Alpha-2A Adrenergic Receptor Subtype Gene Expression in the Intestines of Cocaine-Exposed Rat Embryos

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ABSTRACT

Cocaine has become a popular illicit drug in our society, and pregnant women are not immune from this epidemic. Recently, there have been several references in the literature describing an association between prenatal cocaine exposure and the subsequent development of necrotizing enterocolitis in the neonate, but the mechanism underlying this relationship remains speculative. Because α -2 adrenergic receptors are thought to play a role in the autoregulatory mechanism in the newborn intestine that responds to hypoxia and ischemia, we examined the expression of this receptor in the intestine of embryonic rats exposed to low- and high-dose cocaine *in utero*. Pregnant Sprague Dawley rats were injected daily with either saline, low-dose cocaine, or high-dose cocaine beginning on embryonic day 5 (E 5) and continuing to E 20. Mothers were killed on E 16, E 17, E 18, E 19, and E 20. Embryos were frozen and stored at -80°C . *In situ* hybridization was performed on 20- μm sections with ^{35}S -labeled

oligonucleotide probes specific for the α -2A adrenergic receptor subtype. Densitometric analysis revealed a significant decrease in the α -2A receptor expression in the intestine of both the low-dose and high-dose cocaine-exposed animals compared with controls. This down-regulation was demonstrated by E 17, and continued through the remainder of gestation. These changes may limit the normal adaptation to vasoconstriction, thus exacerbating the already insufficient compensatory mechanisms for responding to ischemic injury, and thus may be one of the important factors predisposing cocaine-exposed infants to necrotizing enterocolitis. (*Pediatr Res* 52: 504–508, 2002)

Abbreviations

NEC, necrotizing enterocolitis
E, embryonic day

The prevalence of cocaine use in women of child-bearing age has increased significantly in recent years, resulting in considerable morbidity and mortality in neonates exposed to cocaine *in utero*. It has been estimated that 17% of pregnant women in an urban setting have used cocaine at some point during pregnancy (1). Cocaine exposure has been associated with many perinatal complications, including placental abruption, prematurity, intrauterine growth retardation, cerebral infarcts, developmental delay, limb reduction defects, and other vascular disruptions (2, 3). In the pregnant mother, cocaine causes an α -adrenergic-mediated mesenteric and uterine vascular constriction resulting in a dose-dependent decrease in uterine blood flow (4). These uterine blood flow changes can jeopardize the fetus by causing profound hypoxemia, resulting

in decreased fetal oxygenation and hypotension. Furthermore, cocaine can cross the placenta and produce levels in the fetus that are up to 10% of the mother's (4). This can lead to fetal tachycardia and hypertension—posing further hazard to the fetus.

Recently, there have been several references in the literature linking *in utero* cocaine exposure and the subsequent development of NEC in the neonate. These include several case reports of either NEC or intestinal infarction following maternal cocaine use (2, 3, 5–8). In addition, several studies have found an increased risk of NEC in cocaine-exposed newborns compared with nonexposed controls (9–12). One of these studies reported that the cocaine-exposed infants with NEC tended to develop more severe disease, were more likely to require surgery, and had a higher mortality rate (13). To date, there is only one animal study linking prenatal cocaine exposure to the subsequent development of NEC in the newborn. Buyukunal *et al.* (14) noted a dose-dependent relationship between *in utero* cocaine exposure and the development of histopathologic changes consistent with NEC in rat pups. This same group reported significant decreases in the perfusion of the uterus,

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placenta, and fetus in cocaine-exposed pregnant rats compared with controls in a more recent study (15). Despite these findings, however, the definitive mechanism underlying the interaction between cocaine exposure and the subsequent development of NEC remains largely speculative.

Cocaine administration is known to cause an increase in the extracellular levels of several neurotransmitters, including norepinephrine, epinephrine, dopamine, and serotonin by blocking their reuptake in the synaptic cleft (16). This then leads to their accumulation at postsynaptic receptor sites, and therefore to activation of adrenergic receptors—the family of G protein-coupled receptors that mediate tissue responses to catecholamines (17). These receptors were originally subdivided into α and β types based on pharmacologic differences; however, more extensive studies have allowed further subclassification into α -1 and α -2 subtypes. Most recently, molecular techniques have led to the cloning of several subtypes of both the α -1 and α -2 adrenergic receptors (17). At least three distinct human α -2 adrenoceptor subtypes have been identified to date: the α -2A, the α -2B, and the α -2C (18). Although linking function to specific receptor subtypes has been difficult due to insufficient subtype-specific ligands (17), there are studies that have associated α -2 adrenergic receptors with the regulation of intestinal motility and intestinal water and electrolyte absorption (19–21). There is also data to suggest that α -2 adrenergic receptors are present on endothelial cells and can mediate vascular relaxation when activated (22). In addition, there is evidence that the α -2 adrenergic receptors may regulate intestinal vasodilation under hypoxic or ischemic conditions (23). Therefore, based on the increased availability of the monoaminergic neurotransmitters after cocaine administration and the role that adrenergic receptors play in maintaining intestinal vasomotor tone, we sought to determine the effect of *in utero* cocaine exposure on the expression of the α -2A adrenergic receptor in the intestine of the fetal rat.

METHODS

Animals. The study was performed with the approval of the Animal Research Committee of the Children's National Medical Center (ARC # 65/97/04). Timed pregnant Sprague Dawley rats were obtained from Harlan Bioproducts for Science (Indianapolis, IN, U.S.A.) at 3 d of gestation and were allowed to acclimate for 2 d before the initiation of the study. The rats were housed under standard conditions and were allowed free access to rat chow and water. Beginning on E 5 and continuing until E 21 (term is generally 21 d), the animals received daily s.c. injections of either saline (control group), 30 mg/kg of cocaine (low-dose group), or 75 mg/kg of cocaine (high-dose group). Before receiving the injections, the rats were anesthetized with inhalational isoflurane.

Tissue preparation. The pregnant rats were killed by decapitation after receiving inhalation isoflurane for anesthesia. Sacrifice occurred at various points during the pregnancy, beginning on E 16 and continuing until E 20. The fetuses were frozen on 2-methylbutane on dry ice and stored at -80°C until ready for use. Frozen sections of these animals were cut on a cryostat, 20 μg thick, and were mounted onto twice subbed

gelatin slides and subsequently frozen at -80°C . Before *in situ* hybridization, the slides were thawed at room temperature for 10 min and were subsequently fixed with 4% formaldehyde in PBS and then processed as described previously (24). The sections were placed in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. They were then dehydrated sequentially in ethanols, 70% for 1 min, 80% for 1 min, 95% for 2 min, and 100% for 1 min. The sections were placed in chloroform for 5 min followed by 100% ethanol for 1 min and 95% ethanol for 1 min. The slides were allowed to air dry before use for hybridization.

In-situ hybridization. The sections were hybridized in a solution of 50% formamide, 1X Denhardt's solution, and a 10% dextran sulfate with 500 $\mu\text{g}/\text{mL}$ of salmon sperm DNA and 100 mM fresh DTT. Each slide (with two to four sections per slide) was incubated with 130 μL of hybridization solution containing $1-3 \times 10^6$ cpm of ^{35}S -labeled probe overnight at 37°C . To prevent drying of the slides, they were covered with parafilm coverslips after probe addition. After the overnight hybridization, the parafilm coverslips were floated off in 1X SSC and the slides were washed in 2X SSC with 50% formamide at 40°C for 1 h with three changes followed by a 1-h wash at room temperature in 1X SSC with two changes. The slides were dipped quickly in water and then 70% ethanol and allowed to air dry before placing them against Amersham β -Max film (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) and exposing them for 11–17 d at room temperature. Control, low-dose, and high-dose slides were placed on the same film. The film was developed in D19 for 4 min followed by stop bath for 2 min, fix for 4 min, and then washed in water for 20 min before air drying.

Probe preparation. Oligonucleotide probes 42–45 bp in length were synthesized on a model 380B DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) and were purified on polyacrylamide gels. The probes were derived from rat cDNA sequences for the α -2A adrenergic receptor subtype. These probes have previously been shown to be specific for the α -2A receptor subtype (25). The sequences are as follows:

5' CTCACACGATGCGCTTTCTGTCGCCACGGCAG-
AGGATCTTCTTGA
5' GAACGTGAAGCGTTTCTCGCGGTTCTGCCTCC-
CGCGCCACCGGAC

For all of the *in situ* studies, a 20X excess of unlabeled probe was used as control. The probe was 3' end-labeled using terminal deoxynucleotidyl transferase (Bethesda Research Labs, Gaithersburg, MD, U.S.A.) and [^{35}S] deoxyadenosine 5' (alpha-thio) triphosphate (PerkinElmer Life Science Products, Boston, MA, U.S.A.) (24). The probe was labeled to a specific activity of $5-15 \times 10^8$ cpm/ μg . Before use, all labeled probes were centrifuged through Sephadex G-25 spin columns (Amersham Pharmacia Biotech) to separate unincorporated ^{35}S -dATP from labeled probe.

Image analysis and statistics. The density of mRNA and ligand binding in the fetal intestine was analyzed by using a MacIntosh-II-based image analysis system (IMAGE, Wayne Rasaband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, U.S.A.). For each section, at

least 10 microscopic fields were analyzed after background was subtracted. Densitometric data were expressed as a mean $\pm$ SD, and then converted to percentage of control, where control was 100%. This was done to allow for comparison between experiments. Statistical analysis was performed using calculation of ANOVA.

RESULTS

There were a total of 39 female rats analyzed in this study. Thirteen were treated with saline, 13 with low-dose cocaine, and 13 with high-dose cocaine. One mother from the low-dose group escaped for 36 h and was euthanized. Nine out of 13 rats from the control group were pregnant (69.2%), 10 out of 12 from the low-dose group (83.3%), and 6 out of 13 from the high-dose group (46.1%). These differences were not statistically significant ($p = 0.9775$). The average number of pups that were delivered was similar among the groups—11.9 in the control group, 11.7 in the low-dose group, and 12.6 in the high-dose group ($p = 0.6687$). We killed the pregnant rats at various points during their gestation to ensure that there was a litter from each gestational age from E 16 until delivery. Thus,

at E 16, there were two litters in the control group, two in the low-dose group, and one in the high-dose group. At E 17, there was one litter each in the control, low-dose, and high-dose groups. At E 18, there were two litters in the control group, and one each in the low- and high-dose group. At E 19, there was one litter in the control group, two in the low-dose group, and one in the high-dose group. At E 20, there were two litters in the control and low-dose groups and one in the high-dose group. The remaining pregnant rats (one from each group) were allowed to deliver. Multiple pups from a single litter from each group were examined from E 16 to E 20.

We found that the α -2A adrenergic receptor subtype was localized in the embryonic rat intestine by E 17. When we quantified the gene expression of this receptor subtype, there was a significant down-regulation in the intestines of cocaine-exposed pups (Fig. 1B, low dose; Fig. 1C, high dose) compared with controls (Fig. 1A). This down-regulation was apparent from E 17, the first point in gestation at which this receptor could be localized, until near term—E 20. This did not appear to be a dose-dependent relationship. Figure 2 demonstrates the average densitometric data of multiple sections from animals

Figure 1. In-situ hybridization for the α -2A adrenergic receptor subtype in the intestine of E 19 rats exposed to saline (A), low-dose cocaine (B), and high-dose cocaine (C) *in utero*. Arrows indicate intestinal signal.

Figure 2. Comparison of the ontogenic expression of the α -2A adrenergic receptor in the intestine of rat pups after maternal exposure to either saline or cocaine. Pregnant rats were injected with either saline (Control group), low-dose cocaine (30 mg/kg) (Low group), or high-dose cocaine (75 mg/kg) (High group) from E 5 to E 20; values are expressed as percentage of control; * $p \leq 0.0001$ vs Control group; † $p = 0.02$ vs Low group; ‡ $p \leq 0.0001$ vs Low group.

of different gestational ages from each treatment group. In E 17 animals ($n = 4$ for each group), the expression of the α -2A receptor was 74.4% of control in the low-dose group ($p < 0.0001$) and 68.9% of control in the high-dose group ($p < 0.0001$). There was also a statistical difference between the low and high groups ($p = 0.02$). In E 18 animals ($n = 4$ for the control group, $n = 5$ for the low- and high-dose groups), the expression of this receptor was 71.6% of control in the low-dose group ($p < 0.0001$) and 90.1% of control in the high-dose cocaine-treated animals ($p = 0.0001$); the difference between the low and high groups was also statistically significant ($p < 0.0001$). In E 19 animals ($n = 5$ for all groups), the expression of the α -2A receptor was 80% of control in the low-dose cocaine group ($p < 0.0001$), and 71.5% of control in the high-dose cocaine group ($p < 0.0001$). The difference between the low- and high-dose groups was also statistically significant ($p < 0.0001$). In E 20 animals ($n = 5$ for all groups), the expression of the α -2A receptor was 69.8% of control in the low-dose group ($p < 0.0001$) and 70.5% of control in the high-dose group ($p < 0.0001$). The difference between the low- and high-dose cocaine groups was not statistically significant ($p = 0.7432$).

DISCUSSION

Our results indicate that the α -2A adrenergic receptor subtype is localized in the neonatal rat intestine, and that it is first evident at a gestational age of 17 d. We also found that chronic *in utero* cocaine exposure substantially altered the gene expression of this receptor subtype in the intestines of fetal rat pups. Furthermore, these changes were perceptible at the earliest gestational age that receptor mRNA expression was identified. In this study, we observed a low rate of pregnancy. The lowest rate was in the high-dose cocaine group, though differences between the groups were not statistically significant. This may be due in part to the fact that we had requested delivery of the pregnant rats relatively early in gestation (E 3), so that we could establish prolonged prenatal cocaine exposure. The company, however, was unable to guarantee preg-

nancy this early in gestation. However, statistical significance could possibly be seen with a larger sample size.

The influence of prenatal cocaine exposure on the expression of the α -2A receptor in the intestine may represent the mechanism that may predispose these newborns to NEC. It has been demonstrated that when the adult intestine is exposed to hypoxia or ischemia, the circulation is capable of escaping to baseline conditions (26). The newborn intestine, however, is less efficient in this autoregulatory response, as there is insufficient dilation of resistance vessels and precapillary sphincters (27). Thus, the immature intestine is more prone to injury. The α -2 adrenergic receptor is thought to play a role in the autoregulatory process in the newborn intestine that responds to hypoxia and ischemia (22). There is evidence that there are actually two mechanisms involved in producing vasodilation in the newborn intestine. One is mediated by nitric oxide, and the other by the α -2 adrenergic receptor (23). The nitric oxide pathway, which is thought to regulate basal vascular tone, may be disrupted in conditions of hypoxia or ischemia, thus rendering the α -2 mediated vascular relaxation more significant (23). The down-regulation of this receptor subtype that we demonstrated in this study may further exacerbate the already inefficient newborn autoregulatory response to intestinal ischemia. Thus, if fewer α -2 receptors are available to produce vasodilation, these cocaine-exposed newborns may be subject to further intestinal injury.

We chose the low and high doses of cocaine (30 mg/kg and 75 mg/kg, respectively) based on the study by Buyukunal *et al.* (14), in which they describe histologic evidence of a dose-dependent intestinal injury in fetal rats after *in utero* cocaine exposure. Interestingly, there did not appear to be a relationship between the *in utero* cocaine dose and the degree of down-regulation of the α -2A receptor in the fetal intestine. One of the difficulties in clinical studies of antenatal cocaine exposure and its consequences for the neonate is controlling for the extent of abuse in the mother, which is widely variable. Our study indicates that higher dosage did not consistently result in the anticipated diminution of expression of this particular receptor in the intestine after cocaine exposure. At the various time points that we examined, high dosage produced either more significant, less significant, or similar changes in this receptor compared with low dosage. Thus, the infant born to a mother who occasionally uses cocaine may be at just as much risk for intestinal injury as the infant born to a mother with a history of heavy cocaine use.

In summary, we conclude that *in utero* cocaine exposure alters the gene expression of the α -2A adrenergic receptor subtype in the neonatal rat intestine. This may represent one of the mechanisms responsible for the increased risk of NEC that may be seen in cocaine-exposed infants. Future studies are needed to examine the histology of the cocaine-exposed newborn intestine after an additional ischemic insult. In addition, these findings demonstrate changes only in the expression of this gene. Further investigation is warranted to examine whether or not this is reflected in functional activity of the gene, and whether other α -2 adrenoceptor subtypes exhibit similar changes after cocaine exposure.

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