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Short Communication

AGE BUT NOT GENDER SELECTIVELY AFFECTS EXPRESSION OF INDIVIDUAL CYTOCHROME P450 PROTEINS IN HUMAN LIVER

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Abstract—Multivariate linear regression analysis was used to examine the influence of age, gender and environmental variables on the hepatic content of cytochromes P450 (CYP, P450) 1A2, 2C, 2E1 and 3A in 71 subjects; 21 with histologically normal livers and 50 with chronic liver disease. There was a clear negative association between age and total P450 content, NADPH-cytochrome c reductase activity and levels of 2E1 and 3A proteins. 1A2 and 2C proteins were unaltered with advancing age. Gender did not influence the expression of any of the CYP proteins. Cigarette smoking was associated with enhanced levels of 1A2, but effects of drug ingestion and alcohol consumption were not apparent in this study, probably because of case selection. It is concluded that age but not gender is a constitutional factor that influences the hepatic content of cytochrome P450 and selected CYP proteins.

Key words: cytochrome P450; CYP 3A; CYP 2C; age; gender; multivariate analysis

Proteins encoded by the CYP† gene superfamily comprise the largest group of enzymes in human liver that modulate the metabolism of drugs and lipophilic endogenous substrates. CYPs 1A, 2C, 2D, 2E and 3A subfamily members are predominant catalysts of drug oxidation. The vast array of therapeutic compounds metabolized by these proteins has been reviewed [1], and the relative contents of major proteins in human liver have been quantified [2].

In addition to the critical importance of pharmacogenetics, other factors may modulate the content and activity of hepatic CYPs. These include constitutional factors such as age and gender, exposure to environmental agents such as drugs, cigarette smoke and alcohol, and disease factors, particularly the presence of liver disease. There are few studies that have examined directly the influence of these factors on hepatic levels of individual P450 proteins in a substantial number of cases. The present report concerns an examination of the influence of age, gender and exposure to xenobiotics on the hepatic contents of CYPs 1A2, 2C, 2E1 and 3A.

Methods

The protocols for experiments on human tissue were approved by the Human Ethics Committees of the Western Sydney Area Health Service. Histologically normal liver (CON) was obtained from 21 patients at the time of hepatobiliary surgery. Cirrhotic liver tissue was obtained at the time of hepatic transplantation. There were 18 cases with cholestatic types of cirrhosis (CHOL), and 32 with hepatic cirrhosis not associated wth cholestasis (HC). The clinical and other characteristics of the patients studied have been described previously [3].

After perusal of patient records, the following data were recorded in a standard format: age, gender, diagnosis, presence and nature of non-hepatic disease and current (previous week) history of cigarette smoking (>5 cigarettes/day) or ethanol consumption (≥10 g/day). All medications consumed in the 2 weeks prior to surgery were recorded. Drugs were classified according to their ability to induce P450 proteins by searching the Medline database.

Twelve of the 21 control subjects were not receiving any medications at the time of surgery, and none of the remainder were taking agents known to induce CYP proteins. Five individuals consumed alcohol (all ≤20 g/day) and three were current smokers. Some patients with cirrhosis were taking drugs known to induce CYP proteins. These drugs included rifampicin (three patients), omeprazole (two patients) and glucocorticoids (14 patients) [3].

Microsomes were prepared by differential ultracentrifugation [4]. Microsomal protein was determined by the method of Lowry *et al.* [5]. Total P450 content was estimated by the method of Omura and Sato [6]. NADPH-cytochrome c reductase activity was assayed by the method of Williams and Kamin [7]. The microsomal contents of the CYPs 1A2, 2C, 2E1 and 3A were determined by immunoprecipitation following SDS-electrophoresis on 7.5% polyacrylamide gels and transfer to nitrocellulose membranes as previously described [3]. The antibodies used have been detailed elsewhere [3]; while the 1A2 and 2E1 antibodies are directed against individual proteins, the 2C and 3A antibodies are likely to recognize all respective subfamily members because of the structural similarities between individual proteins.

Statistical analysis. The influence of age, gender, cigarette smoking, alcohol consumption and drugs on CYP protein levels was examined by multivariate regression analysis. Stepwise procedures were used to identify the best fitting statistical model. Variables analysed included age, sex, current alcohol consumption, cigarette smoking, serum albumin and bilirubin concentration, prothrombin time, the presence of hepatic encephalopathy or ascites, Pugh class of liver disease severity, concomitant sepsis, presence of non-hepatic diseases and the ingestion of drugs known to induce CYP proteins.

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† Abbreviations: CYP, cytochrome P450; CON, control; HC, hepatic cirrhosis without cholestasis; CHOL, cholestatic cirrhosis.

Table 1. Influence of demographic variables on the expression of drug metabolizing enzymes*

Variable	Total P450	Cyt c reductase	1A2	2C	2E1	3A
Age	$P < 0.05$ (neg)	$P < 0.05$ (neg)	0	0	$P < 0.005$ (neg)	$P < 0.01$ (neg)
Gender	0	0	0	0	0	0
Smoking	0	0	$P < 0.05$ (pos)	0	0	0
Alcohol	0	0	0	0	0	0

* Tabulated are the P values for the best fitting multivariate regression model for each outcome. P values have been adjusted for all other variables included in the model (see text). Symbols in parentheses refer to whether, by multiple regression analysis, the variable examined had a negative (neg) or positive (pos) influence on the indicated protein(s). A (0) entry implies that the variable was not associated with the particular outcome (expression of indicated enzyme) measured in the multivariate model.

Since we have shown that liver disease has a major influence on the expression of P450 proteins [3], we first determined whether the effect of a particular constitutional or environmental variable on protein level depended upon the patient group (CON, HC, CHOL). This was achieved by allowing for appropriate interaction terms (variable × group) in the model. None of these interactions was statistically significant. Therefore the total sample ($N = 71$) was analysed allowing for different baseline protein levels in each group.

Analysis was performed on a NEC computer using the SPSS Release 4 package (SSPS Inc, Chicago, IL, U.S.A.). The significance level for all tests was $P < 0.05$. The two sample t test was used to test for differences between smokers and non-smokers with regard to 1A2 expression.

Results

The effects of type of liver disease on total P450 levels, NADPH-cytochrome c reductase activity and immunoquantitation of the four CYP proteins have been previously reported [3]. By multivariate regression analysis there were inverse relationships between increasing age and total P450 levels ($P < 0.05$) and the activity of NADPH-cytochrome c reductase ($P < 0.05$) (Table 1). Total P450 levels declined by 0.02 nmol P450 × mg protein^{-1} (~3.5%) for each decade of life. The corresponding decrease for the reductase activity was 38 nmol cytochrome c reduced/mg protein/min (~9%). After accounting for age effects, there was no gender difference in relation to these variables (Table 1). Similarly, cigarette smoking and alcohol ingestion (Table 1) and drug ingestion (not shown) did not contribute significantly to variability in total P450 or NADPH-cytochrome c reductase activity.

There was no association between age or gender and hepatic microsomal content of 1A2 protein. As expected [8], levels of 1A2 were positively associated with cigarette smoking ($P < 0.05$, Table 1). Mean 1A2 protein values were higher in smokers than in non-smokers (200 ± 80% versus 87 ± 10% of the assay standard, $P < 0.01$), and the highest value for 1A2 content was obtained in a smoker (483% of the assay standard). Age, gender, cigarette smoking and ethanol consumption did not have an independent bearing on hepatic 2C protein content.

There was a negative correlation between age and hepatic content of 2E1 ($P < 0.005$, Table 1). Thus, for each decade of life, microsomal 2E1 content declined by ~5%. Gender, cigarette smoking, and the other variables examined did not influence the level of this isoform. It should be noted, however, that all five patients who consumed ethanol abstained for the two days prior to surgery.

After adjusting for the effects of liver disease, age was shown to influence the level of 3A protein ($P < 0.01$, Table 1). It was calculated that microsomal 3A content declined by ~8% for every decade of life. After adjusting for age, gender did not have an independent effect on 3A protein. Cigarette smoking and alcohol consumption did not influence 3A protein content. Similarly, although 17 patients with liver diseases were taking substances known to induce 3A proteins (usually corticosteroids), hepatic levels of 3A proteins were not significantly different in these cases.

Discussion

The present study provides novel data regarding the effects of age and gender on CYP protein expression in the healthy and diseased human liver. Multivariate regression analysis permitted the relative importance of interactive variables to be identified and quantified. The information obtained extends and, in some instances, differs from findings of previous studies, most of which have employed indirect approaches to drug metabolism. Thus total hepatic P450 levels decreased with advancing age, but this reduction was not observed uniformly for all CYP proteins. In contrast, when the effect of age had been accounted for there was no evident effect of gender on any of the proteins studied.

The most novel finding of this work is that expression of 2E1 and 3A4 proteins were inversely related to age. The content of each of these proteins declined by 5% (2E1) and 8% (3A) for each decade of life indicating that the influence of age was quantitatively important. Since these two proteins, and especially 3A subfamily members [2, 9], are among the most abundant P450s in human liver, the reductions in their expression could account for the decrease in total P450 with age. In contrast, no relationship was discerned between age and the contents of 1A2 and 2C.

Potential sources of bias that could have arisen in the present analysis are inclusion of both patients with liver disease and those with histologically normal livers. If older patients had had more severe cirrhosis, or if more older patients had had cirrhosis then an inadvertent interaction (not detected as statistically significant by the present analysis) could have confounded the results. However, several points are against this interpretation. First, individuals with noncirrhotic livers were older than cirrhotics (for example, 8 of 10 subjects aged more than 60 years had normal livers). Secondly, the indications for liver transplantation were unrelated to age; thus, younger patients had equally severe cirrhosis as older ones. Third, liver disease does not affect expression of individual CYP proteins in the same way as age [3]. Specifically, CYP 1A2

was most affected by liver disease [3] but there was no age effect, and CYP 3A was little altered by some types of liver disease yet was reduced with increasing age. Finally, the same apparent decline in total hepatic P450 and CYP 3A and 2E1 proteins was apparent in the subgroup of 21 subjects with histologically normal livers, although the smaller numbers did not allow significance to be reached in this subgroup. It is concluded that the inclusion of liver disease cases could not explain the present results.

Other studies have examined the influence of age on hepatic monooxygenase reactions catalysed by either 2E1 or 3A [10–12] but these involved relatively small patient numbers and did not immunoquantitate the responsible proteins. In 17 healthy subjects aged 30–75 years, Hunt *et al.* [11] found that hepatic microsomes from subjects older than 60 years metabolized the N-demethylation of *N,N*-dimethylnitrosamine at the same rate as those aged less than 60 years. Catalytic activity using this substrate may not be an optimal index of 2E1 levels because additional proteins can also catalyse the reaction [13], although the masking effect of other forms of P450 on the age-dependent reduction of CYP 2E1 would then have to be dependent on a compensatory increase in the catalytic importance of those enzymes with advancing age. A more important consideration, therefore, may be that by considering age as a dichotomous variable, this earlier (and smaller) study did not have the power to detect an effect on this enzyme at the order of magnitude determined in the present work. In a second study, no effect of age on the 3A-catalysed erythromycin *N*-demethylase activity was detected among 43 individuals [12]; the mode of statistical analysis was binary, even though the data exhibited five-fold variation. We were also unable to find age-related changes in the present study using binary methods of statistical analysis (data not shown), and it therefore seems likely that the use of a more appropriate method of statistical analysis in the present work is the main reason for the apparent disagreement with other investigations. Another study also failed to find an effect of age on expression of 3A and 2C proteins, but protein determinations were performed by semi-quantitative methods and the antibodies used were incompletely characterized [14]. Finally, Brodie *et al.* [15] determined total cytochrome P450 content in liver microsomes from 61 human subjects, about half of whom had liver disease, and there was an apparent negative correlation with age ($r = -0.14$), but this was not significant, possibly due to the interaction of liver disease and other variables.

Pharmacokinetic studies have revealed age-related declines in the clearance or metabolism of midazolam, chlordiazepoxide, lidocaine, quinidine, verapamil and erythromycin [16–21], all of which are oxidized by 3A proteins. Ageing is also associated with other changes that may interfere with drug disposition, including decreased liver volume [22, 23], diminished hepatic blood flow [22, 23], and increased volume of distribution for several drugs [18]. The current data, however, are consistent with the assertion that decreased microsomal 3A protein levels contribute importantly to the decline in 3A-mediated drug metabolism in older individuals. In addition, the observation that NADPH-cytochrome c reductase, another important step in microsomal mixed function oxidase reactions, is equally decreased with age, indicates an additional factor that could diminish the catalytic activity of hepatic CYP proteins in older individuals. Finally, the age-related decrease in liver size [22, 23] would further accentuate the decline in all pathways of drug metabolism *in vivo* among older people.

In contrast to 3A4 and 2E1, the hepatic microsomal contents of 1A2 and 2C proteins were not influenced by age. Compounds metabolized by 1A proteins include phenacetin and certain carcinogenic aromatic amines, while 2C proteins participate in the metabolism of phenytoin, mephenytoin, tolbutamide and warfarin [24]. The finding that warfarin metabolism is unaltered in the elderly [19], is therefore consistent with the present data. It must be acknowledged, however, that different CYP 2C proteins catalyse the oxidation of these individual drugs, and the methodology used to quantify 2C protein(s) in the present work cannot identify changes of individual 2C proteins.

Gender did not appear to contribute to variability in hepatic expression of any of the CYP proteins measured in this study. In the present work, 3A protein was 21% higher in females than in males with normal liver histology, but regression analysis failed to find an independent effect of gender on variability of expression of this protein when age was also considered. This failure to detect any influence of gender on 3A protein contrasts to the reports of Hunt *et al.* [12] and others who found that erythromycin *N*-demethylase activity (3A) was higher in females than males [10, 25]. In none of these reports, however, were the data subjected to multivariate analysis. It is therefore possible that they failed to account for the influence of potentially confounding variables, particularly age.

Cigarette smoking is known to induce 1A2 protein [8], a finding that explains the enhancement of theophylline [26] metabolism in cigarette smokers. The present results are consistent with this observation, and also indicate that smoking does not appear to increase the hepatic content of the other CYP proteins examined. No effect of ethanol consumption on 2E1 levels was detected, but it should be noted that only five subjects consumed alcohol in the week prior to surgery. The inductive effect of ethanol on this protein is likely to be short-lived and abstinence shortly before surgery may therefore account for this finding.

In summary, the results of the present study indicate that age contributes to variability in hepatic content of P450 and to the activity of NADPH-cytochrome c reductase. Moreover, the expression 3A and 2E1 proteins was reduced with age but 1A2 and 2C proteins were not altered. In contrast to the results of some *in vivo* studies, gender was not associated with variability of expression of the hepatic CYP proteins examined in this study.

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