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Identification of an isoform catalyzing the CoA conjugation of nonsteroidal anti-inflammatory drugs and the evaluation of the expression levels of acyl-CoA synthetases in the human liver

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) containing carboxylic acid are conjugated with coenzyme A (CoA) or glucuronic acid in the body. It has been suggested that these conjugates are associated with toxicities, such as liver injury and anaphylaxis, through their binding via trans-acylation to cellular proteins. Although studies on glucuronidation have progressed, studies on CoA conjugation of drugs catalyzed by acyl-CoA synthetase (ACS) enzymes are still in the early stages. This study aimed to clarify the human ACS isoforms responsible for CoA-conjugation of NSAIDs through consideration of the hepatic expression levels of ACS isoforms. We found that among 10 types of NSAIDs, propionic acid-class NSAIDs, namely, alminoprofen, flurbiprofen, ibuprofen, ketoprofen, and loxoprofen, were conjugated with CoA in the human liver, whereas NSAIDs in the other classes, including diclofenac and mefenamic acid, were not. qRT-PCR revealed that among the 26 ACS isoforms, ACSL1 was the most highly expressed in the human liver, followed by ACSM2B. The propionic acid-class NSAIDs were conjugated with CoA by recombinant human ACSL1. The protein binding abilities of the CoA conjugates and the glucuronide forms of propionic acid-class NSAIDs were compared as an index of toxicity. The CoA conjugates had stronger adduct formation with liver microsomal proteins than glucuronides for all 5 propionic acid-class NSAIDs. In conclusion, we found that propionic acid-class NSAIDs could be conjugated to CoA by ACSL1 in the human liver to form CoA conjugates, which likely cause toxicity by protein adduct formation.

Keywords: CoA conjugation, Acyl-glucuronide, Acyl-CoA synthetase, NSAID, Protein binding

1. Introduction

Drugs containing carboxylic acids have been suggested to cause various toxicities, such as anaphylaxis, liver injury, and Stevens-Johnson syndrome (SJS) [1]. It is well known that they are metabolized to acyl-glucuronides, which are likely related to their toxicities because of their instability and tendency to bind to biomolecules [2]. The relevance of acyl-glucuronides to their toxicities has not been fully and experimentally proven, but it has been demonstrated that their instability is highly correlated with the degree of toxicity of the parent compounds [3].

Drugs containing carboxylic acids are also metabolized to acyl-CoA conjugates in addition to acyl-glucuronides. Acyl-CoA conjugation is a thioesterification two-step reaction as follows [4]: (1) the carboxylate ion is replaced with ATP to form acyl adenylate, and then (2) nucleophilic attack of CoA on the carbonyl carbon releases AMP to form a thioester bond with the substrate. These reactions are catalyzed by acyl-CoA synthetase (ACS), which consists of 26 isoforms in humans that are categorized into six groups: short-chain family (ACSS), medium-chain family (ACSM), long-chain family (ACSL), very long-chain family (ACSVL), bubblegum family (ACSBG), and other family (ACSF) [5]. The ACS isoforms are localized in various organelles in cells. For example, ACSS and ACSM are mainly localized in mitochondria, ACSL and ACSVL are mainly localized in the endoplasmic reticulum system, and ACSBG and ACSF are mainly localized in the cytoplasm [5,6,7]. The ACSS, ACSM, ACSL, and ACSVL families are classified by their substrate selectivity, which is based on the length of the carbon chain of the substrate fatty acids C2-4, C4-10, C12-20, and C18-26, respectively. ACSBG is the human ortholog of the bubblegum gene expressed in the Drosophila optic lobe, and the ACSF family deviates from the above five families by phylogenetic tree analysis based on amino acid sequences [4,5]. CoA conjugation in lipid metabolism has been extensively studied, but CoA conjugation of drugs is still incompletely understood.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of pain in various parts of the body, such as the bones, joints, and muscles [8]. NSAIDs often cause

gastrointestinal injury and rarely cause anaphylaxis, SJS, or liver injury as side effects. Suggested possible mechanisms to cause these side effects are the accumulation of arachidonic acid in cells, inhibition of mitochondrial function, and the production of reactive metabolites [9,10]. Many NSAIDs possess carboxylic acids in their structures and are mainly subjected to glucuronidation in humans [11]. It has been reported that acyl-glucuronides are relevant to adverse reactions owing to their instability, although their involvement in toxicity has not been experimentally proven [12]. Recently, Darnell et al. [13] reported that the covalent binding ability of acyl-CoA conjugates of ibuprofen, one of the NSAIDs containing carboxylic acid, via trans-acylation to proteins is higher than that of its acyl-glucuronide, implying the relevance of acyl-CoA conjugation to toxicity. ACS isoforms involved in the acyl-CoA conjugation of drugs and their preferences for various NSAIDs remain to be clarified. In this study, we sought to clarify the ACS isoforms catalyzing acyl-CoA conjugation of NSAIDs by evaluating the expression levels of 26 ACS isoforms in the human liver.

2. Materials and Methods

2.1. Chemicals and Reagents. Alminoprofen, indomethacin, ketoprofen, salicylic acid, sulindac, and valeric acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ibuprofen acyl-glucuronide (IBP Glu) was purchased from Toronto Research Chemical (Toronto, Canada). Adenosine 5′-triphosphate magnesium salt, alamethicin, coenzyme A sodium salt hydrate cofactor for acyl transfer (CoA), diclofenac, flurbiprofen, ibuprofen sodium salt, lauric acid, loxoprofen sodium salt, mefenamic acid, and UDP-glucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO). RNAiso and random hexamers were obtained from Takara Bio (Shiga, Japan). ReverTra Ace and Luna Universal qPCR Master Mix were purchased from Toyobo (Osaka, Japan) and New England Biolabs (Ipswich, MA), respectively. All primers were commercially synthesized at Integrated DNA Technologies (Coralville, IA). Rabbit anti-human ACSL1 and rabbit anti-human ACSM2A antibodies were obtained from Cell Signaling Technology Japan (Tokyo, Japan) and Abcam (Cambridge, MA), respectively. IRDye 680-labeled goat anti-rabbit secondary antibody and Odyssey Blocking buffer were from LI-COR Biosciences (Cambridge, UK). All other chemicals and solvents were of the highest grade commercially available.

2.2. Human Livers. Human liver samples obtained from 28 donors (Table 1) were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan). The use of the human livers was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan). Human liver homogenate (HLH) was prepared by homogenizing the tissue in 100 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol, 100 mM KCl, and 1 mM EDTA (pH 7.4), and pooling samples if necessary. Pooled human liver microsomes (HLM) were prepared from the above HLHs as reported previously [14].

2.3. CoA conjugation of NSAIDs in HLH and recombinant ACSs. The ten NSAIDs shown in Fig. 1, alminoprofen, diclofenac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, loxoprofen, mefenamic acid, salicylic acid, and sulindac, were used as substrates. The reaction mixtures (final volume of 0.2 ml) contained 100 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 150 mM KCl, 0.5 mg/mL pooled HLH (10 donors, No. 1–10 in Table 1), and 3 mM ATP. Ibuprofen sodium salt was dissolved in water, and the other substrates were dissolved in DMSO. The final concentration of the substrates was 500 μM. After preincubation at 37°C for 2 min, reactions were initiated by the addition of 20 μL of CoA (final concentration: 0.3 mM). After a 6-hr incubation at 37°C, the reaction was terminated by the addition of 10 μL of 60% perchloric acid. The mixture was centrifuged at 20,380 g for 5 min, and a 10 μL aliquot of the supernatant was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). An LCMS-8045 (Shimadzu, Kyoto, Japan) equipped with an LC-20AD HPLC system was used. The column was a Develosil ODS-UG-3 (2.0 × 150 mm, 3 μM; Nomura Chemical, Seto, Japan). The flow rate was 0.2 mL/min, and the column temperature was 40°C. Nitrogen was used as the nebulizer gas and drying gas. The operating parameters were optimized as follows: nebulizer gas flow, 3 L/min; drying gas flow, 15 L/min; desolvation line temperature, 300°C; and heat block temperature, 40°C. LC-MS/MS was performed with positive or negative electrospray ionization. For measurement of CoA conjugation from 10 NSAIDs in HLH, CoA conjugates of NSAIDs were monitored in single ion monitoring (SIM). The mobile phase was (A) 10 mM ammonium acetate (pH 5.6) and (B) acetonitrile. The conditions of the mobile phase and *m/z* values are summarized in Table 2. The analytical data were processed using LabSolutions (version 5.82.1; Shimadzu).

For propionic acid-class NSAIDs, further evaluation of CoA conjugation was performed by incubating 50 μM propionic acid-class NSAIDs with recombinant enzymes or HLH after confirmation of linearity in terms of protein concentration and incubation time. The reaction mixtures were the same as described above except for the concentration of HLH. The concentrations of the enzyme sources and the incubation times are shown in Table 3. After incubation, the mixture was centrifuged at 20,380 g for 5 min, and a 10 μL aliquot of the

supernatant was subjected to LC-MS/MS as described above. CoA conjugates were monitored by multiple reaction monitoring (MRM), which was established by performing a product ion scan and voltage optimization (Table 4). The other conditions were the same as described above.

2.4. Real-Time RT-PCR Analysis. Total RNA was extracted from 3 individual human liver samples (Nos. 1–3 in Table 1) using RNAiso, and cDNA was prepared using ReverTra Ace according to the manufacturer's protocols. The ACS mRNA levels were quantified by real-time RT-PCR using Mx3000P (Stratagene, San Diego, CA). A 1 μl portion of the reverse-transcription mixture was added to a PCR mixture containing Luna Universal qPCR Master Mix solution containing 5 pmol primers in a final volume of 20 μL. For 20 out of the 26 isoforms, multiple splicing variants have been registered in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Therefore, primers were designed to amplify all splicing variants in each isoform. The sequences of the primers are shown in Table 4. The PCR conditions were as follows: After an initial denaturation at 95°C for 60 s, amplification was performed by denaturation at 94°C for 20 s, then annealing and extension at the temperature and time shown in Table 5 for 40 cycles. The copy number was calculated by standard curves prepared from the purified PCR product as described previously [15].

2.5. Construction of recombinant human ACSL1 or ACSM2B expressed in baculovirus-infected Sf21 cells. Expression systems of human ACSL1 and ACSM2B were prepared using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's protocol. Human ACSL1 and ACSM2B cDNA were obtained by PCR using cDNA prepared from the human liver and the following primer sets: sense primer (5-ATGCAAGCCCATGAGCTGTT-3) and antisense primer (5-CTAAACCTTGATAGTGG-AATAGAG-3) for ACSL1 and sense primer (5-TTCCGACAGTGCAGGGATTC-3) and antisense primer (5-TGTCTCCTAGACGCCTCACT-3) for ACSM2B. The PCR products

were subcloned into the pTargeT mammalian expression vector (Promega, Maison, WI). The nucleotide sequences were confirmed to be consistent with the reference sequences (accession no. NM_001995.4 and NM_182617.3 for human ACSL1 and ACSM2B, respectively) by DNA sequence analysis (FASMAC, Kanagawa, Japan). The ACSL1 or ACSM2B cDNA in the pTargeT vector was resubcloned into the pFastBac1 vector using *Bam*H I and *Sal* I. The pFastBac1 vector containing ACSL1 or ACSM2B cDNA was transformed into DH10Bac competent cells, and the other steps were performed according to the method reported previously [16]. A homogenate of Sf21 cells infected with the constructed baculoviruses was prepared, and the human ACSL1 and ACSM2B proteins were obtained. The protein concentration was determined according to the method of Bradford [17] using γ-globulin as a standard.

2.6. Immunoblot analyses for ACSL1 and ACSM2B. SDS-PAGE and immunoblot analyses were performed according to a method reported previously [18] with slight modifications. HLH (10 or 50 μg) and Sf21 cell homogenates expressing human ACSL1 (2.5 μg), ACSM2B (15 μg), or mock (15 μg) were separated on 7.5% polyacrylamide gels and electrotransferred onto Immobilon-P (Millipore, Billerica, MA) membranes. The membranes were probed with polyclonal rabbit anti-human ACSL1 or rabbit anti-human ACSM2A antibody and the corresponding fluorescent dye-conjugated secondary antibody. An Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) was used for detection.

2.7. CoA conjugation of lauric acid or valeric acid by HLH and recombinant ACS. CoA conjugation of lauric acid or valeric acid was measured according to a method reported previously [19]. The reaction mixture and assay conditions were the same as described above, except that the concentrations of the enzyme sources (0.1 mg/ml recombinant ACSL1, ACSM2B, or HLH) and incubation times for lauric acid and valeric acid were 10 min and 20 min, respectively. After incubation, the mixture was centrifuged at 20,380 g for 5 min, and a 10 μL aliquot of the supernatant was subjected to LC-MS/MS analysis using the same

systems described above. The column was a Zorbax SB-C18 (2.0 × 50 mm, 3.5 μm; Aglient Technologies, Tokyo, Japan), the flow rate was 0.3 mL/min, and the column temperature was 40°C. Nitrogen was used as the nebulizer gas and the drying gas. The operating parameters were optimized as follows: nebulizer gas flow, 3 L/min; drying gas flow, 15 L/min; desolvation line temperature, 300°C; and heat block temperature, 40°C. The LC-MS/MS was operated in positive electrospray ionization. The CoA conjugates were monitored by MRM. The conditions of the mobile phase and *m/z* values for the detection and collision energies (CE) are summarized in Table 2.

2.8. Synthesis of the CoA conjugate of ibuprofen. To chemically synthesize the CoA conjugate of ibuprofen (IBP CoA), ibuprofen *N*-hydroxysuccinimide (NHS) ester was first synthesized. To a stirred mixture of ibuprofen (500 mg, 2.42 mmol) and NHS (335 mg, 2.91 mmol) in CH_2Cl_2 (2.4 mL), EDCI·HCl (558 mg, 2.91 mmol) was added at room temperature. After stirring for 1 hr, complete consumption of the ibuprofen was confirmed by thin-layer chromatography. The formed ibuprofen NHS ester was purified with a SiO_2 column (eluent: CH_2Cl_2) as a colorless solid (615 mg, 84%), and its structure was confirmed to be identical to that reported previously by ^{1}H NMR [20] [^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29–7.22 (m, 2H), 7.17–7.10 (m, 2H), 4.03 (q, J = 6.4 Hz, 1H), 2.81 (br s, 4H), 2.46 (d, J = 6.8 Hz, 2H), 1.92–1.77 (m, 1H), 1.63 (d, J = 6.8 Hz, 3H), 0.90 (d, J = 6.4 Hz, 6H)].

Next, to a stirred solution of CoA (10 mg, 0.026 mmol) in MeCN/0.5 M aqueous $NaHCO_3$ (1.3 mL/1.3 mL), ibuprofen NHS ester (40 mg, 0.26 mmol) was added at room temperature under an Ar atmosphere. After stirring for 10 hr, the mixture was cooled to 0°C, and an acidic ion exchange resin (DOWEX 50W8, hydrogen form, 500 mg) was added to the mixture. After stirring for 30 min at 0°C, the resin and reaction solution were separated by filtration. The compounds bound to the resin were extracted with water (1 mL, 3 times). The extracts and the former filtrate were combined, and low polar impurities in the mixture were removed by liquid-liquid extraction with AcOEt (10 mL, 3 times). The aqueous phase was freeze-dried to give a colorless solid. The solid was suspended in AcOEt/MeCN (4 mL/4

mL). The suspension was centrifuged at 2,700 g for 5 min, and the supernatant was removed. The washing process with AcOEt/MeCN was repeated 3 times. The remaining solid was dried *in vacuo* to give IBP CoA as a colorless solid (10.0 mg). The product was characterized by ^{1}H NMR [^{1}H NMR (400 MHz, D_2O) δ 8.70 (s, 1H), 8.43 (s, 1H), 7.27 (d, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 2H), 6.23 (d, *J* = 5.6 Hz, 1H), 4.91–4.85 (m, 2H), 4.62 (s, 1H), 4.27 (s, 2H), 4.04 (s, 1H), 4.03 (q, *J* = 6.8 Hz, 1H), 3.88 (dd, *J* = 8.4, 4.8 Hz, 1H), 3.60 (dd, *J* = 9.6, 4.8 Hz, 1H), 3.38–3.26 (m, 4H), 3.10–2.90 (m, 2H), 2.45 (d, *J* = 7.2 Hz, 2H), 2.31–2.22 (m, 2H), 1.88–1.72 (m, 1H), 1.48 (d, *J* = 7.6 Hz, 3H), 0.94 (s, 3H), 0.85 (d, *J* = 6.8 Hz, 6H), 0.81 (s, 3H)], and the product purity was 83% (confirmed by qNMR using sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard). Taking the purity into account, the product yield was 33%.

2.9. Protein adduct formation abilities of IBP CoA and IBP Glu. The protein adduct formation ability of IBP CoA via trans-acylation and that of IBP Glu via glycation were evaluated as follows: incubation mixtures (final volume of 0.2 ml) contained 10 pmol of IBP CoA or IBP Glu, 100 mM Tris-HCl (pH 7.4), 1.0 mg/mL HLH as well as 3 mM ATP and 0.3 mM CoA (for IBP CoA) or 25 μg/mL alamethicin and 2.5 mM UDPGA (for IBP Glu). These mixtures were incubated for 60 min at 37°C, and then the proteins were removed by the addition of 10 μL of 60% perchloric acid and centrifugation at 20,380 g for 5 min. A 10 μL aliquot of the supernatant was subjected to LC-MS/MS analysis. The analytical conditions for the detection of IBP CoA and IBP Glu are shown in Table 2 and Table 6, respectively. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile, as shown in Table 6. Protein adduct formation was calculated with the following equation:

$$\text{Protein adduct formation (\%)} = 100 \times (RA_{(HLH-)} - RA_{(HLH+)})/RA_{(HLH-)}$$

where $RA_{(HLH+)}$ and $RA_{(HLH-)}$ are the remaining amounts of conjugate incubated with and without HLH, respectively.

2.10. Protein adduct formation abilities of propionic acid-class NSAIDs CoA conjugate and acyl-glucuronide. The protein adduct formation ability of the CoA conjugate of propionic acid-class NSAIDs via trans-acylation and that of acyl-glucuronide via glycation were evaluated by not using their authentic standards as follows: For CoA conjugation, incubation mixtures (final volume of 180 μL) contained 50 μM propionic acid-class NSAIDs, 100 mM Tris-HCl (pH 7.4), and 1.0 mg/mL HLM. After preincubation at 37°C for 2 min, reactions were initiated by the addition of 20 μL of 3 mM CoA. After a 60 min incubation at 37°C, the reaction was terminated on ice, and then 100 μL of supernatant containing CoA conjugate was collected after centrifugation at 105,000 g for 60 min. The collected supernatant was mixed with 100 μL of 200 mM Tris-HCl (pH 7.4) and 10 μM triacsin C (ACS inhibitor) with or without 2.0 mg/mL HLM to a final volume of 200 μL. After a 60 min incubation at 37°C, the proteins were denatured by the addition of 10 μL perchloric acid. The mixtures were centrifuged at 20,380 g for 5 min, and a 50 μL aliquot of the supernatant was subjected to LC-MS/MS.

For glucuronidation, the incubation mixtures (final volume of 190 μL) contained 50 μM propionic acid-class NSAIDs, 100 mM Tris-HCl (pH 7.4), 1.0 mg/mL HLM, and 25 μg/mL alamethicin. After preincubation at 37°C for 2 min, the reactions were initiated by the addition of 10 μL of 50 mM UDPGA. After a 60 min incubation at 37°C, the reaction was terminated on ice, and then 100 μL of supernatant containing acyl-glucuronide was collected after centrifugation at 105,000 g for 60 min. The collected supernatant was mixed with 100 μL of 200 mM Tris-HCl (pH 7.4) and 1 mM mefenamic acid (an inhibitor of UGT2B7 and UGT1A9) with or without 2.0 mg/mL HLM to a final volume of 200 μL. After a 60 min incubation at 37°C, the proteins were denatured by the addition of 10 μL perchloric acid. The mixtures were centrifuged at 20,380 g for 5 min, and a 50 μL aliquot of the supernatant was subjected to LC-MS/MS. Protein adduct formation was calculated with the following equation:

$$\text{Protein adduct formation (\%)} = 100 - (100 \times PA_{(HLM+)}/PA_{(HLM-)})$$

where $PA_{(HLM+)}$ and $PA_{(HLM-)}$ are the peak areas of the conjugate in incubation with and without HLM, respectively. It had been preliminarily confirmed that CoA conjugations or glucuronidations of propionic acid-class NSAIDs were inhibited by over 90% by 5 μM triacsin C or 500 μM mefenamic acid, respectively (data not shown).

2.11. Statistical analysis. Data are expressed as the mean ± SD. Comparisons of two groups were made with two-tailed Student's *t*-tests. Correlation analyses were performed using Pearson's test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. CoA conjugation of NSAIDs containing carboxylic acid. To investigate which NSAIDs are conjugated with CoA in the human liver, the formation of CoA conjugates with 10 kinds of NSAIDs was evaluated using HLH as an enzyme source. As a result, the CoA conjugates of alminoprofen, flurbiprofen, ibuprofen, ketoprofen, and loxoprofen, which are propionic acid-class NSAIDs, were detected in positive mode (Fig. 2), whereas those of diclofenac, indomethacin, mefenamic acid, salicylic acid, and sulindac were not detected by SIM or Q1 scan in the range of the predicted $m/z \pm 100$ in either positive or negative electrospray mode (data not shown). Thus, it was demonstrated that CoA conjugates are produced from propionic acid-class NSAIDs.

3.2. Expression levels of 26 ACS mRNAs in human livers. To determine the expression levels of 26 kinds of ACS mRNA in human livers, real-time RT-PCR was performed using cDNA samples from 3 individual livers (Table 7). Among the 26 ACS isoforms, ACSM4, ACSVL2, ACSF1, ACSF2, ACSBG1, and ACSBG2 could not be detected; the absolute hepatic expression levels of the other 20 kinds of ACS isoforms are shown in Table 7. Among them, ACSL1 revealed the highest expression (% in total hepatic ACS levels: 46.6%, 31.2–56.1%), followed by ACSM2B (14.7%, 9.6–20.6%), ACSVL6 (8.8%, 3.1–17.0%), ACSL5 (6.7%, 3.4–12.3%), and ACSVL1 (5.8%, 2.7–10.5%). Thus, ACSL1 and ACSM2B are the major isoforms in the liver, occupying approximately 60% of the total.

3.3. Confirmation of the protein levels and activities of recombinant ACSL1 and ACSM2B. To investigate the possibility that propionic acid-class NSAIDs may be CoA conjugated by ACSL1 and ACSM2B, the most abundant ACS isoforms in the human liver, recombinant human ACSL1 and ACSM2B, were constructed. In the immunoblot analysis, HLH was used as a positive control because the ACS enzymes are located in various organelles, including the plasma membrane, endoplasmic reticulum, mitochondrial

membrane, and mitochondrial matrix [5]. By immunoblot analysis, a band at approximately 70 kDa was observed in recombinant ACSL1, and a band with the same mobility was detected in HLH (Fig. 3A). Lauric acid CoA conjugation by recombinant ACSL1 was approximately 2.5-fold higher than that by HLH (Fig. 3B), which can be explained by the higher expression level of ACSL1 in expression systems than HLH (3.5-fold). Thus, recombinant ACSL1 with a specific activity almost equivalent to ACSL1 expressed in the human liver was established. Two bands were detected for the recombinant ACSM2B. Similarly, two bands were detected in HLH, and the molecular weights of the common bands between recombinant ACSM2B and HLH was 64 kDa, which corresponds to the estimated molecular weight of ACSM2B (Fig. 3C). The intensity of the 64 kDa band in the recombinant ACSM2B was 4.3-fold higher than that in the HLH. Valeric acid CoA conjugation by recombinant ACSM2B was 1.3-fold higher than that in HLH (Fig. 3D). Taken together, we judged that recombinant ACSL1 and ACSM2B were successfully constructed.

3.4. Propionic acid-class NSAID CoA conjugation by recombinant ACSL1 or ACSM2B. We investigated, by using the constructed recombinant enzymes, whether ACSL1 or ACSM2B catalyzes CoA conjugation of propionic acid-class NSAIDs. As shown in Fig. 4, recombinant ACSL1 catalyzed CoA conjugation of alminoprofen, flurbiprofen, ibuprofen, ketoprofen, and loxoprofen at 3.5-, 2.0-, 1.7-, 3.8-, and 3.0-fold higher activities, respectively, than HLH. The CoA conjugates of NSAIDs other than propionic acid-class NSAIDs after exposure to the recombinant enzymes were not detected by SIM (data not shown), consistent with the lack of production of CoA conjugates in HLH. These results suggested that the CoA conjugations of propionic acid-class NSAIDs in the human liver are catalyzed by ACSL1.

3.5. Correlation between ACSL1 protein levels and CoA conjugations for propionic acid-class NSAIDs in 28 individual human liver samples. To confirm the responsibility of ACSL1 for CoA conjugation of propionic acid-class NSAIDs in human liver, the correlations between the ACSL1 protein levels and the CoA conjugations for propionic acid-class

NSAIDs were evaluated using a panel of 28 individual human liver samples (Fig. 5). There were 11.9-fold interindividual variations in ACSL1 protein levels and 59.5-, 12.2-, 26.1-, 23.0-, and 8.8-fold variations in CoA conjugations for alminoprofen, flurbiprofen, ibuprofen, ketoprofen, and loxoprofen, respectively. Significant positive correlations were observed between ACSL1 protein levels and CoA conjugations for alminoprofen ($R = 0.598$, $P < 0.001$), flurbiprofen ($R = 0.623$, $P < 0.001$), ibuprofen ($R = 0.564$, $P < 0.01$), ketoprofen ($R = 0.618$, $P < 0.001$), and loxoprofen ($R = 0.495$, $P < 0.01$). These results supported the proposition that ACSL1 is responsible for the CoA conjugations of propionic acid-class NSAIDs in the human liver.

3.6. Protein adduct formation abilities of the IBP CoA conjugate and glucuronide. The protein adduct formation abilities of IBP CoA and IBP Glu were evaluated by quantification of their unbound amounts in the mixture containing HLH. After the addition of 10 pmol IBP CoA, the remaining amount of IBP CoA was 3.8 pmol in the presence of HLH, whereas it was 4.9 pmol in the absence of HLH (Fig. 6A), which may be due to the instability of IBP CoA in the buffer. Accordingly, the protein adduct formation of IBP CoA was calculated to be 22% by equation (1). The remaining amount of IBP Glu was 4.0 pmol in the presence of HLH, whereas it was 4.2 pmol in the absence of HLH (Fig. 6B). The protein adduct formation of IBP Glu was calculated to be 5%. Thus, it was demonstrated that IBP CoA has a higher protein adduct formation ability than IBP Glu.

3.7. Protein adduct formation abilities of other propionic acid-class NSAID CoA conjugates and glucuronides. CoA conjugates of propionic acid-class NSAIDs other than IBP CoA are not commercially available. As an alternative method, we tried to evaluate the protein adduct formation abilities of the CoA conjugate versus the acyl-glucuronide, which were produced by an *in vitro* metabolism reaction. First, we evaluated the validity of this strategy by examining the binding of IBP CoA and IBP Glu, which were produced by a metabolic reaction with HLM. Protein adduct formations of IBP CoA and IBP Glu were

calculated according to equation (2) and were 28% or 5%, respectively (Fig. 7A), which were close to those obtained using authentic standards of IBP CoA and IBP Glu (Fig. 6). Thus, we judged that this strategy is reliable. Next, the protein adduct formation of CoA conjugates and acyl-glucuronides of the other propionic-acid NSAIDs was evaluated. As shown in Figs. 7B-E, the protein adduct formation of the CoA conjugates of propionic-acid NSAIDs were significantly higher than that of acyl-glucuronides (21% vs −4% for alminoprofen, 45% vs 2% for flurbiprofen, 25% vs −10% for ketoprofen, and 16% vs −15% for loxoprofen, respectively). The negative values for the protein adduct formation observed for the acyl-glucuronides of some NSAIDs is due to the error of the measurement by LC-MS/MS, meaning no adduct formation. Collectively, it was demonstrated that the protein adduct formation ability of the CoA conjugate of propionic acid-class NSAIDs was significantly higher than that of their acyl-glucuronide form.

4. Discussion

NSAIDs containing a carboxylic acid in their structure rarely cause adverse reactions such as anaphylaxis, liver injury, Stevens-Johnson syndrome, gastrointestinal disorders, renal dysfunction, or acute bronchospasm [21]. Some of these have been considered to be due to the covalent binding of the parent drug or its metabolites to cellular proteins [12]. Among their reactive metabolites, acyl-glucuronides have received attention for NSAID-induced toxicity. Recently, it has been reported that the amount of ibuprofen bound to HLM proteins was greater in the presence of CoA than in the presence of UDPGA, and the protein adduct formation of IBP CoA to proteins was 116.3-fold higher than that of IBP Glu [13]. Thus, it has been suggested that acyl-CoA conjugates, rather than acyl-glucuronides, may be responsible for the toxicity.

To examine what kinds of NSAIDs are subjected to CoA conjugation in humans, ten NSAIDs belonging to the anthranic acid, indole acetic acid, phenyl acetic acid, or propionic acid classes were used (Fig. 1). We found that alminoprofen, flurbiprofen, ibuprofen, loxoprofen, and ketoprofen, which are all propionic acid-class NSAIDs, were conjugated with CoA in HLH (Fig. 2). Our results supported earlier study showing that propionic acid-class NSAIDs including ibuprofen and ketoprofen are conjugated with CoA by HLH [22]. It has been reported that a taurine conjugate of ibuprofen was detected in urine after oral intake of ibuprofen in humans [23]. Because CoA conjugation is followed by amino acid conjugation, CoA conjugation of ibuprofen would indeed occur in the human body. In addition, *S*-acyl glutathione conjugate of ibuprofen was detected in rat or human hepatocytes [24]. It has been reported that *S*-acyl glutathione conjugate of ibuprofen is formed from CoA-conjugate of ibuprofen [24]. *S*-Acyl-glutathione conjugate was also formed from diclofenac [25], but in this study, we found that the CoA conjugate was not formed from diclofenac. Thus, *S*-acyl glutathione conjugates would not be necessarily formed from CoA conjugates.

To obtain a clue about the ACS isoform catalyzing CoA conjugation for propionic acid-class NSAIDs, we evaluated the expression levels of the various ACS isoforms in the human

liver by real-time RT-PCR and found that ACSL1 had the highest expression in the human liver (46.6%), followed by ACSM2B (14.7%) (Table 7). Although most isoforms could be quantified, the expression of ACSM4, ACSVL2, ACSBG1, ACSBG2, ACSF1, and ACSF2 were not observed in the human liver. ACSVL2 is expressed in the heart and placenta; ACSBG1 is expressed in the brain, adrenal gland, and testis; ACSBG2 is expressed in the adult testis; ACSF1 is expressed in the kidney, heart, or brain; and ACSF2 is expressed in adipose tissue [5]. Although information about the tissue distribution of human ACSM4 is unavailable, it has been reported that ACSM4 is specifically expressed in the olfactory organ in rats [4]. Thus, they appear to be extrahepatic isoforms.

To investigate whether ACSL1 and ACSM2B, abundant isoforms in the human liver, can catalyze CoA-conjugation of drugs, their recombinant enzymes were constructed. By immunoblot analysis and the measurement of CoA conjugation for lauric acid, we confirmed that a functional recombinant ACSL1 was successfully constructed (Figs. 3A and B). The expression of ACSM2B was observed using an antibody against ACSM2A, whose amino acid sequence identity in the epitope is 97% (195 out of 201 amino acids) identical with that of ACSM2B. Unexpectedly, two bands were detected in the expression system of ACSM2B (Fig. 3C). To explain this, posttranslational modification was considered. A search for glycosylation sites using the NetOGlyc 4.0 Server (Technical University of Denmark) revealed three potential *O*-linked glycosylation sites in ACSM2B. However, treatment with *O*-glycosidase did not change the mobility of the two bands in immunoblot analyses (data not shown). Another possible reason was considered to be the usage of another start codon because an ATG codon is located 51 bp downstream from the start codon according to the mRNA sequence of human ACSM2B (Accession No. NM_182617.4). If this ATG is utilized as a start codon, a protein lacking 17 amino acids at the N-terminus could be produced. Thus, the band with high mobility in recombinant ACSM2B might be derived from this shortened protein.

Two bands were also observed in HLH, and the molecular weight of the band with high mobility was different from that with high mobility in the recombinant human ACSM2B (Fig.

3C). According to the NCBI website (http://www.ncbi.nih.gov/gene/123876), a shorter variant composed of 498 amino acids is also registered for human ACSM2A. This variant lacks 79 amino acids at the N-terminus and can be recognized by the anti-ACSM2A antibody used in this study. The band with high mobility in HLH may be derived from this shorter variant of ACSM2A. The intensity of the band with low mobility in the recombinant ACSM2B was 4-fold higher than that of the corresponding band in HLH, whereas the activity for the formation of the CoA conjugate for valeric acid was almost the same between recombinant ACSM2B and HLH (Fig. 3D). Because human ACSM2A shows the same molecular weight as ACSM2B, the band with low mobility in HLH would be expected to contain both ACSM2A and ACSM2B, which may cause a contradiction between band intensity and activity. Since the constructed recombinant ACSM2B showed efficient CoA conjugation for valeric acid, it was judged that a functional ACSM2B expression system had been successfully developed.

Among the two recombinant ACS isoforms prepared in this study, human ACSL1 showed efficient CoA conjugation for propionic acid-class NSAIDs (Fig. 4), and its responsibility was demonstrated by a positive correlation between ACSL1 protein levels and CoA conjugations for propionic acid-class NSAIDs in a panel of 28 human liver samples (Fig. 5). By comparison of ACSL1 protein levels in HLH and recombinant system by immunoblot analysis (Fig. 3), the contributions of ACSL1 to the CoA conjugations of alminoprofen, flurbiprofen, ibuprofen, ketoprofen, and loxoprofen were calculated to be 99%, 57%, 50%, 109%, and 87%, respectively. Thus, ACSL1 would be a major contributor to the CoA conjugation of three NSAIDs. It has been reported that CoA conjugate of ibuprofen is rapidly epimerized and hydrolyzed to ibuprofen in HLH [22]. The moderate contribution of ACSL1 to the CoA conjugation of ibuprofen may be due to the difference in the efficiencies of epimerization and hydrolysis between HLH and Sf21 cells. This theory may be applied to the moderate contribution of ACSL1 to the CoA conjugation of flurbiprofen.

Possible mechanisms to cause the adverse reactions by NSAIDs include the accumulation of arachidonic acid in cells, inhibition of mitochondrial function, and the production of

reactive metabolites [9,10]. As the former two mechanisms, the direct inhibition of ACS enzymes or fatty acid oxidation enzymes in mitochondrion by NSAIDs, leading to apoptosis or necrosis via ATP depletion, has been proposed [26,27]. As the latter mechanism, the formation of CoA conjugates would be involved. As another mechanism, the excessive consumption of cellular CoA by CoA conjugation reaction, which may cause microvesicular steatosis of the liver [28,29], has been considered, because CoA is required for the formation of acetyl-CoA in glycolysis [30] and in β-oxidation of fatty acids [31]. In addition, a decrease in NADH, which is produced in the tricarboxylic acid cycle driven by acetyl-CoA, may cause a decrease in the production of ATP, which is essential for cellular function [32]. The interindividual variations of CoA conjugations for propionic acid-class NSAIDs were 59.5-fold at the maximum (Fig. 5). Individuals with high ACSL1 activity may be susceptible to adverse reactions, although a possibility could not be denied that other players having other mechanisms for toxicity may also be involved in the susceptibility.

It has been suggested that liver injury is caused by reactive metabolites binding to macromolecules such as proteins and DNA [33,34]; therefore, the protein adduct formation ability of compounds has often been evaluated for toxicity relevance [13,35]. In this study, prior to the evaluation of the adduct formation of the conjugates, the stabilities of IBP CoA and IBP Glu in Tris-HCl buffer were assessed. The amount of IBP Glu was decreased by a 60 min incubation from 10 pmol to 4.2 pmol, even in the absence of any proteins (Fig. 6B). This result is consistent with a previous report showing the instability of IBP Glu in phosphate buffer [3]. The amount of IBP CoA was also decreased from 10 pmol to 4.9 pmol simply by incubation (Fig. 6A); therefore, IBP CoA would also be unstable even in a buffer without any proteins. Next, the adduct formation of IBP conjugates was evaluated by the quantification of unbound conjugates. The protein adduct formation of IBP CoA (22%) was higher than that of IBP Glu (2%) (Fig. 6). In addition, we evaluated the protein adduct formation of the conjugates by an alternative evaluation method with no use of authentic standards, resulting in the protein adduct formation of IBP CoA (28%) and IBP Glu (5%), in which the tendency was similar to that obtained in analyses using authentic standards (Figs. 6 and 7A). Thus, we

judged that this evaluation method was reliable. Darnell et al. [13] reported, by using radioactive ibuprofen, that the binding rates of IBP CoA or IBP Glu produced by metabolic reactions with microsomal proteins were 15% or 0.03%, respectively, which were lower than those obtained in this study. However, the trend of higher adduct formation of IBP CoA than IBP Glu was consistent. For the other propionic acid-class NSAIDs, the evaluation with conjugates produced by the metabolic reaction demonstrated that the covalent binding of the CoA conjugates of alminoprofen, flurbiprofen, ketoprofen, and loxoprofen was significantly higher than that of acyl-glucuronides (Figs. 7B, C, D and E). From these results, it is conceivable that CoA conjugates, rather than acyl-glucuronides, might be relevant to the toxicity of propionic acid-class NSAIDs.

According to LiverTox database (https//www.ncbi.nim.nih.gov/books/ NBK547852/), the likelihood score of liver injury of ibuprofen (A: well known but rare cause of clinically apparent liver injury) is higher rather than that of flurbiprofen and ketoprofen (C: probable rare cause of clinically apparent liver injury). This is unlikely corresponding to the protein adduct formation in Fig. 7. Recommended dosage of ibuprofen, 400–800 mg orally 3 to 4 times daily, is much higher than those of flurbiprofen (50–100 mg 2 to 4 times daily) and ketoprofen (50–75 mg 3 or 4 times daily). This fact supports a theory that daily dose would also be a key factor for the risk of adverse reactions [35,36].

In conclusion, we revealed that ACSL1, the most highly expressed isoform among the ACS family in the human liver, is responsible for the CoA conjugation of propionic acid-class NSAIDs. It was demonstrated that the protein adduct formation of CoA conjugates via trans-acylation was higher than that of acyl-glucuronides. It would be interesting to directly investigate the involvement of ACSL1 in the incidence of adverse reactions induced by propionic acid-class NSAIDs containing carboxylic acid.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

ACS, Acyl-CoA synthetase; ACSBG, ACS bubblegum family; ACSL, ACS long-chain family; ACSM, ACS medium-chain family; ACSS, ACS short-chain family; ACSVL, ACS very long-chain family; CE, Collision energy; HLH, Human liver homogenate; HLM, Human liver microsomes; IBP CoA, CoA conjugate of ibuprofen; IBP Glu, ibuprofen acyl-glucuronide; MRM, Multiple reaction monitoring; NHS, *N*-Hydroxysuccinimide; NSAID, Nonsteroidal anti-inflammatory drug; SIM, Single ion monitoring; SJS, Stevens-Johnson syndrome; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

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Figure Legends

Fig. 1. Chemical structures of 10 NSAIDs containing carboxylic acid investigated in this study. NSAIDs are classified based on their chemical structure.

Fig. 2. LC-MS chromatograms for CoA conjugations for 5 kinds of NSAIDs containing carboxylic acid in human liver homogenate. HLH was incubated with NSAIDs in the absence (A, C, E, G, I) or presence (B, D, F, H, J) of CoA, and each conjugate was monitored by SIM.

Fig. 3. Immunoblot analyses of recombinant human ACSL1 and ACSM2B and evaluation of their activities for fatty acid conjugations. ACSL1 (A) or ACSM2B (C) expression was evaluated by Immunoblot analyses. HLH [10 μg (A) or 50 μg (C)], homogenates of Sf21 cells expressing human ACSL1 (2.5 μg) or ACSM2B (15 μg), and mock homogenate (15 μg) were separated by electrophoresis using 7.5% SDS-polyacrylamide gel. CoA conjugations for lauric acid (B) and valeric acid (D) in HLH and recombinant ACSL1 or ACSM2B were evaluated by LC-MS/MS. Enzyme sources (0.1 mg/mL) were incubated with 30 μM lauric acid (B) or 100 μM valeric acid (D). Each column represents the mean ± SD (n = 3).

Fig. 4. CoA conjugations for propionic acid-class NSAIDs by HLH or recombinant ACSL1 and ACSM2B. CoA conjugations for alminoprofen (A), flurbiprofen (B), ibuprofen (C), ketoprofen (D), and loxoprofen (E) by HLH or recombinant ACSL1 and ACSM2B were evaluated at 50 μM substrate concentrations by LC-MS/MS. Each column represents the mean ± SD (n = 3). ND: Not detected.

Fig. 5. Correlation between ACSL1 protein levels and CoA conjugations for propionic acid-class NSAIDs in a panel of 28 human livers. Correlation analyses between ACSL1

protein levels and CoA conjugations of alminoprofen (A), flurbiprofen (B), ibuprofen (C), ketoprofen (D), or loxoprofen (E) were performed. Pearson's rank correlation method was used. ACSL1 protein levels are relative to the sample with the lowest ACSL1 expression. Each point represents the mean of triplicate determinations.

Fig. 6. Protein adduct formation of CoA conjugate and acyl-glucuronide of ibuprofen. Ten pmol of IBP CoA (A) or IBP Glu (B) was incubated for 60 min at 37˚C in 100 mM Tris-HCl (pH 7.4), 1.0 mg/mL HLH as well as 3 mM ATP and 0.3 mM CoA (A) or 25 μg/mL alamethicin and 2.5 mM UDPGA (B). Proteins were removed by the addition of 60% perchloric acid, and subsequent centrifugation at 20,380g for 5 min. The supernatants were subjected to LC-MS/MS. Each column represents the mean ± SD of triplicate determinations.

Fig. 7. Protein adduct formation of CoA conjugate and acyl-glucuronide of propionic acid-class NSAIDs. Protein adduct formations of CoA conjugate and acyl-glucuronide of ibuprofen (A), alminoprofen (B), flurbiprofen (C), ketoprofen (D), or loxoprofen (E) were evaluated via their productions by *in vitro* metabolic reaction. Each column represents the mean ± SD of triplicate determinations. ***$P < 0.001$.

Table 1. Characterization of 28 donors of livers.

No	Sex	Age (yr)	Ethnicity	Cause of death
1	F	33	Hispanic	Cerebrovascular accident
2	F	47	Caucasian	Intracerebral hematoma
3	F	34	Black	Intracerebral hematoma
4	M	63	Caucasian	Intracerebral hematoma
5	F	32	Hispanic	Subarachnoid hemorrhage
6	M	56	Caucasian	Intracerebral hematoma
7	F	33	Caucasian	Primary brain tumor
8	M	52	Caucasian	Cerebrovascular accident
9	M	53	Caucasian	Intracerebral hematoma
10	M	60	Hispanic	Cerebral apoplexy
11	F	47	Caucasian	S/P code
12	M	36	Caucasian	Intracerebral hemorrhage
13	M	54	Caucasian	Cerebrovascular accident
14	M	62	Asian	Intracerebral hematoma
15	M	36	Caucasian	Head gun wound
16	M	47	Caucasian	Aneurysm
17	M	46	Caucasian	Hypoxia
18	F	35	Hispanic	Intracerebral hemorrhage
19	M	68	Caucasian	Cerebrovascular accident
20	M	16	Caucasian	Traffic accident
21	M	51	Caucasian	Cerebrovascular accident
22	F	52	Asian	Cerebrovascular accident
23	F	59	Caucasian	Traffic accident
24	M	33	Caucasian	Traffic accident
25	M	49	Hispanic	Head injury
26	F	41	Caucasian	Intracerebral hemorrhage
27	M	38	Caucasian	Head injury
28	F	35	Hispanic	Cerebral apoplexy

Table 2. Analytical conditions for fatty acids- or NSAIDs-CoA conjugates by LC-MS/MS.

NSAIDs	Mobile phase	SIM (+ or -)	MRM (+)	Collision energy
	% of B	*m/z*		V
Lauric acid	10-100 (0-4 min) 100 (4-6 min) 10 (6-8 min)		950.25 > 443.40	-40
Valeric acid	15-60 (0-3 min) 60-15 (3-3.5 min) 15 (3.5-5.5 min)		852.20 > 345.30	-35
Alminoprofen	20-90 (0-3 min) 90 (3-6.4 min) 90-20 (6.4-7 min) 20 (7-9 min)	969.05 or 967.05	969.05 > 462.20	-38
Diclofenac	0 (0-1 min) 0-60 (1-3 min) 60 (3-9 min) 60-0 (9-9.5 min) 0 (9.5-11.5 min)	1045.10 or 1043.10		-
Flurbiprofen	20-90 (0-2 min) 90 (2-7 min) 90-20 (7-7.5 min) 20 (7.5-9.5 min)	994.10 or 992.10	994.10 > 487.15	-40
Ibuprofen	20-60 (0-2 min) 60 (2-7 min) 60-20 (7-7.5 min) 20 (7.5-9.5 min)	956.30 or 954.30	956.30 > 449.25	-41
Indomethacin	0 (0-1 min) 0-60 (1-3 min) 60 (3-9 min) 60-0 (9-9.5 min) 0 (9.5-11.5 min)	1107.20 or 1105.20		-
Ketoprofen	20-60 (0-2 min) 60 (2-7.5 min) 60-20 (7.5-8 min) 20 (8-10 min)	1004.10 or 1002.10	1004.10 >497.10	-39
Loxoprofen	20 (0-2 min) 60 (2-5.8 min) 60-20 (5.8-6.3 min) 20 (6.3-8.3 min)	996.05 or 994.05	996.05 > 489.10	-40
Mefenamic acid	5-80 (0-3 min) 80 (3-9 min) 80-5 (9-9.5 min) 5 (9.5-11.5 min)	991.20 or 989.20		-
Salicylic acid	5-90 (0-7 min) 90 (7-9 min) 90-5 (9-9.1 min) 5 (9.1-11.1 min)	888.15 or 886.15		-
Sulindac	0 (0-1 min) 0-45 (1-3 min) 45 (3-9 min) 45-0 (9-9.5 min) 0 (9.5-11.5 min)	1106.20 or 1104.20		-

Table 3. Protein concentration and incubation time of CoA conjugation of propionic acid-class NSAIDs.

Substrate	Enzyme source	Protein concentration (mg/mL)	Incubation time (min)
Alminoprofen	HLH	0.5	10
	Recombinant	0.1	
Flurbiprofen	HLH	0.15	20
	Recombinant		
Ibuprofen	HLH	0.05	45
	Recombinant	0.01	15
Ketoprofen	HLH	0.1	45
	Recombinant		
Loxoprofen	HLH	0.1	15
	Recombinant	0.05	60

Table 4. Primers used for real-time PCR analyses.

Isoforms	Primer	Sequence (5' → 3')	Position
ACSS1	S	CCCAGTTTATCCCAATGCTG	1089-1108
	AS	CCAGGCATCACCGTATTTCA	1184-1203
ACSS2	S	CTGCTTTGTCACCTTGTGTG	1836-1855
	AS	GATTTTCCCTGAGCGGGTTT	1967-1986
ACSS3	S	TTATTTCCGTGTGCTTGCAG	1131-1150
	AS	ATTGTATCCTGGGACGCTTT	1430-1449
ACSM1	S	AGGATTTCACCAGCATCAGG	985-1004
	AS	TCTGTCCCCAGTGTTGTAGA	1123-1142
ACSM2A	S	CCATCTACAGAACTGCGTCA	987-1006
	AS	GTTGCCCTTATCATCTATGAT	1180-1200
ACSM2B	S	CCATCTACAGAACTGCCTCG	987-1006
	AS	GTTGCCCTTATCATCTATAAC	1180-1200
ACSM3	S	CCCTACAAAGAGGAGATCGG	347- 366
	AS	TCTACTGCTGGGGCTAAAAC	526- 545
ACSM4	S	CCTCTGACTCTTGCTGACTTTG	94- 115
	AS	CTCCATTTTACCTCATCCCCT	249- 269
ACSM5	S	CATCTTTCGGCTGCTTGTG	966- 984
	AS	TCGTTTCTTCCCATGAACCA	1396-1415
ACSL1	S	AGAGGATTTGAAGGGTCGTT	1843-1862
	AS	AATGTGATGCCTTTGACCTG	1954-1973
ACSL3	S	ATTCGTCTCCTGTTGTGTGG	1333-1352
	AS	CTAATGGTGCTCCCACTCTG	1485-1504
ACSL4	S	GCAATCAGTAGAAGAGTTGGG	618- 638
	AS	TCAGAGAGTGTAAGCGGAGA	907- 926
ACSL5	S	TGCTGATAGGGAATGTAGAGA	746- 766
	AS	TAGGAGGCACAGGTTTTCTG	900- 916
ACSL6	S	TTAGCATCTCAGGGAATGGG	374- 393
	AS	GCTCCACAATGATCCACTCT	549- 568
ACSVL1	S	CTCTTGCCTTGCGGACTAAA	854- 873
	AS	TCATCACGGACAGGTTCATC	1210-1229
ACSVL2	S	GATTCTGTTCCACAAGGTGT	550- 569
	AS	GGTAGCGACAAAGTTCTCCA	933- 952
ACSVL3	S	ATGTGATCTACCTCGCCCT	1346-1364
	AS	AATGTACTGGAACACCGTCA	1490-1509
ACSVL4	S	GGTTTCAATAGCCGCATCCT	1210-1229
	AS	ATCACCAGCACATCACCAGT	1459-1478
ACSVL5	S	TCAGATCAACCAACAGGACC	1356-1375
	AS	AGCTCATCCATCACTAGCAC	1477-1496
ACSVL6	S	CCTATTCGGATCTGGGAAGT	1586-1605
	AS	GAAGGGTTGCTGGCTTAC	1830-1849
ACSF1	S	GTAAGAAGAACACGCAGATGG	68- 88

	AS	CGATTCCTTTCGATGTGTCC	243- 262
ACSF2	S	ATGTGTGGAGGTGTCATTGC	1204-1223
	AS	GGACTGTTCTCTGTGGTTCC	1300-1319
ACSF3	S	GTGTGATGATGCCTGAGTTC	791- 810
	AS	GGGTAAAATGCCTGTCGTAG	909- 928
ACSF4	S	AGAATAAGCTCAGTGGGAAAGA	1637-1658
	AS	ACTCATCAGGAACCCTCAAA	1719-1738
ACSBG1	S	CATCGCTTATGACTGCTGC	594- 612
	AS	ATCTTGTTTGGAGGAGGTTCT	708- 728
ACSBG2	S	GGCAAGAAACATTGGCTTCA	1044-1063
	AS	AGCTTAGAAAGAACTCGGCA	1215-1234

S: Sense AS: Antisense

Table 5. Conditions of annealing and extension for real-time RT-PCR analysis.

Isoform	Annealing		Extension	
	Temperature (°C)	Time (sec)	Temperature (°C)	Time (sec)
ACSS1	58	30	72	10
ACSS2				
ACSM1	60			5
ACSM2A	51			20
ACSM2B				
ACSM3	58			10
ACSM4				
ACSL1	58			
ACSL3	56			
ACSL4	51			20
ACSL5	58			10
ACSL6				
ACSVL2	58			
ACSVL3	60			
ACSVL4	59			5
ACSVL5	60			10
ACSBG1	58			
ACSBG2				
ACSF1				
ACSF2				
ACSF3				
ACSF4				

Isoform	Annealing and extension	
	Temperature (°C)	Time (sec)
ACSS3	60	30
ACSM5	60	30
ACSVL1	60	30
ACSVL6	65	14

Table 6. Analytical conditions for detection of acyl-glucuronides of NSAIDs.

Substrates	Mobile phase	MRM	Collision energy
	% of B		V
Alminoprofen	20 (0-3 min), 20-90 (3-5 min), 90 (5-9 min), 90-20 (9-9.5 min), 20 (9.5-11.5 min)	396.16 > 174.00 [a]	-35
Flurbiprofen	0-100 (0-7 min), 100 (7-9 min), 100-0 (9-9.5 min), 0 (9.5-11.5 min)	419.00 > 193.00 [b]	11
Ibuprofen	20-90 (0-2 min), 90 (2-8 min), 90-20 (8-8.5 min), 20 (8.5-10.5 min)	380.80 > 193.00 [b]	11
Ketoprofen	0-70 (0-3 min), 70 (3-7.5 min), 70-0 (7.5-8.0 min), 0 (8-10 min)	429.00 > 193.10 [b]	11
Loxoprofen	0-100 (0-10 min), 100 (10-10.5 min), 100-0 (10.5-11 min), 0 (11-13 min)	421.05 > 193.00 [b]	12

[a]Positive mode; [b]Negative mode.

Table 7. ACS mRNA levels in a panel of 3 individual human livers.

Isoform	Sample number			Sample number			
	1	2	3	1	2	3	Average
	10^3 copy/μg total RNA			%			
ACSS1	23.2	30.1	53.0	0.3	0.4	1.6	0.8
ACSS2	229.0	361.0	112.0	2.6	5.1	3.4	3.7
ACSS3	142.0	58.2	13.1	1.6	0.8	0.4	0.9
ACSM1	79.2	59.9	2.9	0.9	0.8	0.1	0.6
ACSM2A	370.0	371.0	73.4	4.2	5.2	2.2	3.9
ACSM2B	1,800.0	1,000.0	313.0	20.6	14.0	9.6	14.7
ACSM3	117.0	111.0	52.9	1.3	1.6	1.6	1.5
ACSM4	ND	ND	ND				
ACSM5	141.0	84.1	12.6	1.6	1.2	0.4	1.1
ACSL1	2,730.0	3,750.0	1,830.0	31.2	52.6	56.1	46.6
ACSL3	54.1	77.4	46.5	0.6	1.1	1.4	1.0
ACSL4	30.3	36.0	7.1	0.3	0.5	0.2	0.4
ACSL5	369.0	244.0	402.0	4.2	3.4	12.3	6.7
ACSL6	5.5	5.4	6.1	0.1	0.1	0.2	0.1
ACSVL1	921.0	300.0	88.4	10.5	4.2	2.7	5.8
ACSVL2	ND	ND	ND				
ACSVL3	51.6	43.0	37.2	0.6	0.6	1.1	0.8
ACSVL4	70.5	51.2	14.1	0.8	0.7	0.4	0.7
ACSVL5	74.8	46.4	66.5	0.9	0.7	2.0	1.2
ACSVL6	1,483.0	464.5	100.2	17.0	6.5	3.1	8.8
ACSF1	ND	ND	ND				
ACSF2	ND	ND	ND				
ACSF3	15.5	13.6	10.8	0.2	0.2	0.3	0.2
ACSF4	33.2	27.5	21.3	0.4	0.4	0.7	0.5
ACSBG1	ND	ND	ND				
ACSBG2	ND	ND	ND				
Total	8,739.9	7,134.3	3,263.0	100	100	100	100

ND: Not detected.

Credit author statement

Identification of an isoform catalyzing the CoA conjugation of nonsteroidal anti-inflammatory drugs and the evaluation of the expression levels of acyl-CoA synthetases in the human liver

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