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GLUCURONIDATION OF OXYGENATED BENZO(A)PYRENE DERIVATIVES BY UDP-GLUCURONYLTRANSFERASE OF NUCLEAR ENVELOPE

Surendra K. Bansal, Jan Zaleski* and Teresa Gessner

Department of Experimental Therapeutics
Roswell Park Memorial Institute
Buffalo, NY 14263

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SUMMARY: Several benzo(a)pyrene phenols, dihydrodiols, epoxides, quinones, and a diolepoxide were tested as possible substrates for UDP-glucuronyltransferase located in rat liver nuclear envelope fraction. Only phenolic derivatives of benzo(a)pyrene served as substrates for the nuclear membrane transferase, under the conditions tested. The specific activities observed in nuclear envelope preparations were greater than or equal to, those of the microsomal fraction indicating that a very effective detoxication mechanism for these phenols is present in the nuclear compartment.

It now appears well established that polycyclic aromatic hydrocarbons (PAH), such as benzo(a)pyrene (BP), require metabolic activation to electrophilic metabolites for their toxicity, mutagenicity, carcinogenicity and binding to tissue macromolecules (1-3). Such electrophilic metabolites of BP are produced through reactions catalyzed by arylhydrocarbon hydroxylases, epoxide hydrases and some spontaneous rearrangements or oxidations, and include phenols, epoxides, dihydrodiols, quinones and diolepoxides (1-5). Formerly, epoxides were considered to be responsible for toxicity and carcinogenicity (6,7), currently diolepoxides, formed from dihydrodiols, are thought to be the ultimate carcinogens (8,9,10). The most potent carcinogenic form of BP has

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Abbreviations: PAH, polycyclic aromatic hydrocarbons; BP, benzo(a)pyrene; 1-OH BP, 1-hydroxy benzo(a)pyrene, similarly for 2-OH BP, 3-OH BP, etc; UDPGA, uridine diphosphoglucuronic acid; NE, nuclear envelope fraction; MCS, microsomal fraction; TLC, thin layer chromatography; DPM, disintegrations per minute.

*Permanent Address: Institute of Biochemistry, University of Warsaw, Al. Zwirki i Wigury, Warsaw 02-089, Poland.

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been shown to be the (+)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro BP (10). Some BP-phenols also have been found to be mutagenic, (11,12) and capable of being metabolized to DNA-binding molecules (12). The quinones can be produced by auto-oxidation of phenols, and 6-OH BP has been shown to form 6,12-, 1,6- and 3,6-diones via a free radical, 6-oxo BP formation (13). These quinones have been observed to be cytotoxic and able to cause strand breakage of nucleic acids (14). Endoplasmic reticulum is the major site for the metabolic activation of PAH (15-17), however, small amounts of such activity have also been observed in nuclei (17-19). The latter may be of special significance because of proximity to nuclear DNA.

It is believed that conjugation reactions are important in the detoxication of the activated metabolites of PAH (12,20,21). Glucuronidation is one such reaction. The ability of rat liver microsomes to conjugate many oxygenated BP derivatives with the glucuronyl moiety of UDP-glucuronic acid, has been reported (22). In this study, we tested rat liver nuclear envelope fraction for its ability to glucuronidate several BP phenols, quinones, epoxides and dihydrodiols, in an attempt to learn which of these compounds can be so detoxified at this important site.

MATERIALS AND METHODS

Chemicals. Uridine diphospho-D-[U-^{14}C]-glucuronic acid, ammonium salt (283 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, IL. Benzo(a) pyrene derivatives were a gift from NCI, Chemical and Physical Carcinogenesis Branch. TLC plates (1mm thick, type PLKF) were purchased from Pierce Chemical Co., Rockford, IL. p-Nitrophenol-β-D-glucuronide, testosterone-β-D-glucuronide, D-saccharic acid-1,4-lactone (saccharolactone), albumin (fraction V of bovine serum), Lubrol WX and β-glucuronidase (type B-10 from bovine liver) were from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Fraction Preparation. Male, ACI strain rats, weighing about 200 g were used throughout. Livers of 3-4 fed rats were combined together for isolation of nuclear envelopes and microsomes. Nuclei were isolated according to the procedure of Widnell and Tata by centrifugation through 2.4 M sucrose (23), with additional washing (19). Nuclear envelope fraction (NE) was then isolated from pure nuclei by method of Kay et al (24). Microsomal fraction (MCS) was separated from the post-nuclear supernatant by centrifugation first at 15,000g for 15 min, then at 105,000 x g for 60 min. The quality of NE was monitored by phase contrast microscopy, and the purity was checked by electron microscopy and by measurement of the activity of ethylmorphine N-demethylase (25).

Protein was determined by a modified Lowry's method (26), after solubilization of membranes in 1% (w/v) Triton X-100, using bovine serum albumin as standard.

Determination of Glucuronidation of Benzo(a)pyrene Derivatives. Glucuronidation of BP derivatives was measured isotopically on the basis of their conjugation with ^{14}C-glucuronic acid donated by UDP-[^{14}C]-glucuronic acid (UDP-^{14}C-GA). The incubation mixture contained, in a total volume of 150 µl: 0.2 mM BP-derivative or other aglycone (added in 25 µl of acetone and evaporated to dryness under nitrogen); 0.1 M tris-maleate buffer, pH 7.4; 1 mM UDP-^{14}C-GA (0.2 µCi); 0.05 or 0.1% (w/v) Lubrol WX in incubations with NE or MCS respectively, and 60 µg of MCS or NE protein. Incubations were performed at 37°C for 15 min, and were stopped by addition of 0.3 ml ethanol. The products were separated on TLC by a general procedure for glucuronide estimation, developed in this laboratory (27) using preparative silica gel TLC plates with butanol:acetone:acetic acid:30% ammonia:water (70:50:18:1.5:60). Metabolites were located by autoradiography, which revealed that in the R_f region of glucuronides (27), two spots were detectable. One faint spot of R_f 0.78 was of equal intensity irrespective of the presence or absence of added BP-derivatives, and the other at R_f 0.81, varied in intensity, depending upon the type of BP-derivative added. Because of their proximity, they were routinely combined for determination of radioactivity. Blank values, due to incubations without added aglycones, were substracted to determine net radioactivity due to metabolites of BP derivatives. Silica gel in R_f region containing the metabolites was scraped into scintillation vials and the radioactivity determined as described elsewhere (27).

β-Glucuronidase Hydrolysis. Ethanol was evaporated under nitrogen from 150 µl aliquots of combined duplicates of incubation mixtures, and 100 µl β-glucuronidase solution (10,000 units/ml) in 0.2 M acetate buffer, pH 4.6 was added. Incubations containing only acetate buffer (controls), or those with 10 mM saccharolactone + β-glucuronidase were treated similarly. After 4 hr incubation at 37°C, 50 µl aliquots were analyzed on TLC as above.

RESULTS

To determine whether nuclear envelope contains glucuronyltransferase activity towards BP oxygenated metabolites, rat liver NE of high purity was prepared. The purity was checked by electron microscopy (28), and possible contamination of NE with MCS was also evaluated enzymatically via the specific activity of ethylmorphine-N-demethylation, a cytochrome-P450 dependent reaction. In NE this amounted only to 10% of the activity present in MCS, therefore, contamination of NE by MCS could have been maximally 10% , however, since NE contains that level of cytochrome-P450 (29), most of the N-demethylase probably was due to NE itself.

Several oxygenated BP derivatives were tested as substrates for glucuronidation by NE enzymes by determining whether there is formation of a ^{14}C metabolite from UDP-^{14}C-GA, which is dependent upon the presence of a parti-

TABLE 1

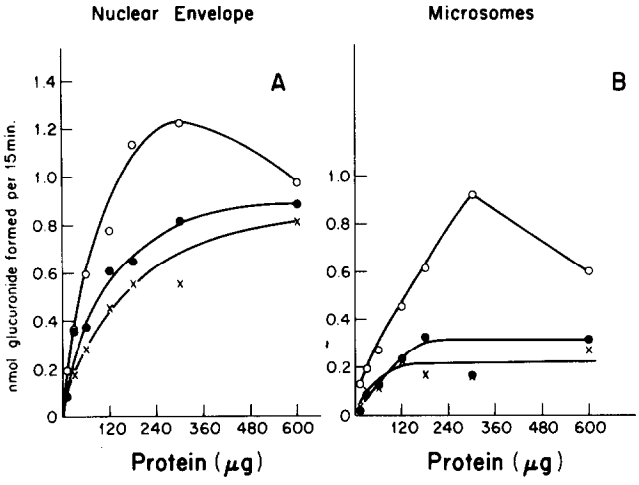
Confirmation of Glucuronide Nature of Metabolites Obtained from Incubations of BP Phenols with UDP-^{14}C-GA and Nuclear Envelope Fraction

BP Derivative	Radioactivity in DPM at R_f of Glucuronides after Various Treatments of Samples*			
	None	Control (buffer)	β-Glucuronidase	β-Glucuronidase + saccharolactone
1-OH	420	337	50	326
2-OH	193	127	58	126
3-OH	315	354	73	286
4-OH	247	217	60	201
5-OH	199	185	44	170
6-OH	444	250	185	274
7-OH	474	407	46	367
8-OH	198	134	44	120
9-OH	322	372	56	321
10-OH	235	297	46	268
12-OH	466	387	94	333
Blank	156	119	36	114

* Experimental details are given under Methods.

cular BP derivative in NE incubations. Incubations containing BP phenolic derivatives consistently yielded radioactive metabolites in the R_f region of glucuronides (e.g. see Table 1). In order to ascertain their glucuronide nature their susceptibility to β-glucuronidase hydrolysis was tested. The results are summarized in Table 1. All metabolites of BP phenols, except for the one derived from 6-OH BP, were almost completely hydrolyzed by β-glucuronidase, and the hydrolysis was prevented by saccharolactone, an inhibitor of β-glucuronidase. Similar results were obtained when such hydrolyses were performed on such metabolites obtained from microsomal incubations (results not shown). Incomplete hydrolysis of the 6-OH BP metabolite by β-glucuronidase cannot be explained at this time, however, it should be pointed out that the metabolite was otherwise quite unstable, since about half of it hydrolysed in buffer without β-glucuronidase (Table 1). Lesser amounts of such spontaneous hydrolysis also occurred in other cases.

Dependence of product formation on the concentration of NE and MCS protein



Dependence of Glucuronidation of some BP-derivatives on the concentration of (A) Nuclear Envelope Protein and (B) Microsomal Protein--The abcissa represents μg protein per 150 μl incubation. Open circles, closed circles and crosses represent reactions carried out in the presence of 9-OH, 3-OH and 10-OH BP respectively. The experimental conditions were as described under Materials and Methods.

was tested using 3-, 9- and 10-hydroxy-BP as substrates. Only near linearity was observed at low concentrations of protein, up to 60 μg protein in 150 μl (Fig. 1). For 9-OH BP a decrease in the activity was observed when the amount of protein in the incubation mixture exceeded 300 μg which could be due to non-specific binding of the BP derivative. In the same experiment, dependence of the reaction on the concentration of several BP-derivatives was tested using 25-200 μM 7-OH, 9-OH, or 10-OH BP, or trans-7,8-dihydrodiol, trans-9,10-dihydrodiol, or 7,8-dihydroepoxide of BP, in incubations containing 300 μg of NE protein and Lubrol WX. The two dihydrodiols, the epoxide and 8-OH BP did not undergo measurable amounts of glucuronidation. However, in the case of 7-, 9- and 10-hydroxy BP the reaction followed Michaelis-Menten kinetics.

In other experiments, glucuronidation of BP derivatives was tested using native, and Lubrol WX activated NE preparations with 0.05% of the detergent which maximally activates NE glucuronidation of several non-carcinogenic substrates (28). Comparisons were made with optimally activated MCS. Assays with standard glucuronyltransferase substrates, p-nitrophenol and tes-

tosterone, present at saturating concentrations (28,30), or at substrating concentrations equal to those of BP derivatives (200 μM), were performed as a check of viability of the enzyme preparations, and for comparisons of the relative activity towards this variety of substrates. The results in Table 2 show that only BP-phenols, served as substrates. Repeated incubations of dihydrodiols, diones, and epoxides, (some carried out up to 60 min), failed to yield measurable amounts of glucuronides of these compounds. Lubrol WX activated to a variable extent (up to 6 fold) glucuronidation of various BP phenols by NE. Since glucuronyltransferase of NE generally does not exhibit much latency (30,31) some of this activation may be due to an enhanced solubilization of the BP derivatives. The relative extent of glucuronidation of various BP- phenols varied with conditions of incubation and with the fraction used. Nevertheless, some phenols appeared to be consistently good substrates (e.g. 3-, 7- and 9-OH BP), and others consistently poor ones (e.g. 2-, 5- and 8-OH); only in detergent activated incubations 1-OH, 6-OH and 12-OH BP appeared to be good substrates. The specific activity of NE preparation was 2-5 fold higher than that of MCS, except for 6-OH BP.

DISCUSSION

UDP-glucuronyltransferase catalyzes the glucuronidation of many substances possessing phenolic or alcoholic hydroxy groups among others (32), but epoxides and diones are not among recognized substrates. However, since Nemoto and Gelboin (22) reported on the microsomal glucuronidation of several BP derivatives, including 4,5- and 7,8-BP oxides, we tested them as possible substrates for NE glucuronyltransferase, and included diones for the sake of completeness. In contrast to those workers, we could not detect measurable amounts of conjugation of epoxides, or dihydrodiols of BP, but the ability of BP-phenols, and inability of BP-quinones to form glucuronides was consistent with their findings. Optimally activated NE and MCS have similar specific activities for p-nitrophenol and testosterone, at optimal concentrations (30)

TABLE 2

A Comparison of Glucuronidation of Various Benzo[a]pyrene Derivatives By Nuclear Envelope and Microsomal Fractions of Rat Liver

Compound (200 μM)	Amount of conjugate (nmoles x mg protein^{-1} x 15 min^{-1})*		
	Nuclear Envelope		Microsomes
	-Lubrol WX	+Lubrol WX	+Lubrol WX
Benzo(a)pyrene Derivative:			
1-OH	1.5	10.3	4.2
2-OH	1.6	1.4	N.D.†
3-OH	5.8 ± 0.3	9.2 ± 3.1	2.3
4-OH	2.0 ± 0	5.4 ± 1.7	1.1
5-OH	N.D.†	4.3 ± 2.2	1.5
6-OH	1.9	11.1	9.6
7-OH	4.9 ± 1.4	15.8 ± 3.3	3.6
8-OH	1.2 ± 0.1	2.3 ± 0.4	N.D.†
9-OH	5.4 ± 1.1	10.3 ± 2.4	4.8
10-OH	2.3 ± 0.7	5.8 ± 1.9	1.2
12-OH	2.3	12.0	2.4
3,6-dione, 7,8-dione, Trans-4,5 dihydrodiol, Cis-4,5 dihydrodiol, Trans-7,8 dihydrodiol, 4,5-dihydroepoxide, 7,8-dihydroepoxide, Trans-7,8 dihydrodiol-9,10 epoxy (anti)	Not Detectable	Not Detectable	Not Detectable
Standard Aglycone:			
p-Nitrophenol ‡	---	27.1	30.6
Testosterone ‡	---	11.0	12.4

* Net activities are tabulated, after substraction of blank values (see Methods) which equaled to 2.9, 5.4 and 5.0 nmoles x mg protein^{-1} x 15 min^{-1}, for NE, NE + Lubrol, and MCS + Lubrol, respectively. Values in the Table are averages of duplicates or mean ± S.E.M. of 3 experiments.

†N.D. means not detectable.

‡At saturating concentrations of aglycones and UDPGA (28,30) the specific activities for p-nitrophenol were 10 to 14 fold higher then at 200 μM, and for testosterone they were 3-4 fold higher.

and at sub-optimal substrate concentrations (Table 2). It was therefore surprising that NE had up to 5 times higher activities towards some BP-phenols than did MCS. This may be indicative of an especially effective detoxication mechanism in NE for this type of substrate.

Virtual inability of NE to glucuronidate dihydrodiols and epoxides may be one factor in their potent carcinogenicity (10). It is also noteworthy that a phenol, such as 2-OH BP, which is resistant to glucuronidation is as potent a carcinogen as BP itself (33). Mutagenicity and carcinogenicity of several BP phenols has been reported (34), and is thought to involve an activation via recycling through a mixed function oxidase system. Conjugation to glucuronides can break this recycling process; especially in NE, in the proximity of genetic apparatus it may represent the last possible barrier to such an activation. The specific activity of BP activating enzymes in NE (29,35), is about 10-100 times lower than that of NE glucuronyltransferase. This indicates that for those BP compounds which are good substrates for NE glucuronidation, a very effective detoxication process is available in that compartment.

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