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GLUCURONIDATION OF CARCINOGENIC ARYLAMINE METABOLITES BY RAT LIVER MICROSOMES

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Abstract—Since 2-acetylaminofluorene (2-AAF), 4-acetylaminobiphenyl (4-AABP) and 2-aminonaphthalene (2-AN) display varying degrees of carcinogenicity in the rat, which is capable of N-acetylating arylamines, an attempt was made to correlate the difference in carcinogenicity of these compounds with the ease of O-glucuronidation of their hydroxamic acids by rat hepatic microsomes, a reaction believed to be a detoxification mechanism. UDP-glucuronosyltransferase activity of rat hepatic microsomes was activated by Triton X-100. Glucuronidation by Triton X-100 activated microsomes of the N-hydroxy derivative of 2-AN was approximately 1.5 and 1.8 times faster than the corresponding derivatives of 2-aminofluorene (2-AF) and 4-aminobiphenyl (4-ABP) respectively. However, glucuronidation of the N-hydroxy-N-acetyl derivative of 2-AN was 40 and 17 times faster than the corresponding derivatives of 2-AF and 4-ABP respectively. Aroclor 1254 and 3-methylcholanthrene, but not phenobarbital, acetanilide and butylated hydroxytoluene, induced the enzyme for the glucuronidation of 2-AN derivatives. The present study (1) demonstrates an inverse relationship between the carcinogenicity of 2-AN, 4-AABP and 2-AAF and the ease of glucuronidation of their hydroxamic acid derivatives, and (2) suggests that, in addition to N- and C-hydroxylation, glucuronidation may play an important role in determining the carcinogenicity of arylamines and arylacetamides in the rat.

Certain arylamines that are suspected to be human bladder carcinogens are carcinogenic in experimental animals (reviewed in Ref. 1). N-Hydroxylation is believed to be the first step in the metabolic activation of arylamines and arylacetamides. The second step involves conjugations of the N-hydroxy metabolites. Glucuronidation appears to be quantitatively the most important type of conjugation involved in the excretion of N-hydroxy metabolites of arylamines and arylacetamides [2]. Hepatic microsomal GT† (EC 2.4.1.17) catalyzes the formation of the N-glucuronides of arylamines and arylhydroxylamines, and the O-glucuronides of arylacethydroxamic acids. In rabbits, as much as 30% of a single oral dose of 2-AAF is excreted in the urine as the O-glucuronide of N-OH-AAF in 24 hr [3]. Rats subjected to an i.p. injection of N-OH-AAF excrete more than 30% of the dose as the O-glucuronide in the bile [4]. More than 50% of N-OH-AN excreted in the urine of rats orally administered 2-AN is excreted as the N-glucuronide [5]. Glucuronic acid conjugation of carcinogenic arylamine derivatives has been shown in several species, including humans [6].

Glucuronic acid conjugates of arylamines [7], arylhydroxylamines [8–10] and arylacethydroxamic acids [11] have been prepared by chemical synthesis or biosynthesis. The N-glucuronides of arylhydroxylamines, which are excreted in the urine, yield in acidic media nitrenium ions that react with macromolecules, suggesting that this type of conjugate may play a role in the genesis of bladder tumors by arylamines [5, 8]. The O-glucuronides of arylacethydroxamic acids react with macromolecules in basic media probably involving base-catalyzed deacetylation of the O-glucuronides [2, 11, 12]. The N-glucuronides of arylhydroxylamines induce repair synthesis of DNA in cultured urothelial cells of several species [13, 14]. However, the O-glucuronide of N-OH-AAF, which is the most reactive O-glucuronide of an arylacethydroxamic acid ever tested [11], does not induce repair synthesis of DNA in the absence of β-glucuronidase [13]. These observations suggest that the N-glucuronidation of arylhydroxylamines may be involved in the transportation of reactive metabolites, whereas the O-glucuronidation of arylacethydroxamic acids may be a detoxification mechanism [13]. Arylamines or arylacetamides differing in aryl moieties display varying degrees of carcinogenicity. 2-AAF is a more potent carcinogen than 4-AABP (comparing data in Refs. 15 and 16), and both produce tumors in various organs in the rat (reviewed in Ref. 1). 2-AN is a weak carcinogen for the rat; it produces bladder tumors only if a very

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† Abbreviations: GT, UDP-glucuronosyltransferase; 2-AAF, 2-acetylaminofluorene; 2-AF, 2-aminofluorene; N-OH-AF, N-hydroxy-2-aminofluorene; N-OH-AAF, N-hydroxy-2-acetylaminofluorene; 4-AABP, 4-acetylaminobiphenyl; 4-ABP, 4-aminobiphenyl; N-OH-ABP, N-hydroxy-4-aminobiphenyl; N-OH-AABP, N-hydroxy-4-acetylaminobiphenyl; 2-AAN, 2-acetylaminonaphthalene; 2-AN, 2-aminonaphthalene; N-OH-AN, N-hydroxy-2-aminonaphthalene; N-OH-AAN, N-hydroxy-2-acetyl aminonaphthalene; DMSO, dimethyl sulfoxide; UDPGA, uridine-5′-diphosphate glucuronic acid; PB phenobarbital; 3-MC, 3-methylcholanthrene; and BHT, butylated hydroxytoluene.

Table 1. Treatment of rats by xenobiotics*

Groups	Treatments
1	Intraperitoneal injection on day 1 with 0.2 ml corn oil
2	Intraperitoneal injection on day 1 with 0.2 ml corn oil containing 75 mg Aroclor (500 mg/kg)
3	Intraperitoneal injection for 4 days with a daily dose of 0.2 ml corn oil containing 3 mg 3-MC (80 mg/kg total dose)
4	Intraperitoneal injection for 4 days with a daily dose of 0.1 ml DMSO
5	Intraperitoneal injection for 4 days with a daily dose of 0.1 ml DMSO containing 12 mg PB (320 mg/kg total dose)
6	Intraperitoneal injection for 4 days with a daily dose of 0.1 ml DMSO containing 75 mg acetanilide (2 g/kg total dose)
7	Intraperitoneal injection for 4 days with a daily dose of 0.1 ml DMSO containing 75 mg BHT (2 g/kg total dose)

* Female CD rats, approximately 150 g, were treated according to the schedules. All the animals were killed on day 5.

large dose is given [17]. To relate this difference in carcinogenicity to the glucuronidation of the hydroxamic acids of these carcinogens, we investigated the glucuronidation of 2-AF, 4-ABP and 2-AN derivatives by rat hepatic microsomes. The inducibility of GT by PB, 3-MC, Aroclor 1254, acetanilide and BHT was also investigated, because these compounds have been shown to modify the carcinogenicities of arylamines and arylacetamides (reviewed in Ref. 1).

MATERIALS AND METHODS

Chemicals. 2-AF, 2-nitrofluorene, 4-ABP, 4-nitrobiphenyl and 2-nitronaphthalene were obtained from the Aldrich Chemical Co., Milwaukee, WI. N-OH-AF, N-OH-AAF, N-OH-ABP, N-OH-AABP, N-OH-AN, and N-OH-AAN were prepared from their corresponding nitro compounds by the method described previously [13]. Reduction of 2-nitronaphthalene by hydrazine at room temperature in the presence of paladium/charcoal yielded 2-AN. Phosphoenolpyruvate, NADH, UDPGA, pyruvate kinase, lactate dehydrogenase, ascorbic acid, BHT, PB, 3-MC, and acetanilide were obtained from the Sigma Chemical Co., St. Louis, MO. Aroclor 1254 was obtained from the Monsanto Chemical Co., St. Louis. MO.

Animal experiments. Female CD rats (Charles River Breeding Laboratories, Wilmington, MA) weighing approximately 150 g were divided into seven groups with four rats each, and treated according to the schedules presented in Table 1. Wayne Lab Blox (Allied Mills, Chicago, IL) and drinking water were given *ad lib.* The rats were killed by ether anesthesia followed by exsanguination on day 5. Livers were homogenized in 0.25 M sucrose solution by a glass–Teflon homogenizer. The homogenate was centrifuged at 9000 *g* for 15 min, and the resultant supernatant fraction was then centrifuged at 105,000 *g* for 1 hr. The microsomal pellets were suspended in 0.25 M sucrose solution and stored at −70°. All the preparation procedures were performed at 4°.

Enzyme assays. The procedure of Mulder and van Doorn [18] was slightly modified for the assay of GT activity. One volume of 2% Triton X-100 was added to 15 vol. of microsomal suspension 20 min before the assay. The reaction mixture contained 0.1 M Tris–HCl, pH 7.3, 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 6 mM UDPGA, 5 mM $MgCl_2$, 5 units of pyruvate kinase, 0.65 unit of lactate dehydrogenase, and 100–400 mg of microsomal protein in a total volume of 0.645 ml. The mixture was added to a cuvette, placed into a Cary 219 spectrophotometer (Varian, Palo Alto, CA), and equilibrated at 37° for 5 min. Arylamines or their N-hydroxylated derivatives were dissolved in ethanol and 0.015 ml of the freshly prepared solution containing desirable amounts of the compounds was added into the reaction mixture. The reaction mixture contained 0.5 mM ascorbic acid only when arylhydroxylamines or amines were used as the substrates. Absorbance at 340 nm was traced immediately following the addition of substrates by an Apple

Table 2. Effect of Triton X-100 on the activity of microsomal UDP-glucuronosyltransferase*

	Glucuronosyltransferase (nmoles · mg^{-1} · min^{-1})		
	N-OH-AN	N-OH-AAN	2-AN
Solubilized	41.0 ± 5.9	47.3 ± 5.9	5.1 ± 0.7
Nonsolubilized	9.0 ± 2.4	26.2 ± 4.9	<1
Activation	4.6X	1.8X	>5X

* One volume of 2% Triton X-100 solution was added to 15 vol. of rat liver microsome 20 min before the assays. The reaction mixture contained 0.1 M Tris–HCl, pH 7.3, 0.3 mM phosphoenolpyruvate, 0.2 mM NADH, 6 mM UDPGA, 5 mM $MgCl_2$, 5 units pyruvate kinase, 0.65 unit lactate dehydrogenase, 100–400 mg microsomal protein, and 0.015 ml ethanol in a total volume of 0.66 ml. The concentrations of N-OH-AN, N-OH-AAN and 2-AN were 0.5, 1.2 and 0.5 mM respectively. The reaction was monitored photometrically by the oxidation of NADH. Data presented are the mean ± S.D. of four rats.

Table 3. Effect of UDPGA concentrations on the glucuronidation of N-OH-AAN*

Conc. of UDPGA (mM)	Glucuronosyltransferase activity (nmoles·mg^{-1}·min^{-1})
2	40.0
4	42.0
6	43.0
8	43.5

* With the exception of UDPGA concentration, the reaction conditions were identical to those described in Table 2. The data are means of duplicate determinations using Triton X-100 treated microsomes.

II computer which was interfaced to the spectrophotometer, and the rate of absorbance change was calculated automatically by an enzyme kinetics program provided by Varian. The rise of substrate-dependent oxidation of NADH was used for the calculation of enzyme activity.

Protein determinations. Protein concentrations were determined by the method described by Bradford [19] using Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin was used as a standard.

RESULTS

Effect of Triton X-100 on GT activity. The assays were conducted using microsomes obtained from the liver of rats that had been treated with DMSO (Group 4, Table 1). Triton X-100 treatment increased the GT activity (Table 2). It is noted that the increase varied with respect to the type of substrate; the increase was greater for N-OH-AN and 2-AN than for N-OH-AAN. Non-enzymatic glucuronidation of these compounds could not be detected under the experimental conditions. The detection limit by this spectrophotometric method was 0.5 nmole/min.

Effect of UDPGA concentrations on GT activity. The GT activity was dependent on the concentration of UDPGA. The optimal concentration of UDPGA was approximately 6 mM for the glucuronidation of N-OH-AAN (Table 3).

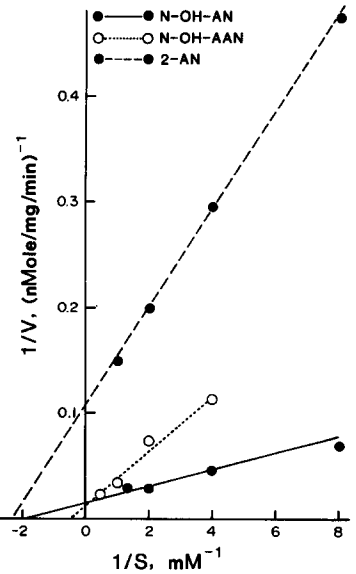

Fig. 1. Lineweaver–Burk plots of the concentration dependence of the velocity of glucuronidation of 2-AN derivatives. With the exception of substrate concentration, the reaction conditions were identical to those described in Table 2. The data are means of duplicate determinations using Triton X-100 treated microsomes.

Effect of concentrations of arylamine derivatives on GT activity. The GT activity was a function of arylamine concentrations (Fig. 1). The double-reciprocal plot indicated that the apparent V_{max} followed the order of N-OH-AAN > N-OH-AN > 2-AN, and the apparent K_m followed the order of N-OH-AAN > N-OH-AN > 2-AN.

Comparison of GT activity for various arylamine derivatives. The abilities of hepatic microsomes of Group 4 rats to catalyze the glucuronidation of 2-AF, 4-ABP and 2-AN metabolites are shown in Table 4. Due to low solubilities of 2-AF derivatives, the concentrations selected for enzyme assays were 0.5 mM for amines and hydroxylamines and 1.2 mM for hydroxamic acids, with the exception of N-OH-AF. This compound was the least soluble and the

Table 4. Glucuronidation of 2-AN, 2-AF, and 4-ABP derivatives*

	Glucuronosyltransferase (nmoles·mg^{-1}·min^{-1})		
	2-AN	2-AF	4-ABP
Hydroxylamine	41.0 ± 5.9 (100)†	26.3 ± 5.7 (100)	22.1 ± 3.0 (100)
Acethydroxamic acid	47.3 ± 5.9 (115)	1.2 ± 0.5 (4.6)	2.8 ± 1.5 (12.7)
Amine	5.1 ± 0.7 (12.4)	6.0 ± 2.1 (22.8)	1.3 ± 1.3 (5.9)

* Rat liver microsomes had been pretreated with 0.125% Triton X-100 for 20 min. The concentration was 0.5 mM for amines and hydroxylamines, with the exception of N-OH-AF. Due to a lower solubility, the concentration for N-OH-AF was 0.35 mM. The concentration for acethydroxamic acids was 1.2 mM. Data presented are the mean ± S.D. of four rats.

† Numbers in parentheses indicate the relative activity between the derivatives of individual amines.

Table 5. Induction of UDP-glucuronosyltransferase by xenobiotics*

Groups	Pretreatment	Glucuronosyltransferase (nmoles · mg^{-1} · min^{-1})		
		N-OH-AN	N-OH-AAN	2-AN
1	Control (corn oil)	27.1 ± 7.6	34.5 ± 7.6	4.0 ± 1.7
2	Aroclor	74.3 ± 7.0†	55.0 ± 3.1†	7.5 ± 0.9†
3	3-MC	91.1 ± 13.7†	55.7 ± 2.0†	6.5 ± 1.4‡
4	Control (DMSO)	41.0 ± 5.9	47.3 ± 5.9	5.1 ± 0.7
5	PB	36.2 ± 9.2	39.6 ± 10.2	6.2 ± 1.7
6	Acetanilide	39.1 ± 10.8	38.2 ± 5.7	4.0 ± 0.8
7	BHT	34.3 ± 5.9	41.3 ± 9.0	4.1 ± 0.8

* Pretreatments were described in Table 1. Data presented are the mean ± S.D. of four rats for each group.
† $P < 0.05$ for Group 1 vs Group 2 and Group 1 vs Group 3.
‡ $P < 0.1$ for Group 1 vs Group 3.

concentration used was 0.35 mM. It is noted that even under the conditions of a greater concentration of hydroxamic acids than hydroxylamines, the glucuronidation rates were remarkably greater for hydroxylamines than for hydroxamic acids of 2-AF and 4-ABP. The differences between N-OH-AF and N-OH-AAF, and between N-OH-ABP and N-OH-AABP, were 22 and 8 times respectively. On the contrary, the rates were approximately equal between N-OH-AN and N-OH-AAN. GT appeared to be less active for amines than for hydroxylamines.

Inducibility of GT by xenobiotics. The results of the effects of pretreatment with Aroclor 1254, 3-MC, PB, acetanilide and BHT on the microsome-catalyzed glucuronidation of N-OH-AN, N-OH-AAN, and 2-AN are shown in Table 5. Only Aroclor 1254 and 3-MC significantly induced the enzyme for the glucuronidation of N-OH-AN and N-OH-AAN ($P < 0.05$). The enzyme for the glucuronidation of 2-AN was significantly induced by Aroclor 1254 ($P < 0.05$) but not by 3-MC ($0.05 < P < 0.1$).

DISCUSSION

This study demonstrated that rat hepatic microsomes catalyzed the glucuronidation of 2-AF, 4-ABP and 2-AN and their N-hydroxy metabolites, and that this glucuronidation activity was much greater for N-OH-AAN than for N-OH-AAF and N-OH-AABP. Both 3-MC and Aroclor 1254, modifiers of arylamine carcinogenesis, induced rat hepatic GT activity for 2-AN derivatives.

Glucuronidation of arylhydroxylamines and arylhydroxamic acids by rat liver microsomes has been reported previously [2, 8, 11, 20, 21]. However, the activities reported are quite varied. The activity for the glucuronidation of N-OH-AN reported by Kadlubar *et al.* [8] is 21 nmoles · (mg protein)$^{-1}$ · min^{-1} for male CD rats, and the activities reported by Bock and Lilienblum [20] are 38 and 9 for male Wistar rats, respectively, with and without the activation by a detergent. Our data are similar to the latter, being 41 and 9, with and without the activation by Triton X-100. The activity for the glucuronidation of N-OH-AAF by untreated liver microsomes reported by Irving [2] is 2 for male or female rats and by Kadlubar *et al.* [8] is 9 for male CD rats, and the activity for Triton X-100 treated liver microsomes of male CD rats reported by Mulder *et al.* [21] is 10. These activities are greater than ours. The differences may be attributed to differences in experimental conditions, such as the strain, age and sex of rats, media used for the preparation of microsomes, pH used for assays, etc. The glucuronidation of arylamines *in vitro* has not been demonstrated previously.

There are at least two molecular species of GT in rat liver [22, 23]; one is inducible by 3-MC (GT_1) and the other by PB (GT_2). Aroclor induces both types of GT [23]. Mouse liver microsomes also have two similar types of GT [24]. Our results are consistent with that of Bock and Lilienblum [20] that 3-MC but not PB induces the enzyme activity for the glucuronidation of N-OH-AN. Aroclor also induces the glucuronidation of these AN metabolites. Thus, arylhydroxylamines and arylhydroxamic acids are the group 1 substrates of GT.

The carcinogenicity of arylamines can be changed by many factors that may affect the metabolisms of arylamines. N- and C-Oxidation appear to be the most important ones. For example, rats that are more susceptible to 2-AAF than 4-AABP excrete more N-hydroxy metabolites of 2-AAF than of 4-AABP after oral administration of these carcinogens [25]. Similarly, dogs that are more susceptible to 4-ABP than 2-AN (comparing data in Refs. 26 and 27) excrete more N-hydroxy metabolites of 4-ABP than of 2-AN [25]. Glucuronidation may also be an important factor. Rats that are able to N-acetylate arylamines [28] may excrete more hydroxamic acids than hydroxylamines. The observations that the O-glucuronide of N-OH-AAF is a much weaker carcinogen than N-OH-AAF [29] and that this glucuronide is not biologically active in intact cells [13] suggest that glucuronidation may be a detoxification reaction for hydroxamic acids. This is consistent with the present finding that there is an inverse relationship between the carcinogenicities of 2-AAF, 4-AABP and 2-AN and the ease of glucuronidation of the hydroxamic acids of these carcinogens by rat liver microsomes.

The mechanisms involved in the modification of the carcinogenicity of arylamines by PB, 3-MC, BHT and acetanilide are perplexing. This is exemplified by the recent finding that BHT inhibits hepatocarcinogenesis, but simultaneously enhances bladder carcinogenesis by 2-AAF in the rat [30].

Although BHT and acetanilide did not enhance hepatic GT activity of arylamine derivatives (Table 5), they increase urinary excretion of the O-glucuronide of N-OH-AAF after oral administration of 2-AAF in the rat [31]. The increase of urinary excretion of this type of conjugate has been attributed previously to the inhibition of hepatocarcinogenesis of 2-AAF by BHT and acetanilide derivatives [31].

Note added in proof. Lilienblum and Bock [32] have recently reported that rat and human liver microsomes catalyze the N-glucuronidation of carcinogenic arylamines.

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