Uridine diphosphoxylose enhances hepatic microsomal UDP-glucuronosyltransferase activity by stimulating transport of UDP-glucuronic acid across the endoplasmic reticulum membrane

Xavier BOSSUYT* and Norbert BLANCKAERT

Laboratory of Biological Chemistry, Department of Molecular Cell Biology, Catholic University of Leuven, Herestraat 49, B-3000 Leuven, Belgium

The UDP-glucuronosyltransferase (UGT) system fulfils a pivotal role in the biotransformation of potentially toxic endogenous and exogenous compounds. Here we report that the activity of UGT in rat liver is stimulated by UDP-xylose. This stimulation was found in native microsomal vesicles as well as in the intact endoplasmic reticulum (ER) membrane, as studied in permeabilized hepatocytes, indicating the potential physiological importance of UDP-xylose in the regulation of UGT. We present evidence that UDP-xylose enhances UGT activity by stimulation of (i) the uptake of UDP-glucuronic acid across the ER membrane and (ii) the elimination of the UDP and/or UMP reaction product out of the ER lumen. UDP-xylose produced a marked *trans*-stimulation of microsomal UDP-glucuronic acid uptake

when it was present within the lumen of the ER. When UDP-xylose was presented at the cytosolic side of the ER, it acted as a weak inhibitor of UDP-glucuronic acid uptake. Likewise, cytosolic UDP-glucuronic acid strongly *trans*-stimulated efflux of intravesicular UDP-xylose, whereas cytosolic UDP-xylose was inefficient in *trans*-stimulating efflux of UDP-glucuronic acid. Microsomal UDP-xylose influx was markedly stimulated by UMP and UDP. Such stimulation was only apparent when microsomes had been preincubated and thereby preloaded with UMP or UDP, indicating that UMP and UDP exerted their effect on UDP-xylose uptake by *trans*-stimulation from the luminal side of the ER membrane.

INTRODUCTION

A major detoxification pathway involves transfer of the polar glucuronic acid group from UDP-glucuronic acid to a hydrophobic acceptor substrate. The resulting glucuronide is more hydrophilic than the parent compound and can be excreted easily [1–5]. Glucuronide formation is catalysed by the UDP-glucuronosyltransferase (UGT) family, a group of isoenzymes located primarily in the hepatic endoplasmic reticulum (ER) membrane [6].

It is widely accepted that the catalytic centre of UGT is located within the ER lumen, and that the transferase latency found with native microsomes could be caused by rate-limiting transport of UDP-glucuronic acid across the microsomal membrane [7–13]. Carrier-mediated transport of UDP-glucuronic acid across the ER has recently been documented [14–16]. Furthermore evidence for carrier-mediated transport of UDP-glucose [17], UDP-*N*-acetylglucosamine [18] and UDP-xylose [19] across the ER has previously been obtained. It has also become clear that transport of nucleotide sugars across the ER membrane plays a crucial role in the regulation of UGT. For example, the stimulation of UGT by UDP-GlcNAc [11,13,20–23] is based on the interaction of transport of UDP-GlcNAc with UDP-glucuronic acid and UMP transport [24]. Intramicrosomal UDP-GlcNAc, on the one hand, stimulates influx of UDP-glucuronic acid and thereby enhances delivery of the donor co-substrate to the catalytic centre of UGT in the lumen of the ER. Cytosolic UDP-GlcNAc, on the other hand, by *trans*-stimulation, helps in the elimination of the UMP reaction product.

We now report that, in contrast with previous reports [25,26],

UDP-xylose stimulates UGT activity. We demonstrate that stimulation of UGT by UDP-xylose is based on the interaction of UDP-xylose with microsomal transport of the UDP-glucuronic acid co-substrate and of the UDP reaction product.

MATERIALS AND METHODS

Chemicals

UDP-[¹⁴C]glucuronic acid (11.1 GBq/mmol) and UDP-[³H]xylose (277.7 GBq/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabelled nucleotides, BSA fraction V and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 4-Methylumbelliferone was from Janssen Chimica (Beerse, Belgium). *Staphylococcus aureus* α -toxin (from strain Wood 46) was purified by a pore glass adsorption procedure [27]. Aqueous solutions were prepared with deionized water.

Isolation and permeabilization of rat hepatocytes

Rat hepatocytes were isolated by the collagenase perfusion method [28]. The cells were maintained in Krebs–Henseleit medium supplemented with 1.2 mM CaCl₂ and saturated with carbogen. The integrity of isolated cells was estimated by determining the exclusion of Trypan Blue and leakage of lactate dehydrogenase. Only cell suspensions with cell viability higher than 90% were used. Selective permeabilization of the plasma membrane was performed by incubating hepatocytes (10 mg of

protein) with 50 μg of *Staphylococcus aureus* α -toxin for 8 min at 37 °C.

Preparation and characterization of microsomes

Rough ER (RER)-derived microsomes were prepared and characterized from male Wistar rat liver as described [14]. Structural intactness of the microsomes, assessed by determination of mannose 6-phosphatase latency [29], was more than 95 % in all preparations used. All microsomes were preincubated for 150 min at 37 °C before uptake studies were started. Such preincubation was performed to remove a luminal pool of endogenous nucleotides, which *trans*-stimulates uptake of nucleotide sugars [14,17,18,30]. All uptake reactions were started immediately after the 150 min preincubation. The long preincubation did not alter the intactness of the microsomal membrane permeability barrier [14].

Permeabilization of microsomal membranes by *Staphylococcus aureus* α -toxin was performed as previously described [31].

Transport measurements and 4-methylumbelliferone glucuronidation assay

Microsomal uptake of radiolabelled nucleotide was assessed by using a rapid ultrafiltration method, as previously described [14,18]. Microsomal 4-methylumbelliferone glucuronidation was measured in the transport assay incubation medium supplemented with 1 mM 4-methylumbelliferone. When glucuronidation was measured by using permeabilized hepatocytes, the assay medium consisted of 20 mM Hepes, 140 mM potassium glutamate, 5 mM glutathione, 0.5 mM EGTA, 9 mM MgCl_2 , 5 % (w/v) dextran 4 (M_r 4000–6000), 2.5 mM MnCl_2 , 25 μM UDP-glucuronic acid and 1 mM 4-methylumbelliferone. The 4-methylumbelliferone was added as an 8 mM solution prepared by diluting a 1.5 M stock solution of 4-methylumbelliferone in DMSO with 1 mM BSA. Glucuronidation reactions were measured at 37 °C. The reaction was started by the addition of 25 μM UDP-glucuronic acid, and 100 μl portions were stopped at 20 s intervals by transferring them into 1 ml of 1 mM trichloroacetate. Extraction of the substrate and determination of the glucuronide product were done as described [32].

Statistics

Results are given as means $\pm$ S.D., with the number of observations in parentheses. Differences between means were analysed by Student's *t* test.

Protein determination

Protein was determined by the protein–Coomassie Blue dye binding method, with BSA as calibration standard [33].

RESULTS AND DISCUSSION

UDP-xylose stimulates glucuronidation assayed with permeabilized hepatocytes

The plasma membrane of hepatocytes was made permeable to molecules of low molecular mass, such as nucleotide sugars, with *Staphylococcus aureus* α -toxin. Because the pores formed by the toxin are too small to allow entry of the toxin itself into the hepatocyte, intracellular membranes such as ER membranes remain unaffected and intact during the treatment with toxin [13]. This procedure permits study of glucuronidation in the intact ER without fragmentation of the membranes.

Figure 1 Effect of UDP-xylose on glucuronidation of 4-methylumbelliferone in rat liver microsomes

Glucuronidation of 4-methylumbelliferone was determined in the absence (●) or presence of 2 mM UDP-xylose, which was added to the incubation mixture 3 min (▼), 9 min (◆) or 15 min (●) before the glucuronidation reaction was started. All data are from one representative experiment out of at least two independent experiments with different microsomal preparations.

Initial rates of 4-methylumbelliferone glucuronidation in α -toxin-permeabilized hepatocytes amounted to 0.78 ± 0.15 nmol/min per mg of protein ($n = 6$) in the absence of UDP-xylose, compared with 2.72 ± 0.7 nmol/min per mg of protein ($n = 6$) in the presence of 2 mM UDP-xylose (results not shown). These observations show that UDP-xylose significantly ($P < 0.001$) stimulates glucuronidation catalysed by UGT present in its native, unperturbed ER environment. This finding indicates that UDP-xylose might play a physiological role in the regulation of UGT.

UDP-xylose stimulates glucuronidation by sealed microsomes

UDP-xylose did not affect glucuronidation of 4-methylumbelliferone when assayed with *Staphylococcus aureus* α -toxin-permeabilized microsomes. Glucuronidation of 4-methylumbelliferone by such permeabilized microsomes amounted to 1.52 nmol per 20 s per mg of protein in the presence of 2 mM UDP-xylose and to 1.59 nmol per 20 s per mg of protein in the absence of UDP-xylose. When assayed with native, sealed microsomes, glucuronidation of 4-methylumbelliferone was stimulated by UDP-xylose. A gradual increase of the stimulation of UDP-glucuronic acid uptake was seen as a function of the time for which the microsomes had been preincubated with UDP-xylose (Figure 1). In the absence of UDP-glucuronic acid, conjugation of 4-methylumbelliferone was not observed (results not shown), indicating that UDP-xylose did not react with 4-methylumbelliferone to produce xylosides. Because UDP-xylose activated glucuronidation by native, sealed microsomal vesicles but not by permeabilized microsomes, we speculated that UDP-xylose stimulates glucuronidation by facilitating microsomal transport of UDP-glucuronic acid.

Effects of UDP-xylose on microsomal uptake of UDP-glucuronic acid

Because both UDP-xylose and UDP-glucuronic acid are transported by a carrier-mediated mechanism across the ER membrane [14,19], we examined whether the observed stimulation

Figure 2 Effect of UDP-xylose on the initial rates of microsomal UDP-glucuronic acid uptake under various conditions

(a) Uptake of 25 μ M UDP-[14 C]glucuronic acid in the absence (○) or presence of 2 mM (▲) or 50 μ M (■) UDP-xylose, added at the same time as UDP-glucuronic acid. (b) Uptake of 25 μ M UDP-[14 C]glucuronic acid in the absence (○) or presence of 2 mM UDP-xylose that had been supplemented 3 min (▼), 9 min (◆) or 15 min (●) before uptake was started. All data are from one representative experiment out of at least two independent experiments with separate microsomal preparations.

of 4-methylumbelliferone glucuronidation by UDP-xylose depended on an effect of UDP-xylose on transport of the UDP-glucuronic acid donor substrate into the ER lumen. Depending on the experimental conditions, UDP-xylose, when incubated with microsomes and UDP-[14 C]glucuronic acid, either slightly inhibited or markedly enhanced microsomal uptake of UDP-glucuronic acid. Figures 2a and 2b show time curves for initial uptake of radiolabel by microsomes incubated with 25 μ M UDP-[14 C]glucuronic acid and unlabelled UDP-xylose under various experimental conditions. UDP-xylose, when added simultaneously and in a 80-fold molar excess over the 25 μ M UDP-[14 C]glucuronic acid substrate, slightly inhibited the early phase of uptake of radiolabel. The initial inhibition of UDP-glucuronic acid uptake was followed by a stimulation of uptake (Figure 2a). No early inhibition of 14 C uptake was observed when UDP-[14 C]glucuronic acid substrate and a low (50 μ M) concentration of unlabelled UDP-xylose were added at the same time as the uptake assay mixture. Under this condition, a stimulation of 14 C uptake was seen after 20 s of incubation (Figure 2a). Enhanced uptake of radiolabelled UDP-glucuronic acid at all time points was observed when microsomes had been preincubated with a high (2 mM) concentration of UDP-xylose

Table 1 *Trans*-stimulation of microsomal UDP-[14 C]GlcA efflux by unlabelled UDP-glucuronic acid, glucuronic acid 1-phosphate or glucuronic acid

Microsomes to which 2 mM UDP-xylose was added during the last 9 min of the preincubation (see the Materials and methods section) were incubated with 25 μ M UDP-[14 C]glucuronic acid. The entry of label was followed as a function of time. Portions were removed at 55 and 90 s. After 60 s, incubation medium (control), UDP-xylose, UDP-glucuronic acid, glucuronic acid 1-phosphate or glucuronic acid (each 400 μ M) were added. Values represent the vesicle-associated radiolabel found after 90 s of incubation expressed as a percentage of the vesicle-associated label found after 55 s of incubation. Each result is the mean $\pm$ S.D. for three determinations in separate microsomal preparations. When indicated by *, the mean is statistically significantly different ($P < 0.0002$) from the mean of the control.

Added nucleotide	Vesicle-associated label after 90 s (% of label after 55 s)
Control	101 $\pm$ 3
GlcA	110 $\pm$ 3
GlcA 1- <i>P</i>	108 $\pm$ 3
UDP-GlcA	81 $\pm$ 3*
UDP-Xyl	114 $\pm$ 9

before adding the radiolabelled substrate. When we examined the effect of UDP-xylose on uptake of radiolabelled UDP-glucuronic acid as a function of the period during which the microsomes were preincubated with 2 mM UDP-xylose before the radiolabelled substrate was added, we observed a gradual increase of the stimulation of uptake as a function of preincubation time (Figure 2b). On the basis of these observations, we speculated that the UDP-xylose-induced stimulation of 4-methylumbelliferone was due to acceleration by UDP-xylose of UDP-glucuronic acid co-substrate uptake in the microsomal vesicles, as a result of *trans*-stimulation of UDP-glucuronic acid influx by internalized UDP-xylose. Only high concentrations of UDP-xylose slightly *cis*-inhibited uptake of UDP-glucuronic acid during the time needed for entry of an amount of UDP-xylose sufficient to produce *trans*-stimulation of UDP-glucuronic acid uptake.

Evidence that UDP-xylose stimulates uptake of intact UDP-glucuronic acid

UDP-glucuronic acid is rapidly and extensively broken down by highly active microsomal nucleotide pyrophosphatase and phosphatase activities [14]. Therefore it was essential to verify that, in the presence of UDP-xylose, microsomal uptake of radiolabel reflected uptake of authentic UDP-glucuronic acid and not of a hydrolysis product, glucuronic acid or glucuronic acid 1-phosphate. We investigated the uptake of 14 C by microsomes preincubated with 2 mM UDP-xylose for 9 min before adding 25 μ M UDP-[14 C]glucuronic acid. If the uptake of radioactivity represented flux of glucuronic acid or of its 1-phosphate derivative, then the addition of an excess of unlabelled glucuronic acid or glucuronic acid 1-phosphate would restrain the uptake of 14 C label. As shown in Table 1, no inhibition was observed when an excess of these hydrolysis products was added to the assay mixture 1 min after the uptake reaction was started. By contrast, a similar concentration of unlabelled UDP-glucuronic acid did *trans*-stimulate the efflux of radiolabel present in microsomes that had been preincubated with UDP-[14 C]glucuronic acid. These observations demonstrate that uptake of label in the presence of the stimulatory agent UDP-xylose did reflect translocation of intact UDP-glucuronic acid rather than of its hydrolysis products.

Figure 3 *Trans*-stimulation of microsomal uptake of UDP-glucuronic acid by UDP-xylose

Microsomes were preloaded with UDP-xylose, after which uptake of 25 μ M UDP-[14 C]glucuronic acid was studied. Preloading consisted of incubating microsomes at 37 °C for 15 min in incubation buffer without (○) or with (●) 5 mM UDP-xylose. The preloading step was followed by re-isolation and washing (twice) of the vesicles by ultracentrifugation (104 000 *g* for 30 min at 4 °C). Each point represents the mean $\pm$ S.D. for three independent observations.

Table 2 *Trans*-stimulation of microsomal nucleotide sugar efflux by unlabelled nucleotide sugar

Microsomes were incubated at 37 °C at time zero with 25 μ M UDP-[3 H]xylose in a total volume of 160 μ l. Microsomal uptake of label was followed as a function of time. Portions (30 μ l) were removed and subjected to ultrafiltration to determine radiolabel taken up by the vesicles at 55 s and 90 s. After 60 s, 20 μ l of UDP-glucuronic acid, UDP-xylose, UMP or UDP, each from a 2 mM stock solution, or 20 μ l of incubation medium (control), was added. Values represent the vesicle-associated radiolabel found after 90 s of incubation expressed as percentage of the vesicle-associated label found after 55 s of incubation. Each result is the mean $\pm$ S.D. for four determinations in separate microsomal preparations. When indicated by *, the mean is statistically significantly different ($P < 0.001$) from the mean of the control.

Added nucleotide	Vesicle-associated label after 90 s (% of label after 55 s)
Control	100 $\pm$ 6
UDP-GlcA	60 $\pm$ 4*
UMP	87 $\pm$ 19
UDP	75 $\pm$ 4
UDP-Xyl	58 $\pm$ 4*

Evidence for a transport system that couples influx of UDP-glucuronic acid to efflux of UDP-xylose

In the experiment shown in Figure 3, RER-derived vesicles were preloaded with UDP-xylose by equilibration with a high (5 mM) concentration of UDP-xylose. After washing and resuspending the microsomes, we studied the uptake of UDP-glucuronic acid in the vesicles by using 25 μ M radiolabelled UDP-[14 C]glucuronic acid substrate. Uptake of UDP-glucuronic acid in such preloaded vesicles displayed a marked overshoot phenomenon, which was not seen in the control vesicles. We found that 908 pmol UDP-[14 C]glucuronic acid equivalents per mg of protein was present in the vesicles when overshooting peaked. A 5-fold UDP-glucuronic acid concentration gradient across the membrane was built up

under these conditions. This experiment demonstrates that the presence of UDP-xylose in the lumen of the microsomal vesicles markedly *trans*-stimulates influx of UDP-glucuronic acid. We next studied the *trans*-stimulation effect of various 5'-uridine nucleotides presented at the cytosolic side on the efflux of UDP-xylose. As is shown in Table 2, efflux of radiolabelled UDP-xylose was affected by extravesicular UMP and UDP. However, this *trans*-stimulation was less pronounced than the marked *trans*-stimulation found with extravesicular unlabelled UDP-glucuronic acid or UDP-xylose. Cytosolic UDP-xylose was inefficient in *trans*-stimulating efflux of intravesicular UDP-glucuronic acid (Table 1). In conclusion, influx of UDP-glucuronic acid was potently *trans*-stimulated by UDP-xylose and efflux of UDP-xylose was *trans*-stimulated by UDP-glucuronic acid. However, UDP-xylose was inefficient at *trans*-stimulating the efflux of intramicrosomal UDP-glucuronic acid. This indicates the presence in the ER of an asymmetric carrier system that facilitates exchange of intravesicular UDP-xylose for extravesicular UDP-glucuronic acid.

Evidence for a transport system that couples influx of UDP-xylose to efflux of UDP or UMP

Next we examined whether intravesicular UDP or UMP could stimulate the influx of radiolabelled UDP-xylose. Preincubating microsomal vesicles with 2 mM UDP or 2 mM UMP for 9 min resulted in a significantly higher UDP-xylose uptake rate than in the control condition in which we tested microsomes that had not been preloaded with UDP or UMP. Uptake rates of 25 μ M UDP-xylose were 27 $\pm$ 6 pmol/min per mg of protein ($n = 4$), 49 $\pm$ 5 pmol/min per mg of protein ($n = 4$) ($P < 0.01$), and 90 $\pm$ 18 pmol/min per mg of protein ($n = 4$) ($P < 0.01$) for respectively the control condition and for the conditions in which the vesicles had been preincubated for 9 min with either 2 mM UMP or 2 mM UDP (results not shown). Such stimulation of UDP-xylose uptake was only apparent when microsomal vesicles had been preloaded with UMP or UDP. When UMP or UDP was added simultaneously and in a 80-fold molar excess over the 25 μ M UDP-xylose substrate, no effect on microsomal uptake of UDP-[3 H]xylose was observed. Under these conditions, the initial UDP-xylose uptake rates were respectively 24 $\pm$ 3 pmol/min per mg of protein ($n = 4$) and 18 $\pm$ 5 pmol/min per mg of protein ($n = 4$). These findings indicated that UMP and UDP exerted their effects on UDP-xylose transport selectively from the luminal side of the membrane.

Efflux of UMP is specifically coupled to influx of UDP-xylose

The flux of UMP out of the microsomes is specifically coupled to the inward flux of UDP-xylose and not to the inward flux of UDP-glucuronic acid. This conclusion is based on the following two observations. UDP-glucuronic acid was not effective in *trans*-stimulating UMP efflux [24], and we did not observe enhanced uptake of UDP-glucuronic acid in microsomes that had been preincubated with UMP for 9 min [24]. These findings demonstrate that there is no direct coupling between UDP-glucuronic acid influx and UMP efflux. Thus UDP-xylose functions as a shuttle compound in helping UDP-glucuronic acid to enter the microsomes and in removing UDP and UMP from the ER lumen.

General comments

UDP-xylose, by *trans*-stimulation, enhances the uptake of UDP-glucuronic acid in the microsomes and delivers the co-substrate

to the catalytic centre of UGT. The reaction products UDP and UMP, formed by hydrolysis of UDP by nucleotide diphosphatase, are transported out of the microsomes in exchange for UDP-xylose, which enters the microsomes. The internalized UDP-xylose then becomes available as a countersubstrate for UDP-glucuronic acid influx. In this way UDP-xylose shuttles between the luminal side and the cytoplasmic side of the microsomes, once in exchange with UMP to enter the microsomes, and once in exchange with UDP-glucuronic acid to leave the microsomes. We postulate that the interaction of two asymmetric transport systems together with intravesicular metabolism of UDP-glucuronic acid forms the mechanism of the UDP-xylose-induced stimulation of glucuronidation.

The effects being ascribed here to UDP-xylose cannot be due merely to its being broken down in microsomes to UDP and UMP, because both UMP and UDP have no, or negligible, effects on microsomal uptake of UDP-glucuronic acid [24].

UDP-GlcNAc and UDP-xylose seem to exert similar effects on UDP-glucuronic acid transport across the ER membrane and may well use the same carrier, as indicated by the following lines of evidence: UDP-xylose inhibits microsomal uptake of UDP-GlcNAc and *trans*-stimulates UDP-GlcNAc efflux from ER vesicles [18].

Unfortunately, no results are available on the UDP-xylose concentration, and biological variation thereof, in rat liver cytosol. Hence it is unclear at present whether this effect of UDP-xylose on hepatic glucuronidation is of physiological significance. By contrast, a physiological role on glucuronidation for UDP-GlcNAc is well established for UDP-GlcNAc [11,13,20–24], which interacts with UDP-glucuronic acid transport by a similar mechanism as reported here for UDP-xylose.

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