Journal of Neurochemistry, 2001, **76**, 1895–1904

Neuroprotective effects of non-steroidal anti-inflammatory drugs by direct scavenging of nitric oxide radicals

Masato Asanuma,* Sakiko Nishibayashi-Asanuma,* Ikuko Miyazaki,* Masahiro Kohno† and Norio Ogawa*

**Department of Neuroscience, Institute of Molecular and Cellular Medicine, Okayama University Medical School, Okayama, Japan*
†Life and Environmental Engineering, Department of Environmental Systems Engineering, Faculty of Engineering, Kochi University of Technology, Kochi, Japan

Abstract

Recently, it has been reported that inflammatory processes are associated with the pathophysiology of Alzheimer's disease and that treatment of non-steroidal anti-inflammatory drugs reduce the risk for Alzheimer's disease. In the present study, we examined nitric oxide radical quenching activity of non-steroidal anti-inflammatory drugs and steroidal drugs using our established direct *in vitro* nitric oxide radical detecting system by electron spin resonance spectrometry. The non-steroidal anti-inflammatory drugs, aspirin, mefenamic acid, indomethacin and ketoprofen directly and dose-dependently scavenged generated nitric oxide radicals. In experiments of nitric oxide radical donor, NOC18-induced neuronal damage, these four non-steroidal drugs significantly prevented the NOC18-induced reduction of cell viability and apoptotic nuclear changes in neuronal cells without affecting the induction of inducible nitric oxide synthase-like immunoreactivity. However, ibuprofen, naproxen or steroidal drugs, which had less or no scavenging effects *in vitro*, showed almost no protective effects against NOC18-induced cell toxicity. These results suggest that the protective effects of the former four non-steroidal anti-inflammatory drugs against apoptosis might be mainly due to their direct nitric oxide radical scavenging activities in neuronal cells. These direct NO· quenching activities represent novel effects of non-steroidal anti-inflammatory drugs. Our findings identified novel pharmacological mechanisms of these drugs to exert not only their anti-inflammatory, analgesic, antipyretic activities but also neuroprotective activities against neurodegeneration.

Keywords: aspirin, indomethacin, ketoprofen, mefenamic acid, nitric oxide, non-steroidal anti-inflammatory drugs.
J. Neurochem. (2001) **76**, 1895–1904.

Non-steroidal anti-inflammatory drags (NSAIDs) exert anti-inflammatory, analgesic and antipyretic activities and are involved in the suppression of prostaglandin synthesis by inhibiting cyclooxygenase (COX), an enzyme that catalyzes the formation of prostaglandin precursors from arachidonic acid (Vane 1971; Engineer *et al*. 1978; Humes *et al*. 1981; Meade *et al*. 1993; Mitchell *et al*. 1993; Furst 1994; Vane 1994). NSAIDs also inhibit activation of neutrophils, which induce inflammation by releasing products other than prostaglandins (Abramson *et al*. 1994). On the other hand, nitric oxide radical (NO·) activates COX-1 and COX-2, increasing prostaglandins, which may aggravate inflammation (Salvemini *et al*. 1993). Although aspirin was found to inhibit platelet activation through an increase in NO· production in peripheral neutrophils (López-Farré *et al*. 1995; López-Farré *et al*. 1996), NSAIDs such as aspirin, sodium salicylate and indomethacin, reduce NO· production induced by lipopolysaccharide (LPS) and/or cytokines by their inhibiting effects on inducible nitric oxide synthase (iNOS) in various peripheral and CNS cells (Amin *et al*.

Received June 14, 2000; revised manuscript received November 2, 2000; accepted December 2, 2000.

Address correspondence and reprint requests to Dr Masato Asanuma, Department of Neuroscience, Institute of Molecular and Cellular Medicine, Okayama University Medical School, 2–5-1 Shikatacho, Okayama 700–8558, Japan. E-mail: asachan@cc.okayama-u.ac.jp

Abbreviations used: carboxy-PTI, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; ESR, electron spin resonance; FITC, fluorescein isothiocyanate; IC_{50}, 50% inhibitory concentration; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO·, nitric oxide radical; NOC7, 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine; NOC18, 2,2′-(hydroxynitrosohydrazino)-*bis*-ethanamine; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; WST-1, 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium.

1995; Farivar *et al*. 1996; Kepka-Lenhart *et al*. 1996; Chen *et al*. 1997; Kwon *et al*. 1997; Sakitani *et al*. 1997; Kim *et al*. 1998; Du and Li 1999; Katsuyama *et al*. 1999; Sanchez de Miguel *et al*. 1999). Several studies have suggested that the inhibitory effects of aspirin and sodium salicylate on iNOS activity are exerted at the level of translational and/or post-translational modulation, since they show no effects on neither iNOS mRNA induction nor nuclear factor-κB (NF-κB) activation, which is a key transcription factor in iNOS gene transcription in response to inflammatory mediators (Amin *et al*. 1995; Kwon *et al*. 1997; Sakitani *et al*. 1997; Katsuyama *et al*. 1999). In contrast with other studies, aspirin and sodium salicylate did inhibit LPS and/or cytokines-induced NF-κB activation (Frantz and O'Neill 1995; Kopp and Ghosh 1994; Weber *et al*. 1995; Grilli *et al*. 1996; Sanchez de Miguel *et al*. 1999) and iNOS mRNA expression (Aeberhard *et al*. 1995; Farivar *et al*. 1996; Sanchez de Miguel *et al*. 1999) at the therapeutic concentration, and consequently reduce NO· production. On the other hand, indomethacin (a non-acetylated NSAID) does not produce any significant inhibitory effects on LPS and/or cytokines-induced NF-κB activation, iNOS activity or NO· production at clinically relevant concentrations (Kopp and Ghosh 1994; Amin *et al*. 1995; Farivar *et al*. 1996; Grilli *et al*. 1996; Kwon *et al*. 1997), despite some reports to the contrary (Aeberhard *et al*. 1995; Chen *et al*. 1997; Du and Li 1999). Thus, there is some controversy concerning the mechanism of inhibition of NO· production by NSAIDs, implying that NSAIDs have multiple action sites on their pathway to exert inhibitory effects against NO·-associated changes by various stimuli (Kepka-Lenhart *et al*. 1996; Lipton 1997). In the present study, we postulated that NSAIDs may have a direct NO· scavenging activity. To elucidate the direct effects of NSAIDs on NO·, we examined NO· quenching activity of NSAIDs and steroidal drugs according to our established methods for direct detection of NO· in an *in vitro* NO·-generating system using NO· trapping reagent and the NO· donor by electron spin resonance (ESR) spectrometry.

Recent studies suggest that inflammatory and immune-mediated processes are associated with the pathophysiology of Alzheimer's disease (McGeer and McGeer 1995; McGeer and McGeer 1999). Several NSAIDs inhibit amyloid β-protein-induced activation of microglia (Gottschall 1996; Netland *et al*. 1998) and reduce the risk for Alzheimer's disease (McGeer *et al*. 1996; Stewart *et al*. 1997; in't Veld *et al*. 1998). We also recently reported that the treatment with NSAIDs, ketoprofen and indomethacin, prevented the late-onset reduction of muscarinic receptors and delayed neuronal death in hippocampal pyramidal neurons after transient forebrain ischemia (Asanuma *et al*. 1997; Kondo *et al*. 1998; Kondo *et al*. 2000). Aspirin and sodium salicylate protect against glutamate-induced neurotoxicity in primary neuronal cultures and hippocampal slices through inhibition of NF-κB activation (Grilli *et al*. 1996). Recently, apoptotic cell death by NO· donor has been reported in neuronal cells (Uehara *et al*. 1999). In order to determine the effects of NSAIDs on NO· in cultured neuronal cells, we also examined in the present study the effects of NSAIDs against NO· donor-induced neuronal damage. We also assessed the morphological apoptotic changes and iNOS induction in neuronal cells after exposure to NO· donor in the presence of NSAIDs, using nuclear staining and immunofluorescein staining.

Materials and methods

Reagents

The NO·-trapping agent, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) was purchased from Labotec (Tokyo, Japan). The NO· donors, 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC7) and 2,2-(hydroxynitrosohydrazino)-*bis*-ethanamine (NOC18) were purchased from Dojin Laboratories (Kumamoto, Japan). The NSAIDs sulpyrine, aspirin, mefenamic acid, indomethacin, ketoprofen, ibuprofen and naproxen were obtained from Wako Pure Chemical Industries (Tokyo, Japan). The steroidal drugs hydrocortisone, prednisolone and dexamethason were also obtained from Wako Pure Chemical Industries. 4-Hydroxy-tempo was purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and L-glutamine were purchased from GIBCO BRL (Rockville, MD, USA). Kanamycin sulfate was obtained from Wako Pure Chemical Industries. Cell counting kit using 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (PMS) was obtained from Dojin Laboratories, Kumamoto, Japan.

ESR spectrometry in NO·-generating system

In the present study, NO· was directly detected in an *in vitro* NO·-generating system using carboxy-PTIO as NO· detecting reagent and the NO· donor, NOC7, by ESR spectrometry, using the method established by our laboratory (Nishibayashi *et al*. 1996). Carboxy-PTIO was dissolved in 250 mM phosphate buffer solution (pH 7.6), and NOC7 was dissolved in 1 mM NaOH. In a test tube, 99.5% ethanol and 5 μM NOC7 were mixed for 30 min at room temperature, and then 5 μM carboxy-PTIO was added to the mixture. The spectra were recorded 10–280 min after the addition of carboxy-PTIO with an ESR spectrometer (JES-FR30, JEOL Co., Tokyo, Japan) using a flat quartz cuvette. The signal intensity was evaluated by the relative peak height of the first signal of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (carboxy-PTI) spin adduct to the intensity of Mn^{2+} signal, which was used as the internal standard to correct for measurement error. Regarding the conditions of the ESR spectrometer to estimate NO·, the magnetic field, power, modulation frequency, modulation amplitude, response time, temperature, amplitude and the sweep time were 335.6 ± 5 mT, 4 mW, 9.41 GHz, 1 × 0.1 mT, 0.1 s, 25°C, 1 × 250 and 1 min, respectively.

NSAIDs or steroids on NO·-generating system

All NSAIDs and steroids were suspended in 99.5% ethanol. The

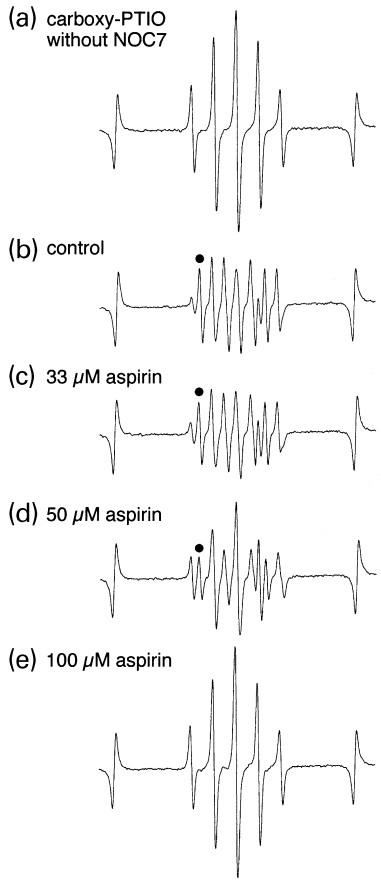


Fig. 1 Effect of aspirin on ESR signals of carboxy-PTIO and carboxy-PTI in NO generating system using NOC7. (a) 5 μM carboxy-PTIO without NOC7; (b) control (99.5% ethanol); (c) 33 μM aspirin; (d) 50 μM aspirin; and (e) 100 μM aspirin. (b–e) At 90 min after the addition of carboxy-PTIO (final concentration: 5 μM) to the mixture of 5 μM NOC7 and 99.5% ethanol or 33 μM, 50 μM, 100 μM aspirin, carboxy-PTI signals were recorded by ESR spectrometer. The dot indicates a monitored carboxy-PTI signal which reflects NO production.

drugs (final concentrations ranging from 333 nM to 333 μM in triplicate) and 5 μM NOC7 were mixed in a test tube, incubated at room temperature for 30 min, and then added with 5 μM carboxy-PTIO. The signal intensity of carboxy-PTI spin adduct was measured 90 min after the addition of carboxy-PTIO under the same ESR conditions as described above.

Cell culture and treatment of NO· donor with NSAIDs or steroids

The rat neuroblastoma cell line, B65 neuronal cells, was obtained from ECACC (Salisbury, Wiltshire, UK). B65 cells were continuously cultured in DMEM, containing 2 mM L-glutamine, 10% FBS, and 60 μg/mL kanamycin. For experiments, cells were plated into 96-well plates or 4-chamber culture slides (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 1.4–1.8 $\times$ 10^4 cells/cm^2, and were maintained in the culture medium at 37°C in a 5% to 95% CO_2–air gas mixture. B65 neuronal cells were grown for 2 days and then exposed to the NO· donor, NOC18 at a final concentration of 250 μM, 500 μM or 1 mM. At the same time, cells were treated with different amounts of non-steroidal or steroidal anti-inflammatory drugs. NOC18 and all examined anti-inflammatory drugs were dissolved in 1 mM NaOH and 99.5% ethanol, respectively. The final concentrations of NaOH and ethanol were maintained at 1 μM and 0.1%, respectively.

Measurement of cell viability

Following 24 h-incubation with NO· donor and anti-inflammatory drugs and exchanging to the culture medium without any drugs, the cell viability in each well of 96-well plate was determined by quantitative colorimetric assay with WST-1 and 1-methoxy PMS (Iwata-Ichikawa *et al*. 1999), a modification of the standard MTT assay. Viability was expressed as a percentage of the cell viability from each control cultures treated with 1 μM NaOH and 0.1% ethanol.

Nuclear staining and immunocytohistochemistry

Nuclear staining by Hoechst dye and immunofluorescein staining for iNOS were also performed using B65 cells on chamber slides, 24 h after the exposure of NOC18 (100, 250 or 500 μM) with or without NSAID: final concentration of aspirin, mefenamic acid, indomethacin or ketoprofen were 500 μM, 2.5 μM, 10 μM or 500 μM, respectively. Cells were fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer) for 10 min. After washing with 10 mM phosphate-buffered saline (PBS), the slides were incubated in 2.5% normal goat serum and 0.1% Triton X-100 in PBS for 20 min, and then exposed to a rabbit purified polyclonal anti-iNOS antibody (diluted 1 : 250 in PBS; BD Transduction Laboratories, Franklin Lakes, NJ, USA) for 18 h at 4°C. After incubation with the primary antibody, sections were washed three times, each for 10 min, in 10 mM PBS before being incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (diluted 1 : 200 in PBS with 1% normal goat serum; Chemicon International Inc., Temecula, CA, USA) for 1.5 h at room temperature. Following washing in PBS (three times, each for 10 min) and removal from chamber walls, nuclear staining was then performed by incubating with 20 μg/mL Hoechst 33342 (*bis*-benzimide; Molecular Probes, Eugene, OR, USA) for 10 min. Slides were rinsed briefly with PBS, air-dried, and then mounted in antifluorescein fading medium (PermaFluor, Immunon, PA, USA). Slides were examined using a fluorescein microscope (Carl Zeiss Axiophot FL, Jena, Germany) using a mercury lamp through 365 nm or 450~490 nm bandpass filter to excite Hoechst 33342 or FITC, respectively. Light emitted from Hoechst 33342 or FITC was collected through 397-nm or 515~565-nm longpass filter, respectively.

Statistical analysis

All data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using one-way ANOVA followed by Fisher's PLSD *post hoc* test. A *p*-value less than 0.05 denoted the presence of a statistically significant difference.

Results

Effects of NSAIDs or steroids on NO·-generating system

NO donor NOC7 is stable in an alkaline solution, and it

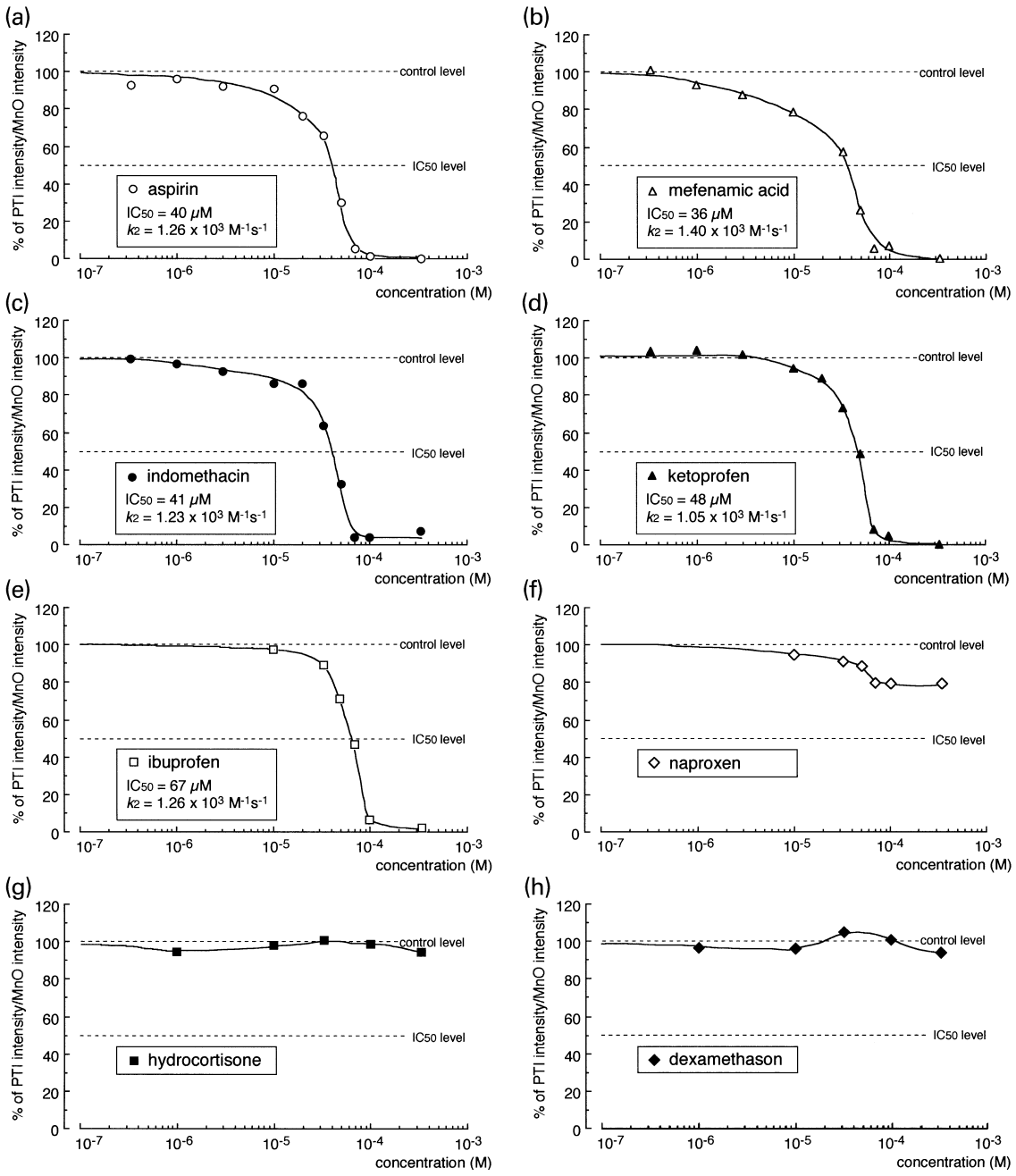


Fig. 2 Effect of aspirin (a), mefenamic acid (b), indomethacin (c), ketoprofen (d), ibuprofen (e), naproxen (f), hydrocortisone (g) and dexamethason (h) on NOC7-induced formation of carboxy-PTI. ESR signals of carboxy-PTI were recorded 90 min after the addition of carboxy-PTIO to the mixture of NOC7 and each NSAID or steroidal drug (final concentration ranging from 333 nM to 333 μM). Each point represents the mean of percentage signal intensity of carboxy-PTI in three independent experiments. Calculated values of IC_{50} and k_2 for each drug are shown in the inset.

starts to quickly produce NO· with a half-life of ~5 min under neutral pH (Harabie and Klose 1993). The ESR signal of carboxy-PTIO is characterized by five waves (1 : 2 : 3 : 2 : 1; $a_N = 0.82$ mT) (Fig. 1a). The generated NO· from NOC7 was oxidized to NO_2 by carboxy-PTIO, and carboxy-PTIO was then transformed to carboxy-PTI indicating NO· generation. In the process of their transformation, carboxy-PTIO and carboxy-PTI present nine mixed waves including two components of each (Figs 1b–d). It is easy to distinguish between the signals of carboxy-PTIO (Fig. 1a) and carboxy-PTI (Figs 1b–d, dot), which reflects NO· production from NOC7, using the ESR spectrometer,

since these radicals have different ***g***-values. The carboxy-PTI shows a seven-wave signal when carboxy-PTIO has been completely convened to carboxy-PTI. Using this procedure, the concentration of NO· can be calculated and it was confirmed by comparison with a suitable standard (4-hydroxy-tempo). After the addition of carboxy-PTIO to the mixture of ethanol and NOC7, the carboxy-PTI signal, which reflects NO· production from NOC7, increased time-dependently and reached a plateau at 100∼270 min (Nishibayashi *et al*. 1996). Based on these results, the following experiments were performed at 90 min after the addition of carboxy-PTIO to a mixture of anti-inflammatory drug and NOC7.

Aspirin at concentrations of between 333 nM and 10 μM showed no scavenging activity in the NO·-producing system. After the addition of 33 μM aspirin, however, NO· quenching began in a dose-dependent manner and at concentrations of 70 μM and higher, aspirin completely scavenged the generated NO· (Figs 1b–e and 2a). Moreover, other NSAIDs, including mefenamic acid, indomethacin, ketoprofen and ibuprofen, also showed NO· quenching activity in a dose-dependent manner (Figs 2b–e, respectively). Interestingly, NO· scavenging activity of ibuprofen appeared at higher concentration (33 μM, Fig. 2e). However, naproxen of NSAID showed no apparent NO· quenching activity at concentrations up to 333 μM (Fig. 2f). These six NSAIDs showed no inhibition of the NO· generation from NOC7, since the sum of the signal intensities for carboxy-PTIO and carboxy-PTI was constant with or without the addition of these drugs.

On the other hand, hydrocortisone and dexamethason of steroidal drugs showed no significant NO· quenching activity at concentrations up to 333 μM (Figs 2g and h). In the present direct NO· detection system, the effects of sulpyrine and prednisolone were not evaluated, since these drugs diminished the signal of carboxy-PTIO itself.

The 50% inhibitory concentrations (IC_{50}) for aspirin, mefenamic acid, indomethacin, ketoprofen and ibuprofen were estimated to be about 40 μM, 36 μM, 41 μM, 48 μM and 67 μM, respectively (Figs 2a and e). To evaluate the kinetics of the reaction between NO· and these NSAIDs, we modified the kinetic competition model based on the obtained results (Mituta *et al*. 1990) that the reduction of carboxy-PTIO to carboxy-PTI was linked and competed with the reaction of drugs with generated NO·. The reactions can be described as:

$$\text{carboxy-PTIO} + \text{NO}\cdot \rightarrow \text{carboxy-PTI} + \text{NO}_2 \quad (k_1)$$

and

$$\text{drug} + \text{NO}\cdot \rightarrow \text{drug-NO}\cdot \quad (k_2)$$

where k_1 and k_2 are the second-order rate constants for each reaction, respectively. The reaction rate constant (k_2) for the drug can be expressed by a simplified equation (Mituta *et al*. 1990):

$$k_2 = k_1 \cdot [\text{carboxy-PTIO}]/IC_{50}$$

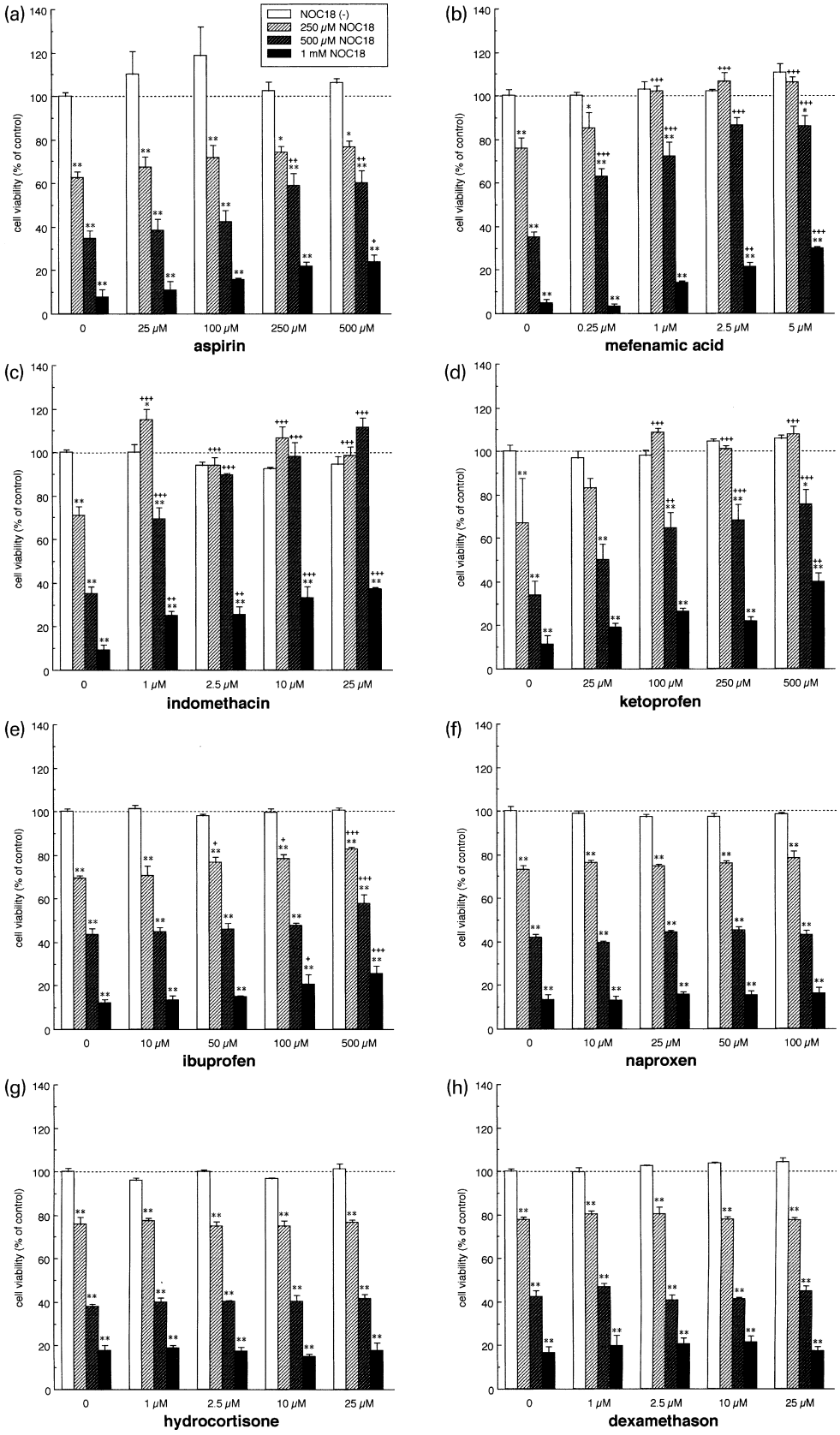
using IC_{50} value for each drug and $k_1 = 1.01 \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}$ based on Hogg *et al*. (Hogg *et al*. 1995). Therefore, we can estimate the value of k_2 for aspirin, mefenamic acid, indomethacin, ketoprofen and ibuprofen to be about $1.26 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$, $1.40 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$, $1.23 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$, $1.05 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$ and $0.75 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$, respectively (Figs 2a–e). In this system, there are no chemical reactions between carboxy-PTIO and these four drugs at any dose.

Effects of NSAIDs or steroids on NO· donor-induced neuronal cell death

NO· donor, NOC18, which slowly and spontaneously releases NO· with a half-life of ∼20 h under neutral pH (Mooradian *et al*. 1995), is known to produce apoptotic cell death in neuroblastoma cell lines (Uehara *et al*. 1999). The effect of NOC18 on cell viability of B65 neuronal cells was assessed 24 h after NOC18 treatment, using the WST-1 assay. Exposure to NOC18 (250 μM, 500 μM and 1 mM) significantly reduced the survival of B65 cells in a dose-dependent manner following 24-h incubation (Fig. 3). We then examined the protective effects of aspirin, mefenamic acid, indomethacin or ketoprofen, which showed marked NO· scavenging effects *in vitro*, against NOC18-induced neurotoxicity in cultured cells. All four NSAIDs significantly and markedly increased cell viability in a dose dependent manner after 24-h incubation with each dose of NOC18. This was particularly true for mefenamic acid and indomethacin (0.25 μM−10 μM) which showed higher protective effects against NOC18-induced cell death (Figs 3b and c). Although 500 μM NOC18 reduced cell viability to 33.9–35.2% of control, treatment with mefenamic acid and indomethacin at 2.5 μM−10 μM almost completely protected against NOC18-induced cell damage. Interestingly, ibuprofen and naproxen, which had less and no *in vitro* quenching effects, showed slightly and no protective effects against NOC18-induced cell death, respectively (Figs 3e and f). Without NOC18 exposure, however, these six drugs themselves did not show any effects on cell viability. We also examined the effects of steroidal drugs, which showed no *in vitro* NO· quenching effects in the present study, on the NOC18 neurotoxicity in neuronal cells. Similar to the results described above for NO· generating system *in vitro*, hydrocortisone and dexamethason showed no effects against NOC18-induced cell death (Figs 3g and h).

NSAIDs on NOC18-induced apoptotic cell damage and iNOS induction

We also examined the nuclear morphological changes following exposure to NOC18 and immunostaining of


(a)
NOC18 (-)
250 μM NOC18
500 μM NOC18
1 mM NOC18
cell viability (% of control)
0
25 μM
100 μM
250 μM
500 μM
aspirin
(b)
cell viability (% of control)
0
0.25 μM
1 μM
2.5 μM
5 μM
mefenamic acid
(c)
cell viability (% of control)
0
1 μM
2.5 μM
10 μM
25 μM
indomethacin
(d)
cell viability (% of control)
0
25 μM
100 μM
250 μM
500 μM
ketoprofen
(e)
cell viability (% of control)
0
10 μM
50 μM
100 μM
500 μM
ibuprofen
(f)
cell viability (% of control)
0
10 μM
25 μM
50 μM
100 μM
naproxen
(g)
cell viability (% of control)
0
1 μM
2.5 μM
10 μM
25 μM
hydrocortisone
(h)
cell viability (% of control)
0
1 μM
2.5 μM
10 μM
25 μM
dexamethason

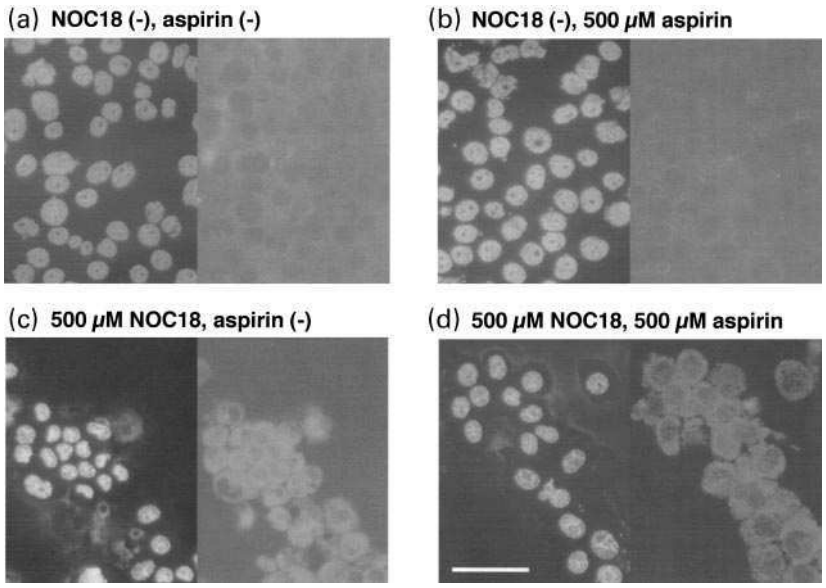


Fig. 4 Representative nuclear staining and immunohistochemistry for iNOS in B65 cells treated with NOC18 and/or aspirin. Cells were incubated with vehicle (a and b) or 500 μM NOC18 (c and d), and with vehicle (0.1% ethanol; a and c) or 500 μM aspirin (b and d) for 24 h. Following the incubation, nuclear staining (each left panel) and immunofluorescein staining for iNOS (each right panel) were performed using Hoechst 33342 dye and polyclonal anti-iNOS Ab with FITC-conjugated secondary Ab, respectively. Scale bar = 50 μm.

iNOS, with or without NSAIDs. Representative stainings in the case of NOC18 (500 μM) and aspirin are shown in Fig. 4. Exposure to NOC18 (250 μM and 500 μM) for 24 h induced pronounced nuclear condensation, which was visualized with the Hoechst dye, in B65 cells (Fig. 4c). All four NSAIDs, aspirin (500 μM; Fig. 4d), mefenamic acid (2.5 μM), indomethacin (10 μM) and ketoprofen (500 μM) inhibited NOC18-induced apoptotic nuclear changes, coinciding with their inhibitory effects on the NOC18-induced reduction of cell viability. However, these drugs themselves showed no apoptotic changes without exposure to NOC18 (Fig. 4b).

Although 100–500 μM NOC18 produced induction of iNOS-like immunoreactivity with 24-h incubation (500 μM NOC18; Fig. 4c), NSAIDs at the final concentration described above did not induce iNOS immunoreactivity (aspirin; Fig. 4b). Furthermore, these NSAIDs showed no effects on NOC18-induced iNOS-like immunoreactivity (aspirin; Fig. 4d).

Discussion

The main findings of our study were: (i) the NSAIDs, aspirin, mefenamic acid, indomethacin and ketoprofen, directly and dose-dependently scavenged generated NO· in our established direct *in vitro* NO· detection system using carboxy-PTIO and NOC7 by ESR spectrometry. Ibuprofen also directly scavenged NO· but at higher concentrations of drug. However, naproxen and other NSAIDs and hydrocortisone and dexamethasone steroidal drugs failed to produce a similar effect. (ii) The former four NSAIDs, which showed marked NO· scavenging effects *in vitro*, significantly prevented NO·-donor, NOC18-induced reduction of cell viability in cultured neuronal B65 cells, while ibuprofen, naproxen and steroidal drugs showed almost no protective effects against NOC18-induced cell toxicity. (iii) The former four NSAIDS in cultured cells without affecting the induction of iNOS-like immunoreactivity.

The pharmacological anti-inflammatory, analgesic and antipyretic effects of NSAIDs are mediated mainly via the suppression of prostaglandin synthesis through the inhibition of COX (Vane 1971; Engineer *et al*. 1978; Humes *et al*. 1981; Meade *et al*. 1993; Mitchell *et al*. 1993; Furst 1994; Vane 1994). Recently, the inhibitory effects of NSAIDs against inflammatory mediator-induced increases in iNOS activity and NO· production have been investigated as another possible pharmacological action of NSAIDs in many studies using various cells (Amin *et al*. 1995; Farivar *et al*. 1996; Kepka-Lenhart *et al*. 1996; Chen *et al*. 1997; Kwon *et al*. 1997; Sakitani *et al*. 1997; Kim *et al*. 1998; Du

Fig. 3 Effect of aspirin (a), mefenamic acid (b), indomethacin (c), ketoprofen (d), ibuprofen (e), naproxen (f), hydrocortisone (g) and dexamethason (h) on NO· donor-induced reduction of cell viability of neuronal cells. Following 24-h incubation with NOC18 (final concentration of 250 μM, 500 μM or 1 mM) and each NSAID or steroidal drug, cell viability of B65 cells was measured by WST-1 colorimetric assay. Each value is the mean ± SEM ($n = 3$) expressed as a percentage of each control cultures treated with vehicles (1 μM NaOH and 0.1% ethanol). $^{*}p < 0.01$, $^{**}p < 0.001$ compared with the control without NOC18 and each anti-inflammatory drug. †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$ compared with NOC18-dose matched control group without each drug (0 μM).

and Li 1999; Katsuyama *et al*. 1999; Sanchez de Miguel *et al*. 1999). However, the mechanisms of the inhibitory effects of NSAIDs on iNOS activity and NO· production are still controversial, as described before: some studies reported the translational and/or post-translational modulation of NSAIDs on iNOS (Amin *et al*. 1995; Kwon *et al*. 1997; Sakitani *et al*. 1997; Katsuyama *et al*. 1999), while others showed the transcriptional inhibition of aspirin and sodium salicylate on iNOS mRNA through the inhibition of NF-κB activation (Aeberhard *et al*. 1995; Kopp and Ghosh 1994; Frantz and O'Neill 1995; Weber *et al*. 1995; Farivar *et al*. 1996; Grilli *et al*. 1996; Sanchez de Miguel *et al*. 1999), but indomethacin showed no significant inhibitory effects on NF-κB activation, iNOS activity or NO· production (Amin *et al*. 1995; Kopp and Ghosh 1994; Farivar *et al*. 1996; Grilli *et al*. 1996; Kwon *et al*. 1997). As postulated by Lipton (1997) that other possible mechanisms of NSAIDs include inhibitory effects on NO·-associated changes; we demonstrated clearly in the present study that NSAIDs have a direct scavenging activity against generated NO· *in vitro* (Figs 1 and 2). The estimated IC_{50} values and reaction rate constant (k_2) for aspirin, mefenamic acid, indomethacin and ketoprofen were about 36–48 μM and about $1.23–1.40 \times 10^3$ M^{-1} s^{-1}, respectively, and those for ibuprofen were 67 μM and 0.75×10^3 M^{-1} s^{-1}, while naproxen and steroidal drugs failed to quench NO· (Fig. 2). Therefore, the former four NSAIDs may have relatively strong and specific NO· scavenging potency. All tested NSAIDs which showed NO· quenching effects have one or two benzene rings, while naproxen, which showed no NO· scavenging activity, has a naphthalene-based structure. Such chemical structure might be related to the differences of NO· scavenging activities. Thus, these direct NO· quenching effects of NSAIDs indicate the novel pharmacological effects of NSAIDs. The effects of sulpyrine and prednisolone on the generated NO·, which were not evaluated in the present study, may be clarified in future studies using other NO· detection system.

In the examination of NO· donor-induced cell loss in cultured neuronal cells, exposure to NOC18 for 24 h dose-dependently reduced cell viability and produced apoptotic nuclear changes in B65 cells (Figs 3 and 4), resembling NOC18-induced apoptotic cell death in neuroblastoma cell lines (Uehara *et al*. 1999). NOC18-induced apoptosis of cultured neuronal cells was significantly prevented by incubation with the test NSAIDs (aspirin, mefenamic acid, indomethacin and ketoprofen) that showed marked NO· scavenging effects *in vitro* (Figs 3 and 4). Thus, we clarified the quenching effects of these four NSAIDs on NO· not only *in vitro* but also in cultured cells in the present study. Their protective effects against apoptosis might be due mainly to their direct NO· scavenging activities in neuronal cells. This discussion is also supported by the present results that ibuprofen and naproxen, which had less and no *in vitro* quenching effects, showed slightly and no protective effects against NOC18-induced apoptosis, respectively (Figs 3e and f). To significantly protect against NOC18 (500 μM)-induced cell death, aspirin, mefenamic acid, indomethacin, and ketoprofen were used at concentrations of 250–500 μM, 0.25–2.5 μM, 1–10 μM, and 100–500 μM, respectively (Figs 3a–d). It is of interest that the inhibiting range of each drug against NOC18-induced cell damage is close to the clinical therapeutic concentration in plasma of each NSAID (Dennis *et al*. 1985; Gilman *et al*. 1993; Ito *et al*. 1994).

Since NSAIDs are reported to block NO· production through their inhibiting effects on iNOS (Amin *et al*. 1995; Farivar *et al*. 1996; Kepka-Lenhart *et al*. 1996; Chen *et al*. 1997; Kwon *et al*. 1997; Sakitani *et al*. 1997; Kim *et al*. 1998; Du and Li 1999; Katsuyama *et al*. 1999; Sanchez de Miguel *et al*. 1999), we also examined the effects of NSAIDs on iNOS expression in the same NOC18-exposed neuronal cells. NO· donor, NOC18-induced iNOS expression in cultured neuronal cells (Fig. 4c) might be caused by the reactive oxygen species-induced promotion of NF-κB (Meyer *et al*. 1993; Schreck *et al*. 1991). In the present study, the four NSAIDs examined, especially aspirin and indomethacin, showed no effects on the marked iNOS expression by NOC18, although they markedly protected against NOC18-induced apoptotic cell death (aspirin; Fig. 4). Furthermore, the level of iNOS expression at low-dose NOC18 (100 μM) was almost similar to that at higher doses of NOC18 (data not shown). These findings indicated the following possible protective mechanism of NSAIDs: even if the examined NSAIDs scavenge most of extracellular NO·, a small amount of the remaining NO· can induce iNOS expression in the neuronal cells, and then NSAIDs may quench the intracellular NO· produced through iNOS induction, suggesting that the protective effects of NSAIDs against NOC18-induced apoptotic nuclear changes might be due mainly to their direct scavenging activities on produced intracellular NO· in neuronal cells. Although *in vitro* NO· quenching activity of aspirin, mefenamic acid, indomethacin and ketoprofen was shown at almost the same concentration (IC_{50}s: 36–48 μM in Fig. 2), different concentrations of each drug exerted the protective effects against NOC18-induced cell damage (Fig. 3), especially mefenamic acid and indomethacin showed higher protective effects at concentrations of 2.5–10 μM against 500 μM NOC18-induced cell death. On the protective effects of mefenamic acid and indomethacin, these might be due not only to direct NO· scavenging effects but also to inhibiting effects on COX activities.

Recent reports showed that NSAIDs have inhibitory effects on inflammatory and immune-mediated responses in microglia (Gottschall 1996; Netland *et al*. 1998; Du and Li 1999) and exert protective effects against Alzheimer's disease in the clinical trials (in't Veld *et al*. 1998; McGeer

and McGeer 1995; McGeer *et al*. 1996; Stewart *et al*. 1997; McGeer and McGeer 1999). Since NO· is well known to be involved in the aggravation of inflammatory responses, the direct NO· scavenging effects of NSAIDs which were identified in the present study might be associated, in part, with their protective effects in inflammatory process in Alzheimer's disease.

NO· formation in the cerebral artery is thought to cause dilatation of cerebral and extracerebral blood vessels with subsequent migraine and other vascular-related headaches; glyceryl trinitrate (an NO· donor) and histamine (which activates endothelial NO· formation) both caused a pulsating headache and this migraine pain was ameliorated by treatment with a NOS inhibitor (Lassen *et al*. 1997; Olesen *et al*. 1994). Some vascular headaches are responsive to NSAIDs (Gilman *et al*. 1993; Goadsby 1999). Therefore, these direct NO· quenching effects of NSAIDs might be involved in the rapid therapeutic effects of NSAIDs for vascular headaches.

In conclusion, we have demonstrated a novel direct effect of NSAIDs, aspirin, mefenamic acid, indomethacin, and ketoprofen as NO· scavengers, and their protective effects on NO· donor-induced apoptotic cell death of neuronal cells in the present study. Taken together with the inhibitory action of NSAIDs on COX and NOS, these findings indicate that NSAIDs have multiple action sites on the pathway at which they exert their inhibitory effects against NO·-associated changes by various stimuli. Furthermore, the direct scavenging effects of NSAIDs against NO· may provide some novel pharmacological mechanisms of NSAIDs to exert not only their anti-inflammatory, analgesic, and antipyretic activities but also neuroprotective activities against neurodegeneration such as Alzheimer's disease.

Acknowledgements

We thank Miss Kumi Sato for her skilful technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Area and Scientific Research (C) from the Japanese Ministry of Education, Science, Sports and Culture, and Grants-in-Aid for Comprehensive Research on Aging and Health, for Research on Brain Science, and for Research on Specific Diseases from the Japanese Ministry of Health and Welfare.

References

Abramson S. B., Leszczynska P. J., Clancy R. M., Philips M. and Weissmann G. (1994) Inhibition of neutrophil function by aspirin-like drugs (NSAIDS): requirement for assembly of heterotrimeric G proteins in bilayer phospholipid. *Biochem. Pharmacol.* **47**, 563–572.

Aeberhard E. E., Henderson S. A., Arabolos N. S., Griscavage J. M., Castro F. E., Barrett C. T. and Ignarro L. J. (1995) Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.* **208**, 1053–1059.

Amin A. R., Vyas P., Attur M., Leszczynska-Piziak J., Patel I. R., Weissmann G. and Abramson S. B. (1995) The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proc. Natl Acad. Sci. USA* **92**, 7926–7930.

Asanuma M., Asanuma S. N., Gómez-Vargas M., Yamamoto M. and Ogawa N. (1997) Ketoprofen, a non-steroidal anti-inflammatory drug prevents the late-onset reduction of muscarinic receptors in gerbil hippocampus after transient forebrain ischemia. *Neurosci. Lett.* **225**, 109–112.

Chen L., Salafranca M. N. and Mehta J. L. (1997) Cyclooxygenase inhibition decreases nitric oxide synthase activity in human platelets. *Am. J. Physiol.* **273**, H1854–H1859.

Dennis M. J., French P. C., Crome P., Babiker M., Shillingford J. and Hopkins R. (1985) Pharmacokinetic profile of controlled release ketoprofen in elderly patients. *Br. J. Clin. Pharmacol.* **20**, 567–573.

Du Z. Y. and Li X. Y. (1999) Inhibitory effects of indomethacin on interleukin-1 and nitric oxide production in rat microglia *in vitro*. *Int. J. Immunopharmacol.* **21**, 219–225.

Engineer D. M., Niederhauser U., Piper P. J. and Sirois P. (1978) Release of mediators of anaphylaxis: inhibition of prostaglandin synthesis and the modification of release of slow reacting substance of anaphylaxis and histamine. *Br. J. Pharmacol.* **62**, 61–66.

Farivar R. S., Chobanian A. V. and Brecher P. (1996) Salicylate or aspirin inhibits the induction of the inducible nitric oxide synthase in rat cardiac fibroblasts. *Circ. Res.* **78**, 759–768.

Frantz B. and O'Neill E. A. (1995) The effect of sodium salicylate and aspirin on NF-κB. *Science* **270**, 2017–2019.

Furst D. E. (1994) Are there differences among nonsteroidal antiinflammatory drugs? Comparing acetylated salicylates, non acetylated salicylates, and nonacetylated nonsteroidal anti-inflammatory drugs. *Arthritis Rheum.* **37**, 1–9.

Gilman A. G., Rall T. W., Nies A. S. and Taylor P. (1993). *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 8th edn. McGraw-Hill, New York.

Goadsby P. J. (1999) Short-lasting primary headaches: focus on trigeminal autonomic cephalgias and indomethacin-sensitive headaches. *Curr. Opin. Neurol.* **12**, 273–277.

Gottschall P. E. (1996) beta-Amyloid induction of gelatinase B secretion in cultured microglia: inhibition by dexamethasone and indomethacin. *Neuroreport* **7**, 3077–3080.

Grilli M., Pizzi M., Memorandum M. and Spano P. (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-κB activation. *Science* **274**, 1383–1385.

Harabie J. A. and Klose J. R. (1993) New nitric oxide-releasing zwitterions derived from polyamines. *J. Org. Chem.* **58**, 1472–1476.

Hogg N., Singh R. J., Joseph J., Neese F. and Kalyanaraman B. (1995) Reactions of nitric oxide with nityonyl nitroxides and oxygen: prediction of nitrite and nitrate formation by kinetic simulation. *Free Radic. Res.* **22**, 47–56.

Humes J. L., Winter C. A., Sadowski S. J. and Kuehl F. A. Jr (1981) Multiple sites on prostaglandin cyclooxygenase are determinants in the action of nonsteroidal antiinflammatory agents. *Proc. Natl Acad. Sci. USA* **78**, 2053–2056.

Ito K., Niida Y., Sato J., Owada E., Ito K. and Umetsu M. (1994) Pharmacokinetics of mefenamic acid in preterm infants with patent ductus arteriosus. *Acta Paediatr. Jpn.* **36**, 387–391.

Iwata-Ichikawa E., Kondo Y., Miyazaki I., Asanuma M. and Ogawa N. (1999) Glial cells protect neurons against oxidative stress via

transcriptional up-regulation of the glutathione synthesis. *J. Neurochem.* **72**, 2334–2344.

Katsuyama K., Shichiri M., Kato H., Imai T., Marumo F. and Hirata Y. (1999) Differential inhibitory actions by glucocorticoid and aspirin on cytokine-induced nitric oxide production in vascular smooth muscle cells. *Endocrinology* **140**, 2183–2190.

Kepka-Lenhart D., Chen L. C. and Morris S. M. J. (1996) Novel actions of aspirin and sodium salicylate: discordant effects on nitric oxide synthesis and induction of nitric oxide synthase mRNA in a murine macrophage cell line. *J. Leukoc. Biol.* **59**, 840–846.

Kim H., Lee E., Shin T., Chung C. and An N. (1998) Inhibition of the induction of the inducible nitric oxide synthase in murine brain microglial cells by sodium salicylate. *Immunology* **95**, 389–394.

Kondo F., Kondo Y., Gómez-Vargas M. and Ogawa N. (1998) Indomethacin inhibits delayed DNA fragmentation of hippocampal CA1 pyramidal neurons after transient forebrain ischemia in gerbils. *Brain Res.* **791**, 352–356.

Kondo F., Kondo Y., Makino H. and Ogawa N. (2000) Delayed neuronal death in hippocampal CA1 pyramidal neurons after forebrain ischemia in hyperglycemic gerbils: amelioration by indomethacin. *Brain Res.* **853**, 93–98.

Kopp E. and Ghosh S. (1994) Inhibition of NF-κB by sodium salicylate and aspirin. *Science* **265**, 956–959.

Kwon G., Hill J. R., Corbett J. A. and McDaniel M. L. (1997) Effects of aspirin on nitric oxide formation and de novo protein synthesis by RINm5F cells and rat islets. *Mol. Pharmacol.* **52**, 398–405.

Lassen L. H., Ashina M., Christiansen I., Ulrich V. and Olesen J. (1997) Nitric oxide synthase inhibition in migraine. *Lancet* **349**, 401–402.

Lipton S. A. (1997) Janus faces of NF-κB: neurodestruction versus neuroprotection. *Nat. Med.* **3**, 20–22.

López-Farré A., Caramelo C., Esteban A., Alberola M. L., Millás I., Montón M. and Casado S. (1995) Effects of aspirin on platelet–neutrophil interactions. Role of nitric oxide and endothelin-1. *Circulation* **91**, 2080–2088.

López-Farré A., Riesco A., Digiuni E., Mosquera J. R., Caramelo C., de Miguel L. S., Millás I., de Frutos T., Cernadas M. R., Montón M., Alonso J. and Casado S. (1996) Aspirin-stimulated nitric oxide production by neutrophils after acute myocardial ischemia in rabbits. *Circulation* **94**, 83–87.

McGeer E. G. and McGeer P. L. (1999) Brain inflammation in Alzheimer disease and the therapeutic implications. *Curr. Pharm. Des.* **5**, 821–836.

McGeer P. L. and McGeer E. G. (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res. Rev.* **21**, 195–218.

McGeer P. L., Schulzer M. and McGeer E. G. (1996) Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. *Neurology* **47**, 425–432.

Meade E. A., Smith W. L. and DeWitt D. L. (1993) Differential inhibition of prostaglandin endoperoxide synthase (cyclo-oxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **268**, 6610–6614.

Meyer M., Schreck R. and Baeuerle P. A. (1993) H2O2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: aP-1 as secondary antioxidant-responsive factor. *EMBO J.* **12**, 2005–2015.

Mitchell J. A., Akarasereenont P., Thiemermann C., Flower R. J. and Vane J. R. (1993) Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Natl Acad. Sci. USA* **90**, 11693–11697.

Mituta K., Mizuta Y., Kohno M., Hiramatsu M. and Mori A. (1990) The application of ESR spin-trapping technique to the evaluation of SOD-*like* activity of biological substances. *Bull. Chem. Soc. Jpn* **63**, 187–191.

Mooradian D. L., Hutsell T. C. and Keefer L. K. (1995) Nitric oxide (NO) donor molecules: effect of NO release rate on vascular smooth muscle cell proliferation *in vitro*. *J. Cardiovasc. Pharmacol.* **25**, 674–678.

Netland E. E., Newton J. L., Majocha R. E. and Tate B. A. (1998) Indomethacin reverses the microglial response to amyloid beta-protein. *Neurobiol. Aging* **19**, 201–204.

Nishibayashi S., Asanuma M., Kohno M., Gómez-Vargas M. and Ogawa N. (1996) Scavenging effects of dopamine agonists on nitric oxide radicals. *J. Neurochem.* **67**, 2208–2211.

Olesen J., Thomsen L. L. and Iversen H. (1994) Nitric oxide is a key molecule in migraine and other vascular headaches. *Trends Pharmacol. Sci.* **15**, 149–153.

Sakitani K., Kitade H., Inoue K., Kamiyama Y., Nishizawa M., Okumura T. and Ito S. (1997) The anti-inflammatory drug sodium salicylate inhibits nitric oxide formation induced by interleukin-1beta at a translational step, but not at a transcriptional step, in hepatocytes. *Hepatology* **25**, 416–420.

Salvemini D., Misko T. P., Masferrer J. L., Seibert K., Currie M. G. and Needleman P. (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl Acad. Sci. USA* **90**, 7240–7244.

Sanchez de Miguel L., de Frutos T., Gonzalez-Fernandez F., del Pozo V., Lahoz C., Jimenez A., Rico L., Garcia R., Aceituno E., Millás I., Gomez J., Farré J., Casado S. and López-Farré A. (1999) Aspirin inhibits inducible nitric oxide synthase expression and tumour necrosis factor-alpha release by cultured smooth muscle cells. *Eur. J. Clin. Invest.* **29**, 93–99.

Schreck R., Rieber P. and Baeuerle P. A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.* **10**, 2247–2258.

Stewart W. F., Kawas C., Corrada M. and Metter E. J. (1997) Risk of Alzheimer's disease and duration of NSAID use. *Neurology* **48**, 626–632.

Uehara T., Kikuchi Y. and Nomura Y. (1999) Caspase activation accompanying cytochrome c release from mitchondria is possibly involved in nitric oxide-induced neuronal apoptosis in SH-SY5Y cells. *J. Neurochem.* **72**, 196–205.

Vane J. R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* **231**, 232–235.

Vane J. (1994) Towards a better aspirin. *Nature* **367**, 215–216.

in't Veld B. A., Launer L. J., Hoes A. W., Ott A., Hofman A., Breteler M. M. and Stricker B. H. (1998) NSAIDs and incident Alzheimer's disease. The Rotterdam Study. *Neurobiol. Aging* **19**, 607–611.

Weber C., Erl W., Pietsch A. and Weber P. C. (1995) Aspirin inhibits nuclear factor-κB mobilization and monocyte adhesion in stimulated human endothelial cells. *Circulation* **91**, 1914–1917.