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Oxidative stress as a mechanism for quinolinic acid-induced hippocampal damage: protection by melatonin and deprenyl

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1 There are differences between the excitotoxic actions of quinolinic acid and N-methyl-D-aspartate (NMDA) which suggest that quinolinic acid may act by mechanisms additional to the activation of NMDA receptors. The present study was designed to examine the effect of a potent antioxidant, melatonin, and the potential neuroprotectant, deprenyl, as inhibitors of quinolinic acid-induced brain damage. Injections were made into the hippocampus of anaesthetized rats, which were allowed to recover before the brains were taken for histology and the counting of surviving neurones.

2 Quinolinic acid (120 nmols) induced damage to the pyramidal cell layer, which was prevented by the co-administration of melatonin (5 nmols locally plus 2×20 mg kg^{-1} i.p.). This protective effect was not prevented by the melatonin receptor blocker luzindole. Neuronal damage produced by NMDA (120 nmols) was not prevented by melatonin.

3 Quinolinic acid increased the formation of lipid peroxidation products from hippocampal tissue and this effect was prevented by melatonin.

4 Deprenyl also prevented quinolinic acid-induced damage at a dose of 50 nmols but not 10 nmols plus 2×1.0 mg kg^{-1} i.p. The non-selective monoamine oxidase inhibitor nialamide (10 and 50 nmols plus 2×25 mg kg^{-1}) did not afford protection.

5 The results suggest that quinolinic acid-induced neuronal damage can be prevented by a receptor-independent action of melatonin and deprenyl, agents which can act as a potent free radical scavenger and can increase the activity of endogenous antioxidant enzymes respectively. This suggests that free radical formation contributes significantly to quinolinic acid-induced damage *in vivo*.

Keywords: Quinolinic acid; kynurenines; melatonin; deprenyl; antioxidants; free radicals; reactive oxygen species; neuroprotection; neurotoxicity; excitotoxicity

Abbreviations: CNS, central nervous system; MAO, monoamine oxidase; MPP^+, methylphenylpyridinium ions; NMDA, N-methyl-D-aspartate; ROS, reactive oxygen species

Introduction

Quinolinic acid is a selective agonist at receptors for the glutamate analogue N-methyl-D-aspartate (NMDA) (Stone & Perkins, 1981; Perkins & Stone, 1983; Stone, 1993) and has become a widely used tool for the study of neuronal damage resulting from activation of these receptors. Since it is an endogenous metabolite of tryptophan, it has also become a focus of interest for understanding the pathological processes underlying neuronal damage in a variety of CNS disorders (Stone, 1993). The levels of quinolinic acid in the CNS increase with ageing (Moroni *et al*., 1984) and following infection with the human immunodeficiency virus or the simian equivalent, when the concentration in cerebrospinal fluid can rise up to 400 fold (Heyes *et al*., 1992), levels substantially greater than those capable of causing neurotoxicity. These amounts correlate with the behavioural, motor and cognitive signs of CNS involvement and decline as viral load is controlled by drugs. Understanding the mechanism of quinolinic acid-induced neuronal damage is, therefore, of some potential clinical importance.

The mechanism by which quinolinic acid produces neuronal damage remains uncertain. As pointed out by Foster & Schwarcz (1991), the neurotoxicity of quinolinic acid is considerably greater than can be accounted for by the activation of NMDA receptors. In electrophysiological studies on several preparations including cortical wedges (Lodge & Martin, 1986; Burton *et al*., 1987) spinal neurones (McLennan, 1984) and hippocampal slices (Stone, 1985), quinolinic acid has proved to be consistently less effective than NMDA, whereas quinolinic acid and NMDA have approximately equal activity as neurotoxins (Foster & Schwarcz, 1991). There are also qualitative differences, as well as pharmacological differences (Perkins & Stone, 1983; Winn *et al*., 1991) between the neurotoxic effects of NMDA and quinolinic acid which suggest the involvement of mechanisms other than NMDA receptor activation.

One such mechanism may involve free radicals. Activation of glutamate receptors is known to induce an influx of calcium ions into neurones which entrains a destructive sequence of events within the cell possibly leading to the generation of reactive oxygen species (Choi, 1987; Hartley *et al*., 1993; Velazquez *et al*., 1997; Atlante *et al*., 1997). Recent work has shown that quinolinic acid may lead to the generation of free radicals since it can induce lipid peroxidation in the brain (Rios & Santamaria, 1991) and neuronal damage can be prevented by spin trap reagents such as α-phenyl-t-butylnitrone (Nakao & Brundin, 1997).

Two agents which have been proposed as inhibitors of neuronal damage are melatonin and deprenyl. Melatonin has been described as the most effective of the endogenous antioxidant molecules, while deprenyl has been shown to

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protect against damage caused by a variety of agents and insults by a mechanism which may involve the scavenging of free radicals and/or the stimulation of antioxidant enzymes (Mytilineou *et al*., 1997a,b; Wu *et al*., 1993; Koutsilieri *et al*., 1994). The present study was designed to examine the possible ability of these compounds to modify damage produced by quinolinic acid in the rat hippocampus.

Methods

Intrahippocampal injections

Male Wistar rats weighing between 200 and 300 g were used in all experiments. All animals were housed singly and provided with free access to food and water. Animals were anaesthetized with chloral hydrate (400 mg kg^{-1}) and placed in a stereotaxic frame. The scalp was incised and a burr hole made through the skull to permit access of the injection needle into the hippocampus at the desired co-ordinates (anteroposterior: 3.0 mm behind the bregma suture, dorsoventral: 2.8 mm below the cortical surface and lateral: 3.0 mm from the midline suture) (Paxinos & Watson 1986). The needle was then inserted and left in place for 2 min before the injection of test agents. The compounds used in this study were all introduced through a 29 gauge stainless steel exploring needle, injections being made in a volume of between 1 and 2 μl at a constant rate of 0.3 μl min^{-1} using a Sage infusion pump. The injection needle was allowed to remain in place for 2 min after ending the injection so as to prevent leakage of drug along the needle track. The scalp was then sutured and the animals left to recover for seven days. All injections were performed unilaterally into the left hippocampus, with separate groups of control animals being injected with vehicle.

Quinolinic acid and NMDA were injected at a dose of 120 nmols, a dose found in preliminary work to induce a sub-maximal destruction of pyramidal neurones while melatonin, deprenyl and nialamide were co-administered at two dose levels by mixing solutions of the individual agents in the appropriate proportions. The concentrations of individual agents were adjusted so that when combined, the total injection volume remained constant. A single intraperitoneal injection of melatonin at a dose of 20 mg kg^{-1} was also administered to all animals receiving intrahippocampal melatonin, immediately after removal of the animal from the stereotaxic frame. A further dose of 20 mg kg^{-1} was given 1 h later. Similarly, animals receiving deprenyl intrahippocampally also received an intraperitoneal injection of 1 mg kg^{-1} on removal from the frame and after a further hour. Nialamide was administered into the hippocampus at doses of 10 and 50 nmols, together with i.p. injections of 25 mg kg^{-1}. These various doses were selected on the basis of previous work with melatonin (Carneiro & Reiter, 1998; Chen & Chuang, 1999; Cho *et al*., 1997; Uz *et al*., 1996), deprenyl (De La Cruz *et al*., 1996) and nialamide (Cousin *et al*., 1986), the latter group demonstrating that doses of 25 mg kg^{-1} produced inhibition of monoamine oxidase (MAO) activity in rats. The protocol of combining central and peripheral injections was devised *ad hoc* on the desire to achieve the highest level of melatonin in the hippocampus consistent with solubility limitations and to maintain a high level of melatonin over the first few hours of quinolinic acid damage. Deprenyl and nialamide were then administered in the same way to achieve a consistent experimental design. Luzindole was administered intrahippocampally at a dose of 1 nmol because of solubility limitations.

Quinolinic acid was dissolved in 0.1 N NaOH and then diluted with 0.9% saline. The pH of the solution was then adjusted using 1 N HCl to between 7 and 7.6 before making up to volume by the addition of further saline. Melatonin was dissolved in ethanol and then diluted with saline and sonicated. NMDA, R(−)-deprenyl hydrochloride and nialamide were dissolved in saline.

Tissue fixing and slicing

Rats were killed by an intraperitoneal overdose of sodium pentobarbitone (60 mg per rat) 7 days after recovery from the intrahippocampal injections. The chest was opened to expose the heart and 20 ml of 0.9% physiological saline was infused *via* a 26 gauge needle inserted into the left cardiac ventricle. This was followed immediately by a solution of 10% formalin buffered to pH 7.2. The brain was then removed and stored in fixative for up to 1 week. A slice of brain, 2 mm thick, was prepared to include the location of the injection track, which was normally apparent from the residual dimpling of the cortical surface produced by the needle penetration. The 2 mm block of brain was dehydrated and impregnated with paraffin wax throughout before embedding in wax. Sections were cut 6 μm thick, mounted on slides and stained with cresyl fast violet.

Sections were subsequently examined under a light microscope and areas CA1, CA2, CA3a, CA3b and CA4 examined for damage. The damage was quantified in the CA3 region by selecting three sections 200–250 μm from the site of the needle track and counting the number of intact, surviving neurones at a magnification of 100×.

For the determination of statistical significance, results were subjected to analysis of variance (ANOVA) followed by post-tests (Dunnett's test for comparison with controls or the Bonferroni test for comparison of all columns). Significance refers to results where $P<0.05$ was obtained.

Lipid peroxidation assays

Hippocampal slices, prepared as above, were homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 20% w v^{-1} homogenate. Aliquots of homogenate (500 μl) were incubated with or without quinolinic acid at concentrations of 10, 100 or 1000 μM at 37°C for 30 min. Melatonin was examined at concentrations of 10, 100 or 1000 μM when added together with the highest concentration of 1000 μM when added together with the highest concentration of 1000 μM quinolinic acid. After incubation the homogenates were centrifuged at 13 000 r.p.m. for 10 min and the concentration of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal measured using a Bioxytech LPO-586 colorimetric assay (R&D systems). The analysis involves the reaction of N-methyl-2-phenylindole with these peroxidation products to form a chromogenic indolic dimer which is estimated spectrophotometrically at 586 nm. All samples were tested in duplicate.

Results

Quinolinic acid induced damage primarily in the CA1, CA3 and CA4 regions of the hippocampus, the CA2 region usually remaining little affected. Normal intact pyramidal neurones have a clearly rounded appearance with a clear nucleus and nucleolus (Figure 1). Quinolinic acid (120 nmols) produced a dose-dependent loss of these normal neurones and resulted in

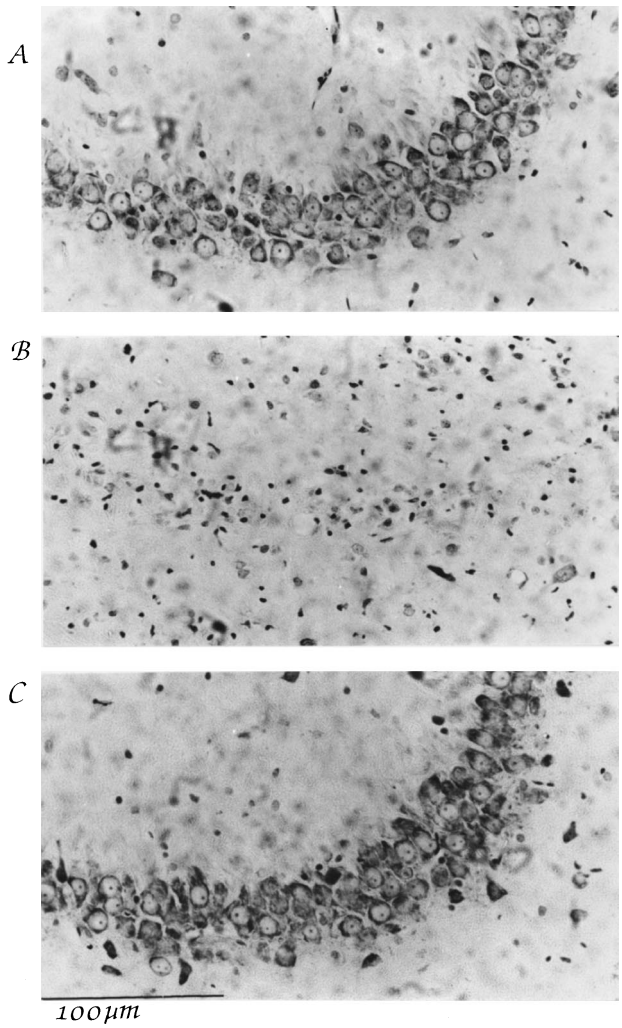

Figure 1 Photomicrographs of the CA3 region of hippocampus in (A) a control animal (B) 7 days following an intrahippocampal injection of quinolinic acid, 120 nmols and (C) 7 days following a combined administration of quinolinic acid 120 nmols and melatonin 5 nmols intracerebrally plus 2×20 mg kg^{-1} i.p. The control hippocampus shows normal healthy neurons with a full, rounded outline containing a clear rounded nucleus and central nucleolus. The damaged section in contrast has no healthy pyramidal neurons, but the field of view is covered by the small dark nuclei of glial cells especially in the central arc representing the CA3 region of former pyramidal neurons. In (C) melatonin has largely protected against quinolinic acid, most pyramidal cells being normal and healthy in appearance. Scale bar 100 μm.

the infiltration of the pyramidal cell layers and surrounding tissue by microglial cells (Figure 1). Of the damaged areas, the CA3 area was selected for the quantification of the damage and protection as described in Methods. The administration of quinolinic acid alone at 120 nmols induced a subtotal loss of neurones in most animals of around 90%, the precise number varying slightly between different series of animals (see Figures).

NMDA (120 nmols) also produced damage in the CA3 region, reducing the number of cells counted from 74 ± 10 to 25 ± 8 ($P < 0.01$; $n = 4$).

Melatonin

The co-administration of melatonin at doses of 1 and 5 nmols tended to prevent the damage induced by quinolinic acid ($F(3,12) = 17.17$; $P = 0.0001$). The mean proportion of surviving neurones seen after 1 nmol of melatonin 25.3% was not significantly different from that produced by quinolinic acid alone (12.6%), and remained significantly different from control brains (Figure 2A). The higher dose of 5 nmols melatonin protected neurones significantly against quinolinic acid and left the CA3 region not significantly different from control animals. The proportion of neurones remaining was

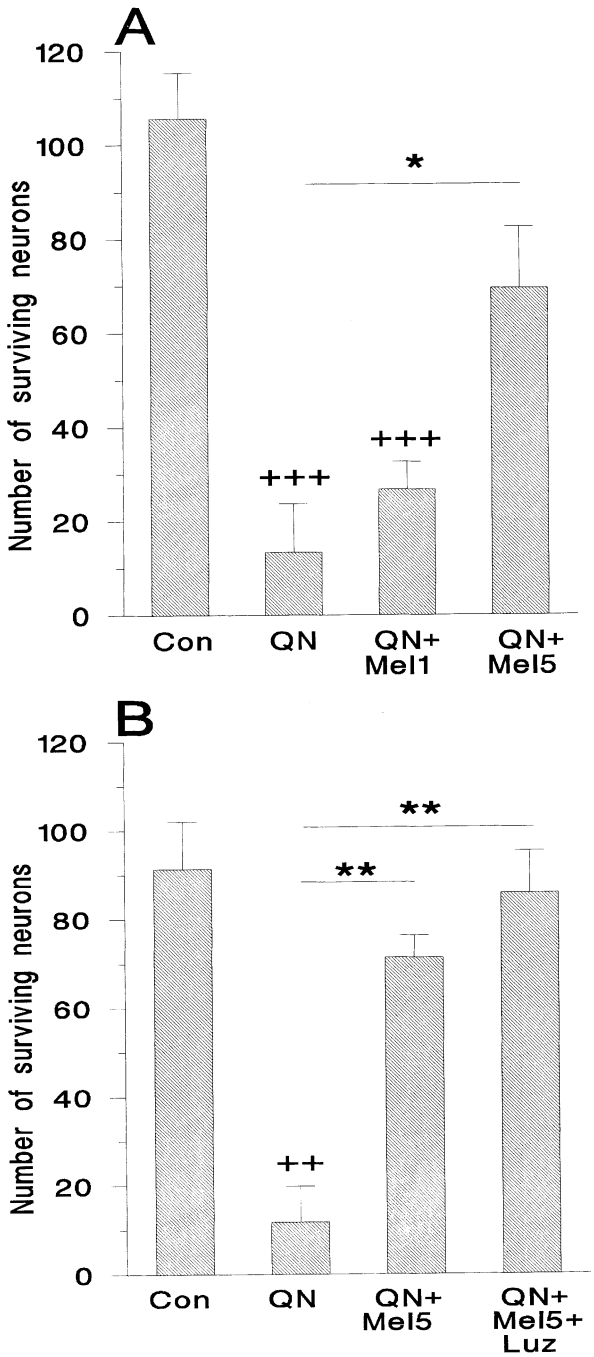

Figure 2 Histograms summarizing the number of neurons surviving after treatment with quinolinic acid 120 nmols (QN) or combined treatments with quinolinic acid and melatonin (1 or 5 nmols plus 2×20 mg kg^{-1} i.p.) (histogram A) or combined treatments with quinolinic acid 120 nmols, melatonin 5 nmols plus 2×20 mg kg^{-1} i.p. and luzindole 1 nmol (histogram B). Four animals were used in each group. Results are shown as mean $\pm$ s.e.mean. Analysis was performed by ANOVA followed by the Bonferroni test for multiple comparisons. *$P < 0.05$; **$P < 0.01$ compared with quinolinic acid alone ($n = 4$). ++$P < 0.01$; +++$P < 0.001$ compared with control.

65.7%. The administration of melatonin alone at this dose did not affect the pyramidal neurone population.

This result was confirmed in a repeat series of animals in which the melatonin receptor antagonist luzindole was included. When administered alone, luzindole had no influence on the damage produced by quinolinic acid, and the antagonist did not prevent the protection afforded by melatonin (Figure 2B). The proportion of neurones surviving after melatonin plus luzindole was slightly greater than that obtained with melatonin itself (93.9% compared with 78.1%) but these values were not significantly different and neither value was significantly different from control animals (Figure 2B).

The administration of melatonin in a dose of 5 nmols did not protect significantly against the damage induced by NMDA (cell count 37 ± 6 compared with the control value of 74 ± 10; $P < 0.05$; ANOVA ($F[2,9] = 9.785$, $P = 0.0055$ followed by Dunnett's test; $n = 4$).

Deprenyl

Deprenyl was administered at doses of 10 and 50 nmols intrahippocampally together with quinolinic acid and with two i.p. injections of 1 mg kg^{-1}. The lower dose showed a clear tendency to reduce the amount of neuronal damage but this did not achieve significance. The higher dose preserved 80.1% of the neurones and resulted in a significant level of protection (Figure 3; $F(3,12) = 16.592$; $P = 0.0001$). The higher dose of deprenyl was also examined in three animals injected with NMDA, 120 nmols, into the hippocampus. No significant protection was seen in these cases.

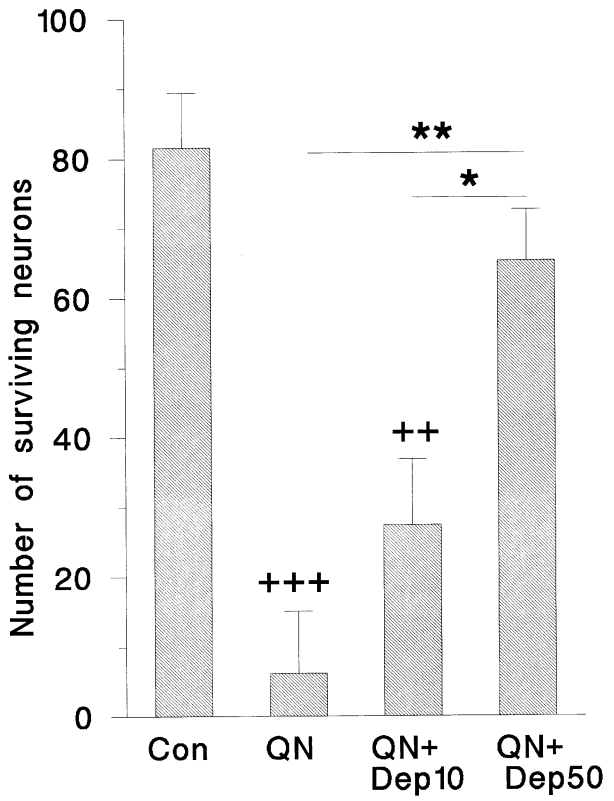

Figure 3 Histogram summarizing the number of neurons surviving after treatment with quinolinic acid 120 nmols (QN) or combined treatments with quinolinic acid and deprenyl 10 or 50 nmols plus 2×10 mg kg^{-1} i.p. Results are shown as mean $\pm$ s.e.mean. Analysis was performed by ANOVA followed by the Bonferroni test for multiple comparisons. Four animals were used in each group. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with quinolinic acid alone. $++P < 0.01$; $+++P < 0.001$ compared with control.

Nialamide

Nialamide was administered at doses of 10 and 50 nmols plus 2×25 mg kg^{-1} together with quinolinic acid, but neither dose changed significantly the amount of neuronal damage.

Lipid peroxidation

The results of measuring the lipid peroxidation products malondialdehyde and 4-hydroxynonenal are summarized in Figure 4. Quinolinic acid induced a concentration-dependent increase in the formation of peroxidation products, with a 60% increase at 1 mM. In the presence of melatonin this increase was prevented, with a concentration of only 10 μM melatonin being sufficient to reduce peroxidation significantly and 1 mM melatonin reducing the amount of lipid peroxidation to control levels (Figure 4).

Discussion

The sensitivity to quinolinic acid of the CA1, CA3 and CA4 regions of the stratum pyramidale is consistent with earlier reports (Schwarcz *et al*, 1984; Foster & Schwarcz, 1991) although the mechanism by which neuronal damage is produced remains uncertain. The present results, showing a clear neuroprotective effect of melatonin, strongly suggest that quinolinic acid-induced damage may be mediated by free radicals, a conclusion consistent with evidence that quinolinic acid can promote lipid peroxidation in brain homogenates (Figure 4; Rios & Santamaria, 1991; Stipek *et al*., 1997) and that quinolinic acid-induced damage can be prevented by spin-trap agents such as α-phenyl-t-butylnitrone (Nakao & Brundin, 1997).

Melatonin has been described as 'the most potent physiological scavenger of hydroxyl radicals found to date' (Hardeland *et al*., 1993; Hardeland & Rodriguez, 1995). Its powerful antioxidant properties are due to its ability to scavenge ROS (Reiter *et al*., 1995) and increase the activity of antioxidant enzymes such as glutathione peroxidase (Barlow-Walden *et al*., 1995). These properties probably underline the protection by melatonin of cultured neurones in the presence of kainate (Giusti *et al*., 1995) or oxidative stress (Cagnoli *et al*., 1995; Lezoualch *et al*., 1996), and of neurones *in vivo* following ischaemia (Cho *et al*., 1997) or the systemic administration of kainate (Uz *et al*., 1996). It has also been shown directly that melatonin can prevent lipid peroxidation induced *in vitro* by kainate, nitric oxide or hydrogen peroxide (Melchiorri *et al*., 1995; Sewerynek *et al*., 1995; Escames *et al*., 1997). Much of the evidence for melatonin's antioxidant activity has been reviewed by Reiter *et al*. (1995).

The ability of melatonin to inhibit the production of ROS by quinolinic acid has been demonstrated here by direct measurement of two of the main products of lipid peroxidation: malondialdehyde and 4-hydroxynonenal.

In addition to its antioxidant status, melatonin acts on at least two varieties of G-protein coupled neuronal receptors. Conceivably these could mediate at least part of the protection by melatonin, especially since one of them, the mt_1 receptor, can depress neuronal activity and is present on cerebral vasculature (Dubocovich 1995). However, luzindole is able to block both subtypes of receptor and its failure to prevent the protection produced by melatonin at a concentration

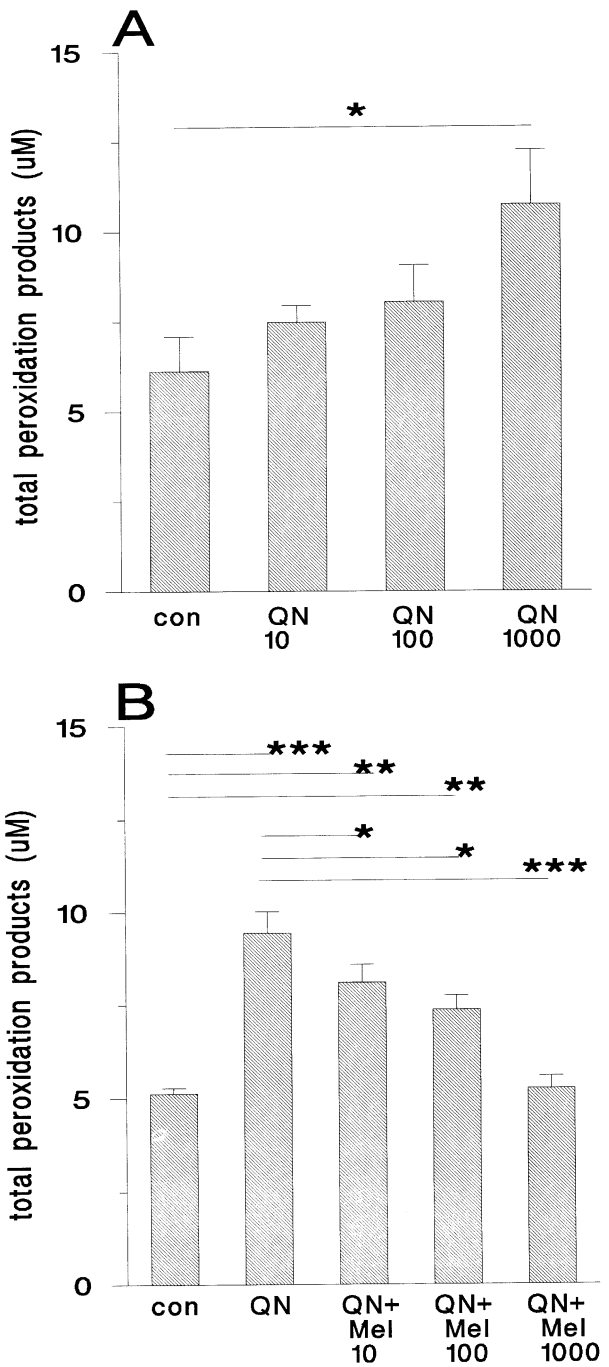

Figure 4 Histograms summarizing the total concentration of malondialdehyde and 4-hydroxynonenal after incubation of hippocampal tissue with quinolinic acid at 10, 100 or 1000 μM (histogram A) or with quinolinic acid 1000 μM together with melatonin at 10, 100 or 1000 μM (histogram B). Results are shown as mean $\pm$ s.e.mean. Analysis was performed by ANOVA followed by the Dunnett test for comparisons with control data (A) or the Bonferroni test for multiple comparisons (B). Four animals were used in each group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ for the differences indicated by the bars.

equivalent to more than 500 times its pA_2 value of 1.7 μM at brain melatonin receptors *in vitro*, (Molinari *et al*., 1996) argues against a receptor-mediated effect. However, it should be noted that there have been no direct demonstrations of the blockade of hippocampal melatonin receptors by luzindole, and our exclusion of a receptor-mediated action is based on this assumption.

The results indicate, therefore, that part of the neuronal damage produced by quinolinic acid is probably attributable to the enhanced formation of free radicals. Part of this effect is likely to be independent of NMDA receptor activation in view of the finding that melatonin was unable to prevent the damage produced by NMDA itself, a result confirming the observation of Giusti *et al*. (1995). Melatonin does not appear to interact directly with NMDA receptors, as it has been shown not to prevent the increase in intracellular calcium produced by activating NMDA receptors in cortical neurones (Cazevieille *et al*., 1997). Since, however, melatonin can prevent the neuronal damage produced by NMDA in culture (Cazevieille *et al*., 1997) it seems possible that the mechanisms of neuronal damage *in vitro* and *in vivo* differ to some extent. Certainly NMDA is able to produce lipid peroxidation under the same *in vitro* experimental conditions as those used here (Santamaria & Rios, 1993).

It is not entirely clear whether the ROS responsible for neuronal damage could be produced by direct chemical interactions between quinolinic acid and normal cellular constituents, or arise secondarily as a result of the activation of NMDA receptors. Quinolinic acid is known to activate NMDA receptors (Stone & Perkins, 1981), resulting in an increase of intracellular calcium (Daniel, 1991). An increase of intracellular calcium load can in turn promote lipid peroxidation (Gutteridge, 1977). The failure of melatonin to prevent the injurious effects of NMDA would indicate that ROS generated as a result of NMDA receptor activation are not sufficient to produce toxicity. The alternative mechanism, of a direct interaction between quinolinic acid and cell constituents without the involvement of a receptor mechanism would, therefore, appear more likely.

However, antagonists at the NMDA receptor are able to prevent much of the toxicity due to quinolinic acid and an explanation of the present data must take this into account. It is possible that the unexpectedly high potency of quinolinic acid in producing damage (Foster & Schwarcz, 1991) is the result of synergism between the activation of NMDA receptors and the formation of free radicals. The combined onslaught on cell membranes may be required for neuronal death. Alternatively, free radicals are themselves known to enhance the release of glutamate from synaptosomal preparations (Pellegrini-Sampietro *et al*., 1988) and this might represent one route by which quinolinic acid could reinforce the activation of NMDA receptors.

A further possibility is that the damage produced by quinolinic acid is partly dependent on the gliosis and inflammatory reaction which occurs in response to excitotoxic challenge. Activated microglia as well as activated macrophages which infiltrate the CNS in the aftermath of insults or lesions are known to produce ROS which could account for some of the neuronal damage *in vivo*. Quinolinic acid could act synergistically with the ROS produced from this source to produce a degree of damage which is dependent on both the activation of NMDA receptors and the oxidative stress imposed by free radical generation. However, quinolinic acid must also be able to generate ROS independently of such cells, in view of the lipid peroxidation which has been noted *in vitro* (Figure 4; Rios & Santamaria, 1991; Stipek *et al*., 1997).

Deprenyl

Deprenyl is of interest as a potential neuroprotectant against a variety of cerebral insults. It has been shown to protect cultured dopamine neurones against glutamate-induced damage which is probably mediated by NMDA receptors

(Mytilineou *et al*., 1997a,b) although it has no activity against binding to NMDA receptors, and reduces hippocampal damage produced by cerebral ischaemia (Paterson *et al*., 1997). It also prevents damage *in vivo* and *in vitro* produced by methylphenylpyridinium (MPP^+) ions (Wu *et al*., 1993; Koutsilieri *et al*., 1994) and reduces the formation of hydroxyl ions which result from perfusion of striatal tissue with MPP^+ (Wu *et al*., 1993).

The mechanism by which deprenyl achieves this protection does not seem to involve monoamine oxidase inhibition (Wu *et al*., 1993; Gerlach *et al*., 1994; Tatton *et al*., 1996), a view which is supported by the present finding that protection was not mimicked by nialamide. The dose of nialamide used here was that shown by Cousin *et al*. (1986) to inhibit brain MAO activity following its intraperitoneal injection.

The actual mechanism of protection remains unclear. In the present study, it failed to prevent significantly the damage produced by NMDA, strengthening the view that the mechanisms of damage by quinolinic acid and NMDA differ. However, *in vitro* studies of cultured neurones have shown that deprenyl can prevent neuronal damage and apoptosis induced by glutamate or NMDA, raising again the possibility that differences also exist in the mechanisms of *in vivo* and *in vitro* toxicity (Mytilineou *et al*., 1997a,b). Deprenyl can suppress the induction of apoptosis in neurones by actions which seem to be exerted at the level of gene transcription (Tatton *et al*., 1996). It also has the ability to promote neuronal regeneration and neuritogenesis (Iwasaki *et al*., 1994; Koutsilieri *et al*., 1994), actions which also imply a site of action within the nucleus. Such effects may contribute to the preservation of neurones after quinolinic acid administration. However, deprenyl also has the ability to promote antioxidant enzyme activity: the chronic administration of deprenyl to rats increases the activities of two of the major antioxidant enzymes, superoxide dismutase and catalase (Carrillo *et al*., 1994a,b,c). It also has been shown to scavenge hydroxyl and peroxyl radicals (Thomas *et al*., 1997). In view of the evidence for a role of free radicals in quinolinic acid-induced damage, the simplest explanation of the effect of deprenyl, therefore, is that it is also working by interfering with ROS generation of levels.

In summary both melatonin, by a receptor-independent action, and deprenyl have the ability to protect against neuronal damage produced by quinolinic acid in the rat hippocampus. The evidence for lipid peroxidation *in vitro*, together with the powerful antioxidant properties of melatonin, suggest that oxidative stress may be a major factor in quinolinic acid-induced damage.

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