

ORIGINAL ARTICLE

Additive Neuroprotective Effects of Creatine and a Cyclooxygenase 2 Inhibitor Against Dopamine Depletion in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) Mouse Model of Parkinson's Disease

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Abstract

There is evidence that both inflammatory mechanisms and mitochondrial dysfunction contribute to Parkinson's disease (PD) pathogenesis. We investigated whether the cyclooxygenase 2 (COX-2) inhibitor rofecoxib either alone or in combination with creatine could exert neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of PD in mice. Both rofecoxib and creatine administered alone protected against striatal dopamine depletions and loss of substantia nigra tyrosine hydroxylase immunoreactive neurons. Administration of rofecoxib with creatine produced significant additive neuroprotective effects against dopamine depletions. These results suggest that a combination of a COX-2 inhibitor with creatine might be a useful neuroprotective strategy for PD.

Index Entries: Inflammation; free radicals; mitochondria; cyclooxygenase; creatine; Parkinson's disease.

Introduction

Although a small number of genetic defects are associated with Parkinson's disease (PD), in most patients, the etiology is unknown. There is increasing evidence that both mitochondrial dysfunction and inflammatory mechanisms may contribute to PD pathogenesis. There is an increase in reactive microglia in the striatum and substantia nigra of patients with idiopathic PD (McGeer et al., 1988). Activated microglia are also found in the substantia nigra of humans dying many yr after exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1999). Increased interleukin

(IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α concentrations are reported in the cerebrospinal fluid and striatum of PD patients (Blum-Degen et al., 1995; Mogi et al., 1996; Muller et al., 1998; Mogi et al., 1994a; Mogi et al., 1994b), and an increase in TNF- α and IL-1 β immunoreactive glial cells has been reported in the substantia nigra (Hunot et al., 1999). Last, an IL-1 β polymorphism was reported to increase the risk of PD (McGeer et al., 2002; Schulte et al., 2002).

Defects in mitochondrial energy metabolism are also implicated in PD pathogenesis. MPTP has been used to model PD both in mice and primates (Beal, 2001). There is substantial evidence that MPTP toxicity involves mitochondrial dysfunction. MPTP is

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metabolized to MPP⁺ by monoamine oxidase B, and is then taken up by the dopamine transporter and accumulates in mitochondria, where it inhibits complex I of the electron transport chain (Gluck et al., 1994; Beal, 2001). Similarly, systemic administration of the complex I inhibitor rotenone produces an animal model of PD (Betarbet et al., 2000).

We previously demonstrated that administration of creatine exerts dose-dependent neuroprotective effects against MPTP neurotoxicity (Matthews et al., 1999). Cyclooxygenase (COX) 2 is induced by cytokines, including IL-1 β and TNF- α , and has been implicated in neurodegeneration in a variety of settings (Almer et al., 2001; Iadecola et al., 2001; Hewitt et al., 2000). Cyclooxygenase converts arachidonic acid to PGH₂, the precursor of PGE₂, and several other prostanoids. COX-1 is expressed constitutively, whereas the expression of COX-2 is induced in inflamed tissue (Smith et al., 2000). Recently it was shown that COX-2 inhibitors exert neuroprotective effects against MPTP toxicity (Teismann et al., 2001; Teismann et al., 2003). In the present experiments, we therefore examined whether creatine could exert additive neuroprotective effects when administered with rofecoxib, one of the two COX-2 inhibitors presently approved for use in man (Fitzgerald and Patrono, 2001).

Materials and Methods

Experimental Animals

Our experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals. Male 3-mo-old Swiss-Webster mice were fed lab chow diets supplemented with 2% creatine, or 0.005% rofecoxib or their combination for 1 wk before MPTP administration. Standard unsupplemented lab chow diet served as a control. We examined 12 mice in each group for neurochemistry and 10 mice in each group for histology. MPTP (20 mg/kg, 5 mL/kg, intraperitoneally) was administered four times at 2-h intervals to the mice. The animals were killed 1 wk after MPTP treatment, and both striata were rapidly dissected on a chilled glass plate and frozen at -70°C. The samples were subsequently thawed in 0.4 mL of chilled 0.1 M perchloric acid and sonicated. Aliquots were taken for protein quantification using a spectrophotometric assay. Other aliquots were centrifuged, and dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in the supernatants by high performance

liquid chromatography (HPLC) with electrochemical detection. Concentrations of dopamine and metabolites were expressed as ng/mg of protein (mean \pm S.E.M.).

MPP⁺ Levels

To determine whether MPTP uptake or metabolism was altered, after 1 wk of supplemented or control diets, MPTP 20 mg/kg was administered intraperitoneally four times at 2-h intervals and mice were killed 90 min after the last injection. MPP⁺ levels were quantified by HPLC with ultraviolet detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid and an aliquot of supernatant was injected onto a Brownlee aquapore X03-224 cation exchange column (Rainin, Woburn, MA). Samples were eluted isocratically with 90% 0.1 M acetic acid and 75 mM triethylamine HCl, pH 2.3, adjusted with formic acid, and 10% acetonitrile (ng/mg protein).

Histological Analysis

Three-month-old Swiss Webster mice were fed a standard lab chow diet (control), or diet containing 2% creatine, 0.005% rofecoxib, or a combination of 2% creatine and 0.005% rofecoxib. After 1 wk, MPTP (20 mg/kg, 5 mL/kg, intraperitoneally) or phosphate-buffered solution (PBS) (5 mL/kg, intraperitoneally) was administered four times at 2-h intervals. One wk after the last MPTP or PBS treatment, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and perfused transcardially with ice-cold 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were removed, postfixed for 2 h in the same fixative, and then cryoprotected in 30% sucrose overnight at 4°C. Serial coronal sections (50 μ m) were cut through the substantia nigra. Three sets consisting of eight sections each, 150 μ m apart were prepared for tyrosine hydroxylase (TH), COX-2, or 4-hydroxynonenal immunohistochemistry using the avidin-biotin peroxidase technique. Briefly, free-floating sections were pretreated with 3% H₂O₂ in PBS for 30 min. The sections were incubated sequentially in (1) 1% bovine serum albumin (BSA)/0.2% Triton X-100 for 30 min; (2) rabbit anti-TH affinity purified antibody (Chemicon, Temecula, CA; 1:2000), rabbit anti-COX-2 (Cayman Chemical, Ann Arbor, MI; 1:1000), or mouse anti-4-hydroxynonenal (1:1000) diluted in PBS/0.5% BSA for 18 h; (3) biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 in PBS/0.5% BSA) for 1 h; and (4) avidin-biotin-peroxidase complex

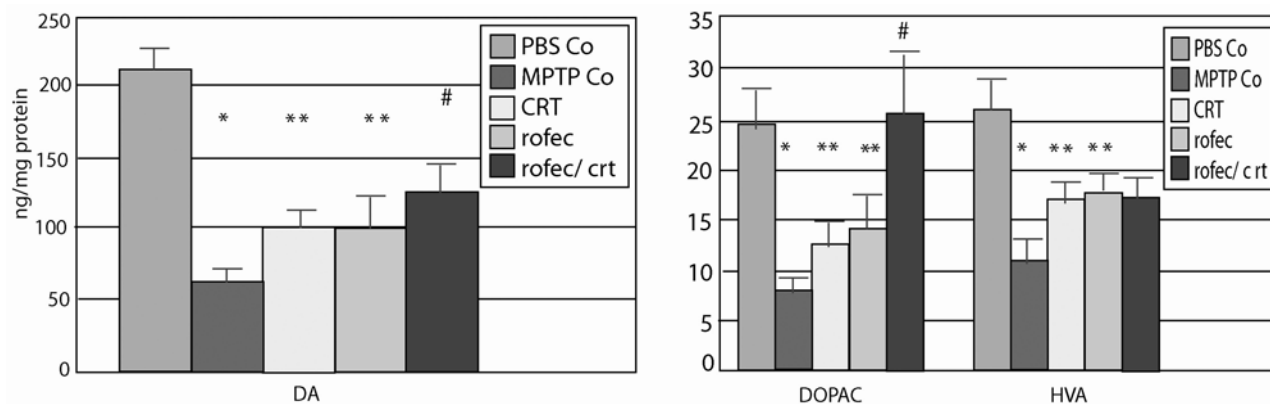


Fig. 1. Effects of MPTP on dopamine, DOPAC, and HVA in mice fed with creatine, rofecoxib, and their combination diet. PBS Co = mice fed with unsupplemented diet and injected with PBS; MPTP Co = mice fed with unsupplemented diet and injected with MPTP; CRT = mice fed with 2% creatine diet; rofec = mice fed with 0.005% rofecoxib; rofec/crt = mice fed with 2% creatine and 0.005% rofecoxib combination diet. * $p < 0.01$, compared with PBS-treated animals on normal diet, ** $p < 0.05$ compared with MPTP-treated mice on normal diet. # $p < 0.05$ compared with MPTP treated mice on creatine or rofecoxib diet.

(Vector; 1:200 in PBS) for 1 h. The immunoreaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) with nickel intensification (Vector) as the chromogen. All incubations and rinses were performed with agitation using an orbital shaker at room temperature. The sections were mounted onto gelatin-coated slides, dehydrated, cleared in xylene, and coverslipped. The numbers of TH-immunoreactive cells in the substantia nigra pars compacta (SNpc) were counted using the optical fractionator method in the Stereo Investigator (v. 4.35) software program (Microbrightfield, Burlington, VT). Results are expressed as the mean \pm S.E.M. Statistical comparisons were made using one-way analysis of variance followed by Newman-Keuls *post hoc* tests.

Results

The effects of administration of MPTP in controls and mice fed with 2% creatine, or 0.005% rofecoxib or their combination are shown in Fig. 1. The dose of MPTP we used (4×20 mg/kg), produced significant dopamine depletion of 69% in mice fed with a regular diet ($n = 12$ /group). Either 2% creatine or 0.005% rofecoxib significantly attenuated the dopamine depletion ($p < 0.01$). In mice fed with creatine and rofecoxib combination, there was an additive neuroprotective effect which was significantly better than either creatine or rofecoxib alone ($p < 0.01$). MPTP produced a significant depletion of

DOPAC and HVA, which was significantly attenuated in mice fed with either creatine or rofecoxib ($p < 0.05$). In the combined treatment group the DOPAC levels were significantly greater than those in the groups with the creatine or rofecoxib alone. A similar additive effect was not seen with HVA, which may be due to experimental variation. The reduced sensitivity to MPTP was not caused by an alteration in uptake or metabolism of MPTP to MPP⁺ because striatal MPP⁺ levels did not significantly differ at 90 min after MPTP administration (MPP⁺ 30.3 ± 3.5 ng/mg protein in controls, 31.6 ± 3.3 ng/mg protein with creatine, and 38.9 ± 4.2 ng/mg protein with rofecoxib). The combination of creatine with rofecoxib also had no significant effect on MPP⁺ levels (34.7 ± 3.6 ng/mg protein).

Histology

In mice fed the control diet, MPTP significantly reduced the numbers of TH-immunostained neurons in the substantia nigra pars compacta as compared with PBS-treated mice by 32% ($p < 0.001$ vs PBS, Figs. 2 and 3) ($n = 10$ /group). In MPTP-treated mice, dietary treatment with 2% creatine or 0.005% rofecoxib significantly increased the number of surviving TH-immunoreactive cells as compared with mice that received the control diet ($p < 0.001$ vs control). The combination of creatine and rofecoxib significantly attenuated the TH-immunoreactive neuronal loss ($p < 0.001$ vs control diet/MPTP) although the neuroprotection was not significantly

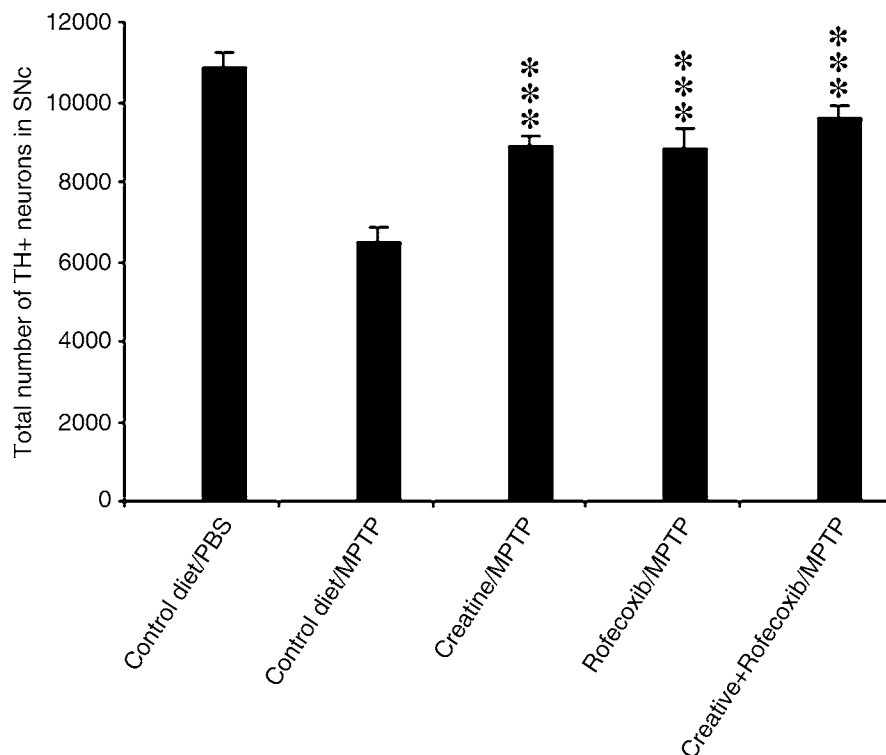


Fig. 2. Effects of creatine and rofecoxib on MPTP-induced loss of TH-immunoreactive neurons in the substantia nigra pars compacta. Cell counts were made using the optical fractionator method. Data are expressed as means \pm S.E.M. ($n = 10$ per group except control diet/PBS, in which $n = 8$). *** $p < 0.001$ vs control diet/PBS, creatine/MPTP, rofecoxib/MPTP, or creatine+rofecoxib/MPTP.

greater than creatine or rofecoxib alone. This was not the result of downregulation of TH expression because adjacent Nissl sections showed identical changes (data not shown).

The pattern of COX-2 immunoreactivity in MPTP-treated mice that received creatine, rofecoxib or its combination was similar to those treated with control diet (data not shown). 4-Hydroxynonenal immunoreactivity increased in the remaining neurons in the SNpc of control diet/MPTP-treated mice compared with the control diet/PBS group (Fig. 4). Creatine, rofecoxib, or its combination appeared to reduce the intensity of 4-hydroxynonenal immunoreactivity in the substantia nigra (Fig. 4).

Discussion

There is substantial evidence implicating both mitochondrial dysfunction and inflammatory mechanisms in PD pathogenesis. Evidence for inflammatory mechanisms comes from a number of studies. There is an increase in activated microglia in the sub-

stantia nigra of idiopathic PD patients as well as patients previously exposed to MPTP (McGeer et al., 1998; Langston et al., 1999). In PD striatum, messenger RNA for the complement components C₁Q and C9 is increased (McGeer et al., 2001). There is an increase in a number of inflammatory cytokines including TNF- α as well as IL1- β within the substantia nigra of PD patients (Mogi et al., 1994a; Mogi et al., 1994b). After administration of MPTP to mice, there is evidence for a microglial reaction (Kohutnicka et al., 1998; Kurkowska-Jastrzebska et al., 1999), which is associated with an increase in proinflammatory cytokines such as IL-1 β (Mogi et al., 1998). Administration of minocycline has neuroprotective effects in the MPTP model, which are associated with a reduction in activated microglia as well as a decrease in mature IL-1 β (Du et al., 2001; Wu et al., 2002).

COX-2 is expressed and regulated in glial cells by cytokines including IL-1 β and lipopolysaccharide (Cao et al., 1994; O'Banion et al., 1996). Chronic infusion of lipopolysaccharide for 2 wk into the sub-

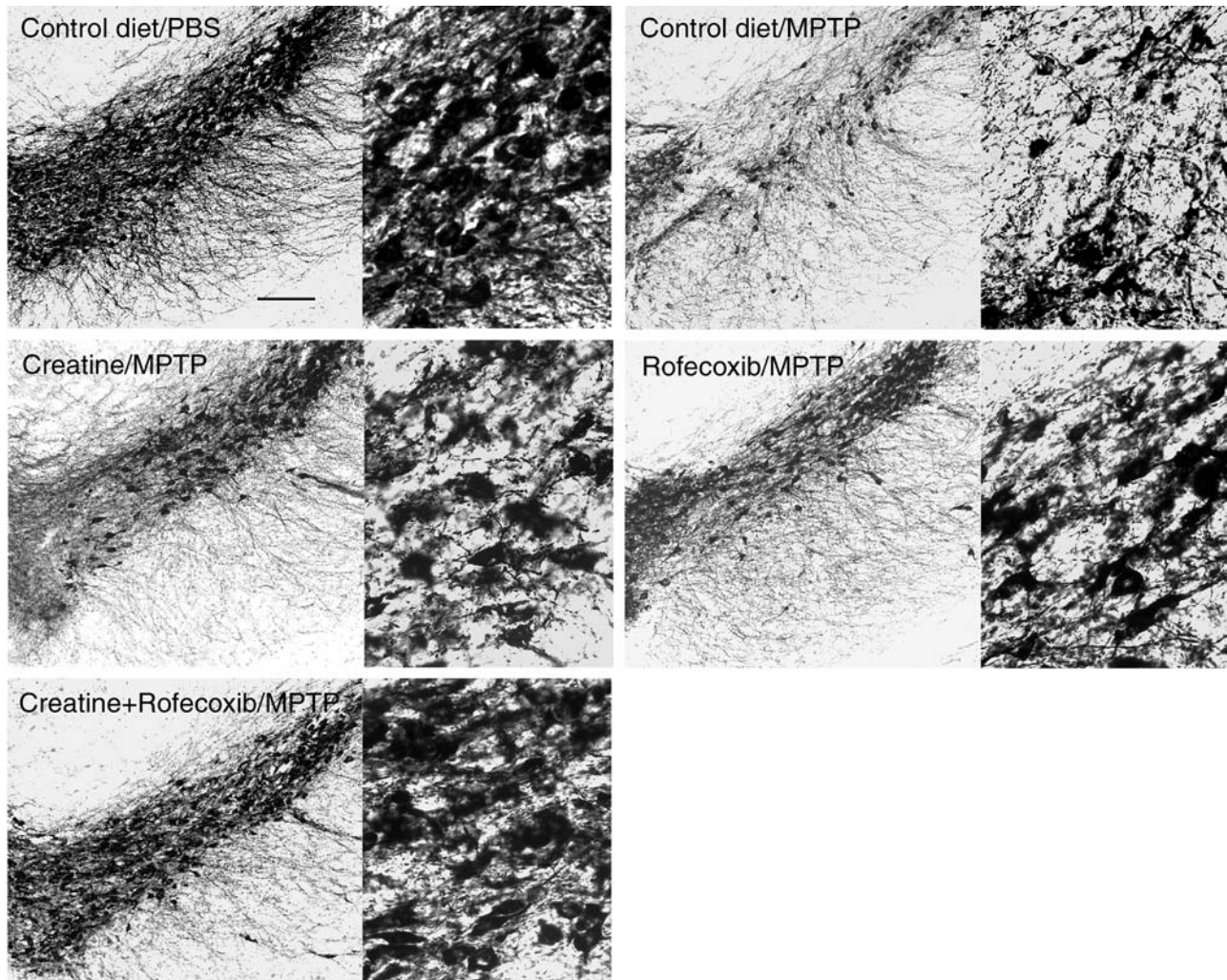


Fig. 3. Low and high magnification photomicrographs of representative TH-immunostained sections through the substantia nigra pars compacta of mice treated with PBS or MPTP that received control diet, creatine, rofecoxib, or a combination of creatine and rofecoxib. A noticeable mitigation of MPTP-induced loss of TH-positive neurons occurred in mice treated with creatine, rofecoxib, or their combination. Scale bar, 200 μ for low magnification photos; 50 μ for high magnification photos.

stantia nigra in rats results in rapid activation of microglia, followed by a delayed and gradual loss of nigral neurons (Gao et al., 2002). COX-2 is also expressed in neurons after excitotoxic lesions, synaptic excitation, cerebral ischemia, and in transgenic mouse models of myotrophic lateral sclerosis (ALS) (Adams et al., 1996; Ho et al., 1998; Planas et al., 1999; Iadecola et al., 2001; Almer et al., 2001). A recent study showed that COX-2 immunoreactivity is expressed in dopaminergic neurons following MPTP as well as in the substantia nigra of PD patients (Teismann et al., 2003). There was no expression in microglia, suggesting that cell autonomous expression of COX-

2 may play a role in its toxicity. The enzyme was active both following MPTP and in PD substantia nigra as evidenced by increased PGE2 levels (Teismann et al., 2003).

Several lines of evidence show that COX-2 contributes to neuronal cell death both in vitro and in vivo. Cyclooxygenase catalyzes the formation of prostaglandins, which involves reduction of a hydroperoxide, resulting in the generation of free radicals. Mice overexpressing COX-2 show increased vulnerability to kainic acid and have elevated lipid peroxidation (Kelley et al., 1999). Neuronal death mediated by N-methyl-D-aspartate is diminished in

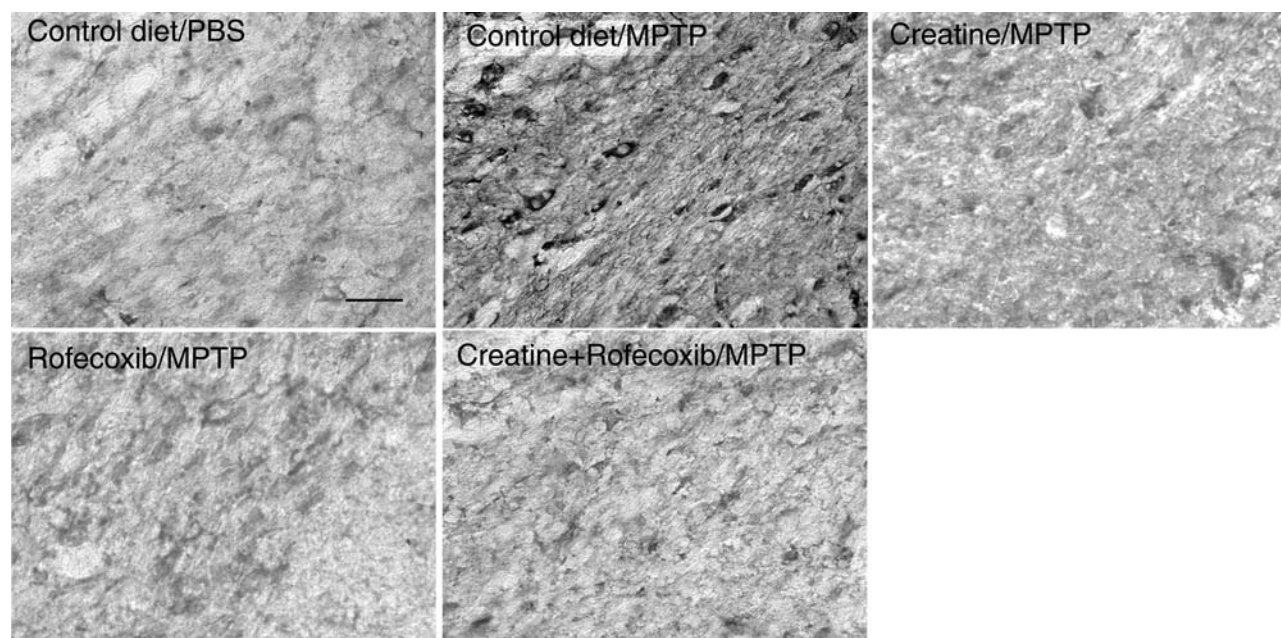


Fig. 4. Photomicrographs of representative 4-hydroxynonenal-immunostained sections through the substantia nigra pars compacta of mice treated with PBS or MPTP, that received control diet, creatine, rofecoxib, or a combination of creatine and rofecoxib. MPTP treatment increased neuronal staining in the substantia nigra pars compacta. Creatine, rofecoxib, or their combination reduced perikaryal HNE immunoreactivity. Scale bar, 50 μ .

a dose-dependent manner by COX-2 inhibitors in primary neuronal cultures (Hewett et al., 2000). Furthermore, transgenic mice that are deficient in COX-2 show reduced susceptibility to N-methyl-D-aspartate-induced excitotoxicity, focal ischemia, and MPTP (Iadecola et al., 2001; Teismann et al., 2003).

We previously showed that mice with a dominant-negative inhibition of interleukin converting enzyme, the known activator of IL-1 β , have a marked attenuation of MPTP induced neurodegeneration (Klivenyi et al., 1999). IL-1 β is known to induce COX-2, a key enzyme involved in the production of both proinflammatory prostanoids as well as reactive oxygen species (Smith et al., 2000). Furthermore, TNF- α activates COX-2 via the JNK pathway, which has also been implicated in MPTP neurotoxicity (Saporito et al., 1999; Saporito et al., 2000).

In the present experiments we found that the selective COX-2 inhibitor rofecoxib exerted significant neuroprotective effects against the MPTP neurotoxicity. This is consistent with a recent study, which showed that the COX-2 inhibitor meloxicam showed protection against MPTP induced cell loss, and also showed significant protection against impairment of locomotor activity and depletion of striatal dopamine (Teismann et al., 2001). Furthermore, another recent study showed that rofecoxib attenu-

ated MPTP-induced loss of tyrosine hydroxylase immunoreactive neurons as well as fibers in the striatum (Teismann et al., 2003).

We also examined whether administration of creatine either alone or in combination with rofecoxib could exert significant neuroprotective effects. Creatine administration can buffer into cellular energy stores as well as inhibit opening of the mitochondrial permeability transition that is linked to both excitotoxic and apoptotic cell death (O'Gorman et al., 1997). Creatine protects creatine kinase from peroxynitrite mediated damage (Wendt et al., 2002) and contributes to reuptake of glutamate into synaptic vesicles (Xu et al., 1996). We previously demonstrated that creatine produces dose-dependent neuroprotective effects, against MPTP induced striatal dopamine depletion as well as loss of tyrosine hydroxylase positive neurons in the substantia nigra (Matthews et al., 1999) and this was confirmed in the present study. Because the primary neuroprotective effects of COX2 inhibitors are anti-inflammatory and antioxidative, whereas the primary neuroprotective effects of creatine are to buffer intracellular energy stores, and both mechanisms are implicated in MPTP pathogenesis, we examined whether they could exert additive neuroprotective effects. The anti-inflammatory drug

minocycline exerts additive neuroprotective effects with creatine in a transgenic mouse model of amyotrophic lateral sclerosis (Zhu et al., 2003). Furthermore, a combination of minocycline, nimodipine, and riluzole exerted additive neuroprotective effects in a transgenic mouse model of amyotrophic lateral sclerosis (Kriz et al., 2003). We previously showed that the combination of coenzyme Q10 with the N-methyl-D-aspartate antagonist remacemide exerts additive neuroprotective effects in a transgenic mouse model of Huntington's disease (Ferrante et al., 2002). There is, therefore, ample precedent that agents targeting different disease mechanisms may exert additive or synergistic neuroprotective effects.

In the present experiments, creatine exerted significant neuroprotective effects against MPTP, and, when combined with rofecoxib, it produced additive neuroprotective effects. The effects of rofecoxib were associated with a reduction in oxidative damage as assessed by 4-hydroxynonenal immunocytochemistry. This is consistent with a recent report that administration of rofecoxib significantly reduced MPTP induced increases in 5-cysteinyl-dopamine, a stable adduct produced by COX-related oxidation of dopamine. Both creatine and potent COX-2 inhibitors have been shown to be safe and well tolerated in man and can, therefore, be readily examined in clinical trials. It is, therefore, possible that a combination of creatine with a COX-2 inhibitor might prove to be useful as a strategy for neuroprotective treatment of PD.

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