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EFFECT OF ISONIAZID ADMINISTRATION ON SELECTED RAT AND MOUSE HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASES AND *IN VITRO* [^{14}C]ACETYLHYDRAZINE-DERIVED COVALENT BINDING

PAUL R. POWELL-JACKSON, J. MICHAEL TREDGER, HEATHER M. SMITH, MICHAEL DAVIS and ROGER WILLIAMS

Liver Unit, King's College Hospital and Medical School, Denmark Hill, London SE5, U.K.

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Abstract—The effect of isoniazid on selected microsomal mixed-function oxidase activities and on the microsomal metabolism of its own metabolite, acetylhydrazine, to a highly reactive compound which covalently binds to intracellular macromolecules was characterised in male C57BL6 mice and male Sprague–Dawley rats. In comparison with controls, isoniazid pretreatment of rats significantly increased the sp. act. of acetanilide 4-hydroxylase and the *in vitro* [^{14}C]acetylhydrazine-derived covalent binding to hepatic microsomes but significantly decreased the sp. act. of benzo[*a*]pyrene hydroxylase and testosterone 16α-hydroxylase. Isoniazid treatment of mice had no effect on any of these parameters except for a significant reduction in sp. act. of testosterone 7α-hydroxylase. Thus the pathway of isoniazid metabolism leading to the formation of reactive metabolites of acetylhydrazine is enhanced by isoniazid pretreatment in rats but not in mice. The presence of similar routes of isoniazid metabolism in man may account for the 8.7–24% incidence of subclinical hepatocellular damage observed in patients receiving isoniazid alone in the chemoprophylaxis of tuberculosis.

Isoniazid (INH) has been used as a "first-line" drug in the treatment of tuberculosis for 30 yr but it is only recently that a number of interactions of INH with the metabolism of other drugs has been described. The major route of metabolism for isoniazid in man is acetylation to acetylisoniazid and is under genetic control so that the proportion of INH acetylated shows wide variation in human populations, which may be divided into two phenotypes, rapid and slow acetylators [1]. As slow acetylators have higher INH blood levels after treatment with the drug, they are not only more likely to develop dose-dependent side-effects, such as peripheral neuropathy [2], but also toxicity to concomitantly administered phenytoin because the hydroxylation of this drug is inhibited by INH [3].

The *in vitro* and *in vivo* administration of INH has been shown to inhibit other mixed-function oxidase activities in man [3–5] and animals [6–9]. In contrast, INH induces a number of anaesthetic defluorinases [10], a finding which might explain the reports of fluorine toxicity in INH treated patients following anaesthesia [11], as well as certain other mixed-function oxidase activities [10].

In man, acetylisoniazid is hydrolysed to isonicotinic acid and acetylhydrazine [12, 13]. Significant amounts of acetylhydrazine are metabolised by the cytochrome P-450 dependent mixed-function oxidase system and it is this pathway which produces the reactive metabolites which have been associated with isoniazid hepatotoxicity [13]. While concomitant treatment of tuberculosis with rifampicin enhances the hepatotoxicity of isoniazid in man and increases the microsomal covalent binding of reactive metabolites of acetylhydrazine in animals [14], it is not known whether the drug stimulates its own metabolism via this pathway.

We have determined in rats and mice the effects of isoniazid pretreatment on the activities of selected mixed-function oxidases which have been found to be useful in the characterisation of a number of microsomal enzyme inducing drugs [15]. We have also studied its effect on the *in vitro* covalent binding of [^{14}C]acetylhydrazine to hepatic microsomes, a measure of the pathway for producing reactive metabolites from acetylhydrazine.

MATERIALS AND METHODS

Chemicals. Isonicotinic acid hydrazine (isoniazid) and *t*-butyl carbazate were purchased from Aldrich Chemicals Co. Ltd., Gillingham, Dorset; other chemicals were obtained from the sources previously described [15]. [4-^{14}C]Testosterone (sp. act. 51 mCi/mmole) and [1-^{14}C]acetic anhydride (sp. act. 27.2 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks. The radiochemical purity of testosterone was >98% on TLC in dichloromethane:acetone (90:50). The isotope was diluted with unlabelled testosterone to 0.417 mCi/mmole before use. Ethylmorphine hydrochloride was kindly provided by May and Baker, Dagenham, Essex, and 6β-, 7α- and 16α-hydroxytestosterones by the Medical Research Council Steroid Reference Collection.

Synthesis of [^{14}C]*acetylhydrazine.* [^{14}C]Acetylhydrazine hydrochloride (0.2 mCi/mmole) was prepared as described by Nelson *et al.* [16] using [1-^{14}C]acetic anhydride. Radiochemical purity was determined by TLC on a preactivated (110° for

60 min) silica gel HF_{254} precoated TLC plate (Merck, Darmstadt, FRG) developed in butan-1-ol:ethanol:0.4 M ammonium hydroxide (4:1:1 by vol.) and was shown to be >95.8%. The melting point was 131–133°.

Animals and pretreatment regimens. Male C57BL6 inbred mice (11 weeks old at death, 18–24 g) were obtained from Bantin and Kingman, Hull, Yorkshire. Male Sprague–Dawley rats (8 weeks old at death) were obtained from OLAC 1976 Ltd., Shaws Farm, Blackthorn, Bicester, OX6 0TP, Oxfordshire. All animals were housed four to a cage on Safety Tread bedding (J. C. Lee and Son, Chase Cottage, 418 Woodham Lane, Weybridge, Surrey) and were fed on Labsure C.R.M. diet (Rank–Hovis–MacDougal, Poole, Dorset) and tap water *ad libitum*. These conditions were maintained for at least two weeks before pretreatment began. Isoniazid (50 mg/kg for rats; 60 mg/kg for mice) in normal saline (10 ml/kg for mice, 4 ml/kg for rats) was given by i.p. injection on 10 consecutive days with the final injection 24 hr before death. Control animals were given vehicle alone.

Tissue preparation. Animals were killed by cervical dislocation between 0800 and 0900 hr and livers were excised into ice-cold KCl-Hepes (1.15% KCl containing 10 mM Hepes buffer, pH 7.6). Washed microsomal suspensions were prepared as previously described [17], using four livers for each pool of mouse microsomes and one liver for each suspension of rat microsomes, and their protein content was determined [18] before making the appropriate dilution for use in the enzyme assays.

Biochemical analysis. Cytochrome P-450 was determined in microsomal suspensions containing 2 mg protein/ml 0.1 M Hepes buffer, pH 7.6 by the method of Omura and Sato [19]. Spectra were recorded on a Cecil Instruments 5095 double-beam spectrophotometer.

Minor modifications as described by Tredger *et al.* [15] of the methods of Bend *et al.* [20], Shimazu [21] and Wattenberg *et al.* [22] were used in the assays for ethylmorphine *N*-demethylase, acetanilide 4-hydroxylase and benzo[*a*]pyrene hydroxylase activities respectively, and of the method of Orton and Philpot [23] for the assay of testosterone hydroxylation.

To determine [^{14}C]acetylhydrazine-derived covalent binding, incubations contained 100 μmole Hepes buffer (pH 8.1), the washed microsomal suspension (6 mg protein) and [^{14}C]acetylhydrazine (2 μmole) in a total vol. of 1.5 ml. The reaction was initiated by the addition of a NADPH generating system (0.5 ml) containing NADP+ (1 μmole), Hepes buffer, pH 8.1 (100 μmole), glucose-6-phosphate (5 μmole) magnesium chloride (10 μmole) and glucose-6-phosphate dehydrogenase (1 unit) to the reaction mixture at 37°. Hepes buffer, pH 8.1 (100 μmole) only was added to the blanks. After incubation for 20 min at 37° the reaction was stopped with cold acetone (2 ml) and by cooling to 4°. The tubes were centrifuged at 1200 *g* at 4° for 3–5 min to form a pellet and clear supernatant. The supernatant was removed by aspiration and the pellet washed by adding 2 ml cold perchloric acid (0.5 M), shaking well and leaving for 12 hr (to improve reproducibility), before recentrifuging at 1200 *g* at 4° for 3–5 min. The washing procedure was repeated with cold perchloric acid (0.5 M) twice and three times with hot (50°) PCA (0.5 M). After removing the supernatant from the last wash, the pellet was dissolved by the addition of 2 ml sodium hydroxide (4 M) and absolute alcohol (0.5 ml). The samples (1 ml) were counted after neutralisation with hydrochloric acid in 10 ml Triton-toluene scintillation cocktail. The counting efficiency was determined by the internal standardisation method and the protein concn of the samples by the method of Lowry *et al.* [18].

Calculation and statistical evaluation of results. Specific activities of ethylmorphine *N*-demethylase and acetanilide 4-hydroxylase were calculated as nmole product (formaldehyde and paracetamol, respectively) per mg microsomal protein. Benzo[*a*]pyrene hydroxylase sp. act. were calculated by measurement of the fluorescence (394 nm excitation, 514 nm emission) of its phenolic metabolites as Relative Fluorescent (RF) units. A standard solution of quinine sulphate (3 μg/ml 0.1 M H_2SO_4) had a RF of 200 units when its fluorescence was measured at 350 nm excitation, 450 nm emission.

Quantitation of the hydroxylated metabolites of testosterone was made by expressing the counts co-chromatographing with the appropriate standard as a percentage of the total applied to the chromatography plate. Calculations of the amounts of testosterone metabolised or hydroxytestosterones produced were made using the appropriate dilutions and by assuming that 1 μmole [^{14}C]hydroxytestosterone was derived from 1 μmole of [^{14}C]testosterone substrate. Allowances were made for the counts appearing in each band in the blanks, i.e. those incubations in which [^{14}C]testosterone was added after termination of the incubation. [^{14}C]Acetylhydrazine-derived covalent binding was expressed as pmole acetylhydrazine equivalents bound per min per mg microsomal protein.

Data are presented as means ± S.E. of four separate determinations for each treatment group as a percentage change compared to the corresponding activity in control animals pretreated with vehicle alone. Student's *t*-test was used to test the null hypothesis. Differences with $P < 0.05$ were considered significant.

RESULTS

Following isoniazid pretreatment a significant reduction (−19%) in cytochrome P-450 content per mg microsomal protein was noted in mice but not in rats (Table 1). In neither species did pretreatment have any significant effect on relative liver wt, or wavelength of the absorption maximum of the reduced cytochrome P-450–CO difference spectrum. The yield of microsomal protein per g of liver was significantly increased (+17%) in mice but not in rats following isoniazid pretreatment.

There were striking differences between the two species in the pattern of enzyme activities modified by isoniazid pretreatment (Fig. 1.). In rats, sp. act. (per mg microsomal protein) of acetanilide 4-hydroxylase and [^{14}C]acetylhydrazine-derived cova-

Table 1. Effects of isoniazid pretreatment on factors associated with the mixed-function oxidase systems of mouse and rat liver

Species	Pretreatment	Relative liver wt g/100 g body wt	Microsomal protein mg/g liver	Cytochrome P-450 Content nmole/mg protein	Cytochrome P-450 Absorption maximum nm
Mouse	Saline	4.90 ± 0.27	17.54 ± 1.17	1.01 ± 0.01	450
	INH	4.98 ± 0.34	20.46 ± 1.74*	0.82 ± 0.05*	450
Rat	Saline	4.11 ± 0.07	23.25 ± 0.99	0.82 ± 0.04	450
	INH	3.98 ± 0.10	25.60 ± 0.53	0.78 ± 0.06	450

* $P < 0.05$ compared to control.

lent binding were increased (+45% and +111% respectively) and activities of benzo[*a*]pyrene hydroxylase and testosterone 16α-hydroxylase were reduced (−30 and −28% respectively). In mice, none of these activities was affected by pretreatment, but testosterone 7α-hydroxylase activity per mg microsomal protein was reduced (−21%). In neither species did isoniazid pretreatment have any effect on the sp. act. of ethylmorphine *N*-demethylase and testosterone 6β-hydroxylase or the total amount of testosterone catabolised.

DISCUSSION

The effects of isoniazid on hepatic microsomal enzyme activity noted in this study were qualitatively different from those of the enzyme inducers, phenobarbitone, β-naphthoflavone (BNF), pregnenolone 16α-carbonitrile and rifampicin reported by us in the same strain of mouse [15] and rat (author's unpublished observations) under similar experimental conditions. There were also differences in the effects of isoniazid (INH) between the two species studies. Such differences have been observed in the effect of other inducing agents on a number of enzyme activities. Thus, phenobarbitone pretreatment exerts different effects on testosterone hydroxylation in the mouse, rat, and rabbit [15, 23] and 3-methylcholanthrene dissimilar effects on benzo[*a*]pyrene hydroxylase activity in the rat and rabbit [24].

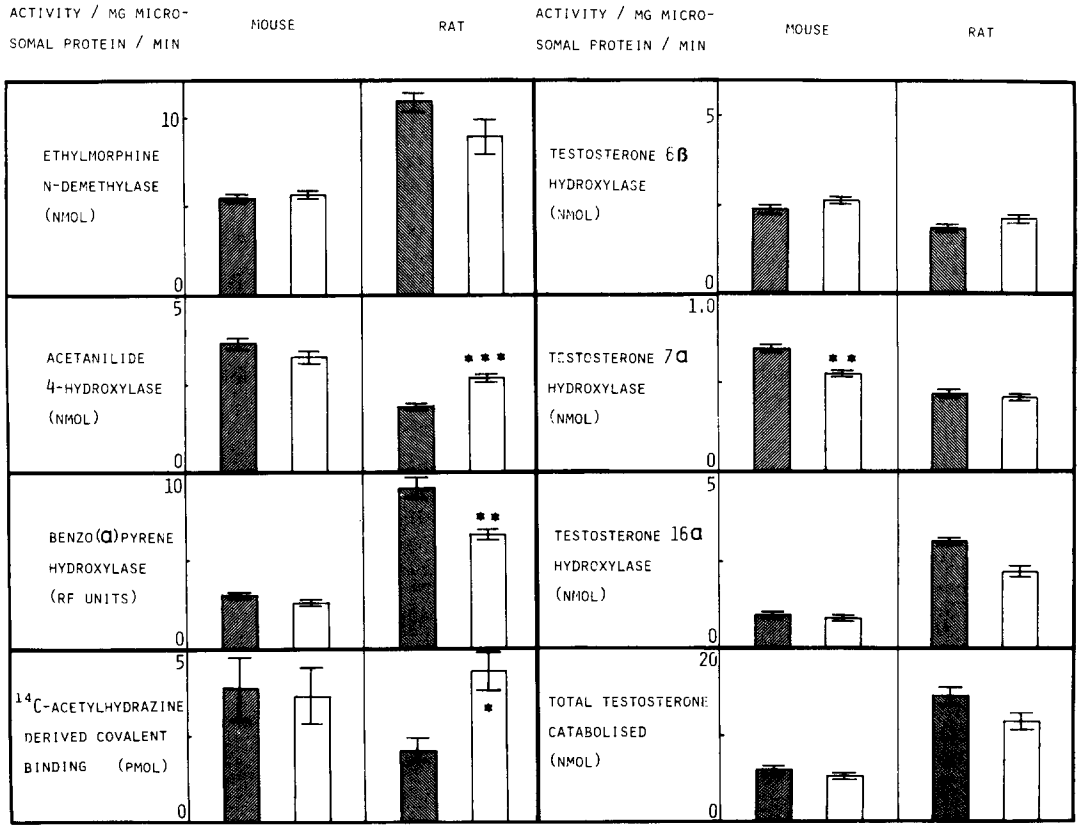

Fig. 1. Effects of isoniazid pretreatment of mice and rats on selected mixed-function oxidase activities and *in vitro* [^{14}C]acetylhydrazine-derived microsomal covalent binding. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's *t*-test.

Marked differences have been reported even between different strains of one species: for example, the effects of BNF on benzo[*a*]pyrene hydroxylase activity in C57BL6 and DBA 2 mice [25]. There are also differences in response between various strains of rats: for example, a shift in the absorption maximum of the reduced cytochrome P-450–CO complex from 450 to 451 nm has been reported following isoniazid treatment in Fischer rats [10] but not in Sprague–Dawley rats, which were used in the present study. The variations in induction of mixed-function oxidase activities observed in different strains may be a direct consequence of such differential P-450 induction. The effect of isoniazid on the mixed-function oxidase system also depends on the mode of administration of the drug. Thus the powerful inhibition of the microsomal metabolism of diphenylhydantoin [3, 7], pentobarbital [8], carisoprodol [8], aminopyrine [8, 26] aniline [26] and acetylhydrazine [9] following the addition of isoniazid *in vitro* contrasts with the effects of long-term administration of isoniazid *in vivo*. Such dissimilar effects on enzyme activities following the acute and chronic administration of other enzyme inducing drugs is well documented [27].

After pretreatment with phenobarbitone, particular strains of rats but not mice are sensitive to hepatotoxicity from acetylhydrazine [28]. Consistent with this is the observation that increases in *in vitro* [^{14}C]acetylhydrazine-derived covalent binding to hepatic microsomes following phenobarbitone pretreatment are correspondingly greater in rats than in mice (author's unpublished observations).

Thus the pathway of isoniazid metabolism leading to the formation of reactive metabolites of acetylhydrazine is enhanced by pretreatment with certain drugs, such as phenobarbitone and isoniazid, in rats but not in mice. The presence of similar routes of isoniazid metabolism in man may account for the 8.7–24% incidence of subclinical hepatocellular damage observed in trials of isoniazid in the chemoprophylaxis of tuberculosis [29–34].

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