

***In vitro* Characterization of the Oxidation of a Pyridinium Metabolite of Haloperidol by Human Placenta: The Effect of Smoking**

Jim Fang^{1,*} and Jiuxue Song¹

¹ College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada.

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Abstract - Purpose. The antipsychotic drug haloperidol can be metabolised to pyridinium metabolites haloperidol pyridinium (HP⁺) and reduced haloperidol pyridinium (RHP⁺). These pyridinium metabolites were proposed to contribute to the extrapyramidal side effects of haloperidol, because they are structural analogues of N-methyl-4-phenylpyridinium (MPP⁺), a well-known neurotoxin. RHP⁺ can be oxidized to HP⁺ by CYP1A1. In the current study, the oxidation of RHP⁺ to HP⁺ was investigated using human placenta microsomal preparations which contain relatively high levels of CYP1A1. **Methods.** Cytochrome P450 isoenzymes responsible for the metabolism of RHP⁺ were characterized in vitro using human placenta microsomal preparations from smokers and non-smokers. **Results.** A comparison of the metabolic activities between smokers and non-smokers suggests that smokers had higher activities for the oxidation of RHP⁺. A selective antibody against CYP1A1 was a partial inhibitor of RHP⁺ oxidase in placenta from smokers but had no effect in placenta from non-smokers. Furafylline and ketokonazole were shown to be stronger inhibitors of the oxidation of RHP⁺ to HP⁺ in liver than in placenta. This seems to indicate important contributions of CYP1A1 and CYP3A7 as compared to CYP1A2 and CYP3A4, respectively, because furafylline and ketokonazole are stronger inhibitors of CYP1A2 and CYP3A4 than CYP1A1 and CYP3A7, respectively. Interestingly, α -naphthoflavone enhanced the metabolic activity in liver microsomes due to its activator effect on CYP3A4. On the other hand, α -naphthoflavone partially inhibited the activity in placenta microsomes, indicating a role played by CYP1A1 or CYP1A2 in the oxidation of RHP⁺ in placenta. **Conclusions.** These data indicate that CYP1A1 plays an important role in the oxidation of RHP⁺ to HP⁺ in placenta from smokers. CYP3A7 and CYP3A4 could also play important roles in the metabolism of RHP⁺ in placenta microsomes.

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INTRODUCTION

The antipsychotic drug haloperidol [4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4'-fluorophenyl)-1-butanone] can induce movement disorders in patients which is a major concern in its clinical use. Haloperidol was shown to be converted to a pyridinium metabolite, 4-(4-chlorophenyl)-1-(4-fluorophenyl)-4-oxobutylpyridinium (HP⁺) (1-4). Further studies identified reduced HP⁺ (RHP⁺) [4-(4-chlorophenyl)-1-(4-fluorophenyl)-4-hydroxybutylpyridinium] in the plasma and urine of haloperidol treated patients (5). Both HP⁺ and RHP⁺ are structurally similar to N-methyl-4-phenylpyridium (MPP⁺), a well-known neurotoxin that causes Parkinson's syndrome in human and experimental animals. As haloperidol induces pronounced and sometimes irreversible movement disorders in its clinical use, it was proposed that the pyridinium metabolites may contribute to these side

effects.

HP⁺ and RHP⁺ has been detected in the brains of patients who have chronically received haloperidol (6). Average brain concentrations of HP⁺ and RHP⁺ were found to be 4.1 and 2.7 pmol/g tissue 14-25 hours post-mortem. It was suggested that HP⁺ is formed mainly in the liver and can pass through the blood-brain barrier (7). Both HP⁺ and RHP⁺ were found to be substrates of organic cation transporters present in the brain suggesting a possible role of active transporters in their passage through the blood-brain barrier (8).

Corresponding Author: Jim Fang, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; E-mail: jim.fang@usask.ca

Numerous studies have been conducted to examine the neurotoxicity of the pyridinium metabolites of haloperidol in the brain. Baboons developed orofacial dyskinesia following chronic treatment with the dehydration product of haloperidol (HTP, 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridine) which is metabolized to HP^+ and RHP^+ (9). The induced orofacial dyskinesia persisted after HTP was ceased and is accompanied by loss of neurons. Intrastriatal microdialysis studies in rat brain showed that perfusion of HP^+ leads to irreversible depletion of striatal dopamine, although HP^+ was less potent than MPP^+ (10). However, intracortical microdialysis studies indicated that the two compounds displayed comparable toxic effects on the cortical serotonergic system (10). In vitro studies shown that HP^+ was a more potent inhibitor of mitochondrial respiration than MPP^+ (10). HP^+ was also found to be toxic to dopaminergic and serotonergic neurons in cultures of embryonic mesencephalic cells and human neuroblastoma SH-SY5Y cells (11, 12). Additionally, HP^+ was found to be a potent inhibitor of dopamine and noradrenaline uptake (13) and a inhibitor of monoamine oxidase (14). The strongest support so far for the "pyridinium hypothesis" of the extrapyramidal side effects of haloperidol comes from a study on 41 psychiatric patients on long-term treatment with haloperidol. It was found that the severity of tardive dyskinesia and parkinsonism is strongly associated with an increased ratio of HP^+ /haloperidol in serum (15). Although other types of antipsychotic drugs such as chlorpromazine also produce movement disorders, severe tardive dyskinesia is found most often for haloperidol and bromperidol (15, 16).

The metabolism of haloperidol has been characterized in vitro using human liver microsomes and recombinant human cytochrome P450 isoenzymes (17, 18). In the experiments with a panel of human liver microsomes from 12 donors, the metabolic activities on haloperidol were correlated with catalytic activities of selective substrates of different P450 isoenzymes and immuno-reactivities toward different P450 isoenzymes. It was found that CYP3A4 activities were correlated to the catalytic activities on most metabolic pathways of haloperidol. This suggests a prominent role for CYP3A4 in the metabolism of haloperidol. Among the recombinant CYP enzymes investigated, CYP1A1, CYP1A2, CYP3A4 were able to oxidize RHP^+ to HP^+ . Interestingly, RHP^+ was shown to be a good substrate of CYP1A1 with activities about 50 times higher than CYP1A2 and

220 times higher than CYP3A4 (17, 18) under the experimental conditions.

While CYP1A1 is not normally present at detectable levels in human liver (19), it is expressed in extrahepatic tissues such as placenta (20), lung and intestine (21). Herein, we report that CYP1A1 plays an important role in the oxidation of RHP^+ to HP^+ in human placenta from smokers. Other enzymes such as CYP3A7 and CYP3A4 may play more important roles in placenta from non-smokers.

MATERIALS AND METHODS

Materials

HP^+ was synthesised as previously described (4). RHP^+ was a gift from Dr. Neal Castagnoli, Jr. (Virginia Tech, VA, USA). Pooled human liver microsomal preparations (HLM161), and human recombinant CYP1A1, CYP3A7 and CYP1A1 anti-serum were purchased from Gentest Corporation (Woburn, MA, USA). Human placentas were obtained from Royal University Hospital (Saskatoon, SK, Canada) and microsomal preparations were prepared according to standard procedure as described previously (4). Protein levels in placenta microsomal preparations were determined using a commercial assay kit (Phenol reagent method for biologic fluids, Sigma diagnostics, St. Louis, MO, USA). The microsomal preparations were adjusted to protein concentration of 0.1 mg/ml for the enzymatic studies.

HPLC analysis

HP^+ and RHP^+ were analysed using a slight modification of an HPLC method described previously (22). Briefly, the system comprised a Waters 515 pump and a Waters 717_{plus} autosampler. A Waters 474 scanning fluorescence detector (excitation wavelength: 300nm; emission: 380nm) was used for the detection of HP^+ and RHP^+ . Signals from the fluorescence detector were collected and processed by a Waters Millennium 32 Chromatography Manager system. A Hypersil CN, 5 μ m column (4.6 x 250 mm) (Phenomenex[®], Torrance, CA, USA) coupled with a SecurityGuardTM guard cartridge system (Phenomenex[®], Torrance, CA, USA) was utilised. The mobile phase consisted of acetonitrile-ammonium acetate buffer (0.1 M)-water (67:10:23 v/v). The mixture was adjusted to pH 5.4 with acetic acid and the solvent was delivered at a flow rate of 1 ml/min.

Enzymatic Studies

Incubation procedures were as follows: 0.1 ml reaction mixtures containing 10 μ l microsomal preparation (recombinant CYP3A7 or human liver microsomes or placenta microsomes from smokers or non-smokers), a cofactor-generating system [consisting of] β -nicotinamide adenine dinucleotide phosphate (1.3 mM), glucose 6-phosphate (3.3 mM), glucose 6-phosphate dehydrogenase (0.4 U/ml) and $MgCl_2$ (3.3 mM) and appropriate concentrations of substrates (in phosphate buffer, 0.1 M, pH 7.4) the reaction mixtures enzyme preparations were incubated at 37 °C. Control incubates contained heat-inactivated microsomes or control microsomes transfected with a control vector. Biological reactions were terminated by the addition of acetonitrile (50 μ l) and denatured proteins were removed by centrifugation. The clear supernatants were subjected to HPLC analysis.

For the panel study, placenta microsomes (10 μ l) from smokers or non-smokers were incubated with 20 μ M RHP⁺ for 60min in the presence of a cofactor-generating system. Biological reactions were terminated by the addition of acetonitrile (50 μ l) and denatured proteins were removed by centrifugation. The clear supernatants were subjected to HPLC analysis. A preliminary study confirmed that the enzymatic activity continue to increase significantly (but not linearly) with time at 60min. This incubation time was used so that enzymatic activities from all placenta samples can be quantified.

Inhibition by CYP1A1 anti-serum

For inhibition by antibody against CYP1A1, microsomal preparations (recombinant CYP1A1 or pooled placenta microsomes from smokers or non-smokers, 10 μ l) were mixed with CYP1A1 anti-serum or normal goat serum and preincubated at room temperature for 30 min. The microsome/antibody mixtures were then placed on ice and the following components added: a cofactor-generating system [β -nicotinamide adenine dinucleotide phosphate (1.3 mM), glucose 6-phosphate (3.3 mM), glucose 6-phosphate dehydrogenase (0.4 U/ml) and $MgCl_2$ (3.3 mM)] and appropriate concentrations of substrates (in phosphate buffer, 0.1 M, pH 7.4). The total volume of reaction mixtures was 0.1 ml. The enzymatic reactions were started by transferring the mixture to a 37 °C water bath and were carried out for 10 min. Biological reactions were terminated by the addition of acetonitrile (50 μ l) and denatured proteins were removed by centrifugation. The clear supernatants were subjected to HPLC analysis.

Inhibition by chemical inhibitors

For inhibition studies, incubation procedures were as follows: 0.1 ml reaction mixtures containing a cofactor-generating system [consisting of] β -nicotinamide adenine dinucleotide phosphate (1.3 mM), glucose 6-phosphate (3.3 mM), glucose 6-phosphate dehydrogenase (0.4 U/ml) and $MgCl_2$ (3.3 mM) and appropriate concentrations of substrates and inhibitors [in phosphate buffer, 0.1 M, pH 7.4]. To the above reactions mixtures were added 10 μ l microsomal preparation (human liver microsomes, placenta microsomes from smokers or non-smokers) and enzymatic reactions were carried out at 37 °C for 60 min. Biological reactions were terminated by the addition of acetonitrile (50 μ l) and denatured proteins were removed by centrifugation. The clear supernatant was subjected to HPLC analysis. The selective inhibitors used are furafylline (CYP1A1/CYP1A2), ketoconazole (CYP3A4), and α -naphthoflavone (CYP1A2). Their concentrations in the incubation mixtures are as shown in Figure 3.

RESULTS

The present study demonstrated catalytic activities present in human placenta microsomes. These activities for the oxidation of RHP were not present in control incubates without microsomes, without the NADPH generating system, without the substrates, or with heat-inactivated microsomal preparation (by incubating in boiling water for 10 min). This confirmed that the observed oxidation of RHP⁺ is catalysed by enzymes requiring NADPH.

A series of studies was conducted to characterize the enzyme(s) responsible for the oxidation of RHP⁺ in human placenta microsomes and the effect of smoking. Firstly, recombinant human CYP3A7 was shown to catalyse the oxidation of RHP⁺ at a rate of 4.90 ± 0.26 pmol/pmol P450/h. This adds to the list of enzymes capable of catalysing this reaction (CYP1A1, CYP1A2, CYP3A4) (17, 18). Secondly, the oxidation of RHP⁺ to HP⁺ was investigated using human placenta microsomal preparations from 12 smokers and 12 non-smokers (Figure 1). A comparison of the metabolic activities between smokers and non-smokers suggests that smokers had slight, but statistically significant higher activities for the oxidation of RHP⁺. Thirdly, an inhibitory antibody against human CYP1A1 was investigated for its ability to inhibit the oxidation of RHP⁺ (Figure 2). The antiserum was shown to be a partial inhibitor of RHP⁺ oxidase in placenta preparations from smokers but had no effect in placenta preparations

from non-smokers. Finally, different chemical inhibitors were investigated for their capacity to inhibit the oxidation of RHP⁺ (Figure 3). Ketoconazole and furafylline were shown to be stronger inhibitors of the oxidation of RHP⁺ to HP⁺

in human liver microsomes than in human placenta microsomes. α -Naphthoflavone enhanced the metabolic activity in human liver microsomes but partially inhibited the activity in placenta microsomes.

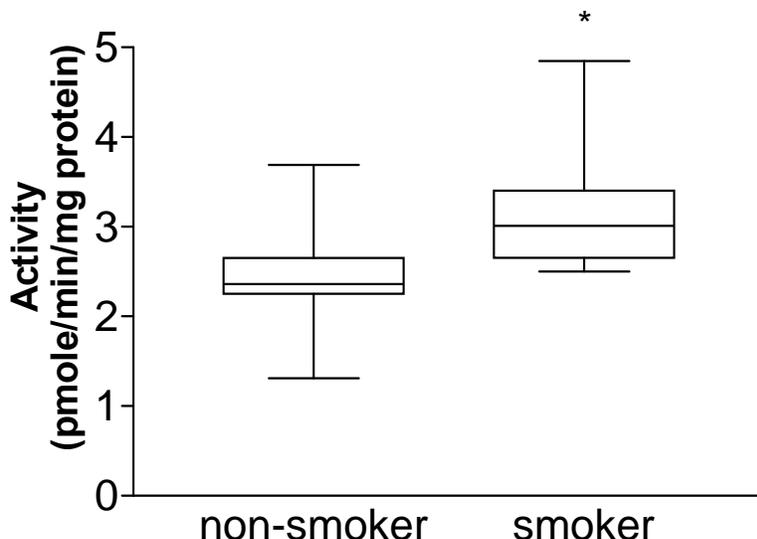


Figure 1. Comparison of the rate of oxidation of RHP⁺ to HP⁺ in placental microsomal preparations from smokers (n=12) and non-smokers (n=12). Box-and-whiskers graph. The box extends from the 25th to the 75th percentile and the line at the middle is the median. The error bars, or whiskers extend down to the lowest value and up to the highest. * P<0.05 (Mann-Whitney U test)

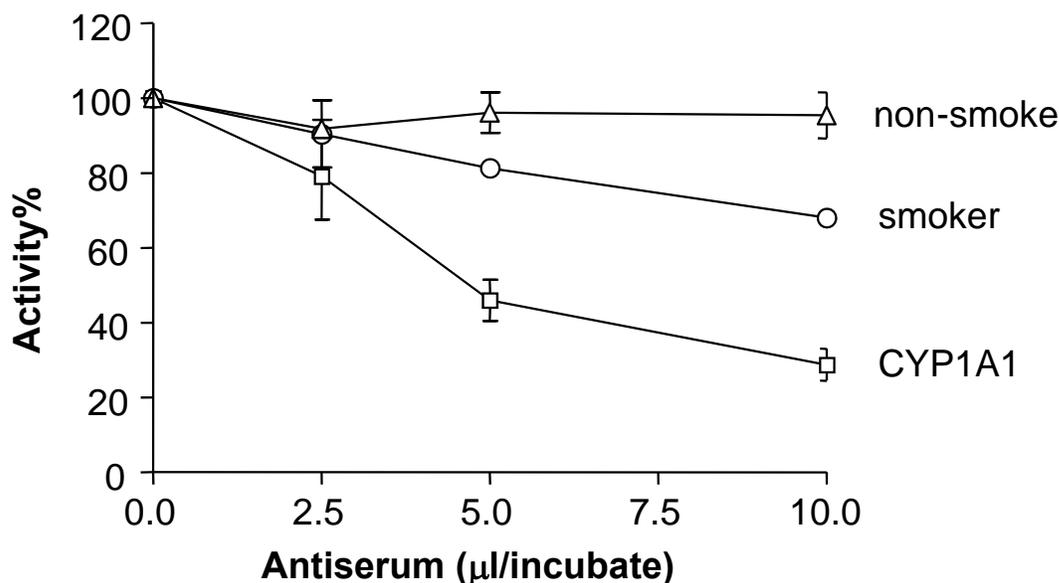


Figure 2. Effects of an antiserum against CYP1A1 on the metabolism of RHP⁺ by recombinant CYP1A1 and human placenta from smokers and non-smokers.

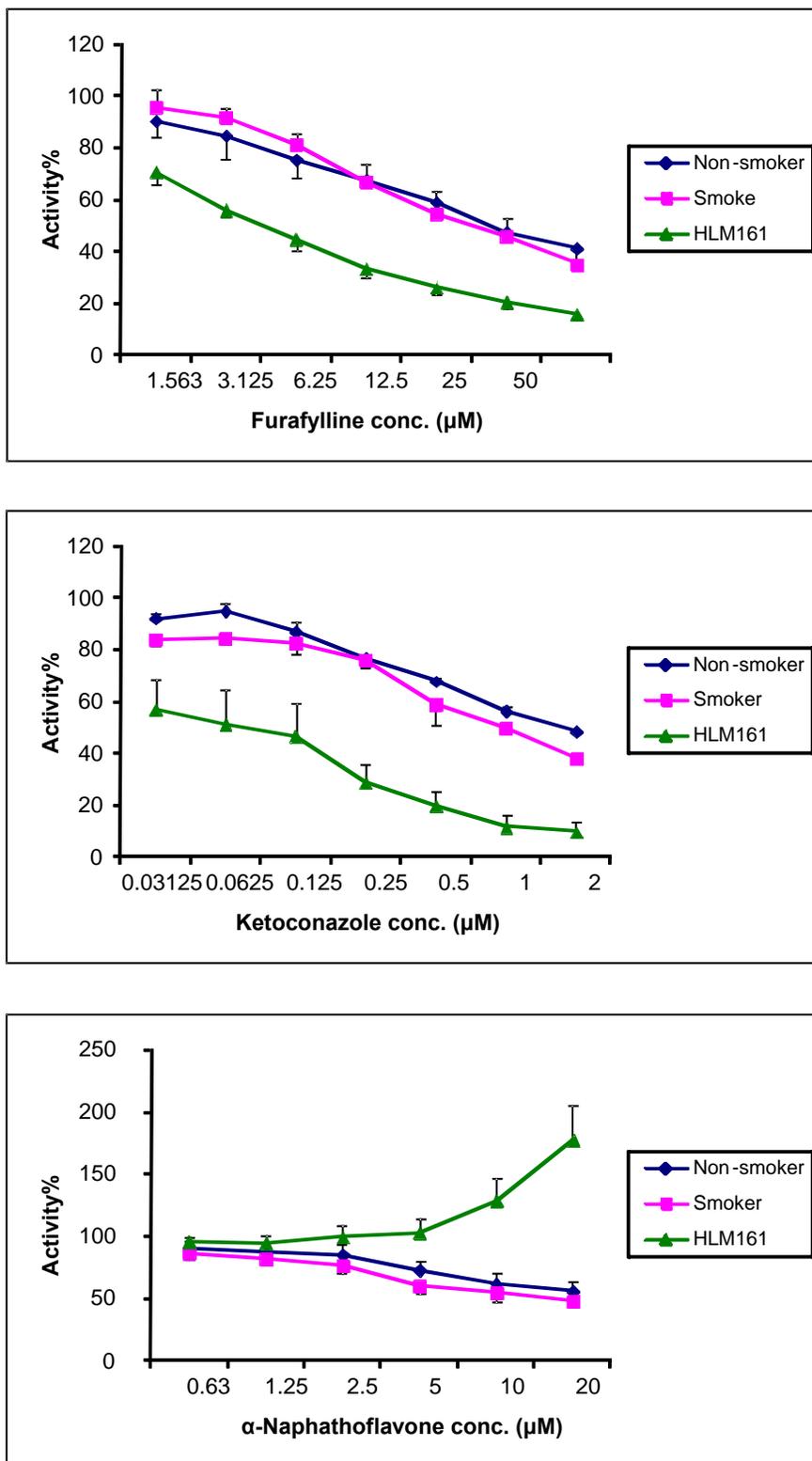


Figure 3. Effects of furafylline, ketoconazole and α -naphthoflavone on the oxidation of RHP⁺ in human liver and placenta from smokers and nonsmokers.

DISCUSSION

CYP enzymes responsible for the oxidation of RHP⁺

RHP⁺ was investigated as a substrate of recombinant CYP1A1, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 (17). Recombinant CYP1A1 showed the highest activity (123.9 pmol/pmol P450/h) followed by CYP3A7 (4.90 pmol/pmol P450/h), CYP1A2 (2.55 pmol/pmol P450/h), and CYP3A4 (0.56 pmol/pmol P450/h). Recombinant CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A5 did not show detectable activity for this metabolic reaction.

In human liver microsomes, CYP3A4 seems to be the dominant enzyme responsible for the oxidation of RHP⁺ to HP⁺. This conclusion is supported by the study with recombinant CYP isoenzymes, a correlation study and the fact that the enzyme activity can be inhibited by ketoconazole, a selective inhibitor of CYP3A4 (17). Although recombinant CYP1A2 is shown to be able to catalyse the oxidation of RHP⁺ to HP⁺, there is not a significant correlation between this catalytic activity and catalytic and immunoactivities of CYP1A2 (17). Thus, it seems that CYP1A2 plays only a minor role in the oxidation of RHP⁺ to HP⁺ in human liver.

Effects of smoking

CYP1A1 is said to be the most important xenobiotic-metabolizing enzyme of the placenta (23). Placenta mRNAs of CYP1A1, CYP1A2, CYP2C, CYP2D6, CYP2E1, CYP2F1, CYP3A4, CYP3A5, CYP3A7 and CYP4B1 have been detected in the first trimester while CYP1A1, CYP2E1, CYP2F1, CYP3A3, CYP3A4, CYP3A5 and CYP4B1 mRNAs are expressed in the full-term placenta (24-26). However, not all of these mRNAs translated into detectable proteins or catalytic activity. CYP1A1 is the only placental CYP enzyme for which significant expression has been conclusively demonstrated in the placenta throughout the entire pregnancy.

In order to further characterize RHP⁺ as a substrate of CYP1A1, more study were carried out using human placenta microsomal preparations from smokers and non-smokers. Placenta microsomal preparations from smokers exhibited higher catalytic activities for the oxidation of RHP⁺ than those from non-smokers (Figure 1). This

elevated enzyme activity is likely due to the induction of CYP1A1 by smoking because the placentas were from women of similar ages. It is well-established that smoking can induce placental CYP1A1 at the mRNA level (27-31) and at protein and enzyme activity level (32-37).

Effects of antibodies against CYP1A1

Selective antibodies against CYP1A1 were used to further characterize the contribution of CYP1A1 to the oxidation of RHP⁺. It was shown that the antiserum could inhibit the oxidation of RHP⁺ in microsomal preparations from smokers and placenta recombinant CYP1A1. The antiserum did not exhibit an inhibitory effect in microsomes from non-smokers (Figure 2). Smoking placenta seems to increase the relative contribution of CYP1A1, making the placenta more sensitive to the inhibition by the antiserum of CYP1A1. Thus, although CYP1A1 plays an important role in the oxidation of RHP⁺ in placenta from smokers, CYP enzyme(s) other than CYP1A1 probably play(s) an important role for the conversion of RHP⁺ to HP⁺ in placenta from non-smokers. mRNAs of CYP1A1, CYP3A3/4, and CYP3A5 were detected in full-term human placenta by reverse transcriptase-polymerase chain reaction (RT-PCR) (20, 38). Immunoblot analysis of microsomes from placentas from early pregnancy revealed immunoreactive bands by an antihuman CYP3A4 antibody (38). Therefore, in addition to CYP1A1, the other contributing enzyme responsible for the oxidation of RHP⁺ could be CYP3A4 or CYP3A7.

Effects of selective inhibitors of CYP

Thus, another series of experiments were carried out to investigate the inhibition of the oxidation of RHP⁺ to HP⁺ by furafylline, ketoconazole, and α -naphthoflavone (Figure 3). Furafylline can inhibit the oxidation of RHP⁺ to HP⁺ in human liver microsomal preparations. In human placenta microsomal preparations, furafylline was shown to partially inhibit the oxidation of RHP⁺ to HP⁺, indicating a role for CYP1A2 and/or CYP1A1 in catalysing this reaction. Furafylline inhibits the oxidation of RHP⁺ to a similar degree in placenta microsomal preparations from both smokers and non-smokers. This may be explained by the fact that smoking selectively induces CYP1A1 and that furafylline is a weaker inhibitor of CYP1A1 as compared to CYP1A2 (39). Furafylline was shown to selectively inhibit CYP1A2 at concentrations

below 20 μM , but can inhibit CYP1A1 at higher concentrations. It was shown that furafylline did not inhibit CYP1A1 in placenta from smokers (40). Inhibition of multiple enzymes with different IC_{50}s leads to a flatter inhibition curve (41). This phenomenon was observed in the inhibition curves of furafylline, ketoconazole, and α -naphthoflavone (Figure 3).

Ketoconazole, a CYP3A4 inhibitor, showed a relatively weak inhibitory effect to the oxidation of RHP^+ in placenta as compared to that in liver (Figure 3). This indicates a less important role of CYP3A4 in the oxidation of RHP^+ in human placenta. The CYP3A enzymes are different in their susceptibility to inhibition by inhibitors. For example, ketoconazole was shown to inhibit CYP3A4 more strongly than CYP3A5 and CYP3A7 (42, 43). It is therefore suggested that CYP3A7 could contribute to the oxidation of RHP^+ in placenta. On the other hand, ketoconazole can strongly inhibit the oxidation of RHP^+ to HP^+ in human liver microsomal preparations. This is consistent with the notion that CYP3A4 is primarily responsible for the oxidation of RHP^+ to HP^+ in human liver.

α -Naphthoflavone was shown to be able to partially inhibit the oxidation of RHP^+ in placenta microsomes (Figure 3). α -Naphthoflavone was also found to be approximately 10-fold more potent as an inhibitor of CYP1A2 than CYP1A1 (44). Interestingly, α -naphthoflavone can significantly enhance the oxidation of RHP^+ in human liver microsomes, but not in placenta microsomal preparations. This suggests that CYP3A4 does not play an important role in the oxidation of RHP^+ in human placenta microsomes. Activation of CYP3A4 by α -naphthoflavone has been observed previously (45), and was attributed to the allosteric effect of α -naphthoflavone (46). Thus, the activation property of α -naphthoflavone on CYP3A4 seems to dominate in human liver microsomes.

Clinical implications

Antipsychotic medications, including haloperidol, are prescribed for pregnant women (47). Most women with a serious psychiatric illness cannot stop taking their medication because this would interfere with their daily activities and care of an infant. Although most toxicological studies were carried out using HP^+ only, there are evidences to indicate that RHP^+ and HP^+ have different

biological properties. For example, RHP^+ was found to be less potent than HP^+ in inhibition of serotonin and dopamine uptake and in releasing preloaded dopamine from striatal synaptosomes (48). RHP^+ and HP^+ also exhibited different selectivity as substrates of human organic cation transporters (8). A clinical study demonstrated that the severity of tardive dyskinesia and parkinsonism is associated with an increased ratio of HP^+ /haloperidol in serum (15). This may be interpreted as evidence that RHP^+ is less toxic than HP^+ .

It is known that pregnancy outcomes could be affected by maternal smoking. For example, lower birth weight and shorter gestational age have been associated with smoking mothers with the CYP1A1 *MspI* variant genotype (*Aa/aa*), which seem to have higher enzyme activity (49, 50). Nevertheless, one should still be cautious to suggest that the modest increase in the oxidation of RHP^+ observed among smokers would have significant clinical consequences.

Taken together, these data seem to indicate that CYP1A1 plays an important role in the oxidation of RHP^+ in placenta from smokers. Other enzymes such as CYP3A7 and CYP3A4 may play more important roles than CYP1A1 in placenta from non-smokers.

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