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NICOTINE METABOLISM IN PREGNANT AND NON-PREGNANT RABBITS

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Abstract

Smoking remains a major public health concern during pregnancy and is associated with numerous adverse effects. Recently the clearance of nicotine (NIC) and cotinine (COT) was shown to be substantially increased in pregnant women compared to non-pregnant controls. The present study investigated the usefulness of the rabbit for studying the molecular basis for the observed changes in NIC and COT disposition during pregnancy. NIC was largely metabolized to COT in rabbit liver microsomes (approximately 50% of total metabolism) with significant amounts of nicotine-N'-oxide and nornicotine also being detected. The conversion of NIC to COT was also detected in rabbit placental and fetal liver microsomes albeit at only a fraction of the rate in adult rabbit liver microsomes. The major products of COT metabolism in rabbit liver microsomes were 5'-hydroxycotinine, cotinine-N'-oxide and norcotinine. Differences between human and rabbit liver were most apparent for COT, with the major human metabolite 3'-hydroxycotinine, being formed at only low levels in rabbit liver microsomes. Pregnancy had no effect on the metabolism of NIC or on the expression of CYP2A6 immunoreactive proteins in rabbit liver microsomes. These studies provide a complete quantitative assessment of NIC metabolism in rabbit liver microsomes and suggest that the rabbit may not be an appropriate animal model to study the effects of pregnancy on NIC and COT metabolism. However, a molecular understanding of these effects is essential for prediction of the pharmacological and toxicological consequences of smoking during pregnancy.

Keywords

Nicotine; Pregnancy; Hepatic metabolism; Cotinine

INTRODUCTION

Cigarette smoking during pregnancy has long been associated with adverse pregnancy outcomes in the mother, fetus and newborn, and continues to be a major public health concern. Numerous studies have demonstrated an association between maternal smoking and low birth weight, premature delivery, placenta previa, placental abruption, an increased incidence of sudden infant death syndrome, and childhood behavioral problems (Ananth, Savitz, & Luther, 1996; Nordentoft et al., 1996; Sundell, 2004; Weitzman, Gortmaker, &

Sobol, 1992). Nicotine (NIC), a major constituent of tobacco, may contribute to some of the harmful effects of smoking during pregnancy (D. A. Dempsey & Benowitz, 2001). Despite knowledge of the harmful effects of smoking, many women continue to smoke throughout pregnancy and postpartum.

The biotransformation of nicotine (NIC) and cotinine (COT) has been described in animals and humans (Hukkanen, Jacob, & Benowitz, 2005; McCoy, DeMarco, & Koop, 1989; Turner, 1975; Tutka, Mosiewicz, & Wielosz, 2005; Williams, Ding, & Coon, 1990; Yi, Sprouse, Bowman, & McKennis, 1977). In the rabbit liver and in humans, metabolism to cotinine is a major pathway of nicotine metabolism (Hukkanen et al., 2005; McCoy et al., 1989). Despite the fact that many NIC and COT metabolites have been identified, relatively little is known about the regulatory aspects of NIC metabolism. NIC metabolites have pharmacological effects (Clark, Rand, & Vanov, 1965; Keenan, Hatsukami, Pentel, Thompson, & Grillo, 1994) and the quantitative determination of NIC metabolites is of particular importance for determination of their contribution to the pharmacological effects resulting from NIC exposure. Differences in the pattern of metabolite formation may contribute to individual variability in NIC elimination (Benowitz & Jacob, 1997).

Recently it has been shown that pregnant women metabolize NIC and COT significantly faster compared to the non-pregnant state (D. Dempsey, Jacob, & Benowitz, 2002). The major enzyme involved in NIC and COT metabolism in humans is CYP2A6 (Hukkanen et al., 2005) and we hypothesized that the expression and/or activity of this enzyme might be altered during pregnancy. Since NIC is commonly abused during pregnancy and human pregnancy alters the rate of NIC metabolism we studied the effects of pregnancy on liver and placental metabolism *in vitro*. The present study was designed (1) to determine the quantitative profile of NIC metabolism in rabbit liver microsomes, (2) to investigate the rabbit as a model for human NIC metabolism during pregnancy and (3) to test whether pregnancy in the rabbit influences the expression of hepatic CYP2A6 immunoreactive proteins.

METHODS

Tissue samples

Livers from near-term pregnant and non-pregnant New Zealand rabbits, fetal livers and placentas were purchased from Pel-Freez (Arkansas Div., Rogers, AR) or obtained through the campus tissue sharing program. Tissue samples were frozen in liquid nitrogen immediately after collection and stored at -80°C until preparation of microsomes.

Preparation of microsomes

Microsomes were prepared from frozen tissue using standard ultracentrifugation techniques and stored at -80°C (Kroetz, Huse, Thuresson, & Grillo, 1997). Cytosolic fractions were isolated from the same preparations. Microsomal and cytosolic protein concentrations were determined using the Pierce BCA protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard. Human liver microsomes and cytosol were purchased from Gentest (Woburn, MA).

Nicotine microsomal metabolism

Microsomal NIC metabolism was measured as previously described (Cashman et al., 1992). Typical reactions used 1 (adult livers and placentas) or 2 mg/ml (fetal livers) microsomal protein, 2 mg/ml rabbit liver cytosol, an NADPH regenerating system using isocitrate dehydrogenase (0.5 IU), 1 mM NADPH (Sigma, St. Louis, MO), and 10 μM S-(−)-NIC (Sigma) in potassiumphosphate buffer at pH 7.4. A stock NIC solution was prepared from

NIC base and the concentration was confirmed by gas chromatography (GC) with nitrogen-phosphorus detection using published methods (P. Jacob, 3rd, Wilson, & Benowitz, 1981). The rabbit cytosol fraction with the highest protein concentration was used as a source of aldehyde oxidase for all of the NIC metabolism assays. Incubations were carried out at pH 7.4 for 30 (adult livers and placentas) or 60 min (fetal livers) in a shaking water bath at 37°C. The reaction was stopped by boiling the sample for 5 min. Reaction conditions were established such that primary and secondary metabolite formation was linear with protein concentration and time. COT formation from NIC was measured in rabbit liver microsomes at varying concentrations of NIC (5–100 µM) and the data were fit to a single enzyme Michaelis-Menten model by nonlinear regression.

Cotinine microsomal metabolism

The metabolism of COT was measured in incubations containing 0.5 mg/ml microsomal protein, a NADPH regenerating system using isocitrate dehydrogenase (0.5 IU), 1 mM NADPH, and 500 µM COT (Sigma, St. Louis, MO). Incubations were carried out at pH 7.4 for 30 min in a shaking water bath at 37°C. The reaction was stopped by boiling the sample for 5 min.

Nicotine and metabolite assays

NIC and COT were assayed by GC using an established method, modified for simultaneous extraction of NIC and COT and determination using a capillary column (P. Jacob, 3rd et al., 1981; P. I. Jacob & Benowitz, 1991). Concentrations of other NIC metabolites were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (P. I. Jacob, Yu, & Benowitz, 2002). Deuterium-labeled internal standards (cotinine d_9 and *trans*-3'-hydroxycotinine- d_7) were added to the incubation mixtures, which were made alkaline with 50% (w/v) tripotassium phosphate, and extracted with methylene chloride. The extracts were acidified by adding methanolic HCl, and evaporated using a Savant centrifugal vacuum evaporator. The evaporated extracts were reconstituted in 10 mM aqueous ammonium formate, and injected into the LC-MS/MS system, which consists of a Hewlett-Packard 1040 HPLC interfaced to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer. Separations were carried out using a Phenomenex Max-RP column (4.6 mm x 150 mm) and a gradient elution of aqueous 10 mM ammonium formate to 10 mM ammonium formate in methanol. Mass spectrometric analyses were performed using atmospheric pressure chemical ionization (APCI) and selected reaction monitoring (SRM) with argon as the collision gas at a pressure of 2.5 millitorr. The metabolites and the transitions that were measured were: cotinine, m/z 177 to 80; nicotine-N'-oxide, m/z 179 to 132; nornicotine, m/z 149 to 130; *trans*-3'-hydroxycotinine and 5'-hydroxycotinine, m/z 193 to 80; cotinine-N'-oxide, m/z 193 to 96; norcotinine, m/z 163 to 80; cotinine- d_9 (internal standard), m/z 186 to 84; and *trans*-3'-hydroxycotinine- d_7 (internal standard), m/z 200 to 84. Quantitation was carried out using the peak area ratios of analyte to internal standard and standard curves constructed using linear regression. Lower limits of quantitation were 10 ng/mL or less for all analytes. For validation of both methods, we use the criteria of Shah et al. (Shah et al., 2000) to validate our methods for precision, accuracy, and to determine the LOQ. These are precision within $\pm 15\%$ and accuracy within $\pm 15\%$ of the expected amount, except at the lower limit of quantitation, for which $\pm 20\%$ is considered acceptable.

CYP content

Total CYP content in microsomes was calculated from the reduced carbon monoxide difference spectra using an extinction coefficient of $91 \text{ mM}^{-1}\text{cm}^{-1}$ (Omura & Sato, 1964). Difference spectra were measured on an Aminco DW-2000 UV-VIS spectrophotometer.

Immunoblotting of CYP proteins

The levels of CYP2A immunoreactive proteins were detected by Western blotting. Hepatic microsomal proteins (10–50 µg) were separated on a 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Trans-Blot, Bio-Rad, Hercules, CA) in 25 mM Tris, 192 mM glycine, and 20% methanol for 30 min at room temperature. Membranes were incubated overnight with a 1000-fold dilution of mouse anti-human CYP2A6 antisera (Gentest, Woburn, MA) followed by a 2000-fold dilution of alkaline phosphatase conjugated anti-mouse IgG (Bio-Rad). Immunoreactive proteins were detected using an alkaline phosphatase conjugate substrate kit (Bio-Rad).

Statistical analysis

Metabolite formation rates are reported as mean $\pm$ SD for samples from three to ten animals. Total CYP concentrations are reported as mean $\pm$ SD for samples from five animals in each group. Statistical significance of differences between groups was evaluated by an unpaired t-test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Aerobic incubation of NIC with rabbit hepatic microsomes in the presence of NADPH resulted in the formation of seven metabolites. The major product of NIC metabolism in rabbit liver microsomes was COT. COT formation at 50 µM NIC increased linearly for up to 30 min in the presence of 0.2–1 mg/ml microsomal protein and a constant concentration (2 mg/ml) of cytosolic protein (data not shown). Cytosolic protein was used as the source of aldehyde oxidase which is required for the first step in COT formation. The concentration of NIC was varied and the COT formation data were fit to a single enzyme Michaelis-Menten model which generated the following parameter estimates: $K_m = 13.2 \pm 4.0$ µM and $V_{max} = 978 \pm 89.2$ pmol/min/mg protein. Cytosolic protein concentrations from 1–10 mg/ml did not significantly alter NIC metabolism (data not shown). The mean rates of COT formation in rabbit liver microsomes are shown in Figure 1A. The rates of COT formation were similar in hepatic microsomes from nonpregnant and pregnant rabbits. COT formation rates were at least twice the rate of the other metabolites.

Greater than 75% of the starting NIC could be accounted for by the appearance of seven metabolites in hepatic microsomes. In addition to COT, only nicotine-N'-oxide and norcotinine were present in significant amounts (16% and 12% of all metabolites in non-pregnant animals, respectively). Four other metabolites of NIC were also detected: norcotinine, cotinine-N'-oxide, 3'-hydroxycotinine, and 5'-hydroxycotinine (Figure 1A). The results from a quantitative assessment of COT metabolism in rabbit liver microsomes are presented in Figure 1B. COT was metabolized to four metabolites. Norcotinine, cotinine-N'-oxide, and 5'-hydroxycotinine were formed in similar amounts and there were no differences in formation rates between liver microsomes from non-pregnant or pregnant rabbits. The rate of formation of 3'-hydroxycotinine was only a fraction of the rate of formation of norcotinine, cotinine-N'-oxide or 5'-hydroxycotinine.

COT formation from NIC was also compared in rabbit adult liver, fetal liver, and placental microsomes (Figure 2). The rate of COT formation was 752 ± 227 pmol/min/mg protein in liver microsomes from non-pregnant rabbits and was much higher than corresponding rates in placental and fetal liver microsomes. In placental microsomes, NIC metabolism to COT was only seven percent of that in liver (53 ± 17.2 pmol/min/mg protein) and COT formation was barely detectable in fetal liver (14.3 ± 7.1 pmol/min/mg protein).

Total P450 content was similar in liver microsomes of pregnant and non-pregnant animals (11.1 ± 4.12 vs. 10.4 ± 1.95 nmol/mg protein, respectively). The CYP content in the rabbit

placental microsomes (3.49 ± 1.99 nmol/mg protein) was only 30% of that in pregnant rabbit liver microsomes and that in fetal liver microsomes (0.96 ± 0.68 nmol/mg protein) was 9% of that in pregnant rabbit liver microsomes.

Western blots of hepatic microsomes with an antibody against human CYP2A6 were used to compare the levels of CYP2A immunoreactive proteins in liver microsomes from non-pregnant and pregnant rabbits. Unfortunately, the cross-reactivity of this antibody with the rabbit CYPs has not been characterized but this antibody is highly specific for human CYP2A6 and shows no cross-reactivity with CYP1A, CYP1B, CYP2B, CYP2C, CYP2D or CYP3A human isoforms. The human CYP2A6 antibody also detects CYP2E1 with a lower mobility than CYP2A6 (Gentest product information). Western blot detection of rabbit liver microsomes with this antibody results in two distinct immunoreactive proteins and we are assuming that CYP2A6 is the upper band (Figure 3). The level of both immunoreactive proteins showed significant intra-rabbit variability, but there was no apparent difference in the level of expression between pregnant and non-pregnant rabbit liver microsomes.

DISCUSSION

The present study evaluated the rabbit as an appropriate animal model to investigate the regulation of NIC during pregnancy. It has been shown that NIC biotransformation can differ among species (Jenner, Gorrod, & Beckett, 1973; Kyerematen, Morgan, Warner, Martin, & Vesell, 1990; Nwosu & Crooks, 1988) but the extent and range of that variability are not clearly determined. An earlier *in vivo* study had shown that NIC is converted extensively to cotinine in the rabbit as it is in humans (P. Jacob, 3rd, Benowitz, Copeland, Risner, & Cone, 1988), suggesting that the rabbit would be a good animal model in which to study the regulation of nicotine metabolism. COT was identified as the major NIC metabolite in rabbit liver microsomes, similar to *in vitro* and *in vivo* studies in humans (Benowitz, Jacob, Fong, & Gupta, 1994; Berkman, Park, Wrighton, & Cashman, 1995; McCoy et al., 1989). COT was also the main NIC metabolite in rabbit lung microsomes (Williams, Shigenaga, & Castagnoli, 1990). We identified six other NIC and COT metabolites in rabbit liver microsomal incubations. Nornicotine and nicotine 1'-N-oxide were formed at the highest rates followed by norcotinine and 5'-hydroxycotinine. Cotinine-N'-oxide and 3'-hydroxycotinine were barely detectable under these conditions. Similar to results from human studies, COT and COT metabolites accounted for 71% of the total NIC conversion in rabbit liver microsomes. The formation of nicotine 1'-N-oxide and nornicotine in rabbit liver microsomes account for 16.4 and 12.1% of the total metabolite production, respectively. In human liver microsomes, nicotine 1'-N-oxide and nornicotine account for 21.3 and 0.5% of total metabolites (Tutka et al., data unpublished). While nicotine 1'-N-oxide and nornicotine account for 12–16% of total NIC conversion in rabbit liver microsomes, <4% of a systemic dose of NIC in chronic smokers is excreted as nicotine 1'-N-oxide and only 0.65% is excreted as nornicotine (Benowitz et al., 1994). This indicates species differences in the minor metabolic pathways for NIC between rabbits and humans.

COT metabolism was also measured directly. COT was efficiently metabolized by rabbit liver microsomes to four major products. Norcotinine, cotinine-N'-oxide and 5'-hydroxycotinine were formed at similar rates in rabbit liver microsomes and account for 93% of COT metabolism. The metabolism of COT to 3'-hydroxycotinine is only a minor pathway in rabbit liver microsomes but is the major COT metabolite in human smokers (Benowitz et al., 1994). Neither 5'-hydroxycotinine or norcotinine were detected in the urine of smokers (Benowitz et al., 1994). Such species differences in the pattern of NIC and COT metabolism suggest the involvement of distinct cytochrome P450 and flavin monooxygenase isoforms in rabbits and humans.

The recent study by Dempsey and coworkers (D. Dempsey et al., 2002) indicates that the metabolism of NIC and COT is increased in pregnant women. NIC clearance was 60% higher and COT clearance was 140% higher in pregnant women compared to non-pregnant women. These data led us to hypothesize that a specific P450 enzyme was induced in the liver of pregnant women. NIC metabolism to COT can be catalyzed by purified rabbit CYP2B4, CYP2C3 and 2A10/2A11 (McCoy et al., 1989; Williams, Ding et al., 1990; Williams, Shigenaga et al., 1990). Consistent with the evidence for CYP2A enzymes in nicotine metabolism in the rabbit, we have found that in the presence of the CYP2A inhibitor coumarin, or the CYP2A6 antibody used in the immunoblotting experiments in this study, COT formation from NIC is strongly inhibited (Tutka et al., data unpublished). In the present study, there were no differences in the hepatic metabolism of either NIC or COT or in the expression level of CYP2A immunoreactive proteins in liver microsomes from pregnant and non-pregnant rabbits. A major objective of the present study was to determine whether the rabbit would be an appropriate model to address the mechanistic basis for changes in NIC and COT clearance during pregnancy. The significant differences in COT metabolism between human and rabbit liver microsomes and the lack of effect of pregnancy on NIC and COT metabolism do not support the use of the pregnant rabbit for these purposes. However, it is possible that in vivo NIC metabolism may not be accurately reflected by our in vitro studies or that smoking itself may influence NIC metabolism in pregnancy. Further studies will need to be carried out to identify a more appropriate model.

In an isolated perfused rabbit lung, NIC was found to be transformed mainly to COT (34%), nicotine 1'-N-oxide (14%), 3-hydroxycotinine (10%) and cotinine 1'-N-oxide (3%) (Aislaitner et al., 1997). Normicotine and norcotinine were detected in only low amounts. With the exception of the lung and nasal mucosa, limited studies have been carried out to characterize the extrahepatic metabolism of NIC (Hukkanen et al., 2005). In the present study, NIC was metabolized at very low rates in fetal liver and placental microsomes relative to adult liver. The relative rates of NIC metabolism in fetal liver and placental microsomes suggest that in the rabbit the placenta may serve as an important protective barrier against NIC exposure in the fetus. It is of interest to understand the importance of placental and fetal metabolism of NIC in humans as well.

In summary, the present study provides a complete quantitative characterization of NIC and COT metabolism in rabbit liver. Although the major metabolic pathway for NIC is identical in human and rabbit, species differences exist in the pattern of COT metabolism and the formation of minor NIC metabolites. The lack of effect of pregnancy on hepatic NIC metabolism discounts the value of the rabbit for elucidating the molecular mechanisms responsible for altered NIC and COT metabolism during pregnancy. Identification of an appropriate animal model to address this and related questions concerning regulation of NIC metabolism will be invaluable in understanding the basis for interindividual differences in the disposition and pharmacological effects of NIC.

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Figure 1. Profile of nicotine (A) and cotinine (B) metabolism in rabbit liver microsomes Microsomal proteins (1 mg/ml) were incubated at 37°C with 20 μ M nicotine or 500 μ M cotinine. Metabolite formation was determined by gas chromatography and liquid chromatography-tandem mass spectrometry as described in the Materials and Methods section. The data shown are the means $\pm$ SD of 8–10 determinations in liver microsomes from non-pregnant (solid bars) and pregnant (hatched bars) rabbits. COT, cotinine; NNO, nicotine-N'-oxide; NORN, nornicotine; 3-OHCOT, 3'-hydroxycotinine; 5-OHCOT, 5'-hydroxycotinine, CNO, cotinine-N'-oxide; NORC, norcotinine.

Figure 2.

Cotinine formation in microsomes from rabbit adult non-pregnant and pregnant liver, placenta and fetal liver. Microsomal proteins (1 mg/ml for adult livers and placenta for 30 min or 2 mg/ml for fetal liver for 60 min) were incubated at 37°C with 20 μ M nicotine. Cotinine formation was determined by gas chromatography as described in the Methods section. The data shown are the means $\pm$ SD of samples from 5–10 rabbits.

Figure 3.

Immunoblotting of CYP2A6 immunoreactive proteins in non-pregnant and pregnant rabbit liver. Microsomal proteins (50 μ g) were separated on a 8% SDS-polyacrylamide gel, transferred to nitrocellulose and blotted with antisera against human CYP2A6. Immunoreactive proteins were detected using an alkaline phosphatase conjugate substrate kit. Each lane represents a liver microsomal sample from an individual rabbit.