Peroxynitrite Stimulates the Pyridine Nucleotide-Linked Ca^{2+} Release from Intact Rat Liver Mitochondria†

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ABSTRACT: Rat liver mitochondria contain a specific Ca^{2+} release pathway which operates when oxidized mitochondrial pyridine nucleotides are hydrolyzed in a Ca^{2+}-dependent manner to ADP-ribose and nicotinamide. We have previously shown that NAD^+ hydrolysis is inhibited by cyclosprin A and is possible only when some vicinal thiols are cross-linked. Here we report that the thiol oxidant peroxynitrite ($ONOO^-$), which can form from nitric oxide (nitrogen monoxide, $NO^•$) and superoxide anion (O_2^-), at low concentrations stimulates the specific Ca^{2+} release pathway. Both peroxynitrite-induced pyridine nucleotide hydrolysis and Ca^{2+} release are inhibited by cyclosporin A, and peroxynitrite is ineffective when pyridine nucleotides are kept reduced. Ca^{2+} release induced by peroxynitrite occurs with maintenance of the mitochondrial membrane potential and is not accompanied by entry of sucrose into mitochondria. The results suggest that peroxynitrite stimulates the specific Ca^{2+} release from intact mitochondria by modifying critical mitochondrial thiols other than glutathione in such a way that hydrolysis of oxidized pyridine nucleotides is achieved. These findings provide further insight into the regulation of Ca^{2+} release from mitochondria by nitric oxide and its congeners.

Changes in the cytosolic Ca^{2+} level control key cellular events, such as muscle contraction, secretion, neuronal activity, and modulation of hormone action. The intracellular Ca^{2+} concentration is regulated by binding of Ca^{2+} to nonmembraneous proteins, by membrane-bound Ca^{2+}-ATPases located primarily in the plasma, nuclear, and endoplasmic reticular membrane, and by mitochondria (Carafoli, 1987). Until recently, mitochondria were regarded mainly as a safety device against potentially toxic increases of cytosolic Ca^{2+} [reviewed in Richter and Kass (1991)] because their affinity for Ca^{2+} was thought to render them insufficient to compete with other membrane-bound transport systems. However, there is now compelling evidence that the mitochondrial contribution to Ca^{2+} buffering is not limited to pathological situations. Rather, it plays an important role in shaping physiological Ca^{2+} transients and in the maintenance of cellular Ca^{2+} homeostasis (Rizzuto et al., 1994; Jouaville et al., 1995) and provides an efficient mechanism for modulating the activity of mitochondrial enzymes upon cell stimulation (McCormack et al., 1990; Richter, 1992).

Mitochondria take up and release Ca^{2+} *via* different pathways. As a consequence, Ca^{2+} can be "cycled" across their inner membrane (Carafoli, 1979). The mitochondrial membrane potential ($\Delta\Psi$),[1] which is maintained either by respiration or by ATP hydrolysis, is the driving force for the uptake of Ca^{2+} by mitochondria. Release of Ca^{2+} occurs either by nonspecific leakage through the inner membrane, *i.e.*, when $\Delta\Psi$ collapses, or by the specific Ca^{2+} release pathway with preservation of $\Delta\Psi$ (Richter et al., 1992). The latter pathway requires intramitochondrial Ca^{2+} and NAD^+, which is hydrolyzed enzymatically to ADP-ribose and nicotinamide [reviewed in Richter and Schlegel (1993)]. NAD^+ hydrolysis is under the control of cyclophilin (*i.e.*, is inhibited by cyclosporin A, CSA) (Schweizer et al., 1993), and is only possible when some vicinal thiols are cross-linked, either by oxidation (Schweizer & Richter, 1994a) or by reaction with phenylarsine oxide (Schweizer et al., 1994).

Peroxynitrite ($ONOO^-$), the product of the reaction between nitric oxide (nitrogen monoxide, $NO^•$) and superoxide anion (O_2^-) (Beckman et al., 1990), reacts with lipids, aromatic amino acids, or metalloproteins, *e.g.*, complex I and II of the respiratory chain and aconitase (Darley-Usmar & Radomski, 1994). Because $ONOO^-$ was also reported to oxidize vicinal thiols to the corresponding disulfide (Radi et al., 1991), we tested whether it can stimulate Ca^{2+} release from mitochondria, and if so, by which mechanism. We now report that peroxynitrite stimulates Ca^{2+} release from isolated rat liver mitochondria by activating the specific Ca^{2+} release pathway.

MATERIALS AND METHODS

Materials. Peroxynitrite was synthesized according to Kissner *et al.* (1995), provided by Drs. J. S. Beckman and R. Kissner, and stored at −80 °C. Its concentration was determined photometrically at 302 nm in 0.1 M sodium hydroxide ($\epsilon_{mM} = 1.67$; Beckman et al., 1994) daily prior to use. Cyclosporin A (CSA) was a gift of Sandoz Pharma Preclinical Research, Basel, Switzerland. It was stored in solid form at −20 °C and dissolved in ethanol immediately prior to use. $^{45}Ca^{2+}$ was from Amersham International, Buckinghamshire, U.K., and [u-^{14}C]sucrose was from New England Nuclear, Boston, MA. All other chemicals were purchased from standard suppliers and were of the highest purity available.

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CSA, cyclosporin A; $\Delta\Psi$, electrical potential across the inner mitochondrial membrane, negative inside; DTT, dithiothreitol; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; arsenazo III, 3,6-bis[(2-arsonophenyl)azo]-4,5-dihydroxy-2,7-naphthalenedisulfonic acid.

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Isolation of Mitochondria. The isolation of rat liver mitochondria was performed by differential centrifugation (Schlegel et al., 1992). The protein content was determined by the Biuret method with bovine serum albumin as standard.

Standard Incubation Procedure. Mitochondria (2 mg of protein/mL) were incubated at 25 °C with continuous stirring and oxygenation in 3 mL of 210 mM mannitol, 70 mM sucrose, and 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.2. The high concentration of Hepes was used to prevent pH changes upon addition of the alkaline peroxynitrite solution.

Determination of Ca^{2+} Uptake and Release by Mitochondria. The standard incubation procedure was followed. After addition of rotenone (5 μM) and potassium succinate (2.5 mM), mitochondria were loaded with Ca^{2+}. Its movement across the inner mitochondrial membrane was monitored by the spectrophotometric or isotope technique (Millipore filtration) (Lötscher et al., 1980a; Schlegel et al., 1992). The former was performed in the presence of 50 μM arsenazo III and the latter with $^{45}Ca^{2+}$. Ca^{2+} was added to give a total load as indicated in the figure legends, and its uptake was allowed to proceed for 2−3 min.

Determination of the Mitochondrial Membrane Potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine. After addition of rotenone (5 μM) and potassium succinate (2.5 mM), they were loaded with Ca^{2+}. $\Delta\Psi$ was determined in an Aminco DW2A spectrophotometer at 511−533 nm (Åkerman & Wikström, 1976). Other compounds were added as indicated in the figure legends.

Spectrophotometric Analysis of Mitochondrial Pyridine Nucleotides. The standard incubation procedure was followed. After addition of rotenone (5 μM) and potassium succinate (2.5 mM), the absorption of mitochondrial pyridine nucleotides was determined in an Aminco DW2A spectrophotometer at 340−370 nm (Schweizer et al., 1993). Other compounds were added as indicated in the figure legends.

Sucrose Entry. Sucrose entry into mitochondria was followed by the isotope technique in combination with filtration (Schlegel et al., 1992). Mitochondria were incubated according to the standard procedure in the presence of [u-^{14}C]sucrose (0.25 μCi/mL) and energized with potassium succinate in the presence of rotenone. Aliquots of 150 μL were withdrawn at the times indicated in the figure legends and analyzed for radioactivity with the Millipore filtration technique described above.

Measurement of Mitochondrial Swelling. Swelling of mitochondria incubated according to the standard procedure was monitored continuously as changes in OD_{540}.

Oxygen Uptake was measured with a Clark-type electrode as described (Frei et al., 1985).

RESULTS

Stimulation by Peroxynitrite of Ca^{2+} Release. Peroxynitrite stimulates Ca^{2+} release from mitochondria as shown by the spectrophotometric (Figure 1A,B) and isotope techniques (Figure 1C). The stimulation is dose-dependent (Figure 1A) and with a given peroxynitrite concentration is dependent on the mitochondrial Ca^{2+} load (Figure 1B). Peroxynitrite was equally effective (result not presented) in inducing Ca^{2+} release from glutathione-depleted (Traber et al., 1992) and glutathione-adequate mitochondria, showing that it does not engage the enzyme cascade glutathione peroxidase/glutathione reductase/energy-linked transhydrogenase (Lötscher et al., 1979). CSA (1 μM) (Figure 1A) or ATP (1 mM) (Figure 1C) completely inhibited the release induced by 500 or 350 μM peroxynitrite, respectively.

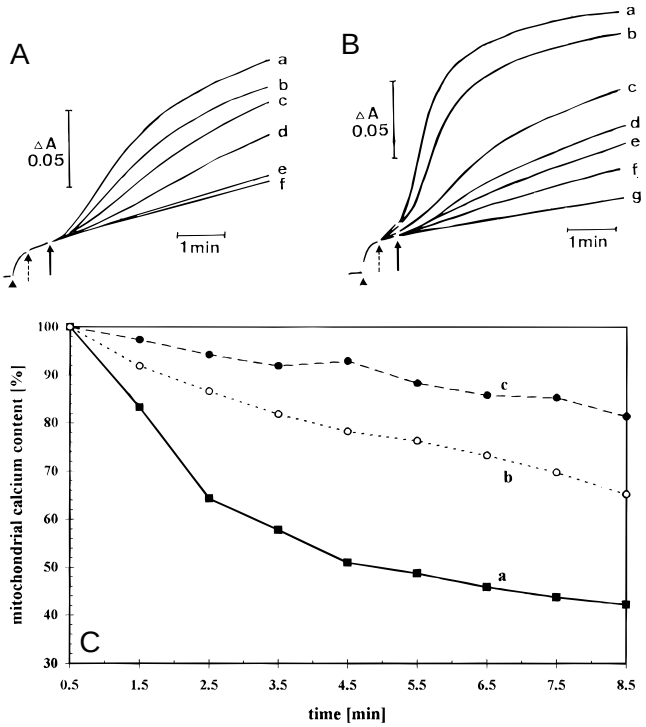

FIGURE 1: Peroxynitrite-stimulated Ca^{2+} release from rat liver mitochondria. (A) Spectrophotometric assay. Mitochondria were incubated according to the standard procedure in the absence (traces a−d) or presence (traces e and f) of 1 μM CSA. After addition of rotenone and potassium succinate they were loaded with 40 nmol of Ca^{2+}/mg of protein. At the arrowhead, ruthenium red (2 nmol/mg of protein), at the dashed arrow, 1 mM acetoacetate, and at the solid arrow, 500 μM (traces a and e), 350 μM (trace b), 200 μM (trace c), or 100 μM (trace d) peroxynitrite were added; trace f, vehicle alone. (B) Spectrophotometric assay. Mitochondria were incubated according to the standard procedure. After addition of rotenone and potassium succinate they were loaded with 60 (trace a), 50 (trace b), 40 (trace c), 30 (trace d), 20 (trace e), 10 (trace f), or 0 (trace g) nmol of exogenous Ca^{2+}/mg of protein. At the arrowhead, ruthenium red (2 nmol/mg of protein), at the dashed arrow, 1 mM acetoacetate, and at the solid arrow, 200 μM peroxynitrite were added. (C) Isotope technique. Mitochondria were incubated according to the standard procedure. After addition of rotenone and potassium succinate mitochondria were loaded with 50 nmol of Ca^{2+}/mg of protein; 3 min thereafter (time 0 min) 10 mM EGTA was added, and 30 s later 1 mM acetoacetate alone (traces a and b) or in combination with 1 mM ATP (trace c) was added. Thirty seconds thereafter (time 1 min), 350 μM peroxynitrite (traces a and c) or its vehicle (trace b) was added. The results shown are the average of three independent experiments. The standard deviation never exceeded 10%.

Intactness of Mitochondria during Peroxynitrite-Induced Ca^{2+} Release. The determination of $\Delta\Psi$ is useful to assess the intactness of mitochondria during and after Ca^{2+} release (Baumhüter & Richter, 1982; Andreeva & Crompton, 1994). Upon addition of peroxynitrite to mitochondria loaded with Ca^{2+} (50 nmol/mg of protein), $\Delta\Psi$ changes depend on whether or not mitochondria are allowed to cycle Ca^{2+} (Figure 2). Thus, $\Delta\Psi$ soon decreases after addition of 500 μM peroxynitrite in the absence of EGTA (trace c) but increases in its presence (trace a), *i.e.*, when Ca^{2+} cycling is prevented. Addition of the uncoupler CCCP completely

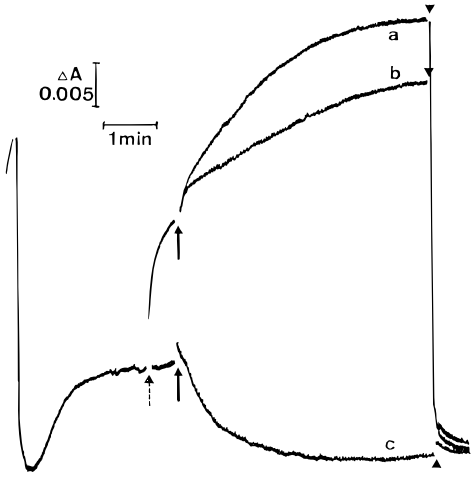

FIGURE 2: Peroxynitrite-induced alterations of the mitochondrial membrane potential. Mitochondria were incubated according to the standard procedure in the presence of 10 μM safranine and rotenone and were energized with potassium succinate. Two and a half minutes after addition of Ca^{2+} (50 nmol/mg of protein), 10 mM EGTA (dashed arrow) (traces a and b) was added, and 30 s later, 500 μM peroxynitrite (traces a and c) or its vehicle (trace b) was added (solid arrows). At the arrowheads, the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (1 μM) was added. The results shown are a typical experiment out of three.

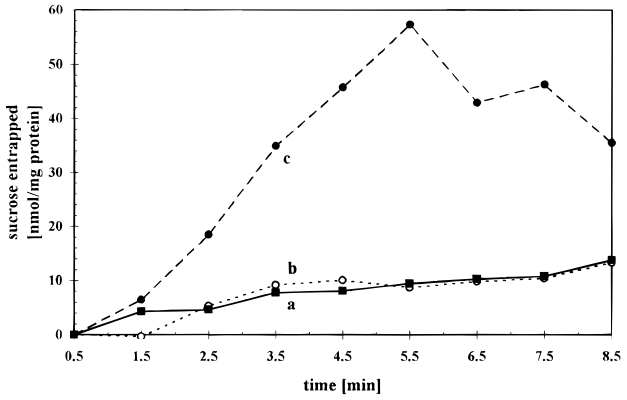

FIGURE 3: Peroxynitrite-induced sucrose entry into mitochondria in the presence or absence of EGTA. Mitochondria were incubated according to the standard procedure and energized with potassium succinate in the presence of rotenone. They were loaded with 60 nmol of Ca^{2+}/mg of protein; 2.5 min thereafter (time 0 min), 10 mM EGTA (lanes a and b) was added, and 30 s later 1 mM acetoacetate was added. Thirty seconds later (time 1 min), 350 μM peroxynitrite (traces a and c) or its vehicle (trace b) was added. Samples were analyzed for sucrose entry as described in the Materials and Methods section. The results shown are the average of three independent experiments. Values of trace c are significantly different from those of traces a and b starting at 3.5 min ($p < 0.01$; ANOVA calculated with Instat 2.05a).

collapses $\Delta\Psi$, showing that mitochondria were fully energized during and after Ca^{2+} release. The rise of $\Delta\Psi$ upon addition of peroxynitrite in the presence of EGTA parallels the loss of previously accumulated Ca^{2+} since the steady-state level of $\Delta\Psi$ depends on the amount of Ca^{2+} in the mitochondrial matrix (Lötscher et al., 1980b). Besides $\Delta\Psi$ determinations, the measurement of sucrose entry into mitochondria is a reliable test for the intactness of the inner mitochondrial membrane (Al-Nasser & Crompton, 1986). Addition of peroxynitrite to Ca^{2+}-loaded mitochondria stimulates sucrose entry into mitochondria (Figure 3, trace c), but EGTA alone (Figure 3, trace b) or peroxynitrite in combination with EGTA (Figure 3, trace a) does not. Also,

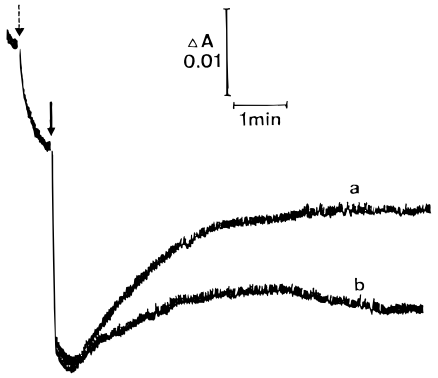

FIGURE 4: Peroxynitrite-induced oxidation of mitochondrial pyridine nucleotides and their re-reduction. Mitochondria were incubated according to the standard procedure in the presence (trace a) or absence (trace b) of 1 μM CSA. Changes in the redox state of mitochondrial pyridine nucleotides were monitored at 340−370 nm. After addition of rotenone and potassium succinate, mitochondria were loaded with 60 nmol of Ca^{2+}/mg of protein. Two and a half minutes after Ca^{2+} uptake, 10 mM EGTA was added, and 30 s later 1 mM acetoacetate (dashed arrow) followed by 500 μM peroxynitrite (solid arrow) was added. The results shown are a typical experiment out of four.

no peroxynitrite-induced mitochondrial swelling occurred under these conditions (results not shown).

Fate of Mitochondrial Pyridine Nucleotides. Ca^{2+} release from intact rat liver mitochondria *via* the specific pathway is linked to the hydrolysis of intramitochondrial oxidized pyridine nucleotides (Richter & Kass, 1991). Therefore, we determined spectrophotometrically to what extent the peroxynitrite-dependent oxidation of pyridine nucleotides is reversible (Figure 4) in order to gauge their hydrolysis (Lötscher et al., 1980a; Schweizer et al., 1993). Peroxynitrite evokes a transient decrease of pyridine nucleotide absorption measured at 340−370 nm in Ca^{2+}-loaded mitochondria (Figure 4). The decrease is almost completely reversible in the presence (trace a) but only partially reversible in the absence (trace b) of 1 μM CSA, indicating that CSA inhibits the hydrolysis of oxidized pyridine nucleotides (Richter et al., 1990) induced by peroxynitrite.

The direct determination of pyridine nucleotide hydrolysis, assayed by nicotinamide release from mitochondria (Lötscher et al., 1980a), revealed that peroxynitrite stimulates in a Ca^{2+}-dependent manner the hydrolysis of oxidized pyridine nucleotides, which is completely inhibited by 1 μM CSA, by 1 mM ATP, or in the absence of Ca^{2+} (not shown).

Since oxidation followed by hydrolysis of pyridine nucleotides is a prerequisite for Ca^{2+} release from intact mitochondria (Richter, 1992), we tested whether the oxidation state of the intramitochondrial pyridine nucleotides influences the peroxynitrite-stimulated Ca^{2+} release (Figure 5). Oxidation of pyridine nucleotides in succinate-energized mitochondria by acetoacetate in the presence of the complex I-inhibitor rotenone strongly stimulates the peroxynitrite-induced Ca^{2+} release (Figure 5A). In contrast, reduction of pyridine nucleotides by β-hydroxybutyrate in the presence of rotenone inhibits Ca^{2+} release stimulated by peroxynitrite (Figure 5B). Figure 5B also shows that β-hydroxybutyrate (trace d) inhibits the Ca^{2+} release induced by ruthenium red alone (trace c), indicating that at least a part of this "spontaneous" Ca^{2+} release occurs *via* the same Ca^{2+}-specific pathway. Again, $\Delta\Psi$ measurements in the presence of acetoacetate or β-hydroxybutyrate corroborate these results:

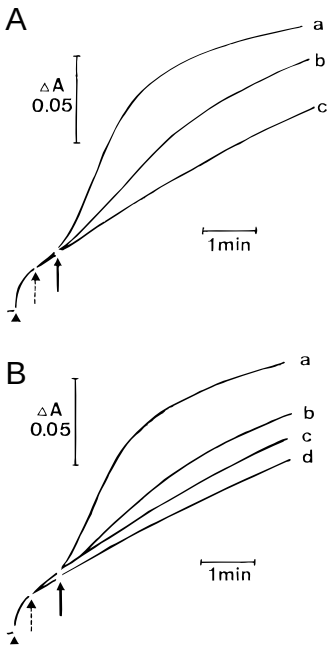

FIGURE 5: Peroxynitrite-stimulated Ca^{2+} release from rat liver mitochondria modulated by the pyridine nucleotide redox state. Mitochondria were incubated according to the standard procedure, and the redox state of their pyridine nucleotides was followed spectrophotometrically. (A) After addition of rotenone and potassium succinate, mitochondria were loaded with 40 nmol of Ca^{2+}/mg of protein. At the arrowhead, ruthenium red (2 nmol/mg of protein), at the dashed arrow, 0.5 mM acetoacetate (traces a and b), and at the solid arrow, 350 μM peroxynitrite (trace a) or its vehicle (traces b and c) were added. (B) After addition of rotenone and potassium succinate, mitochondria were loaded with 60 nmol of Ca^{2+}/mg of protein. At the arrowhead, ruthenium red (2 nmol/mg of protein), at the dashed arrow, 2 mM β-hydroxybutyrate (traces b and d), and at the solid arrow, 500 μM peroxynitrite (traces a and b) or its vehicle (traces c and d) were added. The results shown are a typical experiment out of five.

The rise in $\Delta\Psi$ upon the addition of peroxynitrite in the presence of EGTA is accelerated by acetoacetate and is largely prevented by β-hydroxybutyrate (not shown).

Oxygen Consumption by Rat Liver Mitochondria in the Presence of Peroxynitrite. Addition of up to 500 μM peroxynitrite did not significantly inhibit respiration in succinate-energized mitochondria in the presence of rotenone (not shown). The sharp decrease in the O_2 content of the incubation solution observed upon peroxynitrite addition originates from its reaction with buffer components, *e.g.*, sucrose or Hepes, which form peroxides (Radi et al., 1994; J. S. Beckman, personal communication). The peroxynitrite-stimulated Ca^{2+} release was not dependent on the incubation medium, because Ca^{2+} release was as extensive in a potassium chloride, Tris-based buffer system (100 mM KCl and 20 mM Tris, pH 7.2), in which peroxynitrite does not trigger oxygen consumption (not shown).

DISCUSSION

$NO^{\bullet}$ can combine with O_2^- at a near diffusion-limited rate to form the potent oxidant peroxynitrite ($ONOO^-$) (Beckman et al., 1990; Huie & Padmaja, 1993). The *in vivo* formation of peroxynitrite was recently shown in macrophages and other cells (Ischiropoulos et al., 1992; Carreras et al., 1994; Kooy & Royall, 1994). Its production is implicated in the pathophysiology of diseases such as acute endotoxemia, inflammatory bowel disease, acute lung injury, neurological disorders, *e.g.*, amyotrophic lateral sclerosis, and atherosclerosis (Darley-Usmar et al., 1995; Kooy et al., 1995). Peroxynitrite disrupts mitochondrial Ca^{2+} homeostasis *via* an unknown mechanism (Packer & Murphy, 1994), oxidizes lipids, metalloproteins, and DNA, and nitrates tyrosine residues (Darley-Usmar & Radomski, 1994; deRojas-Walker et al., 1995). Additionally, peroxynitrite was reported to mediate the oxidation of non-protein and protein sulfhydryls (Radi et al., 1991). For example, it reversibly downregulates *N*-methyl-D-aspartate receptor activity by oxidizing sulfhydryl groups of its redox modulatory site (Lipton et al., 1995). Because NAD^+ hydrolysis followed by Ca^{2+} release from intact mitochondria is only possible when some vicinal thiols are cross-linked (Schweizer & Richter, 1994a; Schweizer et al., 1994), we determined whether peroxynitrite can stimulate Ca^{2+} release from mitochondria.

We find that peroxynitrite indeed stimulates Ca^{2+} release from rat liver mitochondria. The organelles remain intact during and after Ca^{2+} release, provided Ca^{2+} cycling is prevented either by inhibiting its re-uptake by ruthenium red or by chelating extramitochondrial Ca^{2+} with EGTA. This is concluded from the observations that (i) $\Delta\Psi$ does not collapse after the addition of peroxynitrite in the presence of EGTA but rather increases in parallel to the loss of Ca^{2+} ions from the mitochondrial matrix, (ii) succinate-driven respiration is not inhibited, (iii) sucrose does not enter mitochondria, nor (iv) do the organelles swell, provided Ca^{2+} cycling is prevented. This unequivocally shows that peroxynitrite stimulates a Ca^{2+}-specific release pathway. Consistent with this, the action of peroxynitrite is effectively blocked by ATP and CSA, known to inhibit the hydrolysis of oxidized pyridine nucleotides (Hofstetter et al, 1981; Schweizer et al., 1993). The finding that the stimulation of Ca^{2+} release is independent of the mitochondrial glutathione status shows that peroxynitrite affects an event distal of the glutathione/pyridine nucleotide−enzyme cascade (Lötscher et al., 1979).

Stimulation of Ca^{2+} release from mitochondria by peroxynitrite is reversed by the addition of 1 mM DTT (not shown). Since Peroxynitrite has a half-life of a few seconds at physiological pH (Beckman et al., 1994), the reversibility of the peroxynitrite-stimulated Ca^{2+} release by DTT corroborates the data showing that NAD^+ hydrolysis is only possible when vicinal thiols are cross-linked (Schweizer & Richter, 1994a; Schweizer et al., 1994). Similarly, the *t*-butylhydroperoxide-induced Ca^{2+} release from intact mitochondria (Lötscher et al., 1980a) is inhibited by DTT (unpublished observation), pig brain NADase is inactivated by DTT as a consequence of the reduction of an essential protein disulfide group (Cayama et al., 1973), and DTT inhibits the NADase activity of CD38 (Kontani et al., 1993), a human leukocyte cell surface antigen.

Hydrolysis of oxidized pyridine nucleotides is a prerequisite for the Ca^{2+}-specific release mechanism. Accordingly, the peroxynitrite-stimulated Ca^{2+} release is accelerated by an increased intramitochondrial NAD^+ content, *i.e.*, after the addition of acetoacetate, and is inhibited by β-hydroxybutyrate, which keeps the pyridine nucleotides in a more reduced state. It should be noted here that between 14% and 43% of the pyridine nucleotides are oxidized in Ca^{2+}-loaded mitochondria in the presence of rotenone and succinate (Lötscher et al., 1979; Frei and Richter, unpublished observation). Pyridine nucleotide hydrolysis is deduced from

the absorption difference at 340−370 nm in the presence and absence of CSA when the peroxynitrite-induced Ca^{2+} release is terminated, and is confirmed by the direct measurement of nicotinamide release from mitochondria.

Our results strongly suggest that peroxynitrite stimulates the specific Ca^{2+} release pathway from intact rat liver mitochondria by oxidizing some critical thiols other than glutathione in such a way that hydrolysis of oxidized pyridine nucleotides is achieved. Such thiols may reside on a protein catalyzing pyridine nucleotide hydrolysis (*e.g.*, a NAD^+ glycohydrolase) or on a molecule regulating such an enzyme. The concentrations of peroxynitrite used in this study are well within the physiological range. Thus, a bolus of 250 μM peroxynitrite is equivalent to the exposure of 1 μM peroxynitrite for only 7 min (Beckman et al., 1994; Radi et al., 1991). Denicola *et al*. (1993) estimated from literature data that the production of peroxynitrite by activated macrophages inside phagolysosomes can reach around 500 μM/min. The stimulation by peroxynitrite of Ca^{2+} release from intact mitochondria, hitherto mainly considered to be a toxic NO congener (Darley-Usmar et al., 1995), indicates that it may also be of importance in physiology.

Binding of $NO^\bullet$ to cytochrome oxidase leads to inhibition of respiration (Cleeter et al., 1994; Schweizer & Richter, 1994b) and consequently to a collapse of $\Delta\Psi$ paralleled by Ca^{2+} release from mitochondria (Schweizer & Richter, 1994b; Richter et al., 1994). Therefore, $NO^\bullet$ and peroxynitrite induce Ca^{2+} release from mitochondria *via* different mechanisms. The concentration of nitric oxide and superoxide anion, the redox milieu, and the antioxidant capacity of the cell will determine which mechanism for mitochondrial Ca^{2+} release will predominate.

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