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ACETALDEHYDE AS A SUBSTRATE FOR ETHANOL-INDUCIBLE CYTOCHROME P450 (CYP2E1)

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Liver microsomes from starved and acetone-treated rats catalyzed NADPH-supported metabolism of acetaldehyde at a rate 8-fold higher than corresponding control microsomes; the V_{max} was about 6 nmol/mg microsomal protein/min and the apparent K_m 30 μM. The reaction was efficiently inhibited by anti-CYP2E1 IgG, but not by control IgG. Reconstituted membranes containing rat CYP2E1 and cytochrome b_5 metabolized acetaldehyde with a V_{max} of 20 nmol/nmol/min and an apparent K_m of 30 μM, whereas CYP2B4 containing vesicles or vesicles without b_5 were ineffective. Gas chromatographic/mass spectrometric analysis of products formed from [$^2H_4$]-acetaldehyde with CYP2E1-containing reconstituted membrane vesicles revealed the formation of acetate as the only detectable product, although other water soluble products were also formed as evidenced from incubations with [1,2-^{14}C]acetaldehyde. The results indicate that CYP2E1 is an aldehyde oxidase and thus metabolizes both ethanol and its primary oxidation product. This might have implications *in vivo* for acetaldehyde metabolism in liver and brain.

Acetaldehyde is used industrially as a solvent in the rubber, tanning, and paper industries. It is one of the major irritants in tobacco smoke and is present in exhausts. Acetaldehyde is formed during ethanol metabolism and small amounts may be produced from carbohydrates by intestinal bacteria (1), by the cleavage of threonine by a cytosolic aldolase (2) or by the actions of deoxypentosephosphate aldolases (3), phosphorylphosphoethanolamine phospholyase (4) or pyruvate dehydrogenase (5).

Weiner has suggested that the level of low K_m mitochondrial aldehyde dehydrogenase is rate-limiting for the metabolism of acetaldehyde at concentrations in the 10^{-4} M range (6). Incubation of liver slices with 200 μM acetaldehyde revealed that low K_m mitochondrial aldehyde dehydrogenase might be responsible for 60 % of the metabolism, whereas high K_m cytosolic aldehyde dehydrogenase metabolized an additional 20% and 20% of the metabolism was due to an undetermined system.

Acetaldehyde is considered to be responsible for many of the toxic effects caused by ethanol (7, 8). The toxicity might be exerted by enhanced free radical activity, glutathione depletion, lipid peroxidation (cf (9)), impairment of mitochondrial oxidative

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phosphorylation, fibrosis and formation of protein adducts leading to enzyme inactivation, or even the production of antibodies against the modified proteins (7, 8).

Evidence have been given to the formation of acetaldehyde protein adducts in hepatic microsomes (10). Western blotting of microsomal proteins from ethanol-treated rats and immunostaining with rabbit anti-acetaldehyde adduct IgG revealed an acetaldehyde-binding protein with *Mr*= 52 000 (10), which was not seen using microsomes from control rats. The results indicated the origin of the protein as ethanol-inducible CYP2E1. One possible reason for CYP2E1 being the major microsomal protein binding to acetaldehyde is that acetaldehyde activation is required for adduct formation and that the reactive species binds at the site of its formation. The present study was therefore performed to evaluate the possible role of ethanol-inducible CYP2E1 in the metabolism of acetaldehyde. Microsomal acetaldehyde metabolism might result in the formation of ethanol, catalyzed by carbonyl reductases present in the microsomes (11), *eg* CYP2E1 or due to a dismutation reaction (12). Both oxidative and reductive pathways of acetaldehyde metabolism were therefore evaluated using gas chromatographic-mass spectrometric analysis.

EXPERIMENTAL PROCEDURES

Materials

Venoject (Terumo) rubber stoppers were generously provided by Mediplast AB, Stockholm. Extractigel (Pierce) was purchased from Tecator, Sollentuna, Sweden. NADPH was from Sigma. Ethanol from the commercial NADPH was removed before the experiments by rotatory evaporation of a 20 mM solution in water. Renex 690 was from Kemiintressen, Solna and EDTA from Fluka. Acetaldehyde, [$^2H_6$]ethanol and other chemicals were purchased from Merck. [$^2H_4$]Acetaldehyde was obtained from A. Hempel GmbH & Co, Düsseldorf, Germany and [1-^{14}C]acetic acid and [1,2-^{14}C]acetaldehyde was from DuPont-NEN Research Products, Dreieich, Germany.

Methods

Animals. Male Sprague-Dawley rats (150-200 g) were obtained from a local farm. Some rats, S/A rats, were treated with acetone (5 ml/kg as a 33% solution) intragastrically for two days as described (13). Control rats received food and water *ad libitum*.

Microsomes. The rats were killed by a blow on the head 18 h after the last treatment, and the livers were homogenized in 2 volumes of cold 10 mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% (w/v) KCl. Microsomes were prepared by ultracentrifugation and washed once before suspension in 50 mM potassium phosphate buffer, pH 7.4, to give a final concentration of 30-60 mg microsomal protein/ml and stored under nitrogen at -70°C. Microsomes were also prepared as previously described (14) from the livers of phenobarbital-treated male rabbits.

Purification of enzymes. Phenobarbital-inducible rabbit liver CYP2B4 was purified from liver microsomes of phenobarbital-treated rabbits as described elsewhere (14). Rat liver ethanol-inducible cytochrome P450, CYP2E1, was purified as previously described (15) essentially according to the procedures of Ryan et al. (16). Rat liver microsomal NADPH-cytochrome P450 reductase was prepared according to methods previously published for rabbit P450-reductase (14, 17).

Electrophoretically homogeneous preparations of rat cytochrome b_5 were obtained by collecting a narrow red band eluting with 0.1 M KCl during DEAE-Sepharose

chromatography of the reductase fraction, as described for rabbit cytochrome b_5 (17). After dialysis against 10 volumes of 10 mM Tris-HCl, pH 8.1, containing 0.1 mM EDTA, the b_5 fractions were applied to a new DEAE-Sepharose column (1x7 cm) equilibrated with the dialysis buffer. The column was washed with large volumes of equilibration buffer until the absorbance of the eluate at 280 nm was below 0.01. Cytochrome b_5 was eluted with the equilibration buffer containing 0.25 M NaSCN and 0.25% sodium deoxycholate. The concentrated fractions were pooled and dialyzed against 2x50 volumes of 20 mM Tris-HCl, pH 7.4, containing 0.2 mM EDTA.

Microsomal lipids were extracted from rat livers according to Bligh and Dyer (18).

Reconstitution of membranous vesicles. Unilamellar membrane vesicles containing NADPH-cytochrome P450 reductase, cytochrome P450 and microsomal lipids at a molar ratio of 2:1:1200 were prepared by the cholate gel filtration method (19) in 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl and 0.1 mM EDTA. Cytochrome b_5 was added to the preformed vesicles to give a concentration equimolar to the P450 content. The vesicles were preincubated at 37°C for at least 15 minutes to attain full incorporation of b_5 into the membranes (20).

Assays. Acetaldehyde metabolism was measured by gas chromatographic determinations of the disappearance of acetaldehyde from the head space of incubations started with NADPH as compared to incubations where NADPH was added after the incubations were stopped. The incubations were performed in 5-ml glass tubes using rat liver microsomes corresponding to 0.5 mg of protein or vesicles corresponding to 0.05 nmol of cytochrome P450, in a total volume adjusted to 1 ml with 50 mM potassium phosphate buffer, pH 7.4. 0.1 mM EDTA was present to inhibit formation of pentane which interferes with the ethanol-inducible CYP2E1 (15). The glass tubes were sealed with gas-tight rubber stoppers and acetaldehyde was injected through the rubber stopper from a freshly prepared stock solution in water. The tubes were preincubated at 37°C for 5 minutes and the reaction was started by the injection of NADPH. The incubations were stopped by the injection of 0.1 ml 3 M HCl, NADPH was added to the blank incubations and the samples were equilibrated at 60°C for 1 h in order to reach an equilibrium of the acetaldehyde distribution between the gas phase and the liquid phase. 1 ml of the gas phase was analyzed utilizing a Shimadzu GC-8A gas chromatograph equipped with a flame ionization detector and a 1.5 m glass column filled with 0.1% SP 1000 on Carbopack C. The column temperature was 70°C and the carrier gas flow was about 45 ml N_2/min. The conditions used yielded retention times for acetaldehyde and ethanol of about 0.8 and 1 minute, respectively.

Possible formation of ethanol and/or acetate was measured by incubating microsomes or vesicles with [$^2H_4$]acetaldehyde. For determination of ethanol, reactions were stopped by the addition of 50 μl 6 M $HClO_4$ to 0.5 ml incubation. The 3,5-dinitrobenzoates were formed after addition of [$^2H_6$]ethanol as an internal standard, and the samples were analyzed by GC/MS (21). The other parts of the incubation mixtures (0.5 ml) were terminated by the addition of 50 μl 3.5 M H_3PO_4. [1-^{14}C]Acetate was added as a standard to check recovery in the micro-vacuum distillation that followed and the labelled acetate was quantitated by GC/MS as the *t*-butyldimethylsilyl derivatives, with propionate as internal standard (22).

Incubations with CYP2E1 vesicles (0.1nmol/inc) were also conducted using [1,2-^{14}C]acetaldehyde. Substrate disappearance and product formation was determined simultaneously. The reaction mixtures were stopped as described above and acetaldehyde was quantified with GC. Subsequently, the residues were neutralized by the addition of 50 μl 6 M NaOH and extracted with two volumes of chloroform:methanol (2:1, v/v). The intermediary precipitate, the chloroform phase and the water phase were taken to dryness under N_2 and subsequently dissolved in 0.5 ml 0.1 M NaOH and the tubes were washed

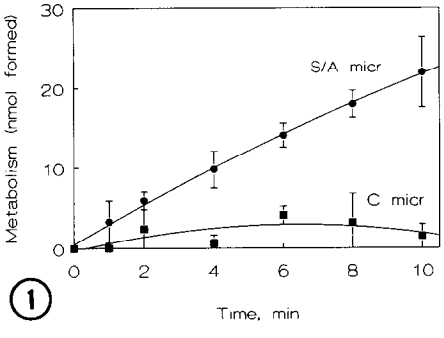
with additional 0.5 ml H_2O. The combined water phases were subjected to liquid scintillation counting.

RESULTS

Acetaldehyde was incubated with liver microsomes from control or starved and acetone-treated (S/A) rats and the rate of metabolism was quantified by measuring the rate of acetaldehyde disappearance using head space gas chromatography. The rate of metabolism using 20 μM acetaldehyde was about 8-fold higher using microsomes from S/A rats, 2.49±0.54 nmol/mg/min, as compared to control microsomes, and was linear for almost 10 minutes (Fig. 1). The apparent V_{max} was 6.1 nmol/mg/min and the apparent K_m was 30 μM (n=4). Rabbit antibodies towards rat CYP2E1 inhibited acetaldehyde metabolism with up to 81±12% (n=4), whereas control IgG had no effect (Fig. 2).

The metabolism of acetaldehyde was also studied in reconstituted unilamellar membrane vesicles containing P450 reductase, CYP2E1 or phenobarbital-inducible rabbit CYP2B4 and cytochrome b_5. Vesicles containing CYP2E1 and b_5 rapidly metabolized acetaldehyde, whereas there was no measurable metabolism with vesicles without b_5 or with vesicles containing CYP2B4 in the presence or in the absence of b_5. Membranes containing 0.05 nmol CYP2E1, incubated with 20 μM acetaldehyde, metabolized the substrate at a rate of 11.1 ±4 nmol/nmol/min (n=3) and the metabolism was linear with time for 10 min. The apparent V_{max} was 20 nmol/nmol/min and the apparent K_m was the same as reached in native microsomes, *i e* approximately 30 μM.

GC/MS analysis of the products formed from incubations of [$^2H_4$]acetaldehyde with microsomes or vesicles showed that no detectable ethanol (less than 5% of acetaldehyde



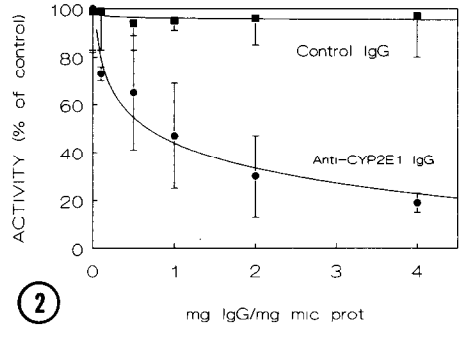


Fig. 1. Time dependence of acetaldehyde metabolism in rat liver microsomes from fasted and acetone-treated rats (S/A-micr) as compared to control rats (C-micr). The curves shown represent the average ± S.D. of four experiments with duplicate samples, performed with 20 μM acetaldehyde, 0.1 mM EDTA and 0.5 mg of microsomal protein per ml.

Fig. 2. Effect of anti CYP2E1-IgG and control IgG on acetaldehyde metabolism in microsomes from starved and acetone-treated rats. The curves represent the average ±S.D. of three experiments with samples in duplicate. The incubations contained 0.5 mg of microsomal protein per ml incubation, 20 μM acetaldehyde and 0.1 mM EDTA. The IgG was added to the incubation mixture 15 minutes prior to the addition of acetaldehyde and the samples preincubated an additional 5 minutes before the reactions were started with NADPH. The incubation time was 6 minutes.

metabolized) was formed. Spectrophotometric analysis revealed that no formaldehyde was formed (not shown). The only detectable product was acetate and in the reconstituted vesicles, the rate of acetate formation was 3.65 ± 0.25 nmol/nmol CYP2E1/ min ($n=5$).

Incubations with CYP2E1 and b_5 containing vesicles were also conducted with [1,2-^{14}C]acetaldehyde (70 μM, 1.2 μCi), when acetaldehyde disappearance, covalent binding to lipids and proteins and formation of water soluble products were determined simultaneously. The results revealed that only small amounts of covalently bound ^{14}C metabolites were formed (<0.2 nmol/nmol, min), whereas the rate of acetaldehyde disappearance was identical to the rate of formation of water soluble metabolites (14 nmol/nmol, min). No water soluble metabolites were detected from incubations with vesicles without b_5 or with CYP2B4 instead of CYP2E1. Incubations which were not neutralized (*cf* Methods) showed no radioactivity in any of the samples, indicating that the acidic forms of the metabolites were volatile.

DISCUSSION

The results presented indicate that CYP2E1 constitutes a microsomal aldehyde oxidase with a high affinity for acetaldehyde. This is supported by the findings that (**a**) liver microsomes from starved and acetone-treated rats, with 8-fold induced levels of CYP2E1 (13), metabolize acetaldehyde at a 8-fold higher rate than control microsomes; (**b**) antibodies against CYP2E1 effectively inhibited microsomal acetaldehyde metabolism; (**c**) reconstituted vesicles containing CYP2E1, P450-reductase and cytochrome b_5, catalyzed acetaldehyde oxidation while no measurable metabolism was found with another P450 (CYP2B4); (**d**) acetate but no detectable amounts of ethanol was formed from [$^2H_4$]-acetaldehyde and CYP2E1 containing vesicles. The identity of the water soluble metabolites not identified as acetate, remains to be investigated.

A mechanism for oxidation of an aldehyde, 11-oxo-tetrahydrocannabinol, by P450 MUT-2 to the corresponding carboxylic acid, involving the hydroxylation of the gem-diol form of the aldehyde, has recently been suggested (23) and could possibly be the mechanism of catalysis also by CYP2E1. Evidence for P450-dependent aldehyde metabolism in rat liver microsomes have previously been provided in a preliminar form by Gans and Werringloer (24) and by Soberman et al. (25).

In experiments where the concentration of acetaldehyde in blood was raised to more than 50 μM by inhibition of aldehyde dehydrogenase in ethanol-treated rats with disulfiram, neural degeneration was seen in the cerebral cortex, olfactory bulbs, the CA1 area of the hippocampus and the cerebellar cortex (26, 27), regions of the brain shown to express CYP2E1 (28). This might be of interest with regard to the present finding of CYP2E1-dependent acetaldehyde metabolism. Furthermore, a similar relationship between CYP2E1 localization and ethanol toxicity is seen in the liver, where only the centrilobular region of the liver acinus, selectively expressing very high levels of CYP2E1 after ethanol treatment (29), is destroyed by the alcohol.

In conclusion, our results show that ethanol-inducible P450 CYP2E1 accompanies the well known aldehyde dehydrogenases in the metabolism of acetaldehyde. The isozyme

specificity is interesting since the same P450 isozyme both metabolizes ethanol and its primary oxidation product, with a much higher affinity for the latter. CYP2E1-dependent metabolism of acetaldehyde might be of toxicological importance *in vivo* since CYP2E1 is expressed in the same areas of brain and liver which undergoes morphological changes after alcohol treatment.

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