Biochemical Pharmacology, Vol. 40, No. 7, pp. 1666–1669, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Effect of normal aging on the activity of human hepatic cytochrome P450IIE1

(*Received* 24 *January* 1990; *accepted* 16 *April* 1990)

In 1978, 11% of the U.S. population was over age 65, yet this age group spent 21% of the national total of $15.1 billion for drugs [1]. In 2030, it is predicted that 40% of national drug expenditures will be made by the elderly [1]. In a survey of an outpatient population over age 60, 83% of patients were taking medications [2]. This widespread medication use in the elderly is associated with a 24% incidence of adverse drug reactions [3]. As the cytochromes P450 metabolize multiple drugs, changes in the activity of these enzymes with normal aging may result in altered drug metabolism [4].

The hepatic cytochromes P450 oxidize lipophilic drugs to water-soluble metabolites for excretion. To date, at least twenty isozymes of the cytochromes P450 have been purified from rat liver microsomes [5, 6]. Homologous isoforms have been identified in human liver. These proteins have been shown to be separate gene products and, thus, true isozymes. These enzymes exist as a superfamily, with each isoform exhibiting distinct substrate specificities [6]. The types and amounts of the various cytochromes P450 determine the rate and pathway of the metabolism of many compounds, including drugs, toxins, and procarcinogens.

In animal studies, the hepatic cytochromes P450 exhibit an aging-related decline in activity [7]. Few studies directly evaluating aging-related alterations in the activity of the human hepatic cytochromes P450 have been performed, to date. However, elderly subjects exhibit decreased clearance of multiple drugs biotransformed by the hepatic cytochromes P450, such as: diazepam, disopyramide, verapamil [8–10], midazolam [11], imipramine, phenytoin, propanolol, and paracetamol [12]. Antipyrine clearance, an activity which has been classically used to determine the activity of the human hepatic cytochromes P450, also decreases significantly with age [13]. However, the urinary excretion of 6β-OH cortisol, which reflects the activity of hepatic cytochrome P450IIIA, is unchanged with aging in healthy men [14]. Clearly, oxidative metabolism in the elderly population must be studied further, as many clinically important xenobiotics are metabolized by these enzymes. For example, human hepatic cytochrome P450IIE1 has been shown to catalyze the following reactions: (a) microsomal ethanol oxidation [15], (b) activation of the procarcinogen *N*-nitrosodimethylamine (NDMA) to its carcinogenic metabolite [16, 17], and (c) conversion of acetaminophen and carbon tetrachloride to hepatotoxic metabolites [18]. In addition, amounts of cytochrome P450IIE1 protein may be increased following exposure to ethanol, fasting, or isoniazid. This isoform is postulated to play a significant role in ethanol-induced liver injury [18]. Therefore, decreases in activity of this isoform with age may prove clinically important.

In addition to potential alterations of the human hepatic cytochromes P450, elderly individuals may experience: (a) an increase in adipose tissue and a decrease in lean body mass relative to total body weight, which may alter the volume of distribution of lipophilic drugs [4]; and (b) decreases in hepatic blood flow [19] which contribute to the reduced clearance of "high-clearance" oxidized drugs, such as lidocaine, imipramine, and midazolam [4]. Hence, direct measurement of hepatic microsomal metabolism *in vitro* will more accurately predict true changes in the activity of the human hepatic cytochromes P450 with aging.

This study sought to define potential aging-related alterations in cytochrome P450IIE1, by examining the activity of this isoform *in vitro*, in liver microsomal samples obtained at surgery from patients age 60–75, and comparing these results to those obtained in liver microsomes prepared in younger subjects, age 30–59. *N*-Nitrosodimethylamine N-demethylation (1.0 mM NDMA DM) was used to profile the activity of human hepatic cytochrome P450IIE1 as this activity has been shown to be specific for this isoform by immunoinhibition assays [16, 17]. The effects of age, sex, obesity, ethanol use, and smoking status on 1.0 mM NDMA DM activity were examined using multivariable linear regression analyses.

Methods

Human liver specimens. Liver specimens were obtained at surgery from twenty consecutive patients undergoing lobectomy, under protocols approved by the Institutional Review Board. No patient received enflurane anesthesia. Patients included in the series had normal serum transaminases and bilirubin levels at the time of liver resection and exhibited normal liver histology. Three of twenty consecutive patients did not meet these inclusion criteria. Relevant medical histories were recorded for each of the remaining seventeen patients (Table 1). Ideal body weight was determined by comparison with charts obtained from the 1979 Build Study of the Society of Actuaries and Association of Life Insurance Medical Directors of America [20]. The liver specimens were transported from the operating room in ice, minced, and immediately frozen in liquid nitrogen and stored at −120°. In batch preparations, the specimens were homogenized, while frozen, in microsomal homogenization buffer, using a Polytron homogenizer, followed by five passes in a Potter–Elvehjem tissue grinder, subjected to differential centrifugation to yield microsomes, and stored at −70° as previously described [21]. Protein concentrations were determined colorimetrically using the method of Lowry *et al.* [22], with bovine serum albumin as the standard.

N-Nitrosodimethylamine N-demethylation. To profile the activity of P450IIE1 in the human liver microsomes, 1.0 mM NDMA DM was measured as follows: 1.0 mg/mL of microsomal protein was incubated in the presence of 0.1 M potassium phosphate buffer, pH 7.4, for 3 min at 37°. The substrate, 1 mM NDMA, was added, and the reaction was initiated with the addition of 1.0 mM NADPH and incubated for 30 min at 37°. Formaldehyde formation was detected by the method of Nash [23]. All reactions were performed in duplicate, with reaction mixtures devoid of NADPH serving as blanks. The extinction coefficient of 8 mM^{-1} was used to calculate the 1.0 mM NDMA DM in nanomoles CHO per milligram of microsomal protein per minute. To determine the interassay coefficient of variation, three separate human liver microsomal samples (with low, midrange and high 1.0 mM NDMA DM values) underwent 1.0 mM NDMA DM determinations, performed in duplicate, on three occasions.

Statistical analysis. Differences between group means were assessed with Student's *t*-test. Multivariable linear regression models were used to evaluate the effect of age on the metabolism of *N*-nitrosodimethylamine, while taking

Table 1. Human liver microsomal samples—Patient histories and cytochrome P450IIE1 activity

Patient No.	Age	Sex	% Ideal body weight	Smoking status	Ethanol use	1.0 mM NDMA DM	Medications
1	30	M	87	Nonsmoker	Nondrinker	0.550	None
2	32	F	111	Unknown	Unknown	0.335	Lorazepam, amitryptyline, prochlorperazine
3	41	M	117	Nonsmoker	Minimal EtOH	0.328	None
4	46	F	?	Nonsmoker	Nondrinker	0.450	None
5	47	F	106	Nonsmoker	Unknown	0.485	Cefazolin
6	49	F	97	5 pack-years*	Nondrinker	0.430	Vibramycin, propanolol, verapamil
7	49	F	106	Ex smoker × 25 years	Nondrinker	0.510	None
8	50	M	104	15–30 pack-years	Nondrinker	0.414	Cimetidine, Maalox, Mylanta
9	56	F	91	20 pack-years	Nondrinker	0.450	Colace
10	61	M	129	20 pack-years	Nondrinker	0.495	Enalapril, trazadone
11	62	M	118	Nonsmoker	Minimal EtOH	0.440	Triazolam
12	65	M	130	15–20 pack-years	Minimal EtOH	0.335	None
13	66	M	119	Nonsmoker	Nondrinker	0.420	None
14	70	F	100	Nonsmoker	Minimal EtOH	0.429	None
15	71	F	154	Nonsmoker	Minimal EtOH	0.360	Digoxin, furosemide, Synthroid, glipizide
16	74	F	103	Nonsmoker	Minimal EtOH	0.450	Diclofenac sodium, Metamucil
17	75	M	93	Nonsmoker	Nondrinker	0.440	Cephalexin, acetaminophen

Human liver microsomal samples were prepared by differential centrifugation from normal human liver specimens obtained at lobectomy. Relevant medical histories were recorded. The 1.0 mM *N*-nitrosodimethylamine N-demethylation (1.0 mM NDMA DM) was measured using 1.0 mg/mL of human liver microsomal protein incubated in the presence of 0.1 M potassium phosphate buffer, pH 7.4, for 3 min at 37°. The substrate, 1 mM NDMA, was added, and the reaction was initiated with the addition of 1.0 mM NADPH and incubated for 30 min at 37°. Formaldehyde formation was detected by the method of Nash [23], using an extinction coefficient of 8 mM^{-1} to calculate the 1.0 mM NDMA DM.

* A pack-year is defined as the equivalent of a package of cigarettes smoked per day for 1 year.

account of potential confounders such as sex, ethanol use, smoking status, and percent ideal body weight.

Results

Medical histories and results of 1.0 mM NDMA DM of human liver microsomal specimens are described in Table 1. Patients ranged in age from 30 to 75 years. Human liver microsomal 1.0 mM NDMA DM was found to vary 2-fold among samples. The intra-assay coefficient of variation for an individual patient was less than 7%. The highest activities were found in patients 1 and 7. Both patients denied the use of pre-operative medications, ethanol, and occupational exposure to solvents or alcohols.

Cytochrome P450IIE1 activity did not change significantly with respect to age, over the range of 30 to 75 years (Table 1). Mean 1.0 mM NDMA DM was 0.424 ± 0.085 nmol CHO/mg microsomal protein/min for those aged 30–59, and 0.421 ± 0.051 nmol CHO/mg microsomal protein/min for patients aged 60–75 ($P = 0.83$). This study possessed at least 80% power to detect a 20% decrease in 1.0 mM NDMA DM in the elderly cohort ($\alpha = 0.05$, one-tailed t-test).

The contribution of age, sex, percent ideal body weight, ethanol use, and smoking status to the prediction of 1.0 mM NDMA DM was assessed using multivariable linear regression analyses. No significant association of 1.0 mM NDMA DM and age, percent ideal body weight, sex, ethanol use, or smoking status was evident.

Discussion

Examination of seventeen human liver microsomal samples, prepared from patients aged 30–75, revealed that the activity of hepatic cytochrome P450IIE1 was unaffected by normal aging. Maintenance of the activity of hepatic cytochrome P450IIE1 during aging is of significant clinical importance, as it catalyzes the microsomal metabolism of ethanol [15], acetaminophen [18], enflurane [18], and 1.0 mM NDMA DM [17].

Multiple drugs have been demonstrated to affect the activity of the hepatic cytochromes P450, either by increasing the amounts of specific isoforms or inhibiting their activity [5]. Amounts of human hepatic cytochrome P450IIE1 protein may increase up to 6-fold in chronic alcoholic patients [15, 17]. No patient in this series described moderate or heavy ethanol use. Supporting these histories, no liver specimen was found to contain evidence of alcohol-induced liver pathology (e.g. Mallory bodies, centrilobular fibrosis, cirrhosis) [24]. The only patient receiving a medication which could potentially affect human hepatic cytochrome P450IIE1 activity was Patient 8, who received cimetidine pre-operatively. Cimetidine has not been reported to affect the amount of hepatic cytochrome P450IIE1 protein, but may inhibit its activity [25]. As cimetidine reversibly binds to the heme prosthetic group of cytochrome P450IIE1, it is unlikely that residual cimetidine would remain associated with the microsomes during preparation. Exclusion of Patient 8 from analyses did not alter significantly the results [with mean 1.0 mM NDMA DM of 0.413 ± 0.092 nmol CHO/mg microsomal protein/min for those aged 30–59, and 0.421 ± 0.051 nmol CHO/mg microsomal protein/min for patients aged 60–75 ($P = 0.84$)].

NDMA is the most prevalent nitrosamine in food and water sources [26]. NDMA has been shown to methylate actively transcribed genes [27]. Activated NDMA is so unstable that metabolites do not pass from organ to organ, so that only organs which metabolize NDMA contain acti-

vated NDMA. This procarcinogen is activated to its carcinogenic product by cytochrome P450IIE1 at the low substrate concentrations (0.5 to 5.0 mM) found in the liver *in vivo* [16, 17]. The stable activity of human hepatic cytochrome P450IIE1 through age 30 to 75 suggests that this isoform may contribute to carcinogenesis throughout adult life.

The clearance of ethanol is performed in concert by hepatic microsomal cytochrome P450IIE1 and cytosolic alcohol dehydrogenase. Alcohol dehydrogenase serves as the major ethanol oxidizing enzyme and exhibits unchanged activity throughout aging [28]. However, in the chronic alcoholic, amounts of cytochrome P450IIE1 protein are increased and contribute importantly to ethanol metabolism [18]. Although microsomal ethanol oxidation was not examined directly, the maintenance of 1.0 mM NDMA DM activity by cytochrome P450IIE1 with aging would suggest that microsomal ethanol oxidation would be similarly maintained in normal aging. This has significant clinical implications, as it is estimated that 1–10% of the elderly population suffers from alcohol abuse [24].

Acetaminophen is a widely used medication, which is cleared largely by Phase II drug metabolism. Phase II drug metabolism undergoes little or no change in normal aging [10]. However, hepatic cytochrome P450IIE1 functions as the major catalyst of microsomal acetaminophen metabolism to its hepatotoxic, electrophilic metabolite [18]. This study suggests that the elderly population would be equally susceptible to the hepatotoxic effects of acetaminophen, although, this was not examined directly.

Increases in total hepatic cytochrome P450 content, as well as the ethanol-inducible cytochrome P450IIE1, have been described recently in a strain of obese rats [29]. In this series, no significant correlation of 1.0 mM NDMA DM and per cent ideal body weight was evident. Conceivably, obesity-related alterations in activity would be detected in a larger population, but this study was not designed to test that hypothesis. In addition, the patients involved in this study ranged from 87 to 154% ideal body weight, and it is possible that patients of greater percent ideal body weight may demonstrate obesity-related alterations in cytochrome P450IIE1 activity. Interestingly, the activity of human hepatic cytochrome P450IIIA, profiled as erythromycin N-demethylation, declined with increases in percent ideal body weight (Hunt CM, unpublished observations).

The ability to examine human liver microsomes *in vitro* provides an invaluable research tool with which to examine aging-related changes of multiple isoforms, free from the constraints and limitations of patients studies (i.e. aging-related changes in absorption, distribution, blood flow, and excretion). To profile the effect of aging on adult human hepatic cytochrome P450IIE1 activity, 1.0 mM NDMA DM was measured in human liver microsomes *in vitro* with respect to age; patients were divided into two groups: (a) aged 30–59, and (b) aged 60–75. Cytochrome P450IIE1 activity was unchanged with age, with mean values of 0.424 ± 0.085 nmol CHO/mg microsomal protein/min in patients aged 30–59 and 0.421 ± 0.051 nmol CHO/mg microsomal protein/min in those aged 60–75 ($P = 0.83$). This study possessed at least 80% power to detect a 20% decrease in 1.0 mM NDMA DM in the elderly cohort ($\alpha = 0.05$, one-tailed t-test). Activity was found to vary 2-fold among individuals, similar to prior published results [30]. Multivariable analyses examining the effects of age, sex, percent ideal body weight, ethanol use, and smoking habits on 1.0 mM NDMA DM failed to reveal significant associations. These results strongly suggest that the activity, and hence drug metabolism, of adult human hepatic cytochrome P450IIE1 is unaltered in normal aging. The stable activity of cytochrome P450IIE1 throughout adulthood has important clinical ramifications, and suggests that the elderly population exhibits similarly preserved microsomal catalysis of ethanol, acetaminophen, and enflurane.

Acknowledgements—This research was supported, in part, by a grant from the John A. Hartford Foundation and by MO1-RR-30, National Center for Research Resources, General Clinical Research Centers Program, N.I.H.

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Biochemical Pharmacology, Vol. 40, No. 7, pp. 1669–1671, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Metabolism of ethyl 2-carbamoyloxybenzoate (4003/2), a prodrug of salicylic acid, carsalam and salicylamide

(*Received* 13 *March* 1990; *accepted* 31 *May* 1990)

Ethyl 2-carbamoyloxybenzoate (4003/2, Fig. 1) is a new anti-inflammatory, analgesic [1] and platelet aggregation inhibitor.* This compound apart from possessing the above useful effects is safe in terms of ulcerogenicity† and toxicity. It seems to be a potentially safer alternative to other salicylates [2] and is presently undergoing clinical evaluation in patients with rheumatoid arthritis.

During preliminary pharmacokinetic studies in rats, rabbits and dogs, a rapid first pass metabolism of 4003/2 was observed,‡ and the presence of three metabolites in plasma was disclosed by the HPLC method.

The present communication describes the production of these three metabolites by incubation of 4003/2 with post-mitochondrial supernatant from rat liver, their isolation, and their identification by comparison of UV, i.r., NMR and MS properties with synthetic samples.

These metabolites were also formed by incubation with post-mitochondrial supernatants from liver of rabbit and dog. An interesting feature observed in this study was that the addition of potassium fluoride to this incubation, inhibited the formation of metabolite *Y*.

* A. Kamal and C. S. Rao, unpublished results.

† Drug Data Report XI: 84, 1989.

‡ A. Kamal, M. V. Rao, A. B. Rao and P. B. Sattur, unpublished results.

Materials and Methods

Chemicals. 4003/2 was synthesized by the reported procedure [1]. All reagents and biochemicals used were commercially available. Solvents were HPLC or spectroscopy grade.

High pressure lipid chromatography. HPLC instrumentation consisted of a Shimadzu liquid chromatograph LC-6A equipped with a SCL-6A controller, SPD-6A Fixed Wavelength UV Monitor as detector, FCV-100B Fraction Collector and a Chromatopac C-R4A Data Processor as recording integrator. The column was 4.6 × 250 mm Ultropac TSK ODS-120A, 5 μm (LKB) for analytical studies and for semi-preparative work the column employed was 20 × 250 mm Shim-pack PREP-ODS, 15 μm (Shimadzu). The eluents were water–methanol (80:20) with 1% acetic acid (v/v). Flow rates were 1.2 mL/min (analytical) and 9.9 mL/min (Semi-preparative). Column effluents were monitored at 254 nm wavelength.

UV and i.r. spectrophotometry. UV spectra were obtained on an LKB Ultrospec K spectrophotometer using methanol as solvent. i.r. spectra were recorded on a Perkin–Elmer 283B spectrophotometer and the samples were taken in KBr pellet form.

NMR and mass spectrometry. ^{1}H-NMR spectra were taken in $CDCl_3$ + d_6-DMSO on a Jeol FX90Q FT NMR spectrometer. TMS was used as an internal reference. EI-MS (electron energy 70 eV, trap current 200 μA) at source