The Effects of Cocaine and Nicotine on Amino Acid Transport across the Human Placental Cotyledon Perfused In Vitro

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ABSTRACT

The inhibitory effects of cocaine and nicotine on placental amino acid transport, as a mechanism contributing to intrauterine growth restriction, were investigated in the in vitro placental perfusion model. Amino acids that represent substrates for known placental transporters were selected: alanine (system A), glutamine (system N), phenylalanine and valine (system l), and arginine (system y^+). Amino acid accumulation on the fetal side was measured in the absence of cocaine or nicotine (n = 7) and in the presence of 1.2 µg/ml cocaine (n = 6), 120 ng/ml nicotine (n = 6), or both (n = 6). Neither cocaine nor nicotine alone significantly inhibited alanine transport, whereas their combination did (P = .02). Significant inhibition of arginine transport was detected with nicotine (P = .007), cocaine (P = .01), and their combination (P = .01), whereas phenylalanine (P = .03, P = .04) and valine (P = .03, P = .04) transport was affected by cocaine and the combination of cocaine and nicotine, respectively. For glutamine, neither cocaine, nicotine, nor their combination had a statistically significant inhibitory effect.

In conclusion, both cocaine and nicotine may contribute to fetal growth restriction by interfering with the activity of amino acids transporters that are necessary to maintain the nutrient gradients associated with normal fetal growth.

Cocaine abuse is widespread, and it is often the drug of choice of a pregnant substance abuser because of its availability, euphoric properties, and addictive nature. The prevalence of fetal exposure to cocaine is reported in various studies to range from 10 to 30% (Bateman et al., 1993; Koren, 1993; Eyler et al., 1998). Intrauterine growth restriction (IUGR) is one of the consistently observed adverse perinatal outcomes associated with cocaine use in pregnancy.

Cocaine users also have significantly greater exposure to other harmful substances. Among cocaine users, more than 85% also smoke cigarettes, compared with approximately 20% among the nonusers (Zuckerman et al., 1989; Neuspiel et al., 1991). As with cocaine, IUGR is one of the most prominent adverse effects associated with maternal cigarette smoking during pregnancy. Some studies have suggested that the concurrent use of cocaine with cigarette smoking may be more harmful to fetal growth than either substance used independently (MacGregor et al., 1987; Zuckerman et al., 1989). A study completed in Toronto by our group documented that babies exposed to both cocaine and cigarette smoke had a mean birth weight that was lower by 800 g, compared with 200 g lower for babies of “smoking only” mothers (Forman et al., 1993).

In cocaine users, it is difficult to define and control other potentially confounding factors on fetal growth such as maternal morbidity, poor maternal nutrition, or inadequate perinatal care. It is even less well understood how IUGR associated with cocaine use in pregnancy might be modified by the presence of cigarette smoking. Nevertheless, available data indicate that IUGR associated with both cocaine use and cigarette smoking in pregnancy may be a result of combined effects of these compounds on fetal growth (Pastrakuljic et al., 1999).

The relatively acute and transient nature of the vasoactive properties of cocaine and cigarette smoke suggest that fetal growth restriction associated with maternal cocaine use and cigarette smoking cannot be attributed solely to their vasoconstrictive properties. Fetal growth depends not only on nutrient provision via the uteroplacental circulation but also on nutrient transport across the syncytiotrophoblast. Studies in animals and in placental vesicles have shown that cocaine and nicotine have the ability to inhibit amino acid transport across the placenta. Therefore, they may directly affect the transfer of amino acids, a mechanism which has been demonstrated in IUGR pregnancies produced by agents other than cocaine and nicotine (Varma and Ramakrishnan, 1991; Pastrakuljic et al., 1999). Cocaine and nicotine interaction with amino acid transport systems in the syncytiotrophoblast may result in a deficit in the transport of amino acids across the placenta.

ABBREVIATIONS: IUGR, intrauterine growth restriction; F/M, fetal/maternal; RIA, radioimmunoassay; hCG, human chorionic gonadotrophin; HBSS, Hanks’ balanced salt solution.

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the placenta. As a consequence, decreased or altered amino acid pools may be available to the fetus. The lack of adequate pools of essential amino acids especially can result in a marked reduction in the rate of fetal growth.

Given the high rates of IUGR in newborns of cocaine users and cigarette smokers, in vitro evidence of amino acid transport inhibition by cocaine may provide the basis for a mechanistic understanding of how cocaine and other drugs of abuse might give rise to IUGR. Therefore, the aim of this study was to determine the inhibitory effect of cocaine, nicotine, and a combination of cocaine and nicotine on the net flux of five amino acids that represent different transport systems in human placenta: alanine (system A), valine and phenylalanine (system L), glutamine (system N), and arginine (system y-).

Materials and Methods

Chemicals. Nicotine hydrogen tartrate salt, (S)-nicotine, MgCl₂, Hanks’ balanced salt solution (HBSS), L-alanine, L-valine, L-arginine, L-phenylalanine, L-glutamine, 5-sulfosalicylic acid, heparin so-placenta was perfused with the addition of 120 ng/ml nicotine, and Hanks’ balanced salt solution (HBSS), L-alanine, L-valine, L-arginine, L-glutamine (0.51 mg/l), L-phenylalanine (0.09 mg/l), and L-valine (system y-). The flux of five amino acids that represent different transport systems was accomplished using a 20-cm ion-exchange column inside a high-temperature reaction vessel. A mixture of 0.1 ml of unknown specimen, standard specimen, or normal control was mixed with 200 ml of a radiolabeled antigen, 125I-benzoylecgonine (Simone et al., 1994). After precipitation with 200 ml of normal rabbit serum and 0.1 ml of goat anti-rabbit IgG serum at room temperature, the double antibody technique was used to separate the antibody-bound nicotine or cotinine from the free analyte (Obach and Van Vunakis, 1990). A mixture of 0.1 ml of [³H]nicotine, 0.1 ml of appropriately diluted serum, 0.1 ml of an unknown sample or 0.1 ml of known standard solution, and isogel Tris buffer (0.15 M NaCl, 0.01 M Tris, 0.1% gelatin, pH 7.4) for a total volume of 0.8 ml was incubated for 1 h at 37°C. The double antibody technique was used to separate bound and free [³H]nicotine by adding 0.1 ml of normal rabbit serum and 0.1 ml of goat anti-rabbit IgG serum at appropriate dilutions and incubating overnight at 4°C. After centrifugation at 1000g, 4°C, for 45 min, the immunoprecipitates were dissolved in 0.1 M NaOH (0.1 ml), and radioactivity was determined after addition of 2.5 ml of scintillation fluid. The radioactivity of the samples was determined using a scintillation counter and was expressed as counts per minute. The technique was sensitive to 0.5 ng/ml.

The RIA procedure for nicotine used [³H]nicotine, an antiserum raised in rabbits, and a goat anti-rabbit gamma globulin (IgG) to separate the antibody-bound nicotine or cotinine from the free analyte (Obach and Van Vunakis, 1990). A mixture of 0.1 ml of [³H]nicotine, 0.1 ml of appropriately diluted serum, 0.1 ml of an unknown sample or 0.1 ml of known standard solution, and isogel Tris buffer (0.15 M NaCl, 0.01 M Tris, 0.1% gelatin, pH 7.4) for a total volume of 0.8 ml was incubated for 1 h at 37°C. The double antibody technique was used to separate bound and free [³H]nicotine by adding 0.1 ml of normal rabbit serum and 0.1 ml of goat anti-rabbit IgG serum at appropriate dilutions and incubating overnight at 4°C. After centrifugation at 1000g, 4°C, for 45 min, the immunoprecipitates were dissolved in 0.1 M NaOH (0.1 ml), and radioactivity was determined after addition of 2.5 ml of scintillation fluid. The radioactivity of the sample was determined using a scintillation counter and was expressed as counts per minute. The technique was sensitive to 0.5 ng/ml.

Oxygen and glucose consumption and lactate production were measured throughout the perfusion experiment to confirm that the tissue maintained its ability for energy metabolism. Preferential secretion of hCG into the maternal circulation was further evidence of placental integrity and viability. Sample Analysis. The pH, pO₂, and pCO₂ of the perfusate samples were measured by using a blood gas analyzer (ABL 330; Radiometer, Copenhagen, Denmark). The perfusate concentrations of lactate, glucose, and hCG as well as tissue hCG content were measured as described previously (Simone et al., 1994). Nicotine and cocaine were measured by RIA assays. The amino acid analyses were performed on an LKB 4145 Alpha Plus System (LKB, Biochrom Limited, Cambridge, England) with norleucine as an internal standard. The separation of the amino acids was accomplished using a 20-cm ion-exchange column inside a column heater. A 20-μl sample containing a mixture of amino acids was loaded into the column and mixed with the ninhydrin reagent, and the mixture was passed through the high-temperature reaction coil. The colored compounds produced from the reaction were detected at 570 nm. The amount of colored compound produced was directly proportional to the quantity of amino acid present in the eluate. The area under the peak indicated the quantity of amino acid present. The coefficient of variation was 1.5%.

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The RIA procedure for cocaine was performed as follows: 25 μl of unknown specimen, standard specimen, or normal control was mixed in test tube with a 100-μl dilution of sheep anti-cocaine antibody (polyclonal) and 200 μl of a radiolabeled antigen, [¹³C]benzoylgonine (Simone et al., 1994). After precipitation with 200 μl of secondary antibody complex and incubation for 2 h at room temperature, samples were centrifuged for 30 min at 2000g. The tubes were decanted, and the pellets containing bound antigen were counted in a gamma scintillation counter; results were expressed as counts per minute. The technique was sensitive to 2 ng/ml, and the dose-response curve was used for calculation of the concentration of unknown specimens.
Results

The preliminary time course studies, with HBSS as a perfusion medium, showed that the placental preparation retained physical integrity and optimal metabolic activity for up to 2.5 h in a closed circuit experimental design. Accordingly, a time period of 140 min was adopted for the amino acid transport studies. Amino acid transport studies in the dually perfused placenta were conducted under four separate experimental conditions: control group, cocaine group, nicotine group, and cocaine and nicotine group. The data for amino acid levels in the fetal and maternal circulations were expressed as fetal/maternal (F/M) concentration ratios. To ensure that placentae used in experiments were naive to nicotine and cocaine, cocaine, nicotine, and cotinine levels were measured in the placental tissue of all placentae before perfusion. None of the placentae tested had detectable levels of either cocaine, nicotine, or cotinine before perfusion.

The mean mass of the perfused cotyledons, the fetal inflow pressure, and the fetal and maternal flow rates are shown in Table 1. Placental glucose and oxygen consumption and lactate production, as indicators of metabolic viability of the placental lobule (Table 1), did not significantly differ between control and experimental groups (Table 2). The preferential secretion of hCG into the maternal circulation was also verified (Table 1) and was not significantly different between control and experimental groups (Table 2).

Using seven placentae in the control group, the transfer of alanine, glutamine, phenylalanine, valine, and arginine toward the fetal circulation increased as a function of time during the observation period of 140 min. As shown in Table 3, the highest accumulation after 140 min on the fetal side of 33 ± 7.6% was observed for arginine. Under control conditions, phenylalanine and valine showed similar accumulations of 13 ± 5 and 13 ± 4%, respectively. Alanine showed an accumulation of 9 ± 1.5%, whereas glutamine showed the lowest accumulation of 7 ± 10%.

In the cocaine group (six placentae), the initial concentrations of cocaine in the maternal and fetal reservoirs were 1.12 ± 0.05 and 1.12 ± 0.15 μg/ml, respectively. In the group of six placentae perfused in the presence of nicotine alone, the initial concentration of nicotine in the maternal reservoir was 119 ± 7 and 118 ± 5 ng/ml in the fetal reservoir. In the group of six placentae perfused with both cocaine and nicotine, the concentration of cocaine in the maternal and fetal reservoirs was 1.2 ± 0.09 and 1.3 ± 0.07 μg/ml, respectively, and the concentration of nicotine in the maternal and fetal reservoirs was 113 ± 17 and 117 ± 20 ng/ml, respectively.

The cocaine and nicotine concentrations used in the in vitro perfusion studies were approximately 3 times higher than that observed in vivo users. This was done to ensure that the effect of cocaine and nicotine on transplacental amino acid transport could be observed within the time frame of the experiment, which is much shorter relative to the in vivo exposure over the entire course of the pregnancy.

As illustrated in Fig. 1, alanine transport toward the fetal circulation was not significantly reduced in the presence of either cocaine alone or nicotine alone. However, the combination of cocaine and nicotine significantly inhibited alanine transport (Table 3). Alanine was the only amino acid that showed a lower accumulation in the presence of both drugs than in the presence of each drug alone.

For glutamine (Fig. 2), neither cocaine, nicotine, nor their combination had a statistically significant inhibitory effect on its accumulation on the fetal side of the placenta. Although not statistically significant, the numerical value for the F/M ratio decreased in all three experimental groups, suggesting a reduction in glutamine transport (Table 3).

The F/M ratios of phenylalanine and valine in control and experimental groups are shown in Figs. 3 and 4, respectively. Cocaine alone significantly reduced phenylalanine and valine transport compared with the controls. In contrast, nicotine alone had no statistically significant inhibitory effect, although there was an apparent decrease in transport of both amino acids. A statistically significant reduction in the transport of both amino acids was also observed in the presence of both cocaine and nicotine, but this reduction is likely to be independent of a nicotine effect (Table 3).

Statistically significant inhibition of arginine transport (Fig. 5) was detected in all three experimental groups with a prominent nicotine effect. However, an additive or synergistic inhibitory effect on arginine accumulation on the fetal side was not observed with the combination of cocaine and nicotine (Table 3).

Discussion

By dually perfusing the placental cotyledon in vitro, the effects of cocaine and nicotine on the transport of amino acids across the human placenta as a mechanism contributing to IUGR seen clinically was investigated. Our hypothesis was based on epidemiological evidence that cocaine use concur-
rent with cigarette smoking in pregnancy may result in even more serious IUGR than either substance used independently. At the present time, the potential combined effect of cocaine and cigarette smoking on fetal growth through their effects on placental amino acid transport has not been elucidated.

Alanine, a system A substrate, was selected for the study for two reasons. First, system A is the dominant transport system in the microvillous membrane of the syncytiotrophoblast that transports neutral amino acids. Second, given that alanine has the highest concentration of all amino acids in the human plasma and is a major fetal gluconeogenic substrate, inhibition of alanine transport toward the fetal circulation may have serious implications on fetal growth (Pastrakuljic et al., 1999).

In the presence of both cocaine and nicotine, the alanine F/M concentration ratio was significantly decreased, suggesting a combined effect of cocaine and nicotine on the system A transporter. Therefore, this mechanism may play a critical role in more serious IUGR in pregnancies exposed to both drugs. Although statistically not significant, an apparent trend of decreased alanine transport was evident with both cocaine alone and nicotine alone. If a greater number of

### TABLE 2
Comparison of physical parameters among the study arms

<table>
<thead>
<tr>
<th>Comparisons (Group)</th>
<th>O$_2$ Consumption</th>
<th>Glucose Consumption</th>
<th>Lactate Production</th>
<th>Inflow Pressure</th>
<th>hCG Secretion</th>
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</thead>
<tbody>
<tr>
<td>Control vs. Nicotine</td>
<td>.63</td>
<td>.64</td>
<td>.18</td>
<td>.39</td>
<td>.83</td>
</tr>
<tr>
<td>Control vs. Cocaine</td>
<td>.20</td>
<td>.73</td>
<td>.32</td>
<td>.54</td>
<td>.78</td>
</tr>
<tr>
<td>Control vs. Coc &amp; Nic</td>
<td>.20</td>
<td>.24</td>
<td>.25</td>
<td>.78</td>
<td>.64</td>
</tr>
</tbody>
</table>

Coc & Nic, cocaine and nicotine.

### TABLE 3
F/M amino acid concentration ratios in the control group, cocaine group, nicotine group, and cocaine and nicotine group

Values are expressed as the mean ± S.E.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.09 ± 0.01</td>
<td>1.00 ± 0.05</td>
<td>NS</td>
<td>1.02 ± 0.03</td>
<td>NS</td>
<td>0.98 ± 0.03</td>
<td>0.02</td>
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<tr>
<td>Glutamine</td>
<td>1.07 ± 0.10</td>
<td>0.86 ± 0.05</td>
<td>NS</td>
<td>0.85 ± 0.05</td>
<td>NS</td>
<td>0.85 ± 0.05</td>
<td>NS</td>
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<tr>
<td>Phenylalanine</td>
<td>1.13 ± 0.05</td>
<td>0.94 ± 0.06</td>
<td>.03</td>
<td>1.01 ± 0.05</td>
<td>NS</td>
<td>0.97 ± 0.04</td>
<td>.04</td>
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<tr>
<td>Valine</td>
<td>1.13 ± 0.04</td>
<td>0.93 ± 0.06</td>
<td>.03</td>
<td>1.04 ± 0.03</td>
<td>NS</td>
<td>0.97 ± 0.04</td>
<td>.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>1.33 ± 0.07</td>
<td>1.07 ± 0.03</td>
<td>.01</td>
<td>0.98 ± 0.08</td>
<td>0.007</td>
<td>1.03 ± 0.06</td>
<td>.01</td>
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</table>

NS, nonsignificant; Ctrl, control; Coc, cocaine; Nic, nicotine; Coc & Nic, cocaine and nicotine.

**Fig. 1.** F/M concentration ratios for alanine in the absence (Control; n = 7) and in the presence of cocaine (n = 6), nicotine (n = 6), and the combination of cocaine and nicotine (Coc & Nic; n = 6). Each bar represents the mean ± S.E. (*P < .05).

**Fig. 2.** F/M concentration ratios for glutamine in the absence (Control; n = 7) and in the presence of cocaine (n = 6), nicotine (n = 6), and the combination of cocaine and nicotine (Coc & Nic; n = 6). Each bar represents the mean ± S.E.
experiments was available to confirm that this trend is significant, it could be of clinical significance. For example, even a small decrease in alanine transport could have a profound effect on fetal growth when exposure is long-term over the course of gestation. The observations that cocaine and nicotine alone could have the potential to inhibit alanine transport and affect the system A transporter are consistent with published data. Cocaine was shown to inhibit alanine transport when added in vitro to microvillous and basal membrane vesicles derived from human placenta, whereas nicotine was shown to inhibit uptake of system A substrates in human placental slices (Barnwell and Sastry, 1983; Dicke et al., 1993, 1994). In contrast, the study by Krishna et al. (1995) showed that concentrations of cocaine likely to be encountered in vivo do not affect alanine transport across the perfused human placenta. A possible explanation for these negative findings is that the placental tissue was exposed to cocaine for only a short period of time (40 min).

The potential adverse impact of cocaine and nicotine on fetal growth was also explored by testing their effects on arginine transport across the placenta. Arginine is primarily transported by system y\(^+\), and given its high capacity, system y\(^+\) is believed to serve as a major cationic transporter on both membranes of the syncytiotrophoblast (Moe, 1995). In addition, the cationic amino acids, such as arginine, have physiological uptakes that barely exceed their accretion rates into fetal proteins. Under normal circumstances, there is a very narrow margin between placental arginine transport capacity and fetal demand, implying that even slight alterations in transporter activity due to exposure to cocaine and nicotine may result in fetal growth restriction.

In this study, a significant decrease in the F/M concentration gradient of arginine was observed with cocaine and with nicotine alone, as well as with their combination. Figure 5 provides evidence that there is no additional inhibition of arginine transport when both cocaine and nicotine were present, suggesting a lack of combined effect. These observations suggest that one of the possible mechanisms by which
cigarette smoking and cocaine affect fetal growth may be impairment of system y⁻. These findings are in contrast with studies in rat placental vesicles that showed no effect of cocaine on arginine transport (Novak et al., 1995). This could be due to species differences that are likely to exist between the human and the rat placenta with regard to amino acid transporters. There are no studies of cocaine or nicotine effect using human placental vesicles. However, a study with lysine, another system y⁻ substrate, showed that high concentrations of cocaine have the potential to inhibit lysine transport in human placental villous fragments (Barnwell and Sastry, 1983), which is in agreement with our findings. Cordocentesis studies in humans also suggest that arginine transport may be significantly depressed in IUGR pregnancies not resulting from cocaine use (Cetin et al., 1996).

Phenylalanine and valine showed a similar pattern of transfer toward the fetal circulation in control and experimental groups of placental. Both amino acids are transported by sodium-independent system l, located on both membranes of the syncytiotrophoblast. In the presence of nicotine alone, no significant inhibition of phenylalanine and valine transport toward the fetal circulation was observed. The F/M concentration ratio showed a statistically significant inhibitory effect of cocaine on the transport of phenylalanine and valine, suggesting that cocaine is a major factor affecting system l when both drugs are used together. Regarding the fact that system l is also responsible for sodium-independent transport of alanine, inhibition of this system by cocaine may significantly contribute to inhibition of alanine transport as well. In addition, the significance of this reduction for fetal growth is that branched-chain amino acids are extensively used by the fetus for energy and protein synthesis, and therefore an impairment of their transplacental transport may result in fetal growth restriction. This hypothesis is confirmed by studies in IUGR pregnancies that consistently show low fetal levels of branched-chain amino acids (Cetin and Pardi, 1994).

For glutamine, a system N substrate, neither cocaine, nicotine, nor their combination had a statistically significant inhibitory effect on its transport toward the fetal circulation. A trend toward a decrease in the F/M concentration ratio in the presence of cocaine and nicotine alone and their combination was evident. However, this apparent inhibition of glutamine transfer lacked statistical significance, possibly due to the limitations of detecting differences with the low rates of glutamine transfer observed in the control group of placenta. The importance of system N inhibition by cocaine is not only in the context of reduced glutamine transport but also on the effect this has on glutamate synthesis. Presence findings suggest that glutamate synthesis is not likely to be affected in drug-exposed fetuses.

Both cocaine and nicotine are potent vasconstrictors, and their vascular effects could also result in reduced placental nutrient transfer to the fetus. Findings from this study clearly show that vasconstriction is not the only cause of amino acid uptake and transfer inhibition. However, when these drugs are abused together, vasconstriction may act to exacerbate other causes of decreased amino acid transfer to the fetus such as drug interaction with amino acid transporters.

In conclusion, the in vitro placental perfusion studies showed a significant relationship between cocaine and decreased amino acid transport of phenylalanine (system l) and arginine (system y⁻) and between nicotine and decreased amino acid transport of arginine (system y⁻). Regarding their combined drug effect, cocaine and nicotine appeared to have a combined inhibitory effect on alanine (system A). Furthermore, cocaine and nicotine alone showed a trend to decrease transport of all five amino acids tested, suggesting that their continuous presence throughout pregnancy may result in fetal amino acid deprivation, further resulting in fetal growth restriction. Further studies are needed to corroborate these findings with in vivo measurements of maternal/fetal and maternal/neonatal concentration ratios of amino acids in growth restricted infants exposed in utero to cocaine, cigarettes, and their combination.

References


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