Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P-450 of rabbit liver microsomes by diverse agents: Ethanol, imidazole, trichloroethylene, acetone, pyrazole, and isoniazid

(cytochrome P-450 isozyme 3a/immunoblot analysis/alcohol oxidation)

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ABSTRACT Isozyme 3a of rabbit liver microsomal cytochrome P-450, also termed P-450mLC, was previously isolated in this laboratory from animals administered ethanol or imidazole, and the purified cytochrome was shown to function in the reconstituted system as an oxygenase in catalyzing the oxidation of ethanol and other alcohols. Although liver microsomes from animals treated in various ways exhibit increased alcohol-oxidizing activity, evidence was not available as to whether this was due to enzyme induction or to other factors influencing the activity. Immunochemical quantitation of P-450 isozyme 3a has now been achieved by use of purified antibody to this cytochrome in NaDodSO₄/PAGE/blotting and dot-blotting techniques. The specific content of isozyme 3a in liver microsomes was found to be increased from 2- to 4-fold by administration of the following agents, in increasing order of effectiveness as inducers: isoniazid, trichloroethylene, pyrazole, ethanol, imidazole, and acetone. Isozyme 3a represents about 5% of the total P-450 in control animals and is increased to as high as 27% by acetone treatment. Isozyme 3a-dependent butanol-oxidation activity, determined by the inhibitory effect of antibody on the various microsomal preparations, was found to increase proportionally with increased content of this cytochrome.

Chronic ethanol consumption by rats results in a proliferation of the hepatic smooth endoplasmic reticulum and an increase in NADPH-cytochrome P-450 reductase and spectrally observable cytochrome P-450 (1-3). The induction of P-450 by ethanol is associated with an increase in the metabolism and, in some instances, in the toxicity of many compounds, including N-nitrosodimethylamine (4-6), acetonaphthen (7-9), aniline (10-12), ethanol (13), and carbon tetrachloride (14-16). The treatment of rabbits with ethanol also results in an increase in hepatic microsomal aniline and ethanol hydroxylaton, but the specific content of microsomal P-450 is not always significantly increased (17). Several years ago in this laboratory, a unique form of P-450, designated isozyme 3a on the basis of its relative electrophoretic mobility, was isolated from ethanol-treated rabbits and characterized (17, 18). More recently, we have purified the same cytochrome from cytochromes treated with imidazole (19).

Purified isozyme 3a, also referred to as P-450mLC (20), exhibits the highest activity of six purified rabbit liver isozymes toward ethanol and other alcohols as well as aniline (18), converts acetonaphthen to a reactive intermediate (21), and is the only P-450 isozyme we have studied that is active in the demethylation of N-nitrosodimethylamine at low substrate concentrations (22). Isozyme 3a is responsible for the increase in oxidation of alcohols and aniline in liver microsomes from ethanol-treated rabbits, as indicated by inhibition of the induced activity with antibody prepared to the purified enzyme (23). Furthermore, the microsomal demethylation of N-nitrosodimethylamine at low substrate concentrations is completely inhibited by the antibody (unpublished data). Increased rates of hydroxylaton of aniline or demethylation of N-nitrosomethylamine have been reported to occur in liver microsomes from rats administered pyrazole (24), acetone (25), or isopropanol (25) or made diabetic with alloxan (26). Isoniazid (27) and benzene (28) have a similar effect in rabbits. We have reported that the aniline hydroxylaton activity of rabbit liver microsomes is increased by treatment of the animals with trichloroethylene, pyrazole, benzene, m-xylene, aniline, or imidazole (29).

Although the studies described above on the activities of microsomes and purified enzyme preparations from animals treated in various ways suggest the occurrence of enzyme induction, more rigorous proof is required to rule out other explanations of changes in catalytic activity. In the present paper, we provide evidence from immunochemical quantitation for an increase in the level of isozyme 3a upon treatment of animals with different agents. These include ethanol and imidazole, used earlier in connection with enzyme isolation, pyrazole and trichloroethylene, administered in a previous study of aniline hydroxylaton, and isoniazid and acetone, which have not been described previously as inducers of isozyme 3a.

MATERIALS AND METHODS

Materials. New Zealand White male rabbits (2.0–2.5 kg) were untreated; given 10% (vol/vol) ethanol for 14 days, 1% (vol/vol) acetone for 7 days, or 0.062% (wt/vol) isoniazid for 10 days in place of the drinking water; or given one of the following intraperitoneally and then killed 24 hours after the last treatment: trichloroethylene (11 mmol/kg) by a single injection, pyrazole (200 mg/kg) by a single injection, or imidazole (200 mg/kg) by injection on each of 4 consecutive days. All animals were fasted for 12–14 hr before death. Pyrophosphate-washed liver microsomes were prepared as described (30) and stored at −70°C at a protein concentration of 20–40 mg/ml in 100 mM Tris acetate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (vol/vol) glycerol. Microsomes prepared and stored in this manner exhibited negligible desferrioxamine-sensitive ethanol oxidation activity in reaction mixtures containing NADPH (23). P-450 isozyme 3a was isolated from ethanol- or imidazole-treated rabbits (17, 19), and antibody to P-450 isozyme 3a was raised in sheep and purified from immune serum as described (23). The small amount of cross-reacting activity toward P-450 isozyme 3b was removed by passing the purified IgG over a
P-450 isozyme 3b-Sepharose 4B column according to the general method of Thomas et al. (31). P-450 isozyme 3b-Sepharose was prepared from the reaction of cytochrome b5 reductase-activated Sepharose 4B (32) with purified P-450 isozyme 3b (33).

Enzyme Assays. P-450 isozyme 3a-catalyzed oxidation of 1-butanol to butyraldehyde was measured by gas chromatography of the head-space gas of the reaction mixtures (23). The reaction mixtures contained microsomes (1 nmol of P-450), 10 mg of sheep anti-3a IgG or preimmune IgG, 30 μmol of butanol, 50 μmol of potassium phosphate buffer (pH 7.6), and 1.0 μmol of NADPH in a final volume of 1.0 ml and were incubated at 30°C. Microsomal P-450 was quantified from the reduced-CO difference spectrum after solubilization with 0.3% Tergitol NP-10 (33).

NaDodSO4/PAGE/Immunoblotting. Microsomes were submitted to NaDodSO4/polyacrylamide slab gel electrophoresis as described (33), with 7.5% acrylamide in the separating gel and pyronin Y as the tracking dye. Proteins were transferred electrophoretically with a Trans-Blot system (Bio-Rad) from the gels to nitrocellulose sheets (Bio-Rad) at 10°C for 90 min at 65 V in a buffer mixture (pH 8.3) containing 20% methanol, 0.01% NaDodSO4, 25 mM Tris, and 192 mM glycerol, as described by Towbin et al. (34). Transfer was complete, as monitored by silver staining (35) of the polycrylamide gels.

Dot Blots by Direct Application of Samples to Nitrocellulose Paper. Microsomal suspensions were diluted with 10 mM Tris Cl buffer (pH 7.4) containing 0.9% NaCl, and purified preparations of isozyme 3a were diluted with 100 mM potassium phosphate buffer (pH 7.4). Samples varying in volume from 100 to 400 μl were pipetted into the wells of a Minifold filtration manifold (Schleicher & Schuell) onto wetted nitrocellulose paper as described by Domin et al. (36).

Immunochromatography. Nitrocellulose sheets containing protein samples from electrophoretic transfer or direct application were incubated overnight at 4°C in 10 mM Tris Cl buffer, pH 7.4/0.15 M NaCl (Tris/NaCl) containing 1% (wt/vol) bovine serum albumin. The sheets were washed twice with Tris/NaCl, anti-3a IgG (1 μg/ml) was added, and incubation was carried out for 1 hr at room temperature. The sheets were washed five times with Tris/NaCl containing 0.05% (vol/vol) Tween 20, followed by two washes with Tris/NaCl, and then incubated in a 1:300 dilution of rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA) in Tris/NaCl. After 30 min at room temperature, washing was again carried out as just described. Peroxidase-conjugated sheep anti-rabbit IgG (Cappel Laboratories) at a 1:5000 dilution was incubated with the nitrocellulose for 30 min at room temperature, followed by washing as described above. The location of peroxidase activity was detected with 4-chloro-1-naphthol and H2O2 as described by Nielsen et al. (37). The reaction was quenched at the end of 30 min by placing the nitrocellulose in water. The sheets were dried, and the intensity of the stain was quantified with a Zeiss soft-laser densitometer.

RESULTS AND DISCUSSION

Fig. 1 shows the results obtained when liver microsomes from untreated or ethanol-, acetone-, or trichloroethylene-treated rabbits were submitted to electrophoresis followed by blotting and immunochromatographic detection procedures. A single stained protein band was observed with the microsomal samples from all four sources. The apparent molecular weight of 51,000 estimated for this band is the same as that previously found for purified isozyme 3a (17) and also corresponds to that estimated from staining patterns in similar experiments with microsomes from pyrazole-, imidazole-, and isoniazid-treated rabbits (results not shown).

Fig. 1. Analysis of rabbit liver microsomes for P-450 isozyme 3a. Each lane was loaded with microsomes from an individual rabbit that was treated with ethanol (ETOH, lanes 1 and 2, 4 μg of protein per lane), untreated (lanes 3–10, 8 μg of protein per lane), treated with acetone (lanes 11–15, 4 μg of protein per lane), or treated with trichloroethylene (TCE, lanes 16–20, 4 μg of protein per lane). Microsomal protein from the NaDodSO4/polyacrylamide gel was electrophoretically transferred to nitrocellulose and immunochromatically stained with anti-3a IgG as described in Materials and Methods.

Taking into account that the microsomes from untreated rabbits were run at twice the level as were microsomes from the treated animals, one can determine visually from the figure that the level of antigen was increased by each compound administered. The crossreactivity of isozyme 3b with anti-3a IgG, observed by radioimmunoassay (23), was also detected on blots of electrophoresed material. The crossreactivity was eliminated by back-absorption of the antibody on an isozyme 3b-Sepharose 4B column. This accounts for the absence of any stain corresponding to isozyme 3b in Fig. 1.

Since the antibody was shown to be specific for isozyme 3a, we adopted the simpler dot-blot methodology described by Domin et al. (36) to quantify this cytochrome in various microsomal samples. As reported by these authors, we found that the range of linearity of the assay was very narrow. Therefore, each of our microsomal preparations was examined at five or more different concentrations and the amount of isozyme 3a was calculated from the slope obtained in a plot of the amount of microsomal protein versus staining intensity. A typical set of results for purified isozyme 3a and for microsomes from untreated, ethanol-treated, and imidazole-treated rabbits is shown in Fig. 2. The quantification of isozyme 3a by this method was compared with the results obtained from blots of electrophoretically fractionated microsomal proteins, where at least four concentrations of microsomes and purified isozyme 3a were examined on each gel and the concentration of microsomal isozyme 3a was calculated from the slope obtained in a plot of the amount of protein versus staining intensity. As shown in Table 1, the two methods gave quite similar values for the isozyme 3a content in microsomes from animals treated in a particular manner.

In view of earlier evidence that treatment of rabbits with a variety of foreign compounds results in an increase in the oxidation of alcohols (as well as in aniline hydroxylation), we expected to find a correlation between the level of isozyme 3a and the rate of alcohol oxidation in the different preparations of liver microsomes. The results of a series of experiments designed to address this question are given in Table 2. Hepatic microsomes were isolated from untreated rabbits...
and from rabbits administered pyrazole, isoniazid, ethanol, acetone, imidazole, or trichloroethylene. The pyrophosphate-washed microsomal preparations were examined for their total content of cytochrome P-450, content of P-450 isozyme 3a, and total butanol-oxidation activity, as well as butanol-oxidation activity inhibited by anti-3a antibody and therefore attributable to the action of this particular P-450 isozyme. Butanol was selected as the test substrate because the turnover numbers are higher than with ethanol and because of the much lower zero-time blanks for butyraldehyde as compared to acetaldehyde. Treatment of the animals with each of the agents resulted in an increase in isozyme 3a content (expressed as nmol/mg of protein), whereas an increase in total P-450 content was observed only with pyrazole and imidazole treatment. This isozyme represents about 5% of the total P-450 in the control animals and is increased to as high as about 27% by acetone treatment. Under the conditions used, isoniazid gave the smallest relative increase in the 3a cytochrome (about 2-fold), whereas imidazole and acetone were the most effective inducers, giving about 4.5- and 4.9-fold increases, respectively. The increase in total butanol-oxidation activity in microsomal suspensions underestimates the extent of induction of isozyme 3a. For example, a comparison of the specific activity (expressed as nmol per min per mg of protein) of microsomes from untreated animals with those after isoniazid and ethanol treatment showed increases of 1.2- and 1.7-fold, respectively. However, when the isozyme 3a-dependent rates were compared, increases of about 2.7- and 4.0-fold were calculated for these two agents. This may be explained by the presence of isozymes of P-450 other than 3a, each of which makes a smaller but significant contribution to butanol oxidation (23). Also shown in Table 2 is the ratio of the increase in isozyme 3a activity to the increase in the isozyme 3a content. The values are similar (1.1–1.3) for the different inducers administered, with the exception of acetone, which gave a ratio of 1.7. It should be emphasized that the effects of the various agents on factors other than P-450 which could influence the catalytic activity, such as the level of the NADPH-cytochrome P-450 reductase or the phospholipid composition of the membrane, may be somewhat different for the various inducers tested.

The increases in both the total microsomal rate of butanol oxidation and the isozyme 3a-dependent rate of oxidation were correlated with the increase in the specific content of isozyme 3a (Fig. 3). Linear regression analysis of the results gave correlation coefficients of 0.96 and 0.97 for total butanol oxidation and isozyme 3a-dependent butanol oxidation, respectively; both lines have slopes of about 3.9 nmol of product formed per min per nmol of isozyme 3a. The similarity of the slopes suggests that the increase in butanol oxidation is due primarily to the increase in the specific content of isozyme 3a and not to an increase in other isozymes that are also known to catalyze butanol oxidation, although at much lower rates (23). Purified preparations of isozyme 3a have turnover numbers for butanol oxidation ranging from 9 to 12 min⁻¹. If isozyme 3a were fully active in

### Table 2. Various agents as inducers of liver microsomal P-450 isozyme 3a

<table>
<thead>
<tr>
<th>Agent administered (no. of animals)</th>
<th>P-450 content</th>
<th>Butanol-oxidation activity</th>
<th>Increase in isozyme 3a activity/increase in isozyme 3a content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total, nmol/mg of protein</td>
<td>Isozyme 3a</td>
<td>Total, nmol/min⁻¹ of protein</td>
</tr>
<tr>
<td>None (8)</td>
<td>2.80 ± 0.22</td>
<td>0.15 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>Isoniazid (3)</td>
<td>2.00 ± 0.17</td>
<td>0.32 ± 0.03</td>
<td>213</td>
</tr>
<tr>
<td>Trichloroethylene (5)</td>
<td>2.74 ± 0.21</td>
<td>0.39 ± 0.08</td>
<td>260</td>
</tr>
<tr>
<td>Pyrazole (5)</td>
<td>3.41 ± 0.17</td>
<td>0.41 ± 0.09</td>
<td>273</td>
</tr>
<tr>
<td>Ethanol (14)</td>
<td>2.45 ± 0.13</td>
<td>0.47 ± 0.04</td>
<td>313</td>
</tr>
<tr>
<td>Imidazole (5)</td>
<td>3.46 ± 0.41</td>
<td>0.67 ± 0.02</td>
<td>447</td>
</tr>
<tr>
<td>Acetone (5)</td>
<td>2.71 ± 0.24</td>
<td>0.73 ± 0.16</td>
<td>487</td>
</tr>
</tbody>
</table>

Hepatic microsomes were isolated from each animal, total P-450 content was determined, and P-450 isozyme 3a was quantified immunochemically by the dot-blot method. The isozyme 3a-dependent rate of butanol oxidation was obtained by subtraction of the rate of butanol oxidation obtained in the presence of optimal levels of anti-3a IgG from the rate obtained in the presence of the same concentration of preimmune sheep IgG. All results are given as the mean ± SEM for each group.
Fig. 3. Correlation between the microsomal content of P-450 isozyme 3a and the rate of butanol oxidation (data taken from Table 2). The average values for total microsomal butanol oxidation and isozyme 3a-dependent butanol-oxidation are plotted for the specific content of isozyme 3a from rabbits that were untreated (○), or treated with isoniazid (□), trichloroethylene (△), pyrazole (●), ethanol (●), imidazole (●), or acetone (●). The solid symbols represent total microsomal butanol oxidation and the open symbols represent the isozyme 3a-dependent butanol oxidation. The lines were determined by linear regression analysis.

The microsomal membrane, the slope of the line in Fig. 3 should be near these values. The lower turnover number of isozyme 3a in microsomes might be a result of the limiting amount of P-450 reductase, as has been reported for other P-450 isozymes (38). Both the total rate of butanol oxidation and the antibody-inhibitable rate were stimulated 2-fold when purified NADPH-cytochrome P-450 reductase was added to microsomes from ethanol-treated rabbits to give a 3:1 molar ratio of reductase to P-450 (data not shown). The stimulation by added reductase, an enzyme which exhibits negligible butanol-oxidation activity alone (23), suggests that the reductase is a limiting component in the membrane. Further experiments will be required to confirm this hypothesis.

These results clearly demonstrate that the treatment of rabbits with structurally diverse compounds results in an increase in the hepatic microsomal concentration of P-450 isozyme 3a. That the induced protein immunchemically quantified is, indeed, isozyme 3a has been confirmed after imidazole treatment by purification of the cytochrome and comparison with the purified ethanol-induced cytochrome (19). Since isozyme 3a was not isolated after treatment of rabbits with the other agents used in the present study, a direct comparison of the purified cytochromes was not possible. However, the observations that a single protein, with an identical mobility to purified form 3a, is identified on immunoblots and that the antibody to form 3a inhibits the induced butanol-oxidation activity strongly support the hypothesis that isozyme 3a is induced by all six compounds. Ingelman-Sundberg and Jörnvall (28) have recently reported that, as judged by radial immunodiffusion, the specific content of this isozyme was increased from 0.17 nmol/mg of protein in untreated rabbits to 0.48 and 0.36 in rabbits treated with imidazole and pyrazole, respectively. Our results agree in general with those values, but the specific content of the total P-450 in our microsomal preparations is considerably higher. This may be the result of different outbred populations of rabbits or of differences in the preparation of the microsomal fractions. We have found, for example, that the specific content of total P-450 is lower in microsomal preparations prepared with a 1.14% (wt/vol) KCl wash than in those prepared with the pyrophosphate wash used in the present study.

The molecular mechanisms by which levels of isozyme 3a are elevated have not been defined. Induction of the protein could occur as a result of an increase in the level of translatable mRNA, stabilization of the existing mRNA, an increase in translation of existing mRNA, or a decrease in the rate of degradation of the isozyme. Direct analysis with cDNA probes indicates that, in the rat, treatment with phenobarbital or 3-methylcholanthrene increases the synthesis of specific mRNA (39, 40). Whether the induction of isozyme 3a involves a similar mechanism or a common hormonal or metabolic change in the animal that indirectly leads to induction remains to be determined.

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