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Activation of Morphine Glucuronidation by Fatty Acyl-CoAs and Its Plasticity: A Comparative Study in Humans and Rodents Including Chimeric Mice Carrying Human Liver

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Summary: The formation of morphine-3-glucuronide (M-3-G, pharmacologically inactive) and morphine-6-glucuronide (M-6-G, active metabolite) by liver microsomes from humans and rodents, including chimeric mice carrying human liver, was evaluated in the presence of fatty acyl-CoAs. Medium- to long-chain fatty acyl-CoAs, including oleoyl-CoAs, at a physiologic level (around 15 μM) markedly enhanced M-3-G formation catalyzed by rat liver microsomes. A separate experiment indicated that 15 μM oleoyl-CoA enhanced 14C-UDP-glucuronic acid (UDPGA) uptake by microsomes. The activation by acyl-CoAs disappeared or was greatly reduced by either pre-treating microsomes with detergent or freezing/thawing the rat liver before preparation. Many of the microsomes prepared from frozen human livers (N = 14) resisted oleoyl-CoA-mediated activation of UDP-glucuronosyltransferase (UGT) activity, including M-6-G formation, which is highly specific to humans. In sharp contrast, the activity of M-6-G and M-3-G formation in freshly-prepared hepatic microsomes from chimeric mice with humanized liver was potently activated by oleoyl-CoA. Thus, acyl-CoAs activate morphine glucuronidation mediated by human as well as rat UGTs. This activation is assumed to be due to the acyl-CoA-facilitated transportation of UDPGA, and microsomes need to maintain the intact conditions required for the activation. The function of UGT appears to be dynamically changed depending on the cellular acyl-CoA level in many species.

Keywords: UDP-glucuronosyltransferase; activation; chimeric mice; fatty acyl-CoA; human liver; morphine

Introduction

Morphine is still an important drug for the relief of moderate to severe pain. This drug is metabolized mainly to its 3- and 6-glucuronides (M-3-G and M-6-G, respectively),1,2) and the former is a major metabolite, with no analgesic activity, in many species.3,4) In contrast, M-6-G exhibits a more potent analgesic effect than the parent morphine.5) The strong analgesic effect of M-6-G is also supported by a clinical study.5) Whereas M-3-G formation occurs ubiquitously in many animal species, M-6-G production is rather specific to humans and the guinea pig.3,4) It is established that UGT2B7 plays a crucial role in the human metabolism of morphine, and this isoform has the ability to produce both glucuronides.7) There are great inter-individual differences in the pharmacological effects of morphine in humans.8) Concerning the mechanism, a recent study has suggested that the combination of genetic polymorphisms in ATP-binding cassette transporter B1 (ABCB1) and μ-opioid receptor correlates
Materials and Methods

Chemicals: Morphine hydrochloride was obtained from Takeda Chemical Industries, Co., Ltd. (Osaka, Japan). UDP-glucuronic acid (UDPGA) (trisodium salt) was purchased from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). [14C]UDPGA (specific activity, 6.67 GBq/mmol) was obtained from Perkin Elmer (Boston, MA). M-3-G and M-6-G were synthesized in our laboratory. Egg yolk L-t-0-phosphatidylcholine (type XI-E) and the following fatty acids and acyl-CoAs were obtained from Sigma-Aldrich (St. Louis, MO): acetyl-CoA sodium salt (C2:0), caproyl-CoA 3Li·3H2O (C6:0), capryloyl-CoA Li·H2O (C8:0), decanoyl-CoA Li·H2O (C10:0), myristic acid/myristoyl-CoA Li (C14:0), palmitic acid/palmitoyl-CoA Li (C16:0), stearic acid/stearoyl-CoA Li (C18:0), oleoyl-CoA Li (C18:1), linoleic acid/linoleoyl-CoA Li (C18:2), and arachidonic acid/arachidonoyl-CoA Li (C20:4). Acetic acid (C2:0), caproic acid (C6:0), capric acid (C10:0), oleic acid sodium salt (C18:1), linolenic acid sodium salt (C18:3), and polyoxyethylene cetlylalcohol ether (Brij 58) were purchased from Nacalai Tesque Co. (Kyoto, Japan). 4-Acetamido-4'-disulfonic acid (SITS) and 4,4'-disulfonic acid (DIDS) were purchased from Sigma-Aldrich. Recombinant UGT2B7 microsomes (Supersomes) were obtained from Gentest, BD Biosciences (Woburn, MA). All other reagents were of analytical grade.

Preparation of microsomes: Normal human liver tissue is obtained when metastatic liver tumors are surgically removed from patients. Liver samples were obtained from 14 Japanese patients who gave their written informed consent to participate in this study. The experimental purpose and design of this study were pre-approved by the Ethics Committee of the Graduate School of Medicine, Kyushu University. The tissue donors were 9 women and 5 men between 38 and 78 years of age (median age: 60 and 69, respectively). Their livers, except for the tumor region, were assumed to function normally because the levels of serum bilirubin (free and conjugated forms) were within the normal range. The liver specimens were stored at −80 °C until the preparation of microsomes. Microsomes were obtained by differential centrifugation, washed once with 1.15% KCl, re-suspended in 0.25 M sucrose, and stored at −80 °C until use.

Animal experiments in this study were conducted under the approval of the Ethics Committee for Animal Experiments of Kyushu University (rat study), or the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board (study with chimeric mice). Male Sprague-Dawley rats (160–180 g) were obtained from Kyudo Co. Ltd. (Kumamoto, Japan). Chimeric mice carrying human liver were produced according to the methods of Tateno et al. All chimeric mouse livers used with the inter-individual variance in pain relief produced by morphine. In addition, it has been demonstrated that a nucleotide change in UGT2B7 promoter significantly alters its promoter activity. However, the genetic polymorphisms mentioned above do not seem to fully explain the differences in the analgesic effect of morphine.

UDP-Glucuronosyltransferase (UGT) is a phase II drug-metabolizing enzyme that catalyzes glucuronidation of both endo- and xenobiotics, including a number of drugs. This enzyme is anchored at its C-terminal to the endoplasmic reticulum (ER) membrane, and the main body, including the catalytic domain, is located within the ER. Because of this orientation, UGT has a diagnostic feature, the so-called latency, whereby treating tissue microsomes with detergent greatly enhances the activity. In addition, UDP-glucuronic acid (UDPGA), a co-substrate, must be supplied from the cytosol to the luminal side of the ER for the UGT reaction to occur. UGT can be inhibited by a number of clinical drugs; however, information about whether endogenous substances modulate UGT function is limited. UDP-N-acetylglucosamine is considered to be one of the endogenous activators of UGT. However, the inhibitory effects of medium- and long-chain fatty acyl-CoAs on UGT have been reported in some detail. In accordance with these reports, Yamashita et al. have also observed that the inhibition of partially purified UGTs by acyl-CoA takes place in a concentration-dependent manner. In all the above studies, the inhibitory effects of fatty acyl-CoA were observed with either partially purified UGT or microsomes under denaturing conditions. However, our previous study has suggested that acyl-CoAs activate, rather than inhibit, the catalytic function of UGT in intact microsomes. More specifically, in rats, the hepatic activity of 4-methylumbelliflorone glucuronidation was activated by acyl-CoAs only when microsomes untreated with detergent were used as the enzyme sources. However, it is largely unknown whether the same thing occurs for human UGT and for substrates other than 4-methylumbelliflorone.

To clarify whether morphine glucuronidation is affected by acyl-CoAs, we initially designed a comparative study using hepatic microsomes from humans and rats. During the course of the present studies, we discovered that microsomes prepared from frozen human livers lose their sensitivity to the acyl-CoA-dependent activation of UGT activity. This finding led us to provide further evidence that the lack of activation is not due to the peculiar features of human UGT. This is because the livers from chimeric mice carrying human liver retained sensitivity to acyl-CoA-dependent activation of M-6-G formation. We describe here the acyl-CoA-produced activation of human UGT and its plasticity. Furthermore, the possible mechanism of the activation is also discussed.
in this study were derived from a single donor. The donor was a 2-year-old Caucasian boy who died in a motor vehicle accident and had no medical history; (Donor # HH170, Lot # 87, Cat # 454504, BD Gentest Cryo human hepatocytes that were prepared on November 12, 2005). The replacement index (RI, %), which is a measure of the ratio of human liver in the whole liver, was evaluated by analyzing the serum level of human albumin (hAlb). Mice with an RI of over 45% were used in this study. The serum level of hAlb was between 2.63 and 7.90 mg/ml. Rat and mouse hepatic microsomes were prepared in a similar way to the preparation of human microsomes. Unless otherwise stated, rat and mouse hepatic microsomes were prepared without freezing the tissues. Microsomal protein was determined by the method of Lowry et al. using bovine serum albumin as a standard.

**Assay of morphine glucuronidation:** Glucuronidation of morphine was assayed according to the method of Oguri et al. with slight modifications. Briefly, incubation mixtures consisted of 50 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, 150 μg l-α-phosphatidylcholine, 5 mM morphine, 2 mM UDPGA, and rat liver microsomes (20 μg protein) in a final volume of 0.3 ml. In the assay of human and mouse liver microsomal activity, the following modifications were introduced: the buffer was changed to 50 mM Tris-HCl buffer (pH 7.8), and 5 mM saccharolactone was added to inhibit β-glucuronidase. In some experiments, microsomes were treated before assay with acyl-CoAs, fatty acids, Brij 58 (0.5 mg/mg microsomal protein), or their combination, on ice for 30 min. Incubation was started by adding microsomes and microsomal protein), or their combination, on ice for 30 min followed by centrifugation at 12,000 rpm and 4°C for 10 min. The resulting supernatant (50 and 100 μL for human/mouse and rat liver microsomes, respectively) was subjected to high-performance liquid chromatography (HPLC).

**HPLC conditions:** M-3-G produced by morphine incubation with rat liver microsomes was analyzed by UV detection under the conditions described elsewhere. Formation of M-3-G and M-6-G from morphine by human/mouse liver microsomes was determined using fluorescence detection according to the method of Antonilli et al. with slight modifications. Chromatographic analysis was carried out using a LaChrome Elite HPLC system equipped with an automatic sampler (model L-2200), pump (L-2130 HTA), and fluorescence detector (L-2485) (Hitachi High-Technologies Co., Tokyo, Japan). Data were stored and processed using D-7000 HPLC System Manager software (version 3.1; Hitachi High-Technologies Co.). Separation was achieved using a Nova-Pak C18 Cartridge (4 μm, 8 × 100 mm) column with an attached guard-Pak pre-column (Waters Co., Milford, MA). The column was eluted with a linear gradient solution prepared by mixing eluent A (100 mM potassium phosphate, pH 3.0) and eluent B [acetoniitrite/100 mM potassium phosphate, 20:80 (v/v), pH 3.0]: the concentration of acetonitrile was increased from 4.8 to 10% for 0 to 10 min and then changed from 10 to 20% during the period 10 to 15 min. The flow rate was set at 0.8 ml/min. Morphine glucuronides were quantified by fluorescence detection (excitation wavelength 210 nm, and emission wavelength 350 nm).

**UGT2B7 genotyping:** The UGT2B7 genotypes were ascertained by sequencing four alleles: UGT2B7*1 (wild type), UGT2B7*2 (802C>T, H268Y), UGT2B7*3 (211G>T, A71S), and UGT2B7*4 (1192G>A, D398N). Genomic DNA was prepared from the peripheral lymphocytes of the liver donors, and the target region where the base substitution is located was amplified by polymerase chain reaction (PCR). A typical reaction mixture (50 μL) for PCR consisted of 100 ng genomic DNA, 1.5 mM MgCl₂, 0.2 mM dNTPs, 5 μL 10× PCR buffer, primers, and 2.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The amplification primers used were UGT2B7*2: 5′-AGACAATGGGGGAAAGCTGACG-3′ (forward, the region spanning 743 and 763 bp from the initiation codon) and 5′-ATCAACATTTGTAAGTGTCAT-3′ (reverse, 825–803 bp); UGT2B7*3: 5′-TTAATTTTCTATACAATCTG-3′ (forward, 23–42 bp) and 5′-ATGAGTGAAATTTCTCCAACCT-3′ (reverse, 272–253 bp) (primer sequences were provided by Dr. Ieiri, Graduate School of Pharmaceutical Sciences, Kyushu University); UGT2B7*4: 5′-CCCTTTGATCCTATTCTACT-3′ (forward, exon 4, −93 to −74 bp) and 5′-CCACCTA-GTGAATAATTTGGTC-3′ (reverse, exon 5, +8 to +30 bp). After initial denaturation at 94°C for 9 min, annealing and extension as described below were repeated for 30 cycles: 94°C for 60 s, 52–56°C for 30–60 s, and then 72°C for 10–80 s. Finally, the mixture was held at 72°C for 10 min. PCR products were analyzed on 2% agarose gels to check their size and were sequenced directly by an ABI 373A automatic sequencer (Applied Biosystems). The sequencing primers were the same as those used in the PCR amplifications.

**Assay of UDPGA uptake into the microsomes:** The transport assay was performed by the published method with slight modifications. Briefly, the reaction mixture (100 μL) consisted of intact microsomes (20 μg protein), 1 μM [14C]UDPGA, 0.25 M sucrose, 10 mM MgCl₂, and 20 mM Tris-HCl, pH 7.4. Microsomes were treated with oleoyl-CoA for 30 min on ice. The reaction (37°C, 2 min) was started by adding membrane vesicles
and stopped by rapid filtration [Unifilter–96 GF/B plate that was adapted to a 96-well Perkin Elmer Filtermate Universal Harvester (Perkin Elmer Inc., Boston, MA)]. The microsomes on the membrane were washed three times with 200 μL ice-cold buffer, and with more than 1 mL of the same buffer (0.25 M sucrose, 10 mM MgCl₂, 20 mM Tris-HCl, pH 7.4, and 1 μM non-radioabeled UDPGA). The filter plate was dried overnight and the bottom was sealed with Packard backing tape before adding 25 μL Microscint 20 (Packard Biosciences Co., Groningen, Netherlands). The radioactivity of [¹⁴C]UDP-GA taken up by the microsomes and collected on the filter was counted in a TopCount microplate scintillation and luminescence counter (Perkin Elmer Inc).

**Data analysis:** Significant differences between groups were assessed by one-way ANOVA with Tukey post-hoc analysis using SPSS Rel. 13.0 software (SPSS, Chicago, IL). Kinetic parameters for the UGT reaction were calculated using SigmaPlot 10.0 software assisted by Enzyme Kinetics 1.3 software (SPSS). In this calculation, the data were fitted to a Michaelis-Menten equation by the nonlinear least-squares method.

**Results**

**Effect of acyl-CoAs on morphine glucuronidation catalyzed by rat liver microsomes:** Prior to our study with human livers, we examined the effect of acyl-CoAs on rat UGT to obtain basic information. When we used rat liver microsomes not treated with a detergent as the enzyme source, oleoyl (C18:1)-CoA and palmitoyl (C16:0)-CoA activated M-3-G formation (Fig. 1A, B). A maximum activation of more than 4-fold that of the control was observed using 15 μM oleoyl- and palmitoyl-CoAs. In contrast, both acyl-CoAs only inhibited UGT activity catalyzed by microsomes pretreated with detergent Brij 58 (Fig. 1A, B). These findings suggest that acyl-CoAs at lower concentrations have the ability to activate morphine glucuronidation mediated by UGTs if the microsomes are intact.

To investigate the specificity of the acyl-CoA-produced modulation of morphine glucuronidation, the effects of 10 acyl-CoAs with different carbon chain lengths were compared using untreated rat liver microsomes (Fig. 2A, B). The results showed that saturated and unsaturated fatty acyl-CoAs with chains consisting of 14 or more carbons exhibited an activating profile, whereas acyl-CoAs with 10 carbons or less failed to activate morphine glucuronidation (Fig. 2A). When we used Brij 58-treated microsomes, acyl-CoAs inhibited M-3-G formation in a concentration-dependent fashion (Fig. 2B). In this case, the magnitude of the inhibitory effect depended on the length of the alkyl chain, and acyl-CoAs with longer chains exhibited stronger effects (Fig. 2B). Since fatty acids can be released from acyl-CoAs, it is possible that fatty acids contribute to the acyl-CoA-dependent activation of morphine glucuronidation. An unsaturated fatty acid, oleic acid (C18:1), enhanced M-3-G-forming activity mediated by rat liver microsomes in a concentration-dependent manner over the range from 20 to 75 μM. In contrast, a saturated fatty acid, stearic acid (C18:0), failed to activate morphine glucuronidation, even at 200 μM (Fig. 3A). Unsaturated fatty acids (C16:1, C18:1, C18:2, and C18:3) at 15 μM had weak activating effects, although arachidonic acid (C20:4) markedly activated morphine UGT at the same concentration (Fig. 3B). However, all of these unsaturated fatty acids caused marked activation at 50 μM. In contrast to the case for unsaturated fatty acids, a series of saturated fatty acids (C2:0–C18:0) did not show any effect, even at 50 μM (Fig. 3B). Thus, because the concentration-dependence in the activation of morphine UGT differs between fatty acids and acyl-CoAs, the activating effect of acyl-CoAs is suggested not to be due to liberated free fatty acids. CoA-SH did not have any effect on UGT function either in the absence or presence of detergent.
Fig. 2. Specificity of acyl-CoAs with regard to the modulation of morphine UGT activity
The microsomes were pre-treated with acyl-CoA (15 and 50 μM) in the absence (A) or presence (B) of Brij 58, and then UGT activity with regard to the 3-hydroxy group of morphine was determined. See also the legend to Fig. 1 for the experimental conditions. Each bar represents the mean ± S.E. of triplicate assays. Significantly different from control without acyl-CoA (*, p < 0.05; **, p < 0.01; ***, p < 0.001). The abbreviations used are: C2:0, acetyl-CoA; C6:0, caproyl-CoA; C8:0, capryloyl-CoA; C10:0, decanoyl-CoA; C14:0, myristoyl-CoA; C16:0, palmitoyl-CoA; C18:0, stearoyl-CoA; C18:1, oleoyl-CoA; C18:2, linoleoyl-CoA; and C20:4, arachidonoyl-CoA; ND, not detected.

Fig. 3. Effect of fatty acids on morphine UGT activity
The UGT activity with regard to the 3-hydroxy group of morphine was measured in the presence of fatty acids. In all assays, rat hepatic microsomes (20 μg protein) were used as the enzyme source and were pre-treated with fatty acid on ice for 30 min prior to assay. Fatty acids were dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide in the incubation mixture was 0.33%. In experiment A, the dose-effect relationship was compared between a saturated fatty acid (C18:0) and an unsaturated fatty acid (C18:1). In experiment B, microsomes were treated with 15 or 50 μM fatty acid. Data represent the mean ± S.E. of triplicate assays. Significantly different from control without fatty acid (**p < 0.01, and ***p < 0.001). The abbreviations used are: (saturated fatty acids) C2:0, acetic acid; C6:0, caproic acid; C10:0, capric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid and (unsaturated fatty acids) C16:1, palmitoleic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; and C20:4, arachidonic acid.

(data not shown). These results suggest that acyl-CoA itself modulates UGT-catalyzed morphine glucuronidation, although free fatty acids may make some contribution to the effect of unsaturated fatty acyl-CoA.

Kinetics of morphine glucuronidation in the presence of acyl-CoAs: We then determined the kinetic parameters for M-3-G formation using rat liver microsomes not treated with detergent in the presence of 15 and 50 μM oleoyl-CoA, which cause activation and inhibition of UGT function, respectively. As shown in Table 1, kinetics analyses were performed involving morphine and UDPGA as substrate and co-substrate, respectively. The reaction by rat liver microsomes exhibited apparent monophasic kinetics with regard to both morphine and UDPGA (data not shown). When the morphine concentration was varied, the apparent Km for M-3-G formation was increased 1.8- and 2.8-fold by 15 and 50 μM oleoyl-CoA, respectively. In contrast, 15 μM oleoyl-CoA increased the velocity 4.3-fold compared with that in the absence of oleoyl-CoA, giving a higher intrinsic clearance ($V_{\text{max}}/K_{m}$). Oleoyl-CoA at 50 μM gave a reduced velocity, resulting in a reduction in intrinsic clearance. Table 1 also shows the kinetic parameters for different UDPGA concentrations. These results demonstrated that the apparent $K_{m}$ was reduced to half the level of the control by 15 μM oleoyl-CoA, whereas the velocity was increased 4.2-fold, resulting in an 8-fold increase in the intrinsic clearance. Although the apparent $K_{m}$ remained unchanged in the presence of 50 μM oleoyl-CoA, this condition reduced the $V_{\text{max}}$ by 50%. These results suggest that oleoyl-CoA enhances UGT-catalyzed morphine glucuronidation mainly due to the increased velocity, although an increase in the affinity of UGT for UDPGA
may also be involved in the activation.

**Effect of acyl-CoAs on UGT-catalyzed morphine glucuronidation in human liver microsomes**: To examine whether oleoyl-CoA activates morphine glucuronidation catalyzed by human liver microsomes, we determined the effect of acyl-CoA using liver tissues from 14 individuals. The results showed that although 15 μM oleoyl-CoA significantly activated both M-3-G and M-6-G formation catalyzed by liver microsomes from 5 donors (HLM# A, C-F), marked activation was observed in only 3 of these 5 donors (Fig. 4A, B). Furthermore, oleoyl-CoA failed to activate morphine glucuronidation in the other 7 samples, despite the absence of detergent treatment (Fig. 4A, B). When we used recombinant UGT2B7, a human morphine UGT, artificially expressed in insect cells (Supersome), oleoyl-CoA inhibited morphine glucuronidation both in the absence and presence of detergent (Fig. 5A, B). The genotypes of the UGT2B7 alleles in the 14 donors described above were: *1/*1 (homozygous of wild-type allele), HLM#A, B, D-F, H, I, K and M; *1/*3, HLM#C, J, L, and N; and *2(*2)(*3)(a new haplotype containing 802C>T and 211G>T), HLM#G. The UGT2B7*T allelle was not found in the samples tested. Of the samples with the homozygous wild-type allele, HLM#A, B, and D-F were sensitive to acyl-CoA-dependent activation. In addition, 2 other samples (HLM#C and G) which were sensitive to oleoyl-CoA-mediated activation had *1/*3 and *2(*2)(*3) alleles, respectively. It is, therefore, unlikely that the activation of morphine glucuronidation depends on the UGT genotype. In contrast to the activation effect, the inhibitory effect of 50 μM oleoyl-CoA on the formation of M-3-G and M-6-G was observed in all microsomes from the 14 donors (Fig. 4A, B).

**Effect of freezing and thawing on the oleoyl-CoA-dependent activation of morphine glucuronidation**: Conceivably, the absence of acyl-CoA-mediated activation in the majority of human liver specimens as well as in heterologously expressed UGT2B7 is attributable to denatured and/or different UGT environments. To address this issue, we examined the effect of freezing/thawing procedures on acyl-CoA-dependent activation of UGT activity using rat livers. The results clearly demonstrated that freezing and thawing of the liver before preparing microsomes markedly reduced the sensitivity of oleoyl-CoA-dependent activation of morphine glucuronidation (Fig. 6). Thus, this seems to be one of the reasons why oleoyl-CoA failed to activate morphine UGT activity in the majority of the human livers. In contrast to the activation, suppression of UGT activity by 50 μM oleoyl-CoA remained unchanged even after liver freezing (Fig. 6). This observation suggests that only the activation by acyl-CoA exhibits plasticity and requires the ER to be intact for it to take place.

**Table 1. Effect of oleoyl-CoA on the kinetic parameters of morphine-3-glucuronide formation by rat hepatic microsomes**

<table>
<thead>
<tr>
<th>Oleoyl-CoA (mM)</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg of protein)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 μM)</td>
<td>0.28 ± 0.05</td>
<td>1.33 ± 0.03</td>
<td>4.75</td>
</tr>
<tr>
<td>15 μM</td>
<td>0.50 ± 0.06</td>
<td>5.71 ± 0.12</td>
<td>11.4</td>
</tr>
<tr>
<td>50 μM</td>
<td>0.79 ± 0.38</td>
<td>0.92 ± 0.09</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Kinetic analysis regarding M-3-G formation was carried out in the absence and presence of oleoyl-CoA. The reaction mixture consisted of rat liver microsomes (200 μg protein), 2 mM UDPGA, 15 or 50 μM oleoyl-CoA, and 5 mM morphine. In kinetics A and B, the concentrations of morphine (0.1–20 mM) and UDPGA (0.1–5 mM) were varied over the ranges indicated. Data were fitted to a Michaelis-Menten equation using the SigmaPlot 10.0 enzyme kinetics module 1.3. Values for $K_m$ and $V_{max}$ represent the estimated value ± S.E.
liver: To evaluate more clearly the effect of acyl-CoA on morphine glucuronidation in humans, we next used chimeric mice with humanized liver as an alternative model. In this experiment, we used microsomes prepared freshly from unfrozen livers. As mentioned in the Introduction, M-6-G-forming activity is regarded as a specific marker for human UGT,⁴ and this activity is very low in mice.⁴ Indeed, the activity of M-6-G formation in the liver microsomes of chimeric mice was found to be comparable with that of human liver microsomes (Figs. 4 and 7). In addition, the activity of M-6-G formation in chimeric mice was correlated with RI, which was estimated from the serum level of hAlb. In contrast, the M-3-G-forming activity exhibited a poor correlation with RI (Fig. 7). The correlation coefficient between M-6-G-forming activity and hAlb was calculated to be 0.747 (p = 0.01 level, Pearson analysis). Absence of correlation between M-3-G forming activity and RI indicates that this glucuronidation activity is mediated by multiple isoforms including UGT2B7 and mouse UGT(s). As shown in Figs. 7A and B, 15 μM oleoyl-CoA activated M-6-G as well as M-3-G formation by all microsomes from 15 chimeric mice except for animals No. 9 (M-3-G) and No. 14 (M-6-G). These results strongly suggest that acyl-CoAs activate the catalysis of both human and mouse UGTs.

Regarding the inhibitory effect of oleoyl-CoA at 50 μM, only 7 of 15 livers were susceptible to the acyl-CoA-dependent inhibition of M-6-G formation (Fig. 7B). In contrast, oleoyl-CoA inhibited the activity of M-3-G formation in 13 livers (Fig. 7A). The significant inhibition of M-3-G formation was observed in most livers of chimeric mice with a high RI. Thus, it is conceivable that human UGT2B7 in the liver of chimeric mice is sensitive to acyl-CoA-dependent inhibition. The reason why a difference in the sensitivity was observed between M-3-G and M-6-G formation activities is unclear. Alternatively, when UGT2B7 baculosomes without detergent-treatment were used, oleoyl-CoA up to 20 μM inhibited M-3-G formation while M-6-G formation was not markedly changed over the range of oleoyl-CoA concentrations used (Fig. 5). No such difference was observed following treatment with 50 μM oleoyl-CoA. Thus, both M-3-G and M-6-G formation were inhibited by 50 μM oleoyl-CoA. However, when the UGT2B7 baculosomes under detergent-treated conditions were used, both M-3-G and M-6-G formation were only inhibited in an oleoyl-CoA-concentration-dependent fashion. It is assumed that the acyl-CoA-induced inhibition of UGT2B7 largely depends on the na-
Fatty Acyl-CoAs Activate Morphine UGT in Human

Fig. 7. Oleoyl-CoA-dependent modulation of morphine UGT activity in liver microsomes of chimeric mice with humanized liver

The UGT activity with regard to M-3-G (A) and M-6-G formation (B) was measured in the absence and presence of oleoyl-CoA. Liver microsomes were prepared from unfrozen livers from 15 chimeric mice and were pre-treated with oleoyl-CoA (15 and 50 μM) on ice for 30 min before assay. Each column represents the mean ± S.E of triplicate assays. As shown beneath panel B, the data are placed in order of RI (Replacement Index, %), a measure of the ratio of human liver in the whole liver. See Materials and Methods for the RI details. White numbers in closed circles indicate male mice and black numbers in open circles mean female mice. Significantly different from control without oleoyl-CoA (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Fig. 8. Effect of oleoyl-CoA on [14C]UDPGA uptake into microsomes

Rat liver microsomes (20 μg) were treated with the indicated concentration of oleoyl-CoA for 30 min on ice, and then [14C]UDPGA uptake was determined by the rapid filtration method as described in Materials and Methods. Each bar represents the mean ± S.E. of triplicate assays. Significantly different from control without oleoyl-CoA (*, p < 0.05; **, p < 0.01).

Fig. 9. Effect of anion-transport inhibitors on the oleoyl-CoA-dependent modulation of morphine UGT activity

Morphine UGT activity was measured in the presence of the indicated concentration of oleoyl-CoA, with and without anion transport inhibitors (SITS and DIDS). Rat liver microsomes were treated with anion-transport inhibitors for 30 min on ice and then the M-3-G-forming activity was determined. Each bar represents the mean ± S.E. of triplicate assays. Significantly different from control in the presence of same concentration of oleoyl-CoA (***, p < 0.001).

Effect of fatty acyl-CoAs on UDPGA uptake into the microsomes:

As shown in the Table 1, a kinetic study using different UDPGA concentrations revealed that the apparent $K_m$ for M-3-G formation catalyzed by rat liver microsomes was reduced to half the level of the control with 15 μM oleoyl-CoA. Therefore, an increase in the affinity of UGT toward UDPGA may also be involved to some extent in the enhancement of morphine UGT activity. Alternatively, if the UDPGA concentration in the lumen of the ER was increased by acyl-CoA treatment, the apparent $K_m$ for UDPGA should decrease. Although the enhancing effect of acyl-CoA seems to be mainly due to an increase in the $V_{max}$ (see above), it is conceivable that fatty acyl-CoA affects UDPGA transport rather than UGT itself to activate glucuronidation. To address this possibility, we examined the effect of acyl-CoA on [14C]UDPGA uptake by rat liver microsomes.

The results showed that UDPGA uptake was enhanced two-fold by 15 μM oleoyl-CoA (Fig. 8). Very little UDPGA transport was observed in the presence of 50 μM oleoyl-CoA, probably because the ER membrane was disrupted by the detergent-like effect of acyl-CoA at higher concentrations (Fig. 8). Therefore, it is assumed that the acyl-CoA-produced activation of glucuronidation is due to an increase in UDPGA concentration in the lumen of the ER. The enhancement of UDPGA uptake to micro-
somes was shown to occur in a fatty acyl-CoA concentration-dependent fashion (Fig. 8). It is possible that oleoyl-CoA interacts with UDPGA transporter to enhance the transport of the obligate factor into the ER. In agreement with this, SITS and DIDS, anion transporter inhibitors capable of inhibiting UDPGA uptake, suppressed UGT activation by 15 μM oleoyl-CoA (Fig. 9). Activation by oleoyl-CoA almost disappeared at the highest concentrations of the inhibitors (Fig. 9). Thus, enhanced UDPGA transport is considered to be, at least in part, involved in the activation of morphine glucuronidation.

**Discussion**

As has long been known, the pharmacological effects of morphine vary markedly between individual humans. However, it is unlikely that polymorphisms of the related genes fully explain the difference. To resolve this issue, we proposed an alternative possibility that protein-protein interaction between UGT2B7 and cytochromes P450 significantly affects the function of the former enzyme. This would be a possible mechanism rationalizing the inter-individual variability in morphine effects which are not fully accounted for by single nucleotide polymorphisms. In addition to this, the present study revealed that acyl-CoA levels have a substantial effect on the catalytic efficiency of UGT with respect to morphine.

Fatty acyl-CoA acts as a detergent. As mentioned before, the catalytic activity of microsomal UGT is greatly enhanced by detergent treatment because of its location within the ER. Considering these findings, one possibility is that acyl-CoA activates UGT function by its effect as a detergent. When rat liver microsomes were used, the maximum activation of morphine UGT by oleoyl- and palmitoyl-CoAs was observed at 15 μM (Fig. 1A, B). Whereas oleoyl-CoA at this concentration did not affect the resistant nature of microsomal UGTs toward trypsin treatment, acyl-CoA at 50 μM caused tryptic digestion of UGTs, even in the absence of detergent. Further, the critical micelle concentration of fatty acyl-CoAs, such as palmitoyl-CoA and oleoyl-CoA, was reported to be between 33 and 80 μM. It is, therefore, evident that acyl-CoA at lower concentrations activates microsomal UGT by a mechanism distinct from its detergent effect. Conversely, as detergent-treated microsomes lose their sensitivity for activation, it is strongly suggested that an intact ER membrane is needed for the activation of UGT-catalyzed morphine glucuronidation by acyl-CoA. In contrast to the case for rat liver microsomes, oleoyl-CoA failed to activate morphine glucuronidation catalyzed by recombinant UGT2B7, despite the absence of detergent. It is well-known that recombinant UGTs expressed heterologously in host cells lose their latency, probably due to the different mode/environment of expression from native enzymes; for example, their activation does not occur even after detergent treatment. This fact also supports the view that acyl-CoA requires intact ER membrane for the activation of UGT.

The total concentration of cellular long-chain acyl-CoAs varies over the range 5–160 μM, depending on the physiological conditions, such as fasting, diabetes, and fat and glucose intake. The level of long-chain acyl-CoAs in rat liver (depending on the feeding conditions) is between 18.8 and 64.4 μM. It has also been suggested that 20–40% of cellular acyl-CoA is present in cytosol. Taking these findings into consideration, the cytosolic pool of acyl-CoA is calculated to be 4–26 μM. It is, therefore, reasonable to suppose that the activation by acyl-CoA at around 15 μM of UGT-catalyzed morphine glucuronidation takes place under normal physiological conditions.

Alternatively, cellular fatty acids are initially converted to fatty acyl-CoA by acyl-CoA synthetases before being used in the metabolic process. Fatty acyl-CoA subsequently serves as an acyl donor for the biosynthesis of both triglyceride and glycerophospholipid in the Kennedy pathway. There are many enzymes involved in the Kennedy pathway, such as glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase, and diacylglycerol acyltransferase (DGAT). An acyl-CoA concentration close to 15 μM, which is needed for morphine UGT activation, is also required by other enzymes in cells, such as GPAT and DGAT. GPAT1 purified from rat liver exhibits a K of 3–15 μM for fatty acyl-CoAs and a K of 0.5–1 mM for glycerol. DGAT2 acts on oleoyl-CoA in the concentration range 0–50 μM. It is, therefore, highly likely that the acyl-CoA concentration of around 15 μM needed for UGT activation is the physiological concentration, because intermediary enzymes work under similar conditions.

At least two mechanisms can be considered for the inhibition of UGT by acyl-CoAs at higher concentrations. One of them is UGT acylation, which can contribute to the inhibition. Another possibility is the inhibitory effect of acyl-CoA itself on UGT. The observation that acyl-CoAs, such as arachidonoyl-CoA, bind to UGT would support this view. As discussed before, acyl-CoA at higher concentrations, renders microsomes permeable, allowing free access to the functional domain(s) of UGT. This seems to be a reasonable explanation for UGT inhibition at higher concentrations of acyl-CoA. Since fatty acids can be released from acyl-CoAs, it cannot be excluded that fatty acids partially contribute to UGT inhibition. Unsaturated fatty acid has been reported to inhibit 4-methylumbelliferone glucuronidation by human kidney microsomes or human recombinant UGT1A9 at 1–3 μM. In marked contrast, our present study demonstrated that unsaturated fatty acids at a concentration greater than 20 μM increase morphine glucuronidation catalyzed by rat liver microsomes (Fig. 3A). Although
the reason for this discrepancy is unknown, the presence of such a difference suggests that the activation or inhibition effect of unsaturated fatty acids may again depend on the nature of the UGT membrane environment.

A kinetic study varying the UDPGA concentration revealed that the apparent \( K_m \) was reduced in the presence of 15 \( \mu M \) oleoyl-CoA compared with the control, whereas in velocity was increased, resulting in a marked increase in morphine glucuronidation efficiency \( (V'_{\text{max}}/K_m) \). This result suggests the enhanced transportation of UDPGA from cytosol into the ER by oleoyl-CoA. The observation that 15 \( \mu M \) oleoyl-CoA enhanced \( [\text{14C}] \) UDPGA uptake by microsomes supports this view (Fig. 8). The above suggestion is also supported by previous evidence from light-scattering measurements showing that preincubation with 10 or 20 \( \mu M \) palmitoyl-CoA makes microsomal vesicles permeable to UDPGA.47) As mentioned previously, the luminal domain of microsomal UGT was resistant to tryptic digestion in the presence of 15 \( \mu M \) oleoyl-CoA.21) Therefore, facilitated uptake of UDPGA in the presence of acyl-CoA appears not to be due to simple disruption of the ER membrane.

It is assumed that UDPGA transporter(s) plays a role in supplying UDPGA from cytosol to the active site of UGTs within the ER for glucuronidation.15) Muraoka et al.31,48) have cloned human UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter (solute carrier family 35, SLC35D1) localized in the ER. This transporter is suggested to supply UDPGA to the ER with concomitant release of UDP-N-acetylgalactosamine. Although the counterpart in rats has not yet been identified, taking all these findings into consideration, it is assumed that acyl-CoA interacts with UDPGA transporter(s) to facilitate the supply of this co-substrate to the lumen of the ER. Histidyl residue(s) may play a role in UDPGA uptake into rat liver ER.49) In connection with this issue, the present study revealed that anion-transport inhibitors (SITS and DIDS) attenuate acyl-CoA-produced morphine UGT activation. These inhibitors have been reported to inhibit the carrier-mediated transport of UDPGA into the ER.15) SITS and DIDS are capable of binding covalently, through their isothiocyanato group, to various amino acid residues including lysine, arginine, histidine, tyrosine, and cysteine, although their binding affinity to the \( \varepsilon \)-amino group of lysine residues of membrane-bound carrier proteins seems to be the highest.50) As SITS and DIDS are membrane impermeable, their binding site is likely to be on the cytosolic side of the UDPGA transporter.51) The above pieces of evidence also support the involvement of UDPGA transporter in the enhancement of morphine glucuronidation by acyl-CoAs.

All human livers used in the present study were frozen before preparing microsomes. As shown in Figure 6, hepatic microsomes from frozen rat liver lost their sensitivity for acyl-CoA-mediated activation of UGT activity. Conversely, hepatic microsomes prepared freshly from humanized mice exhibited activation by oleoyl-CoA, although the same was not observed for the majority of frozen human livers. From these pieces of evidence, it is highly likely that the freezing/thawing procedures, especially tissue freezing, cause an irreversible change in the conditions of the ER membrane so that they are no longer compatible with those needed to regulate UGT function. We were unable to identify the target(s) of freezing damage which leads to the impairment of UGT function. As discussed before, acyl-CoAs may enhance UGT activity by activating UDPGA transporter. Therefore, it is possible that tissue freezing irreversibly impairs the function of this transporter or its regulator(s). It is widely accepted that the early examination of drug pharmacokinetics in humans is a key step in the development of a new drug. Human tissues are often used in this process. However, the present study suggests that frozen human livers should be used with care. This is because, as far as glucuronidation is concerned, its physiological character seems to be lost after tissue freezing. It should be noted that this defect in glucuronidation may be overcome by the use of cryopreserved tissues obtained using a programmed freezer, which was unavailable in this study. Further, the property that UGT activity is activated by 15 \( \mu M \) oleoyl-CoA could act as an index indicating whether prepared liver microsomes have remained intact. On the other hand, as hepatic microsomes prepared freshly from chimeric mice retain their sensitivity toward acyl-CoA, this mouse model involving implantation of human liver has an advantage compared with frozen liver for drug development.

**Conclusion**

In conclusion, this study provided evidence that acyl-CoAs activate morphine glucuronidation in liver microsomes from humans as well as rodents. The data obtained suggest that the activation mechanism involves enhanced transport of UDPGA into the ER. Our findings suggest that the dynamics of nutritional status resulting in changes in the level of endogenous fatty acyl-CoAs greatly affects not only the elimination of morphine but also the formation of the active metabolite M-6-G. We also provided evidence that frozen liver loses sensitivity to acyl-CoAs in terms of UGT activation. The cellular mechanism, which is damaged by freezing, should be identified in future studies.

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