Oxidative damage to mitochondria is mediated by the Ca^{2+} -dependent inner-membrane permeability transition

Naoshi TAKEYAMA,* Nobuaki MATSUO and Takaya TANAKA

Department of Emergency and Critical Care Medicine, Kansai Medical University, Moriguchi, Osaka, Japan

The ability of O_2 metabolites derived from the xanthine–xanthine oxidase system to inhibit mitochondrial function was examined using freshly isolated rat liver mitochondria. Under 2,4-dinitrophenol-uncoupled conditions, mitochondria exposed to free radicals exhibited a significant decrease in O_2 consumption supported by NAD^+ -linked substrates, but showed almost no change in O_2 consumption in the presence of succinate and ascorbate. Oxidative stress caused the loss of intramitochondrial nicotinamide nucleotides, and addition of NAD^+ fully prevented any fall in O_2 consumption with NAD^+ -linked substrates. The activity of electron-transfer complex I (NADH oxidase and NADH–cytochrome *c* oxidoreductase) and the energy-dependent reduction of NAD^+ by succinate were unaltered by oxidative stress. Exposure to free radicals also had an uncoupling effect at all three coupling sites. The degree of mitochondrial swelling was

closely correlated with the inhibition of State-3 oxidation of site-I substrates and with the increase in State-4 oxidation of succinate. The immunosuppressive agent cyclosporin A completely prevented the mitochondrial damage induced by oxygen free radicals (swelling, Ca^{2+} release, sucrose trapping, uncoupling and selective inhibition of the mitochondrial respiration of site-I substrates). The same protective effect was found when Ca^{2+} cycling was prevented, either by chelating Ca^{2+} with EGTA or by inhibiting Ca^{2+} reuptake with Ruthenium Red. These findings suggest that the deleterious effect of free radicals on mitochondria in the present experimental system was triggered by the cyclosporin A-sensitive and Ca^{2+} -dependent membrane transition, and not by direct impairment of the mitochondrial inner-membrane enzymes.

INTRODUCTION

Oxygen-derived free radicals have been implicated in the pathophysiology of cell injury and death [1]. Glutathione depletion [1,2], peroxidation of lipid membranes [3,4], an increase in the cytosolic Ca^{2+} concentration [1,5] and oxidation of protein thiol groups [1,2] have all been suggested to be important factors in cell damage due to oxidative stress. However, despite a growing body of knowledge on this subject, far more information is still needed regarding the essential mechanisms culminating in lethal oxidative cell injury.

One relatively prominent feature of irreversible cell damage caused by oxidative stress is morphological and functional changes in the mitochondria [4,6]. In this context, several studies have investigated the oxidative inactivation of isolated mitochondria after exposure to various toxins and enzymically produced free radicals [3,7–13]. These studies have demonstrated that both mitochondrial matrix enzymes and membrane-bound enzymes are affected by oxidative stress along with membrane damage due to the peroxidation of phospholipids. In addition to this type of irreversible direct oxidative inactivation of mitochondrial components, it is known that mitochondria can release matrix Ca^{2+} on oxidation of internal nicotinamide nucleotides [14–16] and membrane protein thiols [17]. Continuous and excessive pro-oxidant-induced Ca^{2+} release and active Ca^{2+} reuptake via the uniporter can lead to energy dissipation via Ca^{2+} cycling [18], which is likely to bring about a decrease in the mitochondrial membrane potential [7]. Thus it is possible that the adverse effects of oxidative stress on the mitochondria may be elicited by both the direct inactivation of mitochondrial

components and by Ca^{2+} cycling or changes in mitochondrial inner-membrane permeability [19,20].

In this study, to investigate the sequence of events occurring during exposure of mitochondria to oxygen-derived free radicals, we assessed both the function of the mitochondrial respiratory chain (including inner-membrane enzymes) and the degree of Ca^{2+} cycling of alteration in mitochondrial inner-membrane permeability. Our data indicate that the deleterious effect of free radicals on mitochondria is largely due to the opening of the cyclosporin A (CyA)-sensitive and Ca^{2+} -operated non-specific pore and not by direct damage to the mitochondrial inner-membrane enzymes.

MATERIALS AND METHODS

Preparation of mitochondria

Male Wistar rats weighing 180–250 g were used in all experiments. Liver mitochondria were prepared as described previously [21]. EGTA (0.5 mM final concentration) was present during the low-speed centrifugation (400 g) and the first high-speed centrifugation (7000 g), but was excluded during homogenization and washing. Washed mitochondria were suspended in 0.25 M sucrose plus 5 mM Mops (pH 7.4) at a final protein concentration of 30 mg/ml, and were kept on ice and used within 3–5 h. The calcium content in this preparation was 6–8 nmol/mg of protein, as determined by uncoupler-induced release using carbonyl cyanide *p*-fluoromethoxyphenylhydrazone. The protein content of the suspension was determined with a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) using bovine plasma γ -globulin as the standard [22].

Abbreviations used: XO, xanthine oxidase; RuR, Ruthenium Red; CyA, cyclosporin A; DNP, 2,4-dinitrophenol; RCR, respiratory control ratio; SOD, superoxide dismutase.

* To whom correspondence should be addressed.

Xanthine oxidase (XO) treatment of isolated mitochondria

Routinely isolated mitochondria (1 mg/ml) were preincubated for 10 min at 30 °C with 3 munits/ml buttermilk XO (EC 1.1.3.22; Wako Pure Chemicals, Tokyo, Japan) and 200 μ M xanthine in the mitochondrial respiration assay medium (see below). After incubation, free-radical production was terminated by addition of the termination reagents, which comprised 100 μ M oxypurinol, 40 μ g/ml bovine erythrocyte superoxide dismutase (SOD; EC 1.15.1.1; Sigma, St. Louis, MO, U.S.A.) and 40 μ g/ml bovine liver catalase (EC 1.11.1.6; Boehringer, Mannheim, Germany). The concentrations of catalase and SOD used in this mixture totally inhibited hydrogen peroxide formation and cytochrome *c* reduction respectively. The XO activity was determined spectrophotometrically by measuring the conversion of 200 μ M xanthine into urate at 30 °C by monitoring the increase in A_{295} using a millimolar absorption coefficient of 12.3 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ [23]. One unit of XO activity was defined as the amount of enzyme that produced urate at 1 $\mu\text{mol}/\text{min}$ at 30 °C. In several experiments, the XO concentration was varied as specified in the Results section, but the xanthine concentration was always fixed at 200 μ M. In some experiments, catalase, SOD, CyA, EGTA, Ruthenium Red (RuR) and oxypurinol were also included in the preincubation mixture. CyA was a generous gift of Sandoz Pharma AG (Basel, Switzerland). It was dissolved in ethanol at 0.3 mM before addition and the total ethanol concentration was kept below 0.5% by volume. Generation of O_2^- by the xanthine/XO system was measured by monitoring the SOD-inhibitable reduction of ferricytochrome *c* at 550 nm [24]. XO subjected to heat inactivation at 60–70 °C for 10 min was used in control experiments. CyA, RuR and EGTA all had no effect on the generation of O_2^- by the xanthine/XO system used in this study.

Mitochondrial respiration

O_2 consumption by isolated mitochondria exposed to free radicals was measured with a Clark electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) at 30 °C in a temperature-controlled 2 ml chamber with a magnetic flea [21]. The incubation medium consisted of air-saturated 130 mM KCl, 5 mM MgCl_2 , 2 mM KH_2PO_4 , 10 mM Hepes and 2 mg/ml essentially fatty acid-free BSA (pH 7.2), and contained approx. 1 mg/ml mitochondrial protein. Cytochrome *c* (50 μ M) was added after the termination of exposure to free radicals. In some experiments, 1 mM NAD^+ was also added simultaneously with the termination reagents. Then various substrates (10 mM pyruvate plus 2.5 mM L-malate, 20 mM β -hydroxybutyrate, 10 mM succinate plus 2 μ M rotenone or 5 mM ascorbate, plus 0.2 mM *NNNN*-tetramethyl-*p*-phenylenediamine) were added, and State-4 respiration was measured. To initiate State-3 conditions (ADP-stimulated oxidation), 400 nmol of ADP was added. Uncoupled respiration was assessed in the presence of 40 μ M 2,4-dinitrophenol (DNP). The respiratory control ratio (RCR) was calculated as the ratio of State-3 to State-4 O_2 consumption [21].

Enzyme activities and energy-linked reduction of NAD^+ by succinate

Mitochondria were exposed to free radicals for 10 min and the reaction was terminated as described above. Then the mitochondria were rapidly cooled to 4 °C and sonicated (model W185; Branson Sonic Power Co., Danbury, CT, U.S.A.) with three 15 s bursts in an ice-bath. Submitochondrial particles

were subsequently prepared for the assay of the energy-linked reduction of NAD^+ by succinate [25].

Enzyme activity assays and determination of the reversal of electron transport were performed at 30 °C in a final volume of 3 ml using a UV-2100 spectrophotometer (Shimadzu, Kyoto, Japan). NADH oxidase activity was determined by measuring the rotenone-sensitive initial rate of NADH oxidation at 340 nm [26]. Measurements were performed in 100 mM potassium phosphate buffer (pH 7.4) with 160 μ M NADH, and the reaction was initiated by adding 50–200 μ g of sonicated mitochondrial protein. Complex I and III activities were determined by measuring the NADH-dependent reduction of cytochrome *c* from the increase in A_{550} [27]. The assay mixture contained 20 mM phosphate buffer (pH 8.0) with 2 mM KCN, 50 μ M cytochrome *c*, 10 μ M EDTA and 1 mM NADH, and the reaction was initiated by adding 20–70 μ g of sonicated mitochondrial protein.

Energy-linked succinate-mediated reduction of NAD^+ was determined essentially by the method of Ernster and Lee [28]. The reaction mixture contained 180 mM sucrose, 50 mM Tris/HCl (pH 7.4), 6 mM MgSO_4 , 2 mM KCN, 1 mM EDTA, 5 mM succinate, 1 mM NAD^+ and 0.3–1 mg of submitochondrial particle protein. The reaction was initiated by adding 3 mM ATP, and the reduction of NAD^+ was followed by measuring the increase in A_{340} . The increase in A_{340} was reversed by addition of pyruvate and lactate dehydrogenase on completion of the reaction, confirming that it was due to NADH formation.

Measurement of nicotinamide nucleotides

Mitochondria were exposed to free radicals for 10 min and the reaction was terminated as described above. Then the mitochondria were rapidly cooled to 4 °C, and NAD^+ and NADH were extracted by acid and alkaline extraction respectively. The mitochondrial content of nicotinamide nucleotides was then assayed by the method of Williamson and Corkey [29].

Measurement of mitochondrial swelling and Ca^{2+} flux

Swelling was initiated by addition of various concentrations of XO to the 3 ml reaction mixture described under 'XO treatment of isolated mitochondria', and the decrease in A_{540} was recorded continuously at 30 °C.

Movement of Ca^{2+} across the inner mitochondrial membrane was followed spectrophotometrically at 685–675 nm in the presence of 50 μ M Arsenazo III, using a dual-wavelength spectrophotometer (model 557, Hitachi Ltd., Tokyo, Japan) [15].

Determination of sucrose entry into the mitochondria

Sucrose entry and entrapment in the matrix was measured by the radioisotope technique of Al-Nasser and Crompton [30]. Mitochondria were exposed to free radicals in accordance with the standard procedure in the presence of [^{14}C]sucrose at 0.6 $\mu\text{Ci}/\text{ml}$. Samples (200 μl) were withdrawn at the times indicated in the Figures, incubated for 30 s with ice-cold mitochondrial homogenizing buffer (which also contained 5 mM EGTA, 1.5 μ M CyA and 100 μ M oxypurinol), filtered through Millipore filters (0.45 μm pore size) under a vacuum, and rinsed twice with the same buffer. The radioactivity remaining on the filters was determined by liquid-scintillation spectrometry.

Statistical analysis

All data are presented as means $\pm$ S.D. Significant differences among group means were evaluated by one-way analysis of

Table 1 Effect of oxygen-derived free radicals on rat hepatic mitochondrial respiration

Mitochondria were preincubated with xanthine (X; 200 μ M) plus XO (3–6 munits/ml) in the presence or absence of various agents for 10 min at 30 °C. Catalase (CAT; 40 μ g/ml), SOD (40 μ g/ml) or oxypurinol (OXY; 100 μ M) were added 30 s before XO. After free-radical production was terminated, O₂ consumption was measured as described in the Materials and methods section. Values are means $\pm$ S.D. from seven to eight experiments. * P < 0.0001, † P < 0.001, ‡ P < 0.01 compared with the control. § P < 0.0001 compared with X + XO (6 munits/ml).

Treatment	Condition	O ₂ consumption (nmol/min per mg of protein)			
		Pyruvate *	β -Hydroxybutyrate	Succinate	Ascorbate
Control	State 4	13.1 $\pm$ 2.5	15.1 $\pm$ 1.4	30.8 $\pm$ 3.7	50.3 $\pm$ 5.8
	State 3	34.7 $\pm$ 6.7	61.6 $\pm$ 6.5	120.7 $\pm$ 10.6	139.6 $\pm$ 21.6
	RCR	2.7 $\pm$ 0.6	4.1 $\pm$ 0.5	4.0 $\pm$ 0.5	2.8 $\pm$ 0.3
X + XO (3 munits/ml)	State 4	11.5 $\pm$ 1.6	14.7 $\pm$ 1.8	40.9 $\pm$ 5.0	62.8 $\pm$ 7.9
	State 3	22.8 $\pm$ 3.2†	45.0 $\pm$ 5.5*	123.9 $\pm$ 14.2	140.6 $\pm$ 12.2
	RCR	2.0 $\pm$ 0.1‡	3.1 $\pm$ 0.4*	3.1 $\pm$ 0.3†	2.3 $\pm$ 0.2‡
X + XO (6 munits/ml)	State 4	10.7 $\pm$ 1.7	13.5 $\pm$ 1.7	114.9 $\pm$ 10.3*	141.1 $\pm$ 13.7*
	State 3	10.9 $\pm$ 1.6*	13.7 $\pm$ 1.8*	115.7 $\pm$ 10.7	142.9 $\pm$ 13.6
	RCR	1.02 $\pm$ 0.04*	1.02 $\pm$ 0.02*	1.01 $\pm$ 0.01*	1.01 $\pm$ 0.01*
X + XO (6 munits/ml) + CAT	State 4	13.5 $\pm$ 1.4	15.3 $\pm$ 1.7	30.1 $\pm$ 5.9§	49.3 $\pm$ 6.5§
	State 3	38.2 $\pm$ 3.8§	65.1 $\pm$ 10.7§	125.3 $\pm$ 37.4	141.3 $\pm$ 11.7
	RCR	2.8 $\pm$ 0.3§	4.2 $\pm$ 0.5§	4.1 $\pm$ 0.5§	2.92 $\pm$ 0.46§
X + XO (6 munits/ml) + OXY	State 4	10.1 $\pm$ 1.7	14.3 $\pm$ 2.3	29.7 $\pm$ 4.7§	52.3 $\pm$ 7.8§
	State 3	29.9 $\pm$ 7.8§	58.8 $\pm$ 13.7§	123.0 $\pm$ 24.6	145.5 $\pm$ 11.8
	RCR	2.9 $\pm$ 0.4§	4.1 $\pm$ 0.6§	4.1 $\pm$ 0.8§	2.8 $\pm$ 0.5§
X + XO (6 munits/ml) + SOD	State 4	10.3 $\pm$ 1.6	13.4 $\pm$ 1.7	121.7 $\pm$ 12.1	135.3 $\pm$ 15.1
	State 3	10.9 $\pm$ 1.6	13.8 $\pm$ 1.0	123.1 $\pm$ 13.3	136.6 $\pm$ 15.2
	RCR	1.06 $\pm$ 0.07	1.03 $\pm$ 0.04	1.01 $\pm$ 0.02	1.01 $\pm$ 0.01

Table 2 Effect of oxygen-derived free radicals on uncoupled hepatic mitochondrial respiration

The treatment of mitochondria and additions were as described in the legend to Table 1 in the presence of 40 μ M DNP. NAD⁺ (1 mM) was added simultaneously with the termination reagents. Values are means $\pm$ S.D. from seven to eight experiments. * P < 0.0001 compared with the control. † P < 0.0001 compared with X + XO (6 munits/ml). For other abbreviations, see Table 1.

Treatment	O ₂ consumption (nmol/min per mg of protein)			
	Pyruvate	β -Hydroxybutyrate	Succinate	Ascorbate
Control	50.4 $\pm$ 9.5	77.8 $\pm$ 8.0	142.8 $\pm$ 12.7	163.0 $\pm$ 18.4
X + XO (3 munits/ml)	23.0 $\pm$ 3.4*	52.5 $\pm$ 7.9*	142.1 $\pm$ 10.8	165.0 $\pm$ 13.8
X + XO (6 munits/ml)	11.1 $\pm$ 1.6*	14.0 $\pm$ 1.9*	145.1 $\pm$ 11.3	167.1 $\pm$ 10.8
X + XO (6 munits/ml) + SOD	11.8 $\pm$ 2.2	14.0 $\pm$ 1.5	144.5 $\pm$ 13.0	167.5 $\pm$ 15.5
X + XO (6 munits/ml) + CAT	49.8 $\pm$ 6.8†	76.8 $\pm$ 9.2†	141.0 $\pm$ 10.7	166.4 $\pm$ 12.8
X + XO (6 munits/ml) + OXY	49.8 $\pm$ 8.3†	72.5 $\pm$ 10.4†	146.4 $\pm$ 23.9	156.9 $\pm$ 12.1
X + XO (6 munits/ml) + NAD ⁺	47.2 $\pm$ 4.5†	70.3 $\pm$ 3.7†	—	—

variance. If the analysis gave a significant result (P value < 0.05), individual treatment means were further compared by Scheffé's test.

RESULTS

Parameters of mitochondrial respiration

Tables 1 and 2 show some characteristic features of the effect of xanthine plus XO on isolated rat liver mitochondria. For all substrates tested, exposure to various concentrations of XO and 0.2 mM xanthine caused a concentration-dependent decrease in the RCR. There was a 62 $\pm$ 6 % fall in the RCR for pyruvate on preincubation with XO (6 munits/ml) plus xanthine. State-4 respiration was increased dose-dependently, except in the case of NAD⁺-linked substrates. State-4 respiration of pyruvate and β -hydroxybutyrate did not change with the increase in XO. State-3 respiration of pyruvate and β -hydroxybutyrate decreased with the increase in XO, whereas respiration of succinate and ascorbate

were not altered. Exposure to either XO or xanthine alone did not alter State-4 respiration, State-3 respiration or the RCR (results not shown). The effects of preincubation with various concentrations of XO and 0.2 mM xanthine on DNP-induced uncoupled respiration are shown in Table 2. When pyruvate plus malate and β -hydroxybutyrate were used as the substrates, there was a concentration-dependent reduction in the rate of uncoupled respiration, suggesting a major defect in the electron-transport chain. In contrast, with succinate and ascorbate as the substrates, there was no reduction in uncoupled respiration at all concentrations of XO tested. Addition of NAD⁺ (1 mM) fully restored the inhibited respiration of pyruvate plus malate and β -hydroxybutyrate.

The presence of catalase or oxypurinol in the reaction mixture during the incubation with xanthine plus XO completely prevented the occurrence of mitochondrial damage (Tables 1 and 2). On the other hand, SOD had no protective effect. This strongly suggests that extramitochondrial superoxide radicals are not directly responsible for the mitochondrial damage induced by

Table 3 Mitochondrial content of nicotinamide nucleotides after exposure to xanthine plus XO

Mitochondria were incubated as described in Table 1, and then centrifuged and extracted as described in the Materials and methods section. Results are the means $\pm$ S.D. of seven experiments. * $P < 0.0001$ compared with the control. Abbreviations are as in Table 1.

Treatment	Nicotinamide nucleotides (nmol/mg of protein)	
	NAD ⁺	NADH
Control	3.60 $\pm$ 0.36	0.89 $\pm$ 0.09
X + XO (3 munits/ml)	1.65 $\pm$ 0.28*	0.19 $\pm$ 0.13*
X + XO (6 munits/ml)	0.98 $\pm$ 0.19*	0.09 $\pm$ 0.02*
X + XO (10 munits/ml)	0.89 $\pm$ 0.09*	0.09 $\pm$ 0.03*

xanthine plus XO, whereas extramitochondrial hydrogen peroxide and/or by-products such as hydroxyl radicals may contribute to such damage.

Enzyme activities and ATP-dependent reversal of electron flow

After exposure to oxygen-derived free radicals, there was no marked change in the activity of either NADH oxidase or NADH-cytochrome *c* oxidoreductase. After preincubation with 0.2 mM xanthine plus XO (inactivated, or 3, 6 and 10 munits/ml) the activity of NADH oxidase and NADH-cytochrome *c* oxidoreductase was respectively 181 $\pm$ 17.7 and 160 $\pm$ 15.9, 193 $\pm$ 16.5 and 164 $\pm$ 17.5, 199 $\pm$ 14.0 and 158 $\pm$ 21.0 and 199 $\pm$ 20.7 and 159 $\pm$ 19.5 nmol/min per mg of protein (means $\pm$ S.D. for eight mitochondrial preparations). Energy-dependent reduction of NAD⁺ by succinate in submitochondrial particles was also unaffected (reduction of 72.9 $\pm$ 6.9, 75.3 $\pm$ 9.9, 71.8 $\pm$ 6.6, and 77.1 $\pm$ 12.9 nmol of NAD⁺/min per mg of protein after preincubation with 0.2 mM xanthine plus inactivated, 3, 6 and 10 munits/ml XO respectively).

Mitochondrial nicotinamide nucleotides

The mitochondrial content of nicotinamide nucleotides was measured under conditions similar to those used for the measurement of respiration and enzyme activities. It was found that a concentration-dependent depletion of mitochondrial nicotinamide nucleotides occurred after exposure to free radicals (Table 3). Because a proportional increase in the NAD⁺ content was not observed, the decrease in NADH was not simply explained by pro-oxidant-induced oxidation.

Mitochondrial swelling, sucrose entrapment and Ca²⁺ release

When mitochondria were exposed to free radicals under conditions similar to those described above, there was a decrease in light-scattering (A_{540}) caused by their swelling as well as an efflux of Ca²⁺ (Figure 1). As the concentration of XO decreased, the initiation of both Ca²⁺ release and mitochondrial swelling was delayed. Preincubation with catalase or oxypurinol completely blocked Ca²⁺ release and swelling.

Trapping of [¹⁴C]sucrose within the matrix space was used to determine the occurrence of pore formation (the inner membrane was made permeable and then resealed using EGTA and CyA). After exposure to oxygen-derived free radicals, [¹⁴C]sucrose trapping was observed (Figure 1), and it followed a similar pattern to that of mitochondrial swelling and Ca²⁺ release. Thus,

Figure 1 Effect of exposure of free radicals on Ca²⁺ retention, [¹⁴C]sucrose trapping and swelling of mitochondria

Mitochondria were incubated with 0.2 mM xanthine plus XO [10 munits/ml in (a), (d) and (e), 6 munits/ml in (b) and 3 munits/ml in (c)] as described in the Materials and methods section. Catalase (40 μ g/ml; d) and oxypurinol (100 μ M; e) were added 30 s before XO. A_{540} is the apparent absorbance of mitochondrial suspensions at 540 nm, and a downward deflection of the trace indicates mitochondrial swelling. $A_{685-675}$ is the absorbance of arsenazo III, an indicator of the extramitochondrial Ca²⁺ concentration at 685–675 nm. An upward deflection of this trace indicates an increase in the medium Ca²⁺ concentration. □, [¹⁴C]sucrose entry into the mitochondria (nmol/mg of protein). Values for [¹⁴C]sucrose entrapped are means for four determinations on separate mitochondrial preparations. (Standard deviations for the mean values were < 10%).

as the concentration of XO decreased, the initiation of [¹⁴C]sucrose entry was delayed, and no entry of labelled sucrose was observed in the presence of catalase or oxypurinol.

Correlations were analysed between mitochondrial swelling and respiratory function, evaluated as the RCR in the presence of succinate and State-3 respiration of pyruvate plus malate, which were characteristically damaged by free radicals as shown in Table 1. A strong correlation was observed between the extent of mitochondrial swelling and mitochondrial respiratory dysfunction (Figure 2).

Protective effect of RuR, CyA and EGTA

Figure 3 and Table 4 show the effects of CyA, RuR and EGTA

Figure 2 Correlations between mitochondrial swelling and mitochondrial function evaluated as the RCR ($\square$) in the presence of succinate ($r^2 = 0.92$; $P < 0.001$) and State-3 respiration of pyruvate plus malate ($\blacktriangledown$) ($r^2 = 0.93$; $P < 0.001$)

(which interfere with Ca^{2+} mobilization across the mitochondrial inner membrane) on mitochondrial swelling, [^{14}C]sucrose trapping and respiratory capacity. Addition of any of these reagents completely prevented the mitochondrial swelling and [^{14}C]sucrose trapping induced by XO plus xanthine (Figure 3), as well as the respiratory dysfunction (Table 4). In particular, CyA was a potent inhibitor of the mitochondrial swelling induced by oxygen-derived free radicals. Addition of CyA after mitochondrial swelling had commenced promptly inhibited further swelling (trace d in Figure 3).

Both RuR and EGTA can also prevent initial Ca^{2+} uptake from the medium as well as Ca^{2+} cycling. To investigate whether the effect of RuR was mediated by its inhibition of initial Ca^{2+} uptake or subsequent Ca^{2+} release followed by recycling, RuR was added at various intervals after initiation of mitochondrial incubation. Immediately after the mitochondria were added, a decrease in the Ca^{2+} content of the medium was observed as a result of mitochondrial uptake (traces a, b, c, d and f in Figure 4). Addition of RuR completely terminated further Ca^{2+} uptake, and slight Ca^{2+} efflux was subsequently observed. Although initial Ca^{2+} uptake from the medium varied in traces a–e (being largest in a and smallest in e), XO/xanthine-induced mitochondrial swelling was always completely blocked by RuR (traces A–E in Figure 4). These results indicated that the preventive effect of RuR on mitochondrial swelling was not mediated by

Figure 3 Effect of RuR, EGTA and CyA on the swelling (traces a to e) and [^{14}C]sucrose trapping (upper graph) of liver mitochondria in response to 0.2 mM xanthine plus XO (10 munits/ml)

XO was added 2 min after the addition to mitochondria to the sample cuvette. CyA (1.5 μM ; $\bullet$ and a), EGTA (0.5 mM; $\circ$ and b) or RuR (2 μM ; $\blacktriangledown$ and c) was added 30 s before XO. Trace d, 1.5 μM CyA was added as indicated. $\square$ and e, no further additions. Values for [^{14}C]sucrose entrapped are means for four determinations on separate mitochondrial preparations. (Standard deviations for the mean values were $< 10\%$).

Table 4 Effect of RuR, EGTA and CyA on inhibition of mitochondrial respiration by xanthine plus XO

Mitochondria were preincubated with xanthine (X; 200 μM) plus XO in the presence or absence of various agents for 10 min at 30 $^{\circ}\text{C}$. CyA (1.5 μM), EGTA (0.5 mM) or RuR (2 μM) was added 30 s before XO. After oxygen free-radical production was terminated O_2 consumption was measured as described in the Materials and methods section. All values are means $\pm$ S.D. of six to eight experiments. * $P < 0.0001$ compared with the control (X+inactivated XO). $\dagger P < 0.0001$ compared with X+XO (6 munits/ml).

Treatment	RCR			Uncoupled respiration of pyruvate (nmol of O_2 /min per mg of protein)
	Pyruvate	Succinate	Ascorbate	
X + XO (inactivated)	2.84 ± 0.30	3.92 ± 0.32	2.85 ± 0.28	50.5 ± 6.1
X + XO (6 munits/ml)	$1.02 \pm 0.02^*$	$1.01 \pm 0.02^*$	$1.01 \pm 0.01^*$	$10.2 \pm 2.5^*$
X + XO (6 munits/ml) + EGTA	$2.92 \pm 0.31^{\dagger}$	$4.03 \pm 0.59^{\dagger}$	$3.01 \pm 0.22^{\dagger}$	$51.3 \pm 5.3^{\dagger}$
X + XO (6 munits/ml) + RuR	$2.81 \pm 0.27^{\dagger}$	$4.31 \pm 0.43^{\dagger}$	$2.96 \pm 0.19^{\dagger}$	$49.4 \pm 4.9^{\dagger}$
X + XO (6 munits/ml) + CyA	$2.90 \pm 0.30^{\dagger}$	$3.92 \pm 0.42^{\dagger}$	$2.95 \pm 0.28^{\dagger}$	$48.6 \pm 3.7^{\dagger}$

Figure 4 Effect of RuR on swelling (traces A to F) and Ca^{2+} retention (traces a to f) of mitochondria induced by 0.2 mM xanthine plus XO (10 munits/ml)

The experiments were initiated by the addition of mitochondria (1 mg of protein/ml) to the basic medium. Then RuR was added 120 (traces a and A), 90 (traces b and B), 50 (traces c and C), 30 (traces d and D) and 0 s (traces e and E) after mitochondrial addition. RuR was absent for traces f and F. XO was added 2 min after mitochondrial addition in all traces.

inhibition of initial Ca^{2+} uptake, but rather by inhibition of Ca^{2+} cycling.

DISCUSSION

This study showed that oxygen-derived free radicals produced by XO plus xanthine caused mitochondrial damage such as uncoupling at all three coupling sites and the selective alteration of the mitochondrial respiration of site-I substrates. Selective susceptibility of the mitochondrial respiratory chain to inhibition by oxidative stress has been observed previously. Hillered and Ernster [10] studied rat brain mitochondria, Corbisier et al. [9] studied rat liver mitochondria and Malis and Bonventre [13] studied rat renal mitochondria exposed to oxygen free radicals; all their studies suggested that the site of inhibition was the NADH-oxidizing branch of the respiratory chain. Nitrofurantoin produces nitro anion radicals [31] and blocks the respiration of isolated mouse mitochondria by selective inhibition of the complex-I electron-transport system [8]. On the basis of these observations, it is generally assumed that the activity of NADH dehydrogenase is reduced by free radicals. Recently, Zhang et al. assessed the oxidative inactivation of mitochondrial electron-transport-chain components after exposure of bovine heart submitochondrial particles to O_2^- [12]. They demonstrated that O_2^- was a highly efficient inactivator of NADH dehydrogenase, NADH oxidase and ATPase, whereas succinate dehydrogenase and succinate oxidase were only inhibited to a minor degree [12].

In contrast with the assumption that oxidative stress inactivates the mitochondrial inner-membrane enzymes, in particular complex I of the electron-transport system, we proposed that the selective alterations of the mitochondrial respiratory chain caused by free radicals could be a consequence of the loss of NAD^+ and NADH after the development of mitochondrial inner-membrane permeability transition pore. The uncoupling effect of free radicals observed at three coupling sites of oxidative phosphorylation seems likely to cause collapse of the proton electrochemical gradient by destroying the membrane permeability barrier. Our hypothesis is supported by the following evidence observed in this study. (a) Addition of NAD^+ completely prevented the decrease in DNP-uncoupled respiration of pyruvate and β -hydroxybutyrate. (b) The activities of NADH oxidase and NADH-cytochrome *c* reductase were not affected in sonicated mitochondria exposed to various amounts of free radicals. (c) Reversed electron flow and ATPase activity assessed by the energy-dependent reduction of NAD^+ by succinate in submitochondrial particles were unaffected. (d) Accumulation of NADH, which would be observed after complex-I inhibition (i.e. rotenone or amytal treatment), was not seen in this study.

The development of mitochondrial swelling, Ca^{2+} efflux and sucrose entrapment demonstrated that the XO/xanthine system used in this study caused the mitochondrial membrane permeability transition due to CyA-sensitive pore opening. An increase in membrane permeability may lead to the efflux of NAD^+ and NADH from the mitochondria. In addition to such leakage, the hydrolysis of NAD^+ to nicotinamide and ADP-ribose by oxidative stress may be involved in the loss of intramitochondrial nicotinamide nucleotides [16]. The loss of nicotinamide nucleotides leads to selective alteration of the mitochondrial respiration of site-I substrates. This mechanism has long been recognized. In 1955, Hunter and Ford [32] demonstrated that swollen mitochondria induced by phosphate, arsenate, arsenite and various metal ions lost intramitochondrial nicotinamide nucleotides, and that NAD-dependent oxidation was affected as a consequence. In close agreement with our results, they showed that FAD-dependent succinate oxidation was not altered under the same conditions. A recent study by Al-Nasser and Crompton [30] also showed that Ca^{2+} -induced inner-membrane pore opening was associated with loss of nicotinamide nucleotide from the matrix, and that the resultant defective oxidation of site-I substrates could be normalized by addition of exogenous nicotinamide nucleotide.

The essential role of Ca^{2+} in the development of pro-oxidant-induced mitochondrial damage is demonstrated by the finding that chelation of Ca^{2+} with EGTA or blocking of mitochondrial Ca^{2+} uptake by RuR largely repaired the mitochondrial respiratory damage induced by free radicals. This finding agrees with previous studies suggesting that the matrix Ca^{2+} released by oxidative stress can be taken up again by the mitochondria (Ca^{2+} cycling) and that excessive Ca^{2+} cycling may be responsible for mitochondrial damage [7,33]. When Ca^{2+} cycling is prevented, the mitochondrial damage induced by *t*-butyl hydroperoxide [33] and ferric iron complex [7] (swelling, K^+ release and loss of membrane potential) is almost completely reversed.

Ca^{2+} -dependent membrane permeability transition pore and the accompanying functional and structural alterations of the mitochondria may be involved in the pathogenesis of cell injury and death during oxidative stress. Crompton and co-workers [19,20] assumed that mitochondrial membrane transition triggered by oxidative stress played a part in the pathogenesis of ischaemia/reperfusion injury. Excessive cellular uptake of Ca^{2+} and mitochondrial Ca^{2+} overload, low ATP levels and the high P_i during ischaemia may all potentiate pore opening [19]. The

protective effect of the immunosuppressant CyA, a potent inhibitor of pore opening [34,35], against hypoxic [36] and energy-deprived [19] injury of cardiac myocytes, and against *t*-butyl hydroperoxide-induced injury of hepatocytes in a high- Ca^{2+} medium [37], may be explained by inhibition of the pro-oxidant-mediated membrane transition and inhibition of the resultant collapse of the mitochondrial inner-membrane potential.

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