ELSEVIER

European Journal of Pharmacology 330 (1997) 27–34

ejp

Increased brain NAD prevents neuronal apoptosis in vivo

Suman K. Mukherjee, Lori K. Klaidman, Ramona Yasharel, James D. Adams Jr. *

School of Pharmacy, Department of Molecular Pharmacology and Toxicology, PSC 508, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90033, USA

Received 28 April 1997; accepted 2 May 1997

Abstract

Apoptosis is a characteristic form of cell death which has been implicated in neurodegeneration. In this study we document the induction of apoptosis and DNA fragmentation in vivo by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin. MPTP selectively damages dopaminergic neurons in the substantia nigra of the midbrain. It is a potent inducer of oxygen radicals. Nicotinamide, a precursor of NAD, is able to block the apoptosis induced by MPTP. Nicotinamide also quenches some of the radicals formed by xanthine oxidase. Nicotinamide may be of interest in the treatment of neurodegeneration. © 1997 Elsevier Science B.V.

Keywords: Apoptosis; DNA fragmentation; MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); Nicotinamide

1. Introduction

Apoptosis is a delayed form of cell death which is brought about by activation of a suicide program inherent to multicellular organisms. Apoptosis has been implicated in several diseases like cancer, acquired immunodeficiency syndrome and neurodegenerative disorders (Steller, 1995). This study documents the induction of apoptosis in the brain by a neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and discusses the role played by nicotinamide (also called vitamin B_3) which may prevent apoptosis.

It has been shown that MPTP induces parkinsonism in humans and other primates by causing degeneration of dopaminergic neurons within the substantia nigra. Previous studies in our laboratory have shown that MPTP administered to mice can cause swelling (necrosis) as well as shrinkage (possible apoptosis) of dopaminergic neurons (Adams et al., 1989). MPTP can be metabolically converted to an active toxic species, MPP^+ (1-methyl-4-phenylpyridinium ion), by monoamine oxidase B (Adams and Odunze, 1991). MPP^+ is a potent inducer of oxidative stress and generates hydroxyl radicals by redox cycling (Klaidman et al., 1993). MPP^+ is a mitochondrial toxin which inhibits the oxidation of NAD-linked substrates and increases the release of oxygen radicals from mitochondria (Adams et al., 1993; Vyas et al., 1986). MPTP inhibits ubiquinone oxidoreductase, which results in elevated levels of ubisemiquinone, a potent generator of oxygen radicals (Adams and Odunze, 1991; Vyas et al., 1986). MPP^+ has also been shown to cause apoptosis in neuronal cells in culture (Dipasquale et al., 1991).

Apoptosis can be blocked in mildly stressed cells by inhibitors of protein synthesis which increase cell survival. The current study shows that apoptosis can be prevented by nicotinamide which blocks DNA fragmentation and increases NAD levels. The chromatin bound enzyme poly(ADP-ribose) polymerase (PADPRP) is activated by oxidative stress that causes DNA strand breakage (Nosseri et al., 1994). It has been observed that PADPRP, which uses NAD as a substrate, can use up almost all of the intracellular NAD (Berglund, 1994), thereby inducing ATP depletion and cell death. Thus nicotinamide may be able to block the depletion of NAD and ATP. This may result in partial restoration of energy dependent processes inside the cell. Nicotinamide was used in this study to prevent the DNA fragmentation and apoptosis caused by MPTP in vivo.

2. Materials and methods

2.1. Animal model

For all light microscopy experiments, 7 month old C57BL/6 mice were injected twice with 25 mg/kg MPTP

* Corresponding author. Tel.: (1-213) 342-1362; Fax: (1-213) 342-1681.

0014-2999/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0014-2999(97)00171-4

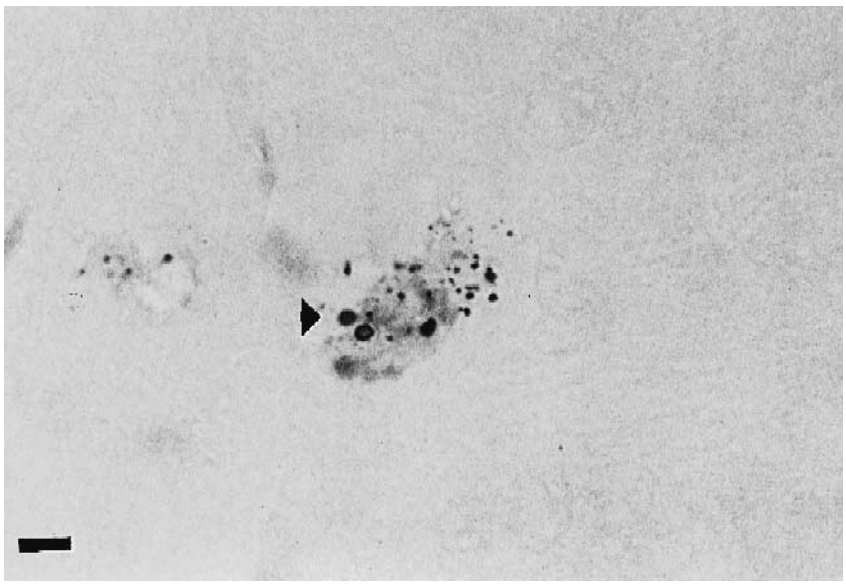


Fig. 1. Midbrain, substantia nigra: an apoptotic neuron (arrow) can be seen 48 h after treatment of the mouse with 25 mg/kg MPTP twice at an interval of 2 h (×600). Dark spots are apoptotic bodies from disintegrated apoptotic cells. Staining was done with the In Situ Apoptosis Detection Kit (Oncor). Bar lengths represent 10 μm. 7 month old C57BL/6 male mice were used.

intraperitoneally (i.p.) at an interval of 2 h. After 2–48 h, the mice were perfused through the left ventricle with phosphate buffered saline (PBS) and then with 4% formaldehyde and 0.2% glutaraldehyde in PBS. The brains were coronally sliced into 50 μm sections in a vibrating microtome. All immunohistochemical studies for the identification of apoptotic cells were done 48 h after injection of MPTP as significantly higher DNA fragmentation values (which may reflect the induction of apoptosis) were observed only after 48 h following administration of MPTP. Necrotic cells were identified within 2–10 h of MPTP administration.

2.2. Histology

To detect individual cells undergoing apoptosis, the In Situ Apoptosis Detection Kit (Oncor) was used. In brief, the tissues were quenched in 2% hydrogen peroxide (H_2O_2) in PBS to deactivate endogenous peroxides. Then two drops of equilibration buffer containing potassium cacodylate were applied. After incubation for 10–15 s, 54 μl of working strength terminal deoxynucleotidyl transferase enzyme was pipetted onto each section and incubated for 1 h in a humidified chamber. The tissues, after incubation, were washed in a prewarmed working strength stop/wash buffer and incubated further for 30 min. Then two drops of anti-digoxigenin-peroxidase were added to the sections. The tissues were again incubated for 30 min at room temperature. H_2O_2 was added to a filtered solution of freshly prepared 3,3′-diaminobenzidine tetrahydrochloride-2-hydrate (DAB) and used to stain sections at room temperature. The tissues were put on slides, air dried, washed in three changes of xylene and mounted with balsam. In this method, the fragmented DNA ends were extended with digoxigenin-dUTP. An anti-digoxigenin antibody then bound to the extension and was detected by a peroxidase reaction.

Tyrosine hydroxylase staining was done to identify dopaminergic neurons in the substantia nigra. The immunoglobulin G (IgG) polyclonal antibodies used were: a rabbit antibody to tyrosine hydroxylase; a sheep antibody to rabbit IgG and a rabbit antibody to horseradish peroxidases with the horseradish peroxidases coupled to the antibody as described previously (Adams et al., 1989). The

Table 1
MPTP induced DNA damage and its prevention by nicotinamide

	Midbrain	Striatum
Control	1.09 ± 0.22	1.09 ± 0.35
Nicotinamide	1.25 ± 0.33	1.21 ± 0.12
MPTP (2 h)	0.91 ± 0.22	0.93 ± 0.06
MPTP (48 h)	1.64 ± 0.46 [a]	1.24 ± 0.24
MPTP + nicotinamide	1.13 ± 0.30	1.26 ± 0.13

C57BL/6 mice were divided into 5 groups with 4 mice in each group. MPTP (25 mg/kg, i.p.) was administered twice at an interval of 2 h to mice: MPTP (2 h) group (brains were removed 2 h after the second injection of MPTP), MPTP (48 h) group (brains were removed 48 h after the second injection of MPTP) and MPTP + nicotinamide group (500 mg/kg nicotinamide was given i.p. 13 h before injection of MPTP and brains were removed 48 h after the second injection of MPTP). MPTP was not administered in the nicotinamide group (brains were removed 48 h after administration of 500 mg/kg nicotinamide i.p.). DNA fragmentation was measured in the midbrain and striatum. Results have been given as means ± S.D. and can be expressed as the amount of DNA damaged per gram of tissue.

[a] Statistically significantly different from corresponding control values by ANOVA and Newman-Keuls test ($P < 0.05$).

tyrosine hydroxylase stained brain slices were mounted on glass slides, dehydrated and coverslipped with balsam.

For electron microscopy, the method employed by Adams et al. (1989) was followed. In this experiment, mice were treated with MPTP for 2–10 h. During this time, both apoptotic and necrotic cells were seen. Following staining for tyrosine hydroxylase, brain slices were postfixed with 2% osmium tetroxide in PBS, dehydrated, embedded in epon-araldite, thin sectioned, mounted on grids and finally stained with uranyl acetate and lead citrate.

2.3. DNA fragmentation

Several reports have documented DNA damage in the brain in Parkinson's disease. A fluorimetric assay was done to detect DNA fragmentation in the midbrain and in the striatum (Mukherjee et al., 1995). C57BL/6 mice were divided into five groups with four animals in each group. Two groups of mice were treated twice with 25 mg/kg MPTP i.p., and 2 h between injections. The brains were removed and sectioned after 2 h for one group and after 48 h for the other group. For a third group of mice, 500

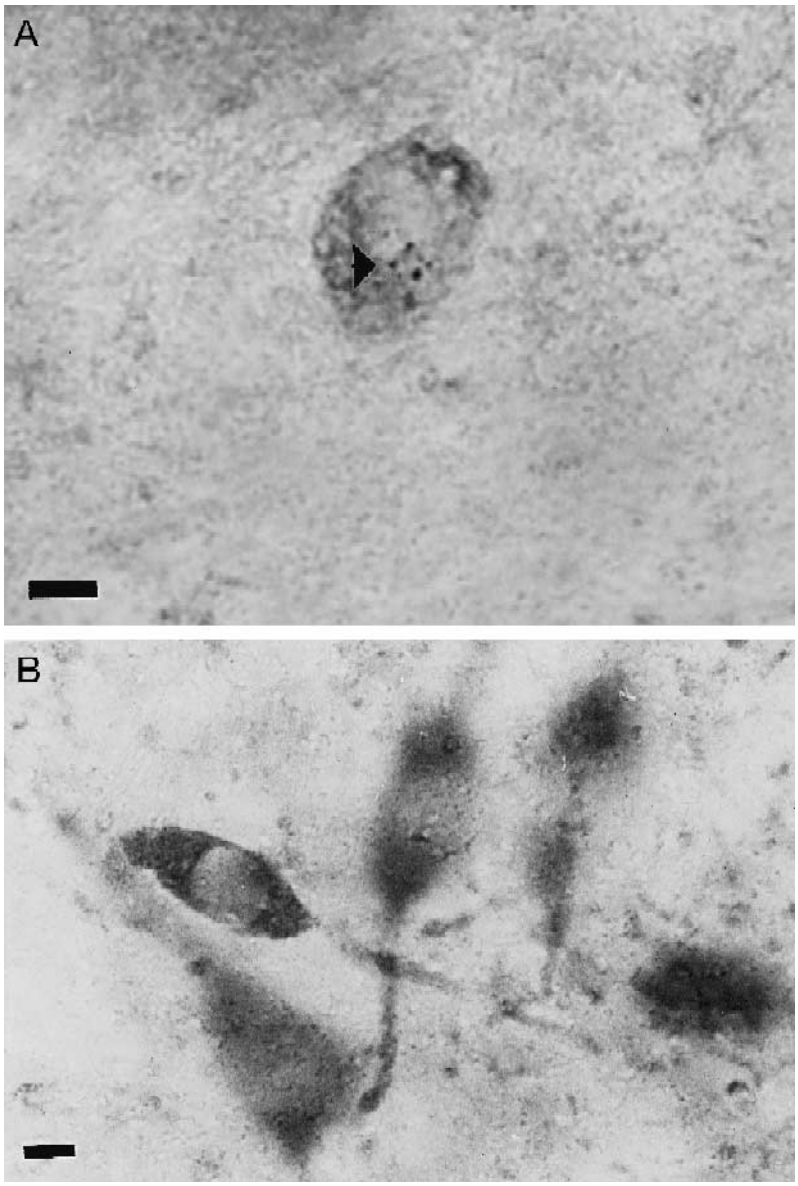


Fig. 2. Midbrain, substantia nigra: in (A), a damaged dopaminergic neuron (arrow) with large vacuoles and a fragmented nucleus forming apoptotic bodies can be seen in a section removed 48 h after treatment with 25 mg/kg MPTP twice at an interval of 2 h (×600). In (B), a section from a mouse injected with 500 mg/kg nicotinamide i.p. alone can be seen (×600). Staining employed tyrosine hydroxylase immunohistochemistry. Bar lengths represent 10 μm. 7 month old C57BL/6 male mice were used.

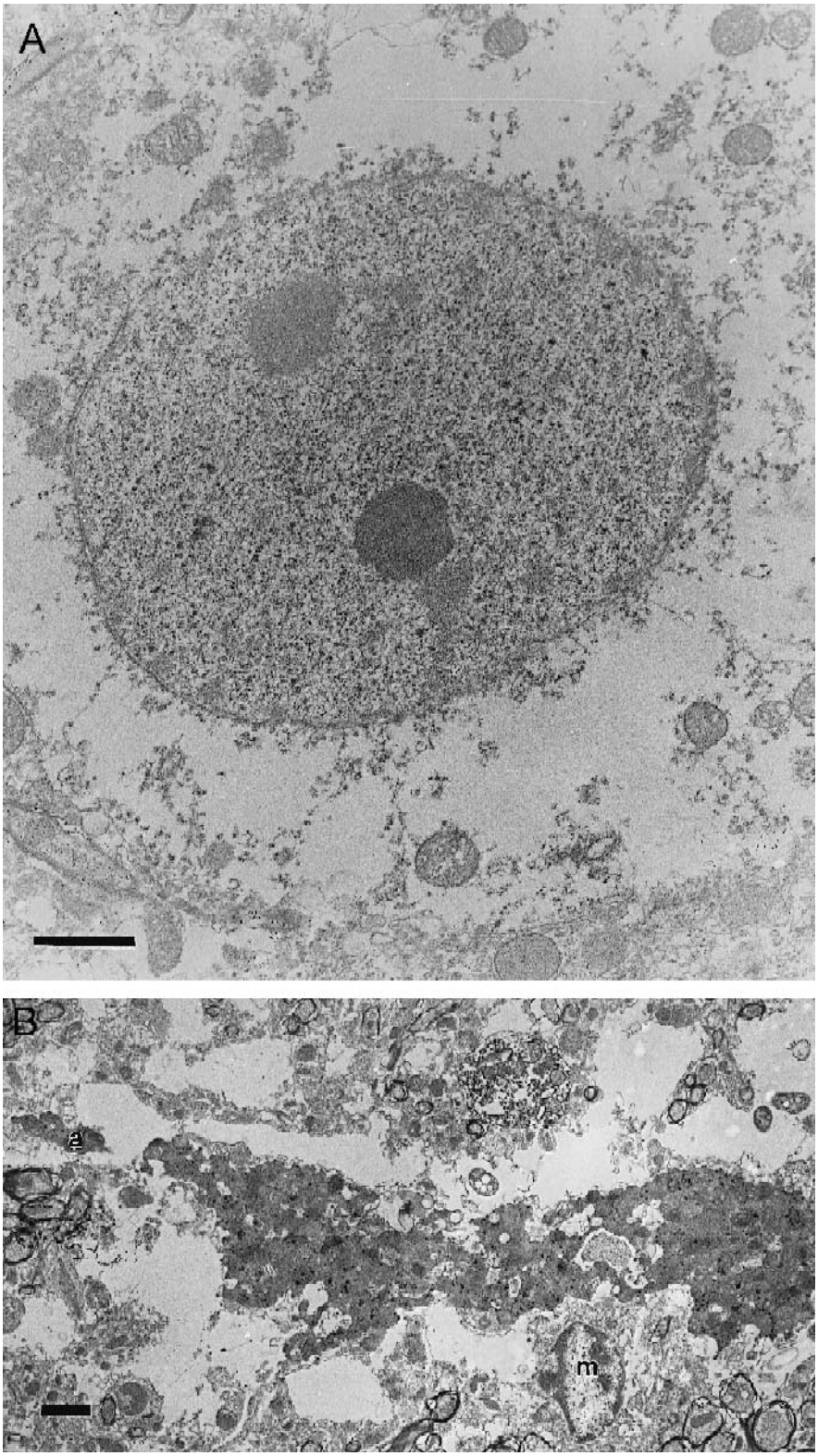
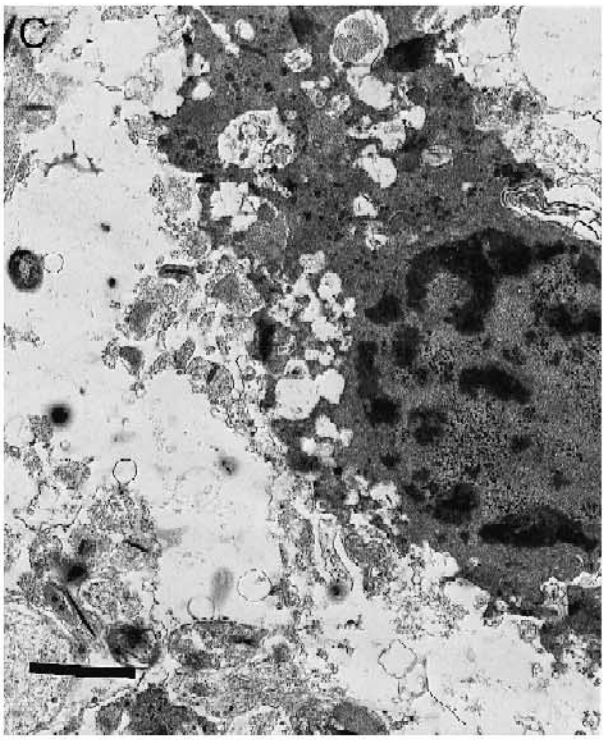
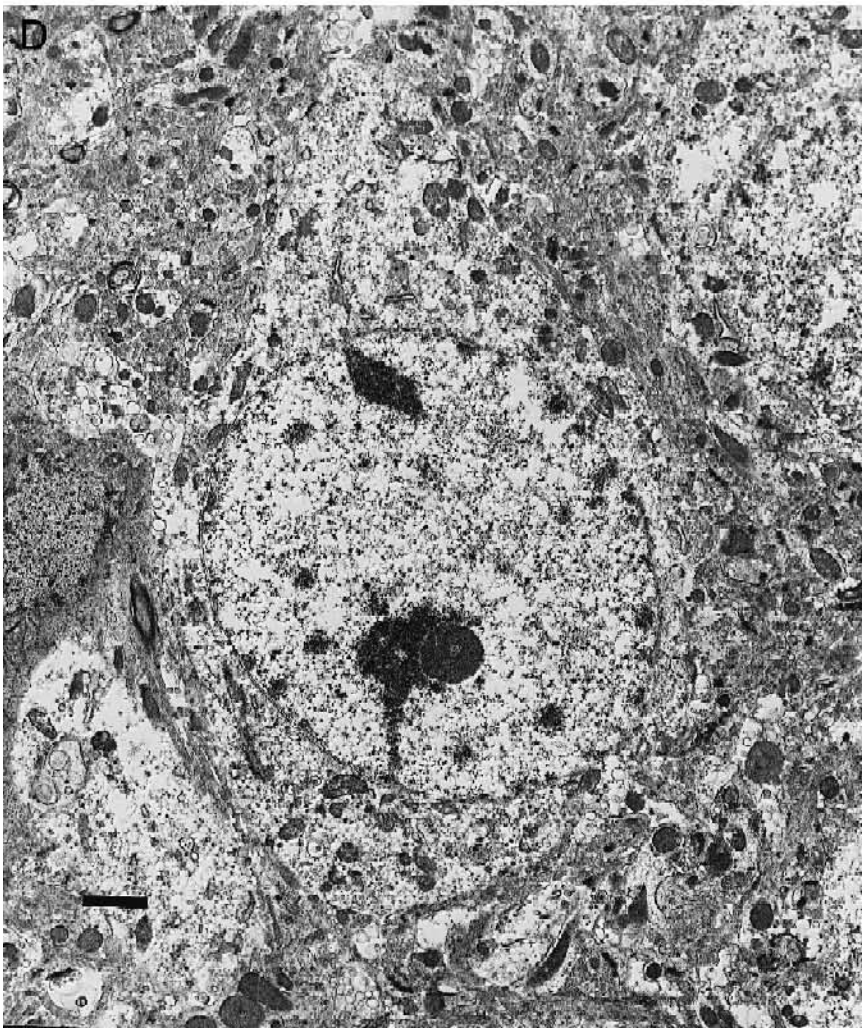


Fig. 3. Midbrain, substantia nigra: in (A) a dopaminergic neuron exhibiting characteristic features of necrosis can be observed (×18 900). The cytoplasm as well as the nucleus are swollen with possible rupture of the nuclear envelope. In (B), an apoptotic dopaminergic neuron can be observed (×7500) with a condensed and vacuolated cytoplasm. Apoptotic bodies can be seen (a), and a microglial cell (m) can also be observed which is probably present to remove the apoptotic cell. In (C), another apoptotic dopaminergic neuron with a condensed nucleus and cytoplasm can be observed (×18 900). (D) is a control dopaminergic neuron (×18 900). Staining employed tyrosine hydroxylase immunohistochemistry. Bar lengths represent 2 μm.


C


D

mg/kg of nicotinamide (i.p.) was administered 13 h prior to MPTP injection. A fourth group of mice was treated only with 500 mg/kg nicotinamide. A fifth group was the control. Brains were removed and dissected as described in Table 1. A volume of 0.2 ml of ice-cold lysing buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% Triton X-100) was added to each section which was centrifuged at $12\,000 \times g$ for 25 min to separate the intact from the fragmented DNA. The supernatant (containing the fragmented DNA) was transferred to a separate tube. Lysing buffer (0.2 ml) was added to the pellet with sonication for 10 s. The amount of DNA in the supernatant and pellet fractions was determined by a fluorimetric method, using the dye Hoechst 33258. DNA damage is normalized by dividing the amount of DNA in the supernatant by the total DNA in the pellet and supernatant and is expressed as the amount of damage per gram of tissue (Mukherjee et al., 1995). The levels of NAD were measured by high pressure liquid chromatography (Klaidman et al., 1995).

2.4. Free radical studies

Electron paramagnetic resonance (EPR) studies were done with 0.1 units/ml xanthine oxidase, 0.5 mM xanthine and 0.1 M 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in 0.1 M glycine buffer (pH 8.3) containing 1 mM diethylenetriaminepentaacetic acid (DETAPAC); 0.1 M nicotinamide and 0.4 M hydrogen peroxide. EPR spectra were measured at 23°C and 100 kHz in a Bruker model ECS 106 spectrometer (Bruker, Karlsruhe, Germany). The EPR conditions were: modulation amplitude 0.483 G, microwave power 20 mW and time constant 1.3 s.

3. Results

3.1. Necrotic and apoptotic morphology

By light microscopy, apoptotic dopaminergic neurons in the midbrain with shrunken and fragmented nuclei were most abundant after 48 h following MPTP administration (Fig. 1). These apoptotic cells were all shrunken with large cytoplasmic vacuoles. However, no apoptotic cells were observed when the mice were treated with 500 mg/kg nicotinamide 13 h before administration of MPTP (Figure not shown). Administration of 500 mg/kg nicotinamide alone for 48 h did not generate any apoptotic cells (Figure not shown).

In the MPTP-treated brains that were stained with a tyrosine hydroxylase antibody, dopaminergic neurons with apoptotic morphology, which had condensed and vacuolated cytoplasms and fragmented nuclei can be clearly observed (Fig. 2A). In the nicotinamide-treated (Fig. 2B) as well as the MPTP- and nicotinamide-treated (500 mg/kg nicotinamide prior to MPTP) mice (Figure not shown), the dopaminergic neurons were normal. No condensed cells with fragmented nuclei were observed.

By electron microscopy, cells having necrotic morphology were found following 2 h of treatment with MPTP (Fig. 3A). In this figure a swollen dopaminergic neuron can be observed that has an inflated nucleus and possibly a ruptured nuclear envelope. A nucleolus has also changed shape. These are characteristic features of necrosis, the cytoplasm and nuclei swell and eventually rupture. As previously reported, the mitochondria appear normal in necrotic dopaminergic neurons following MPTP treatment (Adams et al., 1989). The cytoplasm contains tyrosine hydroxylase, which is identified by the reaction with tyrosine hydroxylase antibody.

On the other hand, following 10–48 h of MPTP treatment apoptotic dopaminergic neurons (Fig. 3B) can be seen. In this figure a condensed dopaminergic neuron is observed which is on the verge of disintegrating into various apoptotic bodies. The cytoplasm is highly condensed and vacuolated, but contains tyrosine hydroxylase that is identified by the antibody reaction. The nucleus is highly condensed. The electron dense chromatin material in the cytoplasm may be nuclear fragments about to be packaged as apoptotic bodies. An apoptotic body can be seen which is separated from the cell, and contains a small amount of possibly nuclear material and perhaps cytoplasmic material. A microglial cell can be observed, that is probably about to remove the apoptotic cell. Fig. 3C is another apoptotic dopaminergic neuron with a condensed nucleus and vacuolated, condensed cytoplasm. The source of the vacuoles is not entirely clear but may be from phagolysosomes which are digesting the remnants of the condensed cytoplasm. Mitochondria appear in the cell and may be somewhat condensed and electron dense. Fig. 3D is a control dopaminergic neuron, with a regular nuclear envelope and tyrosine hydroxylase immunohistochemistry.

3.2. DNA fragmentation and free radicals

Significant DNA fragmentation ($P < 0.05$) was observed after 48 h in the midbrain and not in the striatum after administration of 25 mg/kg MPTP twice (Table 1). Treatment with nicotinamide was found to prevent the MPTP induced DNA fragmentation. An increase in DNA fragmentation was not observed when nicotinamide was administered alone.

It was observed that addition of 0.1 M nicotinamide was able to quench superoxide radicals produced by xanthine oxidase (Fig. 4). 0.1 M nicotinamide was also able to quench hydroxyl radicals generated by the addition of 0.4 M hydrogen peroxide to xanthine oxidase.

3.3. Nicotinamide and NAD levels

Administration of 500 mg/kg nicotinamide for 8 h increased NAD levels from 0.28 ± 0.08 μmol/g to 0.38 ± 0.04 μmol/g in the midbrain and from 0.35 ± 0.04 μmol/g to 0.45 ± 0.04 μmol/g in the striatum. The

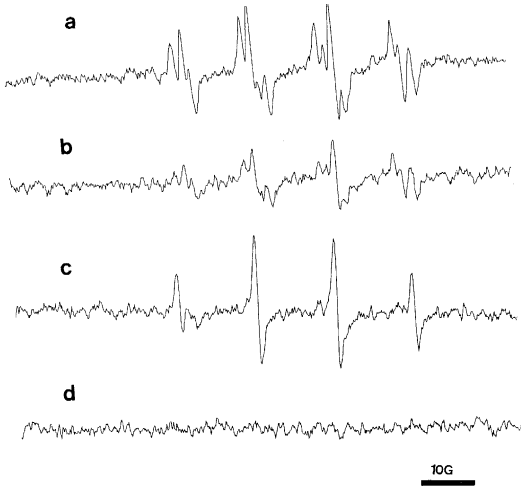


Fig. 4. (a) EPR spectra in the presence of 0.1 units/ml of xanthine oxidase, 0.5 mM xanthine and 0.1 M DMPO in 0.1 M glycine buffer (pH 8.3) containing 1 mM DETAPAC; (b) as in (a), with 0.1 M nicotinamide. Nicotinamide was able to quench the superoxide radicals formed in (a) to some extent; (c) as in (a), with 0.4 M hydrogen peroxide to induce the generation of hydroxyl radical; (d) as in (c), with 0.1 M nicotinamide which was able to quench all radicals.

levels of NAD remained high even 13 h after administration of nicotinamide. A large dose of nicotinamide was used as only a small fraction ultimately reaches the brain.

4. Discussion

4.1. DNA fragmentation and MPTP

In this study the induction of apoptosis and DNA fragmentation by MPTP is reported along with the very interesting observation that nicotinamide seems to be able to prevent both when given prior to neurotoxin administration. DNA may be an important target site for neurotoxins that produce active radical species (Mukherjee et al., 1995). The fragmentation of DNA by MPTP has also been documented in other studies (Dipasquale et al., 1991). Significant DNA fragmentation was observed in the midbrain and not in the striatum following administration of MPTP. It has been shown by previous studies that the midbrain is biochemically different from the striatum in terms of its response to MPTP toxicity (Adams et al., 1989). Midbrain neuronal somas have nuclear DNA not present in striatal terminals. This makes the neurons more susceptible to oxidative stress induced DNA damage than their terminals. Of course, apoptosis of midbrain neurons causes loss of their striatal terminals. The active metabolite of MPTP, which is MPP^+, is actively taken up by the nuclei of dopaminergic neurons (Buu, 1993). Though some of the DNA damage associated with both necrosis and apoptosis may be repaired over time by various enzymes, it is possible that transient DNA damage will affect protein synthesis or mRNA transcription. This may cause disruption of the normal processes in the brain.

4.2. MPTP induction of apoptosis

MPTP administration caused widespread apoptosis in the midbrain. This result is in agreement with previous studies where apoptotic cells had been identified with the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) technique (Mochizuki et al., 1994; Dipasquale et al., 1991; Tatton and Kish, 1997). However, in a recent study (Jackson-Lewis et al., 1995) apoptosis was not detected following treatment with MPTP. In this work no direct experiments (for example, the TUNEL technique or gel electrophoresis to find the characteristic DNA ladder) had been done to detect apoptosis. Therefore it is possible that apoptosis that had been induced may have gone undetected. The cells that stained with the TUNEL technique in the current study were all apoptotic as they all had the characteristic morphology and apoptotic bodies. No necrotic cells were observed at 48 h. All apoptotic cells were found, by oil immersion and electron microscopy, to have condensed cytoplasms and nuclei. Large cytoplasmic vacuoles were frequently present. Apoptotic cells usually have DNA fragments in their cytoplasms indicating possible loss of nuclear membrane integrity.

Electron microscopy was done to observe cells undergoing necrosis and apoptosis. Necrotic cells were observed within 2 h, while apoptotic cells were seen 10 h and later following MPTP treatment. The in situ technique was employed only after 48 h of treatment as this time precludes the presence of necrotic cells. Also higher levels of DNA fragmentation were measured at the end of 48 h which indicates that apoptosis was more pronounced at 48 h. A recent study agrees with this time course, in that following MPTP treatment, mice have the most apoptotic brain cells between 24 and 48 h (Tatton and Kish, 1997).

4.3. MPTP and free radicals

It has been shown in previous studies that MPP^+ can act as a substrate for xanthine oxidase producing $MPP^{\cdot}$, which is the free radical form of MPP^+, and subsequently superoxide and hydroxyl ions (Klaidman et al., 1993). Xanthine oxidase is located in brain endothelial cells and is probably activated during MPTP induced hypoperfusion (Adams et al., 1991). That nicotinamide can act as a scavenger of oxygen derived radicals is supported by other studies (Ledoux et al., 1988), in which nicotinamide was employed to quench radicals generated by alloxan. Additionally, nicotinamide is known to inhibit xanthine oxidase by preventing the conversion of the dehydrogenase form ('D' form) to the oxidase form ('O' form) (Di Steffano and Pizzichini, 1980). Our results demonstrate that nicotinamide inhibits hydroxyl radical production by xanthine oxidase. This may be one mechanism by which nicotinamide prevents MPTP toxicity since xanthine oxidase may be important in MPTP toxicity.

4.4. MPTP, nicotinamide and DNA fragmentation

The exact role of PADPRP and its inhibitors during apoptosis and DNA fragmentation in vivo is still controversial. In some studies a cysteine protease has been observed to destroy PADPRP during the apoptotic process (Nicholson et al., 1995). However, it is clear that a second activation of PADPRP then occurs and is necessary for the induction of apoptosis (Nosseri et al., 1994). This second activation of PADPRP is responsible for a late depression of NAD levels when apoptosis is detected (Morgan, 1995). Nicotinamide can produce a weak in vivo inhibition of PADPRP. However, in our studies, nicotinamide was administered 13 h before MPTP. It is very unlikely that nicotinamide was present to inhibit PADPRP when MPTP was given. Moreover, in a recent study it was observed that PADPRP inhibitors like benzamide and nicotinamide were able to prevent depletion of dopamine following MPTP treatment in mice (Cosi et al., 1996). Additionally, in this study it was observed that nicotinamide caused an increase in 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) levels. This implies that nicotinamide does not act as an inhibitor of monoamine oxidase and may increase dopamine turnover. Other studies (Zhang et al., 1995) have observed that the relative potencies of the PADPRP inhibitors in protecting cells from damage are related to their potencies as inhibitors of PADPRP.

Nicotinamide did produce a long lasting increase in brain levels of NAD, which is a substrate for PADPRP. Restoration and elevation of NAD may be important as NAD is a cofactor in a number of essential biochemical pathways like glycolysis and is also a precursor for ATP synthesis. MPTP is known to decrease ATP levels in cells. NAD can also be converted to NADP and NADPH, which can then be utilized to reduce GSSG to GSH. Therefore, several mechanisms may be important in the inhibition of apoptosis by NAD.

References

Adams, J.D., Odunze, I.N., 1991. Oxygen free radicals and Parkinson's disease. Free Radic. Biol. Med. 10, 161–169.

Adams, J.D., Kalivas, P.W., Miller, C.A., 1989. The acute histopathology of MPTP in the mouse CNS. Brain Res. Bull. 23, 1–17.

Adams, J.D., Klaidman, L.K., Odunze, I.N., Johannessen, J.N., 1991. Effects of MPTP on the cerebrovasculature. Int. J. Dev. Neurosci. 9, 155–159.

Adams, J.D., Klaidman, L.K., Leung, A.C., 1993. MPP^+ and $MPDP^+$ induced oxygen radical formation with mitochondrial enzymes. Free Radic. Biol. Med. 15, 181–186.

Berglund, T., 1994. Nicotinamide, a missing link in the early stress response in eukaryotic cells: a hypothesis with special reference to oxidative stress in plants. FEBS Lett. 351, 1445–1449.

Buu, N.T., 1993. Uptake of 1-methyl-4-phenylpyridinium and dopamine in the mouse brain cell nuclei. J. Neurochem. 61, 1557–1560.

Cosi, C., Colpaert, F., Koek, W., Degryse, A., Marien, M., 1996. Poly (ADP-ribose) polymerase inhibitors protect against MPTP-induced depletions of striatal dopamine and cortical noradrenaline in C57B1/6 mice. Brain Res. 729, 264–269.

Dipasquale, B., Marini, A.M., Youle, R.J., 1991. Apoptosis and DNA degradation induced by 1-methyl-4-phenylpyridinium in neurons. Biochem. Biophys. Res. Commun. 181, 1442–1448.

Di Steffano, A., Pizzichini, M.E., 1980. Nicotinamide and liver xanthine oxidase. Adv. Exp. Med. Biol. 122B, 189–196.

Jackson-Lewis, V., Jakowec, M., Burke, R.E., Przedborski, S., 1995. Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegeneration 4 (3), 257–269.

Klaidman, L.K., Adams, J.D., Leung, A.C., Kim, S.S., Cadenas, E., 1993. Redox cycling of MPP^+: evidence for a new mechanism involving hydride transfer with xanthine oxidase, aldehyde dehydrogenase and lipoamide dehydrogenase. Free Radic. Biol. Med. 15, 169–179.

Klaidman, L.K., Leung, A., Adams, J.D., 1995. High-performance liquid chromatography analysis of oxidized and reduced pyridine dinucleotides in specific brain regions. Anal. Biochem. 228, 312–317.

Ledoux, S.P., Hall, C.R., Forbes, P.M., Patton, N.J., Wilson, G.L., 1988. Mechanisms of nicotinamide and thymidine protection from alloxan and streptozotocin toxicity. Diabetes 37, 1015–1019.

Mochizuki, H., Nakamura, N., Nishi, K., Mizuno, Y., 1994. Apoptosis is induced by 1-methyl-4-pyridinium ion (MPP^+) in ventral mesencephalic-striatal co-culture in rats. Neurosci. Lett. 170, 191–194.

Morgan, W.A., 1995. Pyridine nucleotide hydrolysis and interconversion in rat hepatocytes during oxidative stress. Biochem. Pharmacol. 49 (9), 1179–1184.

Mukherjee, S.K., Yasharel, R., Klaidman, L.K., Hutchin, T.P., Adams, J.D., 1995. Apoptosis and oxidative stress as induced by tertiary butylhydroperoxide in the brain. Brain Res. Bull. 38 (6), 595–604.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vallaincourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T.T., Yu, V.L., Miller, D.K., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, 37–43.

Nosseri, C., Coppola, S., Ghibelli, L., 1994. Possible involvement of poly (ADP-ribose) polymerase in triggering stress-induced apoptosis. Exp. Cell Res. 212, 367–373.

Steller, H., 1995. Mechanisms and genes of cellular suicide. Science 267, 1445–1449.

Tatton, N.A., Kish, S.J., 1997. In situ detection of apoptotic nuclei in the substantia nigra compacta of MPTP treated mice using terminal deoxynucleotidyl transferase labeling and acridine orange staining. Neuroscience 77, 1037–1048.

Vyas, I., Heikkila, R.E., Nicklas, W.J., 1986. Studies on the neurotoxicity of MPTP: inhibition of NAD linked substrate oxidation by its intermediate MPP^+. J. Neurochem. 46, 1501–1507.

Zhang, J., Pieper, A., Snyder, S.N., 1995. Poly (ADP-ribose) synthetase activation: an early indicator of neurotoxic DNA damage. J. Neurochem. 65, 1411–1414.