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The differential effects of superoxide anion, hydrogen peroxide and hydroxyl radical on cardiac mitochondrial oxidative phosphorylation

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Abstract

The involvement of reactive oxygen species (ROS) in cardiac ischemia-reperfusion injuries is well-established, but the deleterious effects of hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) or superoxide anion ($O_2^{\bullet-}$) on mitochondrial function are poorly understood. Here, we report that incubation of rat heart mitochondria with each of these three species resulted in a decline of the ADP-stimulated respiratory rate but not substrate-dependent respiration. These three species reduced oxygen consumption induced by an uncoupler without alteration of the respiratory chain complexes, but did not modify mitochondrial membrane permeability. $HO^{\bullet}$ slightly decreased F1F0-ATPase activity and $HO^{\bullet}$ and $O_2^{\bullet-}$ partially inhibited the activity of adenine nucleotide translocase; H_2O_2 failed to alter these targets. They inhibited NADH production by acting specifically on aconitase for $O_2^{\bullet-}$ and alpha-ketoglutarate dehydrogenase for H_2O_2 and $HO^{\bullet}$. Our results show that $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ act on different mitochondrial targets to alter ATP synthesis, mostly through inhibition of NADH production.

Keywords: *Superoxide anion, hydroxyl radical, hydrogen peroxide, heart mitochondria, NADH, adenine nucleotide translocase*

Introduction

Ischemia results in a rapid disruption of mitochondrial oxygen consumption, which causes a decrease in aerobic ATP synthesis leading to an impairment in normal myocardial function. Restoration of oxygenated blood flow to the ischemic organ can rescue the tissue but, paradoxically, the reperfusion phase can also amplify ischemic damage. The sudden influx of oxygen to the mitochondrial respiratory chain leads to a burst of reactive oxygen species (ROS), which induce oxidative stress and deplete the reducing compounds protecting mitochondria against oxidative insults. In addition, restoration of the mitochondrial membrane potential may drive the cytosolic Ca^{2+} accumulated during ischemia into the mitochondria, leading to Ca^{2+} overload [1]. These conditions favour opening of the permeability transition pore (PTP) [2]. PTP causes disruption of the impermeability of the mitochondrial inner membrane and ultimately complete inhibition of mitochondrial functions. This phenomenon is now considered to play a central role in mitochondria-mediated death pathways [3].

Therefore, inhibition of ROS generation appears to be a relevant pharmacological approach for limiting reperfusion injuries [4–6]. ROS are produced by different mitochondrial enzymes, specifically complexes I and III of the respiratory chain. In normal conditions, $O_2^{\bullet-}$ is immediately eliminated by either

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non-enzymatic or enzymatic (superoxide dismutase) dismutation to hydrogen peroxide (H_2O_2), which is then reduced to water. However, if mitochondria do not succeed in eliminating H_2O_2, it can further decompose to the highly reactive hydroxyl radical ($HO^\bullet$) in the presence of Fe^{2+} [7]. This can occur during the reperfusion of an organ once enzymatic and non-enzymatic antioxidant systems are overwhelmed, and Fe^{2+} is released [8].

While it is well-established that ROS are involved in the induction of cardiac injuries, the deleterious effects of $O_2^{\bullet-}$, H_2O_2 and $HO^\bullet$ on mitochondrial function are not well understood. Previous studies have reported the sensitivity of mitochondrial enzymes to individual oxygen species, but most were performed on purified proteins or on submitochondrial particles [9,10]. In the present study, we separately analysed the effects of $O_2^{\bullet-}$, H_2O_2 and $HO^\bullet$ on the functions of isolated rat heart energized mitochondria and characterized their targets and the mechanisms that result in a decreased capacity of mitochondria to synthesize ATP.

To this end, $O_2^{\bullet-}$ was generated by xanthine plus xanthine oxydase, H_2O_2 was introduced directly in the respiration buffer containing mitochondria and $HO^\bullet$ was produced from H_2O_2 in the presence of ferrous salts.

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Materials and methods

Isolation of mitochondria from rat heart

Hearts were removed from male Wistar rats (250–300 g) obtained from Janvier (Le Genest-St-Isle, France) and immediately immersed and rinsed in ice cold NaCl 0.9%. Hearts ($\approx$ 1 g) were minced and homogenized in 30 ml of cold buffer containing (in mM): mannitol 220, sucrose 70, HEPES 10, EGTA 1, free fatty acid bovine serum albumin (Sigma A-9205) 0.04, pH 7.4 at 4°C with Polytron homogenizer (low setting, 3 s) then with a Potter homogenizer (3 up and down) at 1500 tr/min. Homogenates were centrifuged at 1000 g for 5 min at 4°C and supernatants were centrifuged at 10 000 g for 10 min at 4°C and washed once more by the same procedure. The final mitochondrial pellets were resuspended in 300 µl of homogenization buffer without EGTA and bovine serum albumin to a final concentration of $\sim$ 30 mg/ml. Protein concentrations were measured by the Lowry method [11] using bovine serum albumin as a standard.

Evaluation of mitochondrial respiration

Oxygen consumption was measured at 37°C with a Clark-type electrode (Hansatech, Eurosep, Cergy St Christophe, France). Mitochondria (0.4 mg/ml) were pre-incubated for 3 min in the presence or in the absence of each ROS in a respiration buffer containing (in mM): KCl 100, sucrose 50, HEPES 10, KH_2PO_4 5, pH 7.4 at 37°C.

Respiration was initiated by the addition of 10 mM pyruvate/malate (State 2). After 1 min, ATP synthesis was induced by the addition of 500 µM ADP (State 3 respiration rate). Upon depletion of ADP, the rate of State 4 respiration was measured and the respiratory control ratio (RCR, State 3/State 4) was calculated.

Assay of mitochondrial complex activities

Activities of electron transport chain complexes I, II, III and IV were evaluated after pre-incubation of frozen and thawed mitochondria (0.4 mg/ml) in the presence or in the absence of $O_2^{\bullet-}$, H_2O_2 or $HO^\bullet$ for 3 min at 37°C. Complex I activity was measured at 37°C by monitoring the decrease in fluorescence resulting from the oxidation of NADH (see below). The incubation medium contained 10 mM KH_2PO_4, 1 mM $MgCl_2$, 50 µM decylubiquinone, 250 µM KCN, 1 mg/ml bovine serum albumin. The reaction was started by the addition of 10 µM NADH. Other respiratory chain enzyme activities were measured according to Barrientos [12]. Complex II activity was measured by monitoring the absorbance changes of 2,6-dichloroindophenol (DCIP) at 600 nm. The assay mixture contained 10 mM KH_2PO_4, 1 mM $MgCl_2$, 2 mM EDTA, 2 µM rotenone, 10 mM succinate and 1 mg/ml bovine serum albumin in the presence of 100 µM DCIP. The reaction was started by the addition of 50 µM decylubiquinone. Complex III activity was measured as the rate of cytochrome C reduction at 550 nm. The reaction mixture contained 10 mM KH_2PO_4, 1 mM $MgCl_2$, 2 mM EDTA, 2 µM rotenone, 1 mg/ml bovine serum albumin and 100 µM decylubiquinol. The reaction was started by the addition of 50 µM oxidized cytochrome C. Complex IV activity was performed at 550 nm following the decrease in absorbance resulting from the oxidation of 50 µM reduced cytochrome C. The incubation medium contained 10 mM KH_2PO_4, 1 mM $MgCl_2$ and 2 mM EDTA, pH = 7.4 at 37°C.

Assay of adenine nucleotide translocase activity

Adenine nucleotide translocase (ANT) activity was assessed by measuring the transport of ^{14}C-ADP into mitochondria. Mitochondria (0.4 mg/ml) were suspended in the respiration buffer in a total volume of 200 µl. These samples were pre-incubated for 3 min at 37°C in the absence or in the presence of $O_2^{\bullet-}$, H_2O_2 or $HO^\bullet$, cooled for 20 s at 0°C and finally the transport was initiated by the addition of 8 µM ^{14}C-ADP. After incubation of the suspension for 1 min at 4°C, the reaction was terminated by the addition of 100 µM atractyloside and the suspension was immediately filtered and counted in a liquid scintillation counter.

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Measurement of F1F0-ATPase activity

Mitochondrial F1F0-ATPase activity was determined by measuring the concentration of Pi released by ATP hydrolysis. Briefly, frozen and thawed (three cycles) mitochondria (50 µg/ml) were incubated at 37°C for 3 min in 0.5 ml of a medium containing 50 mM Tris, 5 mM $MgCl_2$ (pH 7.4 at 37°C) in the absence or in the presence of each ROS. ATPase activity was initiated by the addition of 100 µM ATP and the reaction was stopped after 10 min of incubation by the addition of 3% trichloroacetic acid. The solution was centrifuged at 10 000 g for 3 min and Pi concentration was determined against a zero time blank in the supernatant according to the method of Baykov et al. [13]. Mitochondrial F1F0-ATPase activity was defined as the difference between ATPase activity and ATPase activity observed in the presence of 1 µM oligomycin.

Measurement of mitochondrial swelling

Mitochondrial swelling was assessed by measuring the change in absorbance of the mitochondrial suspension at 540 nm using a Jasko model V530 spectrophotometer. Mitochondria (0.4 mg/ml) were incubated at 37°C in 1 ml of the respiration buffer. Mitochondrial swelling was induced by the addition of ROS, Ca^{2+} or ROS + Ca^{2+}.

Exposure of cardiac mitochondria to $O_2^{\bullet -}$, H_2O_2 or $HO^{\bullet}$

The production of $O_2^{\bullet -}$ induced by xanthine + xanthine oxidase was followed by measuring the rate of nitroblue tetrazolium reduction at 560 nm [14] and was evaluated by measuring the concentration of diformazan produced using its molar extinction coefficient (4500 M^{-1} cm^{-1} at 560 mm). To evaluate the effect of $O_2^{\bullet -}$, mitochondria (0.4 mg/ml) were incubated at 37°C in the respiration buffer in the presence of 100 µM xanthine, 70 mU/ml xanthine oxidase and 100 µM of nitroblue tetrazolium for 3 min in a final volume of 1 ml.

To evaluate the effect of H_2O_2, mitochondria (0.4 mg/ml) were incubated at 37°C in the respiration buffer and were exposed to increasing concentrations of H_2O_2 (100–1000 µM) for 3 min in a final volume of 1 ml.

The production of $HO^{\bullet}$ induced by 400 µM H_2O_2 in the presence of 20 µM ferrous ammonium sulphate was followed by measuring the rate of hydroxylation of 1 mM benzoic acid [14]. This reaction produced salicylic acid which was measured spectrofluorometrically (Perkin Elmer LS 50B) at 308 nm (λex; slit 5) and 410 nm (λem; slit 3). In parallel, a salicylic acid standard curve (0.5–10 µM) was carried out to determine its concentration.

To evaluate the effect of $HO^{\bullet}$, mitochondria (0.4 mg/ml) were incubated at 37°C in the respiration buffer and were exposed to 400 µM H_2O_2 in the presence of 20 µM ferrous ammonium sulphate for 3 min in a final volume of 1 ml.

Assay for mitochondrial NAD(P)H levels

NAD(P)H concentration in intact mitochondria (0.4 mg/ml at 37°C) was measured spectrofluorometrically (Perkin-Elmer LS 50B) with excitation and emission wavelengths of 360 nm and 450 nm, respectively, according to Minezaki et al. [15]. Known quantities of NADH were added to 0.4 mg/ml mitochondria for calibration.

Measurement of mitochondrial lipid peroxidation

Lipid peroxidation was determined on mitochondria (0.4 mg/ml) energized with pyruvate/malate (10 mM) and incubated for 10 or 30 min in the absence or in the presence of each ROS by measuring the formation of malondialdehyde (MDA). A mixture of ferrous ammonium sulphate (Fe^{2+}; 50 µM) and ferric ammonium sulphate (Fe^{3+}; 150 µM) was used to induce control lipid peroxidation according to Braughler et al. [16].

Statistical analysis

The data are reported as mean $\pm$ SD. Comparisons between groups were performed among groups using a one-way analysis of variance followed by a Scheffe's test. Significance was accepted when $p < 0.05$.

Results and discussion

Mitochondria is one of the main sources of ROS in the cell [17,18], which renders mitochondrial function particularly sensitive to oxidative damage but the relative contribution of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ is not well-known. Here, we demonstrate that each of these species inhibits the oxidative phosphorylation process by acting on different mitochondrial enzymatic systems.

Effect of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ on oxidative phosphorylation

Loss in the rate of oxidative phosphorylation was measured in the presence of either $O_2^{\bullet -}$, H_2O_2 or $HO^{\bullet}$. This effect was evaluated on intact mitochondria with mixed pyruvate/malate as respiratory substrates to ensure activation of Krebs cycle enzymes. Mitochondria were incubated for 3 min at 37°C with increasing concentrations of H_2O_2 and the phosphorylation process was started by the addition of 0.5 mM ADP. This incubation period was chosen because it induced the least alteration in respiration in control mitochondria. Longer incubation times promoted greater effects which made evaluation of the effects

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of $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ difficult. Moreover, during oxidative stress the first minutes appear critical for the fate of the cell [19]. H_2O_2 induced a concentration-dependent decrease in RCR reaching ~1 for 1 mM H_2O_2 (Table I). This was related to the extensive decrease in State 3 respiration rate, whereas State 4 was not modified. A concentration of 400 μM H_2O_2 was used in all following experiments because it induced a ~50% decrease in State 3 respiration rate. Addition of 20 μM Fe^{2+} to the medium, which promoted the generation of $HO^{\bullet}$ in the presence of H_2O_2 during the incubation period (Figure 1A), amplified this effect (Table I), which was also related to the decrease in State 3 respiration.

To examine the effect of $O_2^{\bullet-}$ exposure, we used the classical enzymatic generating system xanthine + xanthine oxidase. This protocol resulted in constant production of $O_2^{\bullet-}$ in the presence of mitochondria (Figure 1B). We verified that xanthine + xanthine oxidase produced a low concentration of H_2O_2 (≈ 10 % of $O_2^{\bullet-}$ production) in our experimental conditions, using the Amplex Red assay, a highly specific and sensitive probe for H_2O_2 [20]. In addition, we observed that the extra-mitochondrial generation of $O_2^{\bullet-}$ did not modify the formation of H_2O_2 by mitochondria. Indeed, $O_2^{\bullet-}$ inhibited State 3 respiration rate but did not alter State 4 respiration; the same results were observed in the presence of catalase (Table I). This result indicates that the effect observed was due to $O_2^{\bullet-}$ and ruled out a possible effect of H_2O_2 generated by mitochondria.

This specific inhibition of the State 3 respiratory rate and the absence of effect on State 4 respiratory rate by $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ suggested that the integrity of the mitochondrial membrane was not altered. This was confirmed by two additional experiments: (1) no membrane lipid peroxidation was observed after 10 min exposure of mitochondria with these three species (Table II) and (2) $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ did not promote mitochondrial swelling in the absence of Ca^{2+} (Figure 2), confirming that they did not modify mitochondrial membrane permeability. However, in the presence of high Ca^{2+} concentrations, they facilitated mitochondrial swelling (an evidence of permeability alteration). This increase in mitochondrial membrane permeability was due to PTP opening, as it was completely prevented by 1 μM cyclosporine A (not shown), a well-known inhibitor of this channel

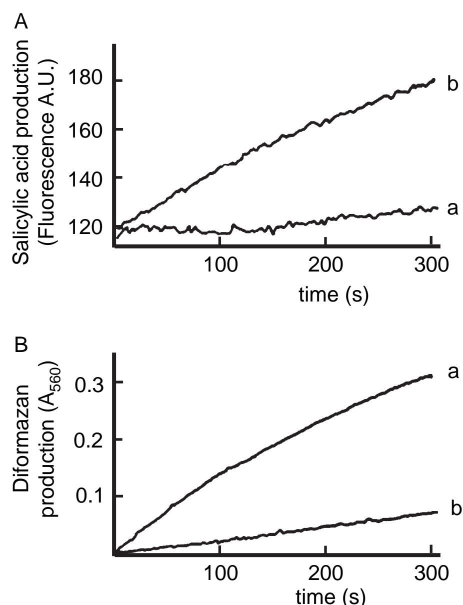

Figure 1. Production of hydroxyl radicals ($HO^{\bullet}$) and superoxide anions ($O_2^{\bullet-}$). (A) The production of $HO^{\bullet}$ induced by the mixture H_2O_2/Fe^{2+} added to heart mitochondria was followed by measuring the rate of hydroxylation of benzoic acid (1 mM) in the presence of 400 μM H_2O_2 (no production, a) or 400 μM H_2O_2 + 20 μM ferrous ammonium sulphate (0.2 μM/min; b). The rate of hydroxylation of benzoic acid was evaluated by measuring the generation of salicylic acid by spectrofluorimetry. (B) The production of $O_2^{\bullet-}$ induced by the mixture xanthine/xanthine oxidase added to heart mitochondria was followed by measuring the rate of nitroblue tetrazolium reduction at 560 nm and was evaluated by determining the production of diformazan (130 nmol/min.mg proteins (a). Alpha-tocopherol (1 mM) was used as a reference inhibiting agent (b).

Table I. Effect of ROS on oxidative phosphorylation. Mitochondria (0.4 mg/ml) were incubated with pyruvate/malate (10/10 mM) for 3 min at 37°C without (control) or with either H_2O_2 (400 μM), H_2O_2 (400 μM) + Fe^{2+} (20 μM) generating $HO^{\bullet}$ or xanthine (100 μM) + xanthine oxidase (70 mU/ml) generating $O_2^{\bullet-}$ and then the respiratory rates were measured. Catalase concentration was 3500 units/mg protein in the reaction medium. Data are means ± SD of five experiments performed in triplicate.

	State 3 (nmol O_2/min/mg protein)	State 4 (nmol O_2/min/mg protein)	RCR
Control	457 ± 40	101 ± 36	4.75 ± 0.50
H_2O_2 (μM)			
100	417 ± 12	105 ± 12	3.98 ± 0.38
200	352 ± 18*	100 ± 8	3.55 ± 0.33*
400	249 ± 22**	92 ± 24	2.81 ± 0.54**
1000	115 ± 15***	94 ± 12	1.22 ± 0.16***
$HO^{\bullet}$	164 ± 24***,†	104 ± 13	1.58 ± 0.16***,†
$O_2^{\bullet-}$	324 ± 33**	111 ± 16	2.92 ± 0.29**
$O_2^{\bullet-}$ + catalase	270 ± 32NS	118 ± 5NS	2.29 ± 0.20NS

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs State 3 control value. †$p < 0.05$ vs H_2O_2 (400 μM). NS: non-significant compared to $O_2^{\bullet-}$ alone.

Table II. Effect of ROS on lipid peroxidation of mitochondrial membranes. Data represent means ± SD of five experiments performed in triplicate.

MDA concentration (nmol /ml/mg protein)	Incubation (10 min)	Incubation (30 min)
Control	19.0 ± 2.28	32.1 ± 2.70
Fe^{2+}/Fe^{3+}	44.02 ± 3.50**	47.8 ± 2.20**
H_2O_2 (400 μM)	18.8 ± 2.60	30.7 ± 6.50
$HO^{\bullet}$	20.4 ± 2.40	40.5 ± 4.34**
$O_2^{\bullet-}$	20.1 ± 3.00	34.8 ± 3.80

**$p < 0.01$ vs their respective control values.

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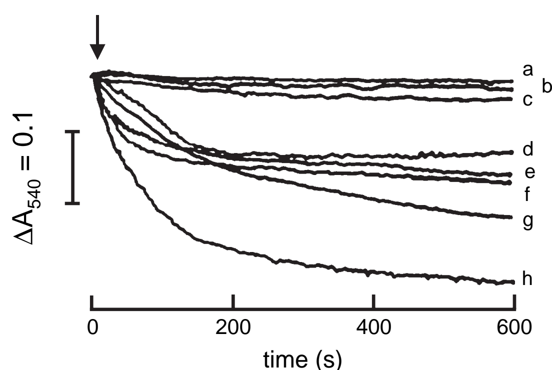

Figure 2. Effect of ROS on mitochondrial swelling. Heart mitochondria (0.4 mg/ml), energized with the mixture pyruvate/malate (10 mM), were incubated in the respiration buffer. After 1 min incubation, swelling was induced (arrow) by either 400 μM H_2O_2 (a), 400 μM H_2O_2+20 μM Fe^{2+} (b), 100 μM xanthine+70 mU/ml xanthine oxidase (c), 100 μM (d) or 400 μM (h) Ca^{2+}. Pre-incubation of the medium for 3 min in presence of 400 μM H_2O_2 (e), 400 μM H_2O_2+20 μM Fe^{2+} (f), 100 μM xanthine+70 mU/ml xanthine oxidase (g) accelerated the swelling induced by 100 μM Ca^{2+} (d). 400 μM Ca^{2+} (h) induced the maximal degree of swelling. The data shown are typical of three such experiments.

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[21]. It should be noted that longer exposure of mitochondria (30 min) showed mitochondrial membrane lipid peroxidation that can explain the alterations of the respiratory chain complexes as observed by some authors after ischemia-reperfusion [22]. This delayed effect could be due to the protective effect induced by endogenous antioxidant compounds until they get exhausted.

Effect of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ on ATP synthesis

The effect of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ on State 3 respiration may result from a direct effect on F1F0-ATPase or may be the consequence of the inhibition of ADP flux across ANT. Figure 3 shows that neither H_2O_2 nor $O_2^{\bullet -}$ altered F1F0-ATPase activity. Only a slight decrease could be observed in the presence of $HO^{\bullet}$ (10%), but this effect was marginally significant and could not explain the strong inhibition of State 3 respiration rate. This absence of effect on F1F0-ATPase is in agreement with those reported for H_2O_2 on rat heart mitochondria [23], on cardiomyocytes [24] or for *t*-butyl hydroperoxide on liver mitochondria [25].

Similarly, a 3 min incubation of mitochondria in the presence of 400 μM H_2O_2 did not modify ANT activity, but $HO^{\bullet}$ and $O_2^{\bullet -}$ inhibited the activity of the carrier, the effect being more marked in the presence of $O_2^{\bullet -}$ (Figure 3). This inhibition of ANT may proceed via the oxidation of critical amino acids, possibly the sulphydryl residue of cysteine, which has been described on ANT isolated from heart mitochondria [26]. It could contribute to the decrease in State 3 respiration rate promoted by $O_2^{\bullet -}$ and $HO^{\bullet}$ and could be particularly relevant in pathological

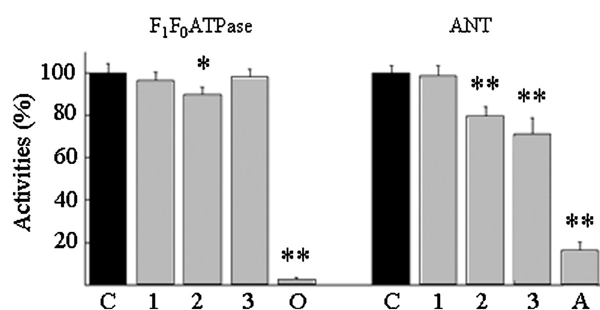

Figure 3. Effect of ROS on ATP synthesis. Heart mitochondria (0.4 mg/ml) were incubated for 3 min in the absence (control, C) or in the presence of either 1: 400 μM H_2O_2; 2: 400 μM H_2O_2+20 μM Fe^{2+} ($HO^{\bullet}$) or 3: 100 μM xanthine+70 mU/ml xanthine oxidase. ROS effect was compared to that of 1 μM oligomycin (O), a specific inhibitor of F1F0-ATPase, and to that of atractyloside (A, 10 μM), a specific inhibitor of ANT. Data are expressed as percentage of their respective control values and represent means ±SD of five experiments performed in triplicate. Control values were 4 nmol Pi/mg protein/min and 1.7 nmol ^{14}C-ADP/mg protein/min for F1F0-ATPase and ANT activities, respectively. *$p<0.05$; **$p<0.01$.

situations where a high activity of F1F0-ATPase is needed to replenish ATP pools. However, this slight ANT inhibition cannot explain the extent of inhibition of respiration, as a strong inhibition of ANT (at least 50%) is required to significantly decrease State 3 respiration rate [27].

Effect of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ on the mitochondrial respiratory chain

We then analysed effect of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ on oxygen consumption on the respiratory chain when the demand of oxygen was increased by the presence of an uncoupling agent, carbonyl cyanide m-chlorophenylhydrazone (CCCP), which removed the contribution of the phosphorylation system. An inhibition of the respiratory chain may not be observable when mitochondria respire during State 4. These three species decreased the extent by which CCCP stimulated mitochondrial respiration, indicating that they limited the activity of the electron transfer chain (Table III). Therefore, we investigated

Table III. ROS decreased the effects of CCCP on mitochondrial oxygen consumption. Mitochondria (0.4 mg/ml) were incubated without (control) or with either H_2O_2 (400 μM), H_2O_2 (400 μM)+Fe^{2+} (20 μM) or xanthine (100 μM)+xanthine oxidase (70 mU/ml). Oxygen consumption was started by pyruvate/malate (PM, 10 mM) and was accelerated by addition of CCCP (10 μM). Data represent means±SD of five experiments performed in triplicate.

	PM	+CCCP (nmol O_2/min/mg protein)	CCCP/PM
Control	118±23	772±60	6.54±0.27
H_2O_2 (400 μM)	96±24	190±62***	1.98±0.57***
$HO^{\bullet}$	106±12	130±26***	1.23±0.38***
$O_2^{\bullet -}$	137±24	338±80***	2.47±0.41***

***$p<0.001$ v respective control values.

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Table IV. Effect of ROS on mitochondrial electron transport complex activities. Mitochondria (0.4 mg/ml) were incubated with pyruvate/malate (10/10 mM) for 3 min at 37°C without (control) or with either H_2O_2 (400 μM), H_2O_2 (400 μM) + Fe^{2+} (20 μM) generating $HO^{\bullet}$ or xanthine (100 μM) + xanthine oxidase (70 mU/ml) generating $O_2^{\bullet -}$ and complex activities (expressed in nmoles/min/mg protein) were measured (see Materials and methods). As inhibiting control value, specific inhibitors were used alone (in absence of ROS) for each complex, i.e. rotenone (1 μM), malonate (10 mM), antimycin A (10 μM) and KCN (10 mM) for complexes I, II, III and IV, respectively. Data are means ± SD of five experiments performed in triplicate.

Complexes	Control	Specific inhibitors	H_2O_2	$HO^{\bullet}$	$O_2^{\bullet -}$
I	69.0 ± 8.80	7.60 ± 0.80	77.2 ± 8.20	69.5 ± 5.00	86.3 ± 9.70
II	86.2 ± 4.00	0.82 ± 0.06	86.3 ± 1.40	85.4 ± 3.50	88.0 ± 2.30
III	2360 ± 130	790 ± 140	2360 ± 40	2280 ± 60	2220 ± 80
IV	2481 ± 182	58 ± 11	2424 ± 116	2375 ± 133	2526 ± 210

their effect on mitochondrial respiratory complexes. As shown in Table IV, the activities of complexes I, II, III and IV of the electron transport chain were not affected by the treatment of mitochondria with these species under the conditions of these experiments.

Effect of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ on NAD(P)H production

When the electron transfer chain is stimulated (by ADP or by an uncoupler), the limitation of its activity may be due to a lack of reducing substrates. Therefore, we determined mitochondrial NAD(P)H production by spectrofluorimetry. It should be noted that the Krebs cycle produces sufficient NADH to sustain basal respiration (State 4 not affected by $O_2^{\bullet -}$, H_2O_2 or $HO^{\bullet}$), but is unable to provide enough NADH when the demand increased (during either ATP production or uncoupling).

Figure 4 shows that addition of H_2O_2 fully oxidized NAD(P)H in mitochondria energized with mixed pyruvate/malate substrates. The addition of Fe^{2+} accelerated this phenomenon. By contrast, $O_2^{\bullet -}$ generation was less effective in promoting NAD(P)H decrease.

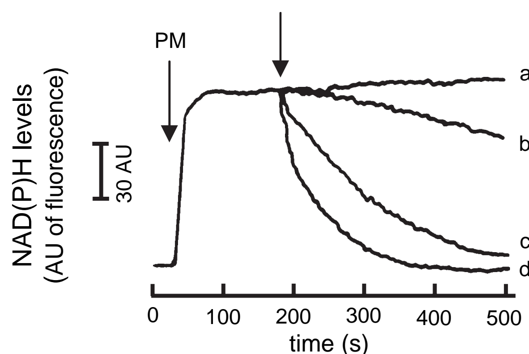

Figure 4. Inhibition of NADH production by ROS in energized heart mitochondria. NADH production was induced by 10 mM pyruvate/malate (PM, arrow) and 3 min later (arrow) 400 μM H_2O_2 (c), 400 μM H_2O_2 + 20 μM Fe^{2+} ($HO^{\bullet}$, d) or 100 μM xanthine + 70 mU/ml xanthine oxidase ($O_2^{\bullet -}$, b) was added. Curve a: no addition. Changes of NADH levels were expressed in arbitrary units (AU) of fluorescence. 30 AU correspond to 1 μM NADH. The calibration of the assay was performed by adding known amounts of NADH. The data shown are typical of three such experiments.

Mitochondria were then incubated for 3 min in the presence of both each species and rotenone to block NADH consumption by complex I. Pyruvate/malate was added to the medium to generate NADH. Under these conditions, $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ slightly decreased the rate, but did not affect total NADH production (Figure 5A). When succinate or malate was used as substrates no effect of $O_2^{\bullet -}$, H_2O_2 or $HO^{\bullet}$ could be observed (not shown). This indicates that the enzymatic systems inhibited by $O_2^{\bullet -}$, H_2O_2 or $HO^{\bullet}$ act upstream to succinate in the Krebs cycle. When pyruvate was used as a substrate, the rate of NADH generation was partially inhibited by H_2O_2 and $HO^{\bullet}$ and totally abolished by $O_2^{\bullet -}$ as observed with fluorocitrate, a specific inhibitor of aconitase (Figure 5B). $O_2^{\bullet -}$ caused the same total NADH inhibition with citrate as a substrate, whereas H_2O_2 and $HO^{\bullet}$ had no effect (Figure 5C). Conversely, when the Krebs cycle was energized by alpha-ketoglutarate in the presence of malonate to block succinate dehydrogenase, a strong inhibition was obtained in the presence of H_2O_2 and $HO^{\bullet}$, and $O_2^{\bullet -}$ was ineffective (Figure 5D).

These data revealed differential effects of each species. $O_2^{\bullet -}$ blocked the citrate-dependent NADH production mediated by aconitase, but did not affect alpha-ketoglutarate dehydrogenase, confirming that aconitase is a major target of this anion in heart mitochondria [23]. Conversely, H_2O_2 did not depress aconitase activity, but strongly inactivated alpha-ketoglutarate dehydrogenase. The addition of Fe^{2+} to the medium did not modify the effect of H_2O_2, indicating that $HO^{\bullet}$ generation did not reinforce the effect of H_2O_2. These data differ from those of Bulteau et al. [28], who reported a partial inactivation of aconitase by H_2O_2; however, they measured the activity of the enzyme after solubilization and isolation, while we evaluated its activity by measuring production of NADH in intact mitochondria. Our study also allows us to approach a controversial issue [17], which is the diffusion of $O_2^{\bullet -}$ across membranes. Our results indicated that $O_2^{\bullet -}$ produced by xanthine + xanthine oxidase outside the mitochondria is able to elicit matrix

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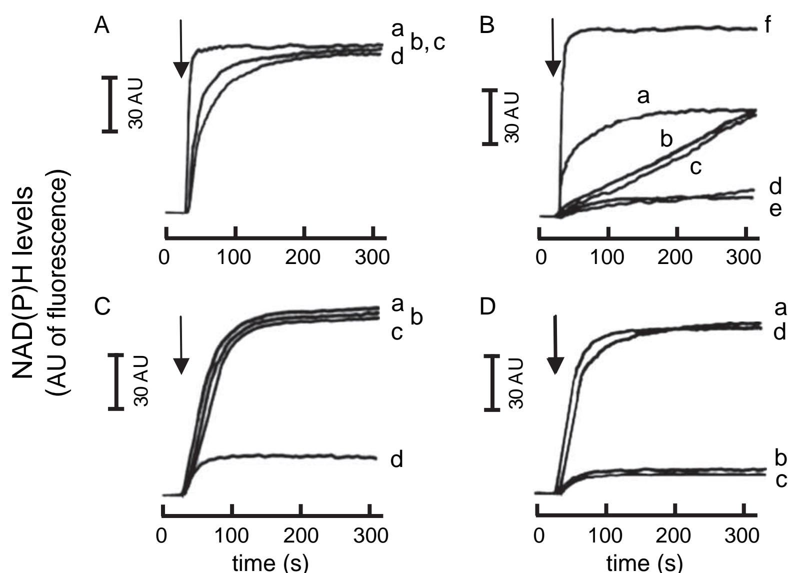

Figure 5. ROS differentially inhibited the production of NADH at the level of the Krebs cycle in heart mitochondria. Mitochondria (0.4 mg/ml) were pre-incubated for 3 min in the absence (control, a) or in the presence of either 400 µM H_2O_2 (b), 400 µM H_2O_2 + 20 µM Fe^{2+} ($HO^{\bullet}$, c) or 100 µM xanthine + 70 mU/ml xanthine oxidase ($O_2^{\bullet -}$, d) and then NADH production was induced by the addition of different substrates (arrow). In all experiments, rotenone (2 µM) was added to the medium to inhibit complex I and thus NADH consumption by the mitochondrial respiratory chain. (A) NADH production was induced by 10 mM pyruvate/malate. (B) NADH production was induced by 10 mM pyruvate. Curve e: effect of 1 mM fluorocitrate. For comparison, curve F showed the generation of NAD(P)H induced by 10 mM pyruvate/malate. (C) NADH production was induced by 10 mM citrate. (D) NADH production was induced by 10 mM alpha-ketoglutarate. Changes of NAD(P)H levels were expressed in arbitrary units (AU) of fluorescence. 30 AU correspond to 1 µM NADH. The calibration of the assay was performed by adding known amounts of NADH. The data shown are typical of three such experiments.

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alterations and thus cross the outer and the inner mitochondrial membranes. One would then predict that the transformation of $O_2^{\bullet -}$ to H_2O_2 by superoxide dismutase and xanthine + xanthine oxidase would only increase the intra-mitochondrial concentration of H_2O_2. However, xanthine + xanthine oxidase did not promote any inhibition of alpha-ketoglutarate dehydrogenase (the target of hydrogen peroxide), but instead induced a complete aconitase inhibition, which was not observed with H_2O_2 under our experimental conditions. This suggests that $O_2^{\bullet -}$ permeates mitochondrial membrane as suggested previously [29,30].

Taken together our data show that the over-production of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ leads to the alteration of ATP synthesis in heart mitochondria by acting specifically on different mitochondrial targets, mainly NADH production. In myocardial ischemia-reperfusion, reperfusion is associated with a burst of ROS which can overwhelm the endogenous antioxidant systems and contributes to the deleterious effect on mitochondria. As the balance between the over-production of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ in these pathological conditions is unknown, it should be interesting to protect mitochondria to use antioxidant agents possessing a broad spectrum of action rather than molecules acting on a particular species. Such agents are rare and, in addition to the difficulty of delivering drugs to mitochondria, this can explain the inefficacy of antioxidant treatment during cardiac ischemia [31].

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