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A NOVEL HAEMOPROTEIN INDUCED BY ISOSAFROLE PRETREATMENT IN THE RAT

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SUMMARY

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis has been used to demonstrate that pretreatment of rats with isosafrole results in the formation of a novel species of cytochrome P-450 (mol. wt. 54,000) quite distinct from that induced by phenobarbitone pretreatment (mol. wt. 50,000) or 3-methylcholanthrene (mol. wt. 58,000).

INTRODUCTION

Many xenobiotics have been found to increase the activity of the hepatic microsomal cytochrome P-450 mediated monooxygenase system (1,2). This increase in monooxygenation has been demonstrated to be a true induction *via* a *de novo* synthesis of cytochrome P-450. It is increasingly apparent that hepatic microsomes contain several forms of cytochrome P-450 which

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differ in their catalytic, spectral and electrophoretic properties (3-6). Inducing agents appear to preferentially increase the amount of one or more species of these cytochrome(s) P-450. Two major classes of inducing agents were early recognized. These are the barbiturate and the polycyclic aromatic hydrocarbon classes which induce the synthesis of cytochrome P-450 and P_1-450 (P-448) respectively (7).

More recently other types of induction have been suggested. For example, it has been shown that polychlorinated biphenyl-induced microsomes have spectral and catalytic properties in common with both P-450 and P_1-450 (8,9). Electrophoretic studies by these workers indicated that polychlorinated biphenyls induce both P-450 and P_1-450 (10). This type of induction has frequently been termed 'mixed', though it is probable that any particular isomer in the polychlorinated biphenyl mixture will only induce one form of P-450.

Isosafrole (4-propenyl-1,2-methylenedioxybenzene) is both an inhibitor and inducer of cytochrome P-450 mediated monooxygenation. As an inducing agent it shares properties with both the barbiturate class and the polycyclic aromatic hydrocarbon class of inducing agents (11-13). The present study attempts to elucidate the type of induction due to isosafrole.

METHODS

Animals and Pretreatment

Male Wistar albino rats (180-200 g) were obtained from the Zentralinstitut für Vesuchstierzucht (Hannover, West Germany) and maintained in clear, solid bottomed polypropylene cages with a steam-treated soft wood bedding (Altromin, G.m.b.H. Lage, West Germany). The ambient temperature was 22-24° and a 12 hr light cycle (0800-2000) was used. Food (Altromin ^{R}, 1314 pelleted diet) and water were allowed *ad libitum*.

Isosafrole (150 mg/kg) and 3-methylcholanthrene (20 mg/kg) were administered by i.p. injection dissolved in corn oil (2.5 ml/kg) and phenobarbitone (80 mg/kg) was injected as a solution in water (5.0 ml/kg). Control animals received corn oil or water alone. Animals were treated as described at 0800 hr on three consecutive days and killed 24 hr later.

Microsome Preparations

Animals were killed by cervical dislocation and the livers rapidly

excised into 1.15% (w/v) KCl, scissor chopped and washed three times with 20 mM Tris-HCl (pH 7.4) containing 1.15% (w/v) KCl using a Potter-Elvehjem homogenizer equipped with a Teflon pestle. Microsomes were prepared from this homogenate as described previously (14). The microsomal pellets were washed with 1.15% (w/v) KCl and resedimented. The final washed microsomes were resuspended in 20 mM Tris-HCl (pH 7.4), containing 250 mM sucrose and 5.4 mM EDTA, to a final concentration of about 1-2 g of liver wet wt/ml. Microsomes were stored at -20° for a maximum of 7 days. The variously induced microsomes were prepared from the pooled livers of 6 rats.

Microsomal protein was determined by the method of Lowry *et al.* (15) using bovine serum albumin standards.

Spectrophotometry

The carbon monoxide and ethylisocyanide spectra of $Na_2S_2O_4$-reduced microsomes (1 mg protein/ml) were obtained by previously described methods (16,17). An extinction coefficient of 91 $mM^{-1}cm^{-1}$ was used for the carboxyferrocytochrome P-450. The generation of the isosafrole metabolite-cytochrome P-450 complex using phenobarbitone-induced microsomes (1 mg microsomal protein/ml), isosafrole (400 μM) and either NADPH (400 μM) or cumene hydroperoxide (200 μM) was carried out as previously described (18). Displacement of the metabolite from isosafrole-induced microsomes (1 mg protein/ml) (19) was elicited by 2-*n*-heptylbenzimidazole (250 μM). A Perkin-Elmer 356 spectrophotometer or an Aminco DW-2 spectrophotometer was used for all measurements.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

A 3 mm slab gel apparatus (Desaga G.m.b.H., Heidelberg, West Germany) was used as described by Dent *et al.* (20). Whole microsomes (3 mg/ml) were solubilized in 62.5 mM Tris-HCl (pH 6.8), containing 15% (v/v) glycerol, 5% (v/v) mercaptoethanol, 2.3% (w/v) sodium dodecyl sulphate and 0.001% (w/v) bromophenol blue, prior to application to the gel. When gels were to be stained for peroxidase activity (21) mercaptoethanol was excluded from the samples.

RESULTS AND DISCUSSION

As illustrated in Table 1, the cytochrome P-450 content of hepatic microsomes was approximately doubled by treatment of rats with phenobarbitone (PB) and 3-methylcholanthrene (3MC). Isosafrole pretreatment appeared to increase the cytochrome P-450 content by only 40%. However, it has been reported (22,23) that treatment of animals or NADPH-supplemented microsomes with methylenedioxyphenyl compounds results in an under-estimation of the cytochrome P-450 content due to inhibition of carbon monoxide binding by a methylenedioxyphenyl metabolite. The isosafrole metabolite-cytochrome P-450 complex, responsible for this methodological artefact, can be dissociated by certain Type I or RI substrates (19,24) allowing measurement of the

TABLE 1

Spectral properties of various microsomal preparations

Pretreatment	Cytochrome P-450 (nmol/mg)	max (nm)	Ethylisocyanide spectra 430/455
Water	0.69	450.0	5.7
Phenobarbitone	1.40	450.8	3.3
Corn Oil	0.59	450.0	5.8
3-Methylcholanthrene	1.26	448.5	1.4
Isosafrole	0.84 (1.66)	449.5 (448.9)	3.9 (2.9)

Values in parenthesis indicate those obtained after dissociation of the isosafrole metabolite-cytochrome P-450 complex using 250 μM 2-heptylbenzimidazole (26).

total cytochrome P-450 content. On dissociation of the complex with 250 μM 2-heptylbenzimidazole (25,26) and subsequent measurement of the cytochrome P-450 content an increase of 181% was found after isosafrole treatment. 2-Heptylbenzimidazole was without effect on the estimates of cytochrome P-450 in control- or in PB- or 3MC-induced microsomes. As expected PB caused a slight red shift in the wavelength of the absorption maximum of the carbon monoxide spectrum of the reduced microsomes, while 3MC treatment resulted in a blue shift. Pretreatment of animals with isosafrole also resulted in a blue shift, which was more pronounced after dissociation of the isosafrole metabolite-complex.

The ethylisocyanide spectrum of $Na_2S_2O_4$-reduced microsomes has been used to classify cytochrome P-450 types. In our studies, isosafrole-induced microsomes appeared to be PB or control-like from their spectra before dissociation of the complex while afterwards the spectral properties resembled those expected of a mixed PB/3MC type of induction. These spectral studies suggested that isosafrole induction may indeed by of the mixed type.

However, on examination of the electrophoretic pattern of the solubilized microsomes it was apparent that this was not the situation. In the 50,000 - 60,000 mol. wt. region one faint and 4 distinct bands were observed in control- (oil or water) microsomes after staining for protein with Coomassie blue (Fig. 1). These bands are numbered 1-5 on the basis of increasing mol. wt. Band 1 (49,000 mol. wt.) was found to run with homogeneous purified epoxide hydratase and did not stain with the peroxidase staining method. This band has been used as a reference point. Band 2 (50,000 mol. wt.) was the densest band observed in control microsomes after staining for protein. Band 3 (54,000) was barely visible while bands 4 and 5 (58,000 and 60,000 mol. wt. respectively) were just observable. In PB-microsomes band 2 (50,000 mol. wt.) was dramatically increased while bands 4 and 5 were unchanged (Table 2). Band 3 was slightly increased in the intensity of staining when compared with control-microsomes. 3MC showed a different pattern of induction. It did not increase band 2; however, a large increase in band 4 (58,000 mol. wt.) was observed. This band is characteristic for the induction of cytochrome P_1-450 by polycyclic aromatic hydrocarbons (10). Band 3 was also slightly increased in intensity when compared with that for control-microsomes. Conversely, when compared with control-microsomes, isosafrole-microsomes only showed a large increase in the intensity of band 3, (54,000 mol. wt.). No intensification was seen in band 4 but band 2 was slightly increased.

3MC and PB preferentially induced cytochrome P-450 apoproteins of 58,000 and 50,000 mol. wt. respectively, while isosafrole induced a haemoprotein with a mol. wt. of 54,000. This haemoprotein was barely observable in control-microsomes. It should be emphasized that the band at 58,000 mol. wt., characteristic of induction of cytochrome P_1-450 by 3MC, was not observed in any of the isosafrole-microsome preparations studied. Although 3MC and PB slightly increased the band at 54,000 mol. wt. as well as their own major bands (58,000 and 50,000 respectively), isosafrole specifically increased the intensity of the band at 54,000 mol. wt.

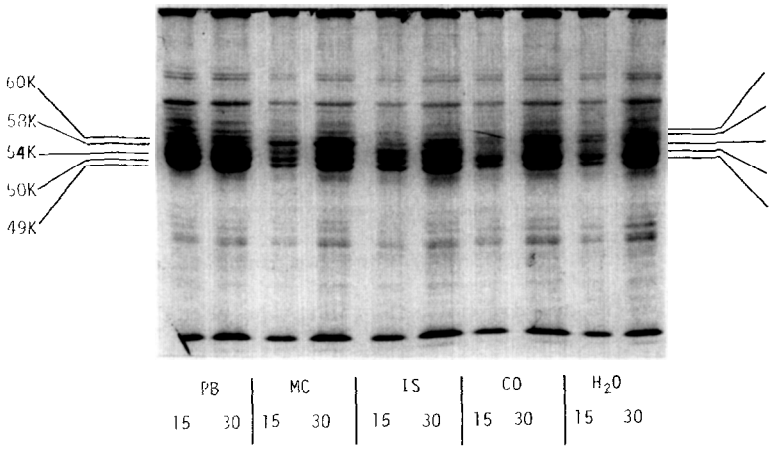

Figure 1. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis of rat hepatic microsomes. PB, MC, IS, CO and H_2O refer to phenobarbitone 3-methylcholanthrene, isosafrole, corn oil control and water control respectively. The figures beneath refer to the amount of microsomal protein (µg) applied to the gel. The gel was stained with Coomassie blue as described in Methods. The arrows at the left denote the positions of 60K, 58K, 54K, 50K and 49K MW proteins; origin is at the top of the gel.

TABLE 2

Changes in the concentration of the 50K, 54K and 58K molecular weight bands following pretreatment with phenobarbitone, 3-methylcholanthrene or isosafrole

Pretreatment	50K	54K	58K
Phenobarbitone	++	±	-
3-Methylcholanthrene	-	+	++
Isosafrole	±	++	-

Signs represent changes in extinction (compared with controls) of the Coomassie Blue-stained gels measured using a Gilford spectrophotometer 2400 at 560 nm (slit width = 2 mm, scanning speed = 2 cm/min).

\+ indicates an increase of between 20-50%

++ indicates an increase of > 50%.

These findings demonstrate that the formation of a novel species of cytochrome P-450 is associated with treatment of rats with isosafrole. To confirm that this 54,000 mol. wt. species was not the result of the tight binding of isosafrole metabolite to 'normal' cytochrome P-450 the gel electrophoresis properties of two further controls were also investigated. Firstly, isosafrole metabolite was completely displaced from microsomes of isosafrole pretreated microsomes prior to running the gel. No change in the electrophoretic pattern compared with non-displaced microsomes was observed i.e. the 54,000 mol. wt. band remained the dominant species. Secondly, phenobarbitone microsomes were incubated with isosafrole in the presence of either cumene hydroperoxide or NADPH under conditions in which maximal *in vitro* generation of isosafrole metabolite complexed to 'normal' P-450 occurs. When these microsomes were run on the gel the major band remained at 50,000 mol. wt. characteristic of phenobarbitone microsomes.

Thus it may be concluded that in rats isosafrole is a novel type of inducing agent causing the formation of a species of cytochrome P-450 quite distinct from those arising from phenobarbitone or 3-methylcholanthrene pretreatment. Preliminary results suggest that the isosafrole induction pattern is also quite distinct from that provoked by pregnenolone-16α carbonitrile (27) or ethanol (4,28).

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