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Nitric oxide enhances MPP^+ inhibition of complex I

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Abstract There is evidence that 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) toxicity is mediated through both inhibition of mitochondrial complex I and free radical generation. 7-Nitroindazole protects against MPTP toxicity in vitro and in vivo, and this appears to be related to its inhibition of nitric oxide ($NO^\bullet$) synthase. We now show that the $NO^\bullet$ generator, glutathione-*N*-oxide, enhances the inhibitory action of 1-methyl-4-phenylpyridinium (MPP^+) on complex I activity in brain submitochondrial particles. We propose that the $NO^\bullet$-induced reversible inhibition of complex IV (cytochrome oxidase) potentiates the MPP^+-induced irreversible free radical-mediated inhibition of complex I. Thus, $NO^\bullet$ may 'prime' the respiratory chain to the effects of MPP^+. These data provide evidence for an interaction between $NO^\bullet$ and MPP^+ at the level of the respiratory chain.

Key words: Mitochondrion; Nitric oxide; Complex I; 1-Methyl-4-phenylpyridinium; Parkinson's disease

1. Introduction

The mechanisms of action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have provided valuable insights into the biochemical abnormalities that might participate in dopaminergic cell death in Parkinson's disease (PD). 1-Methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP, inhibits NADH CoQ_1 reductase (complex I) of the mitochondrial respiratory chain [1], and deficiency of this enzyme has been identified in the substantia nigra of PD brains [2–4]. There is also evidence of oxidative stress and damage in PD substantia nigra [5], and that free radicals may play a role in mediating the effects of MPP^+ toxicity [6–9].

The mitochondrial respiratory chain is responsible for a significant proportion of a cell's superoxide production during aerobic respiration. Inhibition of respiratory chain activity by mitochondrial toxins results in increased O_2 generation [10]. Free radicals themselves are capable of causing respiratory chain defects, primarily involving complexes I and IV in vivo [11,12] and complexes I–III in vitro [13–15]. We have shown in previous studies that if electron transport through the respiratory chain is blocked by inhibition of complex IV, MPP^+ induces a severe, irreversible and selective inhibition of complex I which is mediated via free radical release [7]. $NO^\bullet$ has been shown to be a potent but reversible inhibitor of complex IV [16,17], in addition to inhibiting complex II and to a lesser extent complexes I and III of the respiratory chain [18,19]. Thus, $NO^\bullet$ and MPP^+ might theoretically combine their effects on the respiratory chain to induce irreversible complex I inhibition. MPP^+ and $NO^\bullet$ have been shown to act synergistically in the release of calcium from, and the depolarisation of, isolated mitochondria [20]. It was proposed that the synergism may involve the formation of peroxynitrite promoted by the increased superoxide formation induced by complex I inhibition in the presence of $NO^\bullet$.

Neurones containing neuronal $NO^\bullet$ synthase (NOS) have been demonstrated in the striatum [21] and in the substantia nigra [22], and therefore there is scope for both MPP^+ and $NO^\bullet$ to co-exist in the brain regions associated with PD. Furthermore, 7-nitroindazole, an inhibitor of NOS, has been shown to protect against MPTP toxicity in primates [23] and mice [24]. Although 7-nitroindazole is also an inhibitor of monoamine oxidase-B [25], it still reduces the toxicity of MPP^+, and so must have an action beyond preventing the conversion of MPTP to MPP^+ [26]. We have investigated the combined actions of MPP^+ and $NO^\bullet$ on respiratory chain function to determine if there is a direct interaction between these agents at the level of complex I function.

2. Materials and methods

Unless otherwise stated, reagents were purchased from Sigma Chemicals (Poole, Dorset, UK) or Merck (Dagenham, Essex, UK).

2.1. Purification of rat brain mitochondria and preparation of submitochondrial particles (SMP)

SMP were made from rat forebrains as described previously [19] and the SMP pellet resuspended at approximately 1 mg/ml, frozen in small aliquots and stored at −70°C until used.

2.2. Treatment of SMP with MPP^+ and GSNO

Rat brain SMP were incubated at 0.1 mg/ml for 60 min in a buffer containing 10 mM Tris–HCl, pH 7.4, 320 mM sucrose, 1 mM potassium EDTA, and 0.8 mM NADH and 0.8 mM dithiothreitol (DTT) at 28°C. Glutathione-*N*-oxide (GSNO, from Alexis Corporation, Nottingham, UK) was used as the source of $NO^\bullet$ and was added to reactions just prior to the incubation at concentrations indicated in Section 3. The presence of DTT was required for the generation of $NO^\bullet$ from GSNO. We have previously shown that DTT or decomposition products of GSNO other than $NO^\bullet$ have no effect on mitochondrial respiration [17]. MPP^+ iodide (0.2 mM), 0.3 mM reduced glutathione (GSH), 50 μg/ml catalase, 0.3 M urate, 15 mM reduced oxyhaemoglobin, made from haemoglobin according to Di Iorio [27], and 100 U/ml Mn superoxide dismutase were added as indicated. Aliquots (20 μl) of the incubation were assayed for NADH CoQ_1 reductase activity.

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2.3. Assay of NADH CoQ_1 reductase activity

NADH CoQ_1 reductase (complex I) was assayed spectrophotometrically in a final volume of 1 ml at 28°C [28]. Protein concentrations were determined using the method of Lowry et al. [29] using bovine serum albumin as standard.

2.4. Estimation of rate of production of NO• by GSNO

The production of NO• was analysed using a nitric oxide analyser, model number NOA 280 (Sievers Instruments, Boulder, CO, USA). Briefly, a stream of pure N_2 gas (flow rate 200 ml/min) was passed through a frit into a reaction chamber. This contained 3.4 ml of 10 mM Tris–HCl, pH 7.4, 320 mM sucrose, 1 mM potassium EDTA, 0.8 mM NADH and 0.8 mM DTT at 28°C. Following a 5 min equilibration period, 100 μl of the same buffer containing a solution of GSNO was added to give a final concentration that was 1 mM with respect to GSNO. The gas was taken from the top of the reaction vessel into the detector, where it was mixed with O_3. The resultant activated, NO_2, gave off light as it decayed to its ground state NO_2. This was detected and a reading of NO• in parts per billion was given based on a calibration of the machine with authentic NO•. Data were collected for 1 h and the rate of NO• production determined, using the value of 22.4 l as the molar gas volume at standard temperature and pressure and the flow rate of carrier gas.

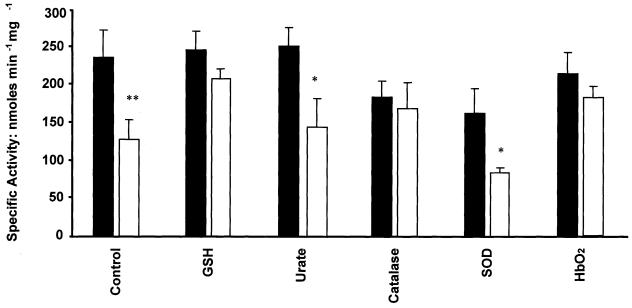

Fig. 2. Rat brain SMP were incubated at 0.1 mg ml^{-1} for 60 min in a sucrose–Tris buffer, pH 7.4, containing 10 mM MPP^+ and 0.8 mM NADH in the presence or absence of 1 mM GSNO. In addition, a variety of reagents were also added separately to the reaction: GSH, urate, catalase, superoxide dismutase or oxyhaemoglobin. The filled bars indicate incubations lacking GSNO; the open bars incubations with GSNO. The results are expressed as the mean of at least three incubations ± S.D. $*P < 0.02$, $**P < 0.001$ relative to the respective control (unpaired *t*-test).

3. Results and discussion

Using 1 mM GSNO as the source of NO•, the rate of production of NO• was calculated to be 0.36 μmol/kg/h at its peak rate (approximately 3 min after addition of 1 mM GSNO), falling exponentially to 0.15 μmol/kg/h after 20 min. There was negligible NO• production thereafter. These figures are in the same range (0.33–0.85 μmol/kg/h) reported for studies on whole rats [30] using an isotopic ($^{16}O_2/^{18}O_2$) ratio method in a closed system. In addition, ex vivo (cryogenic electron paramagnetic resonance) measurements in normal rat brain using spin-trapped NO• gave a control value of 1.1 μmol/kg/h [31].

Incubation of SMP with 1 mM GSNO for 60 min did not affect complex I activity, while 10 mM MPP^+ alone caused a 31% decrease in complex I activity (Fig. 1). This has previously been shown to be due to residual MPP^+ (0.2 mM) in the sample, following dilution into the assay buffer [7]. Co-incubation of 1 mM GSNO and 10 mM MPP^+ induced a 63% inhibition of complex I activity indicating that the inhibitory effects of NO• and MPP^+ on complex I activity are synergistic. MPP^+ interacts reversibly with rotenone binding site(s) within complex I [32] and results in superoxide generation [7,33]. In the presence of complex IV inhibition, however, the MPP^+ inhibition of complex I activity is irreversible and is mediated by free radical damage [7]. While complexes II and III have been identified to be most susceptible to irreversible NO•-induced inhibition [19], NO• also causes a marked reversible inhibition of complex IV activity [17], thereby generating the conditions previously reported for MPP^+ to cause increased free radical production and irreversible complex I inhibition [7]. To determine what role free radicals play in the complex I inhibition reported here, rat brain SMP were incubated with MPP^+ (10 mM), and a range of free radical scavengers in the presence and absence of GSNO (1 mM). As in previous experiments using the complex IV inhibitor cyanide [7], we found that in the presence of NO•, the MPP^+-induced decrease in complex I activity could be prevented by either GSH or catalase (Fig. 2). This suggested that free radical damage was involved in the MPP^+-induced irreversible inhibition of complex I. It is proposed that MPP^+ inhibition of complex I resulted in increased superoxide generation which in turn dismutated to hydrogen peroxide. In the likely event of the presence of free iron, Fenton chemistry would lead to the decomposition of hydrogen peroxide, generating the highly reactive hydroxyl radical and subsequent oxidative damage to neighbouring molecules including complex I itself. HbO_2, which scavenges NO•, was also protective, confirming the requirement for NO• in mediating the complex I inhibition. NO• and $O_2^{\bullet-}$ react at near diffusion rates to form peroxynitrite ($ONOO^-$), a highly reactive and damaging free radical [34] which may contribute to respiratory chain inhibition [35,36]. However, urate, which scavenges $ONOO^-$, did not protect the enzyme activity, suggesting that $ONOO^-$ was not involved directly in the inhibition of complex I. It must be noted however that urate, upon reaction with $ONOO^-$, can give rise to aminocarbonyl radicals [37]. These radicals could attack lipids and thus affect membrane-associated enzymes. The lack of protection by urate therefore must be viewed with caution. The synergistic action of NO• on MPP^+ inhibition of complex I may help explain some of the data on the protective effect of 7-nitroindazole against MPTP toxicity. In the absence of NO•, MPP^+-induced inhibition of

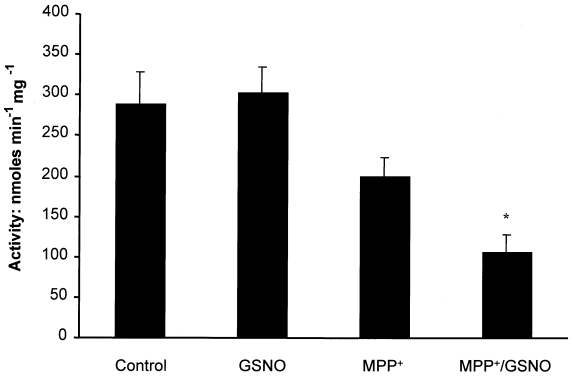

Fig. 1. Rat brain SMP were incubated at 0.1 mg ml^{-1} for 60 min in a sucrose–Tris buffer, pH 7.4, containing NADH (0.8 mM) and combinations of GSNO (1 mM) and MPP^+ (10 mM). The activity of the enzyme NADH CoQ_1 reductase was measured in samples of the incubation mixture. The results are expressed as the mean of at least three incubations ± S.D. $*P < 0.0001$ relative to the control or GSNO alone and $P < 0.002$ relative to MPP^+ alone (unpaired *t*-test).

complex I may be insufficient to lower ATP to the critical levels that may induce cell death. In the brain it is possible that MPP^+ itself may potentiate the interaction of MPP^+ and $NO^\bullet$ by promoting the generation of $NO^\bullet$. The ATP decrease caused by MPP^+ inhibition of complex I could enhance glutamate toxicity through release of the Mg^{2+} blockade of *N*-methyl-D-aspartate receptors. The resulting calcium influx could activate neuronal NOS and $NO^\bullet$ production, resulting in enhanced complex I inhibition by the mechanisms outlined above. As the striatum is richly supplied with NOS positive neurones, and NMDA receptors [38] and MPP^+ enter through nerve terminals [39], the striatum is likely to be the source of greatest $NO^\bullet$ and MPP^+ toxicity. Direct parallels between MPTP toxicity and idiopathic PD must be drawn with care. There is as yet no direct evidence of $NO^\bullet$ involvement in dopaminergic cell death in PD, although there is accumulating evidence to support $NO^\bullet$ generation in the PD brain [40]. Using electron paramagnetic resonance, one study showed increased levels of nitrosyl proteins in PD striatum [41] and nitrotyrosine residues have been identified in Lewy bodies in PD brain. Complex I deficiency in PD brain appears to be confined to, or at least most severe in, the substantia nigra [42]. These factors would suggest that $NO^\bullet$ could contribute to a dying-back degeneration perhaps initiated by a putative MPTP-like toxin that might enter via dopamine terminals in the striatum and be concentrated within the cell body in the substantia nigra.

GSH deficiency has been described in PD substantia nigra [5] and may be an early event in pathogenesis [43]. While the decrease in GSH may be a consequence of free radical generation, it may also reflect the vulnerability of surviving neurones as complex I has been shown to be more sensitive to inhibition at low GSH levels [44]. In addition, we have demonstrated the protective effect of GSH against $NO^\bullet/MPP^+$ toxicity in our in vitro model.

In conclusion, our data provide a novel means of interaction between $NO^\bullet$ and MPP^+ and suggest that complex I inhibition may be a target in a common pathway leading to nigrostriatal neuronal damage.

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