Purification and Partial Characterization of an Indomethacin Hydrolyzing Enzyme from Pig Liver

Keisuke Terashima,[1] Satomi Takai,[1] Yoshiko Usami,[1] Tetsuo Adachi,[1] Tadashi Sugiyama,[2] Yoshihiro Katagiri,[2] and Kazuyuki Hirano[1,3]

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Purpose. Indomethacin is well known to be metabolized via *O*-demethylation and *N*-deacylation. In this paper we found an enzyme involved in the hydrolysis of amide-linkage of indomethacin and partially characterized it as well as its substrate specificity.

Methods. An indomethacin hydrolyzing enzyme was purified to homogeneity from pig liver microsomes using columns of Q-Sepharose, Red-Sepharose and Blue-Sepharose. The enzyme activity was assayed by measuring of p-chlorobenzoic acid liberated from indomethacin by HPLC.

Results. The purified enzyme effectively hydrolyzed the amide linkage in indomethacin but not those in α-naphthylacetate and p-nitrophenylacetate, which are typical substrates for carboxylesterase. The subunit molecular mass of the enzyme was 65 kDa according SDS-polyacrylamide gel electrophoresis. The Michaelis constant (Km) and maximum velocity (Vmax) values for indomethacin were 67.8 μM and 9.02 nmol/min/mg protein, respectively. The amino acid sequence analysis of the enzyme after cyanogen bromide cleavage showed high homology with a mouse carboxylesterase isozyme designated as ES-male. The activity of indomethacin hydrolysis was relatively high in the pig, rabbit and human liver homogenate, but not in those from rat and mouse. On the other hand, purified human liver carboxylesterases pI 5.3 and 4.5, and pig liver carboxylesterases have no catalytic activity for indomethacin.

Conclusions. These results indicate that the hydrolysis of amide-linkage of indomethacin in humans would be associated with an enzyme similar to the indomethacin hydrolyzing enzyme from pig liver microsomes described here.

KEY WORDS: indomethacin hydrolyzing enzyme; carboxylesterase; indomethacin; aniracetam.

INTRODUCTION

Indomethacin (1-(p-chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid) is widely applied as anti-inflammatory and antipyretic agent in adults and as an alternative to surgical ligation of the ductus in treating of patent ductus arteriosus in the premature infant. Indomethacin as well as aspirin is a strong inhibitor of cyclooxygenase (COX) which is the rate limiting enzyme in the production of prostaglandins in the arachidonic cascade (1). The nonsteroidal drug, indomethacin, has several toxic side-effects upon intestinal and gastric mucosa. Indomethacin inhibits both cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) induced in inflamed tissues. Gastrointestinal ulcers caused by indomethacin are probably due to the inhibition of COX-1 (2). Indomethacin undergoes extensive biodegradation via *O*-desmethylation, *N*-deacylation or both (3–5). The respective products of these reactions, desmethyl indomethacin (DMI), deschlorobenzoyl indomethacin and desmethyldeschlorobenzoyl indomethacin have no antiinflammatory activity (6) and are excreted in the urine and bile in the free form and as conjugates. DMI is formed by *O*-desmethylation through the cytochrome P-450 microsomal pathway and is critical to the elimination of indomethacin. In adult male humans, it represents 40–55% of the total drug eliminated in the urine (6).

It has become increasingly important to identify the mechanisms that modulate drug disposition during development to help provide a better prediction of the pharmacological disposition of a given drug and to reduce side effects. In this study, we found an enzyme involved in the hydrolysis of amide-linkage of indomethacin and partially characterized it as well as its substrate specificity.

MATERIALS AND METHODS

Materials

Q-Sepharose Fast Flow and Blue-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), Red-Sepharose was obtained from Kurabo Co. Ltd. (Tokyo, Japan) and α-naphthylacetate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Indomethacin was supplied by Sumitomo Pharmaceutical Co. Ltd. (Osaka, Japan), aniracetam was from Toyamakagaku Co. Ltd. (Tokyo, Japan), and n-propyl-p-hydroxybenzoate as well as ethyl p-hydroxybenzoate were purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Lubrol was from Nacalai Tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade.

Assay of Indomethacin Hydrolyzing Activity

The enzyme activity was assayed at 37°C by measuring the amount of p-chlorobenzoic acid liberated from indomethacin. One hundred microliters of 100 mM Tris-HCl buffer (pH 8.0) and 90 μl of the enzyme solution in 20 mM Tris-HCl buffer (pH 7.4) were mixed and incubated at 37°C for 5 min. The enzyme reaction was initiated by adding 10 μl of 500 μM indomethacin dissolved in ethanol and incubated at 37°C for 1 h. The reaction was terminated with 2 ml of methanol containing 0.555 μM n-propyl-p-hydroxybenzoate as an internal standard. The mixture was centrifuged at 600 × g for 10 min, then the supernatant was evaporated under reduced pressure at 37°C. The residue was dissolved in 200 μl of the HPLC mobile phase and aliquots (40 μl) of the filtrate were applied to reverse phase HPLC (Capcell pak C_{18}, Shiseido, 4.6 × 150 mm) using a mobile phase composed of acetonitrile-0.5% (v/v) phosphoric acid (60:40) at a flow rate of 0.5 ml per min at room temperature. Elution was monitored at 245 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 nmol of p-chlorobenzoic acid from indomethacin per min.

[1] Department of Pharmaceutics Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502, Japan.

[2] Department of Pharmacy, Gifu University Hospital, 40 Tsukasa-machi, Gifu 500, Japan.

[3] To whom correspondence should be addressed.

ABBREVIATIONS: Km: Michaelis constant; Vmax: maximum velocity; Ki: inhibitor constant; COX: cyclooxygenase; TFA: trifluoroacetic acid.

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Hydrolysis of Aniracetam

Twenty microliters of enzyme and 20 μl of 500 μM aniracetam in 0.1 M Tris-HCl buffer (pH 8.0) were mixed and incubated at 37°C for 1 h. The reaction was terminated by adding 100 μl of acetonitrile and mixed with 200 μl of 10 μM ethyl p-hydroxybenzoate in H_2O as an internal standard. Samples (40 μl) were applied to reverse phase HPLC (Develosil ODS-5, 4.6 × 250 mm) using acetonitrile-20 mM trichloroacetic acid (30:70) at a flow rate of 1.5 ml per min at 50°C. Elution was monitored at 254 nm.

Assay of Esterase Activity

Hydrolysis of α-naphthylacetate

The substrate consisted of 2 mM 4-aminoantipyrine, 0.05% Triton X-100 and 1.3 mM α-naphthylacetate in 50 mM Tris-HCl buffer (pH 7.5). Substrate (2 ml) and 10 μl of the enzyme were mixed and incubated at 37°C for 10 min. The enzyme reaction was stopped with 2 ml of 3 mM potassium ferricyanide containing 130 mM boric acid. The absorbance at 500 nm was measured (7).

Hydrolysis of p-nitrophenylacetate

One hundred microliters of 1 M Tris-HCl buffer (pH 8.0) and 100 μl of enzyme were added to 1.8 ml of 1 mM p-nitrophenyl acetate. The reaction rate was determined by measuring the absorbance at 405 nm at 30°C (8).

Determination of Protein

The protein concentration was determined by measuring the absorbance at 280 nm.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were resolved on 9% polyacrylamide gels according to Laemmli (9). The protein was stained with Coomassie Brilliant blue R 250.

Preparation of a Polyclonal Antibody Against Indomethacin Hydrolyzing Enzyme

Rabbit foot pads were injected with 100 μg of purified enzyme in phosphate-buffered saline emulsified in complete Freund's adjuvant. Thereafter, the animal was given three subcutaneous injections at ten day intervals. Antiserum was purified by means of DEAE-cellulose column chromatography after heating at 56°C for 30 min.

Analysis of Amino Acid Sequence

The purified enzyme (100 μg) was dialyzed against distilled water, lyophilized, then cleaved with cyanogen bromide in 70% formic acid at room temperature for 24 h. The reaction mixture was diluted with a 9 fold volume of distilled water and lyophilized. The residue was dissolved in 0.1% trifluoroacetic acid (TFA), applied to a reversed-phase HPLC column (ULTRON 300 C_4, 4.6 mm × 15 cm), and eluted with a linear gradient of 0–60% acetonitrile in 0.1% TFA. Ultraviolet absorption was monitored at 210 nm. The N-terminal amino acid-sequences the of cleaved peptides were analyzed using an Applied Biosystems 473A protein sequencer.

Table 1. Indomethacin Hydrolyzing Activity in Liver Homogenate

Species	Activity (unit/mg protein)
Pig	0.629×10^{-3}
Rabbit	0.880×10^{-3}
Human	0.170×10^{-3}
Rat	N.D.[a]
Mouse	N.D.

[a] N.D.: not detected.

RESULTS AND DISCUSSION

Indomethacin Hydrolyzing Activity in the Mammalian Liver

Indomethacin hydrolyzing activity at the amide-linkage was determined in the liver homogenates. The activity of indomethacin hydrolysis was relatively high in the pig, rabbit and human livers but not in those from rat and mouse, as shown in Table 1. The activity was distributed in the microsomal fraction.

Purification of an Indomethacin Hydrolyzing Enzyme from Pig Liver Microsomes

All procedures were performed at 4°C. Fresh pig liver (40 g) was homogenized in 160 ml of 1.15% KCl at 4°C. After centrifugation at 9,000 × g for 20 min, the supernatant was centrifuged at 105,000 × g for 60 min. The precipitate was suspended in 40 ml of 20 mM Tris-HCl buffer (pH 7.4) mixed with 10 ml of 2.0% (w/v) Lubrol and stirred at 4°C overnight. The mixture was centrifuged at 105,000 × g for 60 min and the supernatant was extensively dialyzed against 20 mM Tris-HCl buffer (pH 7.4) then applied to a column (1.7 × 7.0 cm) of Q-Sepharose Fast Flow equilibrated with 20 mM Tris-HCl buffer (pH 7.4). After washing with the above buffer, the enzyme was eluted with a linear gradient of NaCl up to a concentration of 0.5 M as shown in Fig. 1. The indomethacin hydrolyzing enzyme eluted just before esterase, indicating that this enzyme is not the known carboxylesterase. The active fractions were dialyzed against 20 mM Tris-HCl buffer (pH 7.4), then applied to a column (0.8 × 4.0 cm) containing Red-Sepharose equilibrated with the above buffer. The pass-through fractions were pooled and applied to a column (2.5 × 1.7 cm) containing Blue-Sepharose equilibrated with the

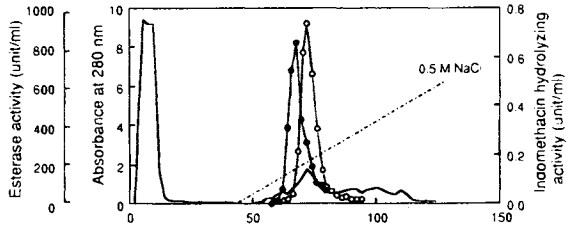

Fig. 1. Chromatography of the indomethacin hydrolyzing enzyme from pig liver. Crude enzyme in 20 mM Tris-HCl buffer, pH 7.4, was applied to a column of Q-Sepharose Fast Flow (1.7 × 7.0 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0–0.5M) in the buffer. Solid and broken lines indicate the absorbance at 280 nm and the NaCl concentration, respectively. Closed and open circles indicate indomethacin hydrolyzing activity (unit/ml) and esterase activity for α-naphthylacetate (unit/ml), respectively.

Table 2. Purification of Indomethacin Hydrolyzing Enzyme from Pig Liver

	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude enzyme (after Lubrol treatment)	807	15.3	0.0190	100
Q-Sepharose Fast Flow	37.0	28.6	0.774	187
Blue-Sepharose	0.525	0.900	1.71	5.87

above buffer. The column was washed extensively with the same buffer containing 0.5 M NaCl, then the enzyme was eluted with the same buffer containing 2.0 M NaCl. The binding affinity of the indomethacin hydrolyzing enzyme to the column was high and a high concentration of NaCl was required to elute it. The active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.4) overnight. The purification procedure is summarized in Table 2. The enzyme was purified about 90-fold with an activity recovery of 5.87%. The yield of the purified enzyme was low, because it is unstable. We therefore developed a faster purification procedure using affinity column chromatography. An indomethacin hydrolyzing enzyme was easily dissolved by 0.4% Lubrol from microsomes, however the activity was inhibited by this detergent. The rise of yield at Q-Sepharose column chromatography may be due to the removal of Lubrol.

As shown in Fig. 2, the purified enzyme migrated as a single protein band with a molecular mass of 65 kDa on SDS-PAGE and formed a single precipitin line against rabbit anti-indomethacin-hydrolyzing enzyme antibody. Concomitantly, the antibody did not produce a precipitin line with the pig liver carboxylesterase known as proline-β-naphthylamidase (10).

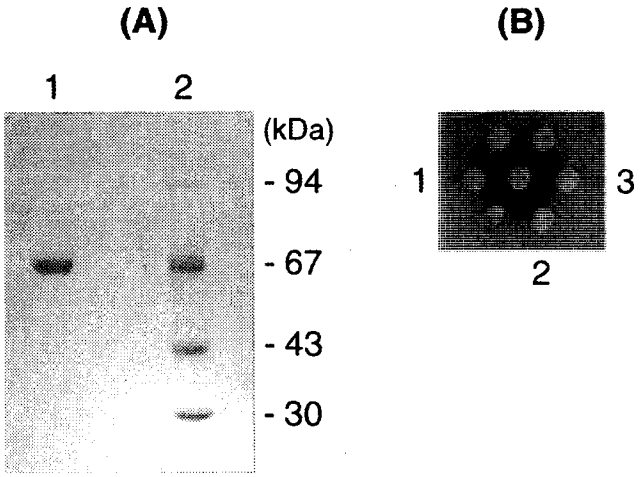

Fig. 2. SDS-PAGE (A) and Ouchterlony (B) of indomethacin hydrolyzing enzyme from pig liver. (A) Lane 1, purified indomethacin hydrolyzing enzyme; lane 2, standard proteins. (B) Center well, rabbit anti-indomethacin hydrolyzing enzyme antibody; wells 1 and 2, purified indomethacin hydrolyzing enzyme; well 3, carboxylesterase purified from pig liver.

Some Properties of the Purified Indomethacin Hydrolyzing Enzyme

Table 3 summarizes the properties of indomethacin hydrolyzing enzyme from pig liver. The enzyme was inhibited by protease inhibitors such as bis-nitrophenyl phosphate (BNPP) and phenylmethyl sulfonyl fluoride (PMSF) but not by EDTA, suggesting that a serine residue is essential for its activity. This enzyme liberated p-chlorobenzoic acid from indomethacin with a Km of 67.8 μM and a Vmax of 9.02 nmol/min/mg protein. The purified enzyme also catalyzed the hydrolysis of the amide-linkage of pyrrolidone in aniracetam with Km and Vmax values of 560 μM and 7.69 nmol/min/mg protein, respectively.

The enzyme had no activity towards α-naphthylacetate and p-nitrophenylacetate, which are typical substrates for carboxylesterase. The hydrolysis of commercially available drugs with an ester- or an amido-linkage in their skeletons was also investigated. The listed drugs except for indomethacin and aniracetam, were not good substrates for this enzyme.

Substrate Specificity

The indomethacin hydrolyzing enzyme hydrolyzed amide-linkages in the pyrrolidone ring of aniracetam to produce anisamidobutyric acid, but not amide-linkages outside the ring. On the other hand, carboxylesterase from pig liver microsome hydrolyzed two amide-linkages in aniracetam to produce anisilic acid and anisamidobutyric acid at a ratio of 2:1 (Fig. 3). The indomethacin hydrolyzing activity was slightly inhibited by aniracetam with a Ki of >500 μM and the activity for aniracetam was competitively inhibited by indomethacin with a Ki of 46.4 μM. The specificity of indomethacin hydrolyzing enzyme is considered to be high compared with that of the carboxylesterase in pig liver.

Table 3. Some Properties of Indomethacin Hydrolyzing Enzyme from Pig Liver

Optimum pH[a]	9.0
pH stability[b]	9.0–11.0
Molecular weight (kDa)	65 (SDS-PAGE)
Inhibitor (1 mM)	**Remaining activity (%)**
BNPP	0
PMSF	0
EDTA	100
Substrate specificity[c]	
α-Naphthyl acetate	—
p-Nitrophenylacetate	—
Indomethacin	+
Aniracetam	+
Nitrazepam	—
Prazosin	—
Fominoben	—
Tiaramide	—
Flutamide	—

[a] For optimum pH analysis, indomethacin hydrolysing activity was determined in 0.1 M buffers with various pH at 37°C for 60 min.
[b] For pH stability analysis, enzyme was incubated in 30 mM buffers with various pH at 30°C for 30 min, and then the remaining activity was determined.
[c] +: hydrolyzed; −: not hydrolyzed. BNPP: bis-nitrophenyl phosphate; PMSF: phenylmethyl-sulfonyl fluoride.

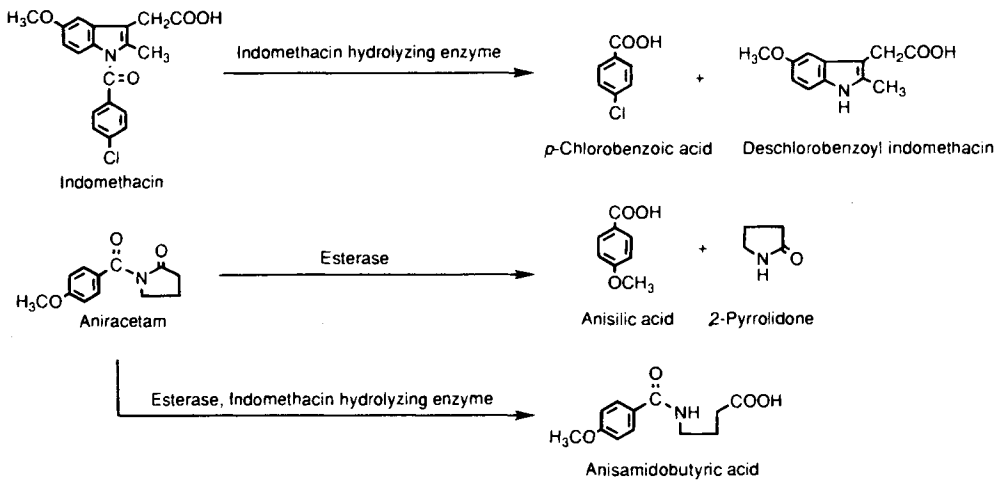

Fig. 3. Hydrolysis of indomethacin and aniracetam by indomethacin hydrolyzing enzyme from pig liver.

Analysis of Amino Acid Sequence

After cyanogen bromide cleavage of indomethacin hydrolyzing enzyme, we isolated two peptide fractions on a C_4 reverse phase HPLC column. The N-terminal sequences (sequences 1 and 2 of 20 amino acids each) of the two peptides were analyzed. The results revealed 70.0 and 55.6% homology with the partial amino acid sequences Pro15-Val34 and Ala481-Trp500 of the mouse carboxylesterase isozyme Es-male (11). Nevertheless, we could not detect indomethacin hydrolyzing activity in the mouse liver, as shown in Table 1. These findings suggested that the indomethacin hydrolyzing enzyme presented here is novel and not a known pig carboxylesterase. However, it may belong to the carboxylesterase superfamily. The substrate specificity shown in Table 3 supported this notion.

Carboxylesterases from the liver and intestinal mucosa of human and other animals (12) have been extensively characterized. Ester-type prodrugs have been developed to increase the bioavailability and the prodrug is generally biotransformed by carboxylesterase to release the mother drug (13–15). However, the indomethacin hydrolyzing enzyme may not contribute to this step because ester-type prodrugs were not suitable substrates for this enzyme on the basis of specificity. Concomitantly, the purified enzyme hydrolyzed indomethacin and aniracetam. Indomethacin is metabolized in humans to the desmethyl and desbenzoyl derivatives (6). Human liver carboxylesterases pI 5.3 and 4.5, rat liver carboxylesterases pI 6.0 and 6.2, and pig liver carboxylesterases have no catalytic activity for indomethacin (data not shown), whereas indomethacin hydrolysing activity was detected in pig and human liver homogenate. These results indicated that the production of the desbenzoyl derivative from indomethacin in humans is associated with an enzyme similar to the indomethacin hydrolyzing enzyme from pig liver microsomes described here.

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