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PRELIMINARY COMMUNICATIONS

ACTIVATION AND INDUCTION OF RAT LIVER MICROSOMAL UDP-GLUCURONYLTRANSFERASE WITH 3-HYDROXYBENZO(a)PYRENE AND N-HYDROXY-2-NAPHTHYLAMINE AS SUBSTRATES

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The ubiquitous environmental pollutant benzo(a)pyrene is metabolized by cytochrome P-450 dependent monooxygenases to various electrophilic intermediates which either spontaneously rearrange to phenols or are converted by epoxide hydratase to dihydrodiols. These primary metabolites may recycle a second time through monooxygenases whereby probably the ultimate carcinogens are formed (1-3). Conjugation with glucuronic acid or sulfate helps to eliminate primary metabolites and thus prevents recycling. A delicate balance between toxifying and detoxifying reactions may be decisive for the accumulation of ultimate carcinogens. In some instances, for example in urinary bladder carcinogenesis caused by 2-naphthylamine, the glucuronide of N-OH-2-naphthylamine is probably the source of the ultimate carcinogen (4).

We studied the glucuronidation of 3-OH-benzo(a)pyrene and N-OH-2-naphthylamine as model compounds in order to evaluate the importance of conjugation in eliminating phenols of polycyclic hydrocarbons and N-hydroxy arylamines. Detailed investigations on the identification of products of these reactions have recently been published (4,5). However in these studies the marked activation and induction of UDP-glucuronyltransferase (GT) has not adequately been taken into account. From our present knowledge about the intracellular control of GT (6) it can be concluded that GT operates in vivo in a latent form which is partly activated by UDP-N-acetylglucosamine. Optimal activation, achieved in vitro for example by detergents, may also be found in vivo following membrane injury (7). Apart from activation

GT is also inducible. However GT reactions are differentially inducible by two prototypes of inducing agents, 3-methylcholanthrene (MC) and phenobarbital (8,9). In studies on differential induction, and similarly in developmental studies (10), substrates of GT can be subdivided into at least two groups. Recently we were able to separate and purify two forms of GT to apparent homogeneity (11). These enzyme forms were characterized by different substrate specificity and differential inducibility by MC and phenobarbital. In this report we demonstrate that activation and induction critically determine the rate of glucuronidation of 3-OH-benzo(a)-pyrene and N-OH-2-naphthylamine. Moreover it is shown that these toxic intermediate belong to the MC-inducible group of substrates of GT.

Materials and Methods. Male Wistar rats (200 g) were treated once i.p. with MC (40 mg/kg, dissolved in olive oil) or with phenobarbital (100 mg/kg; thereafter the animals received 0.1% phenobarbital in their drinking water). Animals were sacrificed on the 4th day after treatment. Liver microsomes were prepared as described previously (8).

Assays of 1-naphthol-GT (12) and morphine-GT (13) were performed as described

Assay of 3-OH-benzo(a)pyrene-GT: It was carried out at 37°C in dim light. In a total volume of 0.5 ml the mixture contained: 0.05 mM 3-OH-benzo(a)pyrene in dimethylsulfoxide (1%,v/v); 0.2 mg microsomal protein; 0.1 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$ and 3 mM UDP-glucuronic acid. When indicated 3 mM UDP-N-acetylglucosamin or Brij 58 (0.05%,w/v; Atlas, Essen) was included. At 0, 2, 5, and 10 min 0.1 ml aliquots of the incubation mixture were transferred to centrifugation tubes containing 0.5 ml icecold methanol. After the addition of 1 ml 0.4 M glycine buffer pH 10.3, and removal of the precipitated protein by centrifugation the fluorescence of 3-OH-benzo(a)pyrene glucuronide was determined at 450 nm, with excitation at 300 nm, using a Farrand spectrophotometer, Mark I. Fluorescence of 3-OH-benzo(a)-pyrene glucuronide was calibrated in similar incubations performed in the presence of UDP-(^{14}C)glucuronic acid (Amersham). The radioactive nucleotide and 3-OH-benzo(a)pyrene-(^{14}C)glucuronide were separated by thin layer chromatography on silica-gel (Merck, Darmstadt) with a solvent mixture of ethylacetate-methanol-water-formic acid (100:25:20:1,v/v (5)). The glucuronide could be readily identifie by both its strong fluorescence and radioactivity. The radioactive glucuronide was scraped off the plate into scintillation vials and counted for radioactivity. The assay was calibrated by comparing the amount of UDP-(^{14}C)glucuronide converted to 3-OH-benzo(a)pyrene-(^{14}C)glucuronide with the fluorescence determined in an aliquo of the same incubation mixture. The fluorescence spectrum of 3-OH-benzo(a)pyrene

glucuronide at pH 10.3 showed maxima at 425 and 450 nm on excitation at 300 nm in agreement with Nemoto et al. (14). The low fluorescence of 3-OH-benzo(a)pyrene at 450 nm was taken into account by subtracting a 0 time blank.

Assay of N-OH-2-naphthylamine-GT (4): The substrate was synthesized (15) and dissolved in dimethylsulfoxide. The solution was discarded when it became yellow-orange. Incubations were carried out under nitrogen. In a total volume of 1 ml the mixture contained: 0.5 mM N-OH-2-naphthylamine, dissolved in dimethylsulfoxide (2%,v/v); 0.1 M Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM EDTA; 0.2 - 1.0 mg microsomal protein and 3 mM UDP-glucuronic acid. When indicated activators were included as described for 3-OH-benzo(a)pyrene-GT. At multiple time points serial aliquots (0.1 ml) were removed from the incubation mixture, and the concentration of N-OH-2-naphthylamine was determined colorimetrically as amyl acetate-extractable Fe^{3+}-reducing equivalents (16). An extinction coefficient of 39 200 cm^2/mol at 535 nm for the Fe^{2+}-bathophenanthroline complex was used for calculation of results.

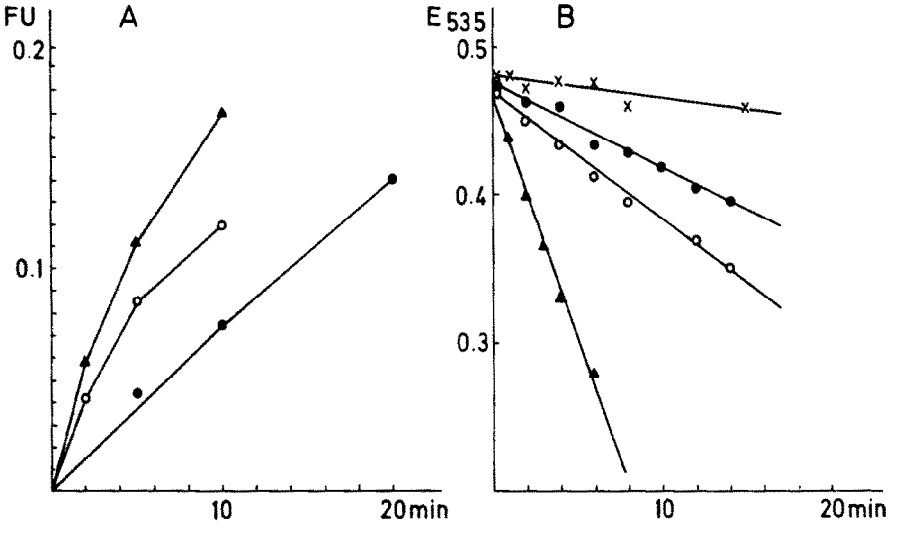

Fig. 1. Effects of UDP-N-acetylglucosamine and Brij 58 on rat liver microsomal GT with 3-OH-benzo(a)pyrene (A) and N-OH-2-naphthylamine (B) as substrates. Liver microsomes from untreated controls were used. (●), native microsomes; (o), addition of UDP-N-acetylglucosamine; (▲), addition of Brij 58; (x), non-enzymatic loss of N-OH-2-naphthylamine. FU = fluorescence units. Values represent the mean of 4 experiments.

Results and Discussion. The glucuronidation of 3-OH-benzo(a)pyrene was studied fluorometrically by measuring the appearance of the glucuronide. The latter could be determined in the presence of 3-OH-benzo(a)pyrene (Fig.1, A). Initial

Table 1. Effects of MC- and phenobarbital-treatment on glucuronidation of 3-OH-benzo(a)pyrene and N-OH-2-naphthylamine in rat liver microsomes

Substrate:	GT (nmol/min/mg protein)[a]		
Additions to assay in vitro	Untreated controls	MC-treatment	Phenobarbital-treatment
3-OH-benzo(a)pyrene:			
None	0.4 ± 0.1	2.6 ± 0.2	1.2 ± 0.1
+ UDP-N-acetylglucosamine	1.0 ± 0.1	3.6 ± 0.5	2.3 ± 0.2
+ Brij 58	1.6 ± 0.1	9.9 ± 1.6	2.7 ± 0.2
N-OH-2-naphthylamine:			
None	3.9 ± 1.1	6.2 ± 1.0	4.2 ± 1.2
+ UDP-N-acetylglucosamine	8.4 ± 0.7	10.5 ± 1.0	6.5 ± 1.2
+ Brij 58	37.6 ± 5.6	102.3 ± 2.9	41.8 ± 5.5
1-Naphthol:			
None	2.8 ± 0.6	7.8 ± 1.9	4.0 ± 0.9
+ UDP-N-acetylglucosamine	10.7 ± 1.3	15.8	12.1
+ Brij 58	75 ± 15	212 ± 49	84 ± 20
Morphine:			
None	1.1 ± 0.2	1.8 ± 0.5	4.0 ± 0.8
+ UDP-N-acetylglucosamine	4.5 ± 0.5	2.2	14.2
+ Brij 58	7.9 ± 0.6	9.9 ± 1.7	18.0 ± 2.8

[a] Values represent the mean ± S.D. of 4 experiments and the mean of 2 experiments where S.D. was omitted.

reaction rates were determined by removing serial aliquots from the incubation mixture. In studies on N-OH-2-naphthylamine glucuronidation the nonenzymatic loss of the reactive substrate had to be subtracted from disappearance rates determined in the presence of UDP-glucuronic acid. This nonenzymatic loss of N-OH-2-naphthylamine was about 2.6 ± 0.5 nmol/min under our conditions (Fig.1,B). Glucuronidation rates of the two toxic intermediates were markedly activated by UDP-N-acetylglucosamine which probably acts as a physiological activator of GT. Full activation was achieved by addition of the nonionic detergent Brij 58. The glucuronidation rate o the intermediates under presumed intracellular conditions appears to be high enoug to efficiently lower the accumulation of these toxic compounds. Moreover rapid conjugation may diminish the marked product inhibition of benzo(a)pyrene hydroxyla by phenols, and may thus stimulate overall metabolism (17).

GT activity towards the two reactive intermediates was markedly enhanced by treatment of rats with MC similar to 1-naphthol-GT, as clearly seen in the fully activated form of the enzyme (Table 1). 1-Naphthol belongs to the MC-inducible group of GT substrates whereas morphine belongs to the phenobarbital-inducible group (7-9). Recently we were able to separate and purify two forms of GT with high specific activity towards 1-naphthol or morphine (11). The glucuronidation of 4-nitrophenol, 3-OH-benzo(a)pyrene and N-OH-2-naphthylamine copurified with that of 1-naphthol. However the glucuronidation of morphine, chloramphenicol and bilirubin which belong to the phenobarbital-inducible group of substrates was not detectable or very low in purified 1-naphthol-GT preparations (18). These observations suggest that 3-OH-benzo(a)pyrene and N-OH-2-naphthylamine are conjugated by the MC-inducible form of GT. Interestingly the formation of 3-OH-benzo(a)pyrene is also chiefly stimulated by MC (19). Hence a regulatory link between cytochrome P-448 dependent monooxygenase and MC-inducible GT would be advantageous for an efficient and 'safe' elimination of a variety of environmental pollutants from the organism. In mice the induction of these phase I and II reactions appears to be genetically linked (20).

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