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Bupropion metabolism by human placenta

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Abstract

Smoking during pregnancy is the largest modifiable risk factor for pregnancy-related morbidity and mortality. The success of bupropion for smoking cessation warrants its investigation for treatment of pregnant patients. Nevertheless, the use of bupropion for the treatment of pregnant smokers requires additional data on its biodisposition during pregnancy. Therefore, the aim of this investigation was to determine the metabolism of bupropion in placentas obtained from nonsmoking and smoking women, identify metabolites formed and the enzymes catalyzing their formation, as well as the kinetics of the reaction. Data obtained revealed that human placentas metabolized bupropion to hydroxybupropion, erythro- and threohydrobupropion. The rates for formation of erythro- and threohydrobupropion exceeded that for hydroxybupropion by several folds, were dependent on the concentration of bupropion and exhibited saturation kinetics with an apparent K_m value of 40 μM . Human placental 11 β -hydroxysteroid dehydrogenases were identified as the major carbonyl-reducing enzymes responsible for reduction of bupropion to threo- and erythrohydrobupropion in microsomal fractions. On the other hand, CYP2B6 was responsible for formation of OH-bupropion. These data suggest that both placental microsomal carbonyl-reducing and oxidizing enzymes are involved in metabolism of bupropion.

1. Introduction

In the United States, 10.7% of women giving birth in 2003 reported smoking during pregnancy [1] despite its association with spontaneous abortion, placental abruption, intrauterine growth restriction, preterm delivery, neonatal mortality, stillbirth and sudden infant death syndrome [2,3]. Smoking during pregnancy is the largest modifiable risk factor for pregnancy-related morbidity and mortality. Although the rate of smoking cessation is high due to counseling and behavioral interventions [4], a significant number of pregnant women (20–30%) fail to achieve this goal [5–7] and hence could benefit from pharmacotherapy.

Pharmacotherapeutic agents successfully used for smoking cessation in non-pregnant women include Nicotine Replacement Therapies (NRTs), bupropion, and more recently, varenicline. However, none of these pharmacotherapies are recommended for pregnant women. The main reason for the limited use of NRTs during pregnancy is the inherent risk of nicotine exposure to the developing fetus [8]. Accordingly, an alternative approach for smoking cessation during pregnancy could be achieved by using a non-nicotinic drug such as bupropion.

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Bupropion is an antidepressant that has also been used to encourage smoking cessation in non-pregnant adults. While the mechanism by which bupropion aids in smoking cessation is unclear, it has the effect of an antagonist at the nicotinic receptor [9].

Bupropion is extensively metabolized in human liver to pharmacologically active hydroxybupropion (OH-bupropion), and a pair of enantiomers—erythrohydrobupropion and threohydrobupropion (Figure 1) [10], and CYP2B6 was identified as the major hepatic enzyme responsible for the biotransformation of bupropion to OH-bupropion [11–13].

The onset of pregnancy is accompanied by changes in maternal physiology [14] to accommodate the development of the feto-placental unit which is viewed as a new compartment for drug distribution. Enzymes in this compartment are responsible for the metabolic pathways necessary for the biosynthesis of several placental-specific hormones (eg, hCG and hPL), for the catabolism of metabolic intermediates as well as for the biotransformation of administered medications [15–17]. The expression and activity of placental metabolic enzymes depends on gestational age, but in general, it is lower than that of hepatic enzymes [18,19]. However, the metabolites formed by placental enzymes are in close proximity to the fetus and are consequently more accessible to the fetal circulation [20, 21]. This may cause unexpected effects on the fetus (favorable or unfavorable) when the metabolites are pharmacologically active.

Cigarette smoke contains more than 5,000 chemicals that could potentially modify the activity of hepatic and extrahepatic metabolizing enzymes. The induction of placental CYP1A1 and uridine diphosphate glucuronosyltransferase (UGT) by maternal cigarette smoking has been extensively studied and is well established [22–25]. Therefore, differences in the metabolic activity of placental enzymes in smoking vs. nonsmoking mothers could affect the bio-disposition of bupropion administered to pregnant women.

Therefore, the aim of this investigation is to determine the metabolism of bupropion in placentas obtained from nonsmoking and smoking women, identify metabolites formed and enzymes catalyzing their formation, as well as the kinetics of the reactions.

2. Material and Methods

2.1. Chemicals and Biological Reagents

OH-bupropion, erythro- and threohydrobupropion were purchased from Toronto Research Chemicals Inc. (North York, Canada). Bupropion hydrochloride, phenacetin, NADP⁺, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, NADH, NADPH, 18 β -glycyrrhetic acid (18 β -GA), flufenamic acid, barbital, and ammonium acetate were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade methanol, acetic acid, 4-methylpyrazole, dicumarol, and menadione were purchased from Fisher Scientific (Fair Lawn, NJ).

Polyclonal antibodies raised against CYPs 2C19, 2D6 and 3A4 were obtained from AbD Serotec (Oxford, UK). Monoclonal and polyclonal antibodies against CYP 2B6, 2C9, 2E1, 2A6, 2C8, 1A2 and 1A1 were purchased from XenoTech, LLC (Lenexa, KS). Rabbit antiserum to human placental aromatase was purchased from Hauptman-Woodward Institute (Buffalo, NY).

2.2. Preparation of subcellular fractions from trophoblast tissue

Human placentas were obtained from nonsmoking and smoking mothers immediately after delivery according to a protocol approved by the Institutional Review Board of the University

of Texas Medical Branch at Galveston. The placentas of smokers were divided into 2 groups: those smoking ≤ 10 cigarettes per day ($n=30$) and those smoking ≥ 20 cigarettes per day ($n=9$).

Villous tissue was dissected, rinsed with ice-cold saline, and homogenized in 0.1 M potassium phosphate buffer pH 7.4 (Ultra Turrax, Staufen, Germany). The homogenate was used to prepare subcellular fractions by differential centrifugation, namely, $10,000 \times g$ pellet for the mitochondrial fraction, $104,000 g$ pellet for the microsomal fraction, and the supernatant for the cytosolic fraction. The mitochondrial and microsomal pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and their protein content was determined by a commercially available kit (BioRad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

2.3. Biotransformation of bupropion by the subcellular fractions

The activity of placental mitochondrial (pool of 33 preparations), microsomal (pool of 29 preparations), and cytosolic (pool of 14 preparations) fractions in metabolizing bupropion was determined. The total reaction volume was 250 μ L of 0.1 M potassium phosphate buffer (pH 7.4). The experimental conditions ensured that the rate of bupropion metabolism was linear with protein concentration and incubation time. Each reaction solution was pre-incubated for 5 min at 37°C and contained 0.25 mg protein of the subcellular fraction and bupropion at a final concentration of 300 μ M ($3 \times K_m$ for hydroxybupropion formation [13]). The reaction was initiated by the addition of an NADPH-regenerating system, made of 0.4 mM NADP⁺, 4 mM glucose 6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, and 2 mM MgCl₂. The reaction components were incubated at 37°C for 40 min. The reaction was terminated by the addition of 25 μ L of 40% trichloroacetic acid (TCA) and placing the tubes on ice. Phenacetin (10 μ L of 2.4 μ g/mL) was added as an internal standard. Extraction of metabolites and internal standard is described below. The effect of bupropion, (0–750 μ M) on reaction velocity was used to construct the saturation curve and to calculate the V_{max} and apparent K_m values.

2.4. Identification of the enzyme(s) catalyzing the hydroxylation of bupropion

Monoclonal and polyclonal antibodies raised against human liver CYP isoforms 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, and rabbit antiserum to human placental aromatase were used to identify the CYP isozymes responsible for the metabolism of bupropion to hydroxybupropion. The reaction volume was 250 μ L of 0.1M potassium phosphate buffer (pH 7.4). Each monoclonal or polyclonal antibody was added to the reaction components at the concentration causing 80% inhibition of the CYP isoform it was raised against. Microsomal proteins (12.5 μ g) were pre-incubated with the antibody at room temperature for 10 min followed by the addition of bupropion at a final concentration of 300 μ M. The reaction was initiated by the addition of the NADPH-regenerating system, incubated for 40 min at 37°C, and terminated by the addition of TCA. In the control reaction, mouse IgG was added instead of antibodies.

2.5. Identification of the enzyme(s) catalyzing the reduction of bupropion

The role of individual carbonyl-reducing enzymes on threo- and erythrohydrobupropion formation by placental microsomal fractions was determined by adding chemical inhibitors selective for each enzyme to the reaction components. The concentration used for each inhibitor was based on its IC_{50} , K_i , or K_m values for specific carbonyl reductases. The following are the inhibitors, the concentrations used, and their selectivity for a carbonyl-reducing enzyme: 4-methylpyrazole, 500 μ M (alcoholdehydrogenase, ADH) [26]; barbital, 500 μ M (aldehyde/aldose reductase) [27]; flufenamic acid 4 μ M (aldo-ketoreductase, AKR) [28]; menadione, 100 μ M (carbonyl reductase, CR) [28]; dicumarol, 500 μ M (quinone oxidoreductase) [29]; and 18 β -glycyrrhetic acid (18 β -GA), 0.1 μ M (11 β -hydroxysteroid dehydrogenase, 11 β -HSD) [30]. Stock solutions of the inhibitors were prepared in 0.5% ethanol in 0.1 M potassium

phosphate buffer (pH 7.4), and an aliquot of each was used to attain the final concentrations as specified above. Each reaction solution contained the following components: inhibitor, bupropion at a final concentration of 40 μM ($\approx K_m$), and the microsomal protein (1 mg of protein/ml 0.1 M potassium phosphate buffer). All components of the reaction solution were preincubated for 10 min at 37°C. The reaction was initiated by the addition of the NADPH-regenerating system, incubated for 40 min at 37°C, and terminated by adding 25 μL of 40% TCA. The control reaction included all the above-mentioned components in 0.1M potassium phosphate buffer without inhibitors.

The IC_{50} of menadione, flufenamic acid, and 18 β -GA for reduction of bupropion by placental microsomes was determined. The concentration of bupropion was 40 μM ($\approx K_m$), and each inhibitor was added in a range of concentrations, namely, menadione (10, 25, 50, 100, 150, 200, and 300 μM), flufenamic acid (1, 2.2, 5, 10, and 50 μM), and 18 β -GA (3, 10, 30, 60, and 100 nM). Each IC_{50} value was calculated from plots of the percent of the product formed (i.e., in the absence of inhibitor) versus the log of its concentration.

2.6. Inhibitory effect of 18 β -glycyrrhetic acid on bupropion reduction

The type of inhibition caused by 18 β -GA, competitive or non-competitive, was determined in the absence and presence of 10, 30, and 60 nM of inhibitor, and the following ranges of bupropion concentrations 20 μM ($1/2 K_m$), 40 μM (K_m), 80 μM ($2K_m$), and 160 μM ($4 K_m$). The data obtained were plotted as the reciprocal of the concentration of metabolite formed versus the reciprocal of bupropion concentration in the absence and presence of inhibitor. The constant of inhibition (K_i) was calculated using the slopes of the primary Lineweaver-Burk plots versus concentrations of 18 β -GA.

2.7. Extraction and recovery of bupropion and its metabolites

Acetonitrile (1.5 mL) was added to the reaction solution, vortexed for 5 min, and centrifuged at 4500 g for 15 min. Acetonitrile from the supernatant was dried at 40°C under a stream of air. The dry residue was reconstituted in 100 μL of the initial mobile phase and filtered through 0.45 μm cellulose membrane filters (Phenomenex, Inc.). An aliquot of 50 μL of each sample was analyzed by HPLC/MS.

2.8. LC/MS analysis

The HPLC system used consisted of a Waters 600E multisolvent delivery system and a 717 plus autosampler controlled by EmpowerTM2 chromatography Data Software (Waters, Milford, Mass). The mobile phase consisted of A: 40% methanol and B: 60% 10 mM ammonium acetate buffer (pH 6.0, adjusted with 0.1M acetic acid). The separation of OH-bupropion, threo- and erythrohydrobupropion was achieved on a Waters Symmetry C₁₈ column (150 $\times$ 4.6mm, 5 μm) connected to a Phenomenex C₁₈ guard column (4 $\times$ 3.0mm) by isocratic elution at a rate of 1.0 mL/min. Detection of the metabolites was achieved by mass spectrometry.

The mass spectrometer (Waters EMD 1000 single-quadrupole; Milford MA) was equipped with an electrospray ion source (ESI) operated in positive mode. Optimal MS parameters are as follows: capillary voltage, 2.2 kV; cone voltage, 40 V; source temperature, 95°C; desolvation temperature, 350°C; desolvation gas flow rate, 450 L/h; cone gas flow rate, 100 L/h. The metabolites and internal standard were monitored by selective ion monitoring (SIM) at m/z 180 for phenacetin (IS), m/z 238 for OH-bupropion and m/z 168 for threo- and erythrohydrobupropion.

The quantitative method of metabolite determination was validated for specificity, linearity, sensitivity, precision and accuracy following the US Food and Drug Administration guideline [31]. The calibration curves of the three metabolites were fit using weighted least squares linear

regression analysis (weighted by y^2) of internal ratio (analyte peak area/IS peak area) versus concentration. The calibration curves of the three metabolites were linear within the test range ($r^2 > 0.98$). The lowest limit of quantification (LLOQ) of OH-bupropion, threo- and erythrohydrobupropion were 1.8, 0.9, and 0.9 ng/mL, and the limit of detection (LOD) of OH-bupropion, threo- and erythrohydrobupropion were 0.18, 0.09, and 0.09 ng/mL, respectively. The inter-day and intra-day accuracy of three metabolites at LLOQ concentration ranged from 83 to 105%, with the relative standard deviation (RSD) less than 11%. The extraction recovery of three metabolites and internal standard were more than 82%.

2.9. Data Analysis

The V_{max} and apparent K_m values for each microsomal preparation were determined using nonlinear regression analysis of the Michaelis-Menten equation (GraphPad Prism 5, Vision 5.01, Graph Pad Software, Inc.).

The difference in the rate of formation of bupropion metabolites between placental preparations obtained from nonsmokers and smokers was determined by Wilcoxon Rank Sum W Test (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

The amount of each metabolite formed in the control for each inhibition experiment was set as 100% and that in the presence of an inhibitor (antibody or chemical) as percent of control. All data are presented as mean $\pm$ S.D. Statistical analysis of the effect of the inhibitors on the formation of bupropion metabolites was accomplished by analysis of variance (ANOVA) with multiple comparisons.

3. Results

3.1. In vitro metabolism of bupropion

Under the experimental conditions used, the amount of OH-bupropion, threo-, and erythrohydrobupropion formed was linear with protein concentration (up to 1 mg/mL) and time (up to 40 min).

Separation of standard compounds of bupropion and its metabolites OH-bupropion, erythro-, and threohydrobupropin was achieved by HPLC-MS according to the method described above. The metabolism of bupropion by human placentas resulted in the formation of three metabolites as identified by their retention times namely, OH-bupropion ($t_R=7.4$ min), erythro- ($t_R=9.0$ min), and threohydrobupropion ($t_R=10.1$ min).

3.2. Metabolism of bupropion by human placental subcellular fractions

The microsomal, mitochondrial and cytosolic fractions of human placentas metabolized bupropion to OH-bupropion, erythro- and threohydrobupropion. The major metabolite formed by all subcellular fractions is threohydrobupropion, which in microsomal fraction accounted for 83% (632 ± 196 pmol/mg.protein) of the total metabolites formed. The amounts of OH-bupropion (19 ± 6 pmol/mg.protein) and erythrohydrobupropion (109 ± 38 pmol/mg.protein) in the same fraction comprised 2.5% and 14%, respectively, of the total metabolites formed (Figure 2).

The microsomal fraction had the highest activity in catalyzing the biotransformation of bupropion and was used to determine the kinetic constants of the reaction (K_m , V_{max} , and K_i). The rate of threo- and erythrohydrobupropion formation by human placental microsomes was dependent on the concentration of bupropion and exhibited saturation kinetics (Figure 3). Analysis of the data obtained revealed that the apparent K_m values of bupropion for formation of threo- and erythrohydrobupropion were similar: 40 μ M. The maximal velocity for

threohydrobupropion formation was approximately 6 times higher than that for erythrohydrobupropion formation (Table 1). The formation of OH-bupropion by microsomes under saturating concentration of bupropion was less than 3% of total metabolites formed.

3.3. Effect of cigarette smoking on metabolism of bupropion by human placentas

The effect of cigarette smoking on the metabolism of bupropion was determined in 3 groups of placental preparations, namely, nonsmokers, women who smoked ≤ 10 cigarettes per day, and women who smoked ≥ 20 cigarettes per day. In all three groups, bupropion was metabolized to threo-, erythrohydrobupropion, and OH-bupropion. However, only in placentas of women who smoked ≥ 20 cigarettes per day the formation of OH-bupropion (550 ± 400 vs 25 ± 10 pmol/mg protein), erythro- (270 ± 170 vs 50 ± 40 pmol/mg protein) and threohydrobupropion (2380 ± 1320 vs 550 ± 470 pmol/mg protein) was significantly increased ($p < 0.01$) as compared to their formation in placentas of nonsmokers (Figure 4).

3.4. Identification of placental enzyme(s) catalyzing hydroxylation of bupropion

Antibodies raised against human liver CYP isoforms and rabbit antiserum to human placental aromatase were used to identify the placental enzyme(s) metabolizing bupropion to OH-bupropion (pool of microsomal preparations from 13 placentas).

In the pool of microsomes, antibodies raised against CYP2B6 and CYP19 caused 50% ($p < 0.01$) and 25% ($p < 0.05$) inhibition of OH-bupropion formation, respectively. The remaining CYP antibodies 1A1, 1A2, 2A6, 2E1, 2D6, 2C8, 2C9, 2C19, and 3A4 did not have an effect on OH-bupropion formation (Figure 5).

This data suggests that CYP2B6, and to a lesser extent, CYP19, are responsible for hydroxylation of bupropion by placental microsomes. The formation of erythro- and threohydrobupropion was not affected by any of the antibodies investigated.

3.5. Identification of placental enzyme(s) catalyzing the reduction of bupropion

Inhibitors known as selective for carbonyl-reducing isoforms were utilized to identify the enzyme(s) catalyzing reduction of bupropion to threo- and erythrohydrobupropion (pool of microsomal preparations from 13 placentas).

The formation of threo- and erythrohydrobupropion was inhibited by 80% ($p < 0.01$) and 70% ($p < 0.01$) by 18β -GA and menadione, respectively. Flufenamic acid caused 50% ($p < 0.01$) inhibition. On the other hand, 4-methylpyrazole, barbital, and dicumarol did not inhibit the formation of threo- and erythrohydrobupropion (Figure 6).

3.6. Effect of 18β -glycyrrhetic acid, menadione, and flufenamic acid on the placental carbonyl-reducing enzymes

The inhibitory effect of 18β -GA, menadione, and flufenamic acid on the reduction of bupropion by placental microsomes was investigated. In the presence of 18β -GA (3–100 nM), menadione (10–300 μ M), and flufenamic acid (1–50 μ M), a concentration-dependent inhibition of threo- and erythrohydrobupropion formation was observed. The IC_{50} for the inhibition of threo- and erythrohydrobupropion by 18β -GA was 53 nM and 59 nM, by menadione 100 μ M and 107 μ M, and by flufenamic acid 5.7 μ M and 6.5 μ M, respectively. The inhibition by 18β -GA was characterized by an increase in apparent K_m values, whereas the V_{max} value was not affected statistically. This is consistent with a competitive-type inhibition between bupropion and 18β -GA (Figure 7). The K_i values for inhibition of threo- and erythrohydrobupropion by 18β -GA were 61 nM and 63 nM (Figure 7 insert), respectively.

4. Discussion

One of the initial steps in developing bupropion for pharmacotherapy of the pregnant smoker is to obtain information on its disposition by human placenta (transplacental transfer, distribution, metabolism, and efflux). The aim of this investigation is to identify the enzymes responsible for the biotransformation of bupropion and the metabolites formed in placentas obtained from nonsmoking and smoking women.

The data obtained in this investigation revealed that human placental subcellular fractions metabolize bupropion to OH-bupropion, threo-, and erythrohydrobupropion (Figure 2). However, the rates of threo- and erythrohydrobupropion formation exceeded that of OH-bupropion by several fold. The biotransformation of bupropion to threo- and erythrohydrobupropion by placental microsomes exhibited saturation kinetics with an apparent *K_m* value of 40 μ M (Table 1). Since formation of threo- and erythrohydrobupropion was also observed during ex vivo placental perfusion of bupropion at concentrations (0.6–1.8 μ M) which correspond to the plasma levels in patients undergoing treatment with 150 mg of bupropion [32], it is also predicted to be formed in vivo.

The three metabolites of bupropion are formed by two different reaction mechanisms. Threo- and erythrohydrobupropion are formed by reduction of the carbonyl group while OH-bupropion is formed by oxidation of the methyl group (Figure 1). The data obtained in this investigation indicate that threo- and erythrohydrobupropion are the major metabolites formed by placental subcellular fractions. Accordingly, the primary metabolic pathway for the metabolism of bupropion in the placenta is reduction of its carbonyl group. This is different from hepatic microsomes, where the major metabolite formed is OH-bupropion [11–13], i.e., the predominant metabolic pathway in the liver is hydroxylation of bupropion. Therefore, the data obtained in this investigation, as well as by others [11–13], revealed tissue-specific differences in the biotransformation of bupropion, which is a substrate of both oxidative and carbonyl-reducing enzymes.

There are four main families of carbonyl-reducing enzymes that are expressed at mRNA level in human placenta, namely, medium-chain dehydrogenases/reductases (ADHs), aldo-keto reductases (AKRs), short-chain dehydrogenases/reductases (includes CRs and 11 β -HSD), and quinone reductases [33]. These enzymes differ in their subcellular localization and their dependence on the cofactors NADH and NADPH [34]. As mentioned above, the reduction of bupropion to threo- and erythrohydrobupropion occurs in the cytosolic, mitochondrial, and microsomal fractions of trophoblast tissue. This suggests that both soluble and membrane-bound forms of the enzyme(s) are involved in the metabolism of bupropion (Figure 2). Moreover, the reduction of bupropion by the cytosolic, mitochondrial and microsomal fractions was dependent on the presence of either NADH or NADPH. However, the presence of NADPH resulted in approximately 50% increase (over NADH) in reaction velocity (data not shown). Since we cannot rule out cross contamination between the subcellular fractions, the identification of the responsible reductases was pursued in the fraction that revealed the highest enzymatic activity, namely, the microsomal fraction.

Two approaches were used to identify the major placental microsomal enzymes responsible for reduction and oxidation of bupropion: chemical inhibitors selective for carbonyl reductases and antibodies raised against CYP isoforms. Inhibition of AKRs (by flufenamic acid), CRs (by menadione), and 11 β -HSD (by 18 β -GA) decreased the formation of threo- and erythrohydrobupropion by 50, 70, and 80%, respectively (Figure 6). On the other hand, inhibition of ADHs and quinone reductases by 4-methylpyrazole and dicumarol, respectively, did not affect threo- and erythrohydrobupropion formation. Taken together, data on the subcellular localization, cofactor dependence (NADH and NADPH), and chemical inhibition,

it can be concluded that placental short-chain dehydrogenases/reductases are the major family of carbonyl-reducing enzymes responsible for the microsomal reduction of bupropion to threo- and erythrohydrobupropion. Furthermore, in this investigation, activity of the enzymes was determined at pH 7.4 which is optimal for 11 β -HSD [35,36]. On the other hand, carbonyl reductases favor an acidic pH of 5.5 to 6.5 [37,38]. The inhibition of threo- and erythrohydrobupropion formation by 18 β -GA was concentration dependent, and revealed IC_{50} (52 nM and 59 nM) and K_i values of (61 nM and 63 nM), respectively. The low nanomolar range indicates its high affinity. Moreover, analysis of the data revealed that inhibition caused by 18 β -GA was of the competitive type.

In humans, there are two isozymes of 11 β -HSD [39,40]: 11 β -HSD1 which is mainly a reductase [41,42] and 11 β -HSD2 which is only an oxidase [40,43]. 18 β -GA is a non-selective inhibitor of both isozymes with IC_{50} values of 779 nM and 257 nM, respectively [44]. Taken together, it appears that the isozymes of 11 β -HSD are the primary carbonyl-reducing enzymes catalyzing the reduction of bupropion in placental microsomes. Unfortunately, further identification/conformation of carbonyl-reducing enzymes is not feasible due to the lack of commercially available isoforms of these enzymes and selective inhibitors for each of them [45].

On the other hand, CYP2B6 and, to a lesser extent, CYP19, were responsible for the formation of OH-bupropion (Figure 5), which is a minor placental microsomal metabolite of bupropion. Although, data on placental protein expression of CYP2B6 remain scarce, mRNA expression has been reported [46,47]. Furthermore, CYP2B6 was identified as the major hepatic enzyme responsible for hydroxylation of bupropion [12,13,48].

A multitude of genetic, environmental, and disease associated factors could affect the activity of hepatic and extrahepatic metabolizing enzymes. For example, polycyclic aromatic hydrocarbons present in cigarette smoke induce both hepatic and extrahepatic enzymes [49] and result in a wide range of inter-individual variations in drug metabolism. Moreover, CYP1A1, the most investigated placental isozyme responsible for the metabolism of numerous xenobiotics, is also induced by maternal smoking [25,50–52].

The data obtained in this investigation revealed that the formation of the three metabolites of bupropion by placental microsomes obtained from women who smoked during pregnancy (≥ 20 cigarettes per day) was significantly higher than in placentas of nonsmokers or occasional smokers (≤ 10 cigarettes per day). This data suggests that components of cigarette smoke could also affect the activity of placental enzymes responsible for the metabolism of bupropion. This is in agreement with an earlier report indicating that smokers and alcoholics who also smoke have higher brain expression of CYP2B6 than non-smokers or non-smoking alcoholics [53].

In summary, data obtained in this investigation revealed that the major pathway for the metabolism of bupropion in human placenta is catalyzed by 11 β -HSD and results in the formation of erythro- and threo- hydrobupropion. On the other hand, in human liver, CYP2B6 is the main enzyme catalyzing the hydroxylation of bupropion to OH-bupropion. These data suggest that different enzymes – depending on tissue localization – may play a primary role in biotransformation of bupropion, which is a substrate of both oxidative and carbonyl-reducing enzymes.

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A list of non-standard abbreviations

V_{max}	maximum velocity
CL_{in}	intrinsic clearance (V_{max}/K_m)
K_m	substrate concentration at 50% of V_{max}
CYP450	cytochrome P450
ADHs	alcohol dehydrogenases
AKRs	aldo-keto reductases
CRs	carbonyl reductases
11 β -HSD	11 β -hydroxysteroid dehydrogenases
18 β -GA	18 β -glycyrrhetic acid

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Figure 1.
Chemical structures of bupropion and its metabolites.

Figure 2. Metabolism of bupropion by placental subcellular fractions. HB, hydroxybupropion; EB, erythro- and TB, threohydrobupropion. Data are presented as mean $\pm$ S.D, n=6.

Figure 3.

Representative saturation curve of threohydrobupropion formation by human placental microsomes. The rate of threohydrobupropion formation was dependent on bupropion concentration and exhibited typical saturation kinetics. Monophasic kinetics was confirmed using Eadie-Hofstee plot (insert) of reaction velocity (v) against $v/[S]$.

Figure 4.

The box plots of distribution of formation of OH-bupropion, erythro- and threohydrobupropion by human placental microsomes obtained from nonsmoking and smoking mothers.

Group 1 – microsomes prepared from placentas (n=20) of nonsmokers; Group 2 – microsomes prepared from placentas (n=30) of women who smoked ≤ 10 cigarettes per day; Group 3 – microsomes prepared from placentas (n=9) of women who smoked ≥ 20 cigarettes per day.

The formation of OH-bupropion, erythro- and threohydroxybupropion was significantly elevated ($p < 0.01$) in placentas obtained from women who smoked ≥ 20 cigarettes per day. The horizontal line inside each box represents the median formation of corresponding metabolite. The 50% of the distribution of metabolites formation lies between lower (25th percentile) and upper (75th percentile) hinges of the boxes demonstrating variability around the median. The “o” indicates the values of mild outliers which is 1.5 to 3 times of box lengths from the upper or lower edges of the box. The “*” indicates the values of extreme outliers which is more than 3 times of box lengths from the upper or lower edges of the box [54].

Figure 5.

Effect of antibodies raised against human CYP450 isozymes on hydroxylation of bupropion by human placental microsomes.

The rate of OH-bupropion formation in the presence of antibodies was expressed as percent of its formation rate (23 ± 3 pmol/mg.protein) in the absence of antibodies (control). Data was presented as mean $\pm$ S.D. of 2 experiments performed in duplicate.

*Statistical significance of $P < 0.05$; ** statistical significance of $P < 0.01$.

Chemical inhibitors against carbonyl reductase

Figure 6.

Effect of chemical inhibitors of carbonyl reductases on bupropion reduction by human placental microsomes.

Each inhibitor was co-incubated with bupropion ($40 \mu\text{M} \approx K_m$) for 40 min at 37°C . The inhibitors are: 4-methylpyrazole (4-MP), barbitol (Bar), flufenamic acid (Flu), dicumarol (Dic), menadione (Men), and 18β -glycyrrhetic acid (18β -GA). The rates of metabolite formation in the presence of inhibitor was expressed as percent of the rates ($480 \pm 39 \text{ pmol/mg.protein}$) in the absence of inhibitors (control). Data was presented as mean $\pm$ S.D. of triplicate experiments.

**Statistical significance of $P < 0.01$.

Figure 7.

Lineweaver-Burk plot of the data on the inhibition by 18 β -GA of bupropion reduction in human placental microsomes. The inset is a secondary plot illustrating bupropion-18 β -GA interactions.

The reciprocal of threohydrobupropion formation is plotted versus the reciprocal of bupropion concentration in the presence and absence of the inhibitor 18 β -GA. Bupropion was used at concentrations 20, 40, 80, and 160 μM . The 18 β -GA did not have an effect on the V_{max} value but increased the apparent K_m of the reaction, indicating competitive inhibition.

TABLE 1

Kinetic parameters for bupropion reduction by human placental microsomes

Human placental microsomes	Threohydrobupropion		Erythrohydrobupropion	
	V_{max}^a	K_m^b	V_{max}^a	K_m^b
HPM 1	12.4	28.1	1.8	29.6
HPM 2	20.5	28.1	3.5	27.8
HPM 3	19.7	37.3	3.4	44.85
HPM 4	10.0	39.6	1.4	26.8
HPM 5	24.5	31.3	4.2	80.2
HPM 6	24.4	70.2	4.3	37.6
Mean±S.D.	18.6±6.1	39.1±16.0	3.1±1.2	41.1±20.3

^a V_{max} in units of picomoles per minute per milligram of protein.^b k_m in units of micromoles per liter.

HPM – human placental microsomal preparation.