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Biochemical and Biophysical Research Communications 345 (2006) 1649–1656
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Fatty acyl-CoA as an endogenous activator of UDP-glucuronosyltransferases

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Received 9 May 2006
Available online 24 May 2006

Abstract

The acyl-CoA-dependent modulation of hepatic microsomal UDP-glucuronosyltransferase (UGT) function in rats was studied. Oleoyl- and palmitoyl-CoAs inhibited UGT activity toward 4-methylumbelliferone in the presence of Brij 58. However, acyl-CoAs enhanced UGT activity in untreated microsomes. A maximum activation of about 8-fold over the control was observed at 15 μM oleoyl-CoA, whereas 50 μM or more oleoyl-CoA had an inhibitory effect on UGT function. Medium- and long-chain acyl-CoAs also exhibited similar effects. On the basis of resistance to tryptic digestion of UGTs, oleoyl-CoA at 15 μM has no ability to change the permeability of the endoplasmic reticulum (ER) membrane, although perturbation of the membrane occurred with 50 μM oleoyl-CoA. *N*-Ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid) abolished the oleoyl-CoA (15 μM)-dependent activation of microsomal UGT. These results suggest that: (1) acyl-CoAs play a role as an endogenous activator of UGTs, and (2) a sulfhydryl group is required for the activation of UGT by physiological concentrations of acyl-CoAs.

Keywords: UDP-glucuronosyltransferase; Acyl-CoA; Intact microsomes; Cysteine residue; Oleoyl-CoA; Palmitoyl-CoA; Activator; Acylation; Inhibitor

Glucuronidation catalyzed by UDP-glucuronosyltransferase (UGT) is one of the detoxification pathways of both exogenous and endogenous compounds. UGT belongs to the glycosyltransferase superfamily which is expressed on the endoplasmic reticulum (ER) membrane [1]. The isoforms of UGT transfer the glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to substrates. The catalytic domain of UGT is localized on the luminal side of the ER with 19–26 amino acids of the carboxy terminal extending into the cytoplasm [2,3]. This extended region is called the cytosolic tail. Many studies have demonstrated that the activity of UGT is modulated by various substances. For example, several drugs have been demonstrated to inhibit glucuronidation [4–6]. UDP-*N*-Acetylglucosamine, one of the endogenous modulators, enhances UGT activity [7]. Early reports have shown that medium- and long-chain fatty acyl-CoAs reduce UGT activities toward various substrates [8–10]. The observation that arachidonoyl-CoA directly binds to several UGT isoforms seems to support the inhibitory effect of acyl-CoAs [8]. In these studies, the enzyme sources used were either partially purified UGTs or rat liver microsomes treated with detergents or a pore-forming agent, alamethicin. As described above, the main body of UGT is located within the ER. This membrane topology gives UGT a diagnostic feature which is called 'latency.' Due to this character, while the activity of UGT is constrained in intact microsomes, it is greatly enhanced by treating microsomes with detergents or alamethicin [11,12]. Based on this, many studies of UGT enzymology have been conducted using detergent/alamethicin-treated

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doi:10.1016/j.bbrc.2006.05.089

microsomes. Accordingly, the effect of acyl-CoAs on UGT activity reported thus far might be an artificial phenomenon not reflecting physiological conditions.

Acyl-CoAs play important physiological roles in a number of cellular reactions, such as the utilization of fatty acids and the syntheses of triglycerides and phospholipids. In addition to these roles, long-chain acyl-CoAs function as a modulator of various enzymes including UGT [13,14]. Thus, it would be of much interest to investigate the effect of acyl-CoAs on UGT under physiological conditions. In this study, we examined the acyl-CoA-dependent modulation of UGT activity in intact microsomes and found for the first time that acyl-CoAs are endogenous activators of UGT.

Materials and methods

Materials. 4-Methylumbelliferone (4-MU), egg yolk L-α-phosphatidylcholine, trypsin, and trypsin inhibitor were purchased from Sigma–Aldrich (St. Louis, MO, USA). The following fatty acids and acyl-CoAs were also obtained from Sigma–Aldrich: caproyl-CoA 3Li · $3H_2O$ (C6:0), capryloyl-CoA Li · H_2O (C8:0), decanoyl-CoA Li · H_2O (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), linoeic acid/linoleoyl-CoA Li (C18:2), linolenic acid (C18:3), and arachidonic acid/arachidonoyl-CoA Li (C20:4). UDPGA trisodium salt and CoA lithium salt were obtained from Wako Pure Chemical Industries, Co. Ltd (Osaka, Japan). Acetic acid (C2:0), oleic acid (C18:1), and 4-methylumbelliferyl-β-D-glucuronide were purchased from Nacalai Tesque Co. (Kyoto, Japan). Microsomes of insect cells transformed with baculovirus carrying human UGT1A6 cDNA (UGT1A6 BACULOSOMES™) were purchased from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade.

Preparation of rat liver microsomes. Animal experiments in this study were conducted with the approval of the Ethics Committee for Animal Experiments of the Graduate School of Pharmaceutical Sciences, Kyushu University. Male Sprague–Dawley rats (body weight, 160–180 g) were obtained from Charles River, Inc. (Yokohama, Japan). All animals were maintained under a constant light–dark cycle (light from 7:00 to 19:00) and fed a standard diet ad libitum for 1 week. Livers from rats were perfused with physiological saline, homogenized in three volumes of 0.25 M sucrose, and centrifuged at 9000*g* for 20 min. The resulting supernatant was further centrifuged at 105,000*g* for 60 min to prepare microsomes. The microsomal pellets were washed once with 1.15% KCl and resuspended in a volume equivalent to the original liver weight of 0.25 M sucrose. The microsomal preparation was divided into aliquots and stored at −80 °C until use.

Measurement of UGT activity. Glucuronidation of 4-MU was assayed fluorometrically by the method of Arias [15] with modifications [16]. In most cases, microsomes were pre-treated with acyl-CoAs, fatty acids, Brij 58 (0.5 mg/mg microsomal protein), and/or SH-modifying reagents on ice for 30 min. Incubation was started by adding microsomes to the incubation solution and performed at 37 °C for 30 min. The incubation mixture consisted of 50 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$, 150 μg L-α-phosphatidylcholine, 0.01–1.0 mM 4-MU, 0.1–5.0 mM UDPGA, and rat liver microsomes (20 μg protein) in a final volume of 0.3 ml. The kinetic parameters with standard deviations were calculated using a non-linear least-square program MULTI [17]. In this procedure, the data were fitted to a Michaelis–Menten equation.

Trypsin digestion of UGTs in rat liver microsomes. Microsomes were pre-treated with oleoyl-CoA or Brij 58 on ice for 30 min as described in Measurement of UGT activity. A 15 μl aliquot of the obtained solution was mixed with 30 μl Buffer A consisting of 75 mM Tris–HCl buffer (pH 7.4) and 15 mM $MgCl_2$ containing trypsin (50 μg/mg microsomal protein). The digestion was performed for 30 min at 20 °C and then 50 μg trypsin inhibitor in 5 μl Buffer A was added to the incubation mixture to terminate digestion. Then, the samples were subjected to Western blotting [18,19] with a non-selective anti-UGT antibody [20] or anti-UGT2B1 antibody [21]. Immunochemical staining was performed with alkaline phosphatase-labeled secondary antibody according to the method of Blake et al. [22]. When trypsin-treated microsomes were used as enzyme sources of glucuronidation assay, trypsin-treated microsomes were further treated with oleyol-CoA and then the UGT activity was assayed.

Protein assay. Protein contents were determined by the method of Lowry et al. [23] using bovine serum albumin as a standard.

Results

Acyl-CoA-dependent modulation of UGT activity: structure–effect relationship and conditions required

The effects of acyl-CoAs on hepatic microsomal UGT activity in the absence and presence of detergent were compared (Fig. 1). In this study, Brij 58 was used as a detergent for the perturbation of the microsomal membrane. The ratio of Brij 58 to microsomal protein (0.5 mg/mg protein) was confirmed to achieve the maximum activation of microsomal UGT activity (data not shown). Dose-dependent inhibition of UGT activity by oleoyl-CoA was observed in Brij 58-pre-treated microsomes. On the other hand, when intact microsomes were used as the enzyme source, oleoyl-CoA enhanced UGT activity toward 4-MU in a dose-dependent manner up to 15 μM. The maximum activation (7.7-fold) was observed at 15 μM oleoyl-CoA. The degree of enhancement was gradually reduced at more than 15 μM, and the UGT activity was inhibited at 50 μM (Fig. 1A). Palmitoyl-CoA exhibited similar results to oleoyl-CoA both in the absence and presence of Brij 58 (Fig. 1B). In marked contrast to rat liver microsomes, insect microsomes expressing a recombinant UGT exhibited a different effect following oleoyl-CoA treatment (Fig. 1C). In this case, oleoyl-CoA reduced dose-dependently the 4-MU UGT activity by both untreated and detergent-treated insect microsomes expressing UGT1A6 which is a major 4-MU UGT in human liver [24]. Oleoyl-CoA failed to enhance the activity even when untreated microsomes were used as the enzyme source. Taken together, these results suggest that acyl-CoAs at lower concentrations activate UGT function, and an intact microsomal membrane or natural conditions are needed for this enhancement.

The specificity of acyl-CoA in the modification of UGT function was further examined using seven acyl-CoAs and intact rat liver microsomes (Fig. 2). Acyl-CoAs having a carbon chain length of 10 or more modulated UGT activity in a similar manner to oleoyl-CoA (C18:1) and palmitoyl-CoA (C16:0), whereas caproyl-CoA (C6:0) and capryloyl-CoA (C8:0) did not cause any activation of UGT at all concentrations examined (Fig. 2). The latter two acyl-CoAs were slightly inhibitory at high concentrations (Fig. 2). These results suggest that medium- and long-chain acyl-CoAs modulate UGT function in a concentration-dependent manner. It is also demonstrated that while the activation needs 10 or more atoms in the acyl-CoA carbon

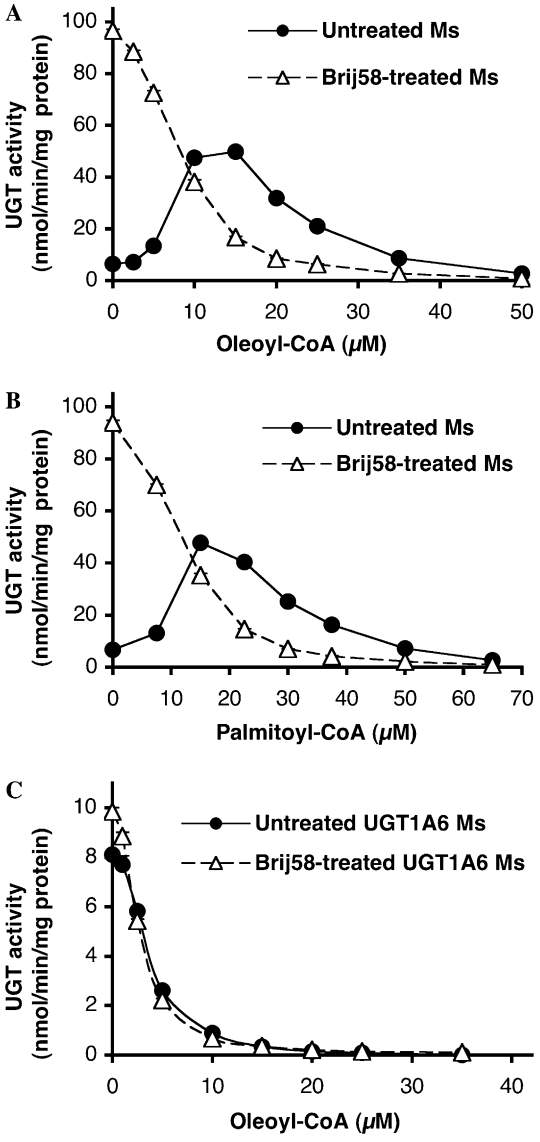

Fig. 1. Acyl-CoA-dependent modulation of 4-MU UGT activity. The UGT activity toward 4-MU was measured in the presence of the indicated concentration of acyl-CoAs. In experiments (A) and (B), rat liver microsomes were pre-treated with oleoyl-CoA (C18:1) and palmitoyl-CoA (C16:0), respectively, in the presence or absence of detergent (Brij 58, 0.5 mg/mg protein). The reaction mixture consisted of microsomes (20 μg protein), 2 mM UDPGA, 0.5 mM 4-MU, and varying concentrations of acyl-CoA. In experiment (C), Sf9 cell microsomes (8 μg protein) expressing a recombinant UGT1A6 were pre-treated with oleoyl-CoA as in the experiment of (A), before assay of 4-MU UGT activity. Each plot represents mean ± SE of triplicate assays.

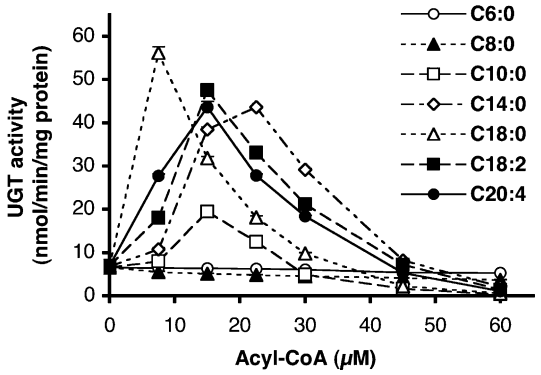

Fig. 2. Specificity of acyl-CoAs with regard to the ability to modulate 4-MU UGT activity. The microsomes were pre-treated with acyl-CoA in the absence of detergent, and then UGT activity toward 4-MU was determined. See also the legend to Fig. 1 for the experimental conditions. Each plot represents the mean ± SE of triplicate assays. The abbreviations used are: C6:0, caproyl-CoA; C8:0, capryloyl-CoA; C10:0, decanoyl-CoA; C14:0, myristoyl-CoA; C18:0, stearoyl-CoA; C18:2, linoleoyl-CoA; and C20:4, arachidonoyl-CoA.

chain, it is independent of the presence or absence of unsaturation in the alkyl chain of the acyl-CoA.

It is of interest to know whether acyl-CoAs directly modulate UGT. Since fatty acids can be released from acyl-CoAs, their effects on UGT were investigated. CoA, another hydrolytic product of acyl-CoA, did not affect UGT at up to 40 μM (data not shown). While unsaturated fatty acids (C16:1, C18:1, C18:2, C18:3, and C20:4) at 20 μM strongly enhanced UGT activity, saturated fatty acids (C2:0, C14:0, C16:0, and C18:0) at the same concentration did not show any effect (Fig. 3A). Dependence on the fatty acid concentration in the modulation of UGT activity was also observed. For example, oleic acid (C18:1), an unsaturated fatty acid, stimulated UGT activity at more than 20 μM and the activity reached plateau at 50 μM. On the other hand, saturated fatty acids [palmitic acid (C16:0) and stearic acid (C18:0)] had no effect on UGT activity even at more than 20 μM (Fig. 3B). These results suggest that acyl-CoA itself modulates UGT activity, although free fatty acid may make some contribution to the effect of unsaturated fatty acyl-CoA.

Kinetic analyses of the effect of oleoyl-CoA on UGT function

The effects of oleoyl-CoA on the kinetic parameters of UGT catalysis were examined using intact microsomes. The concentrations of oleoyl-CoA were fixed to 15 and 50 μM which cause activation and inhibition of 4-MU UGT, respectively. Kinetic analyses were carried out using different concentrations of both 4-MU and co-substrate UDPGA. Table 1 shows the results of kinetic analyses concerning 4-MU. Oleoyl-CoA increased the K_m value for 4-MU approximately 6.5-fold at both concentrations compared with the control. Although the affinity of UGT toward 4-MU was reduced by 15 μM oleoyl-CoA, the V_{max} was increased about 8-fold. The intrinsic clearance (V_{max}/K_m) was comparable between control and 15 μM oleoyl-CoA. Treating with 50 μM oleoyl-CoA reduced the intrinsic clearance to a level 14 times lower than the control. Table 2 shows the results of analyses focusing on UDPGA. Oleoyl-CoA at a low concentration (15 μM) reduced the *K*m value for UDPGA to approximately half that of the control and increased the V_{max} value about 8-fold. Oleoyl-CoA at 50 μM reduced the K_m and V_{max} values to about a quarter of the control. Glucuronidation efficiency was far higher in the reaction with 15 μM oleoyl-CoA than in the control and in the incubation with

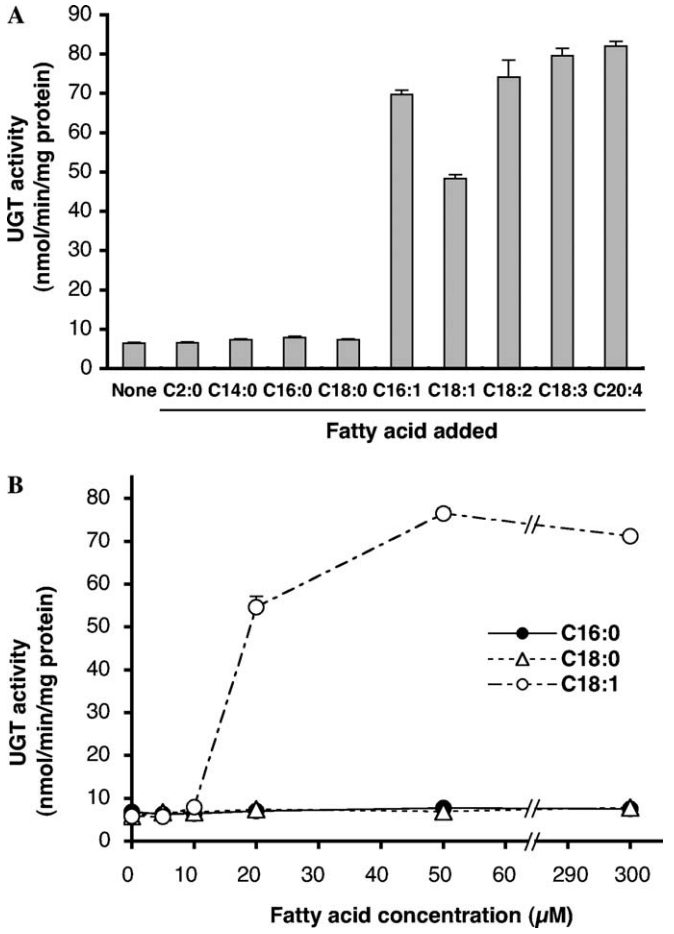

Fig. 3. Effect of fatty acids on 4-MU UGT activity. The UGT activity toward 4-MU was measured in the presence of fatty acids. In all assays, rat hepatic microsomes (20 μg protein) were used as the enzyme source. Fatty acids were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in incubation mixture was 0.33%. In experiment (A), microsomes were treated with each 20 μM fatty acid in the absence of detergent. In experiment (B), the dose–effect relationship was compared between saturated fatty acids (C16:0 and C18:0) and an unsaturated fatty acid (C18:1). Data represent means ± SE of triplicate assays. The abbreviations used are: C2:0, acetic acid; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; and C20:4, arachidonic acid.

Table 1
Effect of oleoyl-CoA on the kinetic parameters of 4-MU glucuronidation by rat hepatic microsomes: kinetics by varying 4-MU concentration

Oleoyl-CoA	K_m (mM)	V_{max} (nmol/min /mg protein)	V_{max}/K_m
Control (0 μM)	0.042 ± 0.003	7.06 ± 0.12	170
15 μM	0.261 ± 0.034	55.69 ± 2.52	247
50 μM	0.280 ± 0.025	3.48 ± 0.12	12

Kinetic analysis of 4-MU UGT activity was carried out in the presence of 0, 15 and 50 μM oleoyl-CoA. In all assays, microsomes (20 μg protein) were used as the enzyme source. The reaction mixture consisted of rat liver microsomes, 2 mM UDPGA, indicated amount of oleoyl-CoA and substrate (4-MU) ranging from 0.01 to 1 mM. Values for K_m and V_{max} represent the optimal value ± SD.

Table 2
Effect of oleoyl-CoA on the kinetic parameters of 4-MU glucuronidation by rat hepatic microsomes: kinetics by varying UDPGA concentration

Oleoyl-CoA	K_m (mM)	V_{max} (nmol/min /mg protein)	V_{max}/K_m
Control (0 μM)	1.51 ± 0.42	11.49 ± 1.28	7.6
15 μM	0.82 ± 0.04	88.08 ± 1.57	107
50 μM	0.37 ± 0.02	2.63 ± 0.03	7.1

Kinetic analysis of 4-MU UGT activity was carried out in the presence of 0, 15 and 50 μM oleoyl-CoA. In all assays, microsomes (20 μg protein) were used as the enzyme source. The reaction mixture consisted of rat liver microsomes, 1 mM 4-MU, indicated amount of oleoyl-CoA and co-substrate (UDPGA) ranging from 0.1 to 5 mM. Values for K_m and V_{max} represent the optimal value ± SD.

50 μM oleoyl-CoA. Taking these results into consideration, oleoyl-CoA-dependent enhancement of UGT activity is thought to be mainly due to the increased velocity. An increased affinity toward UDPGA may also be involved to some extent.

Role of membrane permeability and the UGT cytosolic tail on the acyl-CoA-produced modulation of UGT function

Conceivably, oleoyl-CoA-dependent modulation of UGT activity might be an outcome from the perturbation of the ER membrane. To address this issue, we then examined the effect of oleoyl-CoA on the digestion of microsomes with trypsin. When the ER membrane is sealed, UGT is expected to resist digestion by trypsin. On the other hand, if the ER membrane is rendered permeable, UGT is thought to be digested because of the easy access of trypsin to this enzyme. Accordingly, trypsin-treated microsomes were analyzed by Western blotting using a non-selective anti-UGT antibody (Fig. 4A). As expected, when microsomes were permeabilized with Brij 58, UGTs were digested (Fig. 4A, lane 8). Interestingly, 50 μM oleoyl-CoA

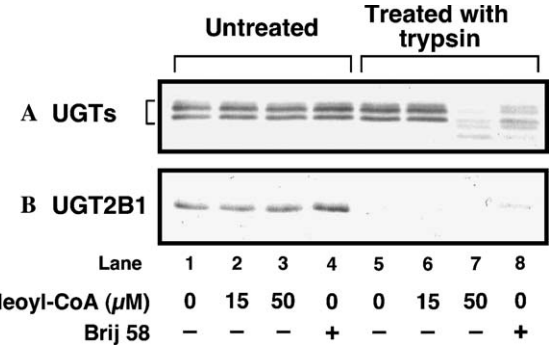

Fig. 4. Effect of oleoyl-CoA on tryptic digestion of microsomal UGTs. Rat liver microsomes (133 μg protein) were treated with oleoyl-CoA for 30 min on ice. The concentration of oleoyl-CoA during tryptic digestion is indicated in the figure. In separate experiments, microsomes were treated with Brij 58 (0.5 mg/mg protein) for 30 min on ice. Then, an aliquot (10 μg microsomal protein) was digested with 0.5 μg trypsin for 30 min at 20 °C and the digestion reaction was stopped by adding trypsin inhibitor (50 μg). Untreated and trypsin-treated samples (2 μg microsomal protein) were subjected to SDS–PAGE (7% gel) followed by immunostaining with anti-low p*I* form UGT antibody (A) or anti-UGT2B1 peptide antibody (B).

treatment of microsomes also caused tryptic digestion of UGTs even in the absence of detergent (Fig. 4A, lane 7). Oleoyl-CoA at 15 μM did not affect the resistant nature of microsomal UGTs toward trypsin (Fig. 4A, lane 6). These results suggest that 50 μM, but not 15 μM, oleoyl-CoA perturbs the ER membrane. Similar results were obtained when the amount of trypsin used was increased from 0.5 to 30 μg (data not shown). When trypsin-treated microsomes were analyzed by Western blotting using an anti-peptide antibody against UGT2B1, no immunoreactive band was observed in any case (Fig. 4B). Since the above antibody specifically recognizes the cytosolic tail of UGT2B1, the result obtained indicates that the cytosolic tail of UGT is removed by trypsin digestion. The data shown in Fig. 4B also indicate that the main bodies of UGTs located inside the membrane remain unaffected even after treatment with 15 μM oleoyl-CoA.

To obtain information about the role of the cytosolic tail of UGT in acyl-CoA-dependent activation of this enzyme, microsomal UGT activity was measured after tryptic digestion (Table 3). In this experiment, trypsin was inactivated by trypsin inhibitor, and then oleoyl-CoA was added to the digested microsomes. Tryptic digestion alone without addition of acyl-CoA increased the activity of microsomal UGT about 2-fold. As described previously, oleoyl-CoA (15 μM) greatly enhanced the UGT activity catalyzed by untreated microsomes. The same was observed when trypsin-treated microsomes were used as the enzyme source; accordingly, oleoyl-CoA-dependent activation of UGT function also occurred for trypsin-treated microsomes (Table 3). Taking these results into consideration, it is suggested that the activation of UGT by oleoyl-CoA does not require the cytosolic tail of UGT.

Involvement of SH-groups on acyl-CoA-produced modulation of UGT function

It has been suggested that arachidonoyl-CoA directly binds to several UGT isoforms probably through the SH moiety [8]. Then, the effect of SH-modifying reagents on oleoyl-CoA-dependent modulation of UGT activity was examined. In this experiment, dithiothreitol (DTT), *N*-ethylmaleimide (NEM), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were used as SH-modifying reagents. While these reagents did not exhibit any significant effect on UGT activity toward 4-MU in the absence of oleoyl-CoA, they facilitated the oleoyl-CoA (50 μM)-produced inhibition of glucuronidation. Since 50 μM oleoyl-CoA perturbs microsomes as described above, the SH-modifying reagents were assumed to penetrate the ER membrane to react with SH-groups located on the luminal side. Treating microsomes with NEM and DTNB markedly reduced the activation of glucuronidation by 15 μM oleoyl-CoA, while DTT did not exhibit such an effect (Fig. 5). These results suggest that oleoyl-CoA targets SH-groups both to activate and inhibit UGT function although the targeted cysteine residue is probably different in each case. The data obtained also suggest that oleoyl-CoA modifies the SH-group in intact microsomes to enhance UGT activity.

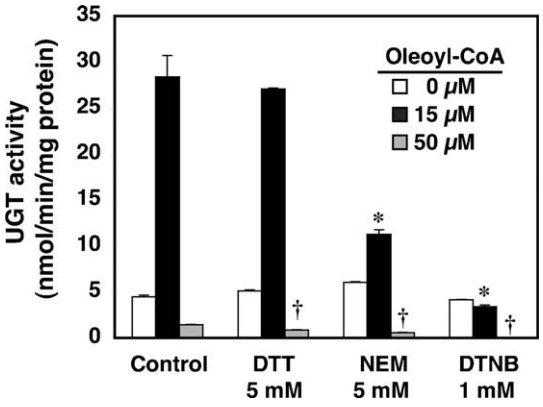

Fig. 5. Effect of SH-modifying reagents on oloeyl-CoA-dependent modulation of 4-MU UGT activity. The UGT activity toward 4-MU was measured in the presence of the indicated concentrations of oleoyl-CoA, with or without SH-modifying reagents. Rat hepatic microsomes were treated with SH-modifying reagents for 30 min on ice. The final concentrations of DTT, NEM, and DTNB during incubation were 5, 5, and 1 mM, respectively. The 4-MU UGT activity in microsomes (20 μg protein) was assayed as described in Materials and methods. Each bar represents the means ± SE of triplicate assays. *Significantly different from control with 15 μM oleoyl-CoA ($p < 0.01$). †Significantly different from control with 50 μM oleoyl-CoA ($p < 0.01$).

Table 3
Effect of trypsin digestion on 4-MU UGT activity

Oleoyl-CoA	UGT activity (nmol/min/mg protein)	
	Untreated	Trypsin-treated
Control (0 μM)	4.09 ± 0.07	9.03 ± 0.09*
15 μM	28.82 ± 0.55*	52.94 ± 0.65†

Rat liver microsomes (96 μg protein) were digested with 4.8 μg trypsin for 30 min at 20 °C and the digestion reaction was stopped by adding trypsin inhibitor (50 μg). Aliquots of untreated and trypsin-treated samples (20 μg microsomal protein) were used as enzymes sources. The UGT activity toward 4-MU was measured in the presence and absence of 15 μM oleoyl-CoA. 4-MU UGT activity was assayed as described in Materials and methods. Each value represents the mean ± SE of triplicate assays.

* Significantly different from the untreated control ($p < 0.001$).

† Significantly different from the trypsin-treated control ($p < 0.001$).

Discussion

In the present study, we have provided evidence for the first time that medium- and long-chain acyl-CoAs activate UGT activity catalyzed by detergent-untreated microsomes. The optimum concentration (7.5, 15 or 22.5 μM) of acyl-CoA-dependent activation differed from the concentration (at least 30 μM) needed for its inhibitory effect (Figs. 1 and 2). Long-chain acyl-CoAs consist of CoA, a hydrophilic moiety, and the hydrophobic region of the acyl chain. It is, therefore, reasonable that long-chain acyl-CoAs act as detergents [25]. Indeed, the critical micelle concentration of palmitoyl-CoA is estimated to be 70–80 μM

[26]. At higher concentrations, acyl-CoAs are thought to inhibit UGT activity in microsomes owing to their detergent-like nature as well as the inhibitory effect on UGTs. The above information suggests that the intact nature of the ER membrane is an important determinant for the acyl-CoAs-dependent enhancement of UGT activity. As shown in Fig. 3, unsaturated fatty acids strongly increased UGT activity, whereas saturated fatty acids and CoA did not exhibit such effects. Inconsistent effects of acyl-CoAs and fatty acids on UGT suggest that the mechanisms governing the effect on UGT function by above two groups of substances are different.

Kinetic analyses demonstrated that acyl-CoAs enhance the velocity of glucuronidation although they reduce the affinity of UGT toward substrate 4-MU. An increase in affinity toward UDPGA and V_{max} on the basis of the UDPGA concentration is also assumed to contribute to the acyl-CoA-produced activation of UGT. It has been suggested that UDPGA is transferred from the cytoplasm to the luminal side of ER by a specific transporter [27]. In fact, human nucleotidyl sugar transporter, which transports both UDPGA and UDP-*N*-acetylglucosamine, was found on the ER membrane [28]. In connection with this, acyl-CoAs might enhance UGT activity through the activation of UDPGA transportation.

While the main body of microsomal UGTs treated with 50 μM oleoyl-CoA were sensitive to tryptic digestion, those of microsomal UGTs treated with 15 μM oleoyl-CoA were resistant (Fig. 4A). These results support the view that acyl-CoAs require an increased membrane permeability to reach the luminal domain of UGT for inhibition. The data reported here and by other workers [8–10] that acyl-CoAs inhibit UGT in detergent-treated microsomes agree with the above assumption. As shown in Fig. 4B, the cytosolic tail of UGT2B1 was truncated by treating detergent-untreated microsomes with trypsin. Such an effect was also observed in the presence of 15 μM oleoyl-CoA. In agreement with this, there are lysine residues, trypsin-sensitive sites, at the putative cytosolic tail of UGT2B1. Although Fig. 4 shows immunoblotting with an antibody specific for the cytosolic peptide of UGT2B1, it is well known that the C-terminal sequence of UGT is relatively conserved [29]. Therefore, the data shown in Fig. 4 suggest that the cytosolic tails of UGTs other than UGT2B1 are also removed by trypsin digestion. Since the trypsin-treated microsomes were activated by 15 μM oleoyl-CoA (Table 3), it is suggested that the cytosolic tail does not play a role in the acyl-CoA-dependent activation of the UGT. However, it should be noted that it is possible that the activation of microsomal UGT occurs through a mechanism whereby acyl-CoAs affect the cytosolic region remaining after tryptic digestion. There are six lysine residues of UGT2B1 at the putative cytosolic tail: namely, K511, K519, K525, K526, K527, and K528. Three cystein residues are also present at C513, C516, and C517. It is of great interest whether these cysteine residues remain after trypsin digestion, because this study indicated the important role of the SH-group in the acyl-CoA-produced activation of UGT (Fig. 5). A previous report has also shown the role of the SH moiety in the binding of acyl-CoAs to UGTs [8]. All isoforms of rat and human UGT other than UGT2A1 have at least one cysteine residue in the cytosolic tail. It is, therefore, possible that acyl-CoA-sensitive cystein residues are present in the trypsin-digested form of UGT2B1 if the most upstream site for the trypsin target is K519.

It remains to be clarified whether UGTs are directly modified by acyl-CoAs. The activity of recombinant UGT1A6 expressed in insect cells was inhibited rather than facilitated by oleoyl-CoA, both in the absence and presence of Brij 58 (Fig. 1C). Such inhibitory effects resemble the effect observed in Brij 58-perturbed rat liver microsomes. Since an intact membrane was assumed to be necessary for oleoyl-CoA-dependent activation of liver microsomal UGT, the absence of an enhancing effect of acyl-CoA on the catalysis by recombinant UGT1A6 might be due to the difference in membrane integrity. In addition, the environment of insect ER differs from that of mammalian cells. Thus, different conditions of recombinant UGT may be the reason why acyl-CoA-dependent activation cannot be observed. Another possibility is that factor(s) other than UGT itself and expression circumstances is a target for acyl-CoA-dependent activation. For example, a transporter of UDPGA mentioned above might be facilitated through such acylation.

In the presence of 50 μM oleoyl-CoA, NEM, DTNB, and DTT inhibited UGT. Since 50 μM oleoyl-CoA seems to disrupt the ER membrane (Fig. 5), the above thiol reagents and oleoyl-CoA appear to have free access to the luminal domain of UGT under these conditions. Site-directed mutagenesis studies have provided evidence that cysteine residues in the luminal domain of both human and rat UGTs play an important role in the function of these enzymes [30–32]. Taken together, it is conceivable that modification of the luminal cysteine residue by NEM and DTNB led to further inhibition of UGT activity in the presence of 50 μM oleoyl-CoA. This mechanism is supported by the finding that NEM and DTNB greatly inhibit microsomal UGT activity in the presence of Brij 58 (data not shown). DTT is likely to facilitate the access of oleoyl-CoA to the free cysteine residue of UGTs by reducing the disulfide bond.

The physiological concentration of long-chain acyl-CoAs in rat liver cytoplasm is estimated to be around 30 μM [33]. Taking this information into consideration, acyl-CoAs would enhance glucuronidation rather than inhibit it under physiological conditions. However, it has been reported that acyl-CoA-binding protein and fatty acid-binding protein serve to buffer the detergent effect of long-chain acyl-CoAs [34]. The authors of above report demonstrated that the intracellular concentrations of free acyl-CoAs are kept at low nanomolar because of the function of these proteins. However, from a homeostatic point of view, the conditions surrounding the ER cannot be

stable. Supporting this idea, it has been demonstrated that the levels of long-chain acyl-CoAs and CoA in rats are increased in a number of conditions such as fasting [33,35,36], diabetes [33,35], and treatment with hypolipidemic drugs [33,36,37]. It has also been shown that the level of CoA is increased by refeeding glucose after a 50-h fast, whereas long-chain acyl-CoAs remain unaffected [38]. Therefore, it is reasonable to suppose that in vivo UGT activity toward various drugs and their metabolites is enhanced as a consequence of elevated levels of long-chain acyl-CoAs produced by the conditions described above.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (Research No. 17590128, recipient Y.I.) from the Japan Society for Promotion of Science.

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