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European Journal of Pharmacology 330 (1997) 221–229

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Inhibition of brain cyclooxygenase-2 activity and the antipyretic action of nimesulide

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Received 9 April 1997; revised 6 May 1997; accepted 7 May 1997

Abstract

The antipyretic action and the mechanism of action of 4-nitro-2-phenoxymethanesulfonanilide (nimesulide), a new nonsteroidal antiinflammatory drug, were investigated in yeast-induced febrile rats. Yeast-injected rats developed marked fever and exhibited an approximately 7-fold increase in brain levels of prostaglandin E_2 and an approximately 2-fold increase in the expression of cyclooxygenase-2 mRNA despite an almost unchanged expression of cyclooxygenase-1 mRNA. Nimesulide produced a dose dependent antipyretic action, which was stronger than that of indomethacin and ibuprofen, and decreased dose dependently the increased brain prostaglandin E_2 levels, whereas it did not influence the expression of cyclooxygenase-2 mRNA. It inhibited markedly the enhanced brain cyclooxygenase activity, primarily cyclooxygenase-2, in vivo and dose dependently increased brain cyclooxygenase activity in vitro. These results suggest that the marked antipyretic action of nimesulide is primarily mediated through the selective inhibition of the activity of brain cyclooxygenase-2 induced under febrile conditions. © 1997 Elsevier Science B.V.

Keywords: Nimesulide; Cyclooxygenase-2; Antipyretic action; Hypothalamus; Yeast-induced hyperthermia; RT-PCR (reverse transcription and polymerase chain reaction)

1. Introduction

Fever is a manifestation often seen in cases of infection by bacteria, viruses and spirochetes, as well as in cases of tumors and traumata. As a possible mechanism of fever, it is suggested that endotoxins released from invading microorganisms may possibly stimulate monocytes and macrophages to inducibly produce febrile cytokines (interleukin-1, interleukin-6, interferon, tumor necrosis factor, macrophage inflammatory protein-1, etc.) (Dinarello et al., 1984; Helle et al., 1988; Nakamura et al., 1988; Wolpe et al., 1988; LeMay et al., 1990). These endogenous pyrogens could be transported by blood to the organum vasculosum laminae terminalis to promote prostaglandin release, which subsequently could react with the preoptic/anterior hypothalamus to change the activity of temperature-sensitive neurons in the hypothalamus (Atkins, 1960; Hori et al., 1991).

Many investigators have already found that nonsteroidal antiinflammatory drugs, such as aspirin and indomethacin, produce antipyretic actions by inhibiting prostaglandin synthesis, in addition to their antiinflammatory and analgesic actions (Ferreira et al., 1971; Vane, 1971; Flower and Vane, 1972). Most nonsteroidal antiinflammatory drugs usually exhibit well-balanced antiinflammatory, analgesic and antipyretic activities. But nimesulide, which is a sulfonanilide derivative, that is, a weakly acidic nonsteroidal antiinflammatory drugs possessing no carboxyl radical in its molecule, has been found to exert a relatively strong antipyretic action compared to its antiinflammatory and analgesic actions. It has been reported that in animal experiments, the antipyretic action of nimesulide is stronger than that of indomethacin and paracetamol, while its antiinflammatory action is approximately the same as that of indomethacin (Tanaka et al., 1992; Ceserani et al., 1993).

It has been demonstrated that the antiinflammatory action of nimesulide may not be due to inhibition of the activity of the constitutive cyclooxygenase-1 present in the stomach, kidneys and seminal vesicle but due to selective inhibition of the activity of the inducible cyclooxygenase-2

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PII S0014-2999(97)00183-0

by various stimuli in the inflammatory cells infiltrating at an inflammatory site (Taniguchi et al., 1995; Tavares et al., 1995). Recently, Kennedy et al. (1993) suggested that the expression of cyclooxygenase-2 mRNA possibly plays an important role in fever. They showed that administration of lipopolysaccharide to rats caused an increase in brain levels of cyclooxygenase-2 mRNA.

In these experiments with yeast-induced febrile rat models, we first studied the relationship between fever and either brain prostaglandin or brain cyclooxygenase-2, and then studied the effects of nimesulide on the inducible expression of cyclooxygenase-2 mRNA in the brain and on the activity of cyclooxygenase enzymes, in order to elucidate the mechanisms of the antipyretic action of nimesulide.

2. Materials and methods

2.1. Animals

Male Wistar rats (Kyudo, Kumamoto, Japan) were raised for more than 3 days and those in good general condition were chosen for the experiments. The animals were raised in rooms kept at a temperature of 23 ± 3°C and at a relative humidity of 55 ± 15%. The rooms were illuminated continuously for 12 h per day and ventilated 10–12 times per h. The animals had free access to water and CE-2 solid diet throughout (Japan Clea, Osaka, Japan).

2.2. Yeast-induced febrile rats

Seven rats weighing approximately 220 g were used per group. A 15% yeast saline suspension (15 ml/kg) was injected subcutaneously into the dorsum, and animals showing an increase of 0.7°C or more in rectal temperature 17 h after yeast injection were chosen for the experiments. Subsequently, test drugs were orally given to the animals, and their rectal temperature was measured using a portable thermocouple thermometer (PTI-200; Unique Medical, Tokyo, Japan) at 1 h intervals for 5 h. According to the method of Clark and Cumby (1975), the fever index was derived from the area under the fever curve for 5 h after test drug administration, and one unit of the fever index was equivalent to a 1°C change lasting for 1 h. Antipyretic ED_{50} values were calculated from the percent inhibition of the fever index of the control group.

2.3. Expression of cyclooxygenase-1 mRNA and cyclooxygenase-2 mRNA in brain

2.3.1. Excision of rat brains

Before and at various time intervals after the yeast injection the animals were decapitated under pentobarbital anaesthesia according to the method of Ferrari et al. (1990). Craniotomy was performed to excise the brains, from which the cerebellums and olfactory bulbs were removed.

2.3.2. Preparation of mRNA

To the excised brain was added 4 ml of extraction buffer (50% guanidine thiocyanate, 0.15 M sodium acetate, 50% acid phenol and 0.35% 2-mercaptoethanol), and the mixture was homogenised using a Polytron PT-10 homogeniser (Brinkmann Instrument, Westbury, NY, USA). Total RNA was isolated by the acid guanidium thiocyanate–phenol–chloroform extraction method as described by Chomczynski and Sacchi (1987).

2.3.3. Reverse transcription and polymerase chain reaction (RT-PCR)

A RT-PCR method was performed according to a modification of the method of Lee et al. (1992). 1 μg of the total RNA was reverse transcribed and amplified according to the instructions of the Takara RNA PCR kit Ver. 2 with AMV reverse transcriptase (RTase) (Takara Shuzo, Tokyo, Japan). The mixture in 1 × RNA PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl) containing 5 mM $MgCl_2$, 1 mM dNTP, 1 U/μl RNase inhibitor, 2.5 μM oligo $(dT)_{20}$-M4 adapter primer and 5 U of AMV RTase (Takara Shuzo) in 20 μl of reaction volume was overlaid with mineral oil and then incubated at 42°C for 60 min. The reaction was terminated by heating at 95°C for 5 min, and the reaction mixture was chilled on ice for 10 min. After addition of the mixture to 1 × RNA PCR buffer containing 4 mM $MgCl_2$, 0.2 μM sense and antisense primer and 2.5 U *Taq* DNA polymerase (Takara Shuzo), 100 μl of cDNA mixture was amplified.

The PCR amplification conditions were as follows: cyclooxygenase-1, 94°C for 2 min for 1 cycle and 68°C for 5 min for 30 cycles; cyclooxygenase-2, 94°C for 2 min for 1 cycle and 55°C for 5 min for 30 cycles; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 94°C for 2 min for 1 cycle and 63°C for 5 min for 25 cycles.

Arrangements of sense primer and antisense primer of cyclooxygenase-1, cyclooxygenase-2 and GAPDH were:

cyclooxygenase-1 sense chain: 5′-TGCAT GTGGC TGTGG ATGTC ATCAA-3′

cyclooxygenase-1 antisense chain: 5′-CACTA AGACA GACCC TGCAT CTCCA-3′

cyclooxygenase-2 sense chain: 5′-ACTCA CTCAG TTTGT TGAGT CATTC-3′

cyclooxygenase-2 antisense chain: 5′-TTTGA TTAGT ACTGT TGGGT TAATG-3′

GAPDH sense chain: 5′-GTGAA GGTCG GTGTG AACGG ATTT-3′

GAPDH antisense chain: 5′-CACAG TCTTC TGAGT GGCAG TGAT-3′

The amplification products were analyzed by 1.2% agarose gel electrophoresis with borate EDTA buffer. After denaturation, the DNA was transferred to a nylon

membrane (Hybond N + ; Amersham, Amersham, UK) by the capillary method and fixed by UV irradiation.

2.3.4. Hybridization

After DNA fixation, the nylon membrane was incubated with hybridization buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 M sodium phosphate buffer, pH 7.2, 7% sodium dodecyl sulphate and 5% formaldehyde) at 65°C for 30 min (prehybridization). Immediately after prehybridization, the hybridization buffer was replaced with a new one, and to the membrane was added a ^{32}P-labelled DNA probe, the arrangements of which are shown below. The membrane was incubated overnight at 65°C.

Labelling of the probes was performed with a random primer DNA labelling kit (cyclooxygenase-1 cDNA probe, Takara Shuzo) and MEGALABEL (cyclooxygenase-2 and GAPDH oligonucleotide probe, Takara Shuzo).

Arrangements of oligonucleotides (cyclooxygenase-2, GAPDH)

cyclooxygenase-2 oligonucleotide probe: 5′-ATCTA GTCTG GAGTG GGAGG CACTT GCATT-3′

GAPDH oligonucleotide probe: 5′-GGCAT CAGCG GAAGG GGCGG AGAGA TGATG ACCCT-3′

After hybridization, the membrane was washed twice for 15 min with washing buffer (1 mM EDTA, 40 mM sodium phosphate buffer and 1% sodium dodecyl sulphate). Then, the half-dried membrane was wrapped and fixed together with an imaging plate (BASIII2025, Fuji Photo Film, Tokyo, Japan) in a cassette to perform autoradiography. After 30 min, the imaging plate was taken out to detect signals using a bioimaging analyser (BAS-2000II, Fuji Photo Film).

2.4. Prostaglandin E_2 levels in brain and hypothalamus

Eight rats weighing approximately 180 g were used per group. A 15% yeast saline suspension (15 ml/kg) was injected into the dorsum, and animals showing an increase of 0.7°C or more in rectal temperature 17 h after yeast injection were chosen for the experiments. Next, test drugs were given to the animals and their rectal temperature was measured using the portable PTI-200 thermocouple thermometer at 1 h before and at 2 h after test drug administration. As shown in Section 2.3.1, the animals were decapitated to excise the hypothalami and the brains, from which the cerebellums and olfactory bulbs were removed. Each tissue was immediately weighed, frozen in liquid nitrogen and homogenised in ice-cooled buffer (0.1 mM EDTA, 7 μM indomethacin and 2.5% ethanol, pH 3.5) using the Polytron PT-10 homogeniser. The homogenate was centrifuged at 10 000 rpm for 10 min. Prostaglandin E_2 in the supernatant was purified by the method of Powell and Chan (1984). 5 ml of the supernatant was applied to a Sep-Pak column previously washed with successive 20 ml portions of ethanol and distilled water. The column was washed with successive 20 ml portions of distilled water, 15% ethanol and petroleum ether and finally eluted with 10 ml of methyl formate. The eluate was evaporated to dryness under a stream of nitrogen, and the prostaglandin E_2 level was assayed by radioimmunoassay (RIA).

2.5. Measurement of cyclooxygenase-2 activity

2.5.1. Preparation of rat brain sample

The test drug was orally given 17 h after the yeast injection, and the animals were decapitated as shown in Section 2.3.1 to excise the brains 2 h after test drug administration. To the excised brains was immediately added 3 ml of lysate buffer (10 mM Tris–HCl buffer, pH 7.8, containing 1% Nonidet P-40, 0.15 M NaCl and 1 mM EDTA). The mixture was placed in a 15 ml centrifuge tube and homogenised using the Polytron PT-10 homogeniser. The homogenate was chilled on ice for 30 min and centrifuged at 4°C at 4000 rpm for 25 min, and the supernatant was used as a brain sample.

2.5.2. Measurement of brain cyclooxygenase activity

The brain sample was diluted 1:25 with lysate buffer. To 500 μl of the diluted brain sample was added 60 μl of lysate buffer containing 10 mM epinephrine, 10 mM phenol and a solution of the test drug in dimethylsulfoxide (Wako Pure Chemicals, Osaka, Japan; final concentration 0.5%). The mixture was chilled on ice for 15 min. To the mixture was added 60 μl of lysate buffer containing 1 mM arachidonic acid, and the mixture was incubated at 37°C for 5 min. The reaction was terminated by adding 250 μl of 1 M HCl, and the mixture was stirred after adding 5 ml of ethyl acetate and centrifuged at 4°C at 3000 rpm for 5 min to collect 4.5 ml of the supernatant ethyl acetate extract. The extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 1 ml of the buffer of the prostaglandin E_2-monoclonal enzyme immunoassay kit (EIA kit; Cayman, Ann Arbor, MI, USA) and used as an assay sample. To assay the existing prostaglandin E_2 level in the brain, a sample not allowed to react with arachidonic acid was prepared in the same manner as above and used for blank determination. The prostaglandin E_2 level was assayed with the EIA kit. The total prostaglandin E_2 level was calculated by multiplying the assay value by the dilution ratio, and the newly produced prostaglandin E_2 level was obtained by subtracting the existing prostaglandin E_2 level (blank value) from the total prostaglandin E_2 level.

2.6. Materials

Test drugs used in the experiments included nimesulide (Lot No. 91522, Helsinn, Chiasso, Switzerland), ibuprofen (Lot No. 625621; Kaken Pharmaceutical, Tokyo, Japan), indomethacin and dexamethasone (Lot No. 19F0018; Sigma, St. Louis, MO, USA). These test drugs were suspended in a solution of 0.5% sodium carboxymethyl-

cellulose (Maruishi Pharmaceutical, Tokyo, Japan) for oral administration. In case of the experiment in vitro, the test drugs were dissolved in dimethylsulfoxide to give a final concentration of 0.5%. The reagents included phorbol 12-myristate 13-acetate, bovine serum albumin (Biochemical Industry, Tokyo, Japan), lipopolysaccharide (Escherichia coli 026:B6, Sigma) and foetal calf serum (Gibco, Gaithersburg, MD, USA). The following reagents were used to assay prostaglandin E_2 cyclooxygenase-1 mRNA and cyclooxygenase-2 mRNA: EIA kit, MEGALABEL, random primer DNA labelling kit Ver. 2, prostaglandin H synthase 1 sense primer, prostaglandin H synthase 1 antisense primer, prostaglandin H synthase 2 oligonucleotide probe, prostaglandin H synthase 2 sense primer, prostaglandin H synthase 2 antisense primer, GAPDH oligonucleotide probe, GAPDH sense primer, GAPDH antisense primer (Takara Shuzo), prostaglandin H synthase 1 cDNA probe (murine) (Cayman), [α-^{32}P] dCTP and [γ-^{32}P] ATP (Amersham).

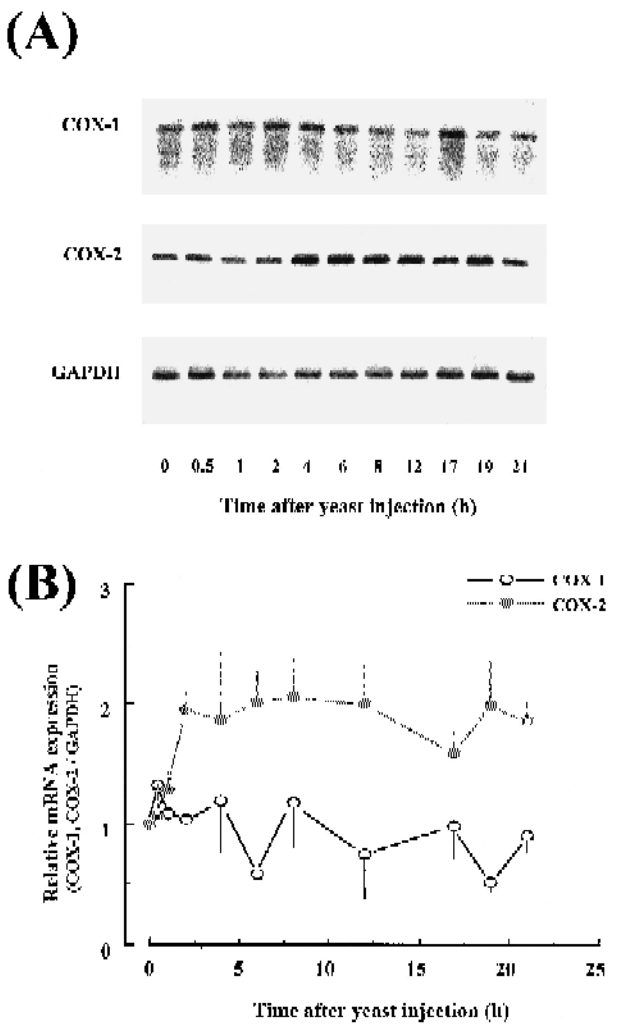

Fig. 1. Time-course of expression of brain cyclooxygenase-1 mRNA and cyclooxygenase-2 mRNA in yeast-induced febrile rats. (A) shows the RT-PCR analysis of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in the brain after the yeast injection. Total RNA was extracted from the brain before and after yeast injection and was hybridized with ^{32}P-labelled murine cyclooxygenase-1 cDNA probe for cyclooxygenase-1 or with ^{32}P-labelled oligonucleotide probe for cyclooxygenase-2. (B) shows the expression of cyclooxygenase-1 mRNA and cyclooxygenase-2 mRNA relative to that of GAPDH mRNA, which was determined with the same RNA sample by densitometry. Each point with a vertical bar represents the mean ± S.E.M. for 3 animals. COX, cyclooxygenase.

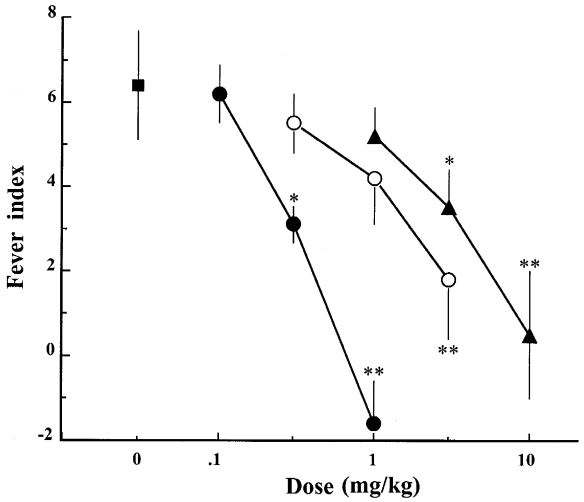

Fig. 2. Antipyretic effects of nimesulide and reference drugs on yeast-induced fever in rats. 15 ml of 15% yeast suspension was subcutaneously injected into the rat dorsum. At 17 h after yeast injection, animals showing an increase of 0.7°C or more in rectal temperature were chosen for the study. Test drugs were orally given, and rectal temperature was measured at 1 h intervals for 5 h after test drug administration. The fever index was derived from the cumulative increases in rectal temperature. ■: control, ●: nimesulide, ○: indomethacin, ▲: ibuprofen. Each point with a vertical bar represents the mean ± S.E.M. for 7 animals. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

2.7. Data and statistical analysis

The results are presented as the means ± S.E.M. for each experiment. The significant differences between experimental groups were analyzed by Dunnett's multiple range analysis. The dose–response regression curve drawn by using the least-squares method was used to calculate the ED_{50} values.

3. Results

3.1. Rectal temperature and expression of brain cyclooxygenase-1 mRNA and cyclooxygenase-2 mRNA in yeast-induced febrile rats

The rectal temperature decreased slightly 1 h after the yeast injection and thereafter it strongly increased. A significant increase was noted from 4 to 8 h. At 8 h, the rectal temperature reached a plateau, which was maintained for 19 h after yeast injection.

Fig. 1A and B show the time course of expression of brain cyclooxygenase-1 mRNA and cyclooxygenase-2

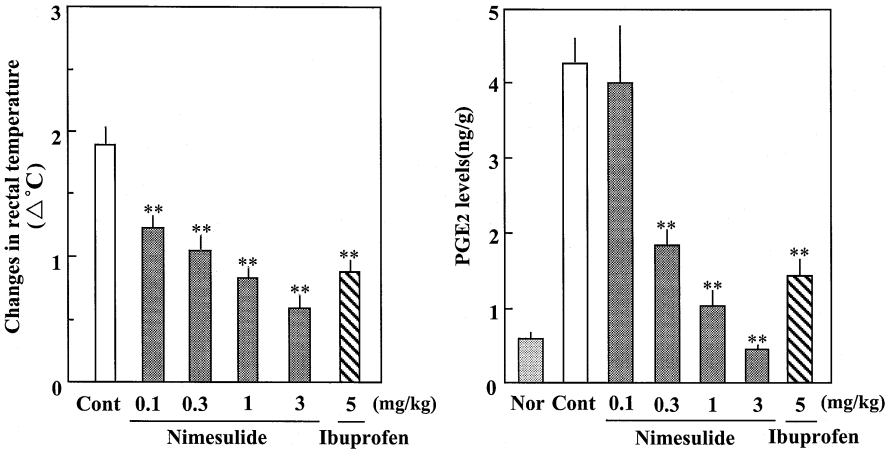

Fig. 3. Effects of nimesulide and ibuprofen on rectal temperature and brain prostaglandin E_2 levels in yeast-induced febrile rats. 15 ml of 15% yeast suspension was subcutaneously injected into the rat dorsum. At 17 h after yeast injection, animals showing an increase of 0.7°C or more in rectal temperature were chosen for the study. Test drugs were orally given, and rectal temperature was measured at 2 h after test drug administration. The animals were decapitated immediately after determining the antipyretic effects of the test drugs, and the heads were frozen in liquid nitrogen. The brains were excised and homogenized in ethanol solution to assay prostaglandin E_2 level by radioimmunoassay. Each column with a vertical bar represents the mean ± S.E.M. for 8 animals. ** $P < 0.01$ vs. control. Cont, control. Nor, normal. PGE_2, prostaglandin E_2.

mRNA in yeast-induced febrile rats. The expression of cyclooxygenase-1 mRNA remained unchanged at all times after the yeast injection, whereas the expression of cyclooxygenase-2 mRNA increased strongly from 2 h and reached a plateau level at 4 h after the yeast injection, which was approximately 2-fold greater than the expression before the yeast injection.

3.2. Antipyretic effects on yeast-induced fever in rats

Fig. 2 shows the antipyretic effects of nimesulide and reference drugs on yeast-induced fever in rats. Nimesulide, indomethacin and ibuprofen produced antipyretic effects on yeast-induced febrile rats in a dose-dependent manner; their ED_{50} values were 0.3, 1.4 and 2.8 mg/kg, respectively.

3.3. Inhibition of prostaglandin E_2 levels in brain and hypothalamus

Fig. 3 shows the effects of nimesulide and ibuprofen on rectal temperature and brain prostaglandin E_2 levels in yeast-induced febrile rats. Nimesulide at doses of 0.1, 0.3, 1 and 3 mg/kg inhibited the brain prostaglandin E_2 levels which were increased by fever, exhibiting an inhibition of 6.2, 57.2, 76.1 and 90.0%, respectively, and ibuprofen at 5

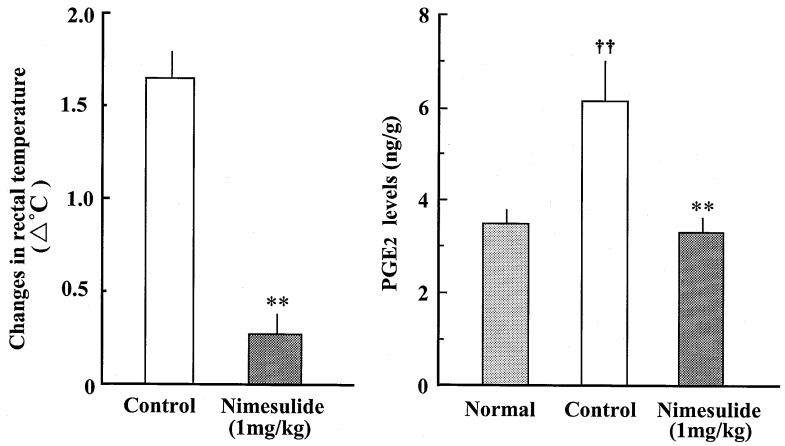

Fig. 4. Effect of nimesulide on rectal temperature and hypothalamic prostaglandin E_2 levels in yeast-induced febrile rats. 15 ml of 15% yeast suspension was subcutaneously injected into the rat dorsum. At 17 h after yeast injection, animals showing an increase of 0.7°C or more in rectal temperature were chosen for the study. Nimesulide was orally given, and rectal temperature was measured at 2 h after test drug administration. The animals were decapitated immediately after determining the antipyretic effects of the test drug, and the heads were frozen in liquid nitrogen. The hypothalami were excised and homogenized in ethanol solution to assay prostaglandin E_2 level by radioimmunoassay. Each column with a vertical bar represents the mean ± S.E.M. for 8 animals. †† $P < 0.01$ vs. normal. ** $P < 0.01$ vs. control.

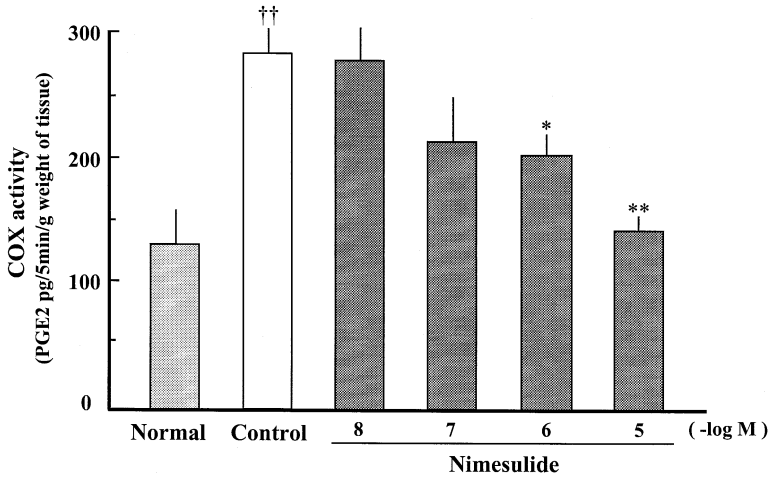

Fig. 5. Effect of nimesulide on brain cyclooxygenase activity in vitro in yeast-induced febrile rats. The brains were collected before and at 17 h after yeast injection and homogenized in Tris–HCl buffer containing EDTA. The homogenate was centrifuged and the supernatant was stored at −80°C. The cyclooxygenase enzyme preparation preincubated with nimesulide was incubated in Tris–HCl buffer containing epinephrine, phenol and arachidonic acid. The reaction was terminated by adding 1 M 213HCl, and the mixture was extracted with ethyl acetate and evaporated to dryness. Prostaglandin E_2 level was assayed with an EIA kit, and the cyclooxygenase activity is presented as prostaglandin E_2 pg/5 min per g wet weight of tissue. Each column with a vertical bar represents the mean ± S.E.M. of 3 determinations. * $P < 0.05$, ** $P < 0.01$ vs. control.

mg/kg caused an inhibition of 53.7%. Nimesulide at 0.1, 0.3, 1 and 3 mg/kg also inhibited the yeast-induced fever, causing an inhibition of 34.7, 44.2, 52.6 and 68.9%, and ibuprofen at 5 mg/kg caused an inhibition of 53.7%.

Fig. 4 shows the effect of nimesulide on rectal temperature and hypothalamic prostaglandin E_2 levels in yeast-induced febrile rats. Nimesulide at the dose of 1 mg/kg inhibited the yeast-induced fever as well as the hypothalamic prostaglandin E_2 levels increased by the fever, exhibiting an inhibition of 83.1 and 106.5%, respectively.

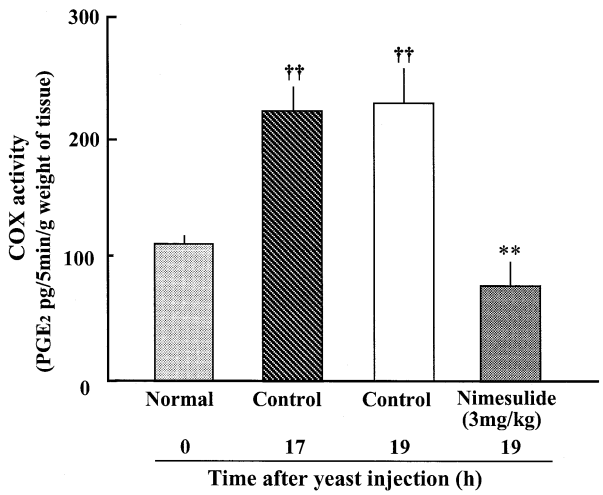

Fig. 6. Effect of orally given nimesulide on brain cyclooxygenase activity in vivo in yeast-induced febrile rats. Nimesulide was orally given to the animals 17 h after yeast injection. The brains were collected before and at 17 and 19 h after the yeast injection and homogenized in Tris–HCl buffer containing EDTA. The homogenate was centrifuged, and the supernatant was used as the source of cyclooxygenase enzymes and diluted to the desired concentrations just before use. The cyclooxygenase enzyme preparation was incubated in Tris–HCl buffer containing epinephrine, phenol and arachidonic acid for 5 min. The reaction was terminated by adding 1 M HCl, and the mixture was extracted with ethyl acetate and evaporated to dryness. Prostaglandin E_2 level was assayed with an EIA kit, and the cyclooxygenase activity was presented as prostaglandin E_2 pg/5 min per g wet weight of tissue. Each column with a vertical bar represents the mean ± S.E.M. for 8 animals. †† $P < 0.01$ vs. normal. ** $P < 0.01$ vs. control (19 h).

3.4. Inhibition of cyclooxygenase activity in vitro and in vivo

Fig. 5 shows the effect of nimesulide on cyclooxygenase activity in supernatants of brains from yeast-in-

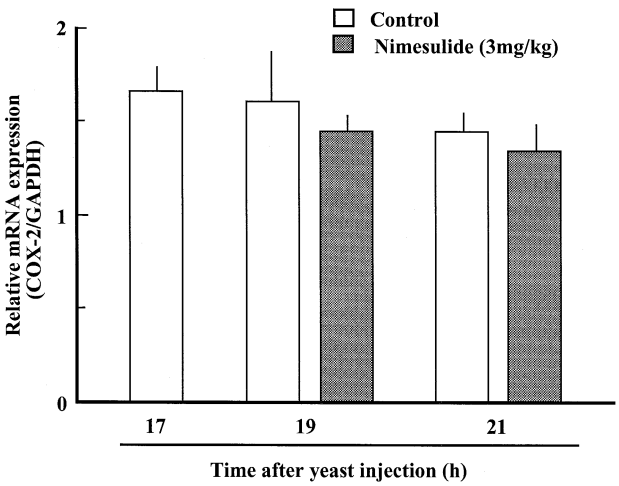

Fig. 7. Effect of nimesulide on the expression of brain cyclooxygenase-2 mRNA in yeast-induced febrile rats. 15 ml of 15% yeast suspension was subcutaneously injected into the rat dorsum. Nimesulide was orally given 17 h after yeast injection. Total RNA was extracted from the brain at 17, 19 and 21 h after yeast injection and hybridized with ^{32}P-labelled oligonucleotide probe for cyclooxygenase-2. The relative expression of cyclooxygenase-2 mRNA to GAPDH mRNA was determined with the same RNA sample by densitometry. Each column with a vertical bar represents the mean ± S.E.M. for 3 animals.

duced febrile rats. At 17 h after the yeast injection, nimesulide inhibited in vitro the activity of cyclooxygenase in brain supernatants prepared from fully febrile rats in a concentration-dependent manner over the concentration range of 10^{-5} to 10^{-8} M.

Fig. 6 shows the effect of orally given nimesulide on brain cyclooxygenase activity in vivo in yeast-induced febrile rats. At 17 h after the yeast injection, nimesulide was orally given to fully febrile rats at the dose of 3 mg/kg, and after 2 h, brain cyclooxygenase activity was measured. Nimesulide strongly inhibited the brain cyclooxygenase activity and reduced it to its normal level.

3.5. Inhibition of expression of cyclooxygenase-2 mRNA in brain

Fig. 7 shows the effect of nimesulide on the expression of brain cyclooxygenase-2 mRNA in yeast-induced febrile rats. Nimesulide at 3 mg/kg exerted almost no influence on the enhanced expression of cyclooxygenase-2 mRNA.

4. Discussion

Many investigators suggest that fever could be induced as a result of the invasion of endogenous pyrogens released from leukocytes into the central nervous system, especially the hypothalamus, where the pyrogens act to produce and release prostaglandins (Stitt, 1986; Coceani et al., 1988). Nonsteroidal antiinflammatory drugs have been found to exert antipyretic actions, inhibiting prostaglandin formation in the hypothalamus (Flower and Vane, 1972; Dinarello and Bernheim, 1981; Scott et al., 1987). However, it is not clear what type of cyclooxygenase enzymes are responsible for prostaglandin synthesis under normal and febrile conditions. Although it has recently been reported that cyclooxygenase-2 can be inducibly expressed during fever in the brain as well as in inflammatory cells such as macrophages (Kennedy et al., 1993), few detailed studies have been reported on cyclooxygenase-2 associated with prostaglandins in the brain.

Nimesulide, one of the nonsteroidal antiinflammatory drugs causing selective inhibition of cyclooxygenase-2, has been found to have a high antipyretic action, as compared with its antiinflammatory action (Tanaka et al., 1992; Ceserani et al., 1993). However, the mechanisms of the antipyretic action of nimesulide have not yet been elucidated. In these experiments, we first confirmed the antipyretic action of nimesulide using a yeast-induced febrile rat model and further studied the inhibitory effect of nimesulide on cyclooxygenase-2 expression as well as on prostaglandin levels, which are increased in febrile rat brains, in order to elucidate the mechanisms of the antipyretic action of nimesulide.

As shown in Fig. 2, nimesulide produced a strong antipyretic action in yeast-induced febrile rats, as compared with indomethacin and ibuprofen. Our experiment with lipopolysaccharide-stimulated rabbits gave similar results, yielding antipyretic ED_{50} values of nimesulide, indomethacin and ibuprofen of 0.4, 11.8 and 35.8 mg/kg, respectively.

In our previous experiment with cyclooxygenase enzyme preparations derived from ram seminal vesicle (used as cyclooxygenase-1) and from sheep placenta (used as cyclooxygenase-2), we found that nimesulide inhibits selectively cyclooxygenase-2 activity (Taniguchi et al., 1995). This finding strongly predicts the results of the present experiments. However, a high dose of nimesulide was required for the inhibition of brain-derived cyclooxygenase activity, as compared with the inhibition of purified cyclooxygenase-2 activity. Since cyclooxygenase enzymes in the microsomal fractions of febrile rat brain consist of a mixture of cyclooxygenase-1 and cyclooxygenase-2 enzymes, the inhibition of cyclooxygenase activity by nimesulide, a selective inhibitor for cyclooxygenase-2 activity, seems to be weakened to some degree. From the above, it is suggested that the potent antipyretic actions of nimesulide come from the selective inhibition of cyclooxygenase-2 activity in the brain, as is the case for inflammatory cells (Kennedy et al., 1993; Tavares et al., 1995).

Indomethacin, known as a non-selective inhibitor of cyclooxygenase activity, caused approximately the same degree of inhibition of cyclooxygenase-2 activity, which is primarily associated with fever, as nimesulide. But taking into account the doses used, its antipyretic effect was definitely weaker than that of nimesulide. Hucker et al. (1966) reported that the reason why relatively higher doses of indomethacin are required to produce an antipyretic action rather than an antiinflammatory action is the poor distribution of indomethacin into the brain.

Since the pK_a of nimesulide is 6.5, it can diffuse into the brain more easily than indomethacin ($pK_a = 4.2$). In order to investigate the distribution of nimesulide in the brain, we orally administered ^{14}C-labelled nimesulide (3 mg/kg) to rats and found that the nimesulide level in the brain was approximately 1 μg eq./g 3 h after administration (data not shown). This tissue level of nimesulide corresponds to levels that caused inhibition of cyclooxygenase-2 activity derived from sheep placenta (Taniguchi et al., 1995; Vago et al., 1995). From these findings, it is suggested that the site of antipyretic action of nimesulide is primarily in the central nervous system.

It is of interest that in these experiments cyclooxygenase-2 mRNA was expressed in the brain even in normal rats. In this connection, Kennedy et al. (1993), Seibert et al. (1994) and Breder et al. (1995) reported the expression of cyclooxygenase-2 mRNA in the normal rat brain, and O'Neill and Ford-Hutchinson (1993) also confirmed the expression of cyclooxygenase-2 mRNA as well as the presence of cyclooxygenase-2-immunoreactive cells in the normal human brain, but their physiological significance remained unexplained. Cyclooxygenase-2 and cyclooxy-

genase-1 in the normal brain may be involved in maintaining body temperature and, additionally, cyclooxygenase-2 may be constantly induced to maintain homeostasis against external stimuli as well as hormone secretions in the body.

In other experiments, we found that nimesulide inhibited the interleukin-1β production at concentrations as high as 10^{-4} M (data not shown). This suggests that inhibition of the production of cytokines, especially interleukin-1β, may not be directly related to the antipyretic action of nimesulide. It has been revealed that during fever development, or after intravenous injection of interleukin-1, prostaglandin E_1 and prostaglandin E_2 can be produced in the central nervous system, especially in the hypothalamus (Feldberg and Gupta, 1973; Dinarello, 1987; Sirko et al., 1989; Dam et al., 1993). This suggests that nimeslide could exert its antipyretic action through inhibition of brain cyclooxygenase-2 activity, which possibly participates in prostaglandin production following interleukin-1 production.

We have now demonstrated that the increased prostaglandin levels in the brain during fever primarily result from cyclooxygenase-2 induced in the brain by fever. From the above results, it is suggested that the antipyretic action of nimesulide is through the selective inhibition of the activity of cyclooxygenase-2, without affecting the expression of cyclooxygenase-2 mRNA in the brain.

Acknowledgements

We would like to thank Professor H. Kamiya, Ph.D., Fukuoka University, Fukuoka, Japan, for constructive comments on the manuscript.

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