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EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ADMINISTRATION ON GLUTATHIONE-*S*-EPOXIDE TRANSFERASE ACTIVITY IN RAT LIVER*

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Abstract—Glutathione-*S*-epoxide transferase activity in rat liver was studied with three epoxide substrates. The specific activities in the high-speed liver supernatant fraction for 3MC 11,12 oxide, styrene oxide and 3,3,3-trichloro-1,2 epoxy propane were 5.7, 86.4 and 165 nmoles/5 min/mg protein, respectively. Phenobarbital (75 mg/kg body wt for 3 days) or 3-methylcholanthrene (20 mg/kg body wt for 2 days) administration to rats resulted in a 40–60% increase in enzyme activity. β-naphthoflavone administration on the other hand was without any effect on glutathione-*S*-epoxide transferase activity.

In animal tissues, polycyclic aromatic hydrocarbons are metabolized at least in part to epoxides [1–5]. These highly reactive oxides are known to have increased biological activity over the parent compounds in producing malignant transformations [6–8]. The oxides are metabolically produced via the action of the mixed function oxidases, principally in liver. The epoxides are detoxified by (a) hydration to the corresponding diol by hepatic epoxide hydrase(s) [9–12] or (b) conjugation with glutathione as catalyzed by a family of enzymes, the glutathione-*S*-epoxide transferases [9, 10, 13–15]. We have studied earlier [16] the effect of PB† and 3MC administration on hepatic epoxide hydrase levels. We now report the effects of PB and 3MC administration on the levels of rat liver supernatant glutathione-*S*-epoxide transferase activity. The results of the present study indicate that the conjugation of the polycyclic hydrocarbon oxides with glutathione is elevated in rat liver by prior administration of these agents.

MATERIALS AND METHODS

Source of enzyme. Male Sprague–Dawley rats were injected with the appropriate inducer and were killed 24 hr after receiving the last injection. The livers from the animals were removed, homogenized in cold 0.25 M sucrose (1:5 w/v), the homogenate was centrifuged at 9000 *g* for 20 min at 5° and the pellet was discarded. The 9000 *g* fraction was further centrifuged at 100,000 *g* for 1 hr at 5°, the resultant supernatant was carefully transferred to another tube and was used as the enzyme source. Protein content of the supernatant was determined by the method of Lowry *et al.* [17] with bovine serum albumin as the reference standard.

Assay of glutathione-S-epoxide transferase with TCPO and styrene oxide. Assays were performed essentially according to the method of Hayakawa *et al.* [18]. [^{35}S]glutathione (500 μCi/3.88 mg) was purchased from Schwarz/Mann and nonradioactive reduced glutathione from Sigma. TCPO and styrene oxide were products of Aldrich Chemicals while 3MC 11,12 oxide was prepared by the method of Sims [19].

Incubation mixtures contained 0.5 μmole of [^{35}S]glutathione (2.3×10^5 cpm), 100 μmoles of pyrophosphate buffer pH 8, 0.6 μmole of TCPO in 5 μl dimethylsulfoxide or 0.5 μmole of styrene oxide in 2 μl ethanol, and enzyme in a total volume of 1 ml. The reaction was started by the addition of the oxide solution. After incubation for 5 min at 22°, the reaction was terminated by the addition of 50 μl of 4 N acetic acid. The conjugates produced during the incubation were adsorbed on 20 mg of activated charcoal. After 30 min at room temperature, the charcoal was collected by centrifugation washed twice with 4 ml of water and the conjugates were eluted twice with 1 ml of methanol–benzene–aqueous ammonia (87:10:3, v/v). The combined eluates were evaporated under nitrogen and then suspended in 50 μl of 50% ethanol and 20 μl was applied to Whatman No. 1 paper. Chromatograms were developed in a descending manner with *n*-butanol–acetic acid–water (12:3:5, v/v). The bands on each chromatogram corresponding to $R_f = 0.37$ and 0.43 for conjugates of SO and TCPO, respectively, were made visible by spraying with ninhydrin. Thereafter, the chromatograms were scanned in a Packard Radiochromatogram Scanner, the radioactive areas on the strips which were ninhydrin-positive were cut out, transferred to a vial containing 10 ml of a toluene–PPO–POPOP scintillation fluid and measured in a Beckman liquid scintillation spectrometer. The per cent radioactivity in the conjugate region was then determined and the nmoles of product were calculated. Incubations carried out in the absence of enzyme served as control. Dimethylsulfoxide at the concentration employed to dissolve TCPO was without effect on enzyme activity.

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† The following abbreviations are employed throughout: PB, phenobarbital; 3MC, 3-methylcholanthrene; TCPO, 3,3,3-trichloro-1,2 epoxy propane; SO, styrene oxide; and βNF, β-naphthoflavone.

Table 1. Enzymatic and nonenzymatic conjugation of glutathione with oxide substrates

Incubation system	nmoles/5 min			
	3MC oxide		SO	TCPO
	22	37		
−Supernatant	0.86	0.94	29	83
+Supernatant	2.82	5.32	107	248

Legend: [^{35}S]glutathione was incubated with the oxide substrates at 22 or 37 for 5 min (SO or TCPO) or 10 min (3MC 11,12 oxide) in the presence or absence of a rat liver supernatant fraction which contained 1.1–1.6 mg protein. The temperature of incubation with SO and TCPO was 22 . The amount of conjugate was determined as described in the text.

Assay of glutathione-S-epoxide transferase activity with 3MC 11,12 oxide as substrate. Assays were performed essentially as described above except the incubations were carried out at 37 for 10 min. The extent of the non-enzymatic reaction at this temperature was the same as at 22 . In addition, the location of the conjugates (R_f = 0.63) was easily ascertained under a u.v. light.

RESULTS

The formation of a glutathione conjugate of 3MC 11,12 oxide, SO and TCPO was tested under enzymatic and nonenzymatic conditions at 22 and 37 (Table 1). The extent of the nonenzymatic reaction is limited and not markedly affected by increasing the temperature from 22 to 37 . The nonenzymatic reaction was highest with TCPO but significantly lower than that of the enzymatic reaction. On the other hand, an increase of 88 per cent in the conjugation of 3MC 11,12 oxide with glutathione was observed in the enzymatic catalysis by raising the temperature from 22 to 37 .

The enzymatic reaction with 3MC 11,12 oxide was linear with respect to (a) supernatant protein, up to 4 mg per assay and (b) time, to 10 min.

The relative activity of glutathione-S-epoxide transferase in the rat liver supernatant fraction was determined with styrene oxide, TCPO and with 3MC 11,12 oxide as substrates. These data are presented in Table 2. Of the three epoxides, TCPO was the most efficacious while 3MC 11,12 oxide was the least reactive.

The effect of prior administration of phenobarbital upon glutathione-S-epoxide transferase activity was ascertained with the three substrates. These data are tabulated in Table 3. Highly significant elevations of 52, 20, and 35 per cent were observed with styrene oxide, TCPO, and 3MC 11,12 oxide, respectively, as substrates.

The administration of 3MC to rats is also attended by an increase in the specific activity of the enzyme in the rat liver supernatant preparation (Table 4).

Table 2. Relative activity of glutathione-S-transferase with various epoxide substrates

Epoxide substrate	nmoles conjugate/5 min/mg protein
Styrene oxide	86.4 ± 2.6 (100)
TCPO	165 ± 5.2 (192)
3MC 11,12 oxide	5.7 ± 0.2 (7)

Enzyme activity was determined with the above epoxides as substrates as described in the text. The incubation was conducted at 22 for 5 min. The data are presented as means ± S.E.M. (8 determinations). The figures within the parentheses represent the relative substrate efficacy with the conjugation of styrene oxide set as 100 per cent.

Table 3. Effect of phenobarbital administration on glutathione-S-epoxide transferase activity

Treatment	nmoles conjugate/5 min/mg protein		
	Styrene oxide	TCPO	3MC 11,12 oxide
Saline	86.4 ± 2.6*	165 ± 5.2	10.8 ± 0.4
Phenobarbital	131 ± 4.9	198 ± 4.4	14.6 ± 0.3
	(P < 0.001)	(P < 0.001)	(P < 0.001)

* Values represent mean ± S.E.M. of eight determinations. Male 50–60 g rats were injected i.p. with phenobarbital, 75 mg/kg body wt. for three days and were killed 24 hr after the last injection. The activity with styrene oxide and TCPO was determined at 22 while that with 3MC 11,12 oxide was determined at 37 . The P values were determined and are indicated within the parentheses.

Table 4. Effect of 3MC administration on glutathione-S-epoxide transferase activity

Treatment	nmoles conjugate/5 min/mg protein		
	Styrene oxide	TCPO	3MC 11,12 oxide
Corn oil	80.5 ± 3.9*	126 ± 8.6	9.5 ± 0.4
3MC, 20 mg/kg	122 ± 3.7	172 ± 4.9	14.5 ± 0.5
3MC, 40 mg/kg	123 ± 2.8	178 ± 2.9	15.7 ± 1.1
3MC, 60 mg/kg	129 ± 4.4	162 ± 10.2	14.6 ± 1.4
3MC, 100 mg/kg	153 ± 5.7	187 ± 3.7	15.8 ± 1.1

* Values represent mean ± S.E.M. of three determinations. The rats were injected at 24 hr intervals with 3MC at the specified dose and were sacrificed 24 hr later. Activity with styrene oxide and TCPO was determined at 22 ; that with 3MC 11,12 oxide at 37 .

Table 5. Effect of 3-methylcholanthrene and β-naphthoflavone administration on glutathione-S-epoxide transferase activity

Treatment	nmoles conjugate/5 min/mg protein		
	Styrene oxide	TCPO	3MC 11,12 oxide
Corn oil	82.6 ± 3.0 (8)*	134 ± 7.1 (4)	9.4 ± 0.3 (8)
3MC 24 hr	112 ± 2.1 (8)	177 ± 3.4 (4)	14.71 ± 1.0 (8)
	(P < 0.001)	(P < 0.001)	(P < 0.001)
3MC 48 hr	118 ± 4.6 (4)	179 ± 5.3 (4)	12.0 ± 0.5 (4)
	(P < 0.001)	(P < 0.001)	(P < 0.001)
β-NF	81.8 ± 3.6	127 ± 4.9 (4)	10.3 ± 0.4
	(P > 0.01)	(P > 0.01)	(P > 0.01)

* Mean ± S.E.M. (number of determinations). Rats were injected with (a) 3MC (20 mg/kg) daily and were sacrificed 24 hr later at the times indicated in the table or (b) β-naphthoflavone (βNF) 100 mg/kg, for two days at 24 hr intervals and were sacrificed 24 hr after the last injection.

After administration of the polycyclic hydrocarbon twice at 20 mg/kg, the specific enzyme activity was elevated 52, 37 and 53 per cent respectively, with styrene oxide, TCPO and 3MC 11,12 oxide as substrates. At the higher dose, i.e., twice at 100 mg/kg, increases of 90, 49 and 66 per cent respectively, were noted.

The effect of a single or of two doses of 3MC at 20 mg/kg body wt upon enzyme activity in the rat liver supernatant fraction was also studied. These data are represented in Table 5. Highly significant increases with all three substrates were observed at these times. On the other hand, β-naphthoflavone administration did not affect the specific activity of glutathione-S-epoxide transferase in liver with any of the substrates.

DISCUSSION

The formation of polycyclic hydrocarbon oxides in biologic systems is of great importance in carcinogenesis as these substances, at least in some instances, may be the ultimate carcinogens [4, 6, 20]. Consequently, the enzymatic pathways for the inactivation of these oxides will play a major role in the potential susceptibility of a tissue to their carcinogenic action. At least two mechanisms for this detoxification are available, epoxide hydrase and glutathione-S-epoxide transferase. We have recently reported on the distribution of the former in rat liver, kidney and lung [12], in its activity in regenerating rat liver and during development [21], and on its blockade by TCPO [21]. We have also shown that the application to mouse skin of TCPO along with 3MC resulted in a considerable reduction in latency time before the onset of tumors, an increase in the per cent of mice exhibiting tumors, and a profound increase in the numbers of tumors per mouse [22].

In the research reported in the present manuscript, we have initiated studies on the activity of the second enzyme system which is capable of catalyzing the inactivation of the polycyclic hydrocarbon oxides, glutathione-S-epoxide transferase. The enzyme (or enzymes) does (or do) indeed catalyze conjugate formation with 3MC 11,12 oxide as substrate but at a considerably lesser extent than with several other substrates employed in this study, namely, TCPO and styrene oxide.

Boyland and Williams [13] and Hayakawa *et al.* [18] have previously reported the interaction of styrene oxide with glutathione as catalyzed by a transferase while Fjellstedt *et al.* [23] have estimated conjugate formation with TCPO as substrate. Using rat liver homogenates, Sims [19] presented evidence for the formation of a glutathione conjugate of 3MC 11,12 oxide.

Considerable evidence has been accumulated which suggests multiple enzymes with transferase activity [24–26]. Furthermore, it has been claimed that one of the transferases, glutathione-S-transferase B, is identical to the intracellular protein which is involved in binding of a number of anions, ligandin [27]. One of the pieces of evidence in this regard is based upon the analogous induction by phenobarbital of both ligandin [28] and of the transferase [27]. We have reported in the present manuscript the elevated activity of the transferase(s) in liver with either

TCPO, styrene oxide or 3MC 11,12 oxide as substrates. In addition, we have also observed an increased activity of this (or these) enzyme(s) after 3MC pretreatment.

It would be of paramount importance to ascertain the relative activities of these enzymes, active in detoxification, in tissues which are susceptible to the action of the polycyclic hydrocarbon, e.g., lung or skin, and the inducibility of these enzymes by barbiturates or other substances. Furthermore, the effects of exogenous glutathione or inhibitors of glutathione synthesis upon the carcinogenic process would be of interest. These studies are presently under way.

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