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UDP-GLUCURONOSYL TRANSFERASE AND THE CONJUGATION OF BENZO(a)PYRENE METABOLITES TO DNA

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SUMMARY: Addition of UDP-glucuronic acid to microsomal incubations containing benzo(a)pyrene caused a dose-dependent conjugation of principally quinone and phenol metabolites. Total benzo(a)pyrene oxidation was also stimulated with a maximum increase at 2 mM UDPGA. In the presence of calf thymus DNA, UDPGA caused a 2.7-fold increase in benzo(a)pyrene diol-oxide modification of DNA, as analyzed by Sephadex LH-20 chromatography. Maximum DNA modification by diol-oxides occurred at a UDPGA concentration which gave the highest level of free benzo(a)pyrene 7,8-dihydrodiol; likewise, the amount of DNA adduct derived from benzo(a)pyrene phenols declined in parallel with levels of free phenol metabolites. The UDPGA-induced increase in benzo(a)pyrene oxidation and concomitant increase in diol-oxide modification of DNA is consistent with removal of product inhibition by glucuronide conjugation of an inhibitory benzo(a)pyrene metabolite.

Chemical carcinogenesis initiated by polycyclic aromatic hydrocarbons requires metabolic activation via microsomal mixed function oxidation (1). There is now a substantial body of evidence that bay-region dihydrodiol-oxides account for the majority of the carcinogenic effect of polycyclic aromatic hydrocarbons (2). DNA which has been exposed *in vitro* to liver microsomal metabolites of benzo(a)pyrene[1] has a small proportion of bases modified by covalent interaction with electrophilic metabolites (3). After enzymic hydrolysis, modified bases have been resolved by Sephadex LH-20 chromatography into several fractions (4,5). The major fractions have been identified as principally purine base conjugates of stereoisomeric BP 7,8-dihydrodiol 9,10-oxides (6,7) and, tentatively, an oxide derivative of BP 9-

[1]Abbreviations used are: BP, benzo(a)pyrene; UDPGA, UDP-glucuronic acid; MC-microsomes, 3-methylcholanthrene-induced rat liver microsomes; BP 7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; BP 7,8-dihydrodiol 9,10-oxide, *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene, *anti* and/or *syn* stereoisomers.

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phenol (3,5,8,9). Significantly, only the former is prominent in DNA isolated from mammalian cells which have been exposed to BP (10,11) even though BP phenols are major metabolites.

Formation of polycyclic aromatic hydrocarbon dihydrodiol-oxides requires a sequence of oxidation of BP at cytochrome P450, hydration at epoxide hydratase followed by a further cytochrome P450-dependent oxidation of the dihydrodiol. A third microsomal enzyme, UDP-glucuronosyl transferase, may remove BP phenols and BP dihydrodiols via glucuronide formation. Recent results (12) also indicate that BP-3-glucuronide may be activated by β-glucuronidase hydrolysis to a product capable of modifying DNA. It is also clear that an important feature of the metabolism of BP is competition between BP and the various BP metabolites for cytochrome P450. In this communication, we provide evidence that such competitive effects within the microsomal membrane play a major role in determining the extent of DNA modification by BP dihydrodiol-oxides, and that the action of UDP-glucuronosyl transferase surprisingly enhances the extent of conjugation of BP 7,8-dihydrodiol 9,10-oxides to DNA.

MATERIALS AND METHODS

[^{3}H] benzo(a)pyrene (56 Ci/mmole) was purchased from Amersham. Calf thymus DNA, UDPGA (sodium salt), and enzymes for hydrolysis of DNA (13) were obtained from Sigma Chemical Company.

The preparation of 3-methylcholanthrene-induced rat liver microsomes, incubation of BP with microsomes, and measurement of BP metabolites by high-pressure liquid chromatography were done as previously described (14,15). Water phases from BP incubations were treated with β-glucuronidase as described (16), using a 3 hr incubation. In DNA labeling experiments, 1 mg of heat-denatured, sonicated DNA (4) was added to standard 1 ml microsomal incubations containing 15 μM [^{3}H] benzo(a)pyrene (2.7 Ci/mmole) and incubated at 37^o for 45 min. The DNA was then extracted with phenol, precipitated with ethanol, and washed as described by King et al. (4). Hydrolysis of DNA to deoxyribonucleosides was done using a 24 hr total incubation as described by Alexandrov et al. (17).

BP-modified deoxyribonucleosides were separated on 20 x 1.0 cm columns (Bio Rad) packed to a height of 18 cm with Sephadex LH-20 equilibrated in 45% MEOH:H_2O (vol/vol). After hydrolysis, 1.8 ml of sample was applied to the column and eluted with a linear gradient of 45% (61 ml) to 75% (65 ml) methanol in water. Ninety 1.4 ml fractions were collected directly into 15 x 45 mm glass mini-vials (Research Products International Corporation) carried in an LKB Redirac fraction collector; 3.5 ml of ACS scintillation cocktail was added and scintillation counting done in a Packard 3320 counter. The column packing was washed in methanol, re-equilibrated, and re-poured after each use.

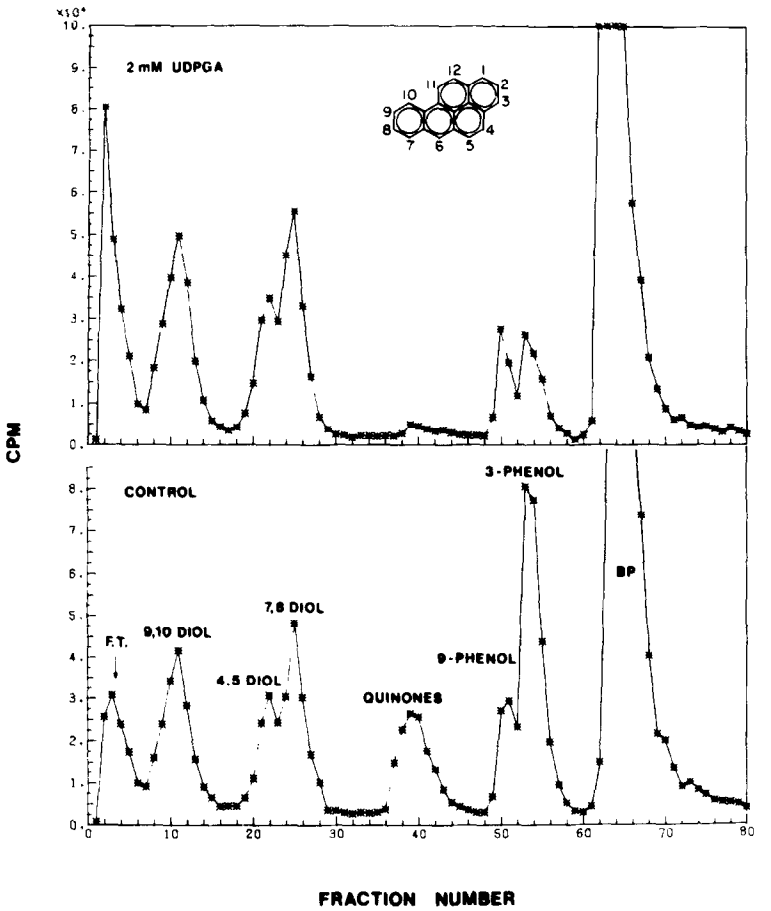

Fig. 1. Effect of UDPGA on distribution of organic-extractable [^{3}H] benzo(a)pyrene metabolites. [^{3}H] BP (15 μM) was incubated with MC-microsomes for 6 min (Control) and, in the presence of UDPGA, added before the 5 min pre-incubation (2 mM UDPGA). Acetone:ethyl acetate extracts were evaporated, resuspended, and analyzed by HPLC. F.T., flow-through fraction. BP retention time was 35 min.

RESULTS AND DISCUSSION

The addition of UDP-glucuronic acid to incubations containing [^{3}H] BP and liver microsomes from MC-induced rats selectively affected levels of organic-extractable metabolites of BP (Fig. 1). In agreement with the results of Nemoto and Takayama (18), we observed large decreases in the levels of BP-quinones and 3-phenol which were half maximal at, respectively, 1.0 and 1.6 mM UDPGA (Fig. 2). However, in contrast to the previously

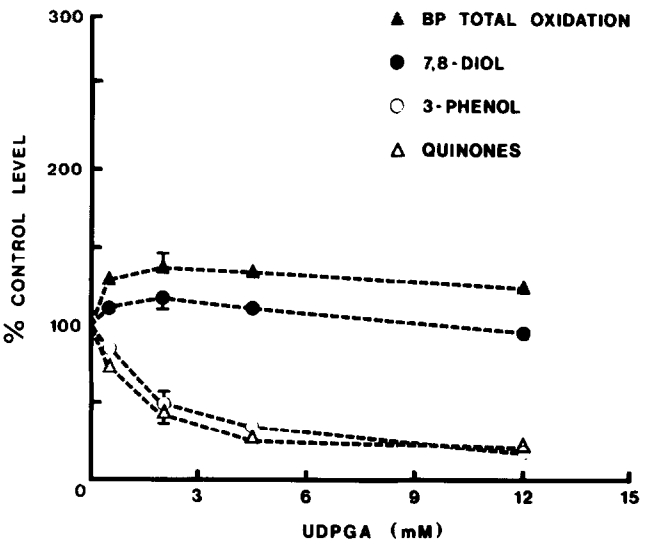

Fig. 2. Relationship between increasing UDPGA concentration and levels of organic-extractable BP metabolites. Control incubation (0 mM UDPGA) contained 15 μM [^{3}H] BP and 0.3 mg/ml MC-microsomes. The incubation was for 6 min with a 5 min pre-incubation. UDPGA was added in water to tubes before pre-incubation. BP total oxidation curve includes water-soluble metabolites. Error bars indicate the standard deviations of the mean metabolite levels. Control specific activity is given in Table I.

reported finding, the 9-phenol level was less sensitive to UDPGA addition, and total BP metabolism increased maximally by 38% at 2 mM UDPGA. A 20% ($p < 0.05$) increase in the level of BP 7,8-dihydrodiol was observed at 2 mM UDPGA, while higher levels of UDPGA decreased this value. When water-soluble products from incubations containing 2 mM UDPGA were treated with β-glucuronidase, subsequent organic extraction and HPLC analysis of the organic extract indicated formation of glucuronides of phenol peak II[2] > quinones > phenol peak I > dihydrodiols (Table I). Trace amounts of dihydrodiols were seen but were not accurately quantifiable. It is presumed that quinones derive from the auto-oxidation of unconjugated BP 6-phenol (19) which here is trapped by glucuronide formation. When compounds released by β-glucuronidase are quantified and added to unconjugated BP

[2]Phenol peak I has been shown to consist of BP 9-phenol, and phenol peak II principally of BP 3-phenol with traces of BP 1- and 7-phenols.

TABLE I

Effect of UDPGA on Distribution of Organic Extractable [^{3}H] Benzo(a)pyrene Metabolites[a]

Metabolites	Control	2 mM UDPGA	β-Glucuronidase[b]
	nmoles/mg/min		% distribution of metabolites
FT[c]	0.33 ± 0.03	1.16 ± 0.09	6
9,10 Diol	1.35 ± 0.06	1.62 ± 0.16	< 5
4,5 Diol	0.60 ± 0.05	0.71 ± 0.04	< 5
7,8 Diol	1.12 ± 0.07	1.32 ± 0.08	< 5
Quinones	1.15 ± 0.06	0.25 ± 0.01	23
9-OH	0.41 ± 0.02	0.33 ± 0.02	6
3-OH	1.72 ± 0.18	0.53 ± 0.06	30
H_2O Phase[d]	0.55 ± 0.03	4.09 ± 0.21	20
Total	7.23 + 0.81	10.02 + 0.90	--

[a] 2 ml incubations containing 0.6 mg MC-microsomes and 15 μM [^{3}H] benzo(a)-pyrene were incubated at 37° for 6 min. Values represent mean and standard deviation of three determinations.

[b] 2 ml H_2O phases remaining after extraction of 2 mM UDPGA tubes (containing 7.4 nmoles product) were bubbled with N_2 to remove acetone and treated with 1700 units β-glucuronidase at 37° for 3 hrs and re-extracted.

[c] Flow-through fraction on HPLC.

[d] Radioactivity representing [^{3}H] release during metabolism was estimated by comparison to parallel incubations using [^{14}C] benzo(a)pyrene and subtracted off. For control incubations, this represented 20% of the radioactivity remaining in the water phase after extraction.

metabolites, it appears that mixed function oxidation of BP is stimulated unselectively in the presence of 2 mM UDPGA.

The effect of UDPGA on the modification of calf thymus DNA by [^{3}H] BP derivatives was examined by means of LH-20 chromatography of DNA hydrolysates according to the method of King et al. (4). UDPGA enhanced conjugation of BP 7,8-dihydrodiol 9,10-oxide to DNA 2.7-fold ($p < 0.01$) (Fig. 3, Peak A) with an optimal increase at 2 mM corresponding to the peak in the level of BP 7,8-dihydrodiol (Fig. 4). Formation of the major DNA conjugate peak (Fig. 3, Peak D) was decreased approximately 35%, in parallel with the decrease in the

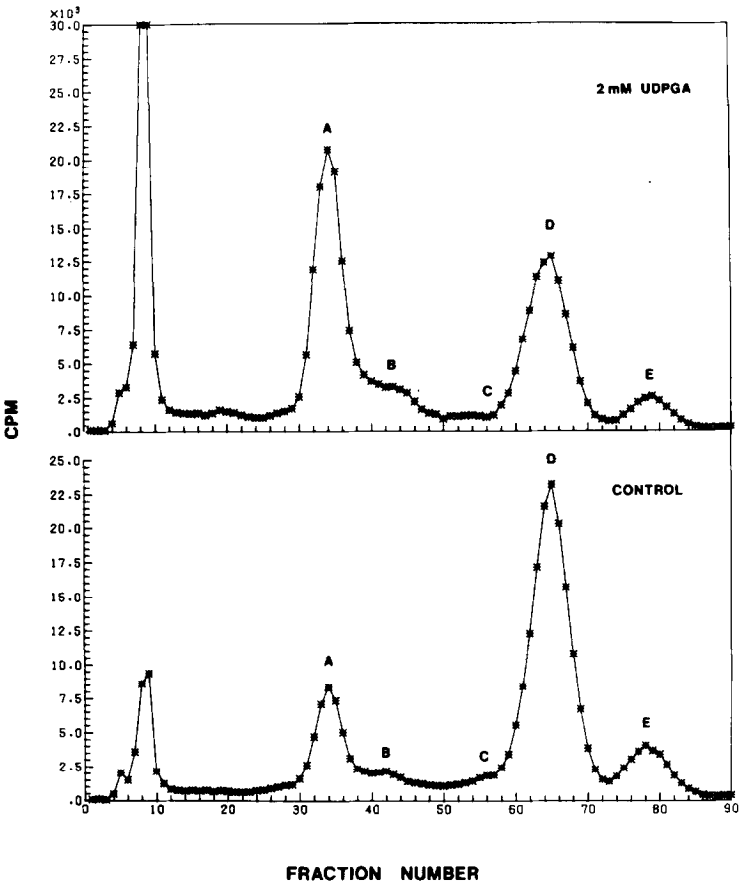

Fig. 3. Effect of UDPGA on the modification of DNA by [^{3}H] BP metabolites. [^{3}H] BP (15 μM) was incubated with 0.3 mg MC-microsomes and calf thymus DNA for 30 min (Control) and, in the presence of UDPGA, added before the 5 min pre-incubation (2 mM UDPGA). Purified DNA was enzymatically hydrolyzed and resolved by LH-20 chromatography as described in Materials and Methods. The 100% control level for peak A represents 35 pmoles diol-oxide bound/mg DNA.

level of BP phenol peak I. The latter observation supports the assignment of Peak D to DNA bases conjugated to a further metabolite of BP 9-phenol (3,5,8, 9).

The most likely explanation of the enhancement of BP metabolism and the resultant increase in DNA modification by BP dihydrodiol-oxides is that certain metabolites of BP inhibit the cytochrome P450 metabolism of both BP and BP 7,8-dihydrodiol. Here, conjugation of these metabolites with glucuronic

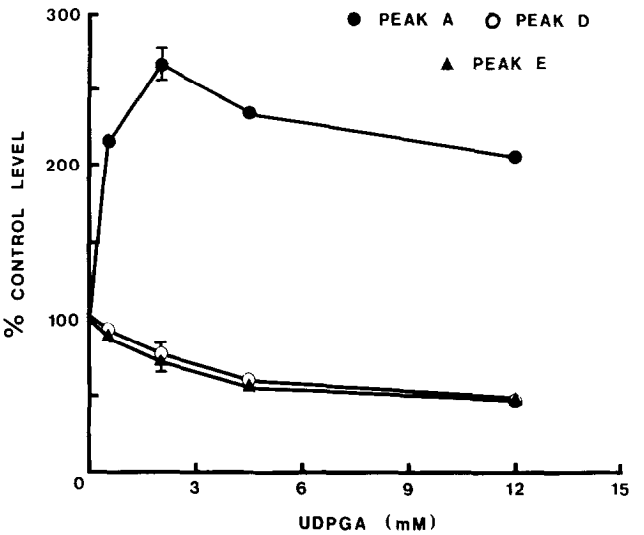

Fig. 4. Relationship between increasing UDPGA concentration and levels of BP metabolite-modified DNA bases. Control incubation (0 mM UDPGA) contained 15 μM [^{3}H] BP, 0.3 mg/ml MC-microsomes, 1 mg/ml calf thymus DNA. Incubation was for 45 min with a 5 min pre-incubation. Error bars indicate the standard deviation of the mean levels.

acid would remove the oxidase inhibitor(s). The most likely candidates are quinones (or 6-phenol) or 3-phenol whose levels are largely depleted by glucuronidation. Preliminary results indicate 60% and negligible inhibition of total BP oxidation in response to, respectively, 5 μM quinone (an equimolar mixture of synthetic 1,6, 3,6, and 6,12 quinones) and 10 μM 3-phenol additions to standard BP incubations. Total modification of DNA by [^{3}H] BP derivatives was similarly inhibited by an addition of 5 μM quinone. By this hypothesis, UDPGA should not affect BP 7,8-dihydrodiol 9,10-oxide modification of DNA when BP 7,8-dihydrodiol is the microsomal substrate. Data in Table II show that indeed UDPGA has no effect on this modification of DNA. However, when unlabeled BP is added so that metabolism of BP occurs concurrently with metabolism of [^{3}H] BP 7,8-dihydrodiol, [^{3}H]-counts in Peak A decrease 4-fold. When correction is made for the small dilution of [^{3}H] BP 7,8-dihydrodiol by formation of unlabeled BP 7,8-dihydrodiol from BP, total modification of DNA by BP 7,8-dihydrodiol 9,10-oxides still decreases by

TABLE II

Microsome-Catalyzed Binding of [^{3}H] BP 7,8-Dihydrodiol Derivatives to Calf Thymus DNA[a]

Incubation	pmoles bound[b]/mg DNA	% Control
Control	375	100
+UDPGA (2 mM)	360	96
+Benzo(a)pyrene (15 μM)	89 (112)[c]	23 (30)[c]
+Benzo(a)pyrene (15 μM) UDPGA (2 mM)	213 (267)[c]	57 (71)[c]

[a] 1 ml incubations containing 0.3 mg MC-microsomes and 6 μM [^{3}H] BP 7,8-dihydrodiol (0.44 Ci/mmol) were incubated for 30 min at 37°. UDPGA was added before the 5 min pre-incubation; benzo(a)pyrene was added immediately before the [^{3}H] BP 7,8-dihydrodiol at time zero. Values represent duplicate determinations.

[b] Pooled radioactivity from fractions 28-46 of LH-20 chromatogram (See Fig. 3).

[c] Corrected for change in [^{3}H] BP 7,8-dihydrodiol specific activity, assuming production of unlabeled BP 7,8-dihydrodiol from BP at a rate of approximately 0.1 nmoles/min (Table I).

over 3-fold. When unlabeled BP is present, UDPGA then stimulates the modification of DNA by [^{3}H] BP 7,8-dihydrodiol 9,10-oxides, presumably by conjugating the oxidase-inhibiting BP metabolite as it is formed. The UDPGA-induced increase (2.4-fold) in modification of DNA resulting from the metabolism of purified [^{3}H] BP 7,8-dihydrodiol (Table II) is essentially the same as the UDPGA-induced increase (2.6-fold) in this modification as a result of BP metabolism (Fig. 4). The remaining inhibition by BP which is insensitive to UDPGA can probably be attributed to competitive inhibition of BP 7,8-dihydrodiol by BP at cytochrome P450.

It seems, therefore, that although UDPGA and glucuronosyl transferase decrease the modification of DNA which has been attributed to phenol-oxides (8,9), this enzyme activity may increase the carcinogenic activity of BP and possibly other polycyclic aromatic hydrocarbons by relieving product inhibition of cytochrome P450. In a simple microsome plus DNA system, relief of the product inhibition is reflected in an increase in BP 7,8-dihydrodiol

9,10-oxide modification of DNA. Preliminary results with cultured hepatocytes[3] showing a large Peak A and a complete lack of Peak D in the presence of normal production of BP phenols would also support this pivotal role of glucuronosyl transferase.

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