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ETHANOL AND ISONIAZID INDUCE A HEPATIC MICROSOMAL CYTOCHROME P-450-DEPENDENT ACTIVITY WITH SIMILAR PROPERTIES TOWARDS SUBSTRATE AND INHIBITORS AND DIFFERENT PROPERTIES FROM THOSE INDUCED BY CLASSICAL INDUCERS*

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Abstract—Male Wistar rats were pretreated with chronic ethanol ingestion (3 weeks), phenobarbital in the drinking water (1 week), beta naphtoflavone or *trans*-stilbene oxide (one daily i.p. injection for 3 days) or isoniazid (one daily i.p. injection for 7 days). All these treatments increased 4-nitroanisole *O*-demethylase activity above the control level. Kinetic studies indicated that ethanol and isoniazid induction gave low apparent $K_{0.5}$ (<0.1 mM), *trans*-stilbene oxide and phenobarbital intermediate (0.1 mM < $K_{0.5}$ < 0.5 mM) and beta naphtoflavone high $K_{0.5}$ (>0.5 mM) for the induced activities. Inhibition studies with 0.1 mM metyrapone, 0.1 mM imidazole or 5 mM 1,3-dioxolane provided further evidence that the induced activities had different properties. Differences were also observed in the subtypes of spectral type II interaction (II_a and II_b) with 0.1 mM imidazole or 0.1 mM metyrapone in the induced microsomes. The results indicate that demethylation of 4-nitroanisole is an effective reaction for distinguishing between three different types of cytochrome P-450 induction. The clinically important hepatotoxins ethanol and isoniazid appeared to induce the same type of cytochrome P-450 activity, and it is speculated that these toxins may predispose to hepatotoxicity by means of this common property.

Hepatic microsomal *O*-demethylation of 4-nitroanisole has been found to increase after pretreatment of rats with phenobarbital and benzo(*a*)pyrene [2] and after DDT and ethanol [3]. Some of these results were recently confirmed in a report from our laboratory [4]. The present work was done to investigate whether the assay could be developed further to yield specific information about induction with three well-defined inducing agents: ethanol, phenobarbital and beta naphtoflavone. *Trans*-stilbene oxide has already been documented as a phenobarbital-type of inducing agent [5, 6], and this inducing agent was included to test whether it could induce a phenobarbital-like 4-nitroanisole *O*-demethylase activity. Isoniazid has been found to be an inducing agent different from phenobarbital and beta naphtoflavone [7], and the induced activity seemed similar to that found after chronic ethanol feeding [8]. Experimental verification of the similarities between ethanol and isoniazid and differences from other inducing treatments could open new experimental approaches for studying the hepatotoxicity of ethanol.

MATERIALS AND METHODS

Chemicals. NADPH was purchased from Sigma (St. Louis, MO). Beta naphtoflavone, metyrapone and *trans*-stilbene oxide were purchased from Aldrich-Europe (Beerse, Belgium). Phenobarbital and isoniazid were purchased from Norsk Medisinaldepot, Norway, and 1,3-dioxolane was purchased from Fluka, (Buchs, Switzerland). Practical grade 4-nitroanisole was purchased from EGA-Chemie (Steinheim, F.R.G.) and purified by three times recrystallization from ethanol–water. The use of organic solvents to bring the nitroanisole into solution was undesirable in the present investigation; hence, working solutions containing 1 mM nominal concentration were prepared by dissolution with continuous stirring and gentle heating in 160 mM Tris–HCl pH 7.7 (25°), 40 mM KCl, 10 mM $MgCl_2$. The solutions were stored at −20° until they were used.

Animals. Male Wistar rats were purchased from Møllegaard (Denmark) and were housed and fed as described previously [9].

Pretreatments. Beta naphtoflavone and *trans*-stilbene oxide, 80 mg/kg [7] and 2 mmoles/kg [6], respectively, were given i.p. as suspensions in sunflower oil once daily for 3 days. The rats were killed on the fourth day. Sodium phenobarbital was given in the drinking water at a concentration of 1 g/l for 5–7 days [10], and the rats were killed without withdrawal of phenobarbital. Isoniazid, 50 mg/kg, was given i.p. as fresh solutions in isotonic saline once daily for 8 days [7], and the rats were killed 24 hr after the last injection. Ethanol was given in a liquid diet [11] for 3 weeks, and the rats were killed without withdrawal of the diet. Control rats were treated with vehicle only, except for the controls for the ethanol group which were treated as

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described previously with lipid as substitute for ethanol [4]. All the rats receiving solid diet were given food until they were killed. The rats were killed by decapitation.

Preparation of microsomes. Postmitochondrial supernatants were prepared as described previously [9], and the microsomes were isolated by ultracentrifugation and washed once by resuspension and recentrifugation. The final resuspension was performed in 20 mM Tris–HCl (pH 7.7 at 25°) containing 20% (v/v) glycerol to a protein concentration of 20–30 mg/ml and stored in small aliquots at −70° until they were used.

Activity studies. Microsomal demethylation of 4-nitroanisole was performed by spectrophotometric monitoring of 4-nitrophenol at 420 nm [2, 4] with a protein concentration of 0.3 mg/ml [12, 13] in 146 mM Tris–HCl (pH 7.4), 36 mM KCl, 9 mM $MgCl_2$ and 2% (v/v) glycerol at eight concentrations of substrate (5, 10, 20, 50, 100, 200, 500 and 900 μM). Incubation volume was 1 ml; incubation temperature was 37°. The reactions were started with 0.2 mM NADPH.

Inhibitor studies. Only water-soluble substances were tested to avoid interference by organic solvents. Two nitrogen-containing inhibitors capable of type II spectral binding to cytochrome P-450 were used: metyrapone, which is a well-documented inhibitor of phenobarbital-induced cytochrome P-450 activities [14], and imidazole, which has been demonstrated by different means to interact with cytochrome P-450 *in vitro* [15, 16]. Both inhibitors were used in 0.1 mM final concentrations: 1 μl of 100 mM solution was added to 1 ml final cuvette volume. 1,3-dioxolane was selected as the third inhibitor because of structural similarities with imidazole. It was used in 5 mM final concentration: 5 μl of a 1 M solution to the final volume of 1 ml. The inhibitor experiments were carried out at three different substrate concentrations: 50, 200 and 900 μM under the standard assay conditions.

Spectral studies. All difference spectra were recorded at the same concentrations of protein and inhibitors in the same buffer and at the same temperature as for the activity measurements. The spectra were traced in the substitution mode, i.e. one cuvette was used; the absolute spectrum of the contents was recorded and stored in the memory of the spectrophotometer. Then the baseline difference spectrum was recorded (i.e. the sample spectrum against the memorized sample spectrum). Subsequently, metyrapone or imidazole was added, and the resulting difference spectrum was recorded against the spectrum stored in the instrument memory. A Perkin-Elmer 557 double wavelength double beam spectrophotometer was used for all activity measurements and all spectral work.

Data evaluation. Statistical analysis was done by analysis of variance [17]. Kinetic analysis was performed graphically [18] in Eadie–Hofstee plots or by means of a calculator program [19] on a Texas Instruments TI 59 programmable calculator.

RESULTS

Kinetics of 4-*nitroanisole* O-*demethylation*

Activities of 4-nitroanisole *O*-demethylase as a function of substrate concentration are shown in Fig. 1. The activity of control microsomes increased with increasing substrate concentrations. The activity was linear with time (not shown), and the activity did not usually exceed 1 nmole/min per mg protein. It was not possible to find one or a few $K_{0.5}$ values in these microsomes because the Eadie–Hofstee plot was hyperbolic (not shown). The activity in ethanol

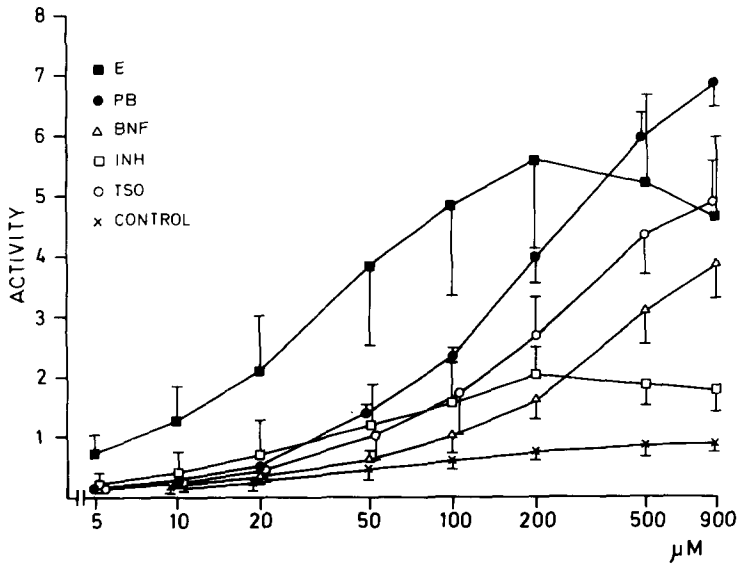

Fig. 1. NADPH-driven microsomal demethylation of 4-nitroanisole as a function of substrate concentration. Mean activity values are given as nmoles/min per mg protein. Standard deviations are indicated. Protein concentration was 0.3 mg/ml. Abbreviations: E, ethanol (N = 7); PB, phenobarbital (N = 6); BNF, beta naphtoflavone (N = 6); INH, isoniazid (N = 5); TSO, *trans*-stilbene oxide (N = 6); CONTROL, control (N = 5). Assay conditions are given in Materials and Methods.

microsomes was higher than the control activity at all tested substrate concentrations and reached a maximum of 5.5 nmoles/min per mg protein at substrate concentrations between 100 and 500 μM. This induced activity also demonstrated substrate inhibition at higher substrate concentrations. The $K_{0.5}$ value for the induced activity of these microsomes was less than 100 μM. The enzyme reaction was linear with time (not shown). The activity of phenobarbital microsomes was higher than control activity at 20 μM and above (Fig. 1) with increasing activity in the entire concentration range used in this investigation. Formal kinetic analysis yielded a $K_{0.5}$ value between 200 and 500 μM and a V_{max} about 9 nmoles/min per mg protein. The induced activity was nonlinear with time at high, but not low, substrate concentrations. Related phenomena have been observed by Imai [20], and has been attributed to inactivation of the enzyme by the generated formaldehyde. The activity in beta naphtoflavone microsomes was higher than the control value only at substrate concentrations above 100 μM (Fig. 1). The activity was linear with time, and the $K_{0.5}$ value was about 650 μM, i.e. near the upper limit of the substrate concentrations used. V_{max} was found to be about 6 nmoles/min per mg protein. The activity in *trans*-stilbene oxide microsomes was also considerably elevated compared to the control value (Fig. 1) with a V_{max} value about 7 nmoles/min per (mg protein). The $K_{0.5}$ value was not significantly different from that of the phenobarbital microsomes.

At high substrate concentrations, the same nonlinearity with time was observed as in phenobarbital-microsomes. The activity in isoniazid microsomes was also elevated compared to the control microsomes (Fig. 1); $K_{0.5}$ was less than 100 μM, and the maximal activity was found around 200 μM. There was also some substrate inhibition at high substrate concentrations. The $K_{0.5}$ was not significantly different from that of the ethanol microsomes, but the V_{max} value was considerably less. The activity was linear with time. So far, the induced activities could be classified into three groups on the basis of interaction with the substrate: the phenobarbital-like group, the ethanol-like group and beta naphtoflavone. Some difficulties were observed: on several occasions, the activities in the induced microsomes could be resolved into two components. One was the "induced" portion that was described above and which was classifiable into three categories. In addition, there was an apparent high-affinity component ($K_{0.5}$ in the range 2–20 μM and an apparent V_{max} below 1 nmole/min per (mg protein) which was corrected for when it was observed in Eadie–Hofstee plots of activities in induced material. It is a possibility that this component represented noninduced portions of the microsomal hemoproteins. This is compatible with the results from control microsomes.

Inhibition of the induced activities

The inhibition with 0.1 mM metyrapone in the induced microsomes is shown in Fig. 2. Ethanol-induced activity was not affected significantly by this agent; beta naphtoflavone-induced activity was

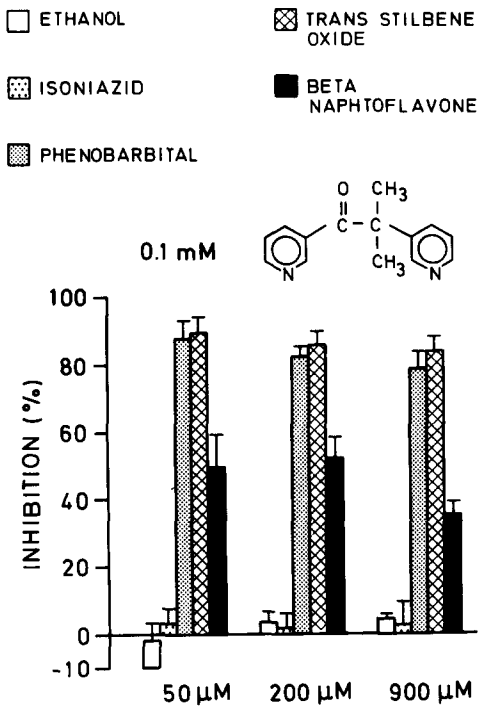

Fig. 2. Metyrapone inhibition of induced 4-nitroanisole demethylation. Three substrate concentrations were used, as indicated. Otherwise, the assay conditions were the same as in Fig. 1. Metyrapone concentration was 0.1 mM. Values are given as mean of three independent experiments. Standard deviations are indicated.

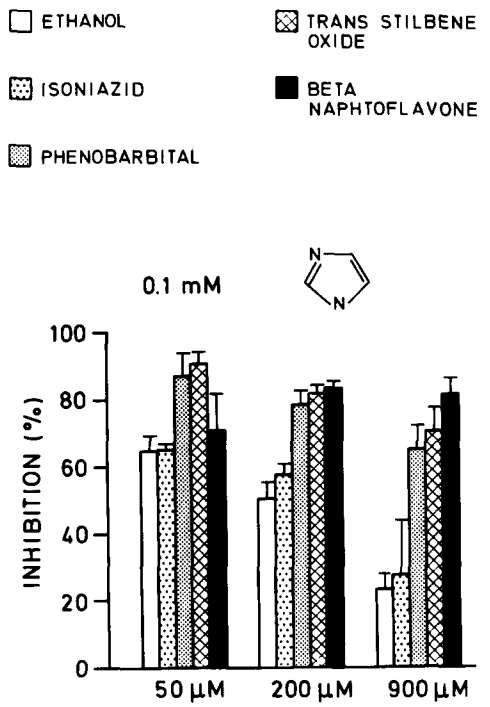

Fig. 3. Imidazole inhibition of induced 4-nitroanisole demethylation. Details are as in Fig. 2. Imidazole concentration was 0.1 mM.

inhibited approximately 50%, and the phenobarbital-induced activity was inhibited between 80 and 90%. These inhibition characteristics were the same at the three tested substrate concentrations. The corresponding results for *trans*-stilbene oxide- and isoniazid-induced microsomes are also shown in Fig. 2. Isoniazid-induced activity was insensitive to metyrapone inhibition, and the *trans*-stilbene oxide-induced microsomes were as sensitive to metyrapone as the phenobarbital microsomes.

The inhibition with 0.1 mM imidazole (Fig. 3) was not so simple. At the lowest substrate concentration (50 μM) there were no striking differences between ethanol-, phenobarbital- and beta naphtoflavone-induced microsomes although phenobarbital-induced microsomes were most sensitive. At the intermediate substrate concentration (200 μM), ethanol-induced activity was significantly less inhibited than phenobarbital- and beta naphtoflavone-induced activities. At the high substrate concentration (900 μM), these three activities were different, with highest inhibition in beta naphtoflavone-induced microsomes, intermediate inhibition in phenobarbital-induced microsomes, and lowest inhibition in ethanol-induced microsomes. As also shown in Fig. 3, isoniazid induction resulted in a pattern resembling ethanol induction, and *trans*-stilbene oxide induction resulted in a phenobarbital-like pattern. In summary, imidazole at 0.1 mM was a real inhibitor for ethanol- and isoniazid-induced activities whereas the same concentration of metyrapone was inactive. Metyrapone was a more effective inhibitor of phenobarbital- and *trans*-stilbene oxide-induced activities than imidazole. The beta naphtoflavone-induced activity was more inhibited by imidazole than by metyrapone.

When 1,3-dioxolane was used as diagnostic inhibitor (Fig. 4), the ethanol-induced activity was most sensitive. At low and intermediate substrate concentrations (50 and 200 μM), the differences between ethanol-, phenobarbital- and beta naphtoflavone-induced microsomes were most marked, with the phenobarbital-induced activity as the least affected one. At the high substrate concentration (900 μM), there was considerable overlap between phenobarbital-induced and beta naphtoflavone-induced activities (Fig. 4). The inhibitory actions on isoniazid-induced and *trans*-stilbene oxide-induced activities were similar to ethanol-induced and phenobarbital-induced activities, respectively (Fig. 4).

The diagnostic inhibitors thus divided the five different types of microsomes into the same three categories as the substrate interaction experiments.

Spectral studies

There was no detectable spectral change in the Soret region of the microsome spectra with 1,3-dioxolane at 5 mM concentration. Metyrapone and imidazole gave type II [21] spectral changes with all microsomes. The absolute values of the spectral changes were different in different microsomal preparations, but the wavelength maxima and minima were reproducible within the same treatment group. In ethanol microsomes, both metyrapone and imidazole gave a type II_a spectral change [21], but imidazole gave an almost threefold larger absorption

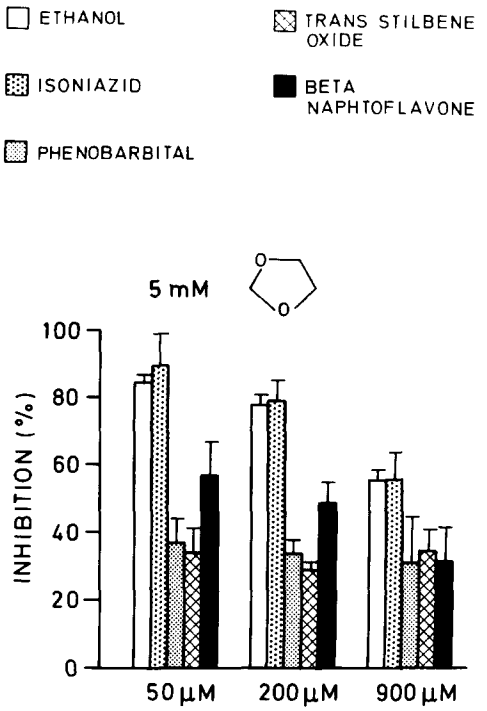

Fig. 4. 1,3-dioxolane inhibition of induced 4-nitroanisole demethylation. Details are as in Fig. 2. 1,3-dioxolane concentration was 5 mM.

decrement at 390 nm. Isoniazid microsomes showed the same type of interactions. Phenobarbital microsomes and *trans*-stilbene oxide microsomes were similar also in this test system, and different from ethanol and isoniazid microsomes, and the beta naphtoflavone microsomes were different from all the others. In ethanol and isoniazid microsomes, the P-450 interacting with metyrapone and imidazole appeared to be mainly high-spin. In the others, there was evidence that the interacting hemoproteins were more of a low-spin nature.

DISCUSSION

The results show clearly that 4-nitroanisole *O*-demethylase activity may be a useful tool for detecting inductions of P-450 hemoproteins. By the use of different concentrations of the substrate, both increased activity and three different classes of activities could be detected after five different pretreatments. Supplementation with inhibitor experiments confirmed the interpretations of the kinetic experiments. In all cases it seemed of value to use relevant positive controls. The value of formal kinetic analysis in microsomal preparations seems questionable [22]. Microsomes contain numerous hemoproteins with different characteristics [23] in a system of lipid/protein vesicles. The difficulties encountered in the formal kinetic analysis of control and induced microsomes serve to illustrate this complexity of the microsomal P-450 dependent activities. For these reasons, simple velocity vs concentration plots appear to be sufficient both for detection of

induction and for determination of kinetic properties with reasonable accuracy, but full quantification of induction is not possible with this simple experimental approach.

The differences in inhibition between imidazole and metyrapone illustrate that general statements about the modifying action of type II agents on P-450 activities are at best inaccurate. Imidazole is a smaller molecule than metyrapone (molecular weight 68 vs metyrapone 226), and the agents have completely different structures. The interactions of these inhibitors with heme iron of different types of cytochrome P-450 were found to be different. The selectivities of the two agents would then have to reside in differences in the protein moieties surrounding the heme and in functional groups of the inhibitors. The absence of spectral interactions between cytochrome P-450 and 1,3-dioxolane and the indisputable inhibitory effect with this agent indicated that inhibition of cytochrome P-450-activities was not dependent on complex binding of heme iron. It was remarkable that the two types of induced hemoproteins with lowest $K_{0.5}$ values (i.e. ethanol-induced and isoniazid-induced cytochrome P-450) showed predominantly high-spin interaction with imidazole and highest degree of inhibition of enzyme activity with 1,3-dioxolane. The different kinetic and inhibitor properties of the three types of induced activities may possibly be explained by different mechanisms of action of the different types of induced cytochrome P-450. The apparent high-spin nature of the ethanol- and isoniazid-induced cytochrome P-450 may be linked to the differences from the other pretreatments with respect to activities and inhibition patterns. If the similarity between isoniazid and ethanol as inducers of hepatic microsomal cytochrome P-450 can be verified, then the toxicological importance of this type of cytochrome P-450 can be studied in an experimental system that does not contain the same metabolic derangements as that of chronic ethanol feeding.

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