Biochemical Pharmacology, Vol. 51, pp. 267–273, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02165-5

Exposure to the Parkinsonian Neurotoxin 1-Methyl-4-Phenylpyridinium (MPP^+) and Nitric Oxide Simultaneously Causes Cyclosporin A-Sensitive Mitochondrial Calcium Efflux and Depolarisation

*Michael A. Packer, Ralf Miesel and Michael P. Murphy**
DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF OTAGO, P.O. BOX 56 DUNEDIN NEW ZEALAND

ABSTRACT. The effect of the parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) together with nitric oxide donors on mitochondrial calcium homeostasis and membrane potential was investigated. Simultaneous exposure of calcium-loaded mitochondria to MPP^+ and nitric oxide donors led to Cyclosporin A-sensitive mitochondrial calcium efflux and depolarisation. When MPP^+ was replaced with the respiratory inhibitor rotenone, mitochondrial calcium efflux and depolarisation also occurred. As both MPP^+ and rotenone induce mitochondrial superoxide formation, the possibility that calcium efflux and depolarisation were due to peroxynitrite formation from reaction of superoxide with nitric oxide was investigated. It was shown that simultaneous exposure of mitochondrial membranes to nitric oxide donors and rotenone led to peroxynitrite formation. The possible roles of nitric oxide, peroxynitrite, mitochondrial depolarisation, and calcium efflux in MPP^+ toxicity are discussed. BIOCHEM PHARMAC 51;3:267–273, 1996.

KEY WORDS. 1-methyl-4-phenylpyridinium (MPP^+); nitric oxide; mitochondrial calcium transport; peroxynitrite; Cyclosporin A; rotenone

The parkinsonian neurotoxin MPTP† is metabolised by striatal glial cells to MPP^+ [1], which selectively kills dopaminergic neurons in primates, leading to symptoms similar to idiopathic Parkinson's disease [2]. The acute toxicity of MPP^+ follows its inhibition of mitochondrial NADH-linked respiration at Complex I, which causes cellular ATP depletion [2, 3]. In addition, MPP^+ induces mitochondrial formation of superoxide radicals (O_2^-) [4–6], and there is considerable evidence for free radical damage in the neurotoxicity of MPTP and MPP^+ [7–11]. Although O_2^- on its own is not very damaging [12, 13] it reacts rapidly with nitric oxide ($^{\cdot}NO$) to form peroxynitrite ($ONOO^-$) [14]. Peroxynitrite is itself a potent oxidant that directly oxidises thiol groups [15]. Peroxynitrite also decays rapidly to form compounds with similar reactivity to nitrogen dioxide and the hydroxy radical, thus damaging proteins, lipids, and nucleic acids [15–19]. Peroxynitrite forms *in vivo* when O_2^- and $^{\cdot}NO$ production are elevated, and has been implicated in ischaemia-reperfusion injury [20, 21], immunocomplex pulmonary oedema [22], pulmonary emphysema [18], neuroexcitotoxicity [23], atherogenesis [24, 25], and familial amyotrophic lateral sclerosis [26]. Nitric oxide is a retrograde messenger in neurotransmission [27, 28], and the background concentration of $^{\cdot}NO$ in the brain is about 10 nM [29]. Therefore, MPP^+ may cause mitochondrial O_2^- formation, which reacts with endogenous $^{\cdot}NO$ to form $ONOO^-$ and this increased oxidative stress may contribute to the neurotoxicity of MPTP and MPP^+. In support of this hypothesis, inhibition of neuronal nitric oxide synthase prevents MPTP neurotoxicity [30].

Oxidative stress causes Cyclosporin A (CsA)-sensitive mitochondrial depolarisation and calcium release by opening a nonspecific pore in the mitochondrial inner membrane, which contributes to oxidant-induced cell death [31–35]. Recently, we have shown that $ONOO^-$ causes CsA-sensitive mitochondrial depolarisation and calcium efflux [36, 37]. Therefore, MPP^+ and endogenous $^{\cdot}NO$ may form $ONOO^-$, leading to mitochondrial depolarisation and calcium efflux contributing to the toxicity of MPP^+. To test this possibility, we determined the effects on mitochondrial polarisation and calcium homeostasis of exposure to MPP^+ and $^{\cdot}NO$ donors simultaneously.

MATERIALS AND METHODS

Materials

MPP^+ and the $^{\cdot}NO$ donors NO-SP and SNAP were from Research Biochemicals Limited, Natick, MA, U.S.A. MPP^+ stock solutions (300 mM) were prepared daily in water, NO-SP stock solutions (50 mM) were prepared in 20 mM NaOH

* Corresponding author. Tel. +64-3-479-7871; FAX +64-3-479-7866.

† *Abbreviations:* DHR, dihydrorhodamine; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $^{\cdot}NO$, nitric oxide; NO-SP, nitric oxide spermine; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; O_2^-, superoxide anion; SOD, CuZn superoxide dismutase (EC 1.15.1.1); $ONOO^-$, peroxynitrite; TPB, tetraphenylboron.

Received 4 July 1995; accepted 28 September 1995.

and used within 3 hours of preparation [38], and SNAP stock solutions (50 mM) were prepared in argon-sparged DMSO immediately before experiments. The NO donor SNP was from BDH (AR grade), and was dissolved in incubation medium immediately before experiments. CsA was a generous gift from Sandoz Pharma Ltd., Basel, Switzerland. Dihydrorhodamine-123 (Molecular Probes, OR, U.S.A.) and rhodamine-123 (Sigma) stock solutions were prepared in argon sparged AR grade dimethylformamide (AR grade) at 28.9 mM and 1 mM, respectively, and stored in the dark at −20°C. Arsenazo III, safranine, and Type III SOD from bovine liver were from Sigma.

Mitochondrial Preparation

Liver mitochondria from fed female Wistar-derived rats of 200–250 g were isolated by standard procedures [39], in medium containing 250 mM sucrose, 1 mM EGTA, and 5 mM Tris-HCl (pH 7.4), washed once in this medium, then washed and resuspended at approximately 50–60 mg protein/mL in medium containing 250 mM sucrose and 5 mM Tris-HCl (pH 7.4) at 4°C. Experiments were carried out within 4 hr of isolation of the mitochondria, and the protein concentration was determined by the biuret assay using BSA as a standard [40]. The endogenous calcium content of mitochondria prepared in this way was 3.8 ± 0.4 nmol/mg protein [41].

Mitochondrial Membrane Fragments

Mitochondrial membrane fragments were prepared from beef heart mitochondria by sonication, followed by washing by successive pelleting and resuspension. Briefly, beef heart mitochondria, isolated by a standard procedure [42], were suspended at 15 mg/mL in ice-cold medium containing 250 mM sucrose, 1 mM EGTA, and 5 mM Tris-HCl (pH 7.4), and 10-mL batches were sonicated on ice (6 × 15 sec of a Branson microprobe 'sonifier', setting 4, with 30 sec gaps). Medium (10 mL) was then added, the suspension centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was ultracentrifuged at 100,000 × *g* for 60 min at 4°C to pellet the membrane fragments. The ultracentrifugation was repeated twice, and the membrane fragments were suspended at 20 mg protein/mL and stored at −80°C.

Standard Incubation

The standard incubation contained 1 mg mitochondrial or 0.75 mg mitochondrial membrane protein/mL in medium containing 195 mM mannitol, 45 mM sucrose, 20 mM HEPES-KOH (pH 7.2), 3.3 mM succinate, 3.3 mM glutamate, and 3.3 mM malate at 25°C, with further additions described in the figure legends. All traces are representative of experiments on at least three separate mitochondrial preparations.

Mitochondrial Calcium Transport

Mitochondrial calcium efflux was determined by incubating mitochondria in a stirred and thermostatted cuvette in medium containing 30 μM arsenazo III and variable amounts of $CaCl_2$. Changes in absorbance of the calcium-sensitive dye arsenazo III were measured with an SLM Aminco *DW2000* double wavelength spectrophotometer at the wavelength pair 675–685 nm [36].

Mitochondrial Membrane Potential

Mitochondria were incubated under standard conditions and their membrane potential was monitored by changes in absorption of safranine (10 μM), measured using an SLM Aminco *DW2000* double wavelength spectrophotometer at the wavelength pair 511–533 nm [43].

Measurement of Peroxynitrite Formation

The formation of $ONOO^-$ was determined from the oxidation of non-fluorescent dihydrorhodamine-123 (DHR) to fluorescent rhodamine-123 [44]. Rhodamine-123 fluorescence was measured in a Perkin-Elmer MPF-3L fluorescence spectrophotometer using an excitation wavelength of 500 nm and an emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively), calibrated with authentic peroxynitrite as previously described [37]. Beef heart mitochondrial membrane fragments (0.75 mg/mL) were incubated in 0.5 mL incubation medium containing 50 μM DHR and 13 μM rotenone or ethanol carrier. At zero time, NADH (50 μM) and NO-SP (200 μM) were added. If NO-SP was omitted, an identical amount of spermine was added from a stock solution in 20 mM NaOH. The background rate of DHR in the absence of rotenone or NO donors was subtracted from all experimental traces. MPP^+ interferes with this assay; therefore, it was not possible to measure $ONOO^-$ production in its presence. Authentic peroyxnitrite was prepared by reaction of acidified hydrogen peroxide with sodium nitrite as described previously [36]. It was not possible to detect $ONOO^-$ formation by isolated mitochondria, probably because of the rapid reaction of $ONOO^-$ with intramitochondrial components such as glutathione; therefore, a mitochondrial membrane preparation was used for these experiments.

Chemiluminescence

The formation of O_2^- by mitochondria was measured as the chemiluminescence caused by the reaction of O_2^- with lucigenin (100 μM) [45] using a Berthold LB953 luminometer (Wildbad, Germany). Rotenone (13 μM) or MPP^+ (1 mM) was added to mitochondria in 0.5 mL incubation medium. Chemiluminesence was initiated by addition of mixtures of glutamate, malate, and succinate (rotenone experiments), or glutamate and malate (MPP^+ experiments) (3.33 mM final concentration of each component), to the mitochondrial suspension within the luminometer. Results are the total amount of chemiluminesence over 60 min, with the spontaneous background chemiluminesence of lucigenin subtracted (n = 3, ± SEM).

RESULTS AND DISCUSSION

Calcium Efflux from Mitochondria

To determine whether MPP^+ and •NO together synergistically cause mitochondrial calcium efflux, calcium-loaded mitochondria were incubated with MPP^+ and the nitric oxide donor NO-SP (Fig. 1A). Incubation with MPP^+ and NO-SP led to complete calcium efflux (Fig. 1A, trace a), which did not occur when either MPP^+ (Fig. 1A, trace c) or NO-SP (data not shown) was used alone. The calcium efflux was blocked by CsA (Fig. 1A, trace b), by O_2^- dismutation with SOD, and by the •NO scavenger oxyhaemoglobin (data not shown). Therefore, •NO and MPP^+ interact synergistically to cause mitochondrial calcium efflux. As calcium efflux is blocked by CsA, it most probably occurs by the oxidant-induced CsA-sensitive pathway. To determine if calcium efflux was due to MPP^+ causing mitochondrial O_2^- formation, experiments were carried out in which MPP^+ was replaced by the respiratory chain inhibitor rotenone (Fig. 1B), which also causes mitochondrial O_2^- formation [46]. Exposure of calcium-loaded mitochondria to NO-SP together with rotenone led to calcium efflux (Fig. 1B, trace a), which was blocked by CsA (Fig. 1B, trace b). Efflux did not occur when either NO-SP or rotenone was used on its own (Fig. 1B, trace c, and data not shown).

To determine if the calcium efflux was dependent on the •NO donor used, SNP was used instead of NO-SP (Fig. 1C). When SNP was used as a •NO donor in the presence of MPP^+, calcium efflux occurred (Fig. 1C, trace a), which was prevented by CsA (Fig. 1C, trace d). This efflux did not occur when either MPP^+ (Fig. 1C, trace b) or SNP (Fig. 1C, trace c) was used in isolation. For these experiments, the lipophilic anion TPB was also present to facilitate MPP^+ transport across the lipid bilayer of mitochondrial inner membrane [6]. The apparent biphasic response with respect to time seen in Fig. 1C, trace a, was reproducible; however, the significance of this finding is unclear. The lag time before onset of calcium efflux in Fig. 1C is 3–4 min longer than that for Fig. 1A. The reason for this difference is unclear; however, it is not due to greater •NO production by NO-SP in Fig. 1A than by SNP in Fig. 1C (data not shown). The delay in onset of calcium efflux caused by SNP may be a nonspecific effect of the high concentration of SNP (2.5 mM) on the mitochondrial calcium efflux pathway itself. In the presence of TPB, lower concentrations of MPP^+ (down to 100 μM, data not shown) were required to cause calcium efflux. Calcium efflux also occurred when the •NO donor SNAP was used in conjunction with MPP^+ (data not shown). Together, the experiments described by Fig. 1 demonstrate that in the presence of •NO donors, the neurotoxin MPP^+ or the respiratory inhibitor rotenone induces mitochondrial calcium efflux synergistically.

Mitochondrial Depolarisation

Oxidant-induced mitochondrial calcium efflux is associated with mitochondrial depolarisation due to the opening of a nonspecific pore in the mitochondrial inner membrane [31], or to increased calcium cycling across the mitochondrial inner

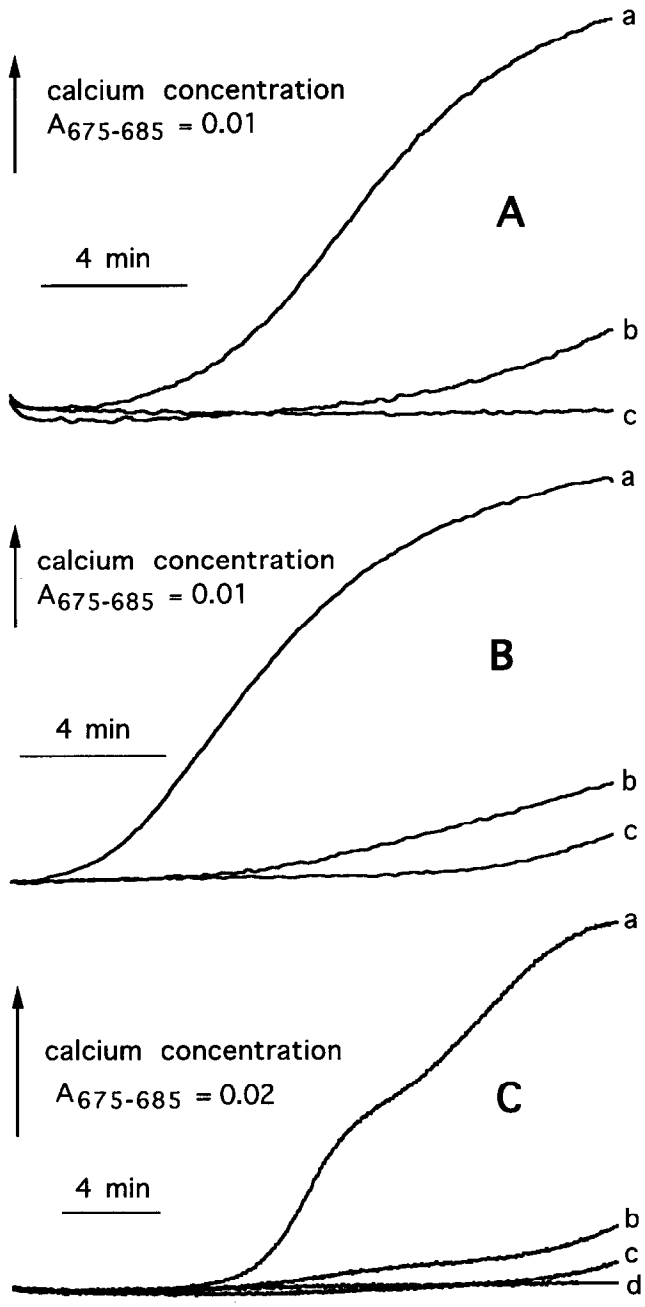

FIG. 1. Mitochondrial calcium efflux following simultaneous exposure to •NO and MPP^+ or rotenone. Traces are shown from 2 min after the start of the incubation when all added calcium had been accumulated by the mitochondria. Panel A: 70 nmol calcium/mg mitochondrial protein and MPP^+ (1 mM) were present for all traces. To these were added NO-SP (200 μM) for traces a and b, and CsA (500 nM) for trace b. No additions were made for trace c. Results similar to trace c were obtained when SOD (250 units/mL), oxyhaemoglobin (50 μM), or NO-SP in the absence of MPP^+ were included in the incubations (data not shown). Panel B: 50 nmol calcium/mg mitochondrial protein were present for all experiments. For traces a and b, rotenone (13 μM) and NO-SP (200 μM) were added. CsA (500 nM) was included for trace b; NO-SP (200 μM) was the only addition for trace c. Incubations with SOD (250 Units/mL) or oxyhaemoglobin (50 μM) gave identical results to trace c (data not shown). Panel C: 70 nmol calcium/mg mitochondrial protein, SNP (2.5 mM), and TPB (10 μM) were present for all traces. MPP^+ (500 μM) and SNP (2.5 mM) were added for traces a and d; CsA (500 nM) was also included for trace d. MPP^+ was present on its own for trace b and SNP was used in isolation for trace c.

membrane [47]. The effect of 'NO donors and MPP^+ or rotenone on mitochondrial membrane potential was measured, and the results of these experiments are presented in Fig. 2. A substantial membrane potential developed on addition of mitochondria to medium containing respiratory substrates. Addition of calcium transiently depolarised the mitochondria due to initial calcium accumulation, followed by a rapid repolarisation to a lower membrane potential due to steady-state calcium cycling. Subsequent addition of MPP^+ (Fig. 2A, trace a) led to mitochondrial depolarisation, which was essentially complete, as shown by the addition of the uncoupler FCCP. Depolarisation did not occur when either 'NO or MPP^+ was used alone (Fig. 2A, traces c and d, respectively), and was prevented by CsA (Fig. 2A, trace b). Addition of EGTA, which chelates external calcium and thus prevents pore opening and calcium cycling, also prevented depolarisation (Fig. 2A, trace e). Depolarisation also occurred when rotenone was used instead of MPP^+ (Fig. 2B, trace a), but did not occur when either 'NO or rotenone was used alone (Fig. 2B trace d and data not shown). This depolarisation was prevented by CsA (Fig. 2B, trace b) or EGTA (Fig. 2B, trace e). Experiments using MPP^+ and the 'NO donors SNP or SNAP, instead of NO-SP, give results similar to those described by Fig. 2A (data not shown).

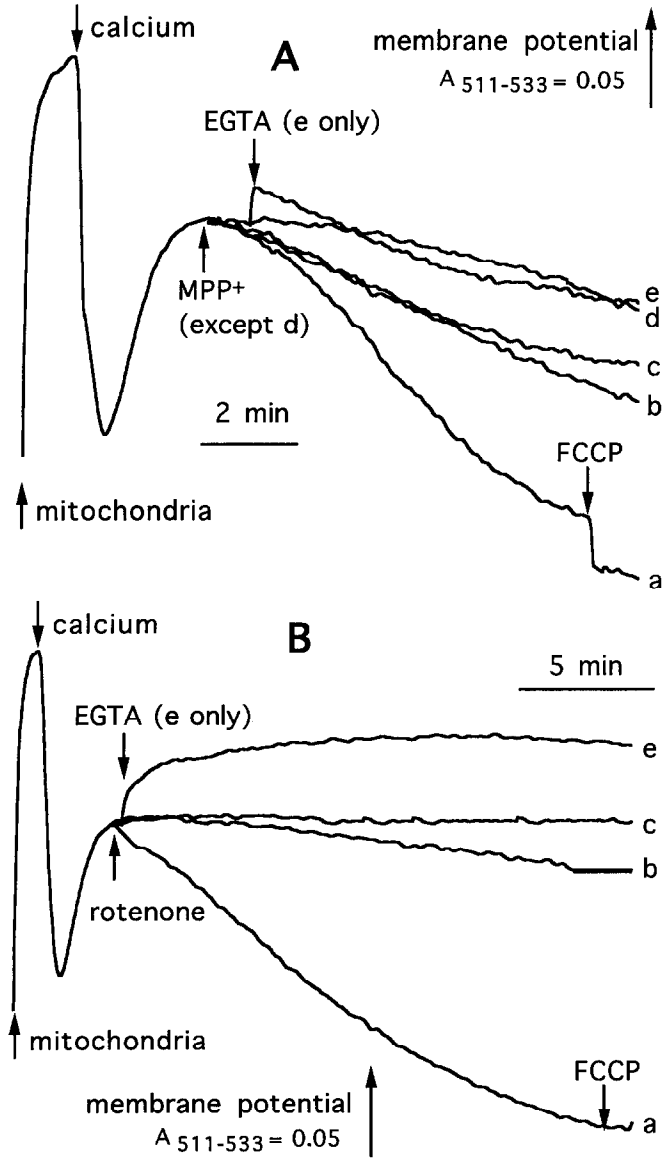

FIG. 2. Mitochondrial depolarisation following exposure to 'NO and MPP^+ or rotenone. Mitochondria were incubated with safranine and 'NO-donors, and $CaCl_2$ (50 nmol calcium/mg) was added 1 min 40 sec later, followed at 4 min by MPP^+ (500 μM, Panel A) or rotenone (13 μM, Panel B). In both panels, traces a show almost complete depolarisation, as shown by the later addition of FCCP (333 nM). CsA (500 nM) was added to traces b; in panel A, trace c MPP^+ was present alone. NO-SP was used on its own in traces d. Spermine dissolved in NaOH (20 mM) together with rotenone was added for the experiment described in Panel B, trace c. For traces e, EGTA (500 μM) was added 20 sec after MPP^+ or rotenone. Addition of FCCP to the experiments described by traces b through e caused depolarisation to the level shown in traces a.

These data suggest a synergistic interaction between MPP^+ or rotenone and 'NO donors, leading to CsA-sensitive mitochondrial depolarisation. The depolarisation is probably associated with calcium efflux (Fig. 1), because depolarisation was prevented by CsA and by chelation of external calcium, which prevents pore opening by removing calcium from an external mitochondrial binding site [48] or by preventing calcium cycling [47].

Detection of Mitochondrial Superoxide Production by Chemiluminescence

Both MPP^+ and rotenone cause mitochondrial O_2^- formation [4, 46]; therefore, the synergistic interaction of MPP^+ or rotenone with 'NO donors may be due in part to O_2^- reacting with 'NO to form $ONOO^-$. To find out if mitochondrial O_2^- formation was increased by MPP^+ or rotenone in our experiments, mitochondria were incubated with rotenone or MPP^+ in the presence of lucigenin, which reacts specifically with O_2^- to form a chemiluminescent product. Addition of MPP^+ or rotenone in the presence of calcium led to an increase in chemiluminescence (Fig. 3). In addition, 'NO donors decreased the chemiluminesence, presumably by $ONOO^-$ formation, thus preventing the detection of O_2^- by lucigenin. Therefore, O_2^- formation in intact mitochondria increases in the presence of MPP^+ or rotenone, in agreement with findings on isolated mitochondrial membranes [4, 46].

Peroxynitrite Formation by Mitochondrial Membranes in the Presence of Nitric Oxide

Part of the synergistic effect of 'NO and MPP^+ or rotenone may follow the formation of the powerful oxidant $ONOO^-$ from the reaction of mitochondrially produced O_2^- with 'NO. To see if this occurred in our experiments, we measured $ONOO^-$ formation by the oxidation of DHR to rhodamine [44]. It was not possible to measure peroxynitrite formation in intact mitochondria, probably because of the high concentration of intra-mitochondrial glutathione. Therefore, experiments were carried out with mitochondrial membrane frag-

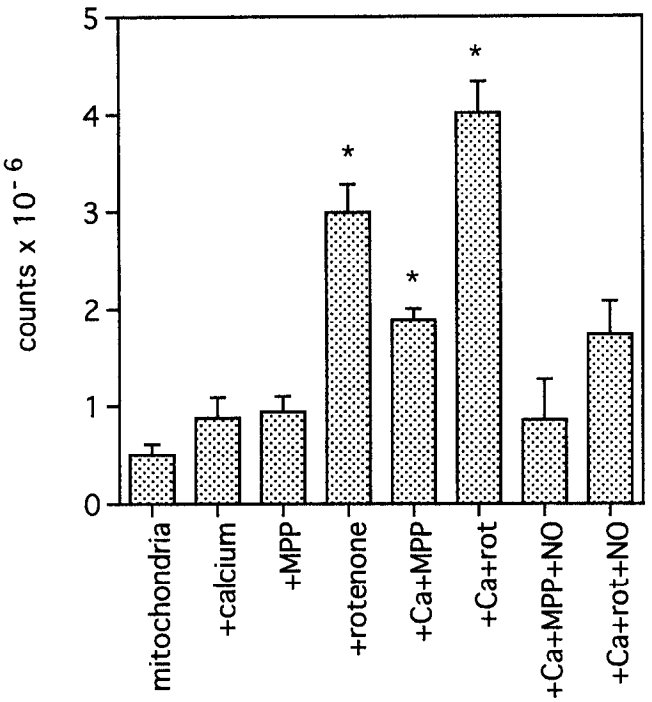

FIG. 3. Stimulation of mitochondrial superoxide formation by MPP^+ or rotenone measured by lucigenin chemiluminescence. Mitochondria were incubated with lucigenin, 1 mM MPP^+, or 13 μM rotenone in standard incubation media in the presence or absence of $CaCl_2$ (70 nmol calcium/mg mitochondrial protein), and lucigenin chemiluminescence was measured. For experiments with MPP^+ in the standard incubation medium, mitochondria were energised with succinate for 12 min before the addition of calcium to allow for complete MPP^+ uptake. Statistical significance was determined using Student's *t*-test, *$P < 0.005$.

ments from which glutathione would have been removed. In the presence of NADH, rotenone, and a •NO donor, the membrane fragments catalysed DHR oxidation, suggesting $ONOO^-$ formation (Fig. 4, trace a). This oxidation was not caused by •NO or rotenone alone (Fig. 4, traces b and c, respectively), as both compounds caused rates of DHR oxidation similar to the background rate.

General Discussion

We have shown that the parkinsonian neurotoxin MPP^+ and the respiratory chain inhibitor rotenone interact synergistically with pharmacologocally active •NO donors to cause mitochondrial depolarisation and calcium efflux. This disruption of mitochondrial function may contribute to the cytotoxicity of MPP^+.

The mechanism by which MPP^+ or rotenone interact with •NO to affect mitochondria is uncertain. However, as this interaction leads to mitochondrial calcium efflux and depolarisation, it is plausible that it is due to increased oxidative stress. In our experiments, MPP^+ and rotenone caused mitochondrial O_2^- formation, which was potentiated by calcium. The mechanism of this potentiation by calcium is unclear, but mitochondrial free radical production is known to be stimulated by calcium [49]. However, O_2^- production in the absence of •NO does not cause mitochondrial depolarisation or calcium efflux. This increased O_2^- production is partially blocked by incubation with nitric oxide donors, suggesting that O_2^- is consumed by reaction with •NO. Together, •NO and O_2^- are known to react forming $ONOO^-$, which would cause substantial oxidative stress, and which is known to cause mitochondrial calcium efflux and depolarisation [36, 37]. In support of this hypothesis, we showed that $ONOO^-$ is formed when rotenone interacts with mitochondrial membrane fragments to produce O_2^- in the presence of •NO donors. Therefore, it is likely that $ONOO^-$ formation contributes to the mitochondrial calcium efflux and depolarisation caused by MPP^+ or rotenone and •NO donors.

The mechanism by which $ONOO^-$ could contribute to mitochondrial calcium efflux follows oxidation of mitochondrial thiols. In support of this, it is known that $ONOO^-$ reacts directly with thiols [15], and oxidation of mitochondrial thiols is known to induce CsA-sensitive mitochondrial calcium efflux. Therefore, the high concentration of mitochondrial glutathione would offer some protection to $ONOO^-$ formation, but may also represent the mechanism by which calcium efflux is induced by this agent. Mitochondrial MnSOD will compete with •NO to react with O_2^- formed by the respiratory chain. However, as the rate of formation of $ONOO^-$ from •NO and O_2^- is effectively diffusion-limited at high concentrations

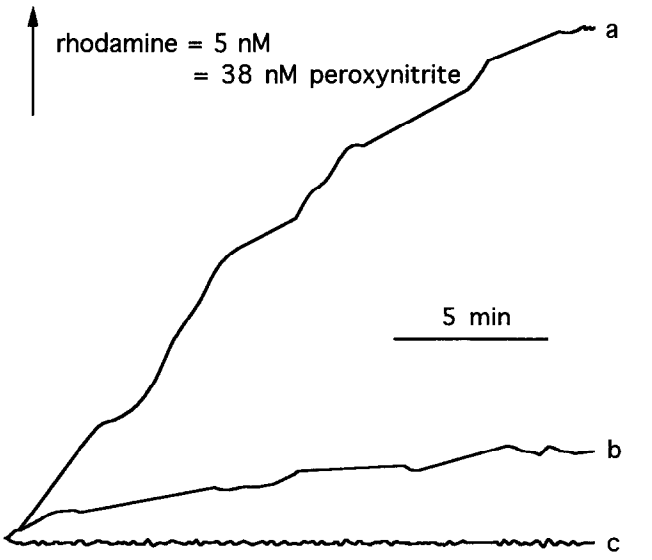

FIG. 4. Peroxynitrite formation by mitochondrial membranes exposed to nitric oxide and rotenone. Mitochondrial membranes were incubated with dihydrorhodamine, and the rate of oxidation of dihydrorhodamine to rhodamine measured to determine peroxynitrite formation. For trace a, rotenone (13 μM) and NO-SP (200 μM) were present. For control experiments, NO-SP (trace b) or rotenone (trace c) were used on their own. Calibration of DHR oxidation with authentic $ONOO^-$ suggested that 6.9 molecules of $ONOO^-$ were required to oxidise 1 molecule of DHR. From this, the flux of $ONOO^-$ produced by the membranes was estimated to be about 16 pmol $ONOO^-$/min/mg membrane protein.

of $^{\cdot}NO$, SOD will offer little protection against $ONOO^-$ formation [12].

In summary, we have shown that MPP^+ or rotenone and nitric oxide donors interact synergistically to cause mitochondrial depolarisation and calcium efflux, which may contribute to MPP^+ cytotoxicity. Part of this synergistic interaction may follow from oxidative stress due to $ONOO^-$ formation. This may arise when MPP^+ interacts with mitochondria to produce $O_2^{\cdot-}$, which reacts with $^{\cdot}NO$ in the brain to form $ONOO^-$.

This research was supported by grants from the Health Research Council of New Zealand, the Neurological Foundation of New Zealand, and the Anderson and Telford Charitable Trust. We thank Carolyn Porteous for expert technical assistance, and Sandoz Pharma Ltd., Basel, Switzerland for a gift of CsA. The generous support of a targeted Ph.D. scholarship from Otago University (MAP) and a post-doctoral research fellowship from Deutscher Akademischer Austauschdienst (RM) are gratefully acknowledged.

References

1. Gerlach M, Riederer P, Przuntek H and Youdim MBH, MPTP mechanisms of neurotoxicity and their implication for Parkinson's disease. *Eur J Pharmacol* **208:** 273–286, 1991.
2. Tipton KF and Singer TP, Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *J Neurochem* **61:** 1191–1206, 1993.
3. Snyder SH and D'Amato RJ, MPTP: A neurotoxin relevant to the pathophysiology of Parkinson's disease. *Neurology* **36:** 250–258, 1986.
4. Hasegawa E, Takeshige K, Oishi T, Murai Y and Minakama S, 1-Methyl-4-phenylpyridinium (MPP^+) induces NADH dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine submitochondrial particles. *Biochem Biophys Res Commun* **170:** 1049–1055, 1990.
5. Cleeter MJW, Cooper JM and Schapira AHV, Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: Evidence for free radical involvement. *J Neurochem* **58:** 786–789, 1992.
6. Ramsay RR and Singer TP, Relation of superoxide generation and lipid peroxidation to the inhibition of NADH-Q oxidoreductase by rotenone, peiricidin A, and MPP^+. *Biochem Biophys Res Commun* **189:** 47–52, 1992.
7. Rossetti ZL, Sotgiu A, Sharp DE, Hadjiconstantinou M and Neff NH, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and free radicals *in vitro*. *Biochem Pharmacol* **37:** 4573–4574, 1988.
8. Przedborski S, Kostic V, Jackson-Lewis V, Naini AB, Simonetti S, Fahn S, Carlson E, Epstein CJ and Cadet JL, Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistent to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J Neurosci* **12:** 1658–1667, 1992.
9. Adams JDJ, Klaidman LK and Leung AC, MPP^+ and $MPDP^+$ induced oxygen radical formation with mitochondrial enzymes. *Free Rad Biol Med* **15:** 181–186, 1993.
10. Olanow CW, A radical hypothesis for neurodegeneration. *TINS* **16:** 439–444, 1993.
11. Zang LY and Misra HP, Generation of reactive oxygen species during the monoamine oxidase-catalyzed oxidation of the neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Biol Chem* **268:** 16504–12, 1993.
12. Beckman JS, Beckman TW, Chen J, Marshall PA and Freeman BA, Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* **87:** 1620–1624, 1990.
13. Halliwell B and Gutteridge JMC, *Free Radicals in Biology and Medicine*, second edition. Oxford University Press, New York, 1991.
14. Saran M, Michel C and Bors W, Reaction of NO with $O_2^{\cdot-}$. Implications for the action of endothelium-derived relaxing factor (EDRF). *Free Rad Res Comms* **10:** 221–226, 1990.
15. Radi R, Beckman JS, Bush KM and Freeman BA, Peroxynitrite oxidation of sulfhydryls. *J Biol Chem* **266:** 4244–4250, 1991.
16. Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin JC, Smith CD and Beckman JS, Peroxynitrite-mediated tyrosine nitration catalysed by superoxide dismutase. *Arch Biochem Biophys* **298:** 431–437, 1992.
17. King PA, Anderson VE, Edwards JO, Gustafson G, Plumb RC and Suggs JA, A stable solid that generates hydroxyl radical upon dissociation in aqueous solutions: reaction with proteins and nucleic acid. *J Am Chem Soc* **114:** 5430–5432, 1992.
18. Moreno J and Pryor W, Inactivation of α1-proteinase inhibitor by peroxynitrite. *Chem Res Toxicol* **5:** 425–431, 1992.
19. Gatti RM, Radi R and Augusto O, Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. *FEBS Lett* **348:** 287–290, 1994.
20. Beckman JS, Ischaemic injury mediator. *Nature* **345:** 27–28, 1990.
21. Matheis G, Sherman MP, Buckberg GD, Haybron DM, Young HH and Ignarro LJ, Role of L-arginine-nitric oxide pathway in myocardial injury. *Am J Physiol* **262:** H616–H620, 1992.
22. Mulligan MS, Hevel JM, Marletta MA and Ward PA, Tissue injury caused by deposition of immune comlexes is L-arginine dependent. *Proc Natl Acad Sci USA* **88:** 6338–6342, 1991.
23. Cazevielle C, Muller A, Meynier F and Bonne C, Superoxide and nitric oxide cooperation in hypoxia/reoxygenation-induced neuron injury. *Free Rad Biol Med* **14:** 389–395, 1993.
24. Darley-Usmar VM, Hogg N, O'Leary V, Wilson MT and Moncada S, The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Rad Res Comms* **17:** 9–20, 1992.
25. Beckman JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM and White CR, Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe-Seyler* **375:** 81–88, 1994.
26. Beckman JS, Carson M, Smith CD and Koppenol WH, ALS SOD and peroxynitrite. *Nature* **364:** 584, 1993.
27. Lowenstein CJ and Snyder SH, Nitric oxide, a novel biological messenger. *Cell* **70:** 705–707, 1992.
28. Bredt DS and Snyder SH, Nitric oxide: A physiologic messenger molecule. *Annu Rev Biochem* **63:** 175–195, 1994.
29. Malinski T, Bailey F, Zhang ZG and Chopp M, Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *J Cereb Blood Flow Metab* **13:** 355–358, 1993.
30. Smith TS, Swerdlow RH, Parker WDJ and Bennett JPJ, Reduction of MPP^+-induced hydroxyl radical formation and nigrostriatial MPTP toxicity by inhibiting nitric oxide synthase. *Neuroreport* **5:** 2598–2600, 1994.
31. Broekemeier KM, Dempsey ME and Pfeiffer DR, Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem* **264:** 7826–7830, 1989.
32. Nicotera P, Bellomo G and Orrenius S, Calcium-mediated mechanisms in chemically induced cell death. *Annu Rev Pharmacol Toxicol* **32:** 449–470, 1992.
33. Richter C, Mitochondrial calcium transport. In: *Molecular Mechanisms in Bioenergetics* (Ed. Ernster L), pp. 349–358. Elsevier, Amsterdam, 1992.
34. Crompton M and Andreeva L, On the involvement of a mitochondrial pore in reperfusion injury. *Basic Res Cardiol* **88:** 513–523, 1993.
35. Gunter TE, Gunter KK, Sheu SS and Gavin CE, Mitochondrial

calcium transport: Physiolgy and pathological relevance. *Am J Physiol* **267:** C313–C339, 1994.
36. Packer MA and Murphy MP, Peroxynitrite causes calcium efflux from mitochondria which is prevented by Cyclosporin A. *FEBS Lett* **345:** 237–240, 1994.
37. Packer MA and Murphy MP, Peroxynitrite formed by simultaneous nitric oxide and superoxide generation causes Cyclosporin A-sensitive mitochondrial calcium efflux and depolarisation. *Eur J Biochem*, in press.
38. Diodati JG, Quyyumi AA and Keefer LK, Complexes of nitric oxide with neucleophiles as agents for the controlled biological release of nitric oxide: Hemodynamic effects in the rabbit. *J Cardiovasc Pharmacol* **22:** 287–292, 1993.
39. Chappell JB and Hansford RG, Preparation of mitochondria from animal tissues and yeasts. In: *Subcellular Components: Preparation and Fractionation*, 2nd edition (Ed. Birnie GD), pp. 77–91, Butterworths, London, 1972.
40. Gornall AG, Bardawill CJ and David MM, Determination of serum proteins by means of the biuret rection. *J Biol Chem* **177:** 751–766, 1949.
41. Reichman N, Porteous CM and Murphy MP, Cyclosporin A blocks 6-hydroxydopamine-induced efflux of calcium from mitochondria without inactivating the mitochondrial inner membrane pore. *Biochem J* **297:** 151–155, 1994.
42. Smith AL, Preparation, properties, and conditions for assay of mitochondria: Slaughterhouse material, small scale. *Methods Enzymol* **10:** 81–86, 1967.
43. Ackerman KEO and Wikstrom MKF, Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett* **68:** 191–197, 1976.
44. Kooy NW, Royall JA, Ischiropoulos H and Beckman JS, Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Rad Biol Med* **16:** 149–156, 1994.
45. Gylenhammar H, Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J Immunol Methods* **97:** 209–213, 1987.
46. Turrens JF and Boveris A, Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* **191:** 421–427, 1980.
47. Richter R and Frei B, Ca^{2+} release from mitochondria induced by prooxidants. *Free Radical Biology & Medicine* **4:** 365–375, 1988.
48. Bernardi P, Veronese P and Petronilli V, Modulation of the mitochondrial Cyclosporin A-sensitive permeability transition pore. I. Evidence for two separate Me^{2+} binding sites with opposing effects on the pore open probability. *J Biol Chem* **268:** 1005–10, 1993.
49. Dykens JA, Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+}: Implications for neurodegeneration. *J Neurochem* **63:**584–591, 1994.