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European Journal of Pharmacology 329 (1997) 181–187

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Aceclofenac blocks prostaglandin E_2 production following its intracellular conversion into cyclooxygenase inhibitors

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Received 4 March 1997; revised 15 April 1997; accepted 22 April 1997

Abstract

Aceclofenac, 2-[(2,6-dichlorophenyl) amino] phenylacetoxyacetic acid, is a novel non-steroidal anti-inflammatory drug. We investigated the effects of aceclofenac on prostaglandin E_2 production by several kinds of human cells. Aceclofenac inhibited interleukin-1β-induced prostaglandin E_2 production by human rheumatoid synovial cells, but had no inhibitory effect on cyclooxygenase-1 or cyclooxygenase-2 activities by itself. We also observed that part of the aceclofenac was converted into diclofenac, the cyclooxygenase-1 and cyclooxygenase-2 inhibitor, when aceclofenac was incubated with human rheumatoid synovial cells. Aceclofenac was also converted into diclofenac and 4′-hydroxy diclofenac by human polymorphonuclear leukocytes and monocytes. 4′-Hydroxy diclofenac suppressed prostaglandin E_2 production specifically by blocking cyclooxygenase-2 activity. These findings suggested that aceclofenac can be metabolized to cyclooxygenase inhibitors (diclofenac and/or 4′-hydroxy diclofenac) by these inflammatory cells. Although detailed examinations in non-inflammatory cells remain to be studied, we concluded that aceclofenac is shown to be a new type of non-steroidal anti-inflammatory drug which is intracellulary converted into active metabolites that inhibit the prostaglandin E_2 production. © 1997 Elsevier Science B.V.

Keywords: Aceclofenac; Cyclooxygenase; Prostaglandin E_2; Polymorphonuclear leukocyte; Monocyte; Synovial cell

1. Introduction

Various kinds of arachidonic acid metabolites, such as prostaglandins, are important mediators of inflammation. Their synthesis is initiated by the release of arachidonic acid from the cell membrane, followed by its conversion into prostaglandin G_2 and H_2 which are metabolized by cyclooxygenase. There are two isoforms of cyclooxygenase (Xie et al., 1991; Kujubu et al., 1991). Cyclooxygenase-1 is constitutively expressed in gastric mucosa (Whittle et al., 1980), vesicular glands (Hemler et al., 1976; DeWitt and Smith, 1988) and platelets (Hammarström and Falardeau, 1977), and plays an important role in maintaining homeostasis. In contrast, the more recently discovered cyclooxygenase-2 was induced in inflammatory cells such as macrophages and rheumatoid synovial cells by stimuli including cytokines and lipopolysaccharide (Ristimäki et al., 1994; Hempel et al., 1994; Crofford et al., 1994), and its expression was inhibited by glucocorticoids (Masferrer et al., 1994b). These observations suggest that cyclooxygenase-2 plays a key role in controlling inflammation. Generally, non-steroidal anti-inflammatory drugs have been demonstrated to inhibit cyclooxygenase activity and to suppress the prostaglandin E_2 production by inflammatory cells such as rheumatoid synovial cells and monocytes (Vane, 1971). However, most non-steroidal anti-inflammatory drugs inhibit not only cyclooxygenase-2 activity at the sites of inflammation but also cyclooxygenase-1 in gastrointestinal tissues, which is not related to inflammation, resulting in the most common side effect of gastric damage (Mitchell et al., 1994; Rainsford, 1975). To reduce these side effects, prodrugs of cyclooxygenase inhibitor or selective inhibitors of cyclooxygenase-2 have been developed (Mishima et al., 1990; Copeland et al., 1994; Masferrer et al., 1994a).

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PII S0014-2999(97)00153-2

Aceclofenac, 2-[(2,6-dichlorophenyl) amino] phenylacetoxyacetic acid, is a novel non-steroidal anti-inflammatory drug developed in Spain (Grau et al., 1991). Aceclofenac has been used clinically in Spain and Portugal, and also in clinical studies in other European countries and Japan. It has shown marked therapeutic effects in rheumatoid arthritis and osteoarthritis (Torri et al., 1994; Ballesteros et al., 1990; Giorgianni et al., 1992), and a good level of tolerability (Bubani, 1988; Giorgianni et al., 1992; Pasero et al., 1994). Aceclofenac also reduced prostaglandin E_2 levels in the synovial fluid of patients with acute knee pain (Cecchettin et al., 1988) and inhibited prostaglandin E_2 production by blood polymorphonuclear leukocytes or mononuclear cells from patients with severe osteoarthritis (González et al., 1994). The structure of aceclofenac suggested that it would be a prodrug of diclofenac. However, the main metabolite of aceclofenac in humans is 4′-hydroxy aceclofenac, and the level of diclofenac, the expected active metabolite, was less than 1% (Yanagawa et al., 1993) or less than 6.7% (Bort et al., 1996) of the level of aceclofenac in human blood after treatment. Therefore, the mechanism of action of aceclofenac is unclear.

In the present study, we hypothesized that aceclofenac is metabolized to cyclooxygenase inhibitor at inflammatory sites and suppresses the prostaglandin E_2 production by inflammatory cells. We therefore investigated the correlation between the effects of aceclofenac and its metabolites on prostaglandin E_2 production, and examined the intracellular metabolism of aceclofenac in several kinds of human cells.

2. Materials and methods

2.1. Materials

Aceclofenac was chemically synthesized by Yakult (Tokyo, Japan). [^{14}C]Aceclofenac and [^{14}C]diclofenac were obtained from Amersham (Amersham, UK). Diclofenac sodium (diclofenac) and indomethacin were purchased from Sigma (St. Louis, MO, USA). 4′-Hydroxy aceclofenac and 4′-hydroxy diclofenac were chemically synthesized by Teikoku Hormone Manufacturing (Tokyo, Japan). These drugs were dissolved in dimethyl sulfoxide before use. Other materials were purchased from the following sources: sheep seminal vesicle microsomes (Eldan Technologies, Jerusalem, Israel); cyclooxygenase-2 isolated from sheep placenta (Cayman, Ann Arbor, MI, USA); arachidonic acid and collagenase type I (Sigma); Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline(−) (PBS(−)), SFM-101 and Hanks' balanced salt solution (Nissui Pharmaceutical, Tokyo, Japan); fetal calf serum (Boehringer-Mannheim, Mannheim, Germany); prostaglandin E_2 enzyme immunoassay kit (Cayman); interleukin-1β (Genzyme, Cambridge, MA, USA); RPMI1640 (Nikken Biomedical Laboratory, Kyoto, Japan); and Ficoll-Paque (Pharmacia, Uppsala, Sweden).

2.2. Assay of cyclooxygenage-1 and -2 activities

Two hundred micrograms of sheep seminal vesicular gland microsomes (cyclooxygenase-1) and 5 units of cyclooxygenase-2 were added to 100 μl of 50 mM Tris-HCl buffer (pH 8.0) containing epinephrine (1 mM) and phenol (2 mM) as cofactors. The reaction mixture was preincubated with various concentrations of aceclofenac, diclofenac, 4′-hydroxy aceclofenac, 4′-hydroxy diclofenac or indomethacin for 5 min at 37°C, and then 100 μM arachidonic acid was added. After incubation at 37°C for 5 min, 10 μl of $FeCl_2$ (22 mM) was added to the reaction mixture, and the preparation was centrifuged at $10000 \times g$ for 5 min. The prostaglandin E_2 content in the supernatant was measured using an enzyme immunoassay kit.

2.3. Culture of human rheumatoid synovial cells

Synovial tissue specimens from patients with rheumatoid arthritis were minced aseptically and digested for 2 h with 0.2% collagenase type I and for 2 h with 0.125% trypsin at 37°C in 5% CO_2. After digestion, the dissociated cells were collected by centrifugation at $500 \times g$ for 10 min, washed with DMEM with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 ng/ml fungizon (Gibco, Grand Island, NY, USA), resuspended in DMEM, and plated in 15 cm culture dishes. The cells were incubated at 37°C in 5% CO_2 for a few days, and non-adherent cells were removed. The adherent cells were used as rheumatoid synovial cells at about the third passage.

2.4. Assay of prostaglandin E_2 production by human rheumatoid synovial cells

The synovial cells were plated in 24-well culture plates at 10^5 cells/well and cultured at 37°C in 5% CO_2. When the cells reached confluency, The DMEM was exchanged for SFM-101 medium supplemented with 200 pg/ml interleukin-1β, 1% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and then various concentrations of aceclofenac, diclofenac, 4′-hydroxy aceclofenac, 4′-hydroxy diclofenac or indomethacin were added. After 24 h in culture at 37°C in 5% CO_2, the amount of prostaglandin E_2 in the culture medium was measured using an enzyme immunoassay kit.

2.5. Preparation of polymorphonuclear leukocytes and monocytes

Heparinized blood from healthy human donors was mixed with an equal volume of 3% dextran in 0.15 M

NaCl, and then allowed to sediment for 30 min at room temperature. The plasma fraction was layered over 15 ml of Ficoll-Paque, and then centrifuged at 380 × *g* for 30 min at room temperature. Polymorphonuclear leukocytes sedimented to the bottom of the tube and the mononuclear leukocyte layer were collected. Erythrocytes in the polymorphonuclear leukocytes sediment were removed by hypotonic lysis, and polymorphonuclear leukocytes were washed twice with Hanks' balanced salt solution. The cells in the mononuclear leukocyte layer were washed twice with PBS(−) at room temperature, resuspended in RPMI1640 medium with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin, and plated in 9 cm culture dishes. After 1 h of culture at 37°C in 5% CO_2, the adherent cells were used as monocytes. Isolated polymorphonuclear leukocytes and monocytes were resuspended in RPMI 1640 medium with 10% fetal bovine serum.

2.6. Assay of aceclofenac metabolism by inflammatory cells

[^{14}C]Aceclofenac (8.0 μM or 1.3 μM; 2.04 GBq/mmol) or [^{14}C]diclofenac (0.02 μM; 1.79 GBq/mmol) was added to cells suspended in RPMI1640 medium or SFM-101 medium at 2.94×10^6 cells/ml or 4×10^5 cells/ml, respectively. After culture at 37°C in 5% CO_2, the cells were washed 3 times with cold PBS(−). To extract [^{14}C]aceclofenac and its metabolites, the cells were ruptured by the addition of 1 ml of acetone, or 1 ml of 0.85 M phosphate solution and 4 ml of hexane/isopropanol (9:1). After centrifugation at 1500 or 2000 × *g* for 10 min, the supernatant was evaporated under a stream of N_2 gas. The residue was then dissolved in acetone or methanol and separated on Silica gel 60 Å (Whatman International, Maidstone, UK) using a solvent system of benzene/acetate ester/acetic acid (18:1:1) or methanol/chloroform/acetic acid (15:85:0.05), respectively, with authentic aceclofenac, diclofenac, 4′-hydroxy acecofenac and 4′-hydroxy diclofenac. After development, the positions of aceclofenac and its metabolites were detected by UV or autoradiography. The parts of the thin layer plates containing aceclofenac and its metabolites were scraped off, and their radioactivities were counted with a liquid scintillation counter (Aloka, Tokyo, Japan).

In some experiments, a high-performance liquid chromatography (HPLC) analysis was performed with an LC-Module 1 (Waters, Milford, MA, USA) with a J'spere ODS-M80 4.6 × 150 mm column (YMC, Kyoto, Japan). The mobile phase, trifluoroacetic acid/acetonitrile (41:59), was delivered at 1.5 ml/min. *p*-Hydroxybenzoic acid was used as the internal standard. Metabolites were detected by ultraviolet adsorption at 276 nm and identified on the basis of their retention times.

3. Results

3.1. Effects of aceclofenac and its metabolites on cyclooxygenase-1 and -2 activities

The main metabolites of aceclofenac in human blood were diclofenac, 4′-hydroxy aceclofenac and 4′-hydroxy diclofenac (Yanagawa et al., 1993; Bort et al., 1996) (Fig. 1). We investigated the effects of these compounds on cyclooxygenase-1 and cyclooxygenase-2 activities. As shown in Fig. 2, aceclofenac and 4′-hydroxy aceclofenac had no inhibitory effect on either cyclooxygenase activity. In contrast, diclofenac inhibited both cyclooxygenase-1 (IC_{50} 3.6 μM) and cyclooxygenase-2 (IC_{50} 0.14 μM) activities. 4′-Hydroxy diclofenac also inhibited cyclooxygenase-2 activity (IC_{50} 2.8 μM) but had no inhibitory effect on that of cyclooxygenase-1. Indomethacin, used as a control, inhibited both cyclooxygenase-1 (IC_{50} 3.4 μM) and cyclooxygenase-2 (IC_{50} 0.97 μM) activities in this system.

3.2. Effects of aceclofenac and its metabolites on interleukin-1β-induced prostaglandin E_2 production by human rheumatoid synovial cells

We investigated the effects of aceclofenac and its metabolites in human blood on interleukin-1β-induced prostaglandin E_2 production by synovial cells from patient with rheumatoid arthritis (Fig. 3). Aceclofenac inhibited prostaglandin E_2 production with an IC_{50} value of 0.051 μM. 4′-Hydroxy aceclofenac also inhibited prostaglandin E_2 production (IC_{50} 0.73 μM), and its effect was weaker than that of aceclofenac. Diclofenac and 4′-hydroxy diclofenac strongly inhibited prostaglandin E_2 production (IC_{50} 0.0015 and 0.0068 μM, respectively). Indomethacin,

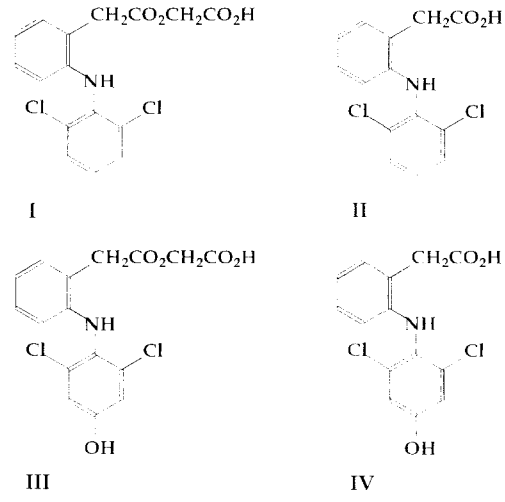

Fig. 1. The structures of aceclofenac and its metabolites in human blood. I, aceclofenac; II, diclofenac; III, 4′-hydroxy aceclofenac; IV, 4′-hydroxy diclofenac.

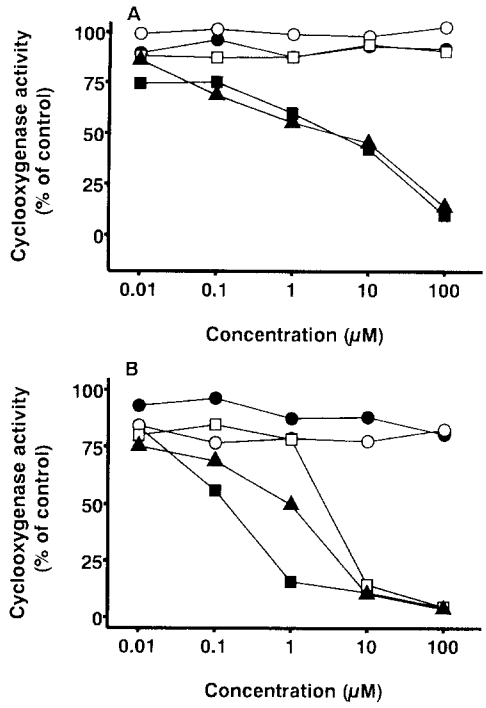

Fig. 2. Effects of aceclofenac and its metabolites on cyclooxygenase-1 (A) and cyclooxygenase-2 (B) activities. Cyclooxygenase-1 or -2 was added to 50 mM Tris-HCl buffer (pH 8.0) containing epinephrine (1 mM) and phenol (2 mM) as cofactors. The reaction mixture was preincubated with aceclofenac (●), diclofenac (■), 4′-hydroxy aceclofenac (○), 4′-hydroxy diclofenac (□) or indomethacin (▲) for 5 min at 37°C, and then 100 μM arachidonic acid was added. After incubation at 37°C for 5 min, the prostaglandin E_2 content was measured by enzyme immunoassay. Data represent the means of duplicate determinations.

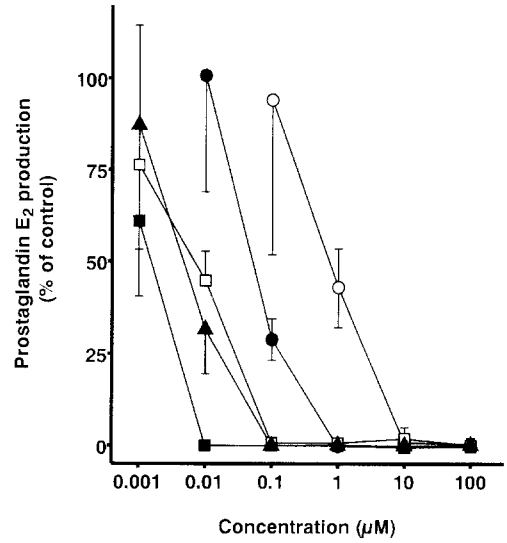

Fig. 3. Effects of aceclofenac and its metabolites on interleukin-1β-induced prostaglandin E_2 production by human rheumatoid synovial cells. Human rheumatoid synovial cells (10^5 cells/ml) were plated in culture medium containing interleukin-1β (200 pg/ml), and then aceclofenac (●), diclofenac (■), 4′-hydroxy aceclofenac (○), 4′-hydroxy diclofenac (□) or indomethacin (▲) was added. After 24 h in culture, the amount of prostaglandin E_2 in the culture medium was measured by enzyme immunoassay. Data represent the means ± S.D. of triplicate cultures.

used as a control, inhibited prostaglandin E_2 production (IC_{50} 0.0047 μM).

3.3. Metabolism of aceclofenac in inflammatory cells

We examined the metabolism of [^{14}C]aceclofenac in polymorphonuclear leukocytes, monocytes from healthy human donors and in synovial cells from patients with rheumatoid arthritis by thin layer chromatography (TLC). As shown in Fig. 4, [^{14}C]aceclofenac in polymorphonuclear leukocytes was metabolized to diclofenac, 4′-hydroxy diclofenac and 4′-hydroxy aceclofenac time dependently, whereas other metabolites were not detected by this system. In addition, the spontaneous degradation of [^{14}C]aceclofenac in the medium was negligible (Fig. 4). After an 18 h culture of polymorphonuclear leukocytes with [^{14}C]aceclofenac, 5.7% and 5.8% of the total radioactivity in the cells were attributable to diclofenac and 4′-hydroxy diclofenac, respectively. In this condition, 0.4% and 3.8% of total radioactivity in the culture medium were attributable to diclofenac and 4′-hydroxy diclofenac, respectively. The amount of conversion of [^{14}C]aceclofenac into each metabolite by monocytes was similar to that of polymorphonuclear leukocytes (Table 1).

Table 1
Metabolism of aceclofenac by PMNs or monocytes

	Untreated [a]	PMNs		Monocytes	
	in medium	in cells	in medium	in cells	in medium
Aceclofenac	97.3	77.7	90.4	79.4	90.1
Diclofenac	0.2	5.7	0.4	6.4	0.8
4′-Hydroxy aceclofenac	1.4	3.4	2.0	2.3	1.4
4′-Hydroxy diclofenac	0.5	5.8	3.8	7.8	4.3

Polymorphonuclear leukocytes (PMNs) or monocytes were plated at a density of 2.94×10^7 cells/dish in culture medium. After 18 h in culture with [^{14}C]aceclofenac (8 μM), [^{14}C]aceclofenac and its metabolites were extracted from cells or medium and determined by thin-layer chromatography.
[a] Culture without cells. Values are the percentages of total radioactivity in medium or in cells.

Table 2
Aceclofenac and its metabolites in human rheumatoid synovial cells

	Aceclofenac			Diclofenac
	in cells		in medium	in cells
	pmol/10^7 cells	% [a]	% [b]	pmol/10^7 cells
Aceclofenac	130.4	89.1	92.0	– [c]
Diclofenac	4.9	3.4	6.4	1.7
4′-Hydroxy aceclofenac	3.4	2.4	1.1	–
4′-Hydroxy diclofenac	0.9	0.6	0.1	0.1

Human rheumatoid synovial cells were plated at a density of 1.6×10^7 cells/dish in culture medium. After 24 h in culture with [^{14}C]aceclofenac (1.3 μM) or [^{14}C]diclofenac (0.02 μM), and IL-1 (200 pg/ml), [^{14}C]aceclofenac, [^{14}C]diclofenac and their metabolites were extracted from cells or medium and determined by thin layer chromatography.
[a] Percentage of total radioactivity in cells.
[b] Percentage of total radioactivity in medium.
[c] Not determined.

Rheumatoid synovial cells also metabolized [^{14}C]aceclofenac to diclofenac (Table 2). When rheumatoid synovial cells were incubated with 1.3 μM of aceclofenac, a concentration which was enough to inhibit interleukin-1β-induced prostaglandin E_2 production completely (Fig. 3), 4.9 pmol of diclofenac was detected in 10^7 cells. In contrast, when rheumatoid synovial cells were incubated with 0.02 μM diclofenac, a concentration which was enough to inhibit interleukin-1β-induced prostaglandin E_2 production completely (Fig. 3), 1.7 pmol of diclofenac was detected in 10^7 cells.

We compared the conversion of aceclofenac in human normal dermal fibroblasts as non-inflammatory cells with that in rheumatoid synovial cells using HPLC. The cells were cultured with 10 μM of aceclofenac for 4 h. After the culture, 0.1 μM of diclofenac (1.0% of aceclofenac) was detected in the culture medium of the normal dermal fibroblasts. We found that 0.7% of the aceclofenac in the medium was degraded to diclofenac spontaneously in this condition. In contrast, 2.3% of aceclofenac in the culture medium was converted into diclofenac by rheumatoid synovial cells after 4 h in culture.

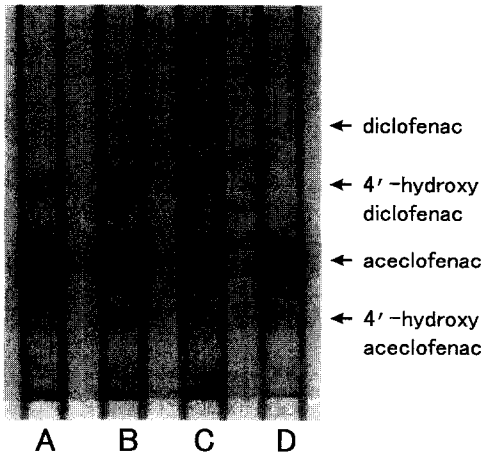

Fig. 4. Identification of [^{14}C]aceclofenac and its metabolites by TLC. Radioactive products were detected by autoradiography as described in Section 2. (A,B,C) Radioactive products after 5 min, 4 h and 24 h in culture of polymorphonuclear leukocytes with 8 μM [^{14}C]aceclofenac, respectively. (D) Radioactive products after the incubation of 8 μM [^{14}C]aceclofenac without cells.

4. Discussion

We investigated the effects of aceclofenac and its metabolites in human blood on cyclooxygenase-1 and cyclooxygenase-2 activities. Aceclofenac and 4′-hydroxy aceclofenac had no inhibitory effect on either cyclooxygenase activity. Diclofenac and 4′-hydroxy diclofenac inhibited cyclooxygenase-2 activity. 4′-Hydroxy diclofenac had no inhibitory effect on cyclooxygenase-1 activity. Aceclofenac and its metabolites also had no inhibitory effect on phospholipase A_2 activity (data not shown). These observations suggested that if the main mechanism of the anti-inflammatory effect of aceclofenac was due to the inhibition of the prostaglandin E_2 production similarly to other general non-steroidal anti-inflammatory drugs, it was necessary for aceclofenac to be metabolized to diclofenac or 4′-hydroxy diclofenac both of which inhibit cyclooxygenase-2 activity.

In this case, aceclofenac was not the normal prodrug because it shows little hydrolysis to diclofenac; the level of diclofenac is less than 1% (Yanagawa et al., 1993) or less than 6.7% (Bort et al., 1996) of the level of aceclofenac in human blood after treatment. We therefore investigated the effects of aceclofenac and its metabolites on prostaglandin E_2 production, and examined the intracellular metabolism of aceclofenac in several kinds of human cells.

Synovial cells from patients with rheumatoid arthritis have been reported to produce high levels of prostaglandin E_2 (Dayer et al., 1976; Mizel et al., 1981), and the prostaglandin E_2 production was increased by stimulation with interleukin-1β (Crofford et al., 1994; Hulkower et al., 1994) and by tumor necrosis factor α (Dayer et al., 1985). Since aceclofenac has shown marked therapeutic efficacy in rheumatoid arthritis (Ballesteros et al., 1990; Giorgianni

et al., 1992), we examined the effects of aceclofenac and its metabolites on interleukin-1β-induced prostaglandin E_2 production by rheumatoid synovial cells. Aceclofenac markedly inhibited the prostaglandin E_2 production, although aceclofenac itself had no inhibitory effect on either cyclooxygenase activity, as mentioned above.

We next investigated the intracellular metabolism of aceclofenac in primary culture systems. Our findings by TLC analysis demonstrated that aceclofenac was partially converted into the cyclooxygenase inhibitor diclofenac, when incubated with rheumatoid synovial cells. The concentrations of aceclofenac used for investigating the intracellular metabolism and the amount of diclofenac produced were enough to completely suppress the interleukin-1β-induced prostaglandin E_2 production by rheumatoid synovial cells. In addition, the plasma maximum concentration of aceclofenac in humans is 18–25 μM (Bort et al., 1996). Thus, we suggest that aceclofenac inhibits prostaglandin E_2 production by an intracellular metabolism in rheumatoid synovial cells.

In the same way, aceclofenac was converted into diclofenac and 4′-hydroxy diclofenac by other human cells, polymorphonuclear leukocytes and monocytes. Aceclofenac has also been reported to inhibit prostaglandin E_2 production by blood polymorphonuclear leukocytes and mononuclear cells from patients with severe osteoarthritis (González et al., 1994). Taken together, these observations suggest that the inhibitory effect of aceclofenac was due to its metabolites in these inflammatory cells.

We also examined the metabolism of aceclofenac by human normal dermal fibroblasts. The conversion of aceclofenac into diclofenac by dermal fibroblasts was 1.0%, whereas the spontaneous degradation of aceclofenac to diclofenac was 0.7%. These results suggest that the conversion of aceclofenac was different among the cell types. However, these findings are preliminary, and further study is necessary.

We demonstrated that aceclofenac has the potential to inhibit prostaglandin E_2 production via its intracellular metabolites diclofenac and 4′-hydroxy diclofenac. 4′-Hydroxy diclofenac generated from aceclofenac suppressed prostaglandin E_2 production specifically by blocking cyclooxygenase-2 activity. Selective inhibitors of cyclooxygenase-2 have attracted attention for their clinical potential in treating inflammation-related diseases such as rheumatoid arthritis. 4′-Hydroxy diclofenac may selectively inhibit cyclooxygenase-2 activity intracellularly. These findings suggested that aceclofenac is a new type of non-steroidal anti-inflammatory drug which blocks prostaglandin E_2 production following its intracellular conversion into cyclooxygenase inhibitors.

Acknowledgements

We wish to thank Drs Shinichi Nishida, Koji Nomoto and Shuichi Tanabe for valuable discussions, and Tsuneo Matsumoto and Kasumi Nagai for excellent technical assistance.

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