Comparison of cerebrovascular effects of intravenous cocaine injection in fetal, newborn, and adult sheep

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COCAINA ABUSE CONTINUES to be a major health problem in the United States (6, 28, 30, 31). Maternal abuse of cocaine has been associated with neonatal stroke, seizures, intracranial hemorrhages, microcephaly, and neurobehavioral abnormalities, but the pathogenesis of these effects remains unknown (4, 6, 7, 14, 28, 31). Newborns may continue to be exposed to cocaine passively via smoke or actively via breast milk. In the adult, cocaine abuse can lead to cerebral hemorrhagic or vasoocclusive injury, resulting in seizures, ischemic attacks, or stroke. Conflicting results from recent animal studies have not yielded a consistent explanation regarding the mechanisms for these cocaine-induced cerebrovascular effects (1, 9, 10, 19, 31). Whereas species and methodological differences may explain some of the conflicting results, we have questioned whether developmental differences may play a role. Therefore, we compared the cerebrovascular responses of a 2 mg/kg iv dose in unanesthetized fetal, newborn, and adult sheep to determine whether the same dose of cocaine without the confounding variable of the uteroplacental circulation would cause cerebral vasodilation at all developmental stages and to determine what, if any, developmental differences there were in the response to cocaine.

METHODS

All surgical procedures and experimental protocols were approved by our institutional Animal Care and Use Committee.

Animals and Surgical Preparation

Fetuses. Eight mixed-breed fetal sheep comprised the fetal group; their responses to a 2 mg/kg iv cocaine injection were reported previously (14). These fetuses ranged from 131 to 136 (133 ± 1) days gestation and weighed 4,424 ± 362 g.

Newborns and adults. Six newborn and 12 adult mixed-breed sheep were used for study. Newborns ranged from 2 to 7 (5 ± 1) days old and weighed 4,733 ± 226 g. Adults were ≥2 yr of age. The animals were brought to the animal care facility on the day before the study and given procaine penicillin (450,000 U im) before surgery. The sheep were anesthetized with pentobarbital sodium (15–20 mg/kg) via a catheter placed percutaneously in the external jugular vein. Additional pentobarbital sodium (1–2 mg/kg) was administered as needed. All cutaneous sites of entry were prepared with an alcohol-betadine solution.

Polyvinyl chloride catheters were placed into the left ventricle and brachiocephalic artery (via the axillary arteries)
and into both femoral arteries and a femoral vein. There was no evidence of arterial insufficiency to the limbs at the time of surgery or on the next day (the day of study). Catheters were flushed and filled with heparinized saline (10 U/ml), sutured to the skin, and placed into a pouch attached to the abdomen.

The sagittal, coronal, and lambdoid sutures were identified, and a shallow burr hole was drilled over the sagittal suture ~0.5 cm anterior to the lambda. The sagittal sinus was identified, and the overlying dura was punctured with a 19-gauge needle. A polyvinyl chloride catheter was then inserted into the sagittal sinus, and its tip was positioned anterior to the confluence of the sinuses to minimize contamination from extracerebral venous blood. The catheter was then flushed and filled with heparinized saline and sutured securely to the scalp. The sheep was then weighed and allowed to recover in the laboratory until it could stand and feed; then it was returned to the animal care facility. On the next day the sheep was returned to the laboratory. Full recovery from anesthesia and surgery was assessed by clinical examination, suckling behavior (in newborns), and baseline arterial blood gas and pH values. Baseline physiological parameters were consistent with previous studies from our laboratory in unanesthetized newborn and adult sheep that had undergone surgery 1 day previously.

**Physiological Measurements**

Regional brain blood flow was measured using the radio-labeled microsphere technique and the least-squares method of differential spectroscopy (13). Approximately 1 × 10⁶ microspheres (0.4 ml) in the fetus and newborn and 2.5 × 10⁶ microspheres (1.0 ml) in the adult, labeled with ¹⁵³Gd, ¹¹⁴In, ¹¹³Sn, ¹⁹³Ru, ⁹⁵Nb, or ⁴⁶Sc (DuPont NEN, Boston, MA), were injected into the left ventricle (in the newborn or the adult) or into the inferior vena cava in the fetus. Microspheres were injected over 30 s, then 5 ml of 0.9% saline were infused. Reference samples were withdrawn from the brachiocephalic artery at a rate of 2.65 ml/min beginning 30 s before the injection and continuing for 1.5 min after the injection was completed. The injections of microspheres were not associated with changes in heart rate (HR), blood pressure, or pulse pressure. This technique provides complete trapping of microspheres by organs, with comparable distribution of spheres in the reference sample (13). After completion of the studies, the animals were killed with an overdose of pentobarbital sodium followed by saturated KCl solution. Catheter positions were checked, and the brain was then removed at the base and divided at the cephalic border of the pons. All supratentorial tissue was pooled, and the radioactivity was counted to calculate cerebral blood flow (CBF). Blood flow was measured separately for the cerebellum and the brain stem (medulla and pons). Radioactivity in blood and tissue samples was determined with a gamma counter (model 5530, Packard Instrument, Downers Grove, IL). All reference blood and tissue samples contained ~400 microspheres.

Blood samples for pH, respiratory blood gases, Hb concentration, O₂ saturation, and hematocrit were withdrawn into heparinized syringes. Blood gas values and pH were measured using a model ABL 30 blood gas analyzer and electrode (Radiometer, Westlake, OH). O₂ saturation and Hb concentration were measured using a hemoximeter (model OSM-3, Radiometer). Mean arterial blood pressure (MAP, referenced to amniotic fluid pressure in fetuses) and HR were continuously monitored (model 2400, Gould Instruments, Oxnard, CA) throughout the experiment.

Blood concentrations of cocaine and its metabolites were measured in arterial blood collected in tubes containing 0.1 ml of enzyme inhibitor (equal parts of a saturated sodium fluoride solution and a 10% solution of glacial acetic acid) per 2.0 ml of blood. The blood was mixed with the inhibitor and centrifuged, and the plasma was stored frozen (~70°C) until analyzed. Analysis for cocaine and its metabolites [ecgonine methyl ester (EME) and benzoylecgonine (BE)] was performed by electron impact gas chromatography-mass spectrometry (multiple-ion monitoring) after extraction with solid-phase extraction cartridges. Deuterated internal standards were used for quantitation. The assay gives a linear response across a concentration range of 3.1–1,000 µg/l. The limit of quantitation by this assay was 1.0 µg/l for each analyte (8).

Blood samples for catecholamines (1.5 ml) were withdrawn into heparinized 3-ml syringes, kept on ice, and then centrifuged at 4°C for 20 min. The plasma was removed, and samples were stored at ~70°C until analyzed. Fetal samples were measured by HPLC with electrochemical detection, as described by Nishijima et al. (20). Samples were purified by alumina absorption. Epinephrine and norepinephrine were oxidized at 650 mV (vs. Ag-AgCl) on a Bioanalytical Systems vitreous carbon working electrode. An integrator quantified catechols by internal standardization. The sensitivity of the assay was 20 ng/l. Newborn and adult catecholamine samples were shipped on dry ice to Dr. Michael Heymann’s laboratory at the University of California, San Francisco, for analysis by HPLC with electrochemical detection (15). Briefly, the thawed plasma samples were extracted by absorption onto acid-washed alumina at pH 8.6, washed, and eluted with 200 µl of 0.1 M perchloric acid. A precise aliquot of the eluate was injected into the HPLC for analysis. The mobile phase consisted of 0.1 M sodium phosphate, 100 mg/ml EDTA, and 80 mg/ml SDS (pH 3.8) flowing at a rate of 1.0 ml/min. The catecholamines were separated on an 8 cm × 4.6 mm ID 3-µm C₁₈ reverse-phase column (ESA, Bedford, MA). The detector (Coulochem Electrochemical Detector, ESA), with a cutoff voltage of 30 mV and detector voltage of ~330 mV, detects catecholamines in amounts of 20–25 pg per injected sample. The average recovery of each catecholamine is quite reproducible from day to day, but the recovery of one catecholamine is quite different from that of another. Thus each assay is run with a complete external (unextracted) and internal (extracted from charcoal-treated plasma) standard curve of five concentrations of each catecholamine of interest. The chromatographs of plasma samples are compared with the internal standard curve to determine plasma catecholamine concentrations. The intra- and interassay coefficients of variation for norepinephrine and epinephrine are 1.4 and 2.7, respectively.

**Experimental Protocol**

On the day of the study, the pregnant ewe or the study animal (newborn or adult) was brought to the laboratory, placed in a specially designed study cart, and allowed 1 h to become accustomed to the surroundings. Six measurements were made during each study. For each measurement, blood samples were drawn first, and then microspheres were injected into the left ventricle (in the newborn or the adult) or the inferior vena cava (in the fetus) while a reference sample was withdrawn from the brachiocephalic artery. Two baseline measurements were obtained 15 min apart. Results from these two measurements were averaged to yield a single “baseline” measurement. At 15–30 min after the second baseline, pure cocaine hydrochloride (2 mg/kg in the newborn
and adult and 2 mg/kg estimated fetal weight; Sigma Chemical, St. Louis, MO) dissolved in 5 ml of 0.9% saline was injected intravenously over 30 s followed by a 5-ml 0.9% saline flush. The 2 mg/kg dose was chosen to standardize the dose with previous studies (32) and because this dose has been reported to be in the range of those used by human cocaine addicts (16, 24, 32). Three complete measurements were obtained at 30 s and at 5 and 15 min (fetus), and a fourth measurement was obtained at 60 min (newborn and adult) after the cocaine injection.

Samples for determination of cocaine, cocaine metabolites (EME and BE), and catecholamines were drawn before administration of cocaine and at 5 and 15 min after the cocaine injection.

Two additional animals in each group were studied using the identical protocol, except, instead of cocaine, they received an injection (5 ml) of 0.9% saline. These animals were used to assess the stability of this awake, unanesthetized preparation over the time course of the study.

Data Analysis and Calculations

Organ blood flow was calculated as follows: (cpm_{organ}/cpm_{ref}) \times reference withdrawal rate (ml/min), where cpm_{organ} and cpm_{ref} represent radioactive counts in the organ and reference samples, respectively. Vascular resistance was calculated as MAP \div organ blood flow. Arterial O_2 content (CaO_2) was calculated as 1.35 \times Hb \times O_2 saturation. Venous O_2 content (CvO_2) was calculated similarly. Cerebral metabolic O_2 consumption (CMRO_2) was calculated as (CaO_2 - CvO_2) \times CBF. Cerebral O_2 delivery was calculated as CaO_2 \times CBF. Cerebral fractional O_2 extraction was calculated as CMRO_2 / O_2 transport.

Differences within groups were tested by ANOVA for repeated measures. If the F test was significant, specific differences were tested with the Newman-Keuls test. An unpaired t-test was used to identify differences within groups, specifically, to identify differences in each timed measurement after cocaine injection from the baseline measurement. Significance was considered at P ≤ 0.05. Values are means ± SE.

RESULTS

Cardiovascular variables (MAP and HR) and arterial blood gases at baseline and in response to cocaine administration are shown in Table 1. In all three groups, cocaine caused an immediate increase in MAP, which returned to baseline in the fetus and newborn after 5 min and remained increased until 15 min in the adult. HR was unchanged in all three groups, as was hematocrit. There was no change in pH in any of the three groups, whereas there was mild persistent hypercapnia and hypoxemia in the fetus and transient hypercapnia and hypoxemia in the adult.

CBF and calculated cerebrovascular resistance at baseline and after intravenous cocaine are shown in Figs. 1 and 2. Cocaine injection caused an increase in CBF in all three groups, but the increase was observed at 5 min in the fetus and newborn (36 ± 7 and 33 ± 4%, respectively), whereas in the adult the increase (19 ± 5%) was observed 15 min after the injection. CBF in
fractional O₂ extraction at 5 min, whereas in the other two groups there was no change in O₂ extraction throughout the study. This increase could be attributed to the decrease in CaO₂ in the fetus. There was no change in animal activity in the fetus or the newborn, i.e., seizure activity, excitability, or changes in behavior. Brief seizure activity was observed in two of the adult animals immediately after the cocaine injection, which led to decreases in CaO₂, CMRO₂, and O₂ transport, with no change in O₂ extraction.

Serum catecholamine, cocaine, and cocaine metabolite levels are shown in Table 3. Cocaine levels increased 5 min after the injection in all three groups. Cocaine was rapidly metabolized to EME in all three groups, with the EME level remaining elevated 15 min after cocaine injection. Cocaine and EME levels were similar in all three groups. However, BE levels were higher in the adult at 5 and 15 min than in the newborn and fetus, and levels were higher in the newborn than in the fetus. Baseline serum catecholamine (norepinephrine and epinephrine) levels (pg/ml) are similar to previous studies (14, 21). Cocaine injection caused an increase in norepinephrine levels in the fetus and newborn but not in the adult; the percent increase was greatest in the fetus. Epinephrine levels increased in the adult but not in the fetus or newborn.

**DISCUSSION**

The major findings of this unique comparative study are that cocaine injection causes cerebral vasodilation and increased O₂ consumption in fetal, newborn, and adult animals immediately after the cocaine injection, with no change in O₂ extraction.

**Table 3. Serum catecholamine levels and cocaine and cocaine metabolite levels at baseline and after cocaine injection**

<table>
<thead>
<tr>
<th></th>
<th>Catecholamines, pg/ml</th>
<th>Cocaine and Cocaine Metabolites, ng/ml</th>
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<tr>
<td></td>
<td>NE</td>
<td>Epi</td>
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<tr>
<td><strong>Fetus</strong></td>
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<tr>
<td>Baseline</td>
<td>355 ± 86</td>
<td>174 ± 51</td>
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<tr>
<td>30 s</td>
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<tr>
<td>5 min</td>
<td>2,989 ± 1,061*</td>
<td>377 ± 80</td>
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<tr>
<td>15 min</td>
<td>3,161 ± 1,297*</td>
<td>326 ± 101</td>
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<tr>
<td>60 min</td>
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<tr>
<td><strong>Newborn</strong></td>
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<tr>
<td>Baseline</td>
<td>125.3 ± 16</td>
<td>16 ± 10.6</td>
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<tr>
<td>30 s</td>
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<tr>
<td>5 min</td>
<td>257.7 ± 73</td>
<td>26.7 ± 18</td>
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<tr>
<td>15 min</td>
<td>215.3 ± 36*</td>
<td>25 ± 19</td>
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<td>60 min</td>
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<tr>
<td><strong>Adult</strong></td>
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<tr>
<td>Baseline</td>
<td>117.7 ± 40</td>
<td>100 ± 30</td>
</tr>
<tr>
<td>30 s</td>
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<tr>
<td>5 min</td>
<td>244 ± 74</td>
<td>385 ± 106*</td>
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<tr>
<td>15 min</td>
<td>246 ± 91</td>
<td>187 ± 37*</td>
</tr>
</tbody>
</table>

Values are means ± SE. NE, norepinephrine; Epi, epinephrine; BE, benzylecgonine; EME, ecgonine methyl ester. *P < 0.05 vs. baseline; †P < 0.05, newborn vs. fetus; ‡P < 0.05, adult vs. newborn and fetus.
adult sheep, but there are developmental differences in the timing of the responses and direct effects of cocaine on systemic oxygenation. The initial cerebral vasoconstriction in the fetus could be a direct effect of the cocaine injection and could subsequently lead to fetal hypoxemia (cerebral vasodilation) and increased CMRO2 (14). We observed a comparative delay in cerebral vasodilation and increased O2 consumption in the adult. Also, EME levels were increased after cocaine administration; this increase could account, in part, for the vasodilation observed in our three groups (22). These findings therefore support the hypothesis that clinical and experimental differences in the cerebral responses to cocaine injection may be explained, in part, by developmental differences.

The mechanism for these developmental differences in the cerebrovascular responses to cocaine is not known. One possibility could be developmental differences in cocaine metabolism. Fetuses and newborns lack the metabolic capabilities of adults, and therefore cocaine levels may remain elevated for a longer period of time, and appearance of cocaine’s various metabolites, all of which are vasoactive, may be delayed (12). Indeed, we found significantly higher BE levels in the adult than in the newborn and fetus, and these BE levels persisted. In isolated sheep cerebral arteries, BE causes vasoconstriction (26). It is possible, therefore, that the higher BE levels in the adult contributed to the delayed cerebral vasodilation we observed. However, differential metabolism could not explain the initial vasoconstriction we observed in the fetus while fetal BE levels were very low.

Another possible explanation for the developmental differences we observed could be differences in CMRO2, either intrinsically or in response to cocaine. 1) We observed an increase in CMRO2 after cocaine injection in all three groups, with the increase occurring later in the adult. 2) CMRO2 is closely coupled with CBF (27). 3) The mechanism whereby cocaine increases CMRO2 is not known but likely is related to increased norepinephrine levels. 4) Cocaine blocks the reuptake of norepinephrine and other neurotransmitters (serotonin and epinephrine) at the synaptic cleft (23). Norepinephrine infusion has been shown to increase CMRO2 and CBF in baboons (18). All three developmental groups showed an increase in norepinephrine concentrations after cocaine injection, but there was a trend toward higher levels in the fetus. This could account for a greater cerebral vasodilatory response to cocaine in the fetus than in the adult.

The most likely explanation for the developmental differences in the timing and extent of responses to cocaine could be the transient hypoxemia that was observed in the fetus (not due to uteroplacental vasoconstriction) and in the adult. In our original fetal report, Iida et al. (14) proposed that the fetal hypoxia could be due to increased brain metabolism associated with cocaine-induced seizure activity or changes in behavioral state. One limitation to this study was our inability to see the fetus during the study and to detect any evidence of subclinical seizure activity in this group. We observed no hemodynamic or blood gas changes suggestive of fetal seizures, but we did not measure electroencephalogram activity during our study, so we may have missed evidence of seizure activity. Indeed, in two of our adult sheep we did observe gross seizure activity, but we did not observe such activity in our newborns. Whatever the cause of the hypoxemia, it was temporally related to the cerebral vasodilation we observed after cocaine injection (e.g., 5 min in the fetus and 15 min in the adult).

Our study results support the results of all our previous studies showing that cocaine injection causes cerebral vasodilation in developing sheep and in cats (10, 11, 14, 21). Others have shown cerebral vasodilation in vivo and in vitro (1, 2, 11, 17, 21, 26, 29). Stein and Fuller (29) reported results similar to our studies, in that they observed an increase in CBF in the rat model as well as a transient increase in blood pressure. Yonetani et al. (33) showed that an acute cocaine injection caused a persistent decrease in cortical O2 pressure with a decrease in CBF and blood pressure. We observed an increase in arterial blood pressure in all three groups; the fetal results were similar to our previous observations after maternal cocaine injection (11). Our group, using a 4 mg/kg dose, previously reported cerebral vasodilation after a direct cocaine injection in near-term fetal sheep and in newborn sheep (14, 21) and vasodilation after cocaine injection in unanesthetized newborn sheep without hypoxemia (21) that was also observed in this study. Others have also shown that cocaine injection is not associated with systemic hypoxemia. Most recently, for example, Burchfield et al. (5) infused cocaine over 30 min into near-term fetal sheep (0.2 mg · kg⁻¹ · min⁻¹ for 30 min = 6 mg/kg, levels ~230 ng/ml) and observed no significant hypoxemia or changes in CBF or O2 delivery. We speculated previously that species and/or methodological differences (route of injection, anesthesia techniques, or timing of measurements) may account for these conflicting results in laboratory animals. Now we could add developmental differences in cerebrovascular or metabolic development to the possible reasons for the conflicting vascular responses to cocaine reported in the literature.

We chose a standard dose of 2 mg/kg rapid intravenous cocaine bolus to compare the cerebrovascular responses at each developmental stage with the same event. This dose is also similar to the dose used by human cocaine addicts (16, 24, 32). However, the metabolism of cocaine is much more rapid in sheep than in humans. In humans the half-life of a single dose of intravenous cocaine in plasma is 48 min (16); in sheep it is 10 min (21). Cocaine and its metabolites have been reported to persist in the urine of adult humans for as long as 60 h after intranasal use (3). Cocaine or cocaine metabolites may have a direct or an indirect effect on the cerebral vascular bed (21, 22). In sheep, cocaine is primarily metabolized to EME; in humans, the major metabolite is BE. EME causes vasodilation in newborn sheep (22) and in isolated cat cerebral arteries (19, 21). EME levels were elevated at 5 min in all three groups.
in our study, and they remained elevated at 15 min. Therefore, differences in cocaine and cocaine metabolism make it difficult to extrapolate our sheep results to humans.

Although there are certain limitations to our studies, we are the first to use an identical unanesthetized experimental protocol to examine the cerebrovascular and metabolic responses to the same dose of any drug at three developmental periods. The sheep is an ideal animal for these studies because of its multicotyledonal-ary placenta, which allows significant fetal and uterine manipulation without precipitating preterm labor or causing fetal distress. In addition, there are numerous previous cerebral physiological studies for comparison at all developmental stages in sheep.

Overall, the results of this unique comparative sheep study confirm that cocaine causes cerebral vasodilation in sheep of all ages, but we have observed developmental differences in the timing and extent of these responses. Our study results support the hypothesis that clinical and perhaps species variability in cocaine's cerebral vascular effects may be due, in part, to developmental differences.

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