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ELEVATION OF HEPATIC EPOXIDE HYDRATASE ACTIVITY BY ETHOXYQUIN IS DUE TO INCREASED SYNTHESIS OF THE ENZYME

Regine Kahl

Department of Pharmacology, University of Mainz
Obere Zahlbacher Strasse 67, D-6500 Mainz, Germany

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SUMMARY. Feeding of the antioxidant ethoxyquin to rats leads to an increase of epoxide hydratase activity in liver microsomes. The apparent half life of the increase is 3-4 days. Elevation of epoxide hydratase activity is also obtained by intraperitoneal treatment of mice with ethoxyquin. This elevation is prevented by concomitant treatment with cycloheximide. When radiolabelled leucine is incorporated into microsomal protein by liver cell fractions from either ethoxyquin-fed or untreated rats, gel electrophoresis reveals that ethoxyquin feeding increases incorporation into epoxide hydratase. These results suggest that the elevation of epoxide hydratase activity by ethoxyquin is due to increased biosynthesis of the enzyme, i.e. enzyme induction.

Evaluation of microsomal epoxide hydratase activity by feeding the antioxidants ethoxyquin, butylated hydroxytoluene and butylated hydroxyanisole has been demonstrated in rodent liver (1-4) and extrahepatic tissues (5,6). Moreover, other enzyme activities involved in the metabolism of foreign compounds have also been shown to increase by feeding these antioxidants: phenobarbital-inducible monooxygenases (7,8,1,4), cytochrome b_5 (8), glucuronosyl transferases (9) and glutathione S-transferase (10). It has been discussed if the protective effect of these antioxidants against some chemical carcinogens including polycyclic hydrocarbons (11-14) is related to such effects on drug metabolizing enzymes. -

The mechanism by which the antioxidants elevate enzyme activities has not yet been elucidated. We have demonstrated that the increase in epoxide hydratase activity in rat liver by antioxidant feeding is related to the increase of a polypeptide in sodium dodecyl sulfate polyacrylamide gels which comigrates with a partially purified epoxide hydratase. This indicates that the elevation is due to increased concentration of the enzyme in the liver and not merely to activation of preexisting enzyme. However, it is not clear if

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the increase in enzyme concentration is due to a protective effect of the antioxidants against enzyme degradation (i.e. stabilization) or to increased synthesis of the enzyme (i.e. induction). It is the aim of the present communication to provide evidence that the elevation of epoxide hydratase activity in rat and mouse liver after feeding of ethoxyquin is due to enzyme induction.

METHODS

For feeding experiments, male Sprague-Dawley rats with an initial body weight of 120 g received a powdered Altromin® diet with 1 % ethoxyquin (kindly donated by Lohmann Tierernährung, Cuxhaven). The animals were maintained on this diet for 14 days and were sacrificed at various intervals after stop of ethoxyquin feeding. For experiments on inhibition of protein synthesis male NMRI mice (20 g) received 25 mg cycloheximide/kg (in 0.9 % NaCl) i.p. at 8 a.m., 2 p.m., 4 p.m. and 10 p.m.; animals were sacrificed at 8 a.m. Preparation of microsomes (2) and determination of epoxide hydratase activity (15, 16) were performed as described previously. [^{14}C]-styrene oxide and [^{3}H]-benzo[a]-pyrene 4,5-oxide were a generous gift from Professor F. Oesch, Mainz.

Cell free protein synthesis was measured by the incorporation of [^{3}H]-leucine into hepatic microsomal protein from either ethoxyquin-fed or untreated rats. The reaction mixture contained in a total volume of 0.5 ml: potassium phosphate buffer (pH 7.4), 12 mM; freshly prepared microsomal protein, 2 mg; freshly prepared 100 000 x g supernatant from the respective livers, 50 µl (approx. 1 mg); $MgCl_2$, 12 mM; ATP, 1.5 mM; GTP, 0.3 mM; phosphoenol pyruvate, 15 mM; pyruvate kinase, 50 µg; sucrose, 75 mM; 2-mercaptoethanol, 6 mM; [^{3}H]-leucine, 16 µM (0.2 mCi). This incubation was similar to that described by Weksler and Gelboin (17) who used the creatine phosphokinase system instead of the pyruvate kinase system. The incubation was carried out for 60 min at 37 °C and stopped by the addition of 1.0 ml 10 % trichloro acetic acid. The protein precipitate was centrifuged down and washed with acetone/0.1 M acetic acid, ethanol and ethyl ether. After drying it was dissolved in 1.0 ml of gel electrophoresis sample buffer (consisting of 0.5 M Tris, pH 6.8, 10 % glycerol, 1 % sodium dodecyl sulfate and 1 % 2-mercaptoethanol); a few drops of 1 N NaOH were added to facilitate dissolving. The solution was heated to 100 °C for a few minutes. 0.05 % bromphenol blue were added before electrophoresis.

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to Laemmli (18); a partially purified rat liver epoxide hydratase (kindly donated by Professor F. Oesch, Mainz) was included as a standard. For densitometric measurement of epoxide hydratase subunit concentration the gels were stained with Coomassie Blue and scanned in a Gilford 2400 spectrophotometer. For measurement of [^{3}H]-leucine incorporation four walls each of a 10 wall slab gel contained the protein from the ethoxyquin experiment and four walls that of the control experiments while two walls contained the standard epoxide hydratase. After electrophoresis, the standard walls and one wall each from the ethoxyquin and control walls were stained for protein while the remaining three walls of each preparation were cut into 0.9 mm slices. The slices were resolved in 30 % H_2O_2 by incubation at 60 °C for 36 hr and radioactivity was quantitated by liquid scintillation counting.

RESULTS

The time dependence of the elevation of epoxide hydratase activity towards the substrates styrene oxide and benzo[a]pyrene 4,5-oxide and increase of the

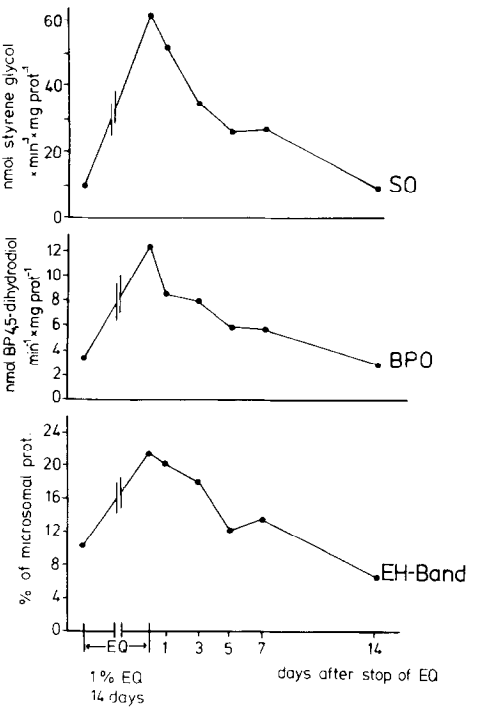


Fig. 1 Decline of the elevation of epoxide hydratase activity in rat liver microsomes and of the epoxide hydratase-containing band in polyacrylamide gels of rat liver microsomes after stop of ethoxyquin feeding. Means of two experiments are plotted. Upper part: enzyme activity towards styrene oxide (SO). Middle part: Enzyme activity towards benzo[a]pyrene 4,5-oxide (BPO). Lower part: Epoxide hydratase (EH)-containing band in sodium dodecyl sulfate polyacrylamide gels given in percentage of total microsomal protein found in the molecular weight region between 200 000 and 15 000. The molecular weight of the standard epoxide hydratase was 49 000.

epoxide hydratase-containing protein band in polyacrylamide gels is demonstrated for rat liver microsomes in Fig. 1 The elevated activity decreases by 50 % within 3-4 days after stop of ethoxyquin. This decrease may reflect the half life of the enzyme. Increased levels are still observed after 7 days; basic values are obtained at 14 days after stop of ethoxyquin feeding.

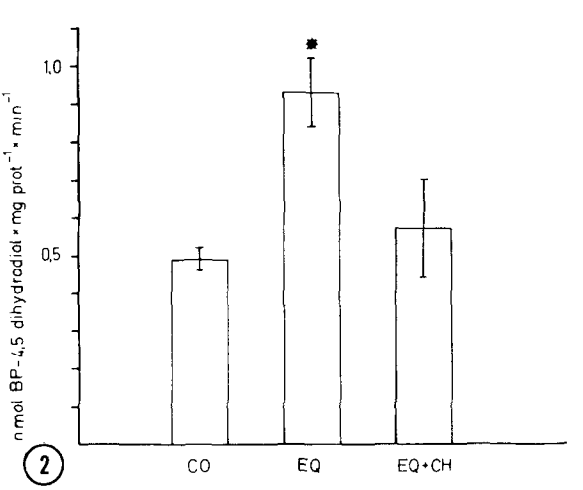
Two types of experiments were performed to test if the elevation of enzyme activity is due to enzyme induction, i.e. increased biosynthesis of epoxide hydratase due to the antioxidant: 1) ethoxyquin treatment during inhibition of protein synthesis, 2) *in vitro* incorporation of radiolabelled leucine by liver cell fractions from ethoxyquin-treated animals.

Feeding of ethoxyquin leads to a very marked but slowly developing increase of epoxide hydratase activity. However, cycloheximide-treated animals will not survive an experimental period much longer than 24 hr. Elevation of enzyme activity within 24 hr was achieved by three i.p. doses of the antioxidant in the mouse but was much less marked than that obtained by feeding. In the rat, no significant ethoxyquin effect was observed after this protocol. Fig. 2 shows that there was no longer an increase in epoxide hydratase activity in mouse liver when the animals were subjected to a combined treatment with ethoxyquin plus cycloheximide. This finding indicates that intact protein synthesis is necessary for the elevation effect and thus provides evidence for a true enzyme induction mechanism.

This conclusion was stressed by the *in vitro* experiment of Fig. 3. For this experiment, microsomes and cell sap were prepared from the livers of either ethoxyquin-fed or untreated rats and were used for a cell free protein synthesis assay. The radioactivity profile after gel electrophoresis of the proteins shows identical label incorporation into most of the separated proteins. However, increased leucine incorporation is observed into two bands in the molecular weight region around 50 000, when cell fractions from ethoxyquin-treated liver were used for the assay. By comparison to the migration distance of the authentic epoxide hydratase one of these bands can be identified as the one containing the enzyme subunit. This finding indicates that ethoxyquin feeding has enabled liver tissue to synthetize epoxide hydratase more rapidly and provides further evidence for true enzyme induction. The other band into which more label is incorporated after ethoxyquin treatment has a molecular weight of about 52 000 and may thus contain the phenobarbital-inducible form of cytochrome P-450.

DISCUSSION

Evidence that ethoxyquin leads to induction of epoxide hydratase in rodent liver is derived from the inhibition of the elevating effect of the antioxidant by an inhibitor of protein synthesis on one hand (Fig. 2) and from in-



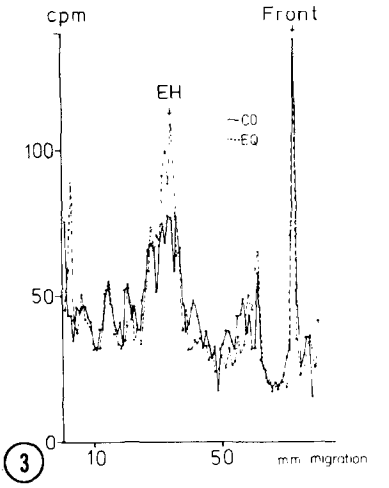


Fig. 2 Prevention of the elevation of epoxide hydratase activity in mouse liver after intraperitoneal administration of ethoxyquin by inhibition of protein synthesis. Values are means ± S.E. (n = 3), * $P \leq 0.05$. The substrate was benzo[a]pyrene 4,5-oxide. BP: benzo[a]pyrene, CO: control, EQ: ethoxyquin, CH: cycloheximide.

Fig. 3 SDS polyacrylamide gel electrophoresis of labelled proteins after incorporation of ^{3}H-leucine *in vitro* hepatic microsomal protein from ethoxyquin-fed and untreated rats. Gels were sliced and processed for measurement of radioactivity. Identification of epoxide hydratase (EH) was achieved by comparison with the migration of an authentic epoxide hydratase present in another wall of the same slab gel. CO: control incubation with microsomes and cytosol of the livers of untreated rats. EQ: incubation with microsomes and cytosol from the livers of ethoxyquin-fed rats.

creased incorporation of leucine *in vitro* into epoxide hydratase on the other hand (Fig. 3). Prevention of elevation by inhibition of protein synthesis has also been demonstrated for another inducer of hepatic microsomal epoxide hydratase, *trans*-stilbene oxide (19), and it is likely that this inducer also acts by enzyme induction.

To measure leucine incorporation we used an *in vitro* method rather than the *in vivo* method employed by Dehlinger and Schimke for the study of cytochrome P-450 induction (20) to avoid reutilization of labelled leucine and interference of effects on turnover. Stabilization should also lead to the accumulation of newly synthetized enzyme but not within the short *in vitro* experiment since the half life of the induced activity was found to be 3-4 days (Fig. 1). Schmassmann and Oesch (19) have tested the epoxide hydratase inducer

trans-stilbene oxide for the duration of its effect and found to be similar (12 days) to that reported here for ethoxyquin, with a similar half life of effect of about 4 days.

It should be an advantage to include epoxide hydratase antibody into the leucine incorporation assay in order to prepurify the labelled enzyme before gel electrophoresis. This has been done in experiments on cell free synthesis of cytochromes P-450 (21,22), and heterologous translation systems have been employed in these experiments. However, in our assay the identification of epoxide hydratase was solely dependent on its molecular weight. Had the enzyme been synthetized as a precursor protein with differing molecular weight, its identification would have been impossible. We therefore decided to provide the messenger with its own microsomal protein processing machinery and with its own cell sap to ensure the synthesis of a final protein identical with that formed *in vivo*. Kumar and Padmanaban (21) have demonstrated that cytochrome P-448 is synthetized in a precursor form with a molecular weight of 59 000 in conventional wheat germ system and that the active form of the enzyme with a molecular weight of 53 000 is only synthetized *in vitro* if microsomal membranes are added to the assay.

Recently, the purification of three different forms of epoxide hydratase from rat liver has been reported (23). These forms differ by immunological properties, by amino acid composition and by substrate specificity but not by molecular weight. It should be interesting to test by differential purification and by immunological methods which of these postulated epoxide hydratase forms is induced by ethoxyquin.

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