Pharmacological Studies on EB-382, a New Non-steroidal Antiinflammatory Agents: Mode of Action of the Analgesic Effects

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The analgesic mechanism of (±)-2-[*p*-[(2-methylallyl)amino]phenyl]propionic acid (EB-382), a new non-steroidal antiinflammatory agent, was studied. EB-382 exerted a potent analgesic effect on yeast-induced hyperalgesia in rats and bradykinin-induced writhing in mice, and its potency was superior to that of ibuprofen. EB-382 had a comparatively weak inhibitory effect on the prostaglandin E_2 production from arachidonic acid in sheep seminal vesicle microsomal fraction *in vitro*. EB-382 exhibited a dose-dependent inhibitory effect on the phospholipase A_2 activity in 3T3 mouse fibroblast cells with a different mechanism from steroidal agents, although indomethacin and ibuprofen did not reveal any significant inhibition. EB-382 did not affect the bradykinin-induced contraction of isolated rat uterus. Intracisternal injection of EB-382 did not affect the acetic acid-induced writhing response in mice. From these results, it is suggested that the analgesic effect of EB-382 in inflammation is exerted through its direct peripheral action, and inhibition of prostaglandin biosynthesis *via* phospholipase A_2, rather than cyclooxygenase.

Keywords EB-382; non-steroidal antiinflammatory agent; analgesic effect; bradykinin; prostaglandin

Introduction

(±)-2-[*p*-[(2-Methylallyl)amino]phenyl]propionic acid (EB-382), a new non-steroidal antiinflammatory agent, was selected from a series of phenylpropionic acid derivatives, in which an amino group is introduced into the structure of ibuprofen on the basis of its potent analgesic and antiinflammatory activities with a wide safety margin.[1] Previous studies[2,3] have already shown that EB-382 displays remarkable antiinflammatory and analgesic effects in spite of its less potent inhibitory activity on prostaglandin biosynthesis [2-4] than that of ibuprofen, and especially exerts a strong inhibitory effect on inflammation which is related to the kallikrein–kinin system. Generally speaking, acidic non-steroidal antiinflammatory drugs exhibit strong inhibition of prostaglandin biosynthesis, and this is considered to represent their main mechanism of action. It was considered therefore that the main mechanism of the analgesic action of EB-382 possibly differed from that of the reference drugs. The present study was undertaken to clarify more precisely the mechanism of the analgesic action of EB-382.

Experimental

Animals Wistar rats of both sexes and male ddY mice were purchased from the Shizuoka Animal Center.

Drugs and Reagents (±)-2-[*p*-[(2-Methylallyl)amino]phenyl]propionic acid (EB-382) was supplied by Bouchara Research Laboratories. The other test compounds used were indomethacin (Sigma), ibuprofen (Sigma), aspirin (Sigma), imipramine (Sigma), [1-^{14}C]arachidonic acid (55.2 mCi/mmol; New England Nuclear), sodium arachidonate (Sigma), bradykinin (Sigma), Brewer's yeast (Sigma), Dulbecco's modified Eagle's salts (DMEM; Dainippon Phamaceutical Co.), and fetal calf serum (FCS; Gibco). All other reagents were of the highest quality commercially available. All test compounds were suspended in 5% arabic gum solution before administration or dissolved in dimethylsulfoxide (DMSO) *in vitro*.

Bradykinin-Induced Writhing A group of 10 male ddY mice weighing about 20 g was used. Employing the method of Burns *et al.*,[5] 10 ml/kg of 0.01% bradykinin was injected intraperitoneally 20 min after oral administration of the test drugs followed by an additional injection of bradykinin (5 ml/kg) 15 min after the first injection. The writhing response was observed for 20 min after the final injection of bradykinin. The drug was considered to be effective when no writhing was observed.

Bradykinin-Induced Contraction of Uterus Female Wistar rats weighing 150—200 g were used. An isolated uterus preparation was suspended in an organ bath filled with Tyrode solution gassed with a mixture of 95% O_2 and 5% CO_2 at 37 °C under a resting tension of about 4 g. The contraction of the preparation induced by bradykinin (3×10^{-8} g/ml) was recorded isotonically with an isotonic transducer (Nihon Koden TD-112S). All test drugs were dissolved in 0.5% DMSO and pretreated 2 min before the application of bradykinin.

Prostaglandin E_2 Biosynthesis of Sheep Seminal Vesicle Microsomal Fraction *in Vitro* Two hundred microliters of a mixed solution which contained 0.2 mg (as total protein) of sheep seminal vesicle microsomal fraction, 1 mM tryptophan, 2 mM glutathione, 0.1 M Tris–HCl (pH 7.6), 8.5 μM [1-^{14}C]arachidonic acid (50 nCi) and each drug dissolved in 0.5% DMSO as final concentration was incubated for 10 min at 37 °C with moderate shaking. The reaction mixture was cooled in ice-chilled water, and extracted twice with 1.5 ml of ethyl acetate. The extract was evaporated under vacuum, dissolved in 0.2 ml of ethanol, and spotted on a silica gel plate (Kieselgel 60 F_{254}, Merck). Thin-layer chromatography was performed according to the technique of Yanagi and Komatsu.[6] The developed prostaglandin E_2 fraction was detected and the radioactivity was counted with a liquid-scintillation counter (Aloka, LSC-900). Each IC_{50} value was calculated graphically from the dose–response curve of inhibition.

Phospholipase A_2 Activity of Mouse 3T3 Fibroblast Cells In accordance with the modified method of Morita *et al.*,[7] a monolayer of mouse 3T3 fibroblast cells was trypsinized with 0.3% trypsin ethylenediamine tetraacetic acid (EDTA). The trypsinized 3T3 cells were disaggregated by gentle pipette mixing, and washed three times with 10% FCS–DMEM. The 3T3 cells were then adjusted to a concentration of 1.2×10^5 cells/ml/dish with 10% FCS–DMEM. One milliliter of this cell preparation was added to each dish and incubated for 24 h in an atmosphere of 5% CO_2 in humidified air. Following the incubation, the cells were washed three times with 1 ml of 10% FCS–DMEM, then 1 ml of fresh 10% FCS–DMEM was added, and [1-^{14}C]arachidonic acid (0.05 μCi/ml) was distributed to each dish. Twenty hours later, the medium was removed and the cells were washed three times with FCS free-DMEM, then 10% FCS–DMEM containing a test drug solubilized in 0.1% DMSO was added. After incubation of the monolayer for 1 or 24 h, the medium was removed. The medium was replaced with fresh 10% FCS–DMEM containing the test drug, and incubated for another 2 h. Following the final incubation, the medium was collected. Aliquots of medium were transferred to counting vials, and the radioactivity of the released [1-^{14}C]arachidonic acid was determined with a liquid-scintillation counter (Aloka, LSC-1000).

Analgesic Effect after Intracisternal Injection of Test Drugs A group of 15 male ddY mice weighing 20—25 g was used. Employing the method of Ueda *et al.*,[8] each test drug was injected intracisternally 15 min before intraperitoneal injection of 10 ml/kg of 0.7% acetic acid. Ten minutes later, the number of writhing responses in mice treated with each test drug was recorded for 10 min.

Statistical Study Student's *t* test was used for statistical analysis of the data. The ED_{50} value and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon.[9]

Table I. Effects of EB-382 and Reference Compounds on the Bradykinin-Induced Writhing Response in Mice

Compounds	Dose (mg/kg, *p.o.*)	*N*	Inhibition (%)	ED_{50} (95%, conf. limits)
EB-382	5	10	30.0	
	10	10	40.0	14.4
	20	10	60.0	(5.4— 36.0)
	50	10	70.0	
Ibuprofen	20	10	30.0	
	50	10	40.0	68.0
	100	10	60.0	(29.4—166.7)
	200	10	70.0	
Phenylbutazone	20	10	30.0	
	50	10	50.0	44.8
	100	10	70.0	(23.6— 85.7)
	200	10	90.0	
Indomethacin	1	10	20.0	
	2	10	30.0	6.8
	5	10	40.0	(2.4— 20.4)
	10	10	60.0	

Table II. Effects of EB-382 on Bradykinin-Induced Contraction of Isolated Rat Uterus

Compounds	Dose (M)	Number of preparations	Contraction (%)
Control		6	100
EB-382	10^{-6}	6	101.0±5.00
	10^{-5}	6	96.7±5.34
	10^{-4}	6	95.8±7.01
Ibuprofen	10^{-6}	6	100.7±5.17
	10^{-5}	6	98.9±5.69
	10^{-4}	6	94.5±5.10
Indomethacin	10^{-6}	6	101.6±5.19
	10^{-5}	6	96.1±6.22
	10^{-4}	6	67.5±9.74[a]
Imipramine	10^{-6}	6	102.4±3.52
	10^{-5}	6	70.5±8.19[a]
	10^{-4}	6	29.2±7.59[a]

Each value shows the mean±S.E. *a*) Significantly different from the control at $p<0.01$.

Table III. Inhibitory Effects of EB-382 and Reference Compounds on Prostaglandin E_2 Biosynthesis in Sheep Seminal Vesicle Microsome Fraction

Compounds	IC_{50} (M)
EB-382	1.9×10^{-4}
Ibuprofen	1.7×10^{-5}
Phenylbutazone	3.4×10^{-5}
Indomethacin	1.3×10^{-7}
Aspirin	4.5×10^{-4}

Table IV. Effects of EB-382 on Phospholipase A_2 Activity of Mouse 3T3 Fibroblast Cells

Compounds	Dose (M)	Inhibition (%)
EB-382[a]	10^{-6}	12.5
	10^{-5}	30.6
	10^{-4}	39.3
Ibuprofen[a]	10^{-4}	6.7
Indomethacin[a]	10^{-4}	6.8
Predonisolone[a]	10^{-4}	1.9
Predonisolone[b]	10^{-7}	17.3
	10^{-6}	26.6
	10^{-5}	30.9

Each value represents the mean of 4—5 experiments. *a, b*) Incubation with drugs for 1 and 24 h, respectively.

Table V. Analgesic Activities of intracisternal Injection of EB-382 on Acetic Acid-Induced Writhing in Mice

Compounds	Dose (μg/kg, i.c.)	Number of animals	Number of writhing responses
Control		30	18.4±1.62
EB-382	10	15	20.3±2.50
	30	15	19.9±2.72
Tiaramide	10	15	12.0±2.25[a]
	30	15	6.6±1.47[b]
Morphine	1	15	0.4±0.19[b]

Each value shows the mean±S.E. *a, b*) Significantly different from the control at $p<0.05$ and $p<0.01$, respectively.

Results

Bradykinin-Induced Writhing EB-382 (5—50 mg/kg, *p.o.*) exhibited a dose-dependent inhibitory effect on the bradykinin-induced writhing response in mice (Table I). The potency of EB-382 was about 4 times higher than that of ibuprofen.

Bradykinin-Induced Contraction of Uterus EB-382 (up to 10^{-4} M) did not affect the bradykinin-induced contraction of the isolated rat uterus (Table II). Indomethacin exhibited a slight but significant inhibition only at a very high concentration (10^{-4} M).

Effect on the Prostaglandin E_2 Biosynthesis of Sheep Seminal Vesicle Microsomal Fraction *in Vitro* EB-382 (10^{-4}—10^{-3} M) exhibited a dose-dependent inhibitory effect on the prostaglandin E_2 biosynthesis from arachidonic acid by sheep seminal vesicle microsomal fraction, and its IC_{50} value was 1.9×10^{-4} M (Table III). The potency of EB-382 was 10 times weaker than that of ibuprofen.

Phospholipase A_2 Activity of 3T3 Fibroblast Cells EB-382 (10^{-6}—10^{-4} M) exhibited a dose-dependent inhibitory effect on the release of arachidonic acid in 3T3 mouse fibroblast cells. Prednisolone showed a dose-dependent inhibition following long-term incubation for 24 h, but did not reveal inhibition after short-term incubation for 1 h. Ibuprofen and indomethacin did not display such inhibition (Table IV).

Analgesic Effect Following Intracisternal Injection of Drugs EB-382 (10 and 30 μg, i.c.) did not affect the acetic acid-induced writhing response in mice, although significant inhibition was observed after i.c. injection of the positive control drugs, tiaramide (30 μg) or morphine (1 μg) (Table V).

Discussion

It was found previously that the analgesic effect of EB-382 was far superior to that of ibuprofen on yeast-induced hyperalgesia in rats using Randall–Selitto's method, in spite of its inferior inhibitory activity on prostaglandin E_2 biosynthesis from arachidonic acid in sheep seminal microsome fraction.[1,2] EB-382 was therefore assumed to have a characteristic pharmacological effect other than inhibition of prostaglandin biosynthesis *via* cyclooxygenase, which has been considered to represent the main mechanism of the analgesic actions of other acidic non-steroidal antiinflam-

matory drugs.[10] Bradykinin is considered to be the most important chemical mediator inducing algesia in the acute phase of inflammation. EB-382 displayed far more potent inhibition of the bradykinin-induced writhing response in mice than ibuprofen. Its potency was about half that of indomethacin. It was inferred therefore that EB-382 effectively exerted an analgesic effect in the acute process of inflammation in which bradykinin is intimately involved. However, EB-382 was considered not to have a direct antibradykinin action, since it could not antagonize the bradykinin-induced contraction of isolated rat uterus. Other reference drugs could not antagonize the contraction either, although a significant inhibition was observed following treatment with a high dose of indomethacin. It has been reported that bradykinin provokes the release of prostaglandins from the phospholipid membrane *via* activation of phospholipase A_2,[11] and in turn the released prostaglandins amplify the algesic effect of the bradykinin.[12] Thus, hyperalgesia is considered to be produced by the interaction of both mediators in inflammation. EB-382 exerted a weak dose-dependent inhibitory effect on the release of arachidonic acid from 3T3 mouse fibroblast cells following short term incubation of 1 h, while indomethacin and ibuprofen failed to show any effect. Prednisolone did not reveal any clear inhibition after incubation for 1 h, but displayed a dose-dependent inhibition after long term incubation of 24 h.

Steroidal antiinflammatory drugs such as prednisolone demonstrate inhibition of phospholipase A_2 activation due to lipocortin production.[13-16] However, the effect is not seen until after a long period of incubation *in vitro*. On the other hand, non-steroidal antiinflammatory drugs such as indomethacin have also been reported to inhibit the phospholipase A_2 of polymorphonuclear leukocytes, and this inhibition may well be important in relation to the effects of the drug on prostaglandin biosynthesis and inflammation.[17] It seems likely therefore that EB-382 exerted its inhibition of activated phospholipase A_2 through a mechanism different from that of the steroidal antiinflammatory drugs. EB-382 was found previously to show potent inhibition of the release of prostaglandins into the pleural cavity in a carrageenin-induced pleurisy model in rats, despite its weak cyclooxygenase inhibition.[18] Accordingly, EB-382 was considered also to inhibit the release of prostaglandin induced through activation of phospholipase A_2 by bradykinin in those experiments, since it has been reported that carrageenin-induced pleurisy may be closely related to the reciprocal action of the kinin and prostaglandin systems, and the released bradykinin provoked release of prostaglandins in the pleural cavity.[19,20] Intracisternal injection of EB-382 did not affect the acetic acid-induced writhing response in mice. The mechanism of action was apparently different from those of tiaramide and morphine. The analgesic effect of EB-382 was confirmed to be related to its direct peripheral action.

The above findings thus suggest that the analgesic effect of EB-382 in inflammation is exerted through its direct peripheral action, and inhibition of prostaglandin biosynthesis *via* phospholipase A_2, other than cyclooxygenase.

Acknowledgement We thank Prof. Sachiko Oh-ishi, Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, and Dr. Masanori Fukushima, Department of Internal Medicine, Aichi Cancer Center, for helpful discussions and technical advice.

References

1) B. Dumaitre, A. Fouquet, C. Perrin, P. Cornu, A. Bucherle, C. Portka, G. Domage and G. Streichenberger, *Eur. J. Med. Chem.*, **14**, 207 (1979).
2) T. Fujiyoshi, K. Ikeda, M. Saito, T. Yamaura, H. Iida, E. Maeda, N. Kase and T. Uematsu, *Nippon Yakurigaku Zasshi*, **87**, 379 (1986).
3) T. Fujiyoshi, H. Iida, M. Saito, K. Ikeda, T. Yamaura and T. Uematsu, *Nippon Yakurigaku Zasshi*, **87**, 527 (1986).
4) T. Fujiyoshi, H. Iida, M. Murakami and T. Uematsu, *Yakugaku Zasshi*, **107**, 170 (1987).
5) R. B. I. Burns, N. J. Alioto and K. E. Hurley, *Arch. Int. Pharmacodyn.*, **175**, 41 (1968).
6) Y. Yanagi and T. Komatsu, *Biochem. Pharmacol.*, **25**, 937 (1976).
7) I. Morita, Y. Nakayama and S. Murota, *Prostaglandins*, **18**, 507 (1979).
8) H. Ueda, H. Amano, H. Shiomi and H. Takagi, *Eur. J. Pharmacol.*, **56**, 265 (1979).
9) J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Ther.*, **96**, 99 (1949).
10) J. R. Vane, *Nature, New Biol.*, **231**, 232 (1971).
11) B. B. Vargaftig and N. D. Hai, *J. Pharm. Pharmacol.*, **24**, 159 (1972).
12) S. H. Ferreira, *Nature, New Biol.*, **240**, 200 (1972).
13) A. Danon and G. Assouline, *Nature* (London), **273**, 522 (1978).
14) R. J. Flower and G. J. Blackwell, *Nature* (London), **278**, 456 (1979).
15) R. J. Flower and G. J. Blackwell, *Nature* (London), **287**, 147 (1980).
16) F. Hirata, E. Schiffman and K. Venkatasubramanian, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2533 (1980).
17) L. Kaplan, J. Weiss and P. Elsbach, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 2955 (1978).
18) T. Fujiyoshi, H. Iida, M. Murakami and T. Uematsu, *Chem. Pharm. Bull.*, **37**, 771 (1989).
19) M. Katori, K. Ikeda, Y. Harada, Y. Uchida, K. Tanaka and S. Oh-ishi, *Agents Actions*, **8**, 108 (1978).
20) K. Yamaki, M. Dozen and S. Oh-ishi, Abstracts of Papers, 5th Kinin International Congress, Tokyo, Nov. 1987, p. 129C.