecific oxidation of essential biomolecules, may comprise both high rates of active oxygen species generation and depressed antioxidant defences (Sies, 1986). Therefore, we have investigated whether the differences in antioxidant capacity found in the mitochondrial fractions are associated with differences in their ROS production. We have found that rate of H_2O_2 release is higher for M_1 fraction and lower for M_{10} fraction, using both succinate and pyruvate/malate as the substrates, and this pattern is not modified by the addition of ADP or antymicin A. This finding indicates clearly that M_1 mitochondria supply a major contribution to oxidative damage of epatocytes during both resting and stimulated respiration. However, information about the possible role played by ROS production in the oxidative damage occuring in the mitochondria can be obtained only if the relationship between their rates of ROS release and production is clarified.

The superoxide anion radical ($O_2^{\bullet -}$) appears to be the first oxygen reduction product generated by electron transport. In mammalian mitochondria $O_2^{\bullet -}$ production occurs at level of the level of a Fe—S center of Complex I (Turrens and Boveris, 1980) or at level of the segment between NADH dehydrogenase and ubiquinone/cytochrome b of Complex III (Loeschen *et al.*, 1971). Then superoxide is converted to H_2O_2 by superoxide dismutase present in the mitochondrial matrix (Loschen *et al.*, 1974). Although $O_2^{\bullet -}$ produced by mitochondria is a stoichiometric precursor of H_2O_2 (Dionisi *et al.*, 1975), the amount of H_2O_2 released extramitochondrially does not correspond to the relative rate of $O_2^{\bullet -}$ generation. Such discrepancy is due to the fact that the rates of H_2O_2 generation can be modified prior to extramitochondrial leakage. Hydrogen peroxide released by intact mitochondria escapes the action of H_2O_2-metabolizing enzymes located in the matrix and hemoproteins or other Fe^{2+} ligands that convert H_2O_2 into more reactive radicals via Fenton reaction. Therefore, the respiratory chain must produce substantially more H_2O_2 than what is detected in intact mitochondria.

Differences in H_2O_2 release by intact mitochondria are often considered indicative of different H_2O_2 production, but such an inference is justified only for mitochondria with the same capacity to remove H_2O_2. Thus, the order found in mitochondrial H_2O_2 release ($M_1 > M_3 > M_{10}$) does not allow us to deduce anything

about the mitochondrial H_2O_2 production by the three fractions. Conversely, the finding that the mitochondria with higher rates of H_2O_2 release are also characterized by higher values of CR indicates that the order of H_2O_2 release depends on differences in the capacities of the mitochondria of the three fractions to produce H_2O_2 during basal, stimulated, and antimycin-inhibited respiration. This indicates that oxidative damage shown in the M_1 fraction is due to both low antioxidant defences and high ROS production.

We are inclined to believe that the difference in ROS production, as well as those in the respiration rates, is linked in great part to the different content in respiratory chain components within mitochondria (Lanni *et al.*, 1996; Venditti *et al.*, 1996). It is well established that, at constant O_2 tension, the rate of mitochondrial $O_2^{\bullet-}$ (and H_2O_2) production is related to the concentration and the degree of reduction of the autoxidizable electron carriers (Chance *et al.*, 1979). In the presence of antymicin, the components of the mitochondrial respiratory chain located on the substrate side of cytochrome b-560 (Barja, 1999) became completely reduced, and their concentration is the only factor affecting ROS production rate. Therefore, the order of the mitochondrial H_2O_2 production ($M_1 > M_3 > M_{10}$), in the presence of antimycin A, mirrors that of respiratory chain components (Lanni *et al.*, 1996). During State 4 respiration H_2O_2 release decreases in all mitochondrial fractions. A further higher decrease, likely associated with a reduction in percentage of O_2 turned on H_2O_2, is shown during State 3 respiration. These results are consistent with the observation that the degree of the respiratory chain reduction is inversely related to the rate of electron flow, and, therefore, it strongly decreases during the State 4 to State 3 transition at the same time that the rate of electron flow is increased (Tzagoloff, 1982). The above analysis could appear inconsistent with our finding that in the M_1 fraction there are low levels of CoQ, whose free radical intermediate, ubisemiquinone ($CoQ^{\bullet-}$), is considered to be the $O_2^{\bullet-}$ generator in the Complex III (Turrens *et al.*, 1985). However, some results indicate that the levels of lipids, phospholipids, cholesterol (Satav *et al.*, 1976), and vitamin E (this paper; Venditti *et al.*, 1999b) are higher in light than in heavy mitochondrial fractions from rat liver. Moreover, some authors have proposed cytochrome *b* instead $CoQ^{\bullet-}$ as the Complex III oxygen radical generator (Nohl and Stolze, 1992).

During resting (State 4) respiration, mitochondria in M_1 fraction, characterized by high content of respiratory chain components, also exhibit O_2 consumption higher than those in M_3 and M_{10} fractions. However, the O_2 consumption is low in all fractions, and, therefore, it is likely that in M_1 mitochondria the reduction degree of autoxidizable carriers is higher and results in a higher H_2O_2 production rate. During ADP-stimulated (State 3) respiration, the decrease of the reduction degree of the respiratory chain components leads to a decrease in ROS production and ratio between H_2O_2 released and O_2 consumed by mitochondria. On the other hand, because the H_2O_2 release is higher in the M_1 fraction, it is likely that, even in this case, the amount of autoxidizable carriers is greater in such a mitochondrial fraction. The fact that during active respiration M_{10} fraction exhibits the lowest H_2O_2 production, although the ratio between H_2O_2 released and O_2 consumed is higher than those of the other fractions, can be explained again by the composition of light fraction. In fact, it contains a quantity of damaged mitochondria with high cytochrome content but low functionality, which likely come from the degradation of M_1 mitochondria (Venditti *et al.*, 1999b), and other cellular organelles like peroxisomes and microsomes (Lanni *et al.*, 1996; Venditti *et al.*, 1996), which are characterized by low O_2 consumption.

The presence of damaged mitochondria can also explain why, notwithstanding the low capacity to generate ROS, M_{10} fraction exhibits oxidative damage comparable to that of the M_1 fraction, which is characterized by high capacity to both produce H_2O_2 and turned it into the highly reactive •OH radical via the Fenton reaction.

It is noteworthy that the characteristics of the mitochondrial fractions do not determine only the extent of oxidative damage from which they are suffering, but also their susceptibility to stressful conditions. This is supported by the fact that M_1 fraction also shows susceptibility to both in vitro oxidative stress and Ca^{2+}-induced permeabilization of the inner membrane higher than other fractions.

The susceptibility to oxidants of the M_1 fraction is documented by the high level of E_{max}, which is dependent on both high cytochrome content (high *a* value) and low antioxidant capacity (low *b* value) of the fraction. In fact, in our system the hemoproteins react with H_2O_2, released by sodium perborate, generating the hydroxyl radical. This, in turn, can either interact in the close vicinity of its site of generation (Gutteridge, 1987; Stadtman, 1993) or be released in solution where it can be intercepted by scavengers. Some of the released radicals escape the scavengers and can interact with detector molecules, thus giving rise to light emission. In vivo, H_2O_2 is produced by autoxidizable components of respiratory chain, and •OH radicals which escape the scavengers interact with membrane lipids, DNA, enzyme system, thus leading to structural alterations and compromising important

cellular function. Heavy mitochondria, which are endowed with greater capacity to produce H_2O_2 and turn it in •OH radicals, are more subjected to the above detrimental changes. Moreover, it is likely that the considerable extent of oxidative process also makes the mitochondria of the M_1 fraction more susceptible to Ca^{2+}-induced permeabilization of the inner membrane.

It is well documented that, in the presence of Ca^{2+}, oxidative alterations of mitochondrial inner membrane protein thiols promote an inner membrane permeabilization referred to as MPT (Vercesi *et al.*, 1997). The pore-opening process seems to require some potential membrane decrease (Bernardi, 1992), whose consequence consists in degradation of the mitochondria since import and assembly of mitochondrial proteins are membrane potential-dependent. Thus, mitochondria which fall to maintain low ROS level and, hence, keep pores open are discarded, whereas low ROS producing organelles survive (Skulachev, 1996). In this frame, it is interesting that the values of the absorbance and membrane potential of the fraction M_{10} before the addition of Ca^{2+} are about the half of the values of M_1 or M_3 fractions. This can be explained by the presence in the M_{10} fraction of light mitochondria endowed with low susceptibility to permeabilization and damaged mitochondria coming from the degradation of M_1 mitochondria, which have reached a high degree of swelling and low membrane potential. Thus, our results support the hypothesis (Venditti *et al.*, 1996) that the same modifications which, during mitochondrial growing-maturation process, leads to an increased mitochondrial functionality, give rise to a series of events resulting after all in mitochondrial degradation.

It has been shown that conditions leading to increased ROS production and Ca^{2+} overload, including exercise and hyperthyroidism, decrease the amount of the heavy mitochondria and increase the amount of degenerating mitochondria in rat liver (Venditti *et al.*, 1999b,c). Moreover, under the same conditions the rate of mitochondrial biosynthesis appears to increase in various tissues (Di Meo *et al.*, 1992; Hamberger *et al.*, 1969). If, as suggested (Davies *et al.*, 1982), the mitochondrial membrane damage is the initiating stimulus to mitochondrial biogenesis, the above results indicate that the susceptibility of the M_1 mitochondria to stressful conditions may have important implications for the dynamics of the mitochondrial population. In fact, it should favor the rapid substitution of oldest mitochondria with neoformed mitochondria. The susceptibility to permeabilization may also have important implications for the regulation of cellular ROS production. It has been suggested that all mechanisms removing $O_2^{\bullet-}$ and its products are hardly sufficient to protect cells from harmful side effects of aerobiosis, because they inactivate already-produced ROS, but do not block their formation (Papa and Skulachev, 1997). Among the mechanisms which might be specialized in such a function, ROS-induced, PTP-mediated mitoptosis, i.e., purification of the mitochondrial population from ROS-overproducing mitochondria has been proposed (Skulachev, 1996). Interestingly, the MPT leads to cytochrome *c* release to the cytoplasm, which appears to be an early event in the apoptotic pathway of cell death (Yang *et al.*, 1997). Such process, which occurs if permeabilization affects a large group of mitochondria, can serve to purify a tissue from cells that produce large amounts of ROS (Skulachev, 1996).

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