Nonsteroidal Anti-Inflammatory Drugs Potentiate 1-Methyl-4-phenylpyridinium (MPP⁺)-Induced Cell Death by Promoting the Intracellular Accumulation of MPP⁺ in PC12 Cells

Norimitsu Morioka, Kei Kumagai, Katsuya Morita, Shigeo Kitayama, and Toshihiro Dohi

Department of Dental Pharmacology, Division of Integrated Medical Science, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan (N.M., K.K., K.M., T.D.); and Department of Dental Pharmacology, Field of Functional Physiology, Branch of Biophysiological Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan (S.K.)

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ABSTRACT

In this study, we investigated the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on 1-methyl-4-phenylpyridinium (MPP⁺)-induced cell death in PC12 cells. Coincubation of PC12 cells with indomethacin, ibuprofen, ketoprofen, or diclofenac, but not aspirin or N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398), significantly potentiated the MPP⁺-induced cell death. In contrast, these NSAIDs had no effect on rotenone-induced cell death. The potentiating actions of these NSAIDs were not suppressed by treatment with phenyl-N-butylnitrone, a radical scavenger; N-acetyl-1-cysteine, an antioxidant; Ac-DEVD-CHO, a selective caspase-3 inhibitor; or 2-chloro-5-nitro-N-phenylbenzamide (GW9662), a selective antagonist of peroxisome proliferator-activated receptor γ. Furthermore, we observed that DNA fragmentation, which is one of the hallmarks of apoptosis, was not induced by coincubation with MPP⁺ and NSAIDs. We confirmed that coincubation of PC12 cells with 30 µM MPP⁺ and 100 µM indomethacin, ibuprofen, ketoprofen, or diclofenac led to a significant increase in the accumulation of intracellular MPP⁺ compared with incubation with 30 µM MPP⁺ alone. In addition, these NSAIDs markedly reduced the efflux of MPP⁺ from PC12 cells. 3-(3-(2-(7-Chloro-2-quinolinyl) ethenyl) phenyl ((3-dimethyl amino-3oxo-propyl) thio) methyl) propanoic acid (MK 571), which is an inhibitor of multidrug resistance proteins (MRPs), mimicked the NSAIDs-induced effects, increasing cell toxicity and promoting the accumulation of MPP⁺. Moreover, some types of MRPs' mRNA were detected in PC12 cells. These results suggest that some NSAIDs might cause a significant increase in the intracellular accumulation of MPP⁺ via the suppression of reverse transport by the blockade of MRP, resulting in the potentiation of MPP⁺-induced cell death.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes a selective degeneration of nigrostriatal dopaminergic neurons and has been investigated extensively as part of an etiological model for Parkinson's disease (Tipton and Singer, 1993; Blum et al., 2001). The neurotoxic effects in vivo of MPTP are dependent on the conversion to its active metabolite 1-methyl-4-phenylpyridinium (MPP⁺) by monoamine oxidase B in glial cells and the uptake into neurons through transporters (Tipton and Singer, 1993; Kitayama et al., 1998). MPP⁺ induces an inhibition of complex I activity of the mitochondrial respiratory chain and cellular ATP depletion and a loss of mitochondrial transmembrane potential, resulting in cell death (Bates et al., 1994).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their anti-inflammatory, antipyretic, and analgesic properties. The molecular basis for the therapeutic effects of NSAIDs is the ability to inhibit cyclooxygenase (COX) activity and thereby suppress the production of prostaglandins (Vane, 1971). In addition to alleviating inflammatory
tion and pain, NSAIDs have in some cases been suggested to have pharmacological effects that are independent of the inhibition of COX activity. For example, aspirin and salicylate inhibit nuclear factor-κB (Kopp and Ghosh, 1994) and the activity of inhibitory κB kinase-β (Yin et al., 1998). Avramovich et al. (2002) demonstrated that NSAIDs can activate extracellular signal-regulated kinase mitogen-activated protein kinase in SH-SY5Y neuroblastoma cells and PC12 cells. Lehmann et al. (1997) indicated that several NSAIDs bind and activate peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor. Moreover, it has been demonstrated in an overexpressing cell system that some NSAIDs, including indomethacin and ibuprofen, inhibit the activity of multidrug resistance protein 4 (MRP4), which is believed to be the active efflux transporter for the release of prostaglandins from the cells that produce them (Reid et al., 2003b).

Several recent studies have reported inconsistent effects of NSAIDs on the neurotoxicity of MPTP or MPP⁺ in vivo. It has been demonstrated that aspirin, a nonselective COX inhibitor, and meloxicam, a selective COX-2 inhibitor, provide neuroprotection from MPTP at the striatal and nigral levels (Teismann and Ferger, 2001). In contrast, other studies have demonstrated that indomethacin and diclofenac, both nonselective COX inhibitors, have no protective effect on MPTP toxicity, whereas celecoxib, which is a specific COX-2 inhibitor, aggravated MPP⁺-induced striatal dopamine depletion in rats (Sairam et al., 2003). The effects of NSAIDs on MPTP- or MPP⁺-induced cell death are therefore controversial. Thus, in the present study, the possible influences of several NSAIDs on the toxicity of neurotoxins in cultured rat PC12 cells were investigated. It was found that indomethacin, ibuprofen, ketoprofen, and diclofenac significantly potentiated the MPP⁺-induced cell death in PC12 cells. These NSAIDs may evoke a significant increase in the intracellular accumulation of MPP⁺ as a result of a blockade of the outflow of MPP⁺ from PC12 cells.

Materials and Methods

Materials. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui (Tokyo, Japan). MPP⁺ iodide, indomethacin, ibuprofen, ketoprofen, diclofenac sodium, phenyl-N-butyl-nitrite (PBN), N-acetyl-l-cysteine (NAC), and rotenone were obtained from Sigma Chemical Co. (St. Louis, MO). N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonylamide (NS-398) and 3-(3′-4′-2′-2′-4′-quinolyl) ethenyl) phenyl (3′-dimethyl amino-3oxo-propyl) thio) methyl) propanoic acid (MK 571) were provided by Cayman Chemical (Ann Arbor, MI). Ac-DEVd-CHO was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), 2-chloro-5-nitro-N-phenylbenzamide (GW9662) was from Tocris Cookson Inc. (Bristol, UK), 2′-(4-idophenyl)-3-(4-nitrophenyl) -2H-tetrazolium (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were from Dojindo Laboratories (Kumamoto, Japan), and [3H]MPP⁺ (2590 GBq/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). All other reagents used were of analytical grade and from Nacalai Tesque (Kyoto, Japan), Katayama Chemical (Osaka, Japan), or Invitrogen (Carlsbad, CA). Indomethacin, ibuprofen, ketoprofen, aspirin, and GW9662 were each dissolved in ethanol. MPP⁺, diclofenac, NAC, and Ac-DEVd-CHO were dissolved in distilled H₂O. NS-398, PBN, rotenone, and MK 571 were dissolved in dimethyl sulfoxide. The final concentrations of all solvents for treatment of the cells, including control cultures, were maintained at 0.25%.

Cell Culture and Drug Treatments. PC12 cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO₂, 95% air at 37°C. All experiments were carried out 24 h after cells were seeded.

MPP⁺ Toxicity Assay. Cells were treated with various concentrations of MPP⁺, either alone or together with NSAIDs, in low-glucose DMEM supplemented with 2% horse serum, 1% fetal calf serum, and penicillin/streptomycin. The quantitative cell toxicity was determined using WST-1, a water-soluble formazan dye. Briefly, after incubation with various concentrations of MPP⁺ for several hours in the absence or presence of test drugs, cells were washed with Krebs-Ringer-HEPES (KRH)-buffered solution. Thereafter, cells were incubated with 100 μl of a solution containing 0.5 mM WST-1 and 20 μM 1-methoxy PMS for 3 h at 37°C. Optical density was measured using a microplate reader at a wavelength of 450 nm (reference wavelength 600 nm).

Detection of DNA Fragmentation. After their incubation with the drugs, cells were washed with phosphate-buffered saline, and DNA was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA). For further, the DNA was subjected to 1.5% agarose gel electrophoresis in Tris-acetate-EDTA buffer. The gel was subsequently stained with 0.5 μg/ml ethidium bromide and visualized under ultraviolet light and photographed.

MPP⁺ Accumulation Assay. Cells were incubated in low glucose and serum DMEM containing 30 μM [3H]MPP⁺ in the absence or presence of NSAIDs for 9 h. After the incubation, the medium was removed, and cells were rapidly washed with an ice-cold KRH solution. Then, they were solubilized with 2 N NaOH for the measurement of radioactivity with a liquid scintillation counter.

MPP⁺ Efflux Assay. Cells were preloaded with 30 μM MPP⁺ in low glucose and serum DMEM at 37°C for 6 h, washed with KRH solution, and then incubated in fresh low glucose and serum DMEM containing test reagents and 100 μM cocaine to prevent the outflow or reuptake of [3H]MPP⁺ via the monoamine transporters. After 3 h, the medium was harvested, and cells were solubilized with 2 N NaOH. The radioactivity in the incubation medium along with that remaining in the cells was measured separately using the liquid scintillation counter. Released [3H]MPP⁺ was expressed as a percentage of total cell count (sum of counts in the incubated medium and in the cells at the end of incubation).

RT-PCR Analysis. Total RNA was isolated from PC12 cells or rat brain using TRIzol reagent (Invitrogen) according to the manufacturer’s directions and used to synthesize cDNA with ReverTra Ace (Toyobo Engineering, Osaka, Japan) and a random hexamer primer. To amplify the several types of rat MRPs mRNA or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, PCRs were performed with rat MRPs-specific primers (MRP1: forward, 5'-GCACTGCTCTTACAATTGG and reverse, 5'-TCTCATTGAAGTGTAGTACAC; MRP2: forward, 5'-GGGATAAATCTCAGTGTG and reverse, 5'-ATAGCTCACAGAGTGT; MRP3: forward, 5'-TAAAGTGTAAGACACCGC and reverse, 5'-CCTCTGCGCAACTGTGAT; MRP4: forward, 5'-GGAACAGTACACTAGCG and reverse, 5'-GTCACCTGCTCAAAGTGTG; MRP5: forward, 5'-GGGACACTTCAGGCTG and reverse, 5'-CTTGAGCGACCACCTTG) or rat GAPDH primers (forward, 5'- GAGCCGAGATTCCCGTACCA and reverse, 5'-CACAGTCTTCTGAGTGGCAAGT). The amplified products were separated on 2% agarose gel and visualized with ethidium bromide.

Statistical Analysis. Data are expressed as the mean ± S.E. of at least three independent experiments. Differences between means were determined using a one-way analysis of variance with a pairwise comparison by the Bonferroni method. Differences were considered to be significant when the P value was less than 0.05.
Results

Effects of NSAIDs on MPP⁺-Induced Cell Death. In the present study, MPP⁺ caused the death of PC12 cells in a time- and dose-dependent manner (Fig. 1 and Fig. 2, left). Previously, we have confirmed that the norepinephrine transporter was expressed in PC12 cells (Ikeda et al., 2001). The cell toxicity of MPP⁺ was almost completely suppressed on coincubation with 1 mM cocaine or 10 μM nisoxetine (data not shown), suggesting that the cell death was induced via the uptake of MPP⁺ from the norepinephrine transporter. Incubation of PC12 cells with 30 μM MPP⁺ significantly induced cell death for 72 h (Fig. 2, left). Treatment with 100 μM MPP⁺ for 24 h induced cell death. Cell viability after incubation for 24, 48, and 72 h with 100 μM MPP⁺ was 68.19 ± 1.09, 34.21 ± 1.33, and 27.21 ± 2.44% (of control), respectively. The effects of NSAIDs on the MPP⁺-induced death of PC12 cells was examined using indomethacin, ibuprofen, ketoprofen, aspirin, diclofenac, and NS-398. At 100 μM, indomethacin, or ibuprofen significantly potentiated the MPP⁺-induced cell death when PC12 cells were coincubated with MPP⁺ for 48 or 72 h but not 24 h (Fig. 2). Figure 3 shows the effects of NSAIDs on the dose-dependent toxicity of MPP⁺. Incubation with 10 or 100 μM indomethacin significantly potentiated MPP⁺-induced cell death (Fig. 3a). At 100 μM, ibuprofen or ketoprofen and diclofenac also significantly potentiated MPP⁺-induced cell death (Fig. 3, b–d). In contrast to these four NSAIDs, aspirin and NS-398 did not enhance the cytotoxicity of MPP⁺ (Fig. 3, e and f). None of the NSAIDs tested alone had any effect on cell viability at the doses used in this study.

Potentiation by NSAIDs of MPP⁺-Induced Cell Death Does Not Involve the Generation of ROS, Caspase Activity, or PPARγ. Several studies have demonstrated that the toxicity of MPP⁺ is related to an increase in the generation of ROS and that there is a crucial association between the production of ROS and NSAIDs-induced apoptosis in many types of cells. Therefore, we investigated the involvement of ROS in the stimulatory effects of indomethacin in PC12 cells, we examined the influence of PBN, a radical scavenger, and NAC, an antioxidant, on the MPP⁺-induced cell death potentiated by indomethacin. At 1 mM, neither PBN nor NAC affected the potentiating action of indomethacin on the MPP⁺-induced cell death (Table 1). In addition, these drugs had no effect on the potentiation by ibuprofen or ketoprofen (data not shown). Thus, these results suggested that ROS is not involved in the potentiation induced by some NSAIDs. A number of observations have indicated that the activation of caspases is involved in the toxicity of MPP⁺. Moreover, caspases have been demonstrated to play pivotal roles in NSAIDs-induced apoptosis in several culture systems.

Therefore, to confirm the involvement of caspases in the stimulatory actions of NSAIDs, we examined the effect of Ac-DEVD-CHO, a selective caspase-3 inhibitor, on the MPP⁺-induced cell death potentiated by three NSAIDs. Ac-DEVD-CHO could suppress neither the cell toxicity induced by MPP⁺ alone nor the MPP⁺-induced cell toxicity enhanced by indomethacin (Table 1), ibuprofen and ketoprofen (data not shown), suggesting that caspase-3 at least is not involved in these events. Furthermore, the effects of indomethacin or ibuprofen on DNA fragmentation in PC12 cells are shown in Fig. 4. Treatment with 30 μM MPP⁺, either alone or together with 100 μM NSAIDs, for 48 h did not induce DNA fragmentation. From these results in PC12 cells, it does not seem that a caspase-3-apoptotic cell death pathway is involved in the cytotoxic effect of MPP⁺ potentiated by these NSAIDs. Recent studies have demonstrated that some NSAIDs induce gene transcription through the activation of PPARγ. We next investigated whether the activation of PPARγ is involved in the MPP⁺-induced cell death potentiated by indomethacin, ibuprofen, or ketoprofen. Coincubation of PC12 cells with 1 or 5 μM GW9662, a selective PPARγ inhibitor, did not suppress the stimulatory effect induced by indomethacin (Table 1). GW9662 had no influence on the actions of the other two NSAIDs either (data not shown). These results suggested that the effects of NSAIDs on MPP⁺-induced cell death is independent of the activation of PPARγ in PC12 cells.

NSAIDs Have No Influence on Rotenone-Induced Cell Death. Neurotoxins are required to be taken up into neuronal cells to initiate their neurotoxic effects. MPP⁺ is thought to be taken up through several transporter systems, especially catecholamine transporters on neurons. Rotenone, another neurotoxin used to develop models of Parkinson’s disease, diffuses freely into neuronal cells, and potently inhibits complex I. Thus, we investigated the effects of some NSAIDs on rotenone-induced cell death in PC12 cells. As shown in Fig. 5, rotenone exhibited a concentration-dependent cytotoxicity in PC12 cells. However, neither indomethacin, ibuprofen, nor ketoprofen affected the rotenone-induced cell death (Fig. 5).

NSAIDs Evolve an Increase in the Intracellular Accumulation of [³H]MPP⁺ through a Decrease in the Efflux of [³H]MPP⁺ from Cells. The specific enhancement of MPP⁺ toxicity may indicate that the stimulatory effect induced by these NSAIDs is dependent on the function of transport systems in the plasma membrane. Thus, to elucidate whether the increase in the accumulation of MPP⁺ is involved in the potentiating actions of some NSAIDs, the effects of three NSAIDs on the amount of MPP⁺ accumulated in PC12 cells were examined. Coincubation of PC12 cells with 30 μM MPP⁺, 10 or 100 μM indomethacin, and 100 μM ibuprofen, ketoprofen, or diclofenac, but not 1 mM aspirin, for 9 h led to a significant increase in the accumulation of [³H]MPP⁺ compared with that in the cells treated with 30 μM MPP⁺ alone (Fig. 6a). Furthermore, the effects of these
NSAIDs on the efflux of MPP⁺ from PC12 cells were examined. Cells were preloaded with [³H]MPP⁺ for 6 h and then the efflux of [³H]MPP⁺ into the medium was measured. When 100 µM indomethacin, ibuprofen, ketoprofen, or diclofenac, but not 1 mM aspirin, was present in the extracellular medium, the release of [³H]MPP⁺ from cells significantly decreased compared with that from vehicle-treated cells (Fig. 6b).

Involvement of MRP in the NSAIDs-Potentiated Cell Toxicity. It has been demonstrated that indomethacin specifically inhibits the MRP activity in human cell lines overexpressing MRP (Draper et al., 1997). Furthermore, Reid et al. (2003b) have suggested that some NSAIDs, including indomethacin, ibuprofen, and ketoprofen, inhibit the activity of MRP4. Thus, to verify the involvement of MRPs in the NSAIDs-potentiated action, we first examined the expression of some types of MRPs mRNA in PC12 cells using RT-PCR analysis. Each cDNA fragment of the expected size (MRP1, 511 bp; MRP3, 766 bp; MRP4, 229 bp; and MRP5, 310 bp) except for MRP2 (868 bp) was amplified after reverse transcription (Fig. 7a). Each amplicon for MRP1, 3, 4, or 5 was also found in rat brain or for MRP2 was found in rat liver (Fig. 7a). Coincubation of PC12 cells with 30 µM MPP⁺ and 20 µM MK 571, which is a MRP inhibitor, for 9 h led to a significant increase in [³H]MPP⁺ compared with incubation with 30 µM MPP⁺ alone (Fig. 7b). The increase is similar to that observed when cells were coincubated with NSAIDs in the present study. Furthermore, 20 or 50 µM MK 571 mimicked the inhibitory effect on the efflux of [³H]MPP⁺ by NSAIDs (Fig. 7c). In addition, the effect of MK 571 on the toxicity of MPP⁺ in PC12 cells was investigated. Treatment with 20 µM MK 571 significantly potentiated the MPP⁺-induced cell death (Fig. 7d).

Discussion

Recently, evidence has emerged that some NSAIDs have effects not correlated with inhibitory action toward COX...
Effects of several kinds of inhibitors on MPP\textsuperscript{+}-induced cell death in PC12 cells

All values indicate the cell viability when PC12 cells were incubated with 30 μM MPP\textsuperscript{+} alone or together with several kinds of inhibitors for 48 h. The data represent the mean ± S.E.M. for four independent experiments.

<table>
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<tr>
<th>Inhibitor Combination</th>
<th>Cell Viability (% of control)</th>
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<tr>
<td>MPP\textsuperscript{+} (30 μM)</td>
<td>82.73 ± 3.61</td>
</tr>
<tr>
<td>+ indomethacin (100 μM)</td>
<td>43.19 ± 4.93</td>
</tr>
<tr>
<td>+ PBN (1 mM)</td>
<td>74.48 ± 5.11</td>
</tr>
<tr>
<td>+ NAC (1 mM)</td>
<td>85.48 ± 7.45</td>
</tr>
<tr>
<td>+ Ac-DEVD-CHO (100 μM)</td>
<td>83.57 ± 1.95</td>
</tr>
<tr>
<td>+ indomethacin (100 μM) + PBN (1 mM)</td>
<td>33.4 ± 1.83</td>
</tr>
<tr>
<td>+ indomethacin (100 μM) + NAC (1 mM)</td>
<td>39.27 ± 0.17</td>
</tr>
<tr>
<td>+ indomethacin (100 μM) + Ac-DEVD-CHO (100 μM)</td>
<td>49.95 ± 0.71</td>
</tr>
<tr>
<td>+ indomethacin (100 μM) + GW9662 (1 μM)</td>
<td>42.59 ± 2.97</td>
</tr>
<tr>
<td>+ indomethacin (100 μM) + GW9662 (5 μM)</td>
<td>47.12 ± 5.92</td>
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isozymes. For example, NSAIDs induced apoptosis in a variety of cells (Lu et al., 1995; Han et al., 2001; Yamazaki et al., 2002). In the present study, the treatment of PC12 cells with NSAIDs alone, such as indomethacin, ibuprofen, ketoprofen, or diclofenac, did not affect the cell viability. However, coin-
cubation with these NSAIDs markedly enhanced the MPP\textsuperscript{+}-
induced cell death. The other NSAIDs used in this study, including aspirin and NS-398, had no effect on the toxicity. Thus, the potentiation effect of these four NSAIDs on the MPP\textsuperscript{+}-induced cell death did not seem to be associated with their ability to inhibit COX enzymes, because all NSAIDs used in this study did not have the potentiating action, and the concentration of NSAIDs required to increase the toxicity of MPP\textsuperscript{+} was several orders of magnitude higher than that required to suppress COX activities. For example, it has been demonstrated that IC\textsubscript{50} values of indomethacin for in-
hibition of human platelet COX-1 and endotoxin-stimulated human monocyte COX-2 were 0.36 ± 0.10 and 0.70 ± 0.20 μM, respectively (Patrignani et al., 1994). Furthermore, several studies have reported the doses of aspirin required to inhibit nuclear factor-κB or inhibitory κB kinase-β are at the millimolar range, much higher than doses used in the present study (Kopp and Ghosh, 1994; Yin et al., 1998).

To better understand the potentiating effects of these NSAIDs on the MPP\textsuperscript{+}-induced cell death, we examined the involvement of ROS in the NSAIDs-potentiated actions. It would seem that the actions of NSAIDs are not associated with an increase in ROS, because neither PBN, a radical scavenger, nor NAC, an antioxidant, had any influence on the NSAIDs-potentiated actions. Moreover, we confirmed that these drugs did not affect the MPP\textsuperscript{+}-induced cell death in PC12 cells. It has been demonstrated that ROS or a ROS-mediated signal plays an essential role in 6-hydroxydopa-
mine-induced but not MPP\textsuperscript{+}-induced cell death in a dopami-
nergic neuronal cell line (Choi et al., 1999, Kim et al., 2001). Seyfried et al. (2000) have suggested that the generation of ROS might not be the primary mechanism of MPP\textsuperscript{+} toxicity in PC12 cells. In contrast, it has been demonstrated that ROS contributed to the apoptotic cell death induced by NSAIDs in cultured gastric cells (Kusuhara et al., 1999). Thus, one possible explanation for the discrepancy is that the mechanism of ROS generation, the susceptibility to ROS or the cell death signal triggered by ROS are cell-specific. Another question arises as to whether the activation of caspases, which is one of the key factors in apoptosis, is associated with the NSAIDs-potentiated effect in PC12 cells. It has been demonstrated that several NSAIDs cause apoptosis through a caspase-dependent cascade (Kusuhara et al., 1998; Klampfer et al., 1999; Pique et al., 2000). In the present study, Ac-
DEVD-CHO, a specific and potent caspase-3 inhibitor, suppressed neither the MPP\textsuperscript{+}-induced cell death nor the NSAIDs-potentiated effects. Consistent with our results, Han et al. (2003) have suggested that caspase-independent cell death pathways operate in primary dopaminergic neu-
rons after MPP\textsuperscript{+} treatment, whereas caspase-dependent pathways are involved in the cell death induced by 6-hydroxydopamine. Moreover, we observed that DNA fragmentation, which is one of the hallmarks of apoptosis, was not induced by incubation of PC12 cells with MPP\textsuperscript{+} alone or together with NSAIDs. Together, NSAIDs are unlikely to potentiate the cell loss induced by MPP\textsuperscript{+} through a general apoptotic cascade in the present system.

Several NSAIDs, including indomethacin and ibuprofen, act as a direct ligand for PPAR\textgamma (Lehmann et al., 1997; Jiang et al., 1998). Recently, evidence has emerged that the direct activation of PPAR\textgamma is involved in the apoptosis induced by NSAIDs in several types of cells (Kusunoki et al., 2002; Yamazaki et al., 2002). In the present study, however, the potentiating effect of NSAIDs on MPP\textsuperscript{+}-induced cell death in PC12 cells was not associated with the activation of PPAR\textgamma, because a selective PPAR\textgamma antagonist GW9662 did not affect the NSAIDs-potentiated action, and 15-deoxy-Δ12,14-prosta-
glandin E\textsubscript{2} or ciglitizone, which are the agonists for PPAR\textgamma, did not mimic the effects of NSAIDs in PC12 cells (our unpublished observation).

![Fig. 4. Effect of NSAIDs on internucleosomal DNA cleavage in PC12 cells. PC12 cells were incubated without (lane 1) or with 30 μM MPP\textsuperscript{+} alone (lane 2) or MPP\textsuperscript{+} plus 100 μM indomethacin (lane 3) or ibuprofen (lane 4) for 48 h. Lane M indicates DNA size markers.](image1)

![Fig. 5. Effects of NSAIDs on rotenone-induced cell death. PC12 cells were incubated with various concentrations of rotenone for 48 h in the absence (○) or presence of indomethacin (100 μM, ▲), ibuprofen (100 μM, △), or ketoprofen (100 μM, ■). Data represent the mean ± S.E.M. (bars) for three to five independent experiments.](image2)
Fig. 6. Effects of NSAIDs on the intracellular accumulation or efflux of [3H]MPP⁺ in PC12 cells. a, effects of NSAIDs on the intracellular accumulation of [3H]MPP⁺ in PC12 cells. PC12 cells were incubated with 30 μM [3H]MPP⁺ for 9 h in the absence (control) or presence of 10 or 100 μM indomethacin (indo) and 100 μM ibuprofen (ibup), ketoprofen (keto), or diclofenac (diclo), or 1 mM aspirin (asp). Data represent the mean ± S.E.M. (bars) for three to five independent experiments. *, P < 0.05; **, P < 0.01 compared with the value for cells treated with 30 μM MPP⁺ alone. b, effects of NSAIDs on the efflux of [3H]MPP⁺ into the culture medium from PC12 cells. Cells were preloaded with 30 μM [3H]MPP⁺ for 6 h. They were then incubated for 3 h in fresh medium alone (control) or containing 100 μM indomethacin (indo), ibuprofen (ibup), ketoprofen (keto), or diclofenac (diclo), or 1 mM aspirin (asp). Data represent the mean ± S.E.M. (bars) for three to five independent experiments. *, P < 0.05; **, P < 0.01 compared with the value in the medium treated with vehicle alone.

Fig. 7. MK 571, an MRP inhibitor, mimics the NSAID-induced effects in PC12 cells. a, RT-PCR analysis of MRPs mRNA expression in PC12 cells. Each lane represents the cDNA fragments of MRPs (MRP1–5; MRP1, 511 bp; MRP2, 868 bp; MRP3, 766 bp; MRP4, 229 bp; and MRP5, 310 bp) amplified from the RNA of PC12 cells (lanes 1, 4, 7, 10, and 13), rat brain (lanes 3, 9, 12, and 15), or rat liver (lane 6). Lanes 2, 5, 8, 11, and 14 indicate the product of PCR with the reverse transcription reaction omitted as a negative control. The integrity of the isolated RNAs was examined using GAPDH-specific primers (lane 1, PC12 cells; lane 2, negative control mentioned above; lane 3, rat brain; and lane 4, rat liver). The cDNA fragment of GAPDH was 330 bp. Lane M indicates size markers (100-bp DNA Ladder). b, effects of MK 571 on the intracellular accumulation of [3H]MPP⁺ in PC12 cells. PC12 cells were incubated with 30 μM [3H]MPP⁺ for 9 h in the absence (control) or presence of 2 or 20 μM MK 571. Data represent the mean ± S.E.M. (bars) for three to five independent experiments. *, P < 0.05; **, P < 0.01 compared with the value for the cells treated with the corresponding dose of MPP⁺ alone. c, effects of MK 571 on the efflux of [3H]MPP⁺ from PC12 cells. After being preloaded as mentioned in Fig. 6b, cells were incubated for 3 h in fresh medium alone (control) or containing 2, 20, or 50 μM MK 571. Data represent the mean ± S.E.M. (bars) for three to five independent experiments. *, P < 0.05; **, P < 0.01 compared with the value for the cells treated with vehicle alone. d, effects of MK 571 on MPP⁺-induced cell death. PC12 cells were incubated with various concentrations of MPP⁺ for 48 h in the absence or presence of MK 571 (2 or 20 μM). Data represent the mean ± S.E.M. (bars) for five independent experiments. *, P < 0.05; **, P < 0.01 compared with the value for the cells treated with the corresponding dose of MPP⁺ alone.
Rotenone is another neurotoxin used to develop models of Parkinson's disease. The mechanisms of action of MPP⁺ and rotenone overlap in many regards. Both reagents inhibit mitochondrial complex I activity, resulting in a reduction in intracellular ATP levels and subsequent cell death. However, the mechanism of cellular entry used by these compounds is different; MPP⁺ is a cationic molecule and enters cells on transporters such as the dopamine and norepinephrine transporter. In contrast, rotenone is a lipophilic molecule and enters cells freely. We found that some NSAIDs did not potentiate the rotenone-induced cell death in PC12 cells. Thus, the mechanisms for MPP⁺-induced cell death with or without NSAIDs are yet to be elucidated, but the present results indicate the possibility that the regulation of the membrane-transport systems of MPP⁺ might be associated with the potentiating actions of NSAIDs. Actually, we demonstrated that incubation of PC12 cells with some NSAIDs significantly increased the intracellular accumulation of [³H]MPP⁺. In contrast, aspirin, which has no influence on the MPP⁺ toxicity, did not affect the intracellular accumulation of [³H]MPP⁺. Moreover, we confirmed that the efflux of [³H]MPP⁺ into the incubation medium from cells preloaded with [³H]MPP⁺ was significantly decreased compared with that from vehicle-treated cells, when the cells were incubated with each NSAID. These results suggest that NSAIDs raise intracellular MPP⁺ concentrations via a blockade of the outflow of MPP⁺ from cells.

It has been demonstrated that indomethacin specifically inhibits the MRPs activity in human cell lines overexpressing MRP (Draper et al., 1997). The MRPs belong to a superfamily of ATP-binding cassette transporters and have been implicated in the resistance of tumor cells to chemotherapeutic drugs (Cole et al., 1992; Borst et al., 2000). In the present study, MRP1, 3, 4, and 5 mRNA were detected in PC12 cells. In addition, MK 571, an MRP inhibitor (Reid et al., 2003a; Rius et al., 2003), mimicked the potentiation of cell toxicity, the enhancement of [³H]MPP⁺ accumulation, and the reduction of [³H]MPP⁺ efflux in response to NSAIDs. Therefore, these results suggest that [³H]MPP⁺ efflux occurs through MRPs expressed in PC12 cells and NSAIDs reduced MPP⁺ efflux by the blockade of MRPs. We could not identify the distinct subtype(s) that contributes to the NSAIDs-induced effects because of lack of MRP subtype-selective inhibitor. Although we could not rule out the possibility that one or some MRPs might contribute to the NSAIDs-induced effects, we have speculated MRP4 is one of the most important factors in the action of NSAIDs for the following reasons. It has been recently demonstrated that MK 571 might selectively inhibit both MRP1 and MRP4 transport (Chen et al., 2001; Dallas et al., 2003). Indomethacin affects some MRPs, including MRP4 (Draper et al., 1997). On the other hand, ibuprofen, ketoprofen, and diclofenac have an inhibitory effect on MRP4 compared with other MRPs (Reid et al., 2003b). Moreover, the concentrations of NSAIDs required to enhance the MPP⁺ toxicity are nearly equal to the IC₅₀ values for these NSAIDs against MRP4 transport (5–50 μM; Reid et al., 2003b). Together, it could be expected that NSAIDs first block the activity of MRPs, including MRP4, and inhibit the efflux of MPP⁺ from PC12 cells, resulting in the retention of a high concentration of MPP⁺ in cells and subsequent potentiating effect of cell toxicity. The cytoplasmic concentration of MPP⁺ in monoaminergic neurons is dependent on the influx of MPP⁺ through plasma membrane monoamine transporters and the sequestration of MPP⁺ into synaptic vesicles through vesicular monoamine transporter. In addition to these well-known transporter systems for MPP⁺, other transporter systems are expected. The present results suggest that MRPs also regulated the concentration of MPP⁺ in a causal relationship with its toxicity in PC12 cells.

In conclusion, our results demonstrated that several NSAIDs potentiate MPP⁺-induced cell death through a blockade of MRPs rather than the inhibition of COXs activities in PC12 cells. Our report provides new insights as follows. 1) Some types of MRPs are expressed in PC12 cells, and these are one of the transport systems involved in discharging intracellular MPP⁺ outside of the cell. 2) Some NSAIDs, including indomethacin, ibuprofen, ketoprofen, and diclofenac, inhibited the activities of MRPs, including MRP4 in PC12 cells. 3) The blockade of MRPs by these NSAIDs led to an increase in the intracellular concentration of MPP⁺ and aggravation of cell toxicity. It has been demonstrated that NSAIDs had inconsistent effects on MPP⁺-induced cell death; a protective, an aggravative, or no effect depending on the drug and cells used or experimental conditions. The present results suggest that differences in the transport systems in cell types and in the action of NSAIDs on these transport systems are critical causes of the discrepancies. NSAIDs are split into various subclasses, including salicylic acid derivatives, indoleacetic acid derivatives, propionic acid derivatives. Therefore, it also might be important to evaluate the effects of each subclass or each compound itself on MRPs.

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References


Address correspondence to: Prof. Toshihiro Dohi, Kasumi 1-2-3, Minami-Hiroshima, Hiroshima 734-8553, Japan. E-mail: todohi@hiroshima-u.ac.jp