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SHORT COMMUNICATION

β-Glucuronidase Latency in Isolated Murine Hepatocytes

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ABSTRACT. The physiological function of microsomal β-glucuronidase is unclear. Substrates may be either glucuronides produced in the lumen of endoplasmic reticulum (ER) or those taken up by hepatocytes. In the latter case, efficient inward transport of glucuronides at the plasma membrane and the ER membrane would be required. Therefore, the potential role of β-glucuronidase in ER was investigated. Isolated mouse hepatocytes and mouse and rat liver microsomal vesicles were used in the experiments. Selective permeabilization of the plasma membrane of isolated hepatocytes with saponin or digitonin resulted in an almost 4-fold elevation in the rate of *p*-nitrophenol glucuronide hydrolysis, while the permeabilization of plasma membrane plus ER membrane by Triton X-100 caused a further 2-fold elevation. In microsomal vesicles, the *p*-nitrophenol glucuronide or phenolphthalein glucuronide β-glucuronidase activity showed about 50% latency as revealed by alamethicin or Triton X-100 treatment. A light-scattering study indicated that the microsomes are relatively impermeable to both glucuronides and to glucuronate. On the basis of our results, the role of liver microsomal β-glucuronidase in the deconjugation of glucuronides taken up by the liver seems unlikely. Hydrolysis of the glucuronides produced in the ER lumen may play a role in substrate supply for ascorbate synthesis or in "proofreading" of glucuronidation. BIOCHEM PHARMACOL **59**;7:801–805, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. endoplasmic reticulum; glucuronide; latency; transport; liver

Glucuronidation is arguably the most important phase II biotransformation reaction in the liver and takes place in the lumen of the ER.¶ The enzyme which hydrolyzes glucuronides, β-glucuronidase, is also found in the same cellular compartment because it is retained in the lumen of the ER, in complex with egasyn [1]. The physiological function of microsomal β-glucuronidase has not been fully defined, although it has been suggested that it plays a role in enterohepatic circulation, because the reabsorbed glucuronides might by hydrolyzed by this enzyme [2]. It removes glucuronate from the conjugated products of biotransformation [3]. An identical, lysosomal β-glucuronidase has an important role in the hydrolysis of steroid hormone glucuronides [4]. The transport of the substrates or products is rate-limiting for several intralumenal enzyme activities of the ER [5]. It has been shown in the case of UGT [6] that this phenomenon is not an artifact caused by the preparation of microsomal vesicles, but can also be demonstrated in intact hepatocytes. The uptake of glucuronides was detected in hepatocytes (for a review see [7]), but the plasma membrane transporter (or transporters) involved has not been characterized or identified as yet. A microsomal antiporter presumably participating in outward glucuronide transport has recently been reported [8], but it also has not been isolated. It is questionable, therefore, if the glucuronides taken up by the liver in the enterohepatic recirculation can be efficiently hydrolyzed by the microsomal β-glucuronidase.

In the present study, the permeability of plasma and microsomal membranes to glucuronides was investigated. Two model compounds were used: low molecular weight *p*-nitrophenol glucuronide and high molecular weight phenolphthalein glucuronide. Membrane permeability was detected by the light-scattering technique and by measuring the latency of the β-glucuronidase enzyme in both microsomal system and isolated hepatocytes.

MATERIALS AND METHODS

Hepatocytes were isolated from male CD-1 mice (20–25 g body weight) by the collagenase perfusion method [9] and incubated as described previously [10]. The animals were

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¶ *Abbreviations:* ER, endoplasmic reticulum; UGT, UDP-glucuronosyltransferase; MOPS, 3-[*N*-morpholino]propanesulfonic acid; and PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid].

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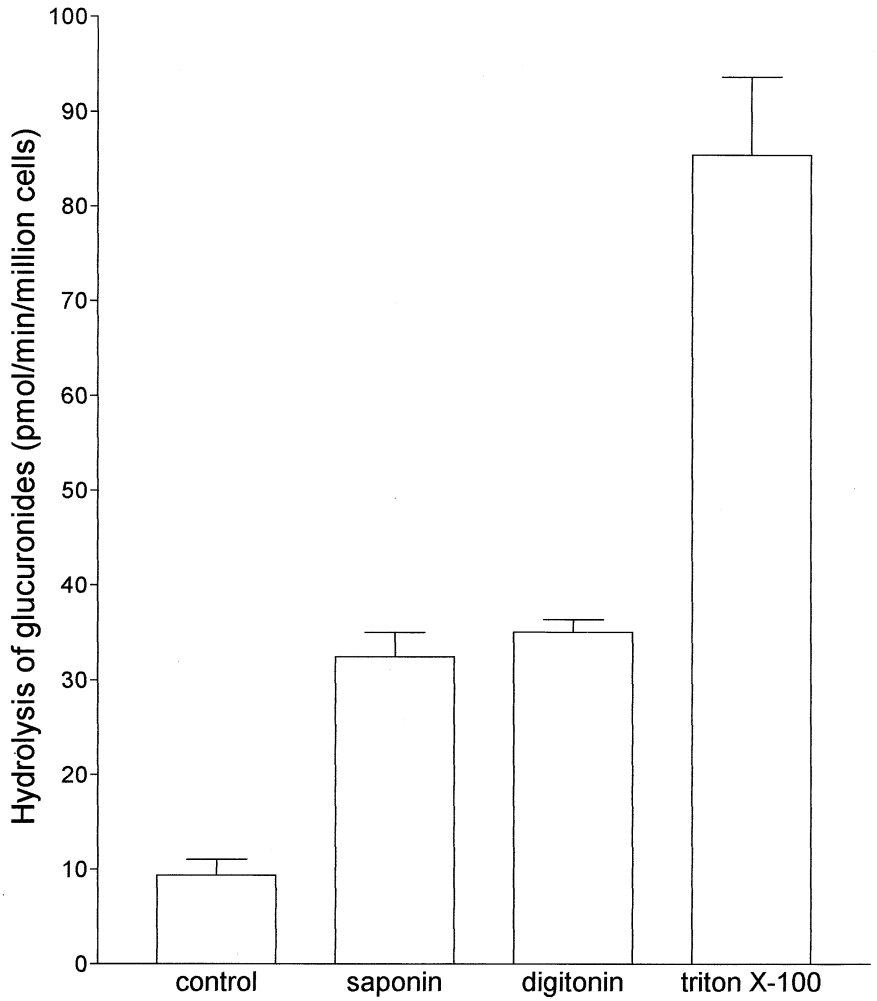

FIG. 1. The latency of β-glucuronidase activity in isolated mouse hepatocytes. Hepatocytes (4–6 × 10^6 cells/mL) from mice starved for 48 hr were permeabilized with saponin (0.01%), digitonin (0.05%), or Triton X-100 (0.05%). β-Glucuronidase activity was detected on the basis of *p*-nitrophenol formation from *p*-nitrophenol glucuronide (0.5 mM). Data are means ± SEM of four experiments.

starved for 48 hr before the experiments to diminish glucuronidation because of the low concentration of UDP-glucuronate in the isolated hepatocytes [11]. Hepatocytes were permeabilized with saponin or digitonin (both selective for the plasma membrane) or with Triton X-100 (permeabilization of plasma membrane plus endomembranes) [6] before the addition of glucuronides.

Rat and mouse liver microsomes were prepared from male Sprague–Dawley rats (180–230 g) and from male CD-1 mice (20–25 g body weight) as described earlier [12] and were stored in liquid nitrogen until use. Microsomes (1–2 mg/mL) were incubated in MOPS/KCl buffer (100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM MOPS, pH 7.2) at 37°. Intactness of the microsomal membrane was ascertained by measuring the latency of mannose 6-phosphatase activity [13]. The latency of mannose 6-phosphatase was higher than 92% in all the preparations used. The microsomal membranes were permeabilized with alamethicin or solubilized with Triton X-100 as reported earlier [14].

Permeability of microsomal vesicles was investigated by the light-scattering technique [15] as described in detail elsewhere [16]. Briefly, microsomal vesicles (0.07–0.1 mg of protein/mL) were equilibrated in a final volume of 2 mL of a hypotonic medium (5 mM K-PIPES, pH 7.0). Then, osmotically induced changes in light scattering were measured following the addition of a small volume (40 μL) of the 1 M solution of the various compounds dissolved in the hypotonic buffer with continuous stirring. Light scattering (400 nm excitation and 400 nm emission wavelengths, 500–500 nm in the case of *p*-nitrophenol glucuronide) was measured in a fluorimeter at room temperature.

β-Glucuronidase activities were measured in cells and microsomal fractions on the basis of aglycon release [8]. Protein concentrations were measured with the protein assay kit from BioRad Laboratories. Collagenase, saponin, digitonin, alamethicin, *p*-nitrophenol glucuronide, and phenolphthalein glucuronide were purchased from Sigma Chemical Co. Male Sprague–Dawley rats were from Nossan, and male CD-1 mice were obtained from Charles River Hungary.

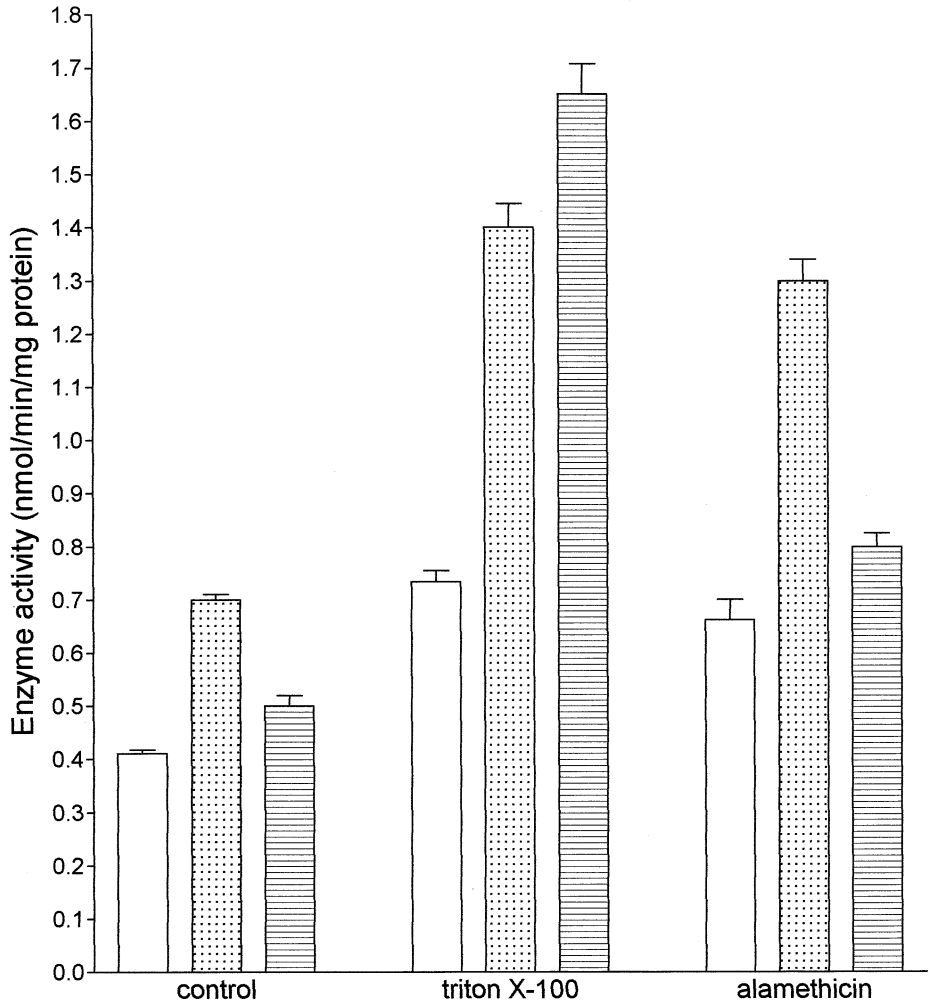

FIG. 2. Effect of Triton X-100 and alamethicin on β-glucuronidase activity in mouse or rat liver microsomal vesicles. Microsomes (1–2 mg/mL) were incubated in MOPS/KCl buffer (pH 7.2) at 37° for 30 min in the presence of 1 mM *p*-nitrophenol glucuronide (clear columns: mouse microsomes; dotted columns: rat microsomes) or phenolphthalein glucuronide (striped columns: rat microsomes). Triton X-100 (0.1%) and alamethicin (0.1 mg/mg protein) were used for permeabilization. *p*-Nitrophenol or phenolphthalein formation was measured. Data are means ± SEM of four experiments.

RESULTS AND DISCUSSION

The rate of *p*-nitrophenol formation from *p*-nitrophenol glucuronide was measured in control and permeabilized cells to study the latency of β-glucuronidase activity in isolated mouse hepatocytes. Selective plasma membrane permeabilization with saponin or digitonin increased β-glucuronidase activity 3- to 4-fold, whereas Triton X-100 permeabilization of all membranes caused an 8- to 9-fold increase in enzyme activity (Fig. 1.). These results suggest that β-glucuronidase activity in the ER is latent. As lysosomal β-glucuronidase may have contributed to the hydrolysis of glucuronides in the hepatocytes, it would appear to be latent as well. Therefore, the effect of various permeabilizing agents on β-glucuronidase activities in mouse and rat liver microsomal vesicles was studied. Triton X-100 and the pore-forming alamethicin increased β-glucuronidase activity almost 2-fold (Fig. 2.). These results agree with the data obtained using isolated hepatocytes.

The findings suggest that microsomal β-glucuronidase is not easily accessible for the extracellular glucuronides; consequently, the permeability of the microsomal membrane was further investigated using the light-scattering technique. The membrane was shown to be poorly permeable to *p*-nitrophenol glucuronide and virtually impermeable to phenolphthalein glucuronide. After addition of alamethicin the vesicles returned to their original size, which indicates that they had become permeable. Triton X-100 was added at the end of the experiments, which further decreases the light-scattering signal because it destroys the vesicular structure. The microsomal membrane was also poorly permeable to glucuronic acid, one of the products of the enzymatic hydrolysis (Fig. 3.).

Microsomal β-glucuronidase seems to play only a minor role in the catabolism of glucuronides taken up by the liver, because both the plasma and ER membranes represent barriers for the glucuronides. It is likely that β-glucuroni-

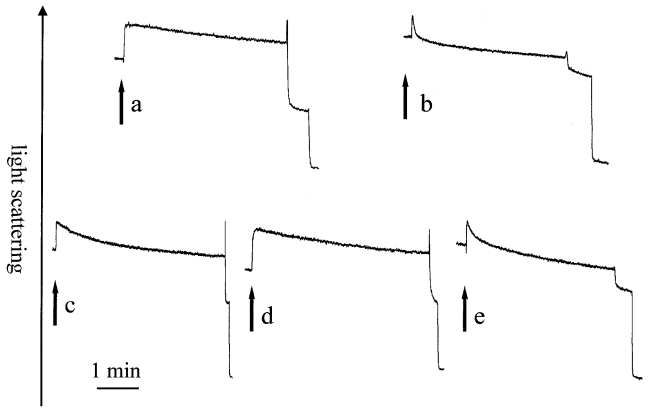

FIG. 3. Osmotically induced shrinking of rat liver microsomal vesicles induced by *p*-nitrophenol glucuronide, phenolphthalein glucuronide, or glucuronate. Rat liver microsomes (70 μg/mL of protein) were equilibrated in a low-osmolarity buffer (5 mM K-PIPES, pH 7) until a stable light-scattering baseline was obtained. Concentrated and neutralized solutions of sucrose (a), glucose (b), *p*-nitrophenol glucuronide (c), phenolphthalein glucuronide (d), or glucuronic acid (e) (0.5 M, in the K-PIPES buffer, pH 7; arrows) were added to 2.0 mL of the microsomal suspensions giving 20 mM final concentration for each. An increase in light scattering indicates shrinkage of microsomal vesicles. The addition of poorly permeable solutes results in a sustained shrinkage. The recovery of initial signal (swelling phase) after the addition of solutes is assumed to reflect their entry into vesicles [13]. Representative traces out of 5–8 similar experiments. At the end of each registration, microsomes were permeabilized with alamethicin, then destroyed by Triton X-100.

dase acts on newly synthesized glucuronides formed in the lumen of the ER. This futile cycle generates glucuronate, the substrate for enzymes of the hexuronic acid pathway present in the hepatic ER of ascorbate-synthesizing species [17, 18]. As a consequence, enhanced glucuronidation is generally accompanied by increased ascorbate production and elevated plasma ascorbate levels in these animals [19].

Rat liver microsomal β-glucuronidase has been shown to have a higher affinity for the acyl than for the ether glucuronides [3]. Xenobiotic acyl glucuronides are often more reactive, less stable, and potentially more toxic products of glucuronidation. It could be suggested that β-glucuronidase plays a selective role in the formation of glucuronides after their synthesis, favoring certain glucuronides for excretion. This can be considered as a kind of proofreading in the glucuronidation process.

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References

1. Zhen L, Baumann H, Novak EK and Swank RT, The signal for retention of the egasyn–glucuronidase complex within the endoplasmic reticulum. *Arch Biochem Biophys* **304:** 402–414, 1993.
2. Whiting JF, Narciso JP, Chapman V, Ransil BJ, Swank RT and Gollan JL, Deconjugation of bilirubin-IXα glucuronides: A physiologic role of hepatic microsomal β-glucuronidase. *J Biol Chem* **268:** 23197–23201, 1993.
3. Brunelle FM and Verbeeck RK, Glucuronidation of diflunisal by rat liver microsomes. Effect of microsomal beta-glucuronidase activity. *Biochem Pharmacol* **46:** 1953–1958, 1993.
4. Zhu BT, Evaristus EN, Antoniak SK, Sarabia SF, Ricci MJ and Liehr JG, Metabolic deglucuronidation and demethylation of estrogen conjugates as a source of parent estrogens and catecholestrogen metabolites in Syrian hamster kidney, a target organ of estrogen-induced tumorigenesis. *Toxicol Appl Pharmacol* **136:** 186–193, 1996.
5. Takahashi T and Hori SH, Alterations in the latency of hepatic microsomal hexose-6-phosphate dehydrogenase under various *in vivo* and *in vitro* conditions. *Biochim Biophys Acta* **672:** 165–175, 1981.
6. Bánhegyi G, Garzó T, Fulceri R, Benedetti A and Mandl J, Latency is the major determinant of UDP-glucuronosyltransferase activity in isolated hepatocytes. *FEBS Lett* **328:** 149–152, 1993.
7. Meier PJ, Eckhardt U, Schroeder A, Hagenbuch B and Stieger B, Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* **26:** 1667–1677, 1997.
8. Bánhegyi G, Braun L, Marcolongo P, Csala M, Fulceri R, Mandl J and Benedetti A, Evidence for a UDP-glucuronic acid/phenol glucuronide antiport in rat liver microsomal vesicles. *Biochem J* **315:** 171–176, 1996.
9. Bánhegyi G, Fulceri R, Bellomo G, Romani A, Pompella A and Benedetti A, Role of a nonmitochondrial Ca^{2+} pool in the synergistic stimulation by cyclic AMP and vasopressin of Ca^{2+} uptake in isolated rat hepatocytes. *Arch Biochem Biophys* **287:** 320–328, 1991.
10. Braun L, Csala M, Poussu A, Garzó T, Mandl J and Bánhegyi G, Glutathione depletion induces glycogenolysis dependent ascorbate synthesis in isolated murine hepatocytes. *FEBS Lett* **388:** 173–176, 1996.
11. Bánhegyi G, Garzó T, Antoni F and Mandl J, Glycogenolysis—and not gluconeogenesis—is the source of UDP-glucuronic acid for glucuronidation. *Biochim Biophys Acta* **967:** 429–435, 1988.
12. Bánhegyi G, Bellomo G, Fulceri R, Mandl J and Benedetti A, Intraluminal calcium of the liver endoplasmic reticulum stimulates the glucuronidation of *p*-nitrophenol. *Biochem J* **292:** 99–104, 1993.
13. Benedetti A, Fulceri R, Romani A and Comporti M, Stimulatory effect of glucose-6-phosphate on the non-mitochondrial Ca^{2+} uptake in permeabilized hepatocytes and Ca^{2+} release by inositol trisphosphate. *Biochim Biophys Acta* **928:** 282–286, 1987.
14. Fulceri R, Bánhegyi G, Gamberucci A, Giunti R, Mandl J and Benedetti A, Evidence for the intraluminal positioning of *p*-nitrophenol UDP-glucuronosyltransferase activity in rat liver microsomal vesicles. *Arch Biochem Biophys* **309:** 43–46, 1994.
15. Meissner G, Ionic permeability of isolated muscle sarcoplasmic reticulum and liver endoplasmic reticulum vesicles. *Methods Enzymol* **157:** 417–437, 1988.
16. Fulceri R, Bellomo G, Gamberucci A, Scott HM, Burchell A and Benedetti A, Permeability of rat liver microsomal membrane to glucose 6-phosphate. *Biochem J* **286:** 813–817, 1992.

17. Horio F, Shibata T, Makino S, Machino S, Hayashi Y, Hattori T and Yoshida A, UDP glucuronosyltransferase gene expression is involved in the stimulation of ascorbic acid biosynthesis by xenobiotics in rats. *J Nutr* **123:** 2075–2084, 1993.
18. Bánhegyi G, Braun L, Csala M, Puskás F and Mandl J, Ascorbate metabolism and its regulation in animals. *Free Rad Biol Med* **23:** 793–803, 1997.
19. Nishikimi M and Yagi K, Biochemistry and molecular biology of ascorbic acid biosynthesis. In: *Subcellular Biochemistry* (Ed. Harris JR), Vol. 25, pp. 17–40. Plenum Press, New York, 1996.