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BENZO(α)PYRENE QUINONES CAN BE GENERATED BY LIPID PEROXIDATION AND ARE CONJUGATED WITH GLUTATHIONE BY GLUTATHIONE S-TRANSFERASE B FROM RAT LIVER.

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SUMMARY: We have investigated the effect of adding purified glutathione S--transferases A, B, and C, to rat liver microsomes metabolizing benzo(α)pyrene. Glutathione S-transferase B was found to be effective in metabolizing benzo-(α)pyrene 1,6 and 3,6 quinones to a water soluble metabolite presumably a glutathione conjugate. Also we show that non-enzymic lipid peroxidation in microsomes by ascorbate and ADP-Fe^{2+} in the presence of benzo(α)pyrene gives rise chiefly to quinones derived from this compound.

INTRODUCTION

Benzo(α)pyrene (BP), a representative of the class of ubiquitous environmental xenobiotics known as polycyclic hydrocarbons, is an extremely potent mutagen and carcinogen. Benzo(α)pyrene is metabolized to carcinogenic reactive intermediates via the cytochrome P-450 system. The production of such intermediates as epoxides and diol epoxides has been extensively investigated and the further metabolism of these substances - e.g., by epoxide hydrolase and glutathione S-transferases - has also received considerable attention. (For general reviews, see references 1-3).

Another group of products arising from the metabolism of BP, at least in vitro, are the 1,6-, 3,6-, and 6,12-quinones, which have been shown to arise in part from autooxidation of the labile 6-hydroxy-BP (4). In the present study we demonstrate that lipid peroxidation also generates quinones from BP.

Abbreviation: BP (Benzo(α)pyrene)
Enzyme: Glutathione S-transferases (E.C.2.5.1.28).

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If quinones or, more likely, semiquinones are indeed reactive intermediates of BP metabolism in vivo, then the further metabolism of these substances is of great interest in connection with the toxic and carcinogenic properties of this xenobiotic. Hitherto, it is known that BP quinones can be recycled through the cytochrome P-450 system or reduced by DT-diaphorase (5) and/or NADPH-cytochrome c reductase (6),also the reduced quinone can be conjugated with glucuronic acid (5). In addition, tracheal explants have been found to yield glucuronide conjugates and other water-soluble metabolites,presumably also conjugates of some sort, of BP quinones (7).

The glutathione S-transferases are an important group of detoxifying enzymes with broad and overlapping substrate specificities for electrophilic, lipophilic substances - including a large number of mutagenic, carcinogenic, or pharmacologically active xenobiotics or metabolites of xenobiotics (for reviews, see 3,8). These enzymes have been shown earlier to be involved in the conjugation of epoxides of BP with glutathione (3,9,10). In the present communication we report that glutathione S-transferase B greatly decreases the accumulation of the 1,6- and 3,6-quinones of BP when added together with glutathione to rat liver microsomes which are metabolizing BP. We also demonstrate that this is not due to an inhibition of lipid peroxidation, as was first suspected since glutathione S-transferase B is known to have glutathione peroxidase activity. Rather, glutathione S-transferase B apparently catalyzes the conjugation of the 1,6- and 3,6-quinones of BP with glutathione. The glutathione S-transferases A and C also catalyze this same reaction, but much less efficiently.

MATERIALS AND METHODS

The total microsomal fraction was prepared as described previously (11) from the livers of 180-200 g male Sprague-Dawley rats which had been starved overnight. The microsomes were washed twice in 0.15 M Tris-Cl, pH 8.0,in order to effectively remove cytosolic proteins (e.g., the cytosolic glutathione S-transferases) (12). The pellets were then resuspended in 0.25 M sucrose, except when lipid peroxidation was to be carried out. In this case the microsomes were resuspended in 0.15 M KCl in order to avoid interference by sucrose in the determination of malonaldehyde.

Incubations of microsomes (0.47 mg protein/ml) with [7,10-^{14}C]-benzo(α)-pyrene (from Amersham-Searle: approximately 1.6 μCi per tube) were carried out in the presence of a NADPH-generating system as described (16). Where indicated, 10 mM glutathione (Sigma) and sufficient amounts of glutathione S-transferase A, B, and C (purified according to references 14,15) to achieve maximal conjugation - i.e., 13,5, and 13 units/ml, respectively - were also present in the incubation mixture. These incubations were carried out at 37° for 60 min.

The total metabolism of BP was determined by an extraction procedure developed earlier in our laboratory (16). Individual metabolites were analyzed using high pressure liquid chromatography as described in reference (17), with the following modifications: the elution rate was 2 ml/min. The gradient used was 55-75% methanol:ethanol (2:1) in water and was applied during a period of 30 min. The radioactivity remaining in the water phase after extracting the sample twice with 4 ml ethyl acetate (minus appropriate controls) was taken as a measure of conjugate formation.

Lipid peroxidation was achieved by the addition of ascorbate and ADP-Fe^{2+} as in (18) to a microsomal suspension (0.74 mg protein/ml), followed by incubation for 20 min at 32°. Where indicated, [7,10-^{14}C]-BP (80 μM), glutathione (10 mM), and/or purified glutathione S-transferases (as above) were also added. The extent of lipid peroxidation was monitored by assaying the amount of malonaldehyde formed (19). The pattern of BP metabolites obtained by lipid peroxidation was determined by high pressure liquid chromatography as described above.

^{14}C-BP-1,6-quinone was prepared enzymically and purified by preparative high pressure liquid chromatography (20). It was diluted with the unlabeled quinone obtained from the National Cancer Institute, USA. To measure the conjugation of this metabolite with glutathione, 5 μM ^{14}C-BP-1,6-quinone (30 nCi/incubation), 10 mM glutathione, and purified glutathione S-transferase A, B, or C were incubated in 50 mM Tris-Cl, pH 7.5, at 37°C under conditions such that the activity was linear with time and protein. Subsequently, the reaction mixture was extracted twice with 4 ml ethyl acetate and the amount of radioactivity remaining in the water phase again taken as a measure of conjugate formation.

Protein was determined according to Lowry et al. (21) with bovine serum albumin as the standard. All chemicals not specified above were obtained from common commercial sources and were of the highest purity available. All experiments were performed at least twice and the results of a typical experiment are shown.

RESULTS AND DISCUSSION

When BP is incubated with rat liver microsomes and an NADPH-generating system, the metabolite pattern shown in Table 1 is obtained. As found by other investigators as well, the single largest metabolite is 3-hydroxy-BP and significant amounts of BP quinones are produced as well. Addition of glutathione to the system reduced the percentage of 4,5-dihydrodiol and, to a lesser extent, of 7,8-dihydrodiol formed. This finding may reflect the fact that the epoxide intermediates from which these metabolites arise can react non-enzymatically with glutathione. Alternatively, such a conjugation might be catalyzed by the microsomal glutathione S-transferase(s) (12,13).

Table 1. The pattern of BP metabolites formed by rat liver microsomes in the presence and absence of purified glutathione S-transferases A, B, and C.

Conditions[a]	Total BP metabolites (nmol)			Metabolites: (% of total metabolites in ethyl acetate phase)							
	in aqueous phase	in ethylacetate phase	More polar	9,10-Diol	4,5-Diol	7,8-Diol	1,6-Quinone	3,6-Quinone	6,12-Quinone	9-OH	3-OH
Control	0.2	8.8	10.3	5.5	6.5	6.2	8.4	14.0	1.6	3.6	44.2
With added glutathione	1.8	9.4	11.5	6.0	3.3	4.9	9.5	12.5	1.3	3.9	45.8
With added glutathione + glutathione S-transferase A	3.1	8.4	12.5	4.2	2.6	4.2	10.4	14.8	1.1	3.8	46.5
With added glutathione + glutathione S-transferase B	6.2	9.2	14.4	4.1	1.1	5.1	2.4	3.8	1.3	5.2	62.6
With added glutathione + glutathione S-transferase C	4.1	7.6	12.7	4.2	1.0	4.8	9.3	14.7	1.4	3.3	48.7
With added glutathione + glutathione S-transferases A + B + C	5.0	8.3	14.1	6.3	2.6	4.9	2.2	2.9	1.1	6.2	59.9
Lipid peroxidation[b]	n.d.	5	17.2	1.2	2.7	2.5	23.2	21.9	11.5	-	4.5

[a]The experimental conditions and analysis by high-pressure liquid chromatography were as described in the Materials and Methods. The "more polar" metabolites, including tetrols, are eluted from the column prior to the diols. The results are the mean of duplicate experiments.

[b]malonaldehyde formed was 30nmol/20 min. n.d. = not determined.

Addition of purified glutathione S-transferase A, B, and C - individually or all three together - to the incubation medium results in about 30 to 50% increase in the total metabolism of benzpyrene. All three transferases further decrease the percentage of 4,5-dihydrodiol produced, but only glutathione S-transferase B reduces the quinone fraction. Both the percentage of 1,6-quinone and 3,6-quinone are reduced by 70-80%, whereas the 6,12-quinone fraction is comparatively unaffected. This decrease in the quinones is accompanied by an apparent increase in the percentage of the 3-hydroxy and 9-hydroxy metabolites. The effect of all three transferases together is largely what might be expected from the use of saturating amounts of these enzymes. In the presence of glutathione S-transferase A, B, or C, but absence of glutathione, the metabolite pattern obtained is identical to that of the control.

There are at least two possible explanations for the decrease in BP 1,6- and 3,6-quinone formation observed in the presence of glutathione S-transferase B, but not with the A and C forms. One simple possibility is that the B form can conjugate these quinones with glutathione, whereas the other two forms catalyze this conjugation only poorly or not at all. The conjugates formed would thus remain behind in the aqueous phase upon extraction of the incubation medium with ethyl acetate for determination of the metabolite pattern. Indeed, as shown in the first column of Table 1, glutathione S-transferase B causes a large increase in the amount of BP metabolites which remain in the aqueous phase. These water-soluble metabolites approximately account for the loss of 1,6-quinone (7.1%) plus 3,6-quinone (8.7%).

This hypothesis was tested directly by incubating isolated BP 1,6-quinone together with purified glutathione S-transferases A, B, and C (Table 2). As can be seen, glutathione S-transferase B is about 40 times more effective in converting the BP-1,6-quinone to a water-soluble product (presumably a conjugate of glutathione) than are the other two transferases. This finding provides, then, a reasonable explanation for the results illustrated in Table 1.

Table 2. Conversion of BP-1,6-quinone (5 μM) to a water soluble form in the presence of glutathione (10 mM9 and glutathione S-transferase A, B, or C.

Enzyme added	water soluble product formed nmol/min per mg protein
Glutathione S-transferase A	0.04 ± 0.004
Glutathione S-transferase B	1.9 ± 0.1
Glutathione S-transferase C	0.05 ± 0.01

This experiment was carried out as described in Materials and Methods. The figures gáven are the means and S.E.M. of 4 determinations. The background obtained when BP-1,6-quinone was simply incubated in buffer in the presence of glutathione, i.e., without added enzyme, was 15 pmol/min and has been subtracted. The background obtained when BP-1,6-quinone was incubated with enzyme in the absence of glutathione was equivalent to 10 pmol/min and has also been subtracted.

It should also be noted that in the incubations containing microsomes and an NADPH-generating system (Table 1), reduction of quinones by DT-diaphorase and/or NADPH-cytochrome c reductase occurs and competes with other metabolic pathways, including conjugation with glutathione. Nonetheless, most of the quinones formed are converted to water-soluble products by glutathione S-transferase B under these conditions. Thus, this might be a relatively effective pathway for the further metabolism of quinones, although there may, of course, be other competing pathways *in vivo*. In addition, it is possible that hydroquinones and, especially, semiquinones may also be conjugated with glutathione.

Another manner in which glutathione S-transferase B might decrease the percentage of BP quinone metabolites recovered is by decreasing their formation, rather than by catalyzing their further metabolism. Glutathione S-transferase B is known to have glutathione peroxidase activity (22) and to inhibit lipid peroxidation (23). Lipid peroxidation might give rise to BP quinones either directly or indirectly via the cytochrome P-450 system. It is known, for instance, that metabolism of BP by the cytochrome P-450 system supported

Table 3. Formation of BP "metabolites" as a result of lipid peroxidation and inhibition of both lipid peroxidation and "metabolite" formation by glutathione S-transferases A, B, and C.

Additions	malonaldehyde nmol/20 min (%)	BP "metabolites" nmol formed/20 min (%)
None[a]	2.2	n.d.
Complete system[b]	29.8 (100)	3.3 (100)
Complete system + glutathione S-transferase A	4.8 (16)	0.3 (9)
Complete system + glutathione S-transferase B	8.1 (27)	1.0 (30)
Complete system + glutathione S-transferase C	6.2 (21)	0.4 (12)

[a]Microsomes and BP in buffer
[b]Microsomes, BP, ascorbate, ADP-Fe^{2+}, and glutathione in buffer
For further experimental details see the Materials and Methods.

by cumene hydroperoxide instead of NADPH gives rise to quinones as the sole metabolites (24).

An experiment shown in Table 3 was performed to test the possibility that glutathione S-transferase B lowers the formation of BP quinones by inhibiting lipid peroxidation. Conditions similar to those used in the experiments shown in Table 1 were employed. Little lipid peroxidation and no detectable formation of benzpyrene quinones occur when microsomes are simply incubated in buffer. Nor is much lipid peroxidation detected when microsomes are incubated together with a NADPH-generating system (not shown). Under conditions where lipid peroxidation does occur (ascorbate + ADP-Fe^{2+}) quite significant amounts of benzpyrene quinones are produced (56% of the metabolites) (Table 1). In addition, an unidentified product eluted between the 9,10 and 4,5-diols accounts for 16% of the total products formed.

Addition of glutathione S-transferases A, B, and C together with glutathione all inhibit both lipid peroxidation and the formation of quinones and

other products (Table 3). However, all three of these enzymes are approximately equally effective in this respect, so that this can not be the mechanism by which glutathione S-transferase B selectively decreases the relative amounts of quinones recovered after microsomal metabolism of BP.

This investigation indicates strongly that the 1,6- and 3,6-quinones of benzpyrene can be conjugated with glutathione and that it is this conjugation which is responsible for the reduction in the relative amounts of these quinones achieved by the addition of glutathione S-transferase B. It has been reported earlier that certain other quinones can also serve as substrates for the glutathione S-transferases (3). It is not at all clear what structure a conjugate formed between a BP quinone and glutathione would have, nor by what mechanism such a conjugate would be formed, and we are presently investigating these questions.

We have also demonstrated that lipid peroxidation in microsomes can result in the formation of BP quinones. However, it is not clear whether reactive intermediates involved in lipid peroxidation oxidize BP directly, or whether lipid peroxides support the oxidation of BP via the cytochrome P-450 system. This is also a question for further investigation, e.g., by carrying out lipid peroxidation with liposomes made from isolated microsomal phospholipids and testing for the formation of BP quinones.

If quinones (or metabolites of quinones, such as semiquinones) are formed *in vivo* as reactive intermediates - for which there are certain indications (25,26) -, then the processes discussed here for the formation (via lipid peroxidation) and further metabolism (via glutathione S-transferase B) of these products may be of fundamental interest in connection with mechanisms of toxicity and carcinogenicity.

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REFERENCES

1. DePierre, J.W. and Ernster, L. (1978) Biochim. Biophys. Acta 473, 149-186.
2. Oesch, F. (1973) Xenobiotica 3, 305-340.
3. Chasseaud, L.F. (1979) Adv. Cancer Res. 29, 175-274.
4. Lorentzen, R.J., Caspary, W.J., Lesko, S.A. and Ts'o, P.O.P. (1975) Biochemistry 14, 3970-3977.
5. Lind, C., Vadi, H. and Ernster, L. (1978) Arch. Biochem. Biophys. 190, 97-108.
6. Shen, A.L., Fahl, W.E., Wrighton, S.A. and Jefcoate, C.R. (1979) Cancer Res. 39, 4123-4129.
7. Mass, M.J. and Kaufman, D.G. (1979) Biochem. Biophys. Res. Commun. 89, 885-892.
8. Jakoby, W.B. (1978) Adv. Enzymol. 46, 383-414.
9. Hayakawa, T., Udenfriend, S., Yagi, H. and Jerina, D.M. (1975) Arch. Biochem. Biophys. 170, 438-451.
10. Nemoto, N., Gelboin, H.V., Habig, W.H., Ketley, J.N. and Jakoby, W.B. (1975) Nature 255, 512.
11. Ernster, L., Siekevitz, P. and Palade, G.E. (1962) J. Cell. Biol. 15, 541-562.
12. Morgenstern, R., Meijer, J., DePierre, J.W. and Ernster, L. (1980) Eur. J. Biochem. 104, 167-174.
13. Friedberg, T., Bentley, P., Stasiecki, P., Glatt, H.R., Raphael, D. and Oesch, F. (1979) J. Biol. Chem. 254, 12028-12033.
14. Askelöf, P., Guthenberg, C., Jakobson, I. and Mannervik, B. (1975) Biochem. J. 147, 513-522.
15. Guthenberg, C., Morgenstern, R., DePierre, J.W. and Mannervik, B. (1980) Biochim. Biophys. Acta 631, 1-10.
16. DePierre, J.W., Moron, M.S., Johannesen, K.A.M. and Ernster, L. (1975) Anal. Biochem. 63, 470-484.
17. Burke, M.D., Vadi, H., Jernström, B. and Orrenius, S. (1977) J. Biol. Chem. 252, 6424-6431.
18. Orrenius, S., Dallner, G. and Ernster, L. (1964) Biochem. Biophys. Res. Commun. 14, 329-334.
19. Bernheim, F., Bernheim, M.L. and Wilbur, K.M. (1948) J. Biol. Chem. 174, 257-264.
20. Jernström, B., Vadi, H. and Orrenius, S. (1978) Chem.-Biol. Interact. 20, 311-321.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
22. Prohaska, J.R. (1980) Biochim. Biophys. Acta 611, 87-98.
23. Burk, R.F., Mayme, J.T. and Lawrence, R.A. (1980) Biochim. Biophys. Acta 618, 35-41.
24. Capdevila, J., Estabrook, R.W. and Prough, R.A. (1980) Arch. Biochem. Biophys. 200, 186-195.
25. Dybing, E., Nelson, S.D., Mitchell, J.R., Sasame, H.A. and Gillette, J.R. (1976) Mol. Pharmacol. 12, 911-920.
26. Pelkonen, O., Boobis, A.R., Yagi, H., Jerina, D.M. and Nebert, D.W. (1978) Mol. Pharmacol. 14, 306-322.