Aromatase Is the Major Enzyme Metabolizing Buprenorphine in Human Placenta

SUJAL V. DESHMUKH, TATIANA N. NANOVSKAYA, and MAHMOUD S. AHMED

Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, Texas

Received April 18, 2003; accepted June 5, 2003

ABSTRACT

Buprenorphine (BUP) is a partial opiate agonist used for treatment of the adult and the pregnant addicted to this class of narcotics. The kinetic parameters for transplacental transfer and the metabolism of BUP during its perfusion in a placental lobule were the subject of an earlier report from our laboratory. The aim of this investigation is to identify and characterize the enzyme catalyzing the metabolism of BUP in term human placenta. Norbuprenorphine (norBUP) is the only metabolite formed as determined by high performance liquid chromatography/mass spectrometry. The activity of the enzyme responsible for BUP metabolism is highest in the microsomal fraction and lowest in the cytosolic, with the mitochondrial in between. Compounds with selective affinity to the enzyme aromatase (CYP 19), namely 4-hydroxyandrostenedione and aminoglutethimide, caused >70% inhibition of norBUP formation. Monoclonal antibodies raised against CYP 19 were the most potent inhibitors of BUP dealkylation. A comparison between the data obtained from the saturation isotherm for BUP dealkylation by placental microsomes and a commercially available system of cDNA-expressed CYP 19 indicated similar kinetic parameters, with apparent \( K_m \) values of 12 ± 4.0 and 14 ± 8.0 \( \mu \)M, respectively. Therefore, aromatase is the major enzyme catalyzing the biotransformation of BUP to norBUP in term human placentas obtained from healthy pregnancies. The minimal involvement of other cytochrome P450 isoforms or enzyme(s) in the metabolism of BUP in placental tissue cannot be ruled out.

Methadone is, and has been for decades, the drug of choice in treatment of the adult and the pregnant opiate addict. In the last decade, data obtained from clinical trials on treatment of the pregnant woman with BUP indicated that the drug was well tolerated by the patient and that the incidence of neonatal abstinence was low to nonexistent (Marquet et al., 1997; Fischer et al., 2000; Eder et al., 2001), suggesting that BUP might be an alternative to methadone for maintenance of this patient population.

BUP levels in the fetal circulation can have a direct effect on its normal growth and development. On the other hand, maternal circulating levels of the opiate could affect placental physiology and, consequently, the fetus. The kinetics for transplacental transfer of BUP, its concentration in the fetal circuit, and effects on placental functions were determined in our laboratory utilizing the technique of dual perfusion of placental lobule (Nanovskaya et al., 2002). Our data indicated that the tissue accumulated 13 times the amount of BUP present in the maternal circuit, and less than 5% of it was metabolized to norBUP. However, in the perfusion system mentioned, BUP was delivered in the intervillous space, thus bypassing the maternal myometrium and endometrium, and its access to the metabolizing enzymes was less. Therefore, it is likely that in vivo metabolism of BUP in the placenta is higher.

In humans, BUP is biotransformed to norBUP by hepatic enzymes during first pass metabolism. The major enzyme responsible for \( N \)-dealkylation of BUP to norBUP in the liver is cytochrome P450 3A4 (CYP 3A4) (Iribarne et al., 1997; Kobayashi et al., 1998). The formation of the glucuronide conjugates of BUP and norBUP was also reported (Cone et al., 1984). During pregnancy, the human placenta plays an important role in the metabolism of endogenous compounds, xenobiotics, and environmental pollutants (Juchau 1980; Contractor, 1983; Nandakumaran et al., 1983, Blanck et al., 1983; Roe et al., 1990; Pienimaki et al., 1997). The expression and activity of various P450 isoforms depends on placental gestation age and tissue maturity but is lower than that in the liver (Hakkola et al., 1996a,b). Aromatase/CYP 19 is one of the cytochrome P450 isozymes with a well established role in the biosynthesis of steroids in human placenta (Thompson

Supported by a grant from the National Institute on Drug Abuse to M.S.A. (DA 13431).

Article publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.103.053199.

ABBREVIATIONS: BUP, buprenorphine; norBUP, norbuprenorphine; P450, cytochrome P450; EFC, 7-ethoxy-4-trifluoromethylcoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; LC/MS, liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance.
and Siiteri, 1974). More recently, the role of aromatase in the oxidative metabolism of xenobiotics by preparations of crude and purified enzymes has been reported (Toma et al., 1996; Osawa et al., 1997). These data were further substantiated by the availability of high specific activity cDNA-expressed CYP 19 that allowed testing the metabolism of several compounds and determining their kinetic parameters (McNamara et al., 1999).

The above underscores the importance of identifying the enzyme responsible for BUP metabolism in human placenta and the effects of prolonged administration of the opiate on its activity. The information obtained is necessary to avoid drug interactions between other therapeutics that may be administered during utilization of BUP for treatment of the pregnant opiate addict. Therefore, the focus of this investigation is to identify and characterize the enzyme responsible for BUP metabolism in term human placenta.

**Materials and Methods**

**Chemicals**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. Acetomintrile was purchased from Fisher Scientific (Fair Lawn, NJ); BUP and norBUP were a gift from the National Institute on Drug Abuse drug supply unit.

**Clinical Material**

All placentas were obtained from term healthy pregnancies after delivery according to a protocol approved by the Institutional Review Board. Every effort was made to exclude placentas of women who abused drugs during pregnancy.

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, 10,000g pellet for the mitochondrial, 104,000g pellet for the microsomal, 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 (Bio-Rad microsomal pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4), followed by incubation for 10 min at 37°C). The incubation was terminated by the addition of 2 ml of ice-cold acetone followed by centrifugation at 7500g for 10 min, and the precipitate was discarded. The inhibition of HFC formation by BUP was determined by utilizing the following inhibitors: the activity of placental mitochondrial, microsomal, and cytosolic fractions in catalyzing the N-dealkylation of BUP to norBUP was determined. The reaction volume was made of 1 ml of 0.1 M potassium phosphate buffer (pH 7.4) and contained 1 mg protein of the subcellular fraction, 50 μM BUP. The components were preincubated for 5 min at 37°C after addition of the NADPH-regenerating system that started the reaction period of 30 min. The latter was made of: 0.4 mM NADP, 4 mM glucose-6-phosphate, 1 mM glucose-6-phosphate dehydrogenase, and 2 mM MgCl₂. The incubation period was terminated by the addition of 100 μl of a 35% (w/v) trichloroacetic acid containing 1 μg/ml terfenadine (internal standard) and centrifuged at 18,000g for 10 min. The amount of norBUP formed in the supernatant was determined by liquid chromatography/mass spectrometry as described below. The control was identical, but the placental fraction was denatured. The dependence of the activity of the microsomal fraction (1 mg/ml) and preparations of human cDNA expressing CYP 19 (40–100 pmol of CYP 19/ml) (Gentest) on the amount of BUP (saturation curve) was determined.

**Enzyme Reactions**

**Monoxygenases.** The activity of these enzymes was determined by the O-deethylation of the substrate 7-ethoxy-4-trifluoromethylcoumarin (EFC) and formation of the fluorescent product 7-hydroxy-4-trifluoromethylcoumarin (HFC). The reaction volume of 1 ml was made of the placentatal subcellular fraction (1 mg of protein), the substrate EFC (50 μM), and NADPH (250 μM) in 0.1 M KPO₄ buffer (pH 7.4), followed by incubation for 10 min at 37°C. The reaction was terminated by the addition of 2 ml of ice-cold acetone followed by centrifugation at 7500g for 10 min, and the precipitate was discarded. The inhibition of HFC formation by BUP was determined by two procedures. In the first, the reaction components and BUP were incubated for 10 min before the substrate EFC was added and in the second, all compounds were added simultaneously.

**N-Dealkylation of BUP.** The activity of placental mitochondrial, microsomal, and cytosolic fractions in catalyzing the N-dealkylation of BUP to norBUP was determined. The reaction volume was made of 1 ml of 0.1 M potassium phosphate buffer (pH 7.4) and contained 1 mg protein of the subcellular fraction, 50 μM BUP. The components were preincubated for 5 min at 37°C after addition of the NADPH-regenerating system that started the reaction period of 30 min. The latter was made of: 0.4 mM NADP, 4 mM glucose-6-phosphate, 1 μM glucose-6-phosphate dehydrogenase, and 2 mM MgCl₂. The incubation period was terminated by the addition of 100 μl of a 35% (w/v) trichloroacetic acid containing 1 μg/ml terfenadine (internal standard) and centrifuged at 18,000g for 10 min. The amount of norBUP formed in the supernatant was determined by liquid chromatography/mass spectrometry as described below. The control was identical, but the placental fraction was denatured. The dependence of the activity of the microsomal fraction (1 mg/ml) and preparations of human cDNA expressing CYP 19 (40–100 pmol of CYP 19/ml) (Gentest) on the amount of BUP (saturation curve) was determined.

**Inhibition of norBUP Formation**

Identification of the P450 enzyme catalyzing the metabolism of BUP to norBUP was achieved by utilizing the following inhibitors: chemicals, i.e., compounds selective for specific P450 isoforms; and monoclonal antibodies against purified human liver P450 isoforms.

**Chemical Inhibitors.** The concentration range used for each inhibitor was based on its IC₅₀, Kₘ, or Kᵥmax values for a specific P450 isoform. The following are the inhibitors, the concentration range, and their corresponding P450 isoforms: a-naphthoflavone, 0.1 μM (CYP 1A); sulfaphenazole, 10 μM (CYP 2C); quinidine, 5 μM (CYP 2D6); 4-methylpyrazole, 50 μM (CYP 2E1); ketoconazole, 2.5 μM (CYP 3A4/CYP 19); troleandomycin, 50 μM (CYP 3A4) (Newton et al., 1995; Bourrie et al., 1996; Pelkonen et al., 1998); 4-hydroxyandrosterone, 1 μM; and aminoglutethimide 10 μM (CYP 19) (Stresser et al., 2000). Concentrated stock solutions of the inhibitors in methanol were prepared, and an aliquot of each was used to attain the final concentration required for each P450 isoform as specified above. Each inhibitor, BUP at a final concentration of 12 μM (=Kᵥmax), and the microsomal preparation in potassium phosphate buffer (1 mg of protein/ml) were preincubated for 5 min at 37°C. The reaction was initiated by the addition of the NADPH-regenerating system and incubated for a period of 30 min. The control reaction contained all the above-mentioned compounds but with 0.5% (v/v) methanol substituting for the inhibitor solution. The effect of 2.5 μM ketoconazole was investigated in commercially available systems expressing either CYP 3A4 or CYP 19 under identical conditions.

**Monoclonal Antibodies.** Monoclonal antibodies against human liver CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, and 3A4/5 and rabbit antiserum to human placental aromatase were utilized to identify/confirm the P450 enzyme responsible for BUP metabolism by placental tissue. A pool of microsomal preparations obtained from 12 placentas was utilized.
In each enzyme assay, 0.1 mg of protein of the microsomal pool was incubated at room temperature with an antibody at its concentration causing 90% inhibition of the P450 isozyme it was raised against. The microsomal pool and the antibodies were incubated for 15 min before BUP was added to attain a final concentration of 50 μM, and the reaction was allowed to continue for another 120 min at 37°C before it was terminated as described above. Mouse IgG replaced the monoclonal antibodies in the control reaction.

Quantitative Determination of the Reaction Products

7-Hydroxy-4-trifluoromethylcoumarin (HFC). The concentration of HFC was determined by fluorescence spectroscopy (CytoFluor, Series 4000 Fluorescence; Applied Biosystems, Foster City, CA) according to the method of Buters et al., (1993). A standard curve for fluorescence intensity of 20 to 2000 pmol/ml HFC, with excitation and emission wavelengths of 450 and 530 nm, respectively, was plotted. The fluorescence intensity at zero time of the incubation period served as a blank.

BUP and norBUP. BUP and norBUP were identified by HPLC according to the method of Iriarte et al. (1997) and as described earlier in detail (Nanovskaya et al., 2002).

Liquid Chromatography (LC) and Mass Spectrometry (MS) Analysis

The amounts of norBUP formed in the reaction mixture were determined utilizing LC/MS. A Spectra system consisting of an AS 3000 autosampler, P 4000 pump (Spectra Physics, San Jose, CA) and a 2 × 30 mm C-18 3-μm Luna column (Phenomenex, Torrance, CA) was used. The mobile phase was made of a linear gradient of acetonitrile/water starting with 10% of the former and ending with 90% at a flow rate of 500 μl/min for a period of 5 min. The ratio was then reverted to 10% acetonitrile for 1 min before the end of the run. The volume of the injected sample was 100 μl. The eluant of the column was coupled to AQA single-quadrupole Navigator LC/MS (Thermo Quest, San Jose, CA). Mass spectral analyses were performed using electrospray in the positive mode with an ionizing voltage of 4.0 kV, and the capillary probe temperature was set at 400°C. Other conditions were as follows: source voltage, 10 V; lens voltage, 1.0 V; ion energy, 5 eV; detector voltage, 750 V; low mass resolution, 25; high mass resolution, 0.

For quantitative determinations, the mass spectrometer was operated in the selected ion monitor mode. Under the above conditions, norBUP (m/z = 413.7) formed a potassium adduct ([M+K]+) that had a relative greater abundance than its molecular ion [M]+ and was used to monitor norBUP.

For quantitative studies, ThermoFinnigan’s Xcalibur data processing software (Thermo Finnigan, San Jose, CA) was utilized. The ratios of norBUP potassium adduct peak area (m/z = 454.7) to that of terfenadine (m/z = 471.7) was plotted against the amount of norBUP. All analyses were carried out in the linear range of the instrument’s sensitivity. Calibration curves were prepared using known amounts of norBUP added to blank samples of the reaction mixture.

HPLC/UV Determination of Estradiol

The method used for determination of 17β-estradiol was as described earlier (Tanguchi et al., 1989) with slight modification. The HPLC system used consisted of a Waters 600E multisolvvent delivery system, a Waters 2487 dual wavelength absorbance detector, and a Waters 717 autosampler controlled by Waters Millennium23 chromatography manager (Waters, Milford, MA). The volume of the sample injected was 200 μl and the C-18 column was a 250 × 4.6 mm Luna 5 μm (Phenomenex). The mobile phase was made of 0.1% triethylammonium in acetonitrile/water (45:55, v/v), and the pH was adjusted to 3.5 by orthophosphoric acid. Isocratic elution was at a flow rate of 1.2 ml/min monitored at a wavelength of 280 nm. The ratio of estradiol peak area to that of the internal standard was used for all calculations of its quantity.

Data Analysis

Data are represented as mean ± S.D. throughout the text. Kinetic parameters were determined by use of nonlinear regression analysis with SPSS Version 11 for Windows (SPSS Science, Chicago, IL). The data were fit to the Michaelis-Menten equation:

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} \]

Statistical analysis of data on the effect of inhibitors on BUP metabolism was carried out using one-way ANOVA with Tukey’s comparison and deemed significant if the p value was <0.05.

Results

Activity of Placental Monoxygenases. Villous tissue of term human placentas (n = 10) was used to prepare the subcellular fractions. The activity of each fraction in catalyzing the deethylation of the marker substrate EFC (50 μM) to HFC was determined. The formation of HFC, under saturating concentrations of EFC, was proportional to the deethylation activity of the subcellular fractions. The mean activity for EFC O-deethylation by the placental subcellular fractions is shown in Table 1. A wide range of values for the deethylation activity of the microsomal fractions activity was observed, with the highest being almost 15 times the lowest, and is attributed to interplacental variations. Therefore, the deethylation activity was arbitrarily divided into two groups, below and above 40 pmol/mg protein · min, and are referred to as “low activity” and “high activity,” with one preparation reaching 300 pmol/mg protein · min in the latter. On the other hand, a smaller range of values for the activity of the enzyme(s) catalyzing the dealkylation of BUP to norBUP was observed in all the subcellular fractions. These differences did not reach statistical significance (at p < 0.05); i.e., no interplacental variations were observed (Table 1).

The activities of placental mitochondrial and microsomal fractions in catalyzing the dealkylation of BUP and the marker substrate EFC were compared. The ratio of mitochondrial to microsomal EFC O-deethylation was equal to 1, and that for N-dealkylation of BUP was 0.5 (Table 1). These data indicate that the dealkylation of BUP by placental mi-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>EFCOD Activity</th>
<th>Mitochondrial Fraction</th>
<th>Cytosolic Fraction</th>
<th>Mean Ratios (Mitochondrial/Microsomal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFC</td>
<td>High</td>
<td>190.9 ± 101.7</td>
<td>154.2 ± 65.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>12.4 ± 0.9</td>
<td>14.1 ± 5.5</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>BUP</td>
<td>N.A.</td>
<td>1.9 ± 0.6</td>
<td>1.1 ± 0.4</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

EFCOD, EFC O-deethylation; N.A., not applicable.

TABLE 1

Activity of the subcellular fractions obtained from placental preparations (n = 10) in catalyzing the O-deethylation of the marker substrate EFC to HFC and the N-dealkylation of BUP to norBUP. The enzymatic activities are expressed in pmol/mg protein · min ± S.D.
crosomal fractions was more active than that by the mitochondrial fractions. Minor contamination of the microsomal fraction with mitochondrial enzymes cannot be ruled out, but a similar observation has been reported earlier (Pasanen et al., 1985) and is addressed further in determination of aromatase activity utilizing its natural substrate, testosterone.

The effect of BUP (10–200 μM) on EFC deethylation was investigated and its IC50 was determined (Fig. 1). The inhibition was concentration-dependent and was only observed in the placental preparations in the high activity group. The IC50 values for BUP in the mitochondrial and microsomal fractions were 129.2 ± 61.1 and 129.4 ± 75.9 μM, respectively. The inhibitory effect of BUP was observed whether the opiate was added before or simultaneously with EFC to the reaction mixture.

Kinetic Parameters for BUP Dealkylation. Our data suggest that the enzyme catalyzing the dealkylation of BUP to norBUP may be present in the three subcellular fractions of placental tissue, with the microsomal fraction having the highest activity. Therefore, a pool of microsomal fractions prepared from six placentas (1–6, Table 2) was utilized to determine the apparent Km and Vmax for the dealkylation of BUP. The enzyme catalyzing the reaction required NADPH. Analysis of the saturation curves for the biotransformation of BUP to norBUP revealed an apparent Km of 12 ± 4.0 μM, a Vmax of 2.9 ± 0.7 pmol/mg protein · min, and an intrinsic clearance (Vmax/Km) value of 0.3 ± 0.1 μl/mg protein · min (Table 2; Fig. 2).

The Effect of Inhibitors Selective for P450 Isoforms on BUP Metabolism. Inhibitors selective for P450 isoforms were utilized to identify the enzyme catalyzing the dealkylation of BUP to norBUP. Ketoconazole (2.5 μM), 4-hydroxyandrostenedione (1.0 μM), and aminoglutethimide (10.0 μM) caused the highest inhibition (70% of control). α-Naphthoflavone and 4-methylpyrazole caused 20% and 40% inhibition at their respective concentrations of 0.1 and 50 μM (Fig. 3). The least amount of inhibition (20%) was caused by 5 μM quindine, whereas sulfaphenazole and troleandomycin did not show any inhibition of BUP metabolism in the concentration range tested.

Ketoconazole is considered an inhibitor of hepatic CYP 3A but is also known to inhibit human placental aromatase (Ayub and Levell, 1988). The effect of ketoconazole on norBUP formation by cDNA preparations expressing CYP 3A4 and CYP 19 was investigated. Ketoconazole (2.5 μM) caused a 90% inhibition of BUP metabolism in both preparations; i.e., it was as effective an inhibitor of CYP 19 as it was of CYP 3A4. Taken together, our data suggest that the human placental microsomal enzyme catalyzing N-dealkylation of BUP to norBUP could be CYP 19.

The Effect of Monoclonal Antibodies on BUP Metabolism. Monoclonal antibodies raised against specific human liver P450 isoforms were used to confirm the identification of the enzyme catalyzing BUP metabolism. A pool of microsomal preparations from 12 placentas was utilized in these experiments. Each monoclonal antibody raised against a specific P450 isoform was tested at its concentration causing 80% inhibition. The highest inhibition observed for BUP metabolism (70%) was observed in the presence of the antibodies raised against CYP 19. Monoclonal antibodies against CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and CYP 3A4/5 did not cause any significant inhibition of norBUP formation (Fig. 4). Data obtained on the effects of the selective inhibitors and monoclonal antibodies on the enzyme-catalyzed dealkylation of BUP indicate that it is CYP 19/ aromatase.

Kinetic Parameters for norBUP Formation by cDNA-Expressed CYP 19. The cDNA-expressed CYP 19 isozyme (Supersomes) catalyzed the dealkylation of BUP to norBUP. Analyses of the saturation curves obtained revealed apparent

### Table 2

<table>
<thead>
<tr>
<th>Microsomal Preparation No.</th>
<th>Km (mM)</th>
<th>Vmax (pmol/mg protein · min)</th>
<th>Intrinsic Clearance (Vmax/Km) (μl/mg protein · min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.7</td>
<td>4.1</td>
<td>0.42</td>
</tr>
<tr>
<td>2</td>
<td>6.8</td>
<td>2.0</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>8.5</td>
<td>2.8</td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td>14.5</td>
<td>2.8</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>13.6</td>
<td>2.7</td>
<td>0.20</td>
</tr>
<tr>
<td>6</td>
<td>16.2</td>
<td>3.1</td>
<td>0.18</td>
</tr>
<tr>
<td>Mean</td>
<td>11.6</td>
<td>2.9</td>
<td>0.27</td>
</tr>
<tr>
<td>S.D.</td>
<td>3.7</td>
<td>0.7</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Fig. 1. The inhibition of EFC O-deethylase activity by BUP. The opiate was preincubated with the respective subcellular fractions for 10 min at 37°C. EFC was then added, and the incubation continued for another 10 min. BUP inhibited the reactions catalyzed by the mitochondrial and microsomal fractions obtained from the high activity group of placentas only.

The apparent Km, Vmax, and intrinsic clearance values for the enzyme-catalyzed N-dealkylation of BUP by microsomal fractions obtained from term human placenta (n = 6).

---

**Desmukh et al.**
Km and Vmax values of 14 ± 8 μM and 0.14 ± 0.1 pmol of norBUP/pmol CYP 19 · min, respectively (Fig. 5); i.e., the apparent Km value obtained is similar to that for the placental microsomal preparation.

Aromatase Activity in the Subcellular Fractions. The activity of aromatase/CYP 19 in placental tissue microsomal and mitochondrial fractions was determined utilizing its natural substrate testosterone conversion to estradiol. The ratio of the enzymatic activity in the mitochondrial to microsomal fractions ranged between 0.32 and 0.56 with a mean value of 0.43. A similar distribution for aromatase activity, as determined by the conversion of androstenedione to estrogens between placental mitochondrial and microsomal fractions, was reported earlier (Pasanen et al., 1985). Taken together, our data indicate that the major enzyme responsible for human placental tissue metabolism of BUP is CYP 19/aromatase.

Discussion

Buprenorphine is an opiate agonist used for treatment of the adult addicted to this class of narcotics; it is well tolerated by the pregnant woman, and the incidence of neonatal abstinence is minimal to nonexistent (Jarvis and Schnoll, 1994; Marquet et al., 1997; Fischer et al., 2000; Lejeune et al., 2001). A recent report from our laboratory indicated that BUP transfer to the fetal circuit is low when administered into the maternal circuit of the dually perfused placental lobe; it accumulates in the tissue and is metabolized to norBUP during the experimental period (Nanovskaya et al., 2002). This report provides data on identification and characterization of the human placental enzyme responsible for the metabolism of BUP to norBUP.

Microsomal CYP 3A4 is the enzyme responsible for the biotransformation of 75% of BUP to norBUP during first-pass metabolism. The remaining 25% is metabolized by CYP 3A5, 3A7, and 2C8, but other minor products may also be formed by CYP 2C18, 2C19, 2D6, and 2E1 (Iribarne et al., 1997; Kobayashi et al., 1998, Moody et al., 2002).

Our working hypothesis was that the enzyme responsible for BUP metabolism in the placenta was an oxygenase. Accordingly, the oxygenase activity of placental subcellular fractions was determined by utilizing the EFC O-deethyla-
tion to the fluorescent product HFC (DeLuca et al., 1988; Buters et al., 1993). Two distinct forms of monooxygenases catalyze this reaction in placental tissue; namely, a constitutive form that includes CYP 19/aromatase (Meigs, 1987) and an inducible CYP 1A1 (Pasanen et al., 1990). The determined EFC O-deethylase activity in placental subcellular fractions revealed a wide range of values for Vmax (Table 1) and was arbitrarily divided into two groups: low activity, < 40pmol/mg protein · min, and the remainder as high activity. A similar observation for O-deethylase activity was reported earlier; the high activity group of placentas was associated with maternal smoking during pregnancy and has been attributed to induction of CYP 1A1 (Pasanen et al., 1990). On the other hand, the activity of our placental preparations in the N-dealkylation of BUP did not reflect the wide range of variability observed for the O-deethyla
tion of EFC (Table 1). Since the term placentas utilized in this investigation were obtained from women who may have been smokers, the involvement of CYP 1A1 in BUP metabolism was excluded.

The highest activity for the enzyme catalyzing the metabolism of BUP to norBUP was in the microsomal fraction (Table 1). The reaction required NADPH and exhibited sat

Fig. 2. A saturation curve for the enzyme catalyzed metabolism of BUP to norBUP. The inset is an Eadie-Hofstee plot of the data.

Fig. 3. The effect of inhibitors selective for various P450 isoforms on the formation of norBUP. Each inhibitor was coincubated with BUP (12 μM = Km) for 30 min at 37°C except troleandomycin, which was preincubated with the microsomes for 15 min before adding BUP. Data are represented as percentage of the control (in the absence of inhibitors). Each concentration of the inhibitor was tested on three placental microsomal preparations. * indicates statistical significance of p < 0.05 as determined by one-way ANOVA with Tukey’s comparison.
microsomal enzyme catalyzing BUP metabolism: the first
involved in biotransformation of the opiate during preg-

is the major site for BUP metabolism, but the placenta is also
(Kobayashi et al., 1998). These data suggest that human liver
trinsic clearance (Table 2) ranged between 0.18 and 0.42
affinities catalyze the reaction. The values obtained for in-
plot of the data revealed monophasic kinetics indicating that

The activity of cDNA-expressed CYP 19 preparations as a function
Fig. 5. The activity of cDNA-expressed CYP 19 preparations as a function
of BUP concentration. All determinations were carried out in triplicate.

ulation kinetics with an apparent $K_m$ of 12 $\mu$M and $V_{max}$ of
2.9 pmol/mg protein $\cdot$ min (Fig. 2; Table 2). An Eadie-Hofstee
plot of the data revealed monophasic kinetics indicating that
either a single enzyme or multiple enzymes with similar
affinities catalyze the reaction. The values obtained for in-
trinsic clearance (Table 2) ranged between 0.18 and 0.42
$\mu$M/mg protein $\cdot$ min (mean = 0.27) and is considerably lower
than that reported for human liver, 17 $\mu$M/mg protein $\cdot$ min
(Kobayashi et al., 1998). These data suggest that human liver
is the major site for BUP metabolism, but the placenta is also
involved in biotransformation of the opiate during preg-

Two types of inhibitors were used to identify the placental
microsomal enzyme catalyzing BUP metabolism: the first
type was chemicals selective for P450 isoforms, and the sec-
ond was monoclonal antibodies raised against purified hu-
man liver enzymes. The most potent inhibitors of norBUP
formation were ketoconazole, 4-hydroxyandrostenedione,
and aminoglutethimide (Fig. 3), suggesting that CYP 3A4
and CYP 19 could be the enzymes catalyzing the reaction. In
our investigations, ketoconazole caused a 70% inhibition of
norBUP formation (Fig. 3) at its concentration of 2.5 $\mu$M that
is selective for its effect on CYP 3A4 (Fabre et al., 1993;
Baldwin et al., 1995; Pelkonen et al., 1998). Ketoconazole
also inhibited the metabolism of BUP by two cDNA prepara-
tions expressing CYP 3A4 and CYP 19. Therefore, our data
confirmed earlier reports that ketoconazole is an inhibitor of
CYP 19 (Ayub and Levell, 1988), with $K_i$ values similar to
those of hepatic microsomal CYP 3A4 (Stresser et al., 2000).
A role for CYP 3A4 as the enzyme catalyzing the metabolism of
BUP by placental microsomal preparations was excluded
because troleandomycin, a selective inhibitor of the isozyme
(Newton et al., 1995), had no effect on norBUP formation.
Taken together, our data on the effects of chemical inhibitors
of the activity of placental microsomes on BUP metabolism
suggest that CYP 19 is the enzyme catalyzing the reaction.

Monoclonal antibodies raised against CYP 19 were the
most potent inhibitor of norBUP formation, confirming the
profile of the chemical inhibitors and the conclusion drawn
(Fig. 4). Moreover, analysis of the kinetics for BUP metabo-
lism by a cDNA-expressed CYP 19 revealed a $K_m$ value simi-
lar to that obtained for the placental enzyme (Fig. 5).

The activity exhibited by the mitochondrial fraction in
metabolizing BUP and our direct determination of testoste-
one aromatization by CYP 19 in both subcellular fractions
are in agreement with earlier reports on the presence of
aromatase activity in the mitochondrial fractions (Finkelstein
et al., 1985; Pasanen et al., 1985).

Our finding that CYP 19 is the enzyme responsible for the
metabolism of BUP in human placenta raises the issue of
drug interactions during pregnancy. Aromatase/CYP 19 is
the enzyme responsible for the conversion of androgens to
estrogens and the metabolism of certain xenobiotics in hu-
man placenta (Meigs, 1987; Toma et al., 1996; McNamara
et al., 1999). It is unclear whether BUP metabolism by placen-
tal microsomes would interfere with the biosynthesis of these
hormones or the detoxification of other drugs.

In summary, data in this report provided evidence that
aromatase/CYP 19 is the enzyme catalyzing the metabolism of
BUP to norBUP in trophoblast tissue obtained from term
human placentas.

Acknowledgments

We appreciate the support of and thank the National Institute on
Drug Abuse drug supply program for providing BUP, Drs. Andrew
Parkinson and Ajay Madan, XenoTech LLC, Kansas City, Kansas for
the generous gift of monoclonal antibodies, and Dr. Yasser Nashed,
University of Missouri, Kansas City School of Pharmacy for assis-
tance with mass spectrometry. The assistance of the medical staff
and the Chairman’s Research Group of the Department of Obstetrics
and Gynecology, University of Texas Medical Branch, is greatly
appreciated.

References

human placental aromatase by imidazole drugs including ketoconazole. J Steroid

Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 3A. Xenobiotica 25:281–270.
DeLuca JG, Dysart GR, Rasnick D, and Bradley MO (1988) A direct, highly sensitive

Address correspondence to Dr. Mahmoud S. Ahmed, Department of Ob- stetrics and Gynecology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0587. E-mail: maahmed@utmb.edu