GLUCURONIDATION AND EXCRETION OF NONYLPHENOL IN PERFUSED RAT LIVER

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ABSTRACT:
Nonylphenol, an environmental estrogenic chemical, is reported to have adverse effects on the reproductive organs of animals. In this study, the metabolism of nonylphenol and that of other alkylphenols in the rat liver was investigated using liver perfusion. Alkylphenols (nonylphenol, hexylphenol, butylphenol, and ethylphenol) were glucuronidated by rat liver microsomes. Nonylphenol was found to be conjugated with glucuronic acid by an isomerase of UDP-glucuronyltransferase, UGT2B1, expressed in yeast AH22 cells. However, when nonylphenol was perfused into rat liver in situ, it was difficult for free nonylphenol and conjugated metabolite to be excreted into the bile or vein, and most of the perfused nonylphenol remained free and as a glucuronide conjugate in the liver tissue, even after 1 h of perfusion. After 1 h of perfusion of the other alkylphenols, most of them were excreted into the bile as glucuronides. Ethylphenol, which has the shortest alkyl chain, was secreted rapidly into both the bile and vein; however, the excretion rates of alkylphenols having longer alkyl chains tended to be slow. MRP-2-deficient Eisai hyperbilirubinemic rats could not secrete alkylphenol-glucuronides into the bile, indicating that alkylphenol-glucuronides are transported by MRP-2 to the bile in normal Sprague-Dawley rats. The results indicate that the kinetics of excretion of alkylphenol-glucuronides into the bile or vein depends on the length of alkyl chain and suggest that nonylphenol-glucuronide formed in the liver cannot be transported by MRP-2.

Environmental estrogenic chemicals such as bisphenol A and nonylphenol, which are contained in many industrial products, can be detected in foods, tap water, and many environmental materials. Nonylphenol is used in a wide variety of detergents and plastics and has been reported to be environmentally persistent (White et al., 1994). The mean daily oral intake of nonylphenol by humans is estimated to be 0.16 mg/day (Muller et al., 1998). Nonylphenol has been shown to be a possible endocrine disrupter due to its estrogenic effects in MCF7 cell proliferation assays (Soto et al., 1991), binding assays to the estrogen receptor (White et al., 1994) and uterotrophic assays in mice (Shelby et al., 1996). Exposure of male rainbow trout (Oncorhynchus mykiss) to four different alkylphenolic chemicals, including nonylphenol, resulted in synthesis of vitellogenin, a process normally dependent on endogenous estrogens, and a concomitant inhibition of testicular growth (Jobling et al., 1996). Male and female ratios of Japanese medaka (Oryzias latipes) in a control group (2:1) and a 100 μg/l nonylphenol treatment group (1:2) were reported to be significantly different (Gray and Metcalfe, 1997). Early neonatal exposure to nonylphenol has been reported to cause dysfunction of postpubertal reproductive function in female rats as well as disrupted development of gonads in male and female rats (Nagao et al., 2000). It has also been reported that nonylphenol administered orally at a dose of 50 mg/kg of body weight induced a significant increase in uterine weight of prepubertal rats and advanced the age of vaginal opening (Laws et al., 2000). Elucidation of the metabolism and fate of nonylphenol is important for estimating the risks of the chemical for animals. Xenoestrogens such as bisphenol A and diethylstilbestrol have been shown to be conjugated with glucuronic acid by a UDP-glucuronosyltransferase isomerase, UGT2B1, in the rat liver (Yokota et al., 1999). Nonylphenol has also been reported to be glucuronidated extensively in the liver of animals such as rainbow trout (Lewis and Lech, 1996; Coldham et al., 1998; Thibaut et al., 1998a,b) and rats (Moffat et al., 2001; Yokota et al., 2002). Since the liver is the main barrier against drugs in the body, identifying or tracing the metabolites of nonylphenol is important for elucidation of the disruptive effects of the compound on the reproductive system.

In this study, we investigated the metabolism and kinetics of nonylphenol and those of other alkylphenols by means of liver perfusion, and we found that nonylphenol was delayed in excretion from the liver and that the length of the alkyl chain is a critical factor for the excretion rate and route out of the liver.

Materials and Methods

Chemicals. 4-Nonylphenol and other alkylphenols were purchased from Kanto Chemical Co. (Tokyo, Japan). High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Labscan Ltd. (Dublin, Ireland). β-Glucuronidase was obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Male young adult Sprague-Dawley (SD) rats (8–13 weeks old) and MRP-2-deficient Eisai hyperbilirubinemic rats (EHBR) were used in all experiments. The rats were housed under standard conditions and given food and water ad libitum before use. The rats were handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University based on

Abbreviations used are: HPLC, high-performance liquid chromatography; SD, Sprague-Dawley; EHBR, Eisai hyperbilirubinemic rats; MRP-2, multidrug resistance-related protein-2; NP-G, nonylphenol-glucuronide; NP, nonylphenol; ER, estrogen receptor.
The liver was perfused for 60 min with Krebs’ solution as a control (A). Nonylphenol perfusion was performed with the same buffer containing 0.050 mM nonylphenol for 5 min and then perfused with Krebs buffer for 55 min (B). Nonylphenol and its metabolites in the liver were extracted with acetonitrile and then analyzed by HPLC after the perfusion as described under Materials and Methods. The enzyme reactions were performed for 0 min (C) and 60 min (D), and the resultant glucuronide conjugate was analyzed by HPLC as described previously (Yokota et al., 1999). Nonylphenol glucuronidation was confirmed by polymerase chain reaction and expressed in yeast AH22 cells as previously described (Yokota et al., 1999). UDP-glucuronosyltransferase isoform UGT2B1 was obtained by reverse transcription-polymerase chain reaction and expressed in yeast AH22 cells, and nonylphenol was glucuronidated by microsomes prepared from the yeast transformant as described under Materials and Methods. The enzyme reactions were performed for 0 min (C) and 60 min (D), and the resultant glucuronide produced by UGT2B1 was analyzed by HPLC. The arrows indicate NP-G and unconjugated nonylphenol (NP).

Expression of UDP-Glucuronosyltransferase 2B1. cDNA of rat UDP-glucuronosyltransferase isoform UGT2B1 was obtained by reverse transcriptase-polymerase chain reaction and expressed in yeast AH22 cells as previously described (Yokota et al., 1999). Nonylphenol glucuronidation was performed using yeast microsome-expressed UGT2B1, and the resultant glucuronide conjugate was analyzed by HPLC as described previously (Yokota et al., 1999).

Surgical Procedure. The rats were anesthetized by intraperitoneal injection of 60% urethane (0.3 ml/100 g of body weight). Whole liver perfusion was performed according to the method reported by Sugano et al. (1978) with slight modifications (Inoue et al., 2001). Briefly, after anesthesia, the abdomen was opened and the liver, portal vein, bile duct, and inferior vena cava were exposed. The common bile duct and the portal vein were cannulated with PE-10 and PE-50 polyethylene tubes (BD Biosciences, San Jose, CA), respectively, and oxygenated Krebs-Ringer buffer, described below, was pumped through the liver via the portal vein. The abdominal vena cava was incised immediately after perfusion, and a dripping polyethylene tube (2 mm i.d., 3 mm o.d.) was inserted. The thorax was then opened and the thoracic vena cava was ligated. The liver was not excised. All experiments were performed in situ. While the animals were still under anesthesia, euthanasia was performed by exsanguination.

Liver Perfusion. Krebs-Ringer buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 1.2 mM NaH2PO4, 1.2 mM Na2SO4, 2.5 mM CaCl2, 25 mM NaHCO3, 10 mM glucose) was used in all experiments. The buffer solution was aerated by 95% O2 and 5% CO2, and the pH was adjusted to 7.4. Ethanol containing alkylphenol was dispersed into the buffer solution, and final concentration of ethanol was 1%. The substrate buffer solution contained alkylphenol in a final concentration of 0.025 or 0.05 mM. These buffer solutions were maintained in a water bath at 37°C. The perfusion system consisted of a peristaltic pump (MP-32N; EYELA, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) and silicone tubes, as described in our previous paper (Inoue et al., 2001). The buffer solution was pumped at a constant rate of 30 ml/min, and the liver perfusion was carried out in a flow-through mode. Preliminary perfusion was done for 15 min, and then the substrate buffer solution was perfused for 5 min, followed by reperfusion of the Krebs-Ringer solution. After perfusion of the substrate buffer, the excised bile and perfusate in the vein were collected at 5-min or 10-min intervals over a 1-h period.

HPLC Analysis of Reaction Products. The bile was dissolved in 40-fold 50% acetonitrile, and perfusate was diluted with an equal volume of acetonitrile; then, the perfusate mixtures were centrifuged for 10 min at 10,000g. The supernatant fractions were analyzed by HPLC (Shimadzu, Tokyo, Japan) according to the method described previously (Yokota et al., 1999; Inoue et al., 2001). Alkylphenols and its metabolites were eluted from HPLC by 75% acetonitrile solution (acetonitrile/water/acetic acid, 75:25:0.1) for nonylphenol, 60% solution for hexylphenol, 50% solution for butylphenol, and 35% solution for ethylphenol. Recordings were made using a C-R8A integrator (Shimadzu, Tokyo, Japan). Alkylphenols and its metabolites retained in the liver after the perfusion were extracted with acetonitrile, the extractions were centrifuged for 30 min at 25,000g, and the supernatants were analyzed by HPLC with the same procedure described above. Alkylphenol-glucuronides were quantitated by the amount of the deconjugated free alkylphenols after β-glucuronidase treatment of the reaction products as previously described (Shibata et al., 2002).
Results

The HPLC profiles of the metabolites of nonylphenol produced in the liver, after the perfusion had been performed as described under Materials and Methods, are shown in Fig. 1. The nonylphenol metabolite extracted from the perfused liver was eluted at an early retention time as a single peak [nonylphenol-β-D-glucuronide (NP-G); 7 min] with slight shoulder peaks (Fig. 1B). The main peak produced by liver microsomes had the same retention time and was reduced by treatment with β-glucuronidase as was previously reported in rainbow trout (Lewis and Lech, 1996; Coldham et al., 1998; Thibaut et al., 1998a,b) (data not shown). A glucuronide conjugate of nonylphenol was produced by UGT2B1 (Fig. 1D), indicating that the main metabolite in the liver perfusion is NP-G. The peak of NP glucuronide was confirmed to be not produced by AH22 yeast microsomes, expressing no UGT2B1, and in the enzyme reactions where the substrate was omitted. The $K_m$ value of the microsomal UDP-glucuronosyltransferase was estimated to be 0.25 mM nonylphenol. UDP-glucuronosyltransferase activities ($V_{max}$) toward nonylphenol and other alkylphenols in rat liver microsomes were assayed, and the estimated enzymatic activities are shown in Fig. 2. UDP-glucuronosyltransferase activity toward nonylphenol showed about the same value as that of ethylphenol. These alkylphenols were injected into the portal veins of rats, and then the bile and perfusate in the veins were sampled and analyzed by HPLC as previously described (Inoue et al., 2001). The resultant metabolites of alkylphenols were mainly glucuronide conjugates (data not shown), as was the case for nonylphenol. The concentrations of the glucuronide conjugates of alkylphenols in the bile and vein during liver perfusion are shown in Fig. 3. After the liver perfusion, alkylphenol-glucuronides were excreted only into the bile (Fig. 3, A–D), and only ethylphenol-glucuronide was excreted into
both the bile and vein within 60 min (Fig. 3E). When 0.05 mM butylphenol and hexylphenol were perfused, these alkylphenols were also glucuronidated in the liver, but these glucuronides were not excreted completely from the liver within 60 min of perfusion (Fig. 3B). Trace amounts of nonylphenol-glucuronide were detected only in the bile (Fig. 3, C and D). The alkylphenols remaining in the livers after 1 h of perfusion were extracted as described under Materials and Methods. The amounts of alkylphenols in the liver and that excreted into bile and vein during 1 h of perfusion are shown in Fig. 4. Recovery of alkylphenols after the perfusions were about 50 to 60% (Fig. 4). One of the reasons for this is that it was supposed that all other alkylphenol metabolites such as hydroxylated alkylphenols and conjugated alkylphenols with sulfate or glutathione remaining in the liver tissue were not extracted with acetonitrile completely, and/or

![Fig. 4. Alkylphenol metabolites in the bile, liver tissue, and vein after 1 h of perfusion in SD rats.](image-url)

Total amounts of alkylphenols and glucuronide conjugates, which were excreted into the bile and vein and remained in the liver during a 60-min period of liver perfusion, are shown. Numerals in the columns indicate the percentages of respective metabolites, free (□) or glucuronide conjugate (□). Data are represented in the upper box (A) when 0.025 mM alkylphenol was injected, in the lower box (B) when 0.050 mM nonylphenol was injected. About 30% of injected alkylphenols were absorbed to the inside of the silicon tubes during the perfusions. Recovery was calculated with the tube absorption of each alkylphenol. Parameters are shown as means ± S.E. (n = 4 animals or n = 3 animals in ethylphenol analysis).
metabolites were observed in the bile (Fig. 5A). Rapid excretion and excretion from the liver. The metabolites of butylphenol and hexylphenol in liver. The transportation of glucuronides into the vein from liver cells, can be transported to the bile by MRP-2. The substrate specificity of MRP-2 has been studied by comparing the transportation activity across the bile canalicular membrane in normal rats with that in transport-deficient rats (Paulusma et al., 1996) or in EHBR, which have a hereditary defect in MRP-2 (Fernandez-Checa et al., 1992; Takenaka et al., 1995; Yamazaki et al., 1996). It has been demonstrated that glutathione conjugates, glucuronides of xenobiotics, sulfates of several bile acids, and some organic anions are substrates for MRP-2, but the mechanism of the transport and the substrate specificity of the transporter have not been clarified yet. In this study, it was found that transportation activity of MRP-2 decreased with increase in the number of alkyl chains of alkylphenols, suggesting that after MRP-2 recognizes or binds to the glucuronic acid anion, the long alkyl chain of alkylphenol-glucuronide across the canalicular membrane into the bile was delayed. These findings suggest that nonylphenol glucuronidation is mediated by UDP-glucuronosyltransferase isofrom(s), such as UGT2B1, with high activity but that the resultant glucuronide having a long (C9) alkyl chain could not be transported by MRP-2.

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Finally, nonylphenol has been shown to reduce the level of CYP1A1 expression in murine Hepa-1c1c7 cells (Jeong et al., 2001) and to inhibit in vitro CYP1A1 activity in rat liver microsomes (Lee et al., 1996) and activities of human cytochrome P450s, including steroidogenic CYP17 activities (Niwa et al., 2002). Oral administration of nonylphenol decreased hepatic testosterone hydroxylation and CYP2C expression level (Laurenzana et al., 2002). Estradiol binding to the estradiol receptor (ER) was significantly inhibited by nonylphenol (Danzo, 1997). These results suggest the possible inhibition of cytochrome P450s and ER functions by nonylphenol, which delays excretion from the liver.
One of the toxicological implications for the delay of excretion of nonylphenol and nonylphenol-glucuronide is estimated to the binding to and inhibition of ER (Tabira et al., 1999); another is the inhibition of MRP-2 which excretes various glucuronides such as estradiol glucuronide (Ito et al., 2001) and bilirubin glucuronide (Kusuhara et al., 1998). The delay of nonylphenol excretion is possible because of the adverse effects of nonylphenol on rat reproductive systems (Laws et al., 2000).

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