Potential neuroprotective effect of ibuprofen, insights from the mice model of Parkinson’s disease

Maciej Świątkiewicz¹, Małgorzata Zaremba¹, Ilona Joniec¹, Andrzej Członkowski¹, Iwona Kurkowska-Jastrzębska²

¹ Department of Experimental and Clinical Pharmacology, Medical University of Warsaw, Krakowskie Przedmieście 26/28, PL 00-927 Warszawa, Poland
² 2nd Department of Neurology, Institute of Psychiatry and Neurology, Sobieskiego 9, PL 02-957 Warszawa, Poland

Correspondence: Maciej Świątkiewicz, e-mail: szwentas@onet.pl

Abstract:
Background: Parkinson’s disease (PD) is one of the most common neurodegenerative diseases. An inflammatory reaction seems to be involved in the pathological process in PD. Prospective clinical studies with various nonsteroidal anti-inflammatory drugs (NSAIDs) have shown that ibuprofen decreases the risk of PD. In the present study we investigated the influence of ibuprofen on dopaminergic neuron injury in the mice model of PD.

Methods: Twelve-month-old male C57Bl mice were injected with MPTP together with various doses of ibuprofen (10, 30 or 50 mg/kg), administered 1 h before MPTP injection for 7 consecutive days. Evaluation concerned dopamine content in the striatum, tyrosine hydroxylase (TH) protein and α-synuclein expression measured 7 and 21 days post MPTP administration (dpa).

Results: MPTP caused injury to dopaminergic neuron endings in the striatum: dopamine content decreased by about 90% 7 dpa and by 85% 21 dpa; TH protein expression diminished by 21% 7 dpa; α-synuclein level decreased by 10 and 26% 7 and 21 dpa, respectively. Ibuprofen administration to mice treated with MPTP significantly increased the level of dopamine in the striatum 7 and 21 dpa. It also prevented TH protein decrease and increased α-synuclein level 21 dpa.

Conclusions: Ibuprofen was shown to protect neurons against MPTP-induced injury in the striatum. The possible mechanism of the neuroprotective effect of ibuprofen might be associated with decreased dopamine turnover and cyclooxygenases inhibition resulting in lower reactive oxygen species formation.

Key words: MPTP, neuroinflammation, neurodegeneration, ibuprofen, α-synuclein, Parkinson’s disease

Introduction

Parkinson’s disease (PD) is one of the most common neurodegenerative diseases. The detailed mechanisms underlying the pathological changes in PD are not clearly understood. An important role in the pathogenesis of progressive neurodegeneration is attributed to microglial and astroglial activation and lymphocytic infiltration, observed in the area of impaired neurons. This local reaction leads to sustained, chronic neuroinflammation, which is suggested to drive the progressive neurodegeneration in PD [2, 26].

The role of inflammation in the pathogenesis of PD and the use of nonsteroidal anti-inflammatory drugs
(NSAIDs) as neuroprotective agents remain areas of intense research. A number of experimental and clinical studies have suggested that NSAIDs may have a therapeutic role in PD [2–4, 15, 16, 18, 22, 23, 25]. The analysis, which combined the results of several large epidemiologic studies, has suggested that the use of non-aspirin NSAIDs is associated with a 15% reduction in the risk of PD. In addition, a greater reduction in the risk has been observed in case of regular (29% reduction) and long-term use of NSAIDs (21% reduction), consistent with a dose-response relation. By contrast, no protective effect has been observed for aspirin or acetaminophen [22]. Prospective clinical studies with NSAIDs have shown that ibuprofen in particular decreases the risk of PD [16]. In a meta-analysis of a large cohort of people in the USA, users of non-aspirin NSAIDs, and in particular ibuprofen, have shown a lower risk of PD than non-users [18]. A meta-analysis of all available prospective studies has also shown that ibuprofen users have an approximately 30% lower risk of PD than non-users [23].

Animal experiments with PD models have shown that several NSAIDs help to protect against dopaminergic neuron degeneration, dopamine (DA) depletion and diminished locomotor activity caused by neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [5, 15, 19, 20, 28, 36, 42]. In our previous studies, also based on the MPTP mice model, ibuprofen dose-dependently alleviated the loss of striatal dopamine without toxic influence on dopaminergic neurons, while other NSAIDs (e.g., indomethacin) appeared to be toxic in high doses [28, 29]. However, results vary across parkinsonian models, and some experiments do not identify specific NSAIDs (e.g., diclofenac, ibuprofen, indomethacin and dexamethasone) as unequivocal neuroprotectants for PD [5, 36].

In the present study, we investigated the influence of ibuprofen on the nigrostriatal system, using one of the best known models of PD – the MPTP mice model. When administered peripherally, MPTP crosses the blood-brain barrier and quite selectively injures dopaminergic neurons of the substantia nigra (SN). This leads to a decrease in DA content and depletion of the number of neuronal endings in the striatum and causes neuroinflammation in the impaired structure. We showed that ibuprofen administered in the phase of acute injury had some protective abilities against MPTP-induced damage and modified dopamine metabolism in the striatum.

**Materials and Methods**

**Animals**

Male C57BL mice (12-month-old) were studied. The animals were housed in plastic cages with free access to food and water. They were maintained on a 12-h light/dark cycle at a temperature of 25 ± 2°C. Experiments were conducted without causing unnecessary harm to the animals and were approved by the Local Ethics Committee.

The mice were divided into 8 groups: an MPTP group (receiving MPTP toxin), a control group (receiving 0.9% solution of NaCl according to the pattern of MPTP administration), three ibuprofen groups – IBF (receiving ibuprofen in a dosage of 10, 30 or 50 mg/kg) and three IBF + MPTP groups (receiving MPTP toxin and ibuprofen in a dosage of 10, 30 or 50 mg/kg). The animals were sacrificed 7 and 21 days after MPTP intoxication (6 animals per time point) by spinal cord dislocation. Their brains were rapidly removed and the striata were dissected and frozen at −80°C.

**MPTP administration**

MPTP-hydrochloride (Sigma, USA) was dissolved in 0.9% solution of NaCl (2 mg/ml) and administered intraperitoneally in four doses of 10 mg/kg each, at one hour intervals, to a total dose of 40 mg/kg.

**Ibuprofen administration**

Ibuprofen (Sigma, USA) was dissolved in 0.9% NaCl solution just before injection. It was administered in three doses of 10, 30 and 50 mg/kg starting one day before MPTP treatment and was given every day for 7 consecutive days.

**HPLC**

The striata were dissected out on an ice-cold plate. Each tissue sample was rapidly weighed and frozen (−80°C) for future analysis. Tissues were homogenized in ice-cold 0.1 M HClO4 and centrifuged (13,000 × g, 15 min) to precipitate proteins. The supernatant was removed, filtered (0.2 µm pore size; Whatman, USA) and examined for dopamine (DA; standard supplied by RBI) and its metabolites content.
3,4-dihydroxyphenylacetic acid (DOPAC; RBI, USA) and homovanillic acid (HVA; Sigma, Germany), using HPLC with electrochemical detection and glassy carbon electrode. The electrochemical potential was set at 0.8 V with respect to the Ag/AgCl reference electrode. The chromatographic system consisted of an autosampler automatic injector (Knauer Basic Marathion), a pump (Mini-Star K-500; Knauer, Germany), and an electrochemical detector (L-3500A; Merck, Germany). The mobile phase comprised 31 mM sodium phosphate (Sigma), 58 mM citric acid (Sigma), 1 mM octane sulfonic acid (Aldrich, Germany), 27 µM ethylenediaminetetraacetic acid (EDTA; Sigma) in deionized (18.3 mΩ) purified water containing 12% acetonitrile (Merck) and 1% methanol (Merck). Monoamines were separated using a C-18 column (250 × 4 mm reverse phase; Nucleosil; 5 µm particle size; Macherey-Nagel, Germany) and a mobile phase flow rate maintained at 0.8 ml/min. The samples were quantified by comparison with standard solutions of known concentration using HPLC software, and the area under the peaks was measured. Data were collected and analyzed using Eurochrom 2000 for Windows (Knauer). Contents of dopamine and its metabolites were expressed as pg/mg fresh tissue.

**Evaluation of MPP⁺ level by HPLC**

In order to assess 1-methyl-4-phenyl-tetrahydropyridinium ion (MPP⁺) level in the striatum, additional groups of mice (five animals each) received three different doses of ibuprofen before MPTP, which was administered according to the standard scheme for the experiment to the total dose of 40 mg/kg. Two hours after the last MPTP injection the mice were killed by cervical dislocation and the striata were prepared as described above. HPLC analysis of MPP⁺ was performed immediately.

The mobile phase contained 0.14 mol sodium dihydrogen phosphate adjusted to pH 2.5 with H₃PO₄ in 1 : 1 of HPLC grade water with 300 ml of acetonitrile, and was delivered at a rate of 0.5 ml/min onto a reversed-phase column (125 × 3 mm with precolumn 5 × 3 mm; Nucleosil 120-3 C-18; Macherey-Nagel, Germany). The UV detector was set at a wavelength of 295 nm. Twenty microliter aliquots were injected into the column. Data were calculated by an external standard calibration.

**Western blot analysis**

The striatum samples were homogenized with a micropestle (Eppendorf) in 1.5 ml of lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% Igepal CA-630, 0.10% sodium dodecyl sulfate, 0.50% deoxycholic acid sodium salt, 0.10 mg/ml phenylmethylsulfonyl fluoride, 1.0 mM sodium orthovanadate, 10 ng/ml aprotinin and 25 ng/ml pepstatin) at 0–4°C. They were subsequently incubated for 30 min on ice. Homogenates were centrifuged at 10,000 × g for 20 min at 4°C. The aliquots of supernatants were taken for total protein analysis (Bradford Reagent, Sigma). For electrophoresis, 60 µg/ml of a homogenized sample was taken and added to SDS sample buffer at a ratio of 1 : 1.

Proteins were electrophoretically transferred to nitrocellulose membranes using the iBlot® Dry Blotting System (Invitrogen). The membranes were subsequently stained to reconfirm equal loading by Ponceau-S staining and washed with distilled water. After blocking for 1.5 h with 5% nonfat dry milk/0.5% Tween-20 in Tris-buffered saline, the membranes were incubated with polyclonal rabbit anti-α-synuclein (1 : 500, Millipore) or polyclonal rabbit anti-tyrosine hydroxylase antibodies diluted in TBST containing 5% nonfat dry milk (1 : 500, Millipore) overnight at 4°C. The membranes were subsequently washed three times (for 10 min. each) in TBST and incubated with secondary antibodies conjugated to horseradish peroxidase electrophoresed on 10% SDS-polyacrylamide gels in Mini Protean II Dual Slab Cell (Bio-Rad) diluted in TBST containing 5% nonfat dry milk (1 : 2,000, Amersham) for 2 h. Peroxidase activity was visualized using the ECL western blotting detection system (Amersham) according to the manufacturer’s instruction. Each gel contained lanes from each time point and control brains. The densities of each band were analyzed with a software program (Syngen) and expressed in terms of their ratio to control lanes.

**Results**

**Dopamine content in the striatum**

Dopamine level decreased markedly to 8% of the control level on the 7th day post MPTP administration.
(dpa) and rose to 15% on the 21st day, indicating the process of dopamine content restoration (Fig. 1). Ibuprofen treatment prevented a decline in the dopamine content measured on the 7th dpa, in the dose of 30 mg/kg alone, and the dopamine level fell to only 16% of the control level (p < 0.05). Although the ibuprofen treatment was stopped on the 7th day, the group of animals receiving ibuprofen showed better dopamine content restoration on the 21st day. The dopamine content represented 15% of the control level in animals treated with MPTP alone and about 25% in animals additionally treated with 10, 30 and 50 mg/kg of ibuprofen (Fig. 1).

Ibuprofen alone increased the dopamine level by about 20% on the 21st dpa in the doses of 30 mg/kg and 50 mg/kg (p < 0.01).

**Dopamine turnover**

The levels of dopamine metabolites DOPAC and HVA were correlated to the dopamine level in every examined group on the 7th and 21st dpa. The dopamine turnover (DOPAC/DA ratio and HVA/DA ratio) was markedly changed by MPTP intoxication and increased 2.2 times (DOPAC/DA) and 7.4 times (HVA/DA) compared to control on the 7th dpa (p < 0.01). The DOPAC/DA ratio returned to the control level on the 21st dpa, while the HVA/DA ratio remained slightly elevated (p < 0.02) (Fig. 2). Ibuprofen alone influenced dopamine turnover, in particular, it increased the DOPAC/DA ratio in the dose of 10 mg/kg (p < 0.03) and led to an insignificant increase in the dose of 30 mg/kg compared to control. It increased also HVA/DA ratio in the doses of 10 mg/kg (insignificantly) and of 30 mg/kg (p < 0.05). Interestingly, on the 21st dpa, we observed diminished DOPAC/DA ratios in the group of animals receiving ibuprofen in doses of 10 and 30 mg/kg, when HVA/DA ratios returned to the control level. When added to MPTP, ibuprofen markedly decreased both the DOPAC/DA and HVA/DA ratios in the doses of 30 and 50 mg/kg compared to the MPTP group on the 7th dpa (p < 0.05). There was no difference between the DOPAC/DA and HVA/DA ratios between the MPTP and IBF + MPTP groups on the 21st dpa, apart from a slightly lower HVA/DA ratio in the IBF50 + MPTP group than in the MPTP group (p < 0.05).

**TH protein expression**

TH protein expression in the striatum decreased after MPTP treatment by 21% on the 7th day (p < 0.02) and returned to the control level on the 21st dpa (Fig. 3). Ibuprofen administration to animals receiving MPTP completely prevented the decline in TH protein content on the 7th dpa when given in the doses of 10 and 30 mg/kg (p < 0.05). Ibuprofen in the dose of 50 mg/kg had no such effect. TH protein expression in the MPTP group was comparable to the control level on the 21st dpa, and ibuprofen did not influence its
level on this day. Ibuprofen administered alone did not change TH expression in the striatum at any time point (Fig. 3).

**α-Synuclein expression**

α-Synuclein expression in the striatum decreased after MPTP treatment by about 10 and 26% compared to the control level on the 7th and 21st dpa, respectively (p < 0.01) (Fig. 4). The group of animals treated with ibuprofen and MPTP showed a slight depletion of α-synuclein content on the 7th day, similar to the MPTP group. However, on the 21st dpa, α-synuclein level was higher in animals receiving 10 and 30 mg/kg of ibuprofen compared to animals treated with MPTP alone (p < 0.05). Ibuprofen administered alone decreased α-synuclein expression only in the dose of 50 mg/kg on the 21st dpa (p < 0.01).

**MPP⁺ evaluation**

MPTP administration resulted in MPP⁺ formation at a level of 4.9 ± 0.54 ng/mg wet tissue weight.
The mice receiving ibuprofen (10, 30 and 50 mg/kg) before MPTP injection produced MPP\textsuperscript{+} levels comparable to those of MPTP alone but with a tendency to increase depending on the ibuprofen dose. The dose of 100 mg/kg increased MPP\textsuperscript{+} production of 69% (p < 0.05) and was thus excluded from other experiments (Fig. 5).

**Fig. 4.** TH protein expression in the striatum in mice intoxicated with MPTP and treated with ibuprofen 7 and 21 days after MPTP administration. Bars show the mean value ± SEM for all groups of animals treated with various doses of ibuprofen, MPTP or ibuprofen with MPTP. Asterisk (*) indicates significant differences compared to control; hash (#) indicates significant difference compared to MPTP group on the same day.

**Fig. 3.** \(\alpha\)-Synuclein protein expression in the striatum in mice intoxicated with MPTP and treated with ibuprofen 7 and 21 days after MPTP administration. Bars show the mean value ± SEM for all groups of animals treated with various doses of ibuprofen, MPTP or ibuprofen with MPTP. Asterisk (*) indicates significant differences compared to control; hash (#) indicates significant difference compared to MPTP group on the same day.
In the present study, we demonstrated that administration of ibuprofen to mice treated with MPTP significantly accelerated the recovery of DA level in the striatum, prevented TH protein depletion and enhanced α-synuclein expression. The protective effect of ibuprofen was not dependent on MPTP conversion to ion MPP⁺, which was responsible for MPTP toxicity. The protection was less evident in the early phase of injury than in the late phase and was dependent on the dose of the drug. Ibuprofen probably diminished the injury of neuron endings in striatum (prevented TH protein depletion), stimulated the phase of recovery and regeneration and in this way prevented MPTP toxicity.

Ibuprofen is commonly known as an anti-inflammatory agent whose main mechanism of action concerns the inhibition of cyclooxygenase (COX) activity and prostaglandins production. Ibuprofen inhibits equally constitutive COX-1 and inducible COX-2 responsible for inflammation. Both COXs have been shown to play an important role in neuroinflammation in the brain. COX-1 is suggested to be an important player in microglial activation and neurodegeneration; COX-2 may mediate neurotoxic or anti-inflammatory effects depending on the stimulus and type of injured cells [1]. The COX-inhibiting mechanism of action may be involved in the protective effect of ibuprofen and other NSAIDs. In some experiments, pretreatment with NSAIDs led to partial or total protection against MPTP toxicity [5, 19, 34, 36, 41, 42]. The mechanisms of their protective effects have been linked with inhibition of inflammation (decreased production of prostaglandins) and with decreased COX-dependent formation of ROS during the acute phase of MPTP injury. Ibuprofen has been shown to protect dopaminergic neurons from 6-OHDA toxicity in doses that block neuronal COX. The effect has been correlated with lower prostaglandin production [11]. On the other hand, MPP⁺ induced injury of dopaminergic neurons has also been prevented by ibuprofen but linked to decreased ROS production [26].

ROS formation is suggested to be involved in progressive dopaminergic neuron damage in PD [39]. DA metabolism generates a huge amount of oxygen species and is likely responsible for the particular susceptibility of dopaminergic neurons to this kind of injury. Some hypotheses suggest that primary neurodegeneration in the nigrostriatal pathways leads to increased dopamine turnover and triggers ROS generation, which in turn contributes to neuronal impairment [17]. MPTP administration directly increases ROS production by inhibiting enzymes of the respiratory chain, and additionally, by increasing dopamine turnover – a compensatory mechanism triggered by DA depletion. Increased ROS formation has been shown to contribute to dopaminergic neuron injury after MPTP administration [41, 42]. We showed that ibuprofen decreased DA turnover in groups of animals receiving MPTP, which was connected with better recovery of DA level in the striatum. It is possible that lower DA turnover results in lower production of ROS, contributing to the protective effect of the drug. Ibuprofen has been shown to decrease ROS produc-

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**Fig. 5.** MPP⁺ content in the striatum measured 2 h after MPTP administration in mice treated with various doses of ibuprofen before MPTP intoxication. Bars show the mean value ± SEM. Significant difference compared to control is indicated by asterisk (*)
tion and damage to dopaminergic neurons injured by manganese [32] or MPP⁺ [26].

Ibuprofen is not known to influence the metabolism of catecholamines. We observed increased MPP⁺ formation when ibuprofen was added to MPTP regimen, which may indicate augmented MAO-B activity. The effect was also visible as an increased DOPAC/DA and HVA/DA ratios on the 7th day in animals receiving ibuprofen alone in the doses of 10 and 30 mg/kg. However, the effect was not so clearly present in animals receiving higher doses of ibuprofen and was reverse in animals treated additionally with MPTP (decreased DOPAC/DA and HVA/DA ratios). The opposite effects of the various doses of ibuprofen on DA metabolism are difficult to explain. One of the possibilities is that ibuprofen may dose-dependently influence the activation of the enzymes involved in dopamine breakdown. Ibuprofen administered to animals with not-injured nigrostriatal system had same tendency to increase dopamine metabolism. After discontinuation of ibuprofen administration, a dopamine metabolism slightly slowed down (decreased DOPAC/DA ratios on the 21st dpa), what may be explained by the mechanism compensating the previous acceleration. On the other hand, ibuprofen in higher doses might inhibit a compensatory enhancement of dopamine turnover in the injured neurons. It has been shown that other NSAID, indomethacin, prevent an increase of dopamine turnover in rat striatum following systemic lipopolysaccharide administration [31].

The interesting observation was that ibuprofen almost completely prevented TH protein depletion on the 7th dpa but did not increased dopamine level on this day. We also noted that the expression of TH protein returned to the control level after MPTP administration together with much smaller increase of the dopamine content. The discrepancy of the restoration of TH protein and dopamine levels after MPTP-caused injury has been noted by other authors and has been explained by the sensitivity of the methods [43]. TH protein restoration, however, does not necessarily mean that the neurotransmitters are already produced. The fast return of TH protein to the control level indicated, that some striatal neurons were only injured and did not died following MPTP treatment.

Apart from the inflammatory reaction, a large part of the process of neurodegeneration in PD is attributed to insoluble inclusion bodies of α-synuclein, which cause dysfunction of DA neurons in the SN and diminish DA content in the striatum. According to some hypotheses, α-synuclein oligomers trigger synapse damage and inhibit the release of neurotransmitter before any injury to the cell occurs, which could explain the early symptoms of PD. However, the physiological function of α-synuclein has not yet to be fully characterized. Recent evidence suggests that α-synuclein is also a constitutive, pre-synaptic protein that regulates synaptic vesicle formation and neurotransmitter release (including DA) [7, 8, 10, 21, 30]. α-Synuclein may also cause the activation of adaptive compensatory mechanisms that protect neurons [9]. During pathological processes, α-synuclein has the ability to bind free radicals, such as free iron or iron-containing radicals and oxidized dopamine, thus preventing their damaging actions [38].

Following MPTP administration in our animal model, α-synuclein level in the striatum was decreased and did not return to the control level as TH protein did on the 21st dpa. This phenomenon indicated that the level of α-synuclein corresponded to the injury to dopaminergic neuron terminals in the striatum, which confirmed its function as a synaptic protein. It also showed that presynaptic endings might suffer from a long-term or even permanent injury after MPTP administration. Ibuprofen treatment prevented TH protein depletion, which could suggest that the majority of neuronal endings in the striatum were not damaged by MPTP treatment in these groups of mice. The decrease in α-synuclein level on the 7th dpa and its subsequent restoration following ibuprofen treatment on the 21st dpa, might also confirm only partial injury to nerve endings. In this context, ibuprofen attenuated the injury, giving neurons a possibility to recover.

Other properties of ibuprofen may also be responsible for its neuroprotective ability. Apart from COX inhibition, ibuprofen has been shown to block cytokine production through direct inhibition of transcription factors, such as NF-κB [12, 14], and indirectly through activation of a peroxisome proliferator-activated receptor-γ (PPARγ) [24, 27]. Pioglitazone and rosiglitazone, compounds that stimulate the activation of PPARγ, have been shown to protect against MPTP-induced neurodegeneration in a model of PD induced in rhesus monkeys [40] and mice [6, 35]. Ibuprofen and other NSAIDs decrease the level of inducible nitric oxide synthase (iNOS) mRNA, which leads to decreased production of NO [37]. Additionally, it has been shown to protect dopaminergic neu-
rons from NO-induced cell death by scavenging NO radical [4] as well as from glutamate toxicity [13]. In conclusion, ibuprofen showed a dose-dependent protective effect against MPTP toxicity in mice. The short-term treatment during the acute phase of injury prevented TH protein depletion and the neurodegeneration of dopaminergic nerve endings in the striatum. These effects were confirmed in our study by faster restoration of DA content and an increase in pre-synaptic protein level (α-synuclein) in the striatum. The possible mechanism of the neuroprotective effect of ibuprofen might be associated with decreased DA turnover and COX inhibition resulting in lower reactive oxygen species formation and less injury. However, further studies are needed to confirm the possible role of ibuprofen as a neuroprotective agent in the model of neurodegenerative disease.

References:


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