Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax

Anatoly A. Starkov, Brian M. Polster and Gary Fiskum

Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland, USA

Abstract

Abnormal accumulation of Ca^{2+} and exposure to pro-apoptotic proteins, such as Bax, is believed to stimulate mitochondrial generation of reactive oxygen species (ROS) and contribute to neural cell death during acute ischemic and traumatic brain injury, and in neurodegenerative diseases, e.g. Parkinson's disease. However, the mechanism by which Ca^{2+} or apoptotic proteins stimulate mitochondrial ROS production is unclear. We used a sensitive fluorescent probe to compare the effects of Ca^{2+} on H₂O₂ emission by isolated rat brain mitochondria in the presence of physiological concentrations of ATP and Mg²⁺ and different respiratory substrates. In the absence of respiratory chain inhibitors, Ca^{2+} suppressed H₂O₂ generation and reduced the membrane potential of mitochondria oxidizing succinate, or glutamate plus malate. In the presence of the respiratory chain Complex I inhibitor rotenone, accumulation

Mitochondrial production of reactive oxygen species (ROS) is thought to contribute significantly to neuronal cell death caused by excitotoxicity and various acute and chronic neurological disorders, e.g. cerebral ischemia/reperfusion and Parkinson's disease (Benzi et al. 1982; Sciamanna et al. 1992; Dykens 1994; Fiskum et al. 1999; Murphy et al. 1999; Fiskum 2000; Nicholls and Budd 2000). Among other factors, mitochondrial accumulation of Ca2+ that occurs in response to these conditions has been reported to promote the generation of ROS (Dykens 1994; Kowaltowski et al. 1995; Kowaltowski et al. 1996; Kowaltowski et al. 1998a; Kowaltowski et al. 1998b; Fiskum 2000; Nicholls and Budd 2000). However, massive mitochondrial Ca2+ accumulation also inhibits the electron transport chain, potentially reducing the flow of electrons necessary for reduction of O₂ to superoxide anion and its metabolites, including H₂O₂ (Villalobo and Lehninger 1980). Mitochondrial Ca²⁺ sequestration also reduces the mitochondrial membrane potential $(\Delta \Psi)$, which is thermodynamically linked to the redox potential of sites in the electron transport chain responsible for production of ROS. Although several reports have of Ca²⁺ stimulated H₂O₂ production by mitochondria oxidizing succinate, and this stimulation was associated with release of mitochondrial cytochrome *c*. In the presence of glutamate plus malate, or succinate, cytochrome *c* release and H₂O₂ formation were stimulated by human recombinant full-length Bax in the presence of a BH3 cell death domain peptide. These results indicate that in the presence of ATP and Mg²⁺, Ca²⁺ accumulation either inhibits or stimulates mitochondrial H₂O₂ production, depending on the respiratory substrate and the effect of Ca²⁺ on the mitochondrial membrane potential. Bax plus a BH3 domain peptide stimulate H₂O₂ production by brain mitochondria due to release of cytochrome *c* and this stimulation is insensitive to changes in membrane potential.

Keywords: apoptosis, BH3 domain, cytochrome *c*, membrane potential, respiration, superoxide.

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demonstrated that Ca^{2+} loading enhances ROS generation in brain (Dykens 1994) and liver (Kowaltowski *et al.* 1995; Kowaltowski *et al.* 1996; Kowaltowski *et al.* 1998a; Kowaltowski *et al.* 1998b) mitochondria, the mechanism responsible for this stimulation remains elusive.

Mitochondrial functions may also be perturbed during acute neural cell injury by redistribution of pro-apoptotic proteins, such as Bax and Bid, to mitochondrial membranes where permeability changes occur that result in release to the cytosol of other pro-apoptotic proteins, e.g. cytochrome c(Fiskum 2000). Release of cytochrome c has been associated with increased cellular oxidative stress (Cai and Jones 1998),

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Address correspondence and reprint requests to Dr Gary Fiskum, Department of Anesthesiology, University of Maryland School of Medicine, 685 W. Baltimore Street, MSTF-5.34, Baltimore, MD 21201, USA. E-mail: gfisk001@umaryland.edu

Abbreviations used: BSA, bovine serum albumin; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; TPP, tetraphenyl phosphonium; ROS, reactive oxygen species.

although a direct cause and effect relationship between release and stimulated mitochondrial ROS generation has not been demonstrated.

One purpose of this study was to clarify the effect of Ca^{2+} uptake on ROS production by isolated brain mitochondria. Experiments were designed to test the hypothesis that Ca^{2+} can either stimulate or inhibit mitochondrial ROS generation, depending on the source of electrons donated to the respiratory chain, and the effects of Ca^{2+} accumulation on the retention of mitochondrial cytochrome *c*. Another aim of the study was to test the hypothesis that mitochondrial ROS production is stimulated by cytochrome *c* release elicited by exposure to Bax and a peptide containing a BH3 cell death domain.

Materials and methods

Reagents

Oligomycin, antimycin A3 and rotenone (Sigma, St Louis, MO, USA) were dissolved in ethanol, and Amplex Red (N-acetyl-3,7dihydroxyphenoxazine; Molecular Probes, Eugene OR, USA) was dissolved in dimethylsulfoxide. All other reagents were purchased from Sigma. All reagents and ethanol were tested and exhibited no interference with the H₂O₂ assay at the concentrations used in our experiments. The sources of full-length human recombinant Bax protein and of the synthetic Bax BH3 domain peptide have been described previously (Fiskum and Polster 2001; Polster *et al.* 2001).

Isolation of brain mitochondria

All animal experiments were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

Non-synaptosomal rat forebrain mitochondria were isolated by the Percoll gradient separation method as described in (Sims 1990). The quality of the mitochondrial preparation was estimated by measuring the acceptor control ratio defined as ADP-stimulated (State 3) respiration divided by resting (State 4) respiration. For these experiments, the incubation medium consisted of 125 mM KCl, 20 mM HEPES (pH 7.0), 2 mM KH₂PO₄, 1 mM MgCl₂, 5 mM glutamate, 5 mM malate, plus 0.8 mM ADP. Oxygen consumption was recorded at 37° C with a Clark-type oxygen electrode. State 3 respiration was initiated by the addition of 0.5 mg per ml rat brain mitochondria to the incubation medium. State 3 respiration was terminated and State 4 initiated by the addition of 1 µM carboxyatractylate, an inhibitor of the ADP/ATP transporter. Only mitochondrial preparations that exhibited an acceptor control ratio greater than 8 were used in this study.

Measurement of H₂O₂

Incubation medium contained 125 mM KCl, 20 mM HEPES (pH 7.0), 2 mM KH₂PO₄, 4 mM ATP, 5 mM MgCl₂, 1 μ M Amplex Red, 5 U/mL horseradish peroxidase superoxide dismutase (HRP) and 40 U/mL Cu,Zn SOD, and was maintained at 37°C, unless stated otherwise. A change in the concentration of H₂O₂ in the medium was detected by fluorescence of the oxidized Amplex Red product using excitation and emission wavelengths of 550 and

585 nm, respectively (Zhou *et al.* 1997). The response of Amplex Red to H_2O_2 was calibrated either by sequential additions of known amounts of H_2O_2 or by continuous infusion of H_2O_2 at 100– 1000 pmol/min. The concentration of commercial 30% H_2O_2 solution was calculated from light absorbance at 240 nM employing $E_{240} = 43.6 \text{ M}^{-1} \times \text{cm}^{-1} 13.6 \text{ M}^{-1} \times \text{cm}^{-1}$; the stock solution was diluted to 100 μ M with water and used for calibration immediately.

Cytochrome c release from mitochondria

Aliquots of mitochondrial suspensions were taken either during or at the end of experiments in which H_2O_2 generation was monitored. Mitochondria were separated from the suspending medium by centrifugation at 14 000g for 5 min. The supernatant was carefully removed and both the supernatant and mitochondrial pellet fractions were immediately frozen and stored at -20° C. Cytochrome *c* concentration was measured in both fractions using an ELISA kit (R & D Systems, Minneapolis, MN, USA). Before measurement, the supernatant and pellet samples were diluted 1 : 40 and 1 : 60, respectively. The release of cytochrome *c* from mitochondria was expressed as the content of cytochrome *c* in the supernatant as a percentage of the total content of cytochrome *c* present in the supernatant plus pellet.

Measurements of $\Delta \Psi$ and extramitochondrial [Ca²⁺]

Qualitative changes in $\Delta \Psi$ were followed using the fluorescence of safranine (5 µM) with excitation and emission wavelengths of 495 nm and 586 nm, respectively (Votyakova and Reynolds 2001). A semiquantitative measurement of $\Delta \Psi$ was estimated from TPP⁺ ion distribution between the medium and mitochondria using a custom-made TPP⁺-selective electrode (Kamo et al. 1979). For these experiments, incubation medium was supplemented with 1.6 µM TPP⁺Cl⁻. Both the TPP⁺-sensitive and reference electrodes were inserted directly into the fluorimeter cuvette, and data were collected using an amplifier and a two-channel data acquisition system, with one channel acquiring the amplified TPP⁺-electrode signal while another was dedicated to the Amplex Red fluorescence signal. The electrode response was calibrated by sequential addition of TPP⁺Cl⁻ in the concentration range 0.2–1.6 μ M, and the mitochondrial $\Delta \Psi$ was calculated as described by Rolfe *et al.* (1994). Alternatively, $\Delta \Psi$ values were calculated by the procedure reported by Rottenberg (1984), assuming that the matrix volume for brain mitochondria is 1.2 µL per mg protein. Both procedures vielded similar results.

Changes in the extramitochondrial medium free Ca^{2+} were followed using the fluorescence of Calcium Green 5N, with excitation and emission wavelengths of 506 nm and 532 nm, respectively.

Results

To measure relatively low rates of H_2O_2 production by brain mitochondria, we used a fluorescent dye/horseradish peroxidase detecting system employing the new peroxidase substrate Amplex Red, based on its high sensitivity and very low background fluorescence in the absence of a biological peroxide-generating system (Fig. 1a, curve 1). We also utilized an incubation medium that contained physiologically relevant concentrations of K⁺, P_i, ATP and Mg²⁺, as



Fig. 1 Effects of Ca²⁺ uptake on ΔΨ and H₂O₂ production by brain mitochondria oxidizing glutamate and malate. Incubation medium maintained at 37°C was supplemented with 5 mM glutamate, 5 mM malate and either 1 μM Amplex Red (a), 100 nM Calcium Green 5N (b) or 5 μM safranin O (c) for measurements of H₂O₂, medium Ca²⁺ concentration or ΔΨ, respectively. Mitochondria (Mito) were added at 0.25 mg per mL mitochondria; Ca²⁺ at 0.2 mM; EGTA at 0.5 mM, oligomycin (Oligo) at 0.5 μg/mL, and rotenone at 0.5 μM. In tracings 1, no additions were made following the addition of mitochondria. In tracings 3, oligomycin was added before the addition of Ca²⁺. In tracings 4, no mitochondria were added. The italicized numbers in (a) represent rates of H₂O₂ production in pmoles per minute per milligram mitochondrial protein.

these components exert dramatic effects on the response of respiring mitochondria to high levels of Ca^{2+} (Murphy *et al.* 1999). Under these conditions, brain mitochondria are resistant to catastrophic bioenergetic and morphological alterations caused by the Ca^{2+} -induced inner membrane permeability transition compared with, for example, liver

mitochondria, but are still susceptible to Ca^{2+} -induced alterations in membrane potential and cytochrome *c* release across the outer membrane (Andreyev and Fiskum 1999).

Figure 1a curve 1 illustrates that isolated rat brain mitochondria respiring on the NAD-linked substrates glutamate and malate produce significant amounts of H2O2 in the absence of added Ca2+ or electron transport chain inhibitors. Under these conditions the medium contaminating free Ca^{2+} (approximately 5 μ M or 20 nmol per mg protein), as measured by the fluorescent indicator Calcium Green 5 N, was accumulated by the added mitochondria and a steadystate medium $[Ca^{2+}]$ was maintained for at least 10–15 min (Fig. 1b, curve 1). Parallel safranin O fluorescent measurements of $\Delta \Psi$ indicated that $\Delta \Psi$ stabilized within 2 min of the mitochondrial addition and remained constant for at least 15 min thereafter, but rapidly dissipated upon addition of the respiratory Complex I inhibitor rotenone (Fig. 1c, curve 1). When brain mitochondria were exposed to a single addition of Ca²⁺ (800 nmol per mg protein), H₂O₂ production was reduced by approximately 60% (193 vs. 448 pmol per mg protein) (Fig. 1a, curve 2). This amount of added Ca²⁺ was accumulated entirely by the rat brain mitochondria, followed by a slow and partial release back into the medium 5-10 min later (Fig. 1b, curve 2). The addition of Ca²⁺ resulted in a transient reduction in $\Delta \Psi$, as expected because mitochondrial Ca²⁺ uptake occurs via an electrophoretic uniporter that draws upon the $\Delta \Psi$ for active Ca^{2+} sequestration. The reduction in $\Delta \Psi$ (Fig. 1, curve 2) stimulates respirationdependent H^+ efflux that, in turn, drives $H_2PO_4^-$ influx via the electroneutral H₂PO₄/OH⁻ antiporter. Thus, electophoretic uptake of Ca²⁺ followed by electroneutral uptake of P_i results in a transient collapse of $\Delta \Psi$ without an increase in pH. Approximately 1 min after the addition of Ca^{2+} , when most of it had been accumulated, $\Delta \Psi$ recovered back to its initial level and remained stable for at least 10 min.

Mitochondrial Ca²⁺ uptake can be driven by electrogenic H^+ efflux mediated by the mitochondrial F_0F_1 ATPase in addition to H⁺ extrusion mediated by the electron transport chain. Experiments were therefore performed in the presence of oligomycin, a specific inhibitor of the mitochondrial ATPase, to determine the influence of ATPase activity on mitochondrial Ca^{2+} uptake, $\Delta \Psi$ and H_2O_2 production. In the presence of oligomycin, the rate of H₂O₂ production before Ca^{2+} addition was slightly higher than in its absence (310 vs. 242 pmol per mg protein). However, following the addition of Ca^{2+} , the rate of H_2O_2 production was approximately 50% of that in its absence (91 vs. 193 pmol per mg protein), and only 20% of that observed in the absence of added Ca^{2+} (91) vs. 448 pmol per mg protein) (Fig. 1a, curve 3). In the presence of oligomycin, mitochondrial Ca²⁺ uptake was initially as, or more, rapid than in its absence but converted to a relatively very slow, sustained rate of net uptake within a minute after the addition of Ca^{2+} (Fig. 1b, curve 3). The presence of oligomycin also resulted in a greater decline in $\Delta \Psi$ upon addition of Ca²⁺ and a sustained reduction in $\Delta \Psi$ for at least 8 min (Fig. 1c, curve 3). The Ca²⁺-dependent reduction in $\Delta \Psi$ was partially reversed by the subsequent addition of the Ca²⁺ chelator EGTA (Fig. 1c, curve 3), as was the Ca²⁺-dependent reduction in H₂O₂ production (Fig. 1a, curve 3). Thus, ATPase-mediated electrogenic H⁺ efflux contributes to the maintenance of $\Delta \Psi$ during and following mitochondrial uptake of large Ca²⁺ loads, but is incapable of completely sustaining $\Delta \Psi$ in the absence of respiration.

Mitochondrial ROS production was much faster with Complex II-linked substrate succinate compared with Complex I-dependent substrates. Within 2 min following the addition of rat brain mitochondria to the incubation medium containing 5 mM succinate, there was a dramatic increase in



Fig. 2 Effects of Ca²⁺ uptake on ΔΨ and H₂O₂ production by brain mitochondria oxidizing succinate. Incubation medium maintained at 37°C was supplemented with 5 mM succinate and either 1 μM Amplex Red (a) or 5 μM safranin O (b) for measurements of H₂O₂ or ΔΨ, respectively. Mitochondria (Mito) were added at 0.125 mg per mL mitochondria; Ca²⁺ at 0.2 mM; EGTA at 0.5 mM, rotenone at 0.5 μM and FCCP at 50 nM. The italicized numbers in panel (a) represent rates of H₂O₂ production in pmoles per minute per milligram mitochondrial protein.

H₂O₂ production (Fig. 2a), at a rate that was seven to eight times faster than that observed in the presence of glutamate plus malate (1700-2000 vs. 250 pmol per mg mitochondrial protein). The delayed activation of succinate-supported H₂O₂ production corresponded to the time at which the $\Delta \Psi$ reached its maximum level following addition of mitochondria to the medium (Fig. 2b) (Korshunov et al. 1997). Subsequent addition of 0.2 mM Ca2+ resulted in an immediate and almost complete inhibition of H₂O₂ production (68 vs. 1760 pmol per mg protein) (Fig. 2a, curve 1). At this level of Ca^{2+} , $\Delta\Psi$ was transiently reduced then recovered, albeit to a level that was lower than that maintained in the absence of added Ca²⁺ (Fig. 2b, curve 1). Whereas EGTA significantly reversed the effect of Ca^{2+} on ROS production and $\Delta\Psi$ in the presence of Complex I-linked substrates, it was unable to reverse the effects of Ca^{2+} in the presence of the Complex II substrate succinate (Figs 2a and b, curves 1).

The Complex I inhibitor rotenone was capable of stimulating mitochondrial H_2O_2 production in the presence of Ca²⁺ but had a negligible effect on the Ca²⁺-induced reduction of $\Delta \Psi$ (Figs 2a and b, curves 1), as expected as rotenone inhibits NAD-linked but not succinate-supported respiration. The rate of H₂O₂ production observed following the addition of Ca²⁺ and then rotenone was still far lower than the initial rate observed in the absence of both agents (469 vs. 1760 pmol per mg protein). This finding verifies the contribution of rotenone-sensitive reversed electron flow through Complex I as a major site of ROS production supported by succinate (Hinkle et al. 1967; Hansford et al. 1997; Korshunov et al. 1997; Turrens 1997; Lass et al. 1998; Votyakova and Reynolds 2001; Liu et al. 2002). However, in the absence of added Ca²⁺, the addition of rotenone resulted in a rate of H₂O₂ production that was substantially lower than that following the addition of Ca²⁺ (264 vs. 469 pmol per mg protein; Fig. 2a, curve 2). This stimulatory effect of Ca^{2+} on succinate-supported H_2O_2 production in the presence of rotenone was further verified by the addition of Ca²⁺ following rotenone, after which the rate of H₂O₂ production increased from 250 to 462 pmol per mg protein (Fig. 2a, curve 3). In contrast to the sustained reduction in $\Delta \Psi$ observed upon addition of Ca²⁺ in the absence of rotenone, mitochondrial Ca2+ uptake in the presence of rotenone resulted in the normal transient drop in $\Delta \Psi$ followed by a complete recovery and a subsequent steady but minor depolarization (Fig. 2b, curve 3). These results further demonstrate that when exposure of mitochondria to high levels of Ca^{2+} results in a reduction in $\Delta\Psi$, ROS production is inhibited. Under conditions in which ROS production is independent of $\Delta \Psi$, e.g. in the presence of rotenone, Ca²⁺ can actually stimulate ROS production.

Experiments were then performed to test the hypothesis that a reduction in $\Delta \Psi$ is a sufficient explanation for the inhibition of H₂O₂ production by Ca²⁺ with either succinate or the NAD-linked substrates glutamate and malate. It is

known that succinate-supported H2O2 production is inhibited by mitochondrial depolarization (Hansford et al. 1997; Korshunov et al. 1997). The dependence of succinatesupported ROS generation on $\Delta \Psi$ is due to the reverse electron transport from Complex II through coenzyme Q to Complex I. The potential energy of $\Delta \Psi$ is necessary to overcome the redox potential difference between Complex I and coenzyme O that promotes Complex I oxidation and coenzyme Q reduction. Although the quantitative relationship between $\Delta \Psi$ and ROS production during succinate-dependent reversed electron transport is established (Korshunov et al. 1997), it has not been determined for ROS generation that occurs during electron transport driven by NAD-linked substrates. This relationship was explored by measuring mitochondrial H₂O₂ production in the presence of glutamate and malate and in the presence of several concentrations of FCCP, a protonophoric uncoupling agent. In order to demonstrate a quantitative relationship, the experimental system utilized the mitochondrial uptake of the lipophilic cation TPP⁺ as a measure of $\Delta \Psi$. The system was also simplified by omitting ATP from the medium and adding the Ca2+ chelator EGTA to prevent possible interference from contaminating Ca²⁺ in the medium. The results shown in Fig. 3 indicated that H₂O₂ production supported by oxidation of NAD-linked substrates is indeed dependent on $\Delta \Psi$, being very sensitive to fluctuations in $\Delta \Psi$ between approximately 150 and 180 mV. These results however, also demonstrate that approximately 30% of the maximal H_2O_2 production is insensitive to $\Delta\Psi$.

The reduction in $\Delta \Psi$ caused by mitochondrial Ca²⁺ accumulation is a sufficient explanation for the inhibition



Fig. 3 Relationship between rate of H₂O₂ production and ΔΨ for brain mitochondria oxidizing glutamate and malate. Mitochondria were added at a concentration of 0.25 mg/mL to the standard incubation medium except that ATP was omitted, MgCl₂ was present at 1 mm, and the medium was supplemented with 0.25 mM EGTA, 1.6 μM TPP⁺Cl⁻, 5 mM glutamate and 5 mM malate. A TPP⁺ electrode was used to measure the medium TPP⁺ concentration both in the absence of FCCP and in the presence of 22, 47, 76 and 110 pmol FCCP per mg mitochondrial protein to modulate the ΔΨ. Values for ΔΨ were calculated as described in Materials and Methods. Values represent means ± SE of four independent experiments.

by Ca^{2+} of H_2O_2 production both with succinate (in the absence of rotenone) or NAD-linked substrates. This effect cannot, however, explain the increase in succinate-supported H_2O_2 generation caused by Ca^{2+} in the presence of the Complex I inhibitor rotenone. We hypothesized that Ca²⁺induced cytochrome c release might be responsible for this phenomenon because cvtochrome c release has been associated with increased oxidative stress in apoptotic cells (Cai and Jones 1998), and cytochrome c is released by brain mitochondria in response to Ca²⁺ uptake under conditions similar to those used in the current study (Andreyev et al. 1998; Andreyev and Fiskum 1999). A comparison between rates of H₂O₂ production and the distribution of cytochrome c between the mitochondria and the suspending medium at the end of the H₂O₂ measurements is provided in Figs 4 and 5 for succinate (in the presence of rotenone) and glutamate plus malate, respectively. The accumulation of Ca²⁺ resulted in a net 10% release of cytochrome c in the presence of succinate and 5% release in the presence of glutamate plus malate. This was accompanied by an approximately 60% increase in H₂O₂ formation with succinate and a greater than 60% reduction in H₂O₂ with glutamate and malate. Thus Ca^{2+} induces the release of mitochondrial cytochrome c in the presence of either malate plus glutamate or succinate but only stimulates H2O2 production when succinate is present as the electron donor and rotenone is present to inhibit reversed electron transport that is driven by $\Delta \Psi$.



Fig. 4 Ca²⁺-induced cytochrome *c* release and stimulation of H₂O₂ production by brain mitochondria oxidizing succinate. Mitochondria were added at a concentration of 0.25 mg/mL to the standard incubation medium maintained at 37°C and supplemented with 5 mm succinate. As in Fig. 2, approximately 3 min later, rotenone (0.2 µm) was added, followed 2 min later by either Ca²⁺ (0.2 mm) or vehicle control (H₂O). The rate of H₂O₂ production was determined by the rate of increase in Amplex Red fluorescence. At approximately 8 min following the addition of Ca²⁺, the suspension was centrifuged and the pellet and supernatant fractions used for ELISA of cytochrome *c* content, as described in Materials and Methods. Values represent the mean ± SE of four independent experiments. **p* < 0.05 versus without Ca²⁺ by Student's *t*-test.



Fig. 5 Ca²⁺-induced cytochrome *c* release and inhibition of H₂O₂ production by brain mitochondria oxidizing glutamate and malate. Mitochondria were added at a concentration of 0.25 mg/mL to the standard incubation medium maintained at 37°C and supplemented with 5 mM glutamate and 5 mM malate. As in Fig. 1, approximately 4 min later, Ca²⁺ (0.2 mM) or vehicle control (H₂O) were added and the rate of H₂O₂ production was determined by the rate of increase in Amplex Red fluorescence. At approximately 10 min following the addition of Ca²⁺, the suspension was centrifuged and the pellet and supernatant fractions were used for ELISA of cytochrome *c* content, as described in Materials and Methods. Values represent the mean ± SE of four independent experiments. **p* < 0.05 versus without Ca²⁺ by Student's *t*-test.

Additional evidence for the direct role of cytochrome crelease in stimulating mitochondrial ROS formation came from experiments in which release was mediated by exposure of brain mitochondria to the pro-apoptotic protein Bax together with a synthesized peptide that contains the BH3 death domain amino acid sequence. We previously demonstrated that this peptide triggers Bax-dependent release of cytochrome c by specifically increasing the permeability of the mitochondrial outer membrane without affecting inner membrane permeability or other components of the electron transport chain (Polster et al. 2001). Figure 6a provides representative fluorescent measurements of H₂O₂ generation by rat brain mitochondria respiring on glutamate and malate in the presence of 100 nM human recombinant full-length Bax and in the absence or presence of 50 µM BH3 peptide. In the absence of the peptide, H₂O₂ produced in the presence of Bax was identical to that in its absence (not shown). In these experiments, ADP was added to the suspension, inducing State 3 respiration. Under these conditions, the redox state of potential sites of ROS production is relatively oxidized and therefore inhibition of electron transport by release of cytochrome c should cause a maximal shift toward a reduced redox state. In the absence of BH3 peptide, H₂O₂ formation increased slowly over approximately 10 min and was stimulated by over 100% upon addition of the Complex I inhibitor rotenone (732 vs. 277 pmol per min per mg protein). The rate of H₂O₂ generation observed 10 min following the addition of the BH3 peptide was 70% greater than the timed control (466 vs. 277 pmol per min per mg



Fig. 6 Stimulation of cytochrome c release and H₂O₂ production by brain mitochondria by addition of Bax plus a BH3 domain peptide. Mitochondria were added at a concentration of 0.125 mg/mL to the standard incubation medium maintained at 37°C and supplemented with either 5 mM glutamate and 5 mM malate or 5 mM succinate. (a) Amplex Red measurements of H₂O₂ generation. At approximately 3 min following addition of brain mitochondria to media containing glutamate and malate and 100 nm Bax (see Materials and methods), 0.1 mm ADP was added to initiate State 3 respiration. Approximately 2 min later, 50 µM BH3 peptide or vehicle control (H₂O) was added. Rotenone (0.2 µm) was added 9 min later to elicit maximal H₂O₂ production. (b) Relationship between H₂O₂ production and release of cytochrome c caused by exposure of brain mitochondria to Bax plus a BH3 peptide. In experiments such as that shown in (a) aliquots of the mitochondrial suspension were removed before and two or three times after addition of the BH3 peptide or vehicle, centrifuged and used for ELISA of cytochrome c release from the mitochondria into the medium. The corresponding rate of H₂O₂ production refers to the rate obtained just before removal of the aliquot for cytochrome c measurement. Experiments were performed either in the presence of 5 mm succinate (○) or 5 mM glutamate and 5 mM malate (■).

protein) and was further stimulated by the addition of rotenone. In the absence of added Bax, BH3 peptide had no effect on ROS production by adult rat brain mitochondria (not shown), consistent with its inability to release cyto-chrome c from these mitochondria in the absence of exogenous Bax (Polster *et al.* 2001). Thus, in contrast to

the inhibition of H_2O_2 production by added Ca^{2+} when NAD-linked respiratory substrates were present (Fig. 1a), the presence of Bax plus a BH3 peptide substantially stimulates mitochondrial H_2O_2 formation. An approximately 70% stimulation of H_2O_2 formation by Bax plus the BH3 peptide was observed in the presence of succinate plus rotenone (not shown). Thus, cytochrome *c* release *per se* stimulates mitochondrial ROS generation in the presence of either Complex I or Complex II respiratory substrates.

As the rate of H_2O_2 production was non-linear during the course of these experiments and as Bax-BH3-mediated cytochrome c release takes several minutes to occur, we took aliquots of the mitochondrial suspension during the course of the H_2O_2 measurements for quantification of cytochrome c distribution to determine if a relationship exists between the extent of release and the rate of H2O2 formation. These aliquots were taken immediately before the addition of the BH3 peptide or vehicle and 5 and 8 min later. The extent of cytochrome c release and the corresponding rates of H_2O_2 generation measured just before sampling using glutamate and malate or succinate (in the presence of rotenone) are presented in Fig. 6b. The H₂O₂ production rate was greater for mitochondria respiring on succinate than on glutamate and malate in the absence of BH3 peptide with background release of 5-12% of the total cytochrome c. However, after 5 min exposure to the BH3 peptide, the extent of cytochrome c release was similar under these two conditions (60-65%), as were the rates of H₂O₂ formation (360-420 pmol per min per mg protein). An additional 3-min exposure to the BH3 peptide resulted in an additional approximately 10% release of cytochrome c and rates of H_2O_2 production that were very similar for the two sets of respiratory substrates (466-520 pmol per min per mg protein). These results indicate that the release of cytochrome c by Bax plus a BH3 peptide results in a dose-dependent stimulation of mitochondrial ROS formation under physiologically relevant in vitro conditions with either succinate or NAD-linked respiratory substrates.

Discussion

The main conclusion that can be drawn from this study is that when $\Delta \Psi$ is reduced with mitochondria respiring on either NAD-linked substrates or succinate in the absence of rotenone, ROS production is inhibited (Figs 1, 2, 3 and 5). Conversely, the release of cytochrome *c* by either mitochondrial Ca²⁺ accumulation or by exposure to Bax and a BH3 domain peptide can significantly stimulate mitochondrial ROS production apparently independently of $\Delta \Psi$ (Figs 2, 4 and 6). These observations were made *in vitro* in the absence of respiratory inhibitors and in the presence of physiologically relevant levels of ATP, Mg²⁺ and other ions that have a profound influence on mitochondrial energy coupling and on mitochondrial responses to raised levels of Ca^{2+} . We therefore believe that these observations may be highly relevant to the effects of both pathological levels of Ca^{2+} and the apoptotic redistribution of Bax and BH3 domain proteins that occurs in response to many forms of neuronal stress.

The modulation of mitochondrial ROS generation observed in the presence of NAD-linked oxidizable substrates is particularly important as these substrates constitute the primary source of fuel for oxidative cerebral energy metabolism. In the presence of ATP, the uptake of Ca²⁺ inhibited H₂O₂ generation (Figs 1 and 5). This inhibition appears to be due to more than one mechanism, as indicated by differences in Ca²⁺-induced mitochondrial depolarization and inhibition of ROS production in the absence and presence of oligomycin (Fig. 1). When ATP turnover was blocked by oligomycin, Ca²⁺ caused a substantial collapse in $\Delta \Psi$ and ~ 60% inhibition of H₂O₂ generation. In the absence of oligomycin, Ca2+ accumulation resulted in no sustained reduction in $\Delta \Psi$ but ROS generation was still inhibited by $\sim 80\%$. As independent experiments performed in the absence of Ca²⁺ and in the presence of different concentrations of the protonophore uncoupler FCCP demonstrated that > 50% of NAD-linked ROS production is sensitive to $\Delta \Psi$ (Fig. 3), we conclude that mitochondrial depolarization is one of the mechanisms by which Ca^{2+} inhibits H_2O_2 formation. Votyakova and Reynolds (2001) reported previously that the presence of an uncoupler does not stimulate NAD-linked mitochondrial ROS production; however, the rates of H₂O₂ generation they observed using the scopoletin method of detection were too low to detect any inhibitory influence of membrane depolarization. The mechanism by which loss of $\Delta \Psi$ reduces mitochondrial H₂O₂ formation probalby involves the oxidation of redox centers, e.g. coenzyme Q or iron-sulfur proteins, which probably mediate the generation of superoxide and therefore H₂O₂.

Ca²⁺-induced mitochondrial depolarization cannot explain the inhibition of NAD-linked H₂O₂ formation observed in the absence of oligomycin as $\Delta \Psi$ was fully preserved (Fig. 1). One possible explanation for this $\Delta \Psi$ -independent inhibition is that Ca²⁺ may impair the flow of electrons within the electron transport chain at a site, most likely within Complex I, that is proximal to the site of ROS generation. Evidence for selective impairment of NADlinked respiration by Ca²⁺ was originally reported for ascites tumor cell mitochondria (Villalobo and Lehninger 1980), and more recently for neural cell mitochondria (Murphy et al. 1996). Although such inhibition could be caused by release of mitochondrial NAD(H) mediated by the Ca²⁺-activated permeability transition (Maciel et al. 2001), it is clear from the maintenance of $\Delta \Psi$ that the permeability transition did not occur under these conditions.

Mitochondrial Ca^{2+} uptake had an even greater inhibitory effect on succinate-driven H_2O_2 production than on

NAD-linked ROS generation (Fig. 2). The explanation for the greater inhibition with succinate is that Ca^{2+} -induced loss of $\Delta \Psi$ deprives mitochondria of the energy required for reverse transport of electrons from Complex II through coenzyme Q to Complex I where sites of succinate-based ROS formation are located (Korshunov *et al.* 1997; Turrens 1997; Lass *et al.* 1998; Liu *et al.* 2002). The most novel observation made with succinate as the respiratory substrate in the presence of rotenone was that Ca^{2+} actually stimulated H_2O_2 production. Under these conditions, Ca^{2+} -induced changes in $\Delta \Psi$ were not expected to affect H_2O_2 production as reverse electron transport is inhibited by the presence of rotenone. Therefore, Ca^{2+} -induced release of cytochrome *c* was pursued as a possible mechanism for its stimulation of H_2O_2 generation.

Quantitative analysis of the extent of cytochrome c released by the end of experiments such as those shown in Figs 1 and 2 indicated that a small but significant percentage of cytochrome c was released following the addition of Ca²⁺ when either glutamate and malate, or succinate were used as respiratory substrates (Figs 4 and 5). Although cytochrome c was released in response to the addition of Ca²⁺ under both conditions, Ca²⁺ only stimulated the production of H₂O₂ in the presence of succinate plus rotenone. The fact that cytochrome c release was observed under these conditions suggested, but did not prove, that it was responsible for the stimulation of H₂O₂ generation by Ca²⁺. Further support for this hypothesis came from experiments performed in the absence of Ca²⁺ but in the presence of Bax and a BH3 death domain peptide.

Ca²⁺-independent stimulation of H₂O₂ production was dependent on the presence of both the human recombinant full-length Bax and the BH3 peptide as neither component alone had any effect on either ROS generation or cytochrome c release (Fig. 6) (Fiskum and Polster 2001; Polster et al. 2001). Release of cytochrome c was accompanied by increased H₂O₂ production in the presence of glutamate plus malate, or succinate plus rotenone. As the release of cytochrome c interrupts the flow of electrons at a site distal to the sites of ROS generation, their redox states shift to a more reduced level. Under these conditions, these redox centers are unaffected by $\Delta \Psi$ because electron transport is severely inhibited by the loss of cytochrome c. Therefore, mitochondrial H₂O₂ production is insensitive to $\Delta \Psi$ when cytochrome c is released by conditions, e.g. the Bax/BH3 system, in which no other electron transport activities are altered.

Although the most plausible explanation for the stimulation of ROS production by the release of cytochrome crelates to the effect of respiratory inhibition on the redox state of superoxide-generating sites proximal to the site of inhibition, other mechanisms may also contribute. One mechanism is the reduction of superoxide scavenging by cytochrome c when it is lost from the mitochondrial intermembrane space to the cytosol (Korshunov *et al.*) 1999). This mode of action is supported by the findings that a substantial fraction of mitochondrial superoxide formation occurs at the outer side of the inner membrane, where cytochrome *c* is normally present in equilibrium with the unbound protein in the intermembrane space at a concentration of 100–700 μ M (Hackenbrock 1966; Hackenbrock 1968). Despite its possible exacerbation of mitochondrial ROS generation, the redistribution of cytochrome *c* into the cytosol has been proposed to help scavenge superoxide or other ROS in that compartment during glutamate excitotoxicity (Atlante *et al.* 2000).

Considerable attention has been focused on the role of raised intracellular Ca2+ in mitochondrial dysfunction and ROS production preceding neural cell death (Fiskum 2000). Recent investigations indicate that the interaction of Bax with BH3 death domain only proteins, e.g. Bid, at the mitochondrial level also contributes significantly to cell death in animal models of acute brain injury and neurodegenerative diseases (Plesnila et al. 2001; Vila et al. 2001). One study demonstrated a relationship between these two forms of stress with Ca2+-induced mitochondrial permeability transition signaling the redistribution of cytosolic Bax to the mitochondrial membrane before cytochrome c release during apoptosis (De Giorgi et al. 2002). Our observations suggest that the stimulation of mitochondrial ROS generation by Bax-mediated cytochrome c release may be particularly important in the pathogenesis of neural cell death. This stimulation can occur in the presence of either Complex I or Complex II respiratory substrates, physiological concentrations of ATP and inorganic ions, and in the absence of respiratory poisons. Development of inhibitors of Bax-mediated cytochrome c release is extremely important as they should both block caspasemediated apoptosis and obstruct the mitochondrial generation of ROS that can promote either apoptotic or necrotic cell death.

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