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Reactive oxygen species induce swelling and cytochrome *c* release but not transmembrane depolarization in isolated rat brain mitochondria

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1 In this study, we have used isolated brain mitochondria to investigate the effects of superoxide anions (O_2^-) on mitochondrial parameters related to apoptosis, such as swelling, potential, enzymatic activity, NAD(P)H, cytochrome *c* release, and caspase activity.

2 Addition of the reactive oxygen species (ROS) generator KO_2 produced brain mitochondrial swelling, which was blocked by cyclosporin A (CSA), and which was Ca^{2+} independent.

3 Calcium induced mitochondrial swelling only at high concentrations and in the presence of succinate. This correlated with the increase in O_2^- production detected with hydroethidine in mitochondrial preparations exposed to Ca^{2+} and the fact that ROS were required for Ca^{2+}-induced mitochondrial swelling.

4 Superoxide anions, but not Ca^{2+}, decreased citrate synthase and dehydrogenase enzymatic activities and dropped total mitochondrial NAD(P)H levels.

5 Calcium, but not O_2^-, triggered a rapid loss of mitochondrial potential. Calcium-induced $\Delta\psi_m$ dissipation was inhibited by Ruthenium Red, but not by CSA.

6 Calcium- and superoxide-induced mitochondrial swelling released cytochrome *c* and increased caspase activity from isolated mitochondria in a CS A-sensitive manner.

7 In summary, superoxide potently triggers mitochondrial swelling and the release of proteins involved in activation of postmitochondrial apoptotic pathways in the absence of mitochondrial depolarization.

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Keywords: Caspase; mitochondrion; excitotoxicity; apoptosis; ROS; brain

Abbreviations: AFU, Arbitrary fluorescent units; CsA, cyclosporin A; DEVD-AFC, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin; MPTP, mitochondrial permeability transitory pore; MnTBAP, Mn(IIItetrakis(4-benzoic acid)porphyrin chloride); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue; Rh 123, rhodamine 123; TCA, trichloroacetic acid; $\Delta\psi_m$, mitochondrial membrane potential

Introduction

Mitochondrial dysfunctions, because of DNA defects, protein mutations or alterations in electron transport (Koutnikova *et al*., 1997) have been linked to neurodegenerative diseases (Sheehan *et al*., 1997; Brown *et al*., 2000) and aging processes (Wallace, 2001). Mitochondria play a central role in different cell death pathways leading to either apoptosis or necrosis (Green & Reed, 1998; Kroemer & Reed, 2000). The first evidence that mitochondria play an important role in cellular death mechanisms came from the observation that '*in vitro*' models required an organelle fraction enriched in mitochondria to induce apoptosis (Newmeyer *et al*., 1994). Apoptosis might involve changes in respiration-dependent mitochondrial potential ($\Delta\psi_m$) and, in some models, formation of a voltage-dependent high conductance multiproteic channel, referred to as mitochondrial permeability transitory pore (MPTP) (Bernardi, 1999). However, there is increasing evidence suggesting that MPTP activation is also important for necrosis activation (Kroemer & Reed, 2000).

The $\Delta\psi_m$ role in apoptosis is still an enigma, as either preservation (Prehn *et al*., 1996) or dissipation (Stout *et al*., 1998) has been shown to block neuronal death. Moreover, the relation between MPTP and $\Delta\psi_m$ remains unclear. There are reports suggesting that for some apoptotical stimuli, MPTP formation is independent of mitochondrial transmembrane depolarization (Madesh & Hajnoczky, 2001). As a result of MPTP opening, mitochondria might lose their membrane permeability control, releasing internal proteins (cytochrome *c*, apoptosis-inducing factor (AIF), SMAC/Diablo, caspase family members, etc.) to the cytoplasm. This release results in the activation of different pathways that lead to cell death. For example, cytochrome *c* participates in the apoptosome formation, and AIF results in chromatin condensation and DNA fragmentation (Susin *et al*., 1999). Although the physiological role of MPTP has not yet been determined, its role as mediator of cell injury and death is generally accepted.

Calcium influx and O_2^- production within the mitochondria prior to cell death are events shared by cells treated with neurotoxic agents like NMDA, veratridine and β-amyloid (Bindokas *et al*., 1996; Jordan *et al*., 2000, 2002; Longo *et al*.,

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2000). Overexpression of proteins that regulate Ca^{2+} and O_2^- levels protect neuronal cultures from neurotoxic stimuli (Jordan *et al*., 1995; Prehn *et al*., 1997). Evidence has been presented suggesting that intracellular O_2^- production during apoptosis injury is Ca^{2+} dependent and from mitochondrial origin (Ankarcrona *et al*., 1995; Bindokas *et al*., 1996; Jordan *et al*., 2000) and either stimuli result in MPTP formation (for review, see Bernardi, 1999). In the present study, we have analyzed the plausible effects of O_2^- and Ca^{2+} on mitochondrial swelling and $\Delta\psi_m$ dissipation using isolated rat brain mitochondria.

Methods

Mitochondria isolation

Mitochondria were isolated from the cortex of adult Sprague–Dawley rats by conventional centrifugation as previously described (Jordán *et al*., 2002). Briefly, tissue was manually homogenized with four strokes with a Teflon pestle in solution I (Sol I) containing 230 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 5 mM HEPES, pH 7.4 on ice. After centrifugation at 4°C ($106 \times g$; 80 s), supernatant was layered onto solution II (460 mM mannitol, 14 mM sucrose, 1 mM EGTA, and 10 mM HEPES, pH 7.4), and centrifuged ($800 \times g$; 3 min). The top layer was then centrifuged ($2000 \times g$; 5 min) and the mitocondrial pellet resuspended in solution III (215 mM mannitol, 71 mM sucrose, 10 mM succinate, and 10 mM HEPES, pH 7.4) and kept on ice until swelling determinations. To exclude that the observed effects were becuase of contaminating synaptosome, we isolated brain mitochondria using a Percoll gradient as previously described (Kristal & Dubinsky, 1997). No differences in KO_2-induced swelling were observed between mitochondria isolated by both the methods.

Permeability transition pore activity

Permeability transition pore opening was assayed spectrophotometrically as previously described (Kristal *et al*., 2000; Galindo *et al*., 2003). Specifically, mitochondria were suspended to reach a protein concentration of 1 mg ml^{-1} in 200 μl of solution III. Changes in absorbance at 540 nm (A_{540}), indicating mitochondrial swelling because of MPTP opening, were followed, after addition of different compounds, using a microplate reader (BioRad, Hercules, CA, U.S.A.). Initial A_{540} values were $\cong 0.8$, and minor differences in loading were compensated by representing the data as the fraction of the initial absorbance measure remaining at a given time. Mitochondrial protein concentrations were quantified spectophotometrically (Micro BCA Protein Reagent Kit, Pierce, Rockford, IL, U.S.A.), with bovine serum albumin as standard.

Monitoring of the mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured in the presence of 0.1 μg ml^{-1}. rhodamine 123 (Rh 123) as described by Emaus *et al*. (1996). The excitation and emission wavelengths for Rh 123 were 503 and 525 nm (slit 3 nm), respectively, using a Perkin-Elmer (luminiscence-spectrophotometer LS50B) fluorimeter.

Assay for mitochondrial NAD(P)H levels

NAD(P)H fluorescence in intact mitochondria (1 mg ml^{-1} at 25°C) was measured spectrofluorimetrically with excitation and emission wavelengths of 340 nm (slit 3 nm) and 460 nm (slit 5 nm), respectively, as previously described (Rover Junior *et al*., 1998). We therefore refer to NAD(P)H, indicating the signal derived from either NADH or NADPH, or both. Under these conditions, an increase in autofluorescence signal indicates an increase in the reduced state of the pyridine nucleotide, NAD(P)H, and a decrease in autofluorescence signal indicates an increased oxidation to $NAD(P)^+$.

Mitochondrial dehydrogenase activity

Mitochondrial dehydrogenase activity was estimated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl) blue (MTT) to its corresponding blue formazan product according to a procedure described elsewhere (Mosmann, 1983). After 30 min incubation of mitochondrial suspension with Ca^{2+} or O_2^-, mitochondria were further incubated for 15 min with 0.5 mg ml^{-1} MTT. At the end of the incubation period, absorbances were read on a microplate reader (BioRad, Hercules, CA, U.S.A.) with a test wavelength of 540 nm and a reference wavelength of 630 nm.

Citrate synthase activity

Citrate synthase (EC 4.1. 3.7) was measured at 410 nm using a microplate reader (BioRad, Hercules, CA, U.S.A.) at room temperature as described by Shepherd & Garland (1969). The reaction buffer contains 100 mM Tris-HCl, pH 8, 0.1 mM acetyl-CoA, 0.2 mM DTNB, 0.1 (V V^{-1}) Triton X-100, and 500 μg protein of mitochondrial suspension in a final volume of 0.2 ml. The assay is initiated by the addition of 10 μl of 20 mM oxalacetate and 10 min later absorbance was read on a microplate reader (BioRad, Hercules, CA, U.S.A.).

Immunoblot analysis

Immunoblot analysis was performed on extramitochondrial extracts from control and treated mitochondrial suspensions. In all cases, mitochondria suspensions were treated with resuspension (control), $CaCl_2$, or KO_2 for 30 min and then centrifuged at 15,000 rpm for 15 min. The supernatants (S100), extramitochondrial fractions, were precipitated by trichloroacetic acid (TCA) (10%, 4°C) overnight and centrifuged ($15{,}000 \times g$;15 min). Pellets were resuspended in 40 μl loading buffer and boiled for 15 min. Samples were loaded onto a 15% SDS-polyacrylamide gel, separated and transferred to a PVDF membrane, which was incubated with mouse monoclonal anticytochrome *c* antibody (Santa Cruz Biotechnology Inc., U.S.A.; 1 : 1000 dilution). The secondary antibody used (1 : 5000 dilution) was a peroxidase-labeled anti-mouse (Promega, Madison, WI., U.S.A.). The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit). Ruthenium Red (5 μM) or cyclosporin A (CsA) (10 μM) was added 15 min before and maintained during $CaCl_2$ or KO_2 treatment.

Assay of caspase enzymatic activity

Mitochondrial suspension aliquots were exposed for 30 min to $CaCl_2$ or KO_2 and then centrifuged at $15{,}000 \times g$ for 15 min. Supernatants, lacking mitochondria, were collected and incubated at 37°C in a buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, 0.1 CHAPS and 10 mM DTT with the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4- trifluoromethylcoumarin (DEVD-AFC) (15 μM in DMSO, Calbiochem System Products) (Jordan *et al*., 1997). Substrate cleavage emitted a fluorescent signal that was quantified in a fluorometer Perkin-Elmer (luminiscence-spectrophotometer LS50B) (excitation 400 nm, emission 505 nm). Enzymatic activity is expressed as arbitrary fluorescent units (AFU). Ruthenium Red (5 μM) or CsA (10 μM) was added 15 min before and maintained during $CaCl_2$ or KO_2 treatment.

Statistical analysis

Quantitative data are expressed as means ± s.e.m. and significance was determined by Student's *t*-test. Statistical significance was considered at the $P<0.05$ level.

Results

Superoxide induces brain mitochondrial swelling in a Ca^{2+}-independent manner

Induction of mitochondrial swelling in isolated brain mitochondria was monitored by following 540 nm absorbance (A_{540}) decrease (Bernardi *et al*., 1999). We used KO_2 as a source of O_2^- anions and corresponding degraded ROS products. KO_2 addition (5 μmol mg^{-1} mit) resulted in an extensive mitochondrial swelling (Figure 1a) that was greatly

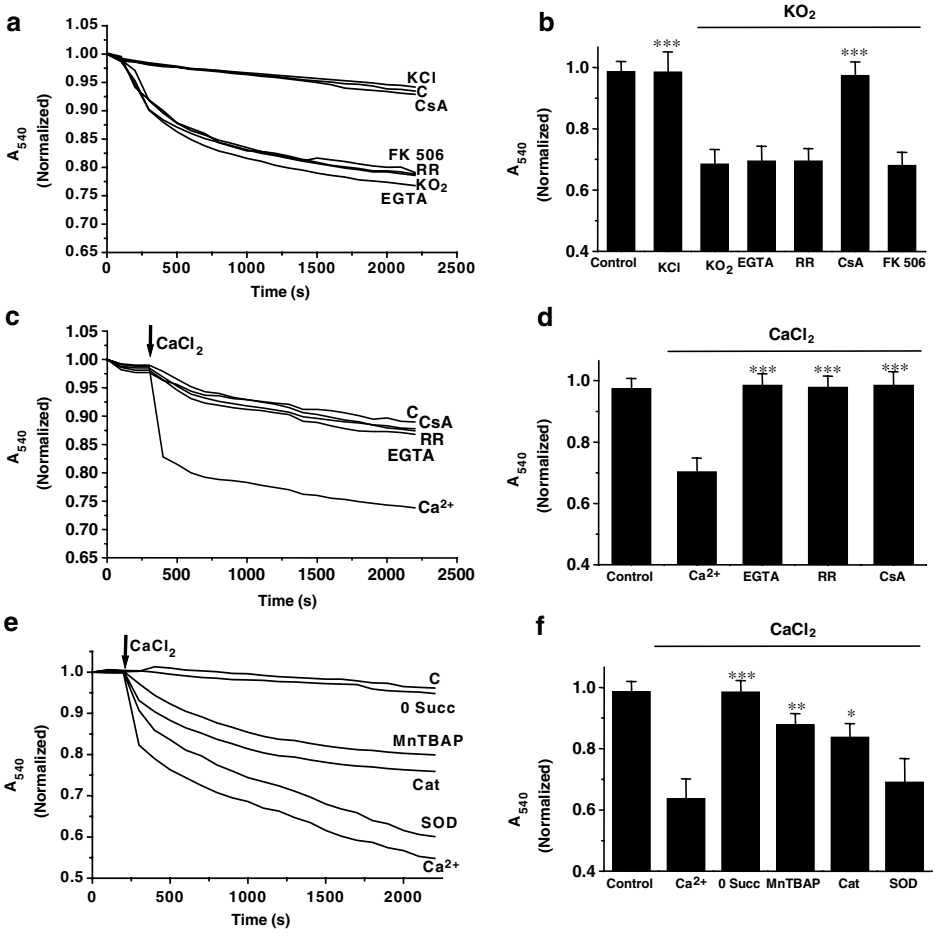

Figure 1 KO_2 and high Ca^{2+} induce mitochondrial swelling. A–B. KO_2 addition (5 μmol mg^{-1} protein; arrow), induces mitochondrial swelling in brain-isolated mitochondria in an MPTP sensitive manner, where Ca^{2+} cycling is not involved. Mitochondrial suspensions were incubated during 15 min with CsA (10 μM), FK 506 (200 nM), Ruthenium Red (RR; 5 μM), or EGTA (1 mM), prior to addition of KO_2. Control (c) and the effect of KCl (5 mM) addition on non-treated mitochondria are also shown. C–D. Calcium addition (15 μmol mg^{-1} mitochondria) induces mitochondrial swelling in brain-isolated mitochondria. Mitochondrial suspensions were incubated during 15 min with CsA (10 μM), Ruthenium Red (RR;5 μM), or EGTA (1 mM) before Ca^{2+} addition. Control non-treated mitochondria (c) are also shown. E–F. Ca^{2+}-induced swelling is ROS and succinate dependent. In absence of succinate (0 Succ), Ca^{2+} failed to induce mitochondrial swelling. Mitochondria were preincubated for 15 min with MnTBAP (10 nM), catalase (Cat;1750 U) or SOD (450 U), prior to Ca^{2+} addition (15 μmol mg^{-1} mit). Control nontreated mitochondria (c) are also shown. Lines represent mean values of one experiment performed by triplicate. Histograms (panels b, d, f) represent mean values ± s.e.m. of normalized A_{540} at 2200 s from at least five different mitochondrial preparations. $^{***}P<0.001$;$^{**}P<0.01$;$^{*}P<0.05$ as compared with KO_2 or Ca^{2+}-treated mitochondria in absence of the drug.

inhibited by the pretreatment and presence of 10 μM CsA, a MPTP blocker, but not by the calcineurin inhibitor FK506 (200 nM), (Figure 1b). Addition of KCl (5 mol mg^{-1} mit) did not result in mitochondrial swelling, confirming that KO_2-induced mitochondrial swelling was because of reactive reactive oxygen species (ROS) and not to potassium (Figure 1a).

Since a variety of chemically different pro-oxidants have been shown to induce Ca^{2+} release from mitochondria and the fact that Ca^{2+} induces mitochondrial swelling in liver mitochondria, we next investigated the existence of a positive feedback between O_2^- anions and Ca^{2+} in mitochondrial swelling. Blockade of Ca^{2+} influx, by the presence of the mitochondrial Ca^{2+} uptake blocker, Ruthenium Red, or by addition of the Ca^{2+} chelator EGTA (1 mM) did not affect KO_2-induced mitochondrial swelling, suggesting that Ca^{2+} does not seem to play a significant role in KO_2-induced mitochondrial swelling (Figure 1c). In contrast to noncerebral tissue, only relatively high Ca^{2+} concentrations induced brain mitochondrial swelling and required the presence of succinate in the mitochondrial resuspension medium, because Ca^{2+} failed to induce mitochondrial swelling in its absence (0 Succ, Figure 1c), while KO_2 did not (data not show). Even at these high Ca^{2+} concentrations, the Ca^{2+} Ca^{2+}-induced mitochondrial swelling was blocked by Ruthenium Red, EGTA, and CsA (Figure 1d). Lower Ca^{2+} concentrations did not produce significant swelling (data not shown), although we cannot exclude that mitochondrial swelling may even be underestimated, as some heavy microsomal membranes could not be entirely separated from mitochondria.

The succinate requirement for Ca^{2+} to induce mitochondrial swelling suggested a role for coenzyme Q as a significant generator of O_2^- (Kowaltowski *et al*., 1995). To test if O_2^- production takes place, in isolated mitochondria, during Ca^{2+} exposures, selective oxidation of hydroethidine, by O_2^-, to ethidium was measured. Brain mitochondria showed a basal O_2^- production rate of 0.0426 ± 0.0072 AFU min^{-1} ($n = 16$). Calcium (15 μmol mg^{-1} mit) caused an increase in the rate of O_2^- production reaching a value of 0.0675 ± 0.0153 AFU min^{-1} ($P < 0.05$; $n = 16$). To check whether this O_2^-, or its derived ROS, produced by Ca^{2+} in mitochondria, participated in Ca^{2+}-induced mitochondrial swelling, we tested the effects of different free radical scavengers. As shown in Figure 1e, MnTBAP, a manganese porphyrin with SOD mimic activity, or catalase addition (1750 U ml^{-1}) significantly attenuated Ca^{2+}-induced mitochondrial swelling, while addition of SOD (450 U ml^{-1}) did not block significantly Ca^{2+}-induced swelling (Figure 1f). Consistently with the utilization of KO_2 as a ROS source, the addition of antioxidant drugs decreased significantly KO_2-induced mitochondrial swelling (data not shown).

KO_2-induced mitochondrial swelling occurs in absence of $\Delta\psi_m$ depolarization

One of the most intriguing questions that remains unanswered is whether mitochondrial swelling produces $\Delta\psi_m$ depolarization or if $\Delta\psi_m$ dissipation precedes mitochondrial swelling. In the next set of experiments, we studied whether Ca^{2+} or O_2^- was able to modify $\Delta\psi_m$. We monitored $\Delta\psi_m$ changes by measuring the release of the cationic membrane-permeant fluorescent probe Rh 123 preloaded into mitochondria. Under these conditions, total fluorescence of mitochondrial suspension will increase if the organelles depolarize. As shown in Figure 2, Ca^{2+}, but not KO_2, induced mitochondrial depolarization. Calcium concentrations required to evoke $\Delta\psi_m$ depolarization were 20-fold lower than those necessary to induce mitochondrial swelling. Mitochondrial depolarization was dependent of Ca^{2+} influx, but independent of MPTP opening, since it was blocked by 15 min pretreatment with Ruthenium Red (5 μM) but not by CsA (10 μM) (Figure 2a). Nevertheless, KO_2 additions failed to influence $\Delta\psi_m$ within the same concentration range that caused mitochondrial swelling (Figure 2b).

Effects of high Ca^{2+} and O_2^- on mitochondrial enzymatic activity and NAD(P)H levels

The next set of experiments were performed to analyze the possible effects of Ca^{2+} and O_2^- on mitochondrial metabolic enzymatic activity.

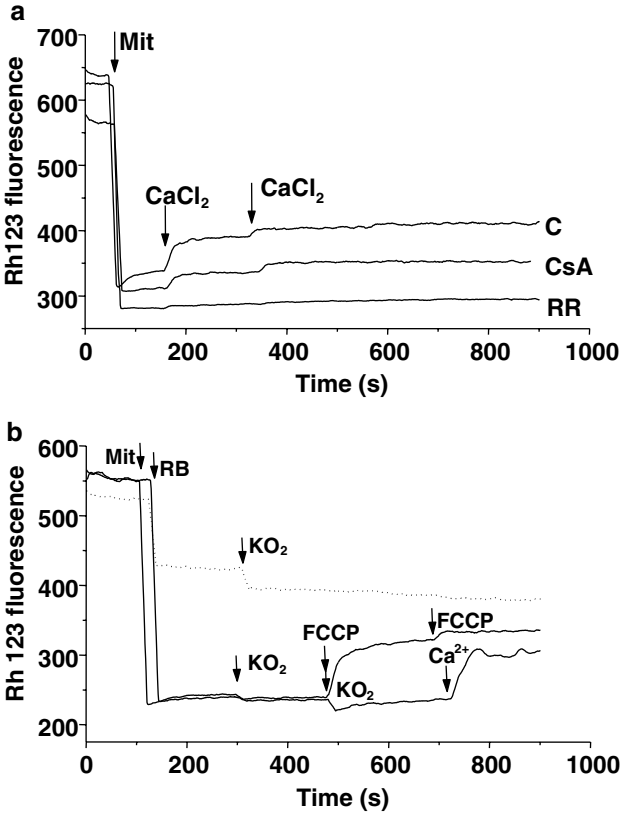

Figure 2 Changes in mitochondrial membrane potential as assessed by Rh 123. Fluorescence excited at 503 nm and emitted at 527 nm was measured in isolated mitochondria. (a) Addition of mitochondria (Mit, 500 μg) caused a decrease in fluorescence intensity because of quenching of Rh 123. $CaCl_2$ (0.75 μmol mg^{-1} mit) was added under control conditions (trace c). In traces RR and CsA, mitochondria were incubated with Ruthenium Red (5 μM, trace RR) or CsA (10 μM, trace CsA) for 15 min and then added to Rh 123 solution. (b) The protocol was similar, but two KO_2 (2.5 and 5 μmol mg^{-1} mit, continuous line) sequential pulses were added. Calcium was added to the incubation buffer to ensure that the mitochondria could be depolarized. Data are expressed as mean values obtained from one experiment performed in triplicate. Similar data were found in at least four different experiments. FCCP (0.1 μM) was added to induce mitochondrial depolarization. Dashed line represents control experiments performed by adding mitochondrial resuspension buffer (RB) and KO_2 solutions in absence of brain mitochondria.

Mitochondrial dehydrogenase activity was measured by determining the formation of MTT into formazan. Under our experimental conditions, control mitochondrial suspension (500 μg protein) had an enzymatic activity of 0.165 ± 0.036 ($n=4$) and was not affected by the treatment with Ca^{2+} (15 μmol mg^{-1} mit) for 30 min (0.145×0.034; $n=4$). However, 30 min incubation of intact mitochondria with KO_2 (5 μmol mg^{-1} mit) produced a marked decrease in dehydrogenase activity (0.052 ± 0.016; $P<0.001$; $n=4$).

Furthermore, the Krebs cycle enzyme citrate synthase was also affected by the presence of O_2^-. Mitochondrial citrate synthase was measured by determining the reaction of free CoA with DTNB. Incubation of brain mitochondria (500 μg protein) with KO_2 (5 μmol mg^{-1} mit) for 30 min, did result in a loss in enzymatic activity (0.175 ± 0.015 ($P<0.001$; $n=5$)) compared to control mitochondrial suspension (0.356 ± 0.026; $n=5$), while Ca^{2+} (15 μmol mg^{-1} mit; 30 min) did not alter citrate synthase activity (0.3494 ± 0.037; $n=5$).

Recently, NAD(P)H has been proposed to act as a direct antioxidant agent (Kirsch & De Groot, 2001). Therefore, we were interested in whether the NAD(P)H levels were modified by these treatments when using either O_2^- or Ca^{2+}. As Figure 3 illustrates, Ca^{2+} (15 μmol mg^{-1} mit) increases NAD(P)H levels in a Ruthenium Red (5 μM, 15 min)-sensitive manner, while, following KO_2 addition (5 μmol mg^{-1} mit), an initial rapid decline in mitochondrial NAD(P)H concentration was observed. Rotenone, a mitochondrial respiratory chain complex I inhibitor (10 μM, Rot) and the mitocondrial uncoupler, FCCP (1 μM, FCCP) were added to estimate the maximal and the minimum nucleotide signal, respectively (Figure 3).

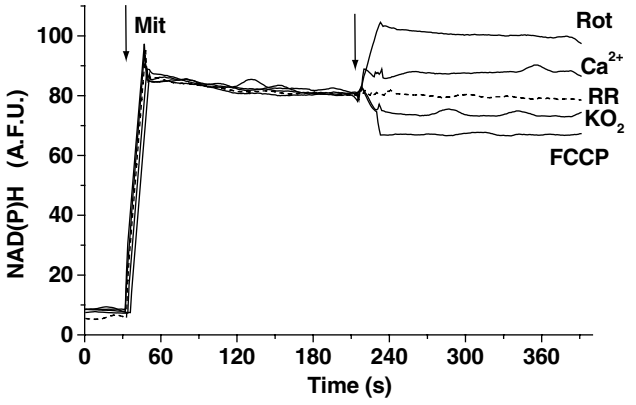

Figure 3 Effect of KO_2 and Ca^{2+} on mitochondrial NAD(P)H levels. NAD(P)H levels were monitored spectrofluorimetrically as described in methods. After 60 s of base line, intact mitochondria (500 μg) were added. Arrow indicates $CaCl_2$ (15 μmol mg^{-1} mit; Ca^{2+}), KO_2 (5 μmol mg^{-1} mit; KO_2), rotenone (10 μM, Rot), and FCCP (1 μM, FCCP) additions. Dashed line represents mitochondria preparation preincubated for 15 min in the presence of Ruthenium Red (5 μM; RR) before $CaCl_2$ addition. One single experiment is shown, but similar data were found in four to six different mitochondrial preparations.

O_2^- induces release of cytochrome c and caspase activity from mitochondria

A consequence of mitochondrial swelling is the release of intramitochondrial proteins like cytochrome *c* to the cytoplasm (Maestre *et al*., 2003). In order to confirm the occurrence of this intramitochondrial protein release in our model, we exposed isolated brain mitochondria for 30 min to $CaCl_2$ (15 μmol mg^{-1} mit) or KO_2 (5 μmol mg^{-1} mit) and tested for cytochrome *c* presence and DEVD-like caspase activity in the extramitochondrial medium. As shown in Figure 4a, direct evidence for cytochrome *c* release from rat brain mitochondria was provided by Western blotting technique of cytochrome *c* in the S100 supernatant, after treating mitochondria with either Ca^{2+} or O_2^-. We next examined whether DEVD caspase activity was released from isolated mitochondria following exposure to Ca^{2+} or KO_2. After these treatments, we performed measurements of the cleavage of a fluorometric caspase substrate, DEVD-AFC (Figure 4b). Increases in DEVD-caspase activity were found in S100 after Ca^{2+} and O_2^- additions (Figure 4b). It is worthy noting that in both assays, O_2^- effect was higher than Ca^{2+}, perhaps because of the the fact that Ca^{2+} might inhibit caspase activity, as seems to happen in Ca^{2+}-mediated neuronal death (Reimertz *et al*., 2001). Consistently with the mitochondrial swelling results, this effect was abolished by CsA, whereas Ruthenium Red was only effective against Ca^{2+} treatments (Figure 4b).

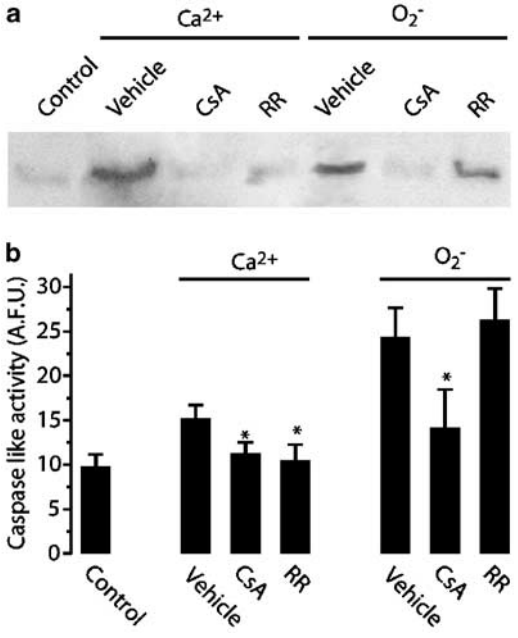

Figure 4 Ca^{2+} and O_2^- addition induced release of cytochrome *c* and caspase activity from mitochondria. (a) Mitochondria were incubated during 30 min with resuspension (control), $CaCl_2$ (15 μmol mg^{-1} mit), or KO_2 (5 μmol mg^{-1} mit) and then centrifuged for 15 min at 15,000 rpm at 4°C (S100; see Methods section). For pharmacological studies, mitochondrial suspensions were pretreated for 15 min with Ruthenium Red (RR, 5 μM) or CsA (10 μM) before and during exposures. (a) Representative gel of S100 protein precipitated overnight with 10% TCA is shown. Pellets were subjected to polyacrylamide gel electrophoresis and immunoblot analysis using an antibody that recognizes cytochrome *c*. (b) S100 supernatant was assayed for caspase activity for 30 min. Data are represented as mean s.d. ($n=4$). *$P<0.05$ as compared with respective controls (vehicle).

Discussion

Changes in Ca^{2+} influx and O_2^- production within the mitochondria prior to cell death are events shared by cells treated with neurotoxic agents such as NMDA, veratridine and β-amyloid (Bindokas *et al*., 1996; Jordan *et al*., 2000; Longo *et al*., 2000). Evidence has been presented that intracellular O_2^- production during apoptosis is Ca^{2+} dependent and of mitochondrial origin (Ankarcrona *et al*., 1995; Bindokas *et al*., 1996; Jordan *et al*., 2000). Herein, we used brain-isolated mitochondria to study the effects of Ca^{2+} and O_2^- on organelle parameters such as swelling, $\Delta\psi_m$, enzymatic activity, NAD(P)H and protein release.

The present data show that superoxide anions and Ca^{2+} produce brain mitochondrial swelling. Moreover, when we analyzed the implication of these two second messengers in mitochondrial swelling, we found that while O_2^--induced swelling was Ca^{2+}-independent, ROS participated significantly in Ca^{2+}-induced mitochondrial swelling. Neither, Ca^{2+} free solution nor the presence of the Ca^{2+} uniporter blocker, Ruthenium Red, modified O_2^--induced mitochondrial swelling. In contrast to noncerebral tissue, and in line with previous reports, only high Ca^{2+} concentration induced mitochondrial swelling (Berman *et al*., 2000; Schild *et al*., 2001; Brustovetsky *et al*., 2002). Moreover, Ca^{2+}, even at these concentrations, required succinate in the resuspension solution to induce brain mitochondrial swelling, suggesting either its requirement for mitochondrial calcium uptake (Votyakova & Reynolds, 2001) or a role for coenzyme Q as a generator of O_2^- (Kowaltowski *et al*., 1995). This correlates well with the increase in superoxide production detected with the specific oxidation sensitive probe, hydroethidine, in mitochondrial preparations exposed to Ca^{2+}. Indeed, Ca^{2+}- induced swelling involved a ROS-dependent component, since the superoxide dismutase mimetic, MnTBAP, and catalase markedly decreased Ca^{2+}-induced swelling.

The membrane permeable SOD mimetic MnTBAP seemed to be more efficient than the poorly membrane permeable SOD to block Ca^{2+}-induced mitochondrial swelling. This difference can be explained, in terms of product compartmentalization by either of the following reasons: (i) O_2^- formed inside the mitochondria does not pass through mitochondrial membranes and SOD was less efficient, since it was added to the extramitochondrial compartment; (ii) SOD poorly penetrates into the mitochondria being unable to intercept the generated O_2^-; and (iii) excess O_2^- was degraded to H_2O_2 by MnSOD and exceeded mitochondrial catalase capacity to detoxify it. Consistent with the latter reason, mitochondria treatment with catalase significantly reduced Ca^{2+}-induced swelling (Figure 1c).

We have also measured the effects of O_2^- and supraphysiological Ca^{2+} on mitochondrial metabolic activity. Superoxide anions, or its related ROS, compromised citrate synthase and dehydrogenase enzymatic activities. These effects seem to be more related to oxidation of the enzymes than to mitochondrial swelling, since Ca^{2+} did not modify them. Our observations might indicate that ROS are the second messengers involved in the decrease of these enzymatic activities showed in several neurodegenerative models, like mice deficient in MnSOD (Melov *et al*., 1999). Moreover, KO_2 additions dropped total mitochondrial NAD(P)H levels. This is interesting because NAD(P)H, besides acting indirectly as an antioxidant in the reduction of GSSG to GSH, operates as an antioxidant in the matrix space, both by scavenging toxic free radicals and repairing biomolecule-derived radicals (Kirsch & De Groot, 2001). Interestingly, Ca^{2+} increased NADPH autofluorescence. This is consistent with the enhanced activity of Ca^{2+}-dependent intramitochondrial enzymes, described in dissociated mouse sensory neurons (Duchen, 1992). This increase was blocked by the addition of Ruthenium Red, suggesting the dependence of the response on mitochondrial Ca^{2+} uptake (Duchen, 1992).

Under our experimental conditions Ca^{2+}, but not O_2^-, produced a rapid loss of $\Delta\psi_m$ that was inhibited by Ruthenium Red, suggesting that Ca^{2+} mediates this mitochondrial effect after its uptake into the mitochondria. Indeed, our study reveals that Ca^{2+}-triggered $\Delta\psi_m$ depolarization appeared to be MPTP independent, since CsA did not block this depolarization. These results are in agreement with observations on mitochondrial swelling that postulate that Ca^{2+} acts through a voltage sensor, while ROS-induced swelling results from an oxidative action (Petronilli *et al*., 1993a, b). It has also been shown that CsA has no effect on $\Delta\psi_m$ depolarization after strong depolarizations (Brustovetsky & Dubinsky, 2000).

The lack of O_2^- effects on $\Delta\psi_m$ observed here are consistent with the fact that mitochondrial depolarization is not required for the induction of apoptosis in models where an overproduction of O_2^- or the release of cytochrome *c* takes place (Petronilli *et al*., 1993a, b; Kluck *et al*., 1997; Vander Heiden *et al*., 1997; Krohn *et al*., 1998; Chiu & Oleinick, 2001) like hippocampal neurons treated with staurosporine (Krohn *et al*., 1999) or neurons subjected to growth factor withdrawal (Kluck *et al*., 1997). In other models, protein release happens before $\Delta\psi_m$ depolarization, indicating that disruption of the outer membrane, but not the inner mitochondrial membrane, is the primary structural alteration (Vander Heiden *et al*., 1997), and that the electron-transport chain can maintain the $\Delta\psi_m$ even after cytochrome *c* has been released (Goldstein *et al*., 2000). Moreover, it has been described that O_2^--induced cytochrome *c* release does not produce $\Delta\psi_m$ loss (Madesh & Hajnoczky, 2001) and in other models as RAW 264.7 cells, ROS-induced cytochrome *c* release is accomplished without relevant changes of $\Delta\psi_m$ (Hortelano *et al*., 1999).

In agreement with the above data, both Ca^{2+}-and O_2^--induced swelling resulted in the release of mitochondrial intermembrane proteins. We found that, after mitochondrial swelling, the release of a cytochrome *c* and the activation of DEVD-caspase took place in isolated mitochondria. This release was sensitive to the presence of CsA suggesting that it was mediated by MPTP. This protein release has been observed in neurodegenerative models, such as focal ischemia and Parkinson's disease (Namura *et al*., 2001; Viswanath *et al*., 2001) and has been correlated with the point-of-no-return for cell death pathways (Hirsch *et al*., 1998). Although the synaptosome contamination can overestimate DVED-like caspase activity, our observations are consistent with previous results from Yuan *et al*. (2001) which showed that mitochondrial caspases are regulated independently of the cytosolic pool of caspases, and that staurosporine treatment led to preferential autoprocessing of caspase-9 associated with mitochondria. On the other hand, Ca^{2+}-induced smaller cytochrome *c* and DVED-like caspase activity release from mitochondria than KO_2, perhaps throughout calpain-I activation that has been shown to inhibit cytochrome *c* release and caspase activation in a cell-free system (Lankiewicz *et al*., 2000).

In summary, our data provide direct information and detailed evidence on how superoxide acts on mitochondria to initiate apoptotic pathways, and that these changes are initiated in the absence of mitochondrial depolarization. However, more work is necessary to identify the mechanism that could explain how MPTP might be activated without loss of mitochondrial potential.

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