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Peroxynitrite causes calcium efflux from mitochondria which is prevented by Cyclosporin A

Michael A. Packer, Michael P. Murphy*

Department of Biochemistry, University of Otago, PO Box 56, Dunedin, New Zealand

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Abstract

Superoxide reacts with nitric oxide to form peroxynitrite, a potent oxidising agent which may contribute to tissue damage in pathological situations such as inflammation and ischaemia/reperfusion. One mechanism by which oxidative stress damages tissues is the induction of a specific Cyclosporin A-sensitive mitochondrial calcium efflux pathway. Here we show that peroxynitrite induces calcium efflux from mammalian mitochondria and that this efflux is blocked by Cyclosporin A. These data suggest that disruption of mitochondrial calcium efflux may contribute to tissue damage when superoxide and nitric oxide are present together in vivo.

Key words: Peroxynitrite; Mitochondrial oxidative damage; Calcium efflux; Cyclosporin A; Mitochondrial permeability transition

1. Introduction

Nitric oxide ($^{\bullet}NO$) is formed in many mammalian tissues where it acts as a chemical messenger [1] and superoxide ($O_2^{\bullet-}$) is a by product of normal metabolism [2]. The reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$ produces the potent oxidant peroxynitrite ($ONOO^-$) [3,4] which contributes to tissue damage in inflammation and ischaemia/reperfusion where significant amounts of $^{\bullet}NO$ and $O_2^{\bullet-}$ are produced [4,5]. At 37°C, the pK_a of $ONOO^-$ is 6.8 and the half life of its protonated form, peroxynitrous acid, is less than 1 second [6]. Peroxynitrous acid spontaneously cleaves to produce the powerful oxidants nitrogen dioxide and the hydroxyl radical [4,7]. The $ONOO^-$ anion itself oxidises thiols to disulfides [7] and therefore formation of $ONOO^-$ from $^{\bullet}NO$ and $O_2^{\bullet-}$ may lead to oxidative damage in vivo by a number of mechanisms.

Mitochondria are an important target for oxidative damage by $ONOO^-$ since they produce $O_2^{\bullet-}$ [2]. Therefore exposure to $^{\bullet}NO$ can lead to the formation of $ONOO^-$ within mitochondria [6]. Furthermore, it has been shown that $^{\bullet}NO$ and $ONOO^-$ disrupt mitochondrial function [6,8]. In addition to directly inactivating mitochondrial enzymes [9,10], many oxidants disrupt mitochondrial calcium metabolism by inducing a specific mitochondrial calcium efflux pathway [11]. This calcium efflux is associated with, or leads to, the opening of a non-specific pore in the mitochondrial inner membrane which depolarises the mitochondrion [11,12]. The mechanisms of induction of calcium efflux and pore opening are not known, but Cyclosporin A (CsA) blocks this pathway [11,12]. While the role of calcium efflux and pore opening is unclear, it may contribute to cell damage following oxidative stress as CsA protects cells from oxidants [13].

We have investigated whether or not oxidative damage by $ONOO^-$ disrupts mitochondrial calcium transport. To do this we measured calcium efflux and swelling in isolated rat liver mitochondria exposed to $ONOO^-$ and found that $ONOO^-$ induces mitochondrial calcium efflux and opening of the inner membrane pore, both of which can be prevented by CsA.

2. Materials and methods

2.1. Materials

Arsenazo III, superoxide dismutase and catalase were from Sigma. $^{45}CaCl_2$ was from Amersham. Cyclosporin A was a kind gift from Sandoz Pharma Ltd., Basel, Switzerland.

2.2. Preparation of mitochondria

Liver mitochondria were prepared from fed female Wistar rats (150–200 g) by homogenisation and differential centrifugation [14] in medium containing 250 mM sucrose, 5 mM Tris and 1 mM EGTA adjusted to pH 7.4 with HCl. The final wash was in medium without EGTA. The protein concentration was determined by the biuret method using BSA as a standard [15].

2.3. Preparation of peroxynitrite

Peroxynitrite was prepared at 4°C by a variation of published procedures [4,16]. Briefly, 5 ml 0.6 M sodium nitrite was mixed with 5 ml acidified hydrogen peroxide (0.7 M H_2O_2 in 0.6 M HCl) in a simple flow reactor before running into 5 ml rapidly stirred 1.5 M NaOH. Residual hydrogen peroxide was degraded by incubation with MnO_2 (2 g) for 30 min, which was subsequently removed by filtration. Freeze fractionation of this solution at −20°C formed an upper, concentrated yellow band of $ONOO^-$ which was removed. These solutions, containing 90–140 mM $ONOO^-$ ($\varepsilon_{302\,nm} = 1670\ M^{-1} \cdot cm^{-1}$ [17]), were frozen at −80°C and used within 4 days of preparation. A 'late quenched' solution, differing only in that it contained no $ONOO^-$, was prepared by reacting acidified peroxide with nitrite for 10 min, before addition to NaOH. Any $ONOO^-$ formed would have decomposed before addition of NaOH.

*Corresponding author. Fax: (64) (3) 479 7866.

Abbreviations: CsA, Cyclosporin A; $^{\bullet}NO$, nitric oxide; $ONOO^-$, peroxynitrite anion; $O_2^{\bullet-}$, superoxide.

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2.4. Mitochondrial incubation conditions

Mitochondrial incubations were at 25°C in medium containing 195 mM mannitol, 25 mM sucrose and 40 mM K-HEPES (pH 7.2) to which 13 μM rotenone and 500 nM CsA, or an equivalent volume of ethanol vehicle, had been added. For spectroscopic measurement of calcium efflux, an SLM Aminco *DW 2000* dual wavelength spectrophotometer was used to measure the difference in absorbance between 675 nm and 685 nm of the calcium-sensitive dye Arsenazo III. Mitochondria (2 mg protein/ml) and Arsenazo III (30 μM) were added to 3 ml medium in a thermostatted and stirred cuvette and after 2 min 60 nmol $CaCl_2$/mg protein was added and followed 1.5 min later by K-succinate (3.33 mM). Peroxynitrite (250 μM) was added 6 min later. Control experiments demonstrated that adding concentrations of $NaNO_2$, NaOH or NaCl equivalent to the maximum that may have been added with the $ONOO^-$ preparation did not induce calcium efflux. The high concentration of K-HEPES (40 mM) buffered pH changes on addition of the alkaline $ONOO^-$ solution to less than 0.1 unit.

To measure calcium efflux using $^{45}Ca^{2+}$, mitochondria (1 mg protein/ml) were incubated in 4 ml of rapidly stirred medium containing 3.33 mM K-succinate, $CaCl_2$ (60 nmol/mg protein) and $^{45}Ca^{2+}$ (210,000 DPM/ml). A 1 ml sample was taken after 3 min to quantitate mitochondrially-accumulated calcium and immediately after this K-EGTA (0.5 mM), or K-EGTA and $ONOO^-$ together, were added to induce calcium efflux. Seven minutes later triplicate 1 ml samples were taken to measure the amount of calcium remaining in the mitochondrial matrix by scintillation counting, as described previously [18].

Mitochondrial swelling was measured by incubating mitochondria (1 mg protein/ml) with 60 nmol $CaCl_2$/mg protein and 3.33 mM K-succinate in a thermostatted cuvette and A_{540} was measured continuously as described previously [18].

The traces shown in Figs. 1, 2 and 4 are representative of experiments which were repeated on a minimum of 3 different mitochondrial preparations. Statistical significance was determined by a two-tailed Student's *t*-test.

3. Results

3.1. Peroxynitrite causes calcium efflux from mitochondria

When 250 μM $ONOO^-$ was added to a suspension of calcium-loaded mitochondria, the increase in $A_{675-685}$ indicated that $ONOO^-$ rapidly induced calcium efflux (Fig. 1, trace a). This efflux was blocked by CsA (Fig. 1, trace b). Higher (500 μM) and lower (125 μM) concentrations of $ONOO^-$ than that added in Fig. 1 also caused calcium efflux. Addition of an equivalent volume of 'late quenched' control preparation, which is similar in composition to the $ONOO^-$ solution but does not contain $ONOO^-$, did not cause calcium efflux (Fig. 1, trace c). Addition of hydrogen peroxide (100 μM or 1.5 mM) to the calcium-loaded mitochondria did not induce calcium efflux and in the presence of catalase (50 units/ml), or superoxide dismutase (50 units/ml) addition of $ONOO^-$ caused calcium efflux similar to that shown in trace a of Fig. 1. These findings agree with an earlier report that hydrogen peroxide does not cause calcium efflux from mitochondria because of the considerable catalase contamination of these preparations [19].

In further control experiments shown in Fig. 2, $ONOO^-$ was added 3 min prior to the mitochondria. Because of its short half life, $ONOO^-$ would have completely decomposed by the time the mitochondria were added. It can be seen that following the prior addition

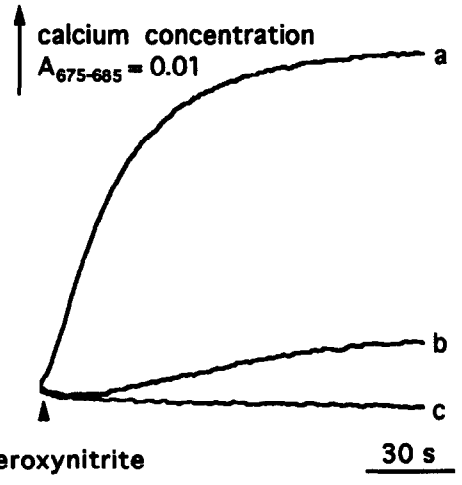

Fig. 1. Peroxynitrite causes calcium efflux from energised mitochondria which can be inhibited by Cyclosporin A. In trace a, mitochondria were incubated as described in section 2 and peroxynitrite (250 μM) was added to induce calcium efflux. For the experiment shown in trace b, calcium efflux was blocked by Cyclosporin A (500 nM). Addition of an equivalent volume of 'late quenched' solution containing no peroxynitrite (trace c) did not cause calcium efflux.

of $ONOO^-$ (Fig. 2, trace c) calcium was accumulated and retained. The calcium uptake which occurred when no additions had been made was the same as that shown in Fig. 2, trace c. Therefore the components in the $ONOO^-$ preparation which caused calcium efflux are short-lived. Addition of hydrogen peroxide (3.0 mM) at the start of the incubation resulted in traces identical to trace c in Fig. 2. In contrast, addition of a high concentration of the more stable *tert*-butyl hydroperoxide, which induces calcium efflux from calcium-loaded mitochondria, prevented calcium uptake (trace a) and at a lower concentration (trace b), partial accumulation of calcium occurred followed by efflux.

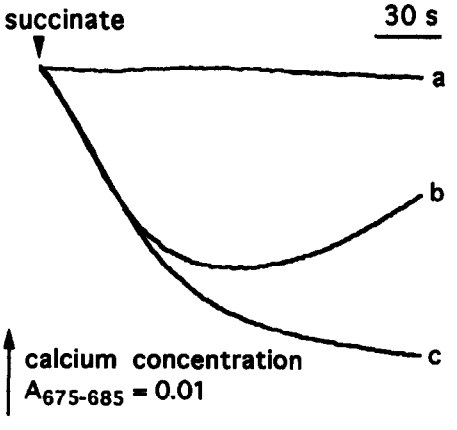

Fig. 2. Peroxynitrite has no effect on calcium accumulation if it is added prior to mitochondria. Incubations were carried out as described in the materials and methods section. *tert*-Butyl hydroperoxide (1.45 mM or 250 μM, traces a and b respectively) or peroxynitrite (250 μM, trace c) were added three minutes prior to mitochondria, followed 30 s later by K-succinate (3.33 mM).

In the experiments described in Fig. 1, some of the calcium released from the mitochondria would have been reaccumulated, allowing calcium to cycle across the mitochondrial inner membrane. Complementary experiments were done measuring calcium efflux under conditions where calcium reaccumulation was prevented. In these experiments (Fig. 3), mitochondria were loaded with $^{45}Ca^{2+}$ and then EGTA was added, in conjunction with $ONOO^-$, to prevent calcium uptake after it has been released from the mitochondria [20]. The amount of $^{45}Ca^{2+}$ retained by the mitochondria, after addition of EGTA and $ONOO^-$, was then measured. In Fig. 3 it can be seen that addition of EGTA alone leads to efflux of calcium, as has been shown previously [18], but this background efflux was increased significantly by addition of $ONOO^-$. In the presence of CsA the stimulation of calcium efflux by $ONOO^-$ was abolished. The efflux caused by addition of the control 'late quenched' preparation which lacks $ONOO^-$, along with EGTA, was the same as that caused by EGTA alone. Therefore, in agreement with the data presented in Fig. 1, these data show efflux of calcium on addition of $ONOO^-$ and inhibition of this efflux by CsA.

3.2. *Peroxynitrite causes calcium loaded mitochondria to swell*

Many oxidants cause calcium-loaded mitochondria to swell by opening a pore in the mitochondrial inner membrane [11]. Mitochondrial swelling decreases light scattering which can be measured as a decrease in absorbance. Addition of $ONOO^-$ to calcium loaded mitochondria caused them to swell (Fig. 4, trace a) and this swelling was blocked by CsA (Fig. 4, trace b), giving an experimental trace identical to that obtained if no addition was made (data not shown). As a positive control phosphate was added (Fig. 4, trace c) and shown to cause swelling which was also blocked in the presence of CsA (Fig. 4, trace d), as has been reported previously [21].

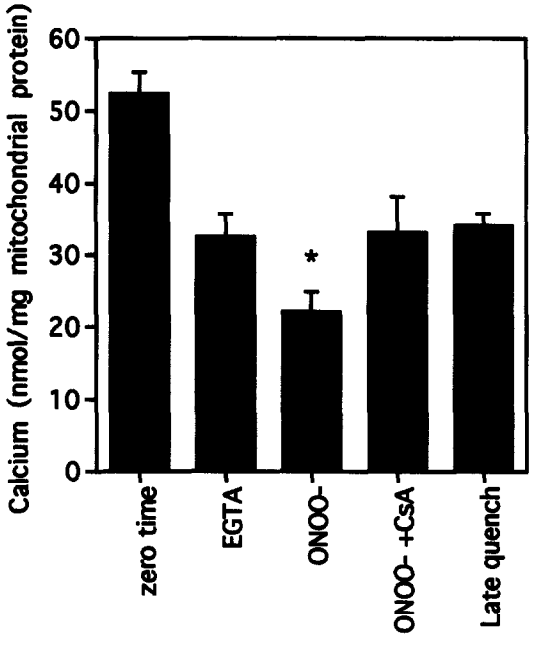

Fig. 3. Measurement of peroxynitrite-induced calcium efflux using $^{45}Ca^{2+}$. Mitochondria were incubated and sampled as described in the materials and methods section. Zero time shows the amount of calcium in the mitochondra prior to additions of $ONOO^-$ while the other measurements show the amount of calcium in the mitochondria after various additions. Data are the mean ± S.E.M. of experiments on at least 3 different mitochondrial preparations. *Indicates a significant difference ($P < 0.05$) between efflux in the presence and absence of Cyclosporin A. Cyclosporin A did not affect efflux caused by EGTA alone.

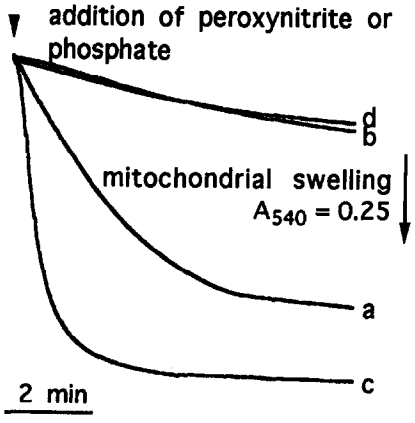

Fig. 4. Peroxynitrite causes mitochondrial swelling which can be inhibited by Cyclosporin A. Mitochondria were incubated as described in section 2. Volume changes due to mitochondrial swelling were measured by a decrease in A_{540}. Addition of peroxynitrite (250 μM) caused swelling (trace a) and this swelling was inhibited by Cyclosporin A (500 nM trace b). Addition of K-phosphate (5 mM) caused swelling (trace c) which was also blocked by Cyclosporin A (trace d).

4. Discussion

Figs. 1 and 3 show that $ONOO^-$ causes calcium efflux from calcium loaded mitochondria both when calcium can cycle across the mitochondrial inner membrane and when this calcium cycling has been prevented. In both situations this calcium efflux can be prevented by CsA. Appropriate control experiments, such as those shown in Fig. 2, indicate that calcium efflux is caused by a short-lived component of the $ONOO^-$ solution, therefore it is reasonable to infer that $ONOO^-$ or its breakdown products, the hydroxyl radical and nitrogen dioxide, are the causative agents. As mannitol, an efficient hydroxyl radical quencher, is present at high concentrations in these experiments, it is probable that it is the oxidation of mitochondrial thiols by the $ONOO^-$ anion itself that leads to calcium efflux [7]. This is consistent with the proposed sequence of events by which other oxidants induce mitochondrial calcium efflux by oxidising glutathione which in turn leads to oxidation of nicotinamide nucleotides and calcium efflux [12]. Efflux of calcium from mitochondria is associated with the opening of a

pore in the mitochondrial inner membrane which depolarises the mitochondrion [11, 12]. The swelling experiments shown in Fig. 4 indicate that $ONOO^-$ leads to opening of this pore and that this opening can be prevented by CsA. Further experiments are now being carried out to determine whether the continuous production of $^{\bullet}NO$ and $O_2^{\bullet-}$ at the levels occurring in vivo brings about calcium efflux and mitochondrial swelling. In summary, we have shown that peroxynitrite induces calcium efflux and opening of a non-specific pore in the mitochondrial inner membrane. This disruption of mitochondrial calcium transport may contribute significantly to tissue oxidative damage under conditions where $ONOO^-$ is formed in vivo from $^{\bullet}NO$ and $O_2^{\bullet-}$.

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