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Role of enzymatic and non-enzymatic processes in H_2O_2 removal by rat liver and heart mitochondria

Paola Venditti · Gaetana Napolitano · Sergio Di Meo

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Abstract We compared the capacity of rat liver and heart mitochondria to remove exogenously produced H_2O_2, determining their ability to decrease fluorescence generated by H_2O_2 detector system. In the absence of substrates, liver and heart mitochondria removed H_2O_2 at similar rates. Respiratory substrate addition increased removal rates, indicating a respiration-dependent process. Moreover, the rates were higher with pyruvate/malate than with succinate and in heart than in liver mitochondria. Generally, the changes in H_2O_2 removal rates mirrored those of H_2O_2 release rates excluding the possibility that endogenous and exogenous H_2O_2 competed for the removing system. This idea was supported by the observation that the heaviest of three liver mitochondrial fractions exhibited the highest rates of both H_2O_2 release and removal. Pharmacological inhibition showed tissue-linked differences in antioxidant enzyme contribution to H_2O_2 removal which were consistent with the differences in antioxidant system activities. The enzymatic processes accounted only in part for net H_2O_2 removal and the non-enzymatic ones participated to H_2O_2 scavenging to a degree that was higher for heart than for liver mitochondria. The idea that non-enzymatic scavenging was due in great part to hemoproteins action was consistent with observation that the concentration of cytochromes, in particular cytochrome c, was higher in heart mitochondria. Indirect support was also obtained by a technique of enhanced luminescence, utilizing the capacity of cytochrome c/H_2O_2 to catalyze the luminol oxidation, which showed that luminescence response to an oxidative challenge was higher in heart mitochondria.

Keywords H_2O_2 removal · Mitochondria · Catalase · Glutathione peroxidase · Thioredoxin · Cytochromes

P. Venditti (✉) · G. Napolitano · S. Di Meo
Dipartimento di Biologia, Università di Napoli "Federico II",
Via Mezzocannone 8, 80134 Napoli, Italy
e-mail: venditti@unina.it

Introduction

Aerobic organisms are exposed to potentially harmful action of reactive oxygen species (ROS) generated as by-products of normal physiological processes (Halliwell and Gutteridge 2006). Although a number of cellular ROS sources, located in sub-cellular organelles and cytosol, have been identified (Freeman and Crapo 1982), mitochondrial respiratory chain appears to be the quantitatively more important ROS generator (Brand et al. 2004). Over 90 % of the oxygen consumed by a mammal undergoes a concerted tetravalent reduction to produce water in a reaction catalyzed by cytochrome oxidase. However, the direct electron transfer to oxygen, instead of to the next electron carrier, generates the superoxide anion radical ($O_2^{\bullet-}$), whose spontaneous and enzymatic dismutation yields hydrogen peroxide (H_2O_2).

Mitochondrial capacity to produce ROS is generally evaluated by determining the rate of H_2O_2 release by intact mitochondria in the presence of respiratory substrates. However, such a determination does not allow to deduce anything about the rate of H_2O_2 production, because H_2O_2 generated within mitochondria is partially removed by H_2O_2-detoxyfying systems (Brand et al. 2004). Because H_2O_2 readily crosses biological membranes, it is reasonable to think that mitochondria are able also to remove H_2O_2 exogenously produced. Surprisingly, few data are available on such a mitochondrial detoxifying capacity. Previously, we standardized a method providing an evaluation of the ability of mitochondria from rat tissues to scavenge H_2O_2 produced by glucose oxidase-catalyzed glucose oxidation (Venditti et al. 2001). Because the H_2O_2-linked fluorescence inhibition was not linearly related to the amount of mitochondria, the capacity of H_2O_2 removal was expressed as equivalent concentration of a substance able to interfere with H_2O_2 detection. Our results suggested that H_2O_2 removal depended on both H_2O_2-metabolyzing enzymes and iron ligands, that converted

H_2O_2 in $^{\bullet}OH$ radical via Fenton reaction (Halliwell and Gutteridge 1990).

Subsequent study, dealing with ability of brain mitochondria to remove exogenously supplied H_2O_2 reported that malate/glutamate supplemented mitochondria are able to remove H_2O_2 at a rate higher than that found with succinate-supplemented mitochondria (~6.4 and 3.3 nmol/min/mg protein, respectively) (Zoccarato et al. 2004). It was also reported that H_2O_2 removal is largely dependent on glutathione peroxidase (GPX) and glutathione reductase (GR), while other removers, including hemoproteins, account only for~20 % of mitochondrial detoxifying activity (Zoccarato et al. 2004). Conversely, other authors (Drechsel and Patel 2010) reported that the thioredoxin/peroxiredoxin (TrxR/Prx) system is the major contributor to H_2O_2 removal in brain mitochondria, whereas, in liver mitochondria, catalase (CAT) is the main contributor to H_2O_2 removal, which does not seem to depend upon a respiration-driven process.

Because scant attention was turned to hemoproteins contribution to H_2O_2 removal, in this study we provide an evaluation of the mitochondrial capacity to remove H_2O_2 and the relative relevance of the detoxifying action linked to enzymatic and non-enzymatic processes using two tissues, such as rat liver and heart, which are characterized by different cytochromes content (Venditti et al. 1996).

Materials and methods

Animals

Male Wistar rats 60 days old, supplied by Nossan (Correzzana, Italy), were used in these experiments. The animal treatment was in accordance with the guidelines set forth by the Animal Care Review Committee of the University "Federico II" of Naples.

Preparation of mitochondria

Mitochondrial fractions were isolated by centrifugation at 3,000*g* from rat liver and heart as previously described (Venditti et al. 2003b). Liver mitochondrial subpopulations at 1,000, 3,000, and 10,000*g*, designed as M_1, M_3, and M_{10}, respectively, were also isolated according to Venditti et al. (1996). The protein content in the mitochondrial preparations was determined, upon solubilisation in 0.5 % deoxycholate, by the biuret method (Gornall et al. 1949) with bovine serum albumin as a standard.

The integrity of mitochondrial preparations, isolated at 3,000 *g*, was controlled determining the ADP/O ratio in the presence of succinate as the substrate. Mitochondrial O_2 consumption was monitored at 30 °C by an Hansatech respirometer in 1.0 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 mitochondria per mL and succinate (10 mM), plus 5 μM rotenone (Rot) as substrate, in the absence (State 4) and in the presence (State 3) of 500 μM ADP.

H_2O_2 release and removal

Mitochondrial H_2O_2 removal and H_2O_2 release were assessed following the changes 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).

Measurements of H_2O_2 release rate were performed as previously described (Venditti et al. 2003a). Preliminary experiments were performed in which reaction mixtures, containing between 0.2 and 1.2 mg of liver mitochondrial proteins and succinate or pyruvate/malate, were used.

For measurements of mitochondrial H_2O_2 removal rate a modification of a previously reported method was used (Venditti et al. 2001). In brief, the peroxide was exogenously generated by glucose oxidation catalysed by glucose oxidase (GOX). Reaction of PHPA oxidation was started by adding 10 μl of 80 μg/mL GOX to a mixture containing 0.2 mg/mL PHPA, 6 U/ml HRP, 5 mM glucose in the same medium used for H_2O_2 release determination.

Measurements on non-respiring mitochondria were performed by adding 10 μL of mitochondrial samples, containing 0.2 mg of proteins and incubated for 5 min at 30 °C, to reaction medium.

Measurements on respiring mitochondria were performed by adding 10 μL of mitochondrial samples, containing 0.2 mg of proteins and incubated for 5 min at 30 °C in the presence of succinate, or succinate plus rotenone, or pyruvate malate, to reaction medium in which final concentrations of succinate, rotenone, pyruvate and malate were 10 mM, 5 μM, 10 mM and 2.5 mM, respectively. 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, additions of 10 μL of liver mitochondrial samples, containing between 0.2 and 1.2 mg of proteins, followed by succinate or pyruvate and malate additions, were performed.

Pharmacological inhibition was used to assess the contribution of mitochondrial enzymatic systems towards H_2O_2 removal in the heart and liver. Malathion, auranofin and sodium azide, at concentrations 0.1 mM, 1 μM, and 1 mM, respectively, were used to inhibit the activities of GPX, TrxR and CAT, respectively.

Preliminary experiments showed that the concentrations used for the inhibitors of the antioxidant enzymes did not inhibit HRP. Moreover, the lack of sodium azide interference with the system detecting H_2O_2 in our

conditions was supported by the results reported by Ortiz de Montellano et al. (1988).

Antioxidant system activities

The coupled activities of GR/GPX and TrxR/Prx were assessed by following the decrease of NADPH absorbance at 340 nm (Wendel 1981; Chae et al. 1999). The reaction was supplemented with glutathione (2 mM) or *E.Coli* recombinant Trx (5 μM). Catalase activity was determined by the method of Aebi (1984).

Cytochrome content

For determination of cytochrome content, mitochondria were solubilised by addition of suitable aliquots of 20 % Triton X-100 in 1.0 M potassium phosphate buffer, pH 7.4, to 1 mL of suspensions. The volume was made to 2 mL with the above buffer and then to 10 mL with isolation medium. Samples in the reference cuvettes were oxidized by the addition of a few crystals of potassium ferricyanide and those in the experimental cuvettes were reduced by the addition of a few milligrams of sodium dithionite. The difference spectra of cytochrome were recorded using a Hitachi (Model U-2000) double-beam spectrophotometer. Cytochrome content was calculated by using the wavelength pairs and extinction coefficients as given by Estabrook and Holowinsky (1961).

Cytochrome c content

Samples were prepared by diluting 10 μL of mitochondrial suspension containing 1.5 mg/mL of protein with 5 μL of 3 % SDS, 30 % glycerol, 15 % β-mercaptoethanol 0.1 % bromophenol blue, 0.187 M Tris base, pH 6.8, boiled for 5 min before loading on the gel, and electrophoresed through 6 % stacking and 12 % running SDS-PAGE gel. Gel was run in the mini protean equipment (Bio-Rad) for about 1 h at constant voltage (25 V).

Separated mitochondrial proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with a 1:1,000 dilution of a rabbit polyclonal antibody (H-104) to cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2.5 % non-fat dry milk, 10 % Tween 20.

Antibody binding was detected by carrying out secondary antibody incubation using peroxidase-conjugated anti first IgG antibody (Santa Cruz Biotechnology) diluted 1:5,000. Secondary antibody was detected using the ECL system according to the manufacturer's recommendation (Santa Cruz Biotechnology).

The blots were stripped by treating them for 10 min with 0.2 M NaOH followed by 5-min wash with H_2O and two 5-min washes with 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.1 % Tween 20. The blots were again blocked for 30 min with 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2.5 % non-fat dry milk, 10 % Tween 20, washed as above, and incubated for 2 h with a 1:2,000 dilution of anti-Voltage-Dependent Anion Channel (VDAC) antibody (Calbiochem, San Diego, CA, USA) in blocking solution. VDAC was used for loading standardization. To compare protein expression levels between cardiac and hepatic mitochondria, a standard sample of liver mitochondria was run on each gel and values obtained for both tissues were then compared with the liver sample that was assigned a value of 1.

Response to oxidative challenge

Mitochondrial susceptibility to oxidative challenge was determined as previously described (Venditti et al. 1999). 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 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, UK).

Data analysis

The data, expressed as means ± standard error, were analyzed with unpaired Student's *t* test or two way analysis of variance method, followed by Bonferroni post-test to determine the statistical significance between means. Probability values (P)<0.05 were considered significant. In Fig. 5 the results of the experiments are presented as sample curves.

Results

The values of the ADP/O ratios, 2.1±0.2 and 1.9±0.2 for liver and heart mitochondria, respectively, showed that the integrity of the mitochondria obtained at 3,000 g was not substantially reduced by the isolation procedures.

The utilization of reaction mixtures containing increasing amounts of mitochondrial proteins led to a progressive increase in the rates of whole H_2O_2 release sustained by succinate or pyruvate malate (unreported data), which was, however, associated to a progressive fall in the specific rate of H_2O_2 release (nmol H_2O_2/min/mg protein) (Fig. 1).

The addition of increasing amounts of mitochondrial proteins to the H_2O_2 generation system, constituted by glucose and glucose oxidase, caused a progressive fall in the rate of change of the fluorescence generated by the system detector

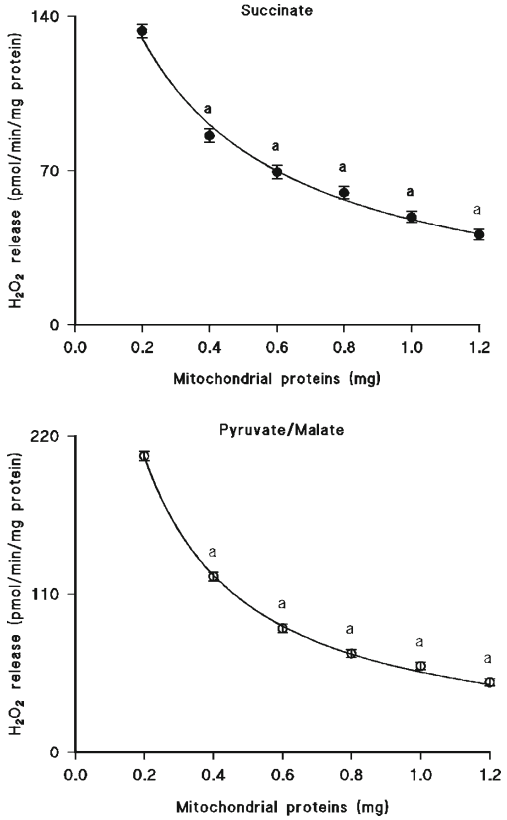

Fig. 1 Effect of increasing amount of liver mitochondria (mg of mitochondrial protein) on rate of specific H_2O_2 release (pmol/min/mg protein) in the presence of succinate or pyruvate/malate. The data are expressed as means ± SEM of six experiments. One rat was used for each experiment. [a] significant vs. sample at the closest lower concentration. The level of significance was chosen as $P<0.05$

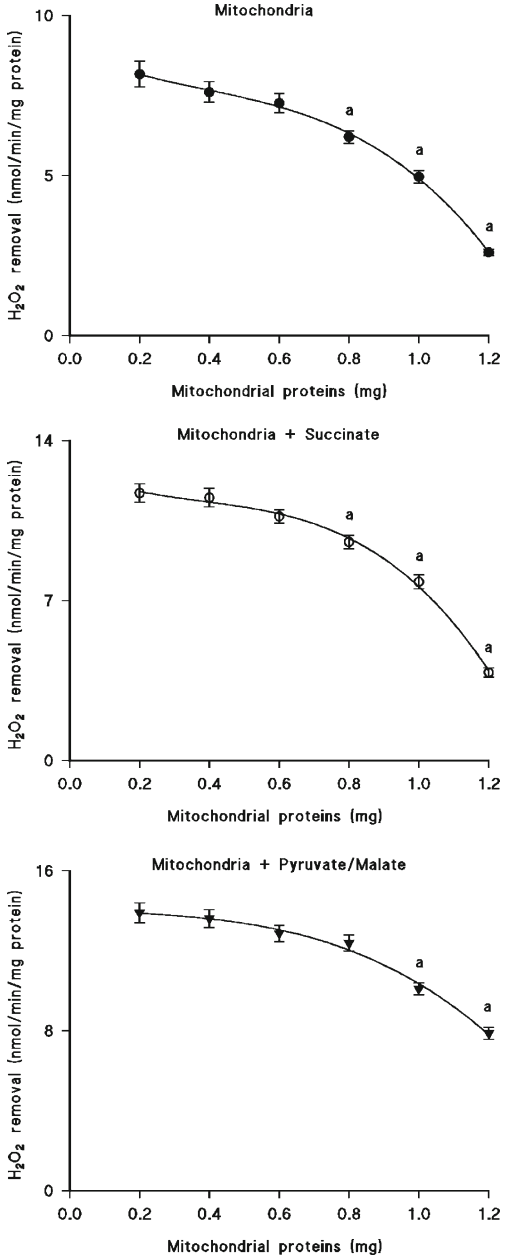

Fig. 2 Effect of increasing amount of liver mitochondria (mg of mitochondrial protein), in the presence and in absence of respiratory substrates, on rate of H_2O_2 removal (nmol/min/mg protein). The data are expressed as means ± SEM of six experiments. One rat was used for each experiment. [a] significant vs. sample at the closest lower concentration. The level of significance was chosen as $P<0.05$

for the hydroperoxide. Analogous fall was obtained when mitochondria were supplemented with succinate or pyruvate/malate (Fig. 2).

The lack of a linear relationship between H_2O_2-linked fluorescence and amount of mitochondrial proteins made necessary the utilization of a fixed concentration (0.1 mg/mL) to compare the capacity of liver and heart mitochondria to remove exogenous H_2O_2.

The results of such comparison, summarized in Table 1, show that, under resting conditions in the absence of substrates, heart mitochondria consumed H_2O_2 at higher rate than liver mitochondria. In mitochondria from both tissues, removal rates increased following addition of respiratory substrates (State 4), such as succinate, which feeds electrons to the respiratory chain via Complex II (succinate dehydrogenase), and pyruvate/malate, which feeds electrons via Complex I (NADH dehydrogenase). Upon addition of pyruvate/malate, removal rates were higher than upon addition of succinate. The presence of ADP (State 3) did not cause increase in both succinate and pyruvate/malate-dependent removal rates, whereas significant decreases were produced by addition of rotenone to succinate supplemented mitochondria. During both State 4 and State 3 respiration, removal rates were higher in heart mitochondria in presence of pyruvate/malate, but not in the presence of succinate.

Heart mitochondria exhibited higher rates of H_2O_2 release than the liver ones in all experimental conditions except State 3 of respiration of succinate-supplemented mitochondria. In both tissues, addition of ADP to succinate or pyruvate/malate supplemented mitochondria led to decreases in rates of H_2O_2 release. The ADP effect is due to slowing down of the electron

Table 1 Rates of H_2O_2 removal by liver and heart mitochondria

Preparations	Tissue	
	Liver	Heart
M	8.17±0.06	10.38±0.10[a]
M, succinate	12.04±0.13[b]	12.52±0.09[b]
M, succinate + rotenone	9.00±0.38[c]	10.66±0.22[a,c]
M, succinate + ADP	12.42±0.80[b]	12.44±0.47[b]
M, pyruvate/malate	13.89±0.13[b,d]	16.27±0.29[a,b,d]
M, pyruvate/malate + ADP	14.02±0.85[b,d]	16.77±0.43[a,b,d]

Values are means ± SEM. For each value eight rats were used. Rate of mitochondrial H_2O_2 removal is expressed in nmol/min/mg protein.

M mitochondria

[a] Significant vs. liver

[b] Significant vs. M of the same tissue

[c] Significant vs. M + the same substrate

[d] Significant vs. succinate-supplemented M in the same respiration state

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

flow, which leads to decrease in reduction degree of autoxidizable carriers, by which H_2O_2 release depends. Rates of H_2O_2 release were also decreased by addition of rotenone to succinate supplemented mitochondria (Table 2).

In Table 3 the comparison between rates of H_2O_2 removal and H_2O_2 release by liver mitochondrial subpopulations are reported. The results show that removal rates are higher in M_1 than in M_3 and M_{10} fraction irrespective of respiratory substrate. Moreover, in all mitochondrial fractions the rates were higher with pyruvate/malate than with succinate. Even rates of H_2O_2 mitochondrial release were higher with pyruvate/malate in all fractions, and reached the highest and lowest values in M_1 and M_{10} fractions, respectively.

Table 2 Rates of H_2O_2 release by liver and heart mitochondria

Substrate	Tissue	
	Liver	Heart
Succinate	153.7±4.1	223.5±7.6[a]
succinate + rotenone	106.4±1.5[b]	157.1±4.7[a,b]
succinate + ADP	94.29±1.7[b]	110.8±3.4[b]
pyruvate/malate	230.9±5.8[c]	254.3±6.7[a,c]
pyruvate/malate + ADP	104.7±4.5[b]	124.6±3.5[a,b]

Values are means ± SEM. For each value eight rats were used. Rate of H_2O_2 mitochondrial release is expressed in pmol/min/mg protein

[a] Significant vs. liver

[b] Significant vs. mitochondria + the same substrate

[c] Significant vs. succinate-supplemented mitochondria in the same respiration state

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

Table 3 Rates of H_2O_2 release and removal by mitochondrial subpopulations from rat liver

Substrate	Subpopulations		
	M_1	M_3	M_{10}
Removal			
Succinate	12.27±0.67	9.01±0.74[a]	8.94±0.29[a]
Pyruvate/malate	15.30±0.49[c]	11.56±0.82[a,c]	11.37±0.77[a,c]
Release			
Succinate	163.10±3.20	151.54±3.30[a]	105.73±1.24[a,b]
Pyruvate/malate	233.04±3.15[c]	206.29±4.35[a,c]	181.87±3.46[a,b,c]

Values are means ± SEM. For each value eight rats were used. Rates of mitochondrial H_2O_2 release and removal are expressed in pmol/min/mg protein and nmol/min/mg protein, respectively

[a] Significant vs. M_1

[b] Significant vs. M_3

[c] Significant vs. succinate

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

The percent reduction of H_2O_2 removal by enzyme inhibitors and antioxidant system activities in liver and heart mitochondria are reported in Table 4. The results show that the contribution of CAT and GPX to H_2O_2 removal is higher in liver mitochondria, whereas the TrxR one is higher in heart mitochondria.

The tissue-linked differences in the contribution to H_2O_2 removal of the antioxidant enzymes were supported by the differences in antioxidant system activities since those of CAT and GR/GPX were higher in liver mitochondria, whereas that of TrxR/Prx was higher in heart mitochondria.

As shown in Fig. 3, mitochondrial cytochrome content was higher in heart than in liver. Accordingly, the cytochrome c

Table 4 Percent reduction of H_2O_2 removal by enzyme inhibitors and antioxidant system activities in mitochondria from rat liver and heart

Tissue	Antioxidant enzyme or system		
	CAT	TrxR	GPX
Reduction			
Liver	31.88±0.48	18.48±0.11[b]	22.56±0.26[b,c]
Heart	13.35±0.82[a]	22.37±0.37[a,b]	14.40±0.40[a,c]
	CAT	TrxR/Prx	GR/GPX
Activity			
Liver	184.4±1.0	39.3±0.9	41.3±1.2
Heart	58.66±0.9[a]	61.9±0.7[a]	16.5±0.2[a]

Values are means ± SEM. For each value eight rats were used

[a] Significant vs. liver

[b] Significant vs. CAT

[c] Significant vs. TrxR

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

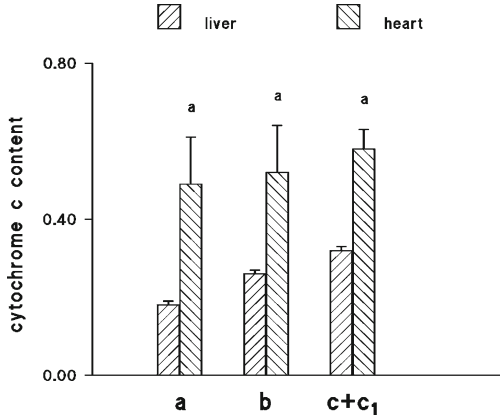

Fig. 3 Cytochrome content of liver and heart mitochondria. The cytochrome content is expressed as nmol/mg of mitochondrial protein. a, b and c+c1 indicate cytochrome a, cytochrome b, and cytochrome c+c1, respectively. Values are means ± SEM of eight experiments. One rat was used for each experiment. [a] significant vs. liver. The level of significance was chosen as $P<0.05$

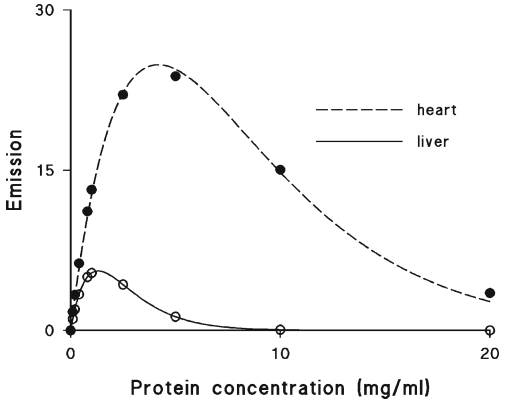

Fig. 5 Response to in vitro oxidative challenge of liver and heart mitochondria. Mitochondrial susceptibility to stress was evaluated by determining the variations, with concentration of mitochondrial proteins, of light emission from a luminescent reaction. Emission values are given a percentages of an arbitrary standard (44 ng ml^{-1} peroxidase). The curves were computed from experimental data by equation $E=a\cdot C/\exp(b\cdot C)$

level, determined by western blotting, was higher in cardiac mitochondria (Fig. 4).

The results of the experiments performed on liver and heart mitochondria to determine the luminescence response to oxidative challenge show that in both tissues light emission (E) is a function of protein concentration (C), which can be described by the same equation $E=a\cdot C/\exp(b\cdot C)$, in which a and b are two constants. As shown in Fig. 5, the light emission and, in particular, emission maximum (E_{max}) are higher in heart mitochondria. Moreover, examination of parameters determining light emission shows that the values of a and E_{max} are higher in heart mitochondria while the value of b is higher in liver mitochondria (Table 5).

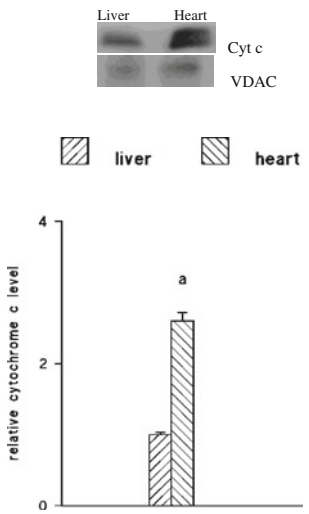

Fig. 4 Levels of cytochrome c protein expression in rat liver and heart mitochondria. Mitochondrial proteins were isolated and analysed using Western blot analysis. A representative result of 3 independent experiments is shown. Values are means ± SEM of three experiments. One rat was used for each experiment. VDAC was used for loading standardization. Ratios of band intensities to VDAC band intensities were compared with a standard sedentary untreated sample that was assigned a value of 1. [a] significant vs. liver. The level of significance was chosen as $P<0.05$

Discussion

Since the early papers demonstrated that mitochondria produce ROS in the presence of respiratory chain inhibitors (Boveris et al. 1972; Loschen et al. 1971), the idea that the mitochondrial electron transfer chain is one of the major cellular generators of ROS has received wide consensus (Kowaltowski et al. 2009). Conversely, a few studies have addressed the possibility that mitochondria might be not only a source but also a sink of ROS under physiological conditions (Venditti et al. 2013). The fact that this aspect of intracellular ROS metabolism has been widely neglected is surprising as a clear indication of the scavenging mitochondrial ability comes from the observation that specific rate of H_2O_2 mitochondrial release (pmol/min/mg protein), evaluated by horseradish peroxidase-based detection system, is lower in media containing greater amounts of mitochondrial proteins.

Table 5 Parameters characterizing the response to oxidative challenge of mitochondria from rat liver and heart

Tissue	Parameters		
	a	*b*	E_{max}
Liver	11.4±0.7	0.76±0.05	5.51±0.33
Heart	16.2±1.5[a]	0.24±0.02[a]	24.82±1.17[a]

Values are means ± SEM. For each value eight rats were used

The relation between light emission and mitochondrial protein concentration is described by the equation $E=a\cdot C/\exp(b\cdot C)$

[a] significant vs. liver. The level of significance was chosen as $P<0.05$

This phenomenon can be explained by a competition between the mitochondrial peroxidases and the detection system for H_2O_2 trapping, so that the increase in mitochondrial protein concentration reduces the probability that H_2O_2 is removed by hydroperoxide detecting system. In other words, the apparent decrease in the rate of mitochondrial H_2O_2 release, found by increasing mitochondrial protein amount, should indicate that: i) a fraction of H_2O_2, which gets out of mitochondria, escaping removal, is then processed by surrounding mitochondria, ii) a more significant fraction of H_2O_2 released by a mitochondrion is intercepted by other mitochondria when their number increases. With regard to this, an interplay between peroxide production and its removal has been hypothesized. Indeed, according to Zoccarato et al. (2004), in a mixed mitochondrial population, some mitochondria, that are H_2O_2 producers, have a low peroxidase activity, so that their H_2O_2 is released to be taken up by mitochondria with higher peroxidase activity. Although the hypothesis is plausible, results reported in this paper suggest that it is possible that the same mitochondrial fraction is strong H_2O_2 producer and has high peroxidase activity.

Analyzing the mitochondrial ability to remove H_2O_2 exogenously generated by glucose-glucose oxidase system, we found that increasing amounts of mitochondrial proteins induced not linear decrease in the H_2O_2-linked fluorescence in the presence and in the absence of respiratory substrates. The same course of the dose–response curve was obtained using higher enzyme amount (unreported data). For this reason, we used a protein concentration low as 0.1 mg/mL, which was also used for measurements of H_2O_2 release rate.

Our results show that the capacity of cardiac mitochondria to scavenge H_2O_2 exogenously produced is generally higher than that of hepatic mitochondria. In mitochondria from both tissues, such a capacity is accentuated by respiratory substrates, in particular pyruvate and malate.

A feature of the GPX/GR and Trx/TrxR and Prx systems is that, for their activity, they need NADPH, which in mitochondria can be regenerated by NADH-supported $NADP^+$ reduction via energy dependent trans-hydrogenation. Thus, the increase in H_2O_2 removal in respiring mitochondria can be attributed to the generation of NADPH necessary to promote enzyme-mediated H_2O_2 removal, and the lack of change after ADP addition suggests that, during State 4, NADPH levels are sufficient to such a promotion. Moreover, the higher effect of the respiration on H_2O_2 removal by heart mitochondria, can be due to their lower content of GSH (Moreira et al. 2011) (and likely Trx), which are more rapidly depleted making the respiration essential for further H_2O_2 detoxification.

The higher net H_2O_2 removal rate with pyruvate/malate could depend on a lower mitochondrial peroxide production, supplying a lower amount of endogenous H_2O_2 in competition with exogenous H_2O_2 for the H_2O_2-removing system and/or on a higher activation of such a system.

To distinguish between these possibilities we monitored H_2O_2 release and found that this was higher with pyruvate/malate than with succinate and in heart than in liver mitochondria. Although the determination of H_2O_2 release rate does not supply information on the true value of H_2O_2 production rate, it allows us to assume that heart mitochondria are more effective H_2O_2 producers than liver mitochondria, particularly in the presence of pyruvate/malate, thus excluding the possibility that endogenous and exogenous H_2O_2 compete for the removing system. This was confirmed by the observation that the addition of rotenone to succinate-supplemented mitochondria, which reduces H_2O_2 release, stopping that occurring at Complex I due to the reverse electron from coenzyme Q (Ernster and Lee 1967), also reduces mitochondrial removal rate stopping NADH formation at Complex I.

The idea that mitochondria, which are strong H_2O_2 producers, can also have high peroxidase activity was further supported by the examination of three liver mitochondrial subpopulations provided with different respiratory activities and different capacities to release H_2O_2 (Venditti et al. 2002). Indeed, we found that the heaviest (M_1) fraction not only exhibited the highest rate of H_2O_2 release, but also the highest capacity to remove H_2O_2.

Pharmacological inhibition of antioxidant enzymes showed a different contribution of catalase, glutathione peroxidase, and thioredoxin reductase to H_2O_2 detoxification in liver and heart. Furthermore, catalase and TrxR accounted for the higher H_2O_2 detoxification in liver and in heart, respectively. Such results were consistent with those concerning antioxidant system activities.

Enzymatic processes accounted only in part for net respiration-driven H_2O_2 removal. The extent of such a removal was higher for liver mitochondria (72.9 %) than for heart mitochondria (50.1 %), indicating that non-enzymatic processes participate in H_2O_2 scavenging to a degree that is higher for heart (49.9 %) than for liver (27.1 %) mitochondria.

Mitochondrial levels of compounds, such as CoQ_{10} and vitamin E, are higher in heart than in liver (Venditti et al. 2003b), whereas those of other reducing compounds, such as GSH (Moreira et al. 2011), NADPH and NADH (Jacobson and Kaplan 1957) are lower in heart. Although GSH, NADPH, and NADH levels have been tested under purification conditions likely different from ours, it is conceivable that the ratios between reducing agent levels in cardiac and hepatic mitochondria have not been reversed in such conditions and, therefore, such agents are not responsible for higher non-enzymatic H_2O_2 removal by heart mitochondria. Conversely, it is possible that such a capacity depends on cytochromes, which exhibit mitochondrial levels higher in heart than in liver (Di Meo et al. 1996) and are able to react with H_2O_2, even though the molecular mechanisms involved in such a process are not fully understood. Some information is available about the action of cytochrome c, a small globular hemoprotein

which is located in high concentrations (0.5–5 mM) in the intermembrane space. A part of cytochrome c, which is loosely attached to the inner membrane, participates in electron transport, mediates superoxide removal, and prevents oxidative stress (Korshunov et al. 1999; Semak et al. 2005). The other part, which is tightly bound to membrane, accounts for the peroxidase activity (Kagan et al. 2004), through which it can oxidize organic molecules in the presence of H_2O_2 (Radi et al. 1991a). The reaction of H_2O_2 with hemoproteins, such as cytochrome c, produces highly reactive ferrylheme species that are capable of oxidizing biomolecules and initiating lipid peroxidation (Radi et al. 1991a, 1991b). H_2O_2 oxidizes cytochrome c to a peroxidase compound I-type intermediate (an oxoferryl heme species), which is able to oxidize GSH, NADH, 2′,7′ dichlorofluorescin, 5,5-dimethyl-1-pyrroline *N*-oxide, and ascorbate (Lawrence et al. 2003). The peroxidase activity of cytochrome c may compete with other mitochondrial and perixosomal H_2O_2-scavenging enzymes to control H_2O_2 levels at the expense of intracellular reductants (Kagan et al. 2005). Furthermore, the reaction of cytochrome c with H_2O_2 may lead to release of iron ions which may react with H_2O_2 generating $^{\bullet}OH$, the most powerful oxidizing species, through a Fenton-like reaction.

We found that mitochondrial cytochrome content, and in particular that of cytochrome c, is higher in heart than in liver, but unfortunately the lack of a system able to selectively inhibit H_2O_2 removal by cytochromes did not allow to determine the contribution of such hemoproteins to mitochondrial H_2O_2 removal. However, we obtained an indirect evidence that heart mitochondria are provided with a higher capacity for cytochrome-linked H_2O_2 removal, using a technique of enhanced luminescence (Venditti et al. 1999), which utilizes the ability of cytochrome c/H_2O_2 to catalyze the oxidation of luminol (Radi et al. 1991a). In fact, the examination of parameters characterizing light emission indicates that heart mitochondria have higher capacity to induce chemiluminescent reaction (higher E_{max} value), which, in turn, is due to higher a value and lower b value in the equation $E=a \cdot C/\exp(b \cdot C)$, describing the dose–response curves. The former parameter depends on levels of substances able to induce luminol oxidation, such as cytochromes, which lead to formation of oxoferryl species and $^{\bullet}OH$ radicals. Conversely, the latter depends on levels of substances able to reduce light emission, removing H_2O_2, scavenging $^{\bullet}OH$ radicals, or competing with luminol for oxoferryl reaction (Venditti et al. 1999).

In this light, our results are consistent with the observation that heart mitochondria have higher levels of cytochromes and a lower effectiveness of antioxidant defence system than liver mitochondria. Moreover, they supply a strong support to the idea that hemoproteins affect in relevant measure the capacity of heart mitochondria to remove hydrogen peroxide.

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