Carrier-mediated transport of uridine diphosphoglucuronic acid across the endoplasmic reticulum membrane is a prerequisite for UDP-glucuronosyltransferase activity in rat liver

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UDP-glucuronosyltransferases (EC 2.4.1.17) is an isoenzyme family located primarily in the hepatic endoplasmic reticulum (ER) that displays latency of activity both in vitro and in vivo, as assessed respectively in microsomes and in isolated liver. The postulated luminal location of the active site of UDP-glucuronosyltransferases (UGTs) creates a permeability barrier to aglycone and UDP-GlcA access to the enzyme and implies a requirement for the transport of substrates across the ER membrane. The present study shows that the recently demonstrated carrier-mediated transport of UDP-GlcA across the ER membrane is required and rate-limiting for glucuronidation in sealed microsomal vesicles as well as in the intact ER of permeabilized hepatocytes. We found that in both microsomes and permeabilized hepatocytes a gradual inhibition by N-ethylmaleimide (NEM) of UDP-GlcA transport into the ER produced a correspondingly increasing inhibition of 4-methylumbelliferone glucuronidation. That NEM selectively inhibited the UDP-GlcA transporter, without affecting intrinsic UGT activity, was demonstrated by showing that NEM had no effect on glucuronidation in microsomes or hepatocytes with permeabilized ER membrane. Additional evidence that UDP-GlcA transport is rate-limiting for glucuronidation in sealed microsomal vesicles as well as in the intact ER of permeabilized hepatocytes was obtained by showing that gradual selective trans-stimulation of UDP-GlcA transport by UDP-GlcNAc, UDP-Xyl or UDP-Glc in each case produced correspondingly enhanced glucuronidation. Such stimulation of transport and glucuronidation was inhibited completely by NEM, which selectively inhibited UDP-GlcA transport.

INTRODUCTION

Conjugation with glucuronic acid enhances the water-solubility of a vast array of endogenous compounds and xenobiotics, thereby facilitating their excretion into bile and urine [1–4]. Glucuronide formation is catalysed by the UDP-glucuronosyl-transferase (UGT) system, a superfine family of isoenzymes located primarily in the hepatic endoplasmic reticulum (ER), where they are deeply embedded in the membrane [5]. Two functional properties apply to each of the UGT isoenzymes in native microsomal preparations: first, UGT activity is markedly latent in native microsomes [6]; secondly, UDP-GlcNAc [7–10] and UDP-Xyl [11] enhance UGT activity severalfold in native microsomes. These properties also seem to apply to UGT in the intact hepatocyte [8]. It has been postulated, but remains unproved, that these properties are related to compartmentation. The catalytic centre of the UGTs has a luminal location [1,9,12–18]. Such topology creates a permeability barrier for substrate access to the active site of the UGT and necessitates translocation across the ER membrane of the hydrophilic donor substrate, UDP-GlcA, which is synthesized in the cytosol [19]. There is evidence for carrier-mediated transport of both UDP-GlcA and UDP-GlcNAc across the ER membrane [20–23]. It has also been demonstrated that UDP-GlcA transport is markedly stimulated by UDP-GlcNAc by a trans-stimulation mechanism, whereby UDP-GlcNAc shuttles back and forth across the membrane [24]. Collectively, these findings are consistent with the so-called compartmentation model for regulation of UGT activity. This model postulates that transmembrane transport of UDP-GlcA is rate-limiting for UGT activity in the ER, thereby explaining UGT latency in native microsomes and in vivo, as shown in the isolated liver. By taking into account that UDP-GlcNAc enhances the transport of UDP-GlcA into the ER, this hypothesis can also explain the stimulatory effect of UDP-GlcNAc on glucuronidation. However, direct evidence to support this model is still lacking because there have been no reports on the comparison of UGT activities with UDP-GlcA transport rates measured under the same conditions in microsomal vesicles. Moreover, the direct dependence of glucuronidation by intact ER membranes, either in native well-sealed microsomes or in isolated hepatocytes, on recently identified carrier-mediated transport of UDP-GlcA has not been demonstrated.

The present study was undertaken to elucidate the role of carrier-mediated transport of UDP-GlcA across the ER membrane in UGT activity. We examined (1) whether the glucuronidation rate specifically attributable to sealed microsomal vesicles quantitatively corresponds to the microsomal UDP-GlcA uptake rate, (2) whether selective and gradual inhibition or stimulation of UDP-GlcA uptake results in equal impairment or enhancement of glucuronidation by correctly sealed ER vesicles, and (3) whether glucuronidation in hepatocytes depends on, and is determined by, UDP-GlcA transport across the ER membrane.

EXPERIMENTAL

Chemicals

UDP-[14C]GlcA (11.1 GBq/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). 4-Methylumbelliferone (4-MU) was from Janssen Chimica (Beerse, Belgium). BSA

Abbreviations used: 4-MU, 4-methylumbelliferone; ER, endoplasmic reticulum; NEM, N-ethylmaleimide; UGT, UDP-glucuronosyltransferase.

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fraction V, Heps and all unlabelled nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *Staphylococcus aureus* α-toxin (from strain Wood 46) was purified by a pore glass adsorption procedure [25]. All other chemicals were of analytical reagent grade quality. Aqueous solutions were prepared with deionized grade water.

**Preparation, characterization and treatment of microsomes**

Microsomes were prepared from male Wistar rat liver and characterized as described [20]. The structural intactness of the microsomes was verified by determination of mannose-6-phosphatase latency [26], which was more than 95% in all preparations used.

When labile phosphate esters (e.g. ATP) were present in the incubation medium, we used a radioactive assay for measurement of mannose-6-phosphatase activity [27]. The microsomal vesicles were completely disrupted by incubation with the detergent Chapso for 60 min at 0 °C, as previously described [26]. Stable permeabilization of the microsomal membrane by using *S. aureus* α-toxin (125 µg of toxin/mg of protein) was produced as reported elsewhere [28]. Loading microsomes with nucleotide sugars was done as follows. The vesicles were first incubated for 150 min at 37 °C. This preincubation was performed to dissipate a luminal pool of endogenous nucleotides, which when present inside the vesicles trans-stimulates the microsomal uptake of nucleotide sugars [16,20,23]. Thereafter the vesicles were incubated at 37 °C in the absence (control vesicles) or in the presence of 5 mM UDP-GlcNAc, 5 mM UDP-Xyl or 5 mM UDP-Glc. This was followed by re-isolation and washing (twice) of the vesicles by ultracentrifugation (104000 g for 30 min at 4 °C) [29].

**Isolation and permeabilization of rat hepatocytes**

Rat hepatocytes were isolated by the collagenase perfusion method [30]. The cells were maintained in Krebs–Henseleit medium supplemented with 1.2 mM CaCl₂ and saturated with carbogen. Cell viability was estimated by determining the exclusion of Trypan Blue and the leakage of LDH. Only cell suspensions with a cell viability higher than 90% were used. Selective permeabilization of the plasma membrane was performed by incubating 10 mg of protein hepatocytes with 50 µg of *S. aureus* α-toxin for 8 min at 37 °C. To measure the total UGT activity in the hepatocytes, the cells were sonicated twice for 10 min with an exponential probe sonicator (20 kHz, amplitude 10 µm). Thereafter the preparation was incubated for 5 min in 2.6 mM Chapso.

**Various assays**

Microsomal uptake of radiolabelled nucleotide was assessed by a previously reported rapid filtration technique [20]. 4-MU glucuronidation was assayed as follows. The enzymic uptake mixture consisted of the so-called transport assay incubation medium [20] supplemented with 1 mM 4-MU. This 4-MU was added as an 8 mM solution, prepared by diluting a stock solution of 1.5 M 4-MU in DMSO with 1 mM BSA. The glucuronidation assay was started by the addition of 25 µM UDP-GlcA and stopped by transferring 100 µl portions into 1 ml of 1 mM trichloroacetic acid. Extraction of unreacted substrate and determination of the glucuronide were done as described [31]. Protein was determined by the protein–Coomassie Blue dye binding method with BSA as calibration standard [32].

**RESULTS AND DISCUSSION**

**Determination of UGT activities selectively in the correctly sealed ER vesicles of native microsomal preparations**

During the preparation of native microsomes, some vesicles become disrupted or leaky. We describe the well-sealed, right-side-out vesicles as correctly sealed ER vesicles and the remaining ER structures as perturbed microsomes. In our microsomal preparations, perturbed microsomes amounted to less than 5% of the total microsomes, as assessed by the assay of mannose-6-phosphatase latency [26]. Although perturbed microsomes form only a minor fraction of the total membranes in native microsomal preparations, they contribute substantially to the basal glucuronidation activity. To determine the specific activity of UGT selectively in the fraction of perturbed microsomes, we permeabilized all ER membranes in the microsomal preparation by using the pore-forming α-toxin from *S. aureus*. The 4-MU-UGT activity (mean ± S.D.) in these fully permeabilized microsomes amounted to 8773 ± 440 (n = 3) pmol/min per mg of protein, which averages 31 times the glucuronidation activity [254 ± 73 (n = 3) pmol/min per mg of protein] found in our native microsomal preparations. The glucuronidation rate in correctly sealed microsomes was then estimated by subtracting from the basal rate the glucuronidation activity contributed by the perturbed microsomes [33] as assessed by the mannose-6-phosphatase latency. For the above-mentioned case of 4-MU glucuronidation, we calculated that if 1.4% of the native microsomal vesicles were disrupted, then these broken vesicles accounted for 50% of the basal glucuronidation activity. This demonstrates that the contribution by broken vesicles to the glucuronidation activity in native microsomal preparations must be taken into account when UDP-GlcA transport activities are compared with glucuronidation activities. Moreover, a small error in the estimation of the degree of membrane disruption can lead to a considerable error in estimating the contribution of the disrupted microsomes to the basal glucuronidation activity.

**Selective inhibition of microsomal UDP-GlcA transport inhibits glucuronidation by correctly sealed microsomes**

We examined the effects of variable degrees of inhibition of microsomal UDP-GlcA transport on UGT activity in native microsomes. N-Ethylmaleimide (NEM) was used to inhibit UDP-GlcA transport selectively. This compound effectively inhibits microsomal UDP-GlcA transport [20] and only minimally affects the intrinsic UGT activity [34]. In the absence and the presence of 2.5 mM NEM, the 4-MU glucuronidation activity (mean ± S.D.) in α-toxin-permeabilized microsomes was respectively 8773 ± 440 (n = 3) and 7853 ± 574 (n = 3) pmol/min per mg of protein. A spectrum of degrees of impairment of UDP-GlcA transport was produced by pretreating the microsomes with different concentrations of NEM. Figure 1 shows that there existed a remarkably close parallelism between the changes in transport activity and the alterations in activity of UGT in native microsomes. At high concentrations of NEM, UDP-GlcA uptake was almost completely restrained, whereas a rest activity of UGT remained, reflecting the enzyme activity attributable to the perturbed microsomes. By subtraction of the UGT activity attributed to the perturbed vesicles from the basal UGT activity found in native preparations, we calculated the UGT activity originating specifically from correctly sealed vesicles. Figure 1 (broken line) shows that (1) such corrected values for enzyme activity were correlated with UDP-GlcA transport rates and (2) the gradually increasing inactivation of
UDP-GlcA transport resulted in a gradual abolition of the glucuronidation reaction. These results imply that UDP-GlcA transport is a prerequisite for glucuronidation by correctly sealed vesicles.

**Importance of UDP-GlcA transport for glucuronidation by permeabilized hepatocytes**

To assess the importance of UDP-GlcA transport for glucuronidation in intact, unfragmented ER, we determined the glucuronidation of 4-MU under various conditions in permeabilized hepatocytes. The plasma membrane of hepatocytes was permeabilized for small-molecular-mass molecules with S. aureus α-toxin. As shown in Figure 2, NEM inhibited the glucuronidation of 4-MU in isolated hepatocytes in a concentration-dependent manner. In contrast, NEM did not affect the glucuronidation of 4-MU by sonicated hepatocytes in which the ER membranes were fully disrupted. The total glucuronidation of 4-MU in sonicated hepatocytes amounted to 2.3 nmol/min per mg of protein in the absence of NEM and 2.4 nmol/min per mg of protein in the presence of NEM.

**Table 1** Effect of preloading microsomes with UDP-GlcNAc, UDP-Xyl or UDP-Glc on UDP-GlcA uptake and on 4-MU glucuronidation

Control microsomes (non-preloaded microsomes) and microsomes preloaded with UDP-Glc, UDP-Xyl or UDP-GlcNAc (see the Experimental section) were used to study the uptake of UDP-GlcA and the glucuronidation of 4-MU. Uptake and glucuronidation were measured in the absence (−NEM) or the presence (+NEM) of 2.5 mM NEM, which was added 1 min before the reaction was started. Glucuronidation and UDP-GlcA transport rates postulated to depend specifically on the NEM-inhibitable carrier for UDP-GlcA in the ER membrane were calculated by subtracting the rates found in the presence of NEM from the rates in the absence of NEM. These calculated values are shown under ‘carrier-dependent’. Results represent the means ± S.D. for three determinations in different microsomal preparations.

<table>
<thead>
<tr>
<th>Loading condition</th>
<th>4-MU glucuronidation (pmol/min per mg of protein)</th>
<th>UDP-GlcA uptake (pmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−NEM</td>
<td>+NEM</td>
</tr>
<tr>
<td>Control</td>
<td>304 ± 21</td>
<td>301 ± 10</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>408 ± 82</td>
<td>290 ± 64</td>
</tr>
<tr>
<td>UDP-Xyl</td>
<td>1283 ± 100</td>
<td>267 ± 20</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>1513 ± 141</td>
<td>272 ± 10</td>
</tr>
</tbody>
</table>
indicating that UDP-GlcNAc activated glucuronidation by acti-
stimulation was totally abolished by NEM (results not shown),
presence of 2 mM UDP-GlcNAc. This UDP-GlcNAc-induced
of UDP-GlcNAc and 2.2 nmol

Figure 2 Effect of NEM on 4-MU glucuronidation by permeabilized
hepatocytes

S. aureus α-toxin-permeabilized hepatocytes (7 mg/ml protein) were preincubated at 37 °C for
3 min with various concentrations of NEM. Thereafter the glucuronidation of 4-MU was
determined. The figure shows, as a function of the NEM concentration in the medium, the
residual glucuronidation rate expressed as a percentage of the UGT activity found in the absence
of NEM. Each data point represents the mean ± S.E.M. for five independent experiments.

mg of protein in the presence of 10 mM NEM. Together these
results indicate that UDP-GlcA transport is a necessary and rate-
limiting step in the overall glucuronidation by the intact, unfrag-
mented ER.

As observed with microsomes, UGT activity could be stimu-
lated by preincubating the permeabilized hepatocytes with UDP-
GlcNAc. The glucuronidation of 4-MU by permeabilized hepat-
ocites amounted to 1.3 nmol/min per mg of protein in the absence
of UDP-GlcNAc and 2.2 nmol/min per mg of protein in the
presence of 2 mM UDP-GlcNAc. This UDP-GlcNAc-induced stimulation was totally abolished by NEM (results not shown),
indicating that UDP-GlcNAc activated glucuronidation by activ-
ating UDP-GlcA transport.

The observation that UDP-GlcNAc is able to elicit 97 % of the
total UGT activity in permeabilized hepatocytes illustrates the
potential physiological role of UDP-GlcNAc in the regulation of
UGT.

It is unlikely that the low latency of 4-MU-UGT activity is due
to a disrupted ER environment in permeabilized hepatocytes because mannose-6-phosphatase latency was 96.8 ± 0.1 % and 4-
MU-UGT activity was completely blocked by NEM. The latency
calculated from the 4-MU-UGT activity found after inhibition
of UDP-GlcA transport with NEM was 99 % and thus compara-
table to mannose-6-phosphatase latency.

In summary, the selective inhibition or stimulation of UDP-
GlcA transport by NEM or UDP-GlcNAc resulted in inhibition
or stimulation respectively of glucuronidation by permeabilized
hepatocytes.

Collectively, our findings with microsomal preparations and
isolated hepatocytes support the hypothesis that the recently
identified carrier-mediated transport of the co-substrate UDP-
GlcA across the ER plays a pivotal and regulatory role in overall
hepatic glucuronidation in vivo.

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REFERENCES

1 Dutton, G. J. (1980) in Glucuronidation of Drugs and Other Compounds, CRC Press,
Boca Raton, FL
5 Burchell, B., Nebert, D. W., Nelson, D. R., Bock, K. W., Iyanoji, T., Jansen, P. L. M.,
Lancet, D., Mulder, G. J., Chowdhury, R. J., Siest, G. et al. (1991) DNA Cell Biol. 10,
487–494
1293–1297
Metabolism and Disposition of Endo- and Xenobiotics (Bock, K. W., Gerok, W.,
328, 149–152
12 Scragg, I. M., Arion, W. J. and Burchell, B. (1985) in Advances in Glucuronide
Conjugation (Matern, S., Bock, K. W. and Gerok, W., eds.), pp. 390–391, MTP Press,
Lancaster
1441–1449
617–620
267, 11380–11385
19 Coates, S. W., Gurney, Jr., T., Sommers, L. W., Yeh, M. and Hirschberg, C. B. (1980)
J. Biol. Chem. 255, 9225–9229
108, 183–192
22 Radominska, A., Berg, C., Treat, S., Little, L. J., Lester, R., Gollan, J. L. and Drake,
907–913
4901–4907

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