Induction of Cytochrome P450 2A6 Expression in Humans by the Carcinogenic Parasite Infection, Opisthorchiasis Viverrini

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Abstract
The purpose of this study was to examine in vivo the activity of cytochrome P450 (CYP) 2A6, an enzyme capable of activating carcinogens, including N-nitrosodimethylamine, in humans with the carcinogenic liver fluke infection, opisthorchiasis viverrini, before and after treatment with the antiparasitic agent, praziquantel. Coumarin hydroxylase activity of CYP 2A6 was assessed by administering a probe drug, coumarin, and measuring its metabolite, 7-hydroxycoumarin, in urines collected between 0–2 h and 2–4 h of 106 people with varying intensities of Opisthorchis viverrini infection. Five individuals who did not excrete any detectable 7-hydroxycoumarin (and have a genetic defect probably leading to an absence of catalytic activity of the CYP 2A6 protein) were excluded from analysis. Infected people excreted an average of 22.7 μmol of 7-hydroxycoumarin in the first 2 h after taking the drug, whereas the mean of the uninfected group was 19.4 μmol; this difference did not reach statistical significance (P = 0.10). However, a highly significant increase in CYP 2A6-related activity was observed in infected individuals who also had radiological evidence of biliary fibrosis (28.1 μmol) compared to those without (19.4 μmol; P = 0.01). Reassessments of coumarin hydroxylase activity of CYP 2A6 made 2 months after praziquantel treatment showed highly significant reductions in the amount of 7-hydroxycoumarin excreted among the infected groups but no difference in the uninfected group. These results suggest that expression of CYP 2A6 is induced among chronically infected people who also have fibrosis of the intrahepatic bile duct. As already demonstrated in an animal model and now observed in humans for the first time, this increase in CYP 2A6-related enzyme activity may represent an important mechanistic link between inflammatory products of chronic liver fluke infection (e.g., DNA alkylation damage from endogenously formed N-nitrosamines) and the high risk of cholangiocarcinoma faced by infected individuals.

Introduction
Humans are exposed to potentially carcinogenic nitrosamines that often include NDMA3 from both exogenous and endogenous sources (1). Endogenous synthesis of these suspected carcinogens can occur in the stomach and in inflamed tissue. In the stomach, nitrosamines are generated from precursors, nitrate/nitrite, and amines, which are abundant in food and the environment (2, 3). In contrast, in inflamed tissue, nitrosamines are possibly generated from the reaction between amines present in the tissue (e.g., dimethylamine) and nitroating agents derived from nitric oxide via oxidation of arginine by inducible nitric oxide synthase (4, 5). Evidence that the latter synthetic route occurs during immune responses to some pathogens, e.g., the liver fluke, Opisthorchis viverrini, is accumulating, suggesting a role for endogenously generated nitrosamines in inflammation-associated cancer (6–9).

Metabolic activation of NDMA to form the DNA methylating agent is mediated by CYP 2E1 and CYP 2A6 in humans and appears to be essential for its carcinogenicity (10–13). The activity of these enzymes is independently regulated, but both vary with genetic and environmental factors. Importantly, experimental infection with hepatitis B virus, Opisthorchis viverrini, and various types of chemically induced liver damage are reported to induce rodent CYP 2A5 (orthologous to human 2A6) and carcinogen metabolism by this enzyme (14, 15). It is not known whether these infections induce the same response in humans.

The model of cholangiocarcinoma associated with the liver fluke, Opisthorchis viverrini, is useful for the study of nitrosamine carcinogenesis in humans (16). The incidence of this usually rare cancer in the endemic area in Northeast Thailand, where 30 to 40% of the population is infected, is extremely high, with a 2–3-fold higher incidence among males than females (17, 18). Cross-sectional studies have revealed radiological signs of this cancer in a very high proportion (4.2%) of heavily infected individuals (18). Liver fluke infection enhances the synthesis of nitric oxide and endogenous nitrosation, and certain parasite-specific immune responses are associated with higher levels of NDMA excretion after alcohol consumption (6–9).4

Sex-associated variation in CYP activity has been sug-

1 The abbreviations used are: NDMA, N-nitrosodimethylamine; CYP, cytochrome P-450; 7-HC, 7-hydroxycoumarin; epg, eggs per gram.

4 M. R. Haswell-Elkins, S. Satarug, P. Sithithaworn, et al., Specific immune responses to the carcinogenic parasite infection, Opisthorchis viverrini, correlate with synthesis and excretion of N-nitrosodimethylamine by infected men. submitted for publication.
gested as an explanation for sex differences in the incidence of some cancers, including fluke-associated bile duct cancer. Kirby et al. (15) demonstrated that the activity of CYP 2A6 is elevated in male, but not female, hamsters infected with this liver fluke. Immunohistochemical analysis demonstrated that this elevated expression in hamsters was localized in hepatocytes close to the inflammatory lesions of the bile duct where the flukes reside. Differential expression of CYP enzymes metabolizing nitrosamines may in part explain the increased susceptibility of infected males to carcinogetic transformation of the biliary epithelium. However, there are no data in humans to support this suggestion.

Noninvasive, in vivo methods to determine CYP 2E1 and 2A6 activities use probe drugs, chlorzoxazone, and coumarin (19, 20). Coumarin is rapidly metabolized to 7-HC by CYP 2A6 and excreted in urine. Metabolites of NDMA are not detectable.

We have undertaken a comprehensive study to measure the activity of CYP 2E1 and 2A6 among Thai males and females with no or moderate to heavy liver fluke infection. This report presents the results of CYP 2A6 assessments before and 2 months after curative treatment with the drug praziquantel.

Materials and Methods

Study Group Selection and Sample Collection. The individuals in the sample group chosen for this study were participants in a large survey of liver fluke infection in four provinces of Northeast Thailand. Stool samples were collected, processed by the quantitative formalin ethyl acetate concentration technique, and O. viverrini epg were determined by microscopy (21). Twelve villages with relatively high levels of O. viverrini infection were selected from a total of 42 as a source of subjects. The selection criteria for individuals included not having taken praziquantel treatment within the previous 2 years. All eligible individuals with heavy infection (>6000 epg, n = 18) were invited, together with one to two (depending on availability) age- and sex-matched individuals with moderate infection (range, 1000–6000 epg) and with no infection (0 epg). In total, 106 men and women participated (Table 1), which required staying together for 4 days in five groups of 15-24 people, consuming a controlled, low nitrate diet and refraining from smoking. Written informed consent to participate was provided by each subject, and ethical procedures recommended by ethics committees in Thailand and Australia were followed.

Each subject underwent an abdominal ultrasound examination by the same radiologist who was unaware of the infection status of the individual to assess hepatobiliary status, including measurements of gallbladder dimensions and height of the left hepatic lobe at the abdominal aorta, as described previously (18, 21, 22). Portal vein radicule echoes were scored as positive when the radiologist observed an increased prominence of echoes along the portal triad. A second stool was collected and examined to confirm infection status, and a morning urine specimen was cultured for bacterial infection. On separate days and after at least 24 h on the controlled diet, 15 mg of coumarin (Venalot; Schaper & Brummer, Germany) and a single tablet of Parafon, containing 250 mg of chlorzoxazone, were administered to each participant after overnight fasting and first morning urination. The method described by Rautio et al. (20) for CYP 2A6 assessments was closely followed for both drugs. All voided urine was collected, and three pools were made for each subject, representing total output over a period of 0–2 h and 2–4 h, then 4–24 h after drug administration. Because not every subject could provide a urine specimen, the number of minutes between the consumption of the drug and the last urine voided and total urine volume for each individual within the collection periods were recorded. Aliquots of urine were stored frozen till analysis; those of the initial visit were analyzed for 7-HC within 2 months, but the posttreatment samples were examined 1 year after collection. Blood samples were also collected 30 min after administration of each drug.

On the initial visit, those positive for fluke infection were treated with praziquantel at 40 mg/kg body weight. All participants were invited for reassessments 2 months later, and the process was repeated, including stool examination to detect reinfection. Although 96 (90.5%) returned, the coumarin test was given to only 85 people due to insufficient supply of the drug.

Laboratory Analysis of Samples. The concentration of creatinine in all urine specimens was determined by standard methods. Analysis of urine for 7-HC was carried out by spectrophotometry after treatment of urine with β-glucuronidase to liberate 7-HC from glucuronide conjugation and extraction with chloroform (20). Only a subset of 4–24 h specimens was tested because they contained a small amount of the metabolite.
4-h period ranged from 0 (the zero activity group, see above), hydroxylase activity, from a total of 85 participating minus 4 quantel. Groups are stratified according to their liver fluke infection (determined respectively, whereas those of the 2-4-h period were nearly period were 21.3 mol (3.5 mg) and 45.1 mol (7.4 mg), CYP gene, and they are excluded from analysis. An additional carried out in our laboratory has revealed mutations in the amount of two groups, using paired analysis for pre- and post- treatment comparisons of the same individuals and unpaired analysis when comparing different groups. ANOVA was used when three or more groups were compared and when multivariate analysis was used to control for covariates. Correlations were tested using Pearson’s r correlation coefficient.

Results
Among the 106 subjects tested overall, five did not excrete detectable levels of 7-HC in any of the urine specimens on the first or second assessment. DNA analysis of these individuals carried out in our laboratory has revealed mutations in the CYP 2A6 gene, and they are excluded from analysis. An additional 10 people did not provide a urine sample between 60 and 120 min after taking the drug and are also excluded from pretreatment analysis, leaving a sample size of 91. The posttreatment sample consists of 7-HC data on 80 individuals with coumarin hydroxylase activity, from a total of 85 participating minus 4 with no activity and 1 who did not provide urine in the 60–120-min interval.

The mean and maximum amounts of 7-HC in the first 2-h period were 21.3 μmol (3.5 mg) and 45.1 μmol (7.4 mg), respectively, whereas those of the 2–4-h period were nearly identical (20.8 and 45.1 μmol; Table 1). The average level of 7-HC detected in 4–24-h urine specimens from 21 individuals tested was 0.56 μmol. Amounts of 7-HC excreted in the first 4-h period ranged from 0 (the zero activity group, see above), 6.1 μmol (lowest individual with detectable activity), to 72.5 μmol (maximum), with an average of 42.1 μmol.

Levels of 7-HC detected in the 0–2-h urines of the follow-up (posttreatment) assessments were significantly lower (26%; t tests; T = 4.8, df 70; P < 0.001) than those of the initial assessment, whereas 2–4-h levels were approximately 5% lower (T = 1.9, df 68; P = 0.06). The averages for the 2-, 4-, and combined 4-h periods were 15.8, 19.8, and 35.7 μmol, respectively.

Infected individuals excreted slightly higher levels of 7-HC in the 0–2-h period than those uninfected in pretreatment assessments; but this difference did not reach statistical significance (T = 1.65, P = 0.10). ANOVA models incorporating other variables revealed no significant differences between the sexes or with age. However, infected individuals who also had enhanced portal vein radicle echoes (probably representing biliary fibrosis) excreted significantly higher 7-HC than those uninfected (T = 2.6, df 54; P = 0.014) and those infected but without fibrosis (T = 2.2, df 49; P = 0.03), a trend observed in both males and females (Table 1; Figs. 1 and 2). This group also excreted higher levels of creatinine in 0–2-h urines. The levels of 7-HC in 2–4-h urines of the pretreatment samples varied little with infection/fibrosis status.

Follow-up assessments revealed no significant difference in 7-HC excretion related to previous infection/fibrosis status (F = 0.51, df 2.75; P > 0.05). Significant drops in 7-HC levels in 0–2-h urines occurred after treatment in the infected (paired samples t test, T = 3.6, df 26; P = 0.001) and most strikingly in the infected plus fibrosis group (paired t test, T = 5.0, df 14; P < 0.0001; Fig. 2). There were no significant drops in 7-HC levels in 2–4-h urines of any group (P > 0.05).

There was a close correlation between total μmol 7-HC in the 0–2-h urine specimens and the rate of 7-HC excretion (total amount divided by the number of minutes between coumarin ingestion and the last urine voided within the collection period) within individuals (Pearson’s correlation, r = 0.93, P <

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**Fig. 1.** Mean μmoles of 7-HC detected in urines collected between 0 and 2 h after ingestion of coumarin in the first assessment, before treatment with praziquantel. Groups are stratified according to their liver fluke infection (determined by stool examination) and fibrosis (determined by ultrasonography) status and by sex with a total sample size of 91. ■ females; ● males.

**Fig. 2.** Mean μmoles of 7-HC detected in urines collected between 0 and 2 h after ingestion of coumarin in the first assessment (■) and in the second assessment (●) made 2 months after treatment of those infected with praziquantel. Groups are stratified according to their liver fluke infection (determined by stool examination) and fibrosis (determined by ultrasonography) status. Only those who participated in both assessments are included, giving a total sample size of 73.
Fig. 3. Relationship between μmoles of 7-HC detected in urines and the number of minutes between ingestion of coumarin and the last urine specimen collected during initial, pretreatment assessments (a) and posttreatment assessments (b). The markers and lines differentiate the infection/fibrosis status of the individuals, and the value of $r^2$ ($R^2$) indicates the strength of the linear relationship between the points and best fit lines for each group.
larger adjusted liver heights than those without. This difference approached statistical significance ($F = 3.4, df 1.89; P < 0.06$).

**Discussion**

The increased urinary excretion of 7-HC within 2 h after administration of coumarin and its disappearance after anthelminthic treatment, which we have detected in liver fluke-infected individuals with evidence of fibrosis along the intrahepatic bile ducts, is likely to be the result of induced expression of hepatic CYP 2A6. These molecular epidemiological data are supported by experimental studies showing that exposure and hepatic damage caused by a wide range of liver inflammatory agents generate conditions where induction of CYP 2A6 is observed (14, 15). Thus, inflammation-related induction of this specific cytochrome is in contrast to the well-documented effects of parasitic infection on total CYP activity, which may decrease during infection (23).

Individuals with liver fluke infection show considerable variation in their immune response to parasite antigens; the immunological variation may in part determine the different disease manifestations, including biliary fibrosis and bile duct cancer, caused by infection (21, 22, 24). The dual requirement for biliary fibrosis as well as active infection suggests that enhanced CYP 2A6 activity is derived from chronic inflammatory processes occurring in an infected person and not from parasite-derived CYP 2A6. Although the biliary fibrosis is not likely to have completely reversed within 2 months after treatment, the loss of enhanced CYP 2A6 activity suggests that active inflammation (perhaps mediated via cytokines), and not simply fibrosis, is required for maintenance of the induced state.

It is unlikely that the observed differences between groups are a result of variation in the absorption of coumarin, renal clearance of 7-HC, usage of drugs capable of inducing CYP 2A6, or other non-fluke-associated variables because the recovery of 7-HC at 2–4 h and in posttreatment assessments showed no variation between infection groups. The statistical analysis of 7-HC versus time as shown in Figs. 3 and 4 clearly indicate that urine collection at 120 min after ingestion of coumarin would be most desirable but is not practical. The stronger time dependence of 7-HC levels among those with infection and fibrosis than among those without probably reflects both the higher amount of 7-HC generated per minute as well as the longer time to reach a local concentration of coumarin sufficient to achieve saturation of the enzyme in the liver. Levels did not continue to increase during the 2–4-h period, probably because of dispersion of the unmetabolized drug after its “first pass” in the liver. Experimental data also confirm that liver fluke infection induces expression of CYP 2A6. The immunohistochemistry study carried out by Kirby et al. (15) demonstrated an increase in the expression of CYP 2A5 (equivalent to CYP 2A6 in humans) in liver fluke-infected hamsters. The increased expression of 2A5 was localized and detected only in the hepatocytes in inflamed areas of the fluke-infected liver. These sites may be equivalent to the fibrotic areas (detected radiologically as enhanced portal vein radicles) shown here to be associated with increased CYP 2A6 activity in humans. Furthermore, these lesions were associated with liver enlargement, which was also a determinant of 7-HC activity. Thus, if the increase in CYP 2A6 activity detected here is similarly localized to inflammatory sites where nitric oxide, NDMA, and other nitrosamines are endogenously formed and reaching a high local concentration, the likelihood of DNA damage may be dramatically enhanced. Indeed, this assumption
is supported by our observations on Opisthorchis-infected hamsters exposed to aflatoxin B1 (a high affinity substrate of CYP 2A5). High levels of aflatoxin B1 binding to DNA took place in areas of the liver where CYP2 A5 expression was high (15).

In contrast to the hamster studies (15), no sex difference in coumarin hydroxylation was observed in humans. However, we have shown in population-based ultrasound studies that males have nearly 3-fold higher odds of having portal vein radicle echoes, the factor shown here to determine whether CYP 2A6 expression is increased during infection, than females of similar age and intensity of infection (22). Thus, the increased risk of cholangiocarcinoma experience by males compared to females may be closely linked with enhanced susceptibility to biliary fibrosis, which may cause or may result from induction of CYP 2A6 activity.

The five individuals who excreted no 7-HC were analyzed for their CYP 2A6 genotype (25). A genetic defect was found in the CYP 2A6 gene and is a likely cause of the zero activity. This finding also confirms our earlier observations that the coumarin test is a highly specific probe for CYP 2A6, since it is unlikely that any other cytochrome that was capable of metabolizing coumarin in vivo was simultaneously inactive in those individuals. The chlorzoxazone test revealed that all five had normal CYP 2E1 activity.

In summary, we present in vivo evidence that the activity of CYP 2A6 is enhanced among people with moderate and heavy liver fluke infection who also have fibrosis of the intrahepatic bile duct. This induction may play an important role in the activation of nitrosamines, e.g., NDMA, generated within inflamed tissue of the biliary tract, and the malignant transformation of proliferating biliary epithelial cells. This sequence of events occurring within a small foci in the biliary tract may partially explain the very high frequency of cholangiocarcinoma arising in the liver fluke-infected biliary tract. This study has provided a unique opportunity to explore the relative importance of genetic versus environmental factors influencing the phenotype of an individual CYP in a human cancer model.

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