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ETHOXYQUIN FEEDING TO RATS INCREASES LIVER MICROSOME-CATALYZED FORMATION OF BENZO(A)PYRENE DIOL EPOXIDE - DNA ADDUCT

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SUMMARY. The ability of rat liver microsomes to catalyze the formation of benzo(a)pyrene 7,8-diol-9,10-epoxide - DNA nucleoside adduct was increased threefold by feeding 0.5 % ethoxyquin to the animals. Microsomal epoxide hydratase activity was enhanced in parallel by a factor of 3 while aryl hydrocarbon hydroxylase activity was not induced. Liver microsomes from rat pretreated with 3-methylcholanthrene produced an increased proportion of diol epoxide - DNA adduct when ethoxyquin had been fed to the animals. The main chromatographic peak formed by microsomes from 3-methylcholanthrene treated rats which contains DNA adducts of secondary benzo(a)pyrene phenol metabolites is reduced when the animals had received ethoxyquin.

Feeding of the antioxidant ethoxyquin to experimental animals has been reported to exert a protective action against tumor formation due to benzo(a)pyrene and dimethylbenz(a)anthracene (1). The mechanisms involved are not yet clear but may be related to ethoxyquin effects on the metabolic activation of the carcinogens. The compound inhibits hepatic microsomal monooxygenase activity in vitro (2, 3, 4) and reduces the formation of mutagenic benzo(a)pyrene metabolites (4). Pretreatment with ethoxyquin induces cytochrome P 450-dependent reactions in liver microsomes (5, 3); however, aryl hydrocarbon hydroxylase is not induced (3) Moreover, we have recently shown that ethoxyquin feeding induces epoxide hydratase in rat liver microsomes (3, 6), and this may influence the metabolite profile of benzo(a)pyrene and the pattern of DNA nucleoside adducts formed. The benzo(a)pyrene 7,8-diol-9,10-epoxideshave been shown to be ultimate mutagens (7, 8) and diol epoxide - DNA adducts have been detected under in vivo conditions (9, 10, 11). Since the formation of these metabolites is dependent on epoxide hydratase activity it may be enhanced by ethoxyquin feeding in spite of the reported protective effects of the antioxidant against benzo(a)pyrene (1). The

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present communication provides evidence that this is indeed the case in an in vitro test system for DNA adduct formation.

METHODS

Male Sprague-Dawley rats received a powdered Altromin^{R} diet to which 0.5 % ethoxyquin (kindly donated by the Deutsche Naarden GmbH, Hamburg) was added. The feeding period was 14 days. Normal pellet diet was then given for 24 hr and was withdrawn 12 hr before sacrifice. For 3-methylcholanthrene induction the animals received 3 i.p. injections of 20 mg/kg in oil in 12 hr intervals, the last injection given 36 hr prior to sacrifice. Liver microsomes were prepared as described previously (3). Enzyme activities were measured according to published methods (12, 13).

Calf thymus DNA (Sigma Chemie, München) was incubated with liver microsomes in the presence of 6 µM ^{3}H benzo(a)pyrene (Amersham Buchler, Braunschweig, diluted to a specific activity of 20 µCi/nmol and purified before use) as described previously (14). The DNA was then isolated, extensively washed and hydrolyzed by the protocol given by Pelkonen et al. (14). The digest was chromatographed on Sephadex LH 20 columns by elution with a 30 to 100 % water:methanol gradient as described by Baird and Brookes (15). 5.1 ml fractions were collected and an aliquot of every second fraction was analyzed for radioactivity. For quantitation of peaks, baseline radioactivity as found at fraction 60 was subtracted.

RESULTS

The profile of benzo(a)pyrene metabolite-DNA nucleoside adducts formed in vitro is shown in Fig. 1. Sephadex LH 20 chromatography resolves the adducts into 3-4 peaks which have previously been identified (16, 17, 10, 14). Peak 1 contains the diol epoxide adducts (16, 10). This peak is enhanced when liver microsomes from ethoxyquin-pretreated animals are used for metabolic activation; this is found if no additional monooxygenase inducer had been given (Fig. 1a) and if concomitant 3-methylcholanthrene induction had been performed (Fig. 1b), a situation by which total adduct formation is very much increased.

Enhanced diol epoxide adduct formation, though not consistent with a protective role of ethoxyquin against benzo(a)pyrene carcinogenesis (1), may be explained by the fact that ethoxyquin markedly induces hepatic epoxide hydratase activity (Tab. 1). In contrast to the threefold increase in epoxide hydratase activity aryl hydrocarbon hydroxylase is not induced and its induction by 3-methylcholanthrene is inhibited by ethoxyquin. Tab. 2 shows that the proportion of the diol epoxide adduct peak as related to the total radioactivity eluted from the column is also enhanced by a factor of 3. Since total binding of benzo(a)pyrene equivalents is

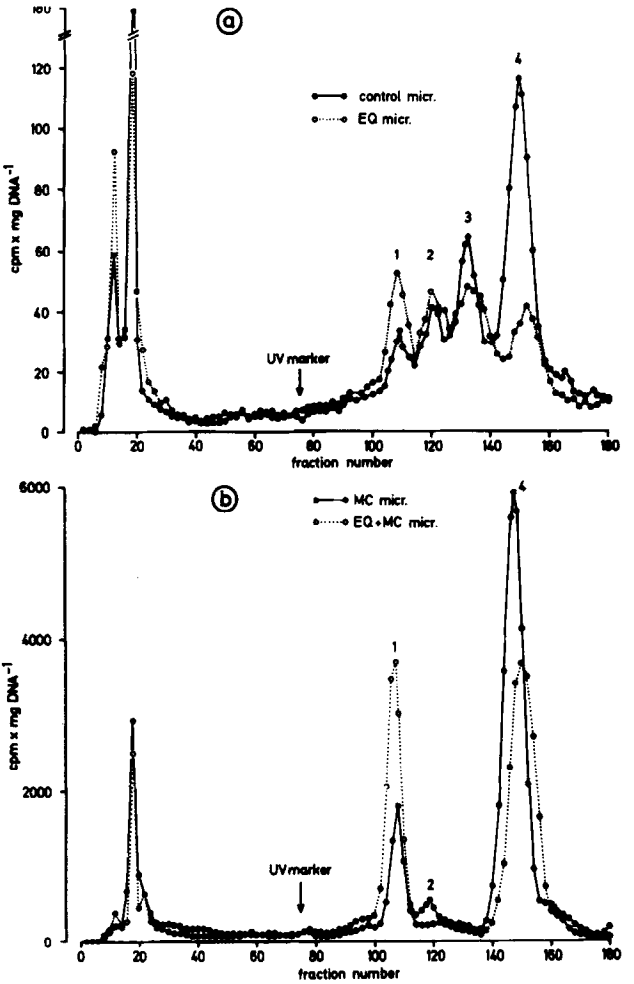

Fig. 1 Elution profile from Sephadex LH 20 chromatography of hydrolyzed calf thymus DNA after incubation with 6 µM ^{3}H-benzo(a)pyrene and liver microsomes from variously pretreated rats. Elution was performed with a 30 to 100 % water:methanol gradient. The UV marker was 4-(p-nitrophenyl)pyridine. EQ: ethoxyquin pretreatment (0.5 % in food for 14 days). MC: 3-methylcholanthrene pretreatment (3 x 20 mg/kg i.p.).

not significantly influenced, this means that threefold amounts of benzo(a)pyrene equivalents are present in the form of adduct 1 when ethoxyquin microsomes had been used for metabolic activation instead of control microsomes.

A threefold increase in proportion of adduct 1 is also found when animals treated with ethoxyquin plus 3-methylcholanthrene instead of 3-methylcholanthrene only had been used as the donor animals for the activating system (Tab. 3); since

Table 1: Induction of epoxide hydratase in rat liver microsomes by ethoxyquin feeding

Pretreatment	EH activity (nmol styrene glycol x mg prot.$^{-1}$ x min^{-1})	AHH activity (nmol 3-hydroxybenzo-(a)pyrene x mg prot.$^{-1}$ x min^{-1})
none	9.6 ± 0.8	0.66 ± 0.08
EQ	34.5 ± 2.1*	0.48 ± 0.02
MC	8.6 ± 0.7	1.98 ± 0.16
EQ + MC	28.4 ± 0.5**	1.28 ± 0.17***

EH: epoxide hydratase; AHH: aryl hydrocarbon hydroxylase; EQ: ethoxyquin (0.5 % in food for 14 days); MC: 3-methylcholanthrene (3 x 20 mg/kg i.p.); values are means ± S.E.M. (n = 3 - 6); * $P < 0.001$ against no treatment group, ** $P < 0.001$ against MC group, *** $P < 0.05$ against MC group.

Table 2: Benzo(a)pyrene metabolite-DNA nucleoside adducts formed during incubation with liver microsomes from untreated and ethoxyquin-fed rats.

	no treatment	EQ
peak 1 (%)	4.5 ± 0.4	13.6 ± 1.6*
peak 2 (%)	7.3 ± 2.0	10.0 ± 2.6
peak 3 (%)	16.1 ± 2.8	14.4 ± 4.8
peak 4 (%)	15.0 ± 4.6	15.6 ± 1.9
total binding (cpm x 10^4/mg DNA)	1.4 ± 0.7	1.7 ± 0.5

EQ: ethoxyquin (0.5 % in food for 14 days). Sephadex LH 20 chromatography of DNA digests. Peaks are expressed as % of total radioactivity eluted. Baseline radioactivity as found at fraction 60 was subtracted from all values. Values are means ± S.E.M. (n = 3).

* $P < 0.001$ against no treatment group.

Table 3: Benzo(a)pyrene metabolite-DNA adducts formed during incubation with liver microsomes from rats pretreated with 3-methylcholanthrene or ethoxyquin plus 3-methylcholanthrene.

	MC		EQ+MC	
	exp. 1	exp. 2	exp. 3	exp. 4
peak 1 (%)	13.9	9.5	39.9	31.4
peak 2 (%)	2.9	2.2	11.9	4.6
peak 3 (%)	1.6	n.d.	n.d.	0.9
peak 4 (%)	81.6	64.7	35.0	46.0
total binding (cpm x 10^4/mg DNA)	57.1	32.5	10.9	24.2

MC: 3-methylcholanthrene (3 x 20 mg/kg i.p.), EQ: ethoxyquin (0.5 % in food for 14 days). Sephadex LH 20 chromatography of DNA digests. Peaks are expressed as % of total radioactivity eluted. Baseline radioactivity as found at fraction 60 was subtracted from all values. n.d.: not detected.

total binding is reduced the absolute amount of diol epoxide adduct formed is not much increased under these conditions. The proportion of adduct 1 is increased by use of 3-methylcholanthrene-stimulated instead of control microsomes as expected from HPLC analysis of benzo(a)pyrene metabolite pattern (18).

While the increase in peak 1 shown in Fig. 1a is representative for the 3 experiments performed this is not the case for the reduction in peak 3 and 4. Peak 3 is partially due to the K-region epoxide adduct (14), and induction of epoxide hydratase by ethoxyquin might be expected to decrease its formation. However, Tab. 2 reveals that the slight decrease observed was statistically not significant. Small amounts of adduct 3 if any are found when microsomes from 3-methylcholanthrene-treated animals are used (Tab. 3).

Peak 4 has been shown to contain secondary metabolites of benzo(a)pyrene phenols (17, 14). This peak was extremely variable when control animals were used for microsome preparation (Tab. 2); statistically, no influence of ethoxyquin feeding was observed. However, in the experiments with induced monooxygenase a more consistent view was obtained. Peak 4 was the main peak with 3-methylcholanthrene microsomes as the activating system but its proportion was reduced by ethoxyquin feeding; about equal peak heights were observed for adduct 1 (diol epoxides) and adduct 4 (phenol oxides) after treatment with ethoxyquin plus 3-methylcholanthrene (Fig. 1b; Tab. 3). In view of decreased total binding this means a marked reduction of the amount of metabolites present in the form of adduct 4.

Peak 2 appears unaltered by ethoxyquin in Fig. 1a and Tab. 2. This peak has been postulated to contain secondary benzo(a)pyrene quinone metabolites (14). Its proportion but not the absolute amounts formed seem to increase after treatment with ethoxyquin plus 3-methylcholanthrene instead of 3-methylcholanthrene only (Tab. 3).

DISCUSSION

Induction of epoxide hydratase without concomitant induction of monooxygenase is difficult to obtain (19). A limiting role of this enzyme for the formation of

active carcinogen metabolites is therefore not readily assessed. The potent epoxide hydratase inducer ethoxyquin used in this study is not completely selective since it also induces desalkylation reactions (2, 3). Aryl hydrocarbon hydroxylase is not induced, but detailed information on the benzo(a)pyrene metabolite pattern in liver microsomes from animals fed ethoxyquin has not been published. The present results suggest that in rat liver microsomes epoxide hydratase activity may be limiting for the production of the 7,8-diol-9,10-epoxides because the amount of diol epoxide adduct is increased threefold. On the other hand, reduced formation of the 4,5-oxide adduct might be expected to occur by induction of the enzyme. The present study failed to unequivocally detect such reduction.

Increased formation of the diol epoxide adduct is certainly not a finding to be expected for a substance which has been claimed to reduce tumorigenesis induced by benzo(a)pyrene (1). The protective effect of antioxidants (1, 20, 21) has been related to various actions of these compounds on the metabolic activation of polycyclic hydrocarbons. Monooxygenase activity is inhibited in vitro (22, 23, 3, 6) but this potency is markedly reduced (3) or no longer detectable (6) if cytochrome P 448 has been induced and may thus play a minor role in carcinogenesis if testing with high doses of polycyclic hydrocarbons. Treatment with phenolic antioxidants has been claimed to alter the metabolite pattern of benzo(a)pyrene in mouse liver with the percentage of diol formation decreasing (24), and to reduce the ability to catalyze covalent binding to DNA (20, 21, 4, 6).On the other hand, butylated hydroxytoluene has been observed to enhance the formation of the 7,8-dihydrodiol in vitro (25). The phenolic antioxidants are also inducers of rat liver epoxide hydratase (6). We have tried to detect increased diol epoxide adduct formation after feeding of butylated hydroxytoluene; however, in spite of effective epoxide hydratase induction, only marginal increases of this adduct were found. It is at present not clear if there exist differences in the action of the tested antioxidants on benzo(a)pyrene metabolism. We have preliminary evidence that both of them induce epoxide hydratase also in rat lung and kidney microsomes (Kahl and Wulff, unpublished observations). Increased diol

epoxide formation may thus also occur in lung which is one of the target organs of benzo(a)pyrene. In an animal in a carcinogenesis test, induction of cytochrome P 448 will probably take place by the high doses of carcinogens used. In this situation, the absolute amount of diol epoxide adduct formed is not as markedly increased as in animals with a constitutive monooxygenase population. The main adduct then is the adduct 4 containing secondary phenol metabolites. This adduct has not been detected in human bronchial explants (9) and hamster embryo cells (10) but we have recently observed that it is formed in isolated perfused rat lung (11). Its biological properties are not sufficently known to speculate on a role in carcinogenesis; however, it cannot be excluded that a reduced capacity of the liver to produce this adduct is related to the protective action of ethoxyq Moreover, effects of ethoxyquin on events occurring later in the development of benzo(a)pyrene-induced cancer may counteract a potency to increase the primary DNA lesion.

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