Inhibition of Nitric Oxide Synthase Enhances Cocaine's Developmental Toxicity: Vascular and CNS Effects

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Ischemia and/or reperfusion injury from free radicals may cause cocaine's toxicity, including its effect upon neurobehavioral development. We previously used salicylate to measure hydroxyl free radicals in chick embryos exposed to cocaine. The combination was more toxic than cocaine alone. We postulated that salicylate enhanced the vasoconstriction and toxicity via inhibition of compensatory processes (eg by inhibition of the synthesis of vasodilatory prostanoids and/or nitric oxide). A nontoxic dose of $N(G)$-nitro-L-arginine methyl ester (L-NAME) was used to inhibit nitric oxide synthase to test this hypothesis. In one experiment, cocaine was injected every 1.5 h (total dose = 67.5 mg/kg egg) on day 15 of development, 1 h after injection of L-NAME (200 mg/kg egg) to determine viability and hatchability, which are measures of toxicity. Another experiment measured diameters of blood vessels after L-NAME injection, followed by NaCl or cocaine infusion (0.23 mg/egg/min; total dose = 67.5 mg/kg egg) at 15 and 5 min afterwards. Lastly, brains of embryos pretreated with L-NAME before cocaine injections were analyzed for nitric oxide synthase activity. Cocaine decreased viability and hatchability. L-NAME enhanced cocaine's effect upon both parameters. Blood vessel diameters were decreased by cocaine after 15 min of infusion. L-NAME + cocaine caused a decrease in vessel diameter as soon as 5 min into the infusion and was greater with time, compared with other groups. Enzyme activity in brains was decreased only in the L-NAME + cocaine group. Thus, inhibition of nitric oxide synthesis interferes with the embryos' capacity to mount a compensatory vasodilatory response.
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INTRODUCTION

Human and animal studies have reported cerebrovascular accidents (ie hemorrhage and/or ischemia) in adults, as well as in offspring of cocaine-using mothers (Levine *et al*, 1991; Frank *et al*, 1999; Kapur *et al*, 1991; Akoka *et al*, 1999). *In utero*, exposure to cocaine has been shown to cause various detrimental consequences including, but not limited to congenital malformations and postnatal neurobehavioral changes (Young *et al*, 1992; Plessinger and Woods, 1993; Vorhees *et al*, 1995).

When sodium salicylate was injected into chicken eggs with embryos to determine if cocaine's developmental toxicity might be due, in part, to elevated free radical production, we observed evidence of enhanced toxicity in E18 embryos caused by the combination (Castelli *et al*, 2001). We also reported that salicylate, in combination with cocaine, can exacerbate cocaine's toxic effect upon younger (ie E12) chicken embryos, manifest as hemorrhages and reduced hatchability in the presence of hydroxyl free radicals (Venturini and Sparber, 2001). Recently, we demonstrated that salicylate enhanced the vasoconstrictive effect of cocaine and that the degree of vasoconstriction was inversely related to embryonic brain-inducible nitric oxide synthase mRNA concentration (Mendoza-Baumgart *et al*, 2004). We hypothesized that this enhanced vascular and other toxic effects of cocaine by salicylate was owing to the blockade of compensatory mechanisms, such as production of prostacylin and/or nitric oxide, which are both powerful vasodilators (Shimokawa *et al*, 1988; Vane and Botting, 1998; Ignarro, 2002). In addition to its effect upon prostanoid synthesis, salicylate has been reported to decrease nitric oxide formation by interfering with the post-transcriptional, translational, and post-translational processing of nitric oxide synthase (Amin *et al*, 1995; Ryu *et al*, 2000). We proposed that the increase in brain synthase mRNA caused by the combination of salicylate + cocaine may have been the result of a positive feedback in an effort to compensate for, or counteract the decrease in the synthesis of nitric oxide in the periphery or brain. The present study was carried out to determine the consequence of direct

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synthase inhibition, with and without cocaine, upon viability, hatchability (ie toxicity), extraembryonic blood vessel diameters, as a parameter of vascular function, and to determine the effect of synthase inhibition, with and without cocaine, upon chick embryo brain enzymatic activity. To test the hypothesis discussed above, essentially the same treatment protocols used in the salicylate experiments were used for these experiments. Nontoxic doses of salicylate were used as a reactant to 'trap' any hydroxyl free radicals generated as a result of ischemia and reperfusion. These experiments were carried out to determine if cocaine's developmental effects were also enhanced by pretreatment with a nontoxic dose of $N(G)$-nitro-L-arginine methyl ester (L-NAME) in order to inhibit nitric oxide synthase enzymatic activity directly.

METHODS

Subjects

Fertilized chicken (*Gallus domesticus*) eggs (White Leghorn × White Leghorn) were obtained from Midwest Poultry and Hatchery (Dassel, MN). The eggs were stored overnight at 14–16°C to synchronize embryo development. They were then set in a rotating forced air incubator/hatcher (Humidaire, New Madison, OH) with the temperature set at 37.5°C and the relative humidity at 58–60%. The day eggs were placed in the incubator was designated as embryonic day 0 (E0). On E14, the day before injection/infusion of drugs, the eggs were candled and treatment was assigned randomly to eggs with viable embryos. An injection site for drug administration was marked about 2.0 cm below the air cell, avoiding membrane-bound blood vessels that could be observed during the candling procedure. The shell surface at the injection site was sterilized with a small drop of 2% iodine tincture and immediately wiped off with a gauze pad moistened with 70% ethanol. A 1.2-mm diameter dental burr and a small drill were used to drill injection holes without puncturing the underlying membrane. Holes were covered with a small piece of transparent plastic tape (3 M, St Paul, MN).

Drugs

Cocaine HCl was kindly provided by the National Institute on Drug Abuse. L-NAME was purchased from Sigma (St Louis, MO). All drugs were dissolved in filtered (Acrodisc 0.2 µm filter, Pall Corp, Ann Arbor, MI) distilled water just before their use. The dose of cocaine, as the HCl salt, was based on previous experiments in our laboratory, within the range that produces moderate to severe effects, including reduced hatchability of chick embryos. Based on our preliminary toxicity studies, the L-NAME dose used was devoid of toxicity (ie reduced hatchability) when administered alone. Avian isotonic NaCl (0.85% (w/v) was used for control injections and infusions.

Protocols

See Figure 1a–c for a visual description of the separate experimental protocols.
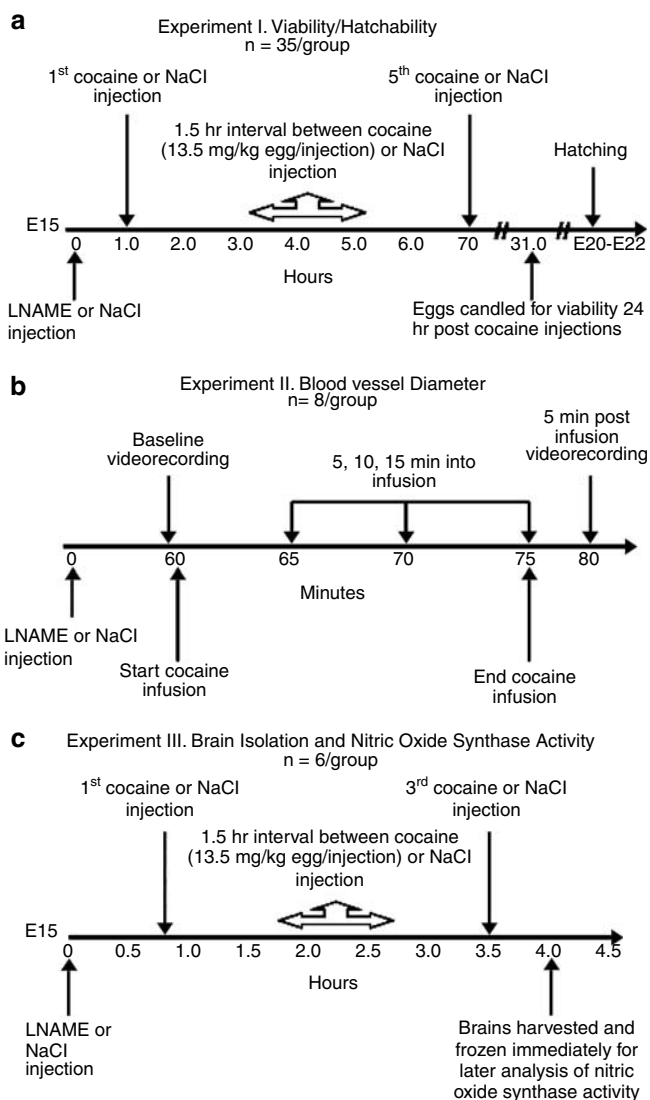
Figure I Experimental design. (a) The viability and hatchability experiment; (b) the blood vessel experiment; and (c) the brain isolation and nitric oxide synthase activity experiment.

Viability and hatchability. On E15, 35 eggs per treatment group were injected with L-NAME (200 mg/kg egg or 10 mg/egg) or NaCl 1 h before five injections of 13.5 mg cocaine/kg egg (average egg weight = 50 g; total dose of 67.5 mg/kg egg) or NaCl. Volume of injection was 20 µl and injections were administered at 1.5 h intervals. We have used this injection protocol in the past as a 'model' of embryonic/fetal exposure as a consequence of binge cocaine use by pregnant women (Castelli *et al*, 2001; Schrott and Sparber, 2001; Venturini and Sparber, 2001). After 24 h, eggs were candled for viability and were then returned to the incubator. On E18, eggs were transferred to the hatcher compartment, which was checked three times daily for new hatchlings, starting on E20 until E22. Treatment groups are as follows: NaCl + NaCl, NaCl + cocaine, L-NAME + NaCl, and L-NAME + cocaine (Figure 1a).

Blood vessel diameter. On E15, eggs were injected with L-NAME (200 mg/kg egg) or NaCl followed 1 h later by

infusion of NaCl or cocaine (67.5 mg/kg egg; 0.225 mg/egg/min) during 15 min. Treatment groups ($n = 8$/group) consisted of: NaCl + NaCl, NaCl + cocaine, L-NAME + NaCl, and L-NAME + cocaine. The procedure and equipment used are described in detail elsewhere (Zhang et al, 1998; Mendoza-Baumgart et al, 2004). Briefly, the eggshell above the aircell was removed and the chorioallantoic membrane was made transparent with a drop of mineral oil so as to visualize, record, and measure the apparent diameter of extraembryonic vitelline blood vessels before, during, and after infusion of NaCl or cocaine in eggs previously injected with NaCl or L-NAME. A 1 cm long, 250-mm-diameter, 3.0 silk suture, previously soaked in mineral oil, was placed near the desired blood vessel as a reference (Figure 1b).

Brain isolation and nitric oxide synthase activity. Thirty minutes after the third injection of cocaine (ie after 40.5 mg cocaine/kg egg) or NaCl, whole embryo brains were rapidly externalized and immediately frozen in liquid nitrogen and stored at -80°C (Figure 1c). We took brains after the third injection instead of after five injections because we had observed L-NAME-enhanced vasoconstriction after acute infusions of 22.5–45 mg cocaine/kg egg, at times when cocaine did not yet cause significant vasoconstriction (ie within 5–10 min after starting cocaine infusions). Thus, we wanted to determine if L-NAME + cocaine caused a greater reduction in enzyme activity after a dose of cocaine comparable to that which caused significant vasoconstriction, before the vasoconstriction caused by cocaine itself was manifest. Brains were thawed in two volumes (weight of tissue/volume of buffer) of ice-cold homogenization buffer composed as 25 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM ethyleneglycolbis(-aminoethylether)-N,N,N',N'-tetraacetic acid, all reagents purchased from Sigma (St Louis, MO). Brains were homogenized using a tissue homogenizer (STD1810; Tekmar, Cincinnati, OH) and homogenates were centrifuged at 20 000g for 15 min at 4°C using a refrigerated centrifuge (J2-21 Beckman, Fullerton, CA). After centrifugation, supernatants were transferred to fresh 1.5 ml Eppendorf tubes and kept on ice until incubation (Figure 1c).

Measurement of nitric oxide synthase catalytic activity is based upon conversion of arginine into citrulline during the production of nitric oxide. The method of Bredt and Snyder (1990), with modifications for optimizing use with embryonic chicken brain, was used. Reaction mixtures were prepared on ice as follows: 25 mM Tris-HCl (pH 7.4), 3 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 0.1 mM nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 1 mM calcium chloride, and 1 mM magnesium diacetate (Sigma). In 1.5 ml microcentrifuge tubes, 40 μl of brain homogenates were added to 40 μl of reaction mixture and samples were incubated for 10 min at 30°C in a Dubnoff shaking metabolic incubator (Precision Scientific, Chicago, IL) to warm the mixture and convert any residual endogenous arginine to citrulline. Radiolabeled substrate was then added to samples. Arginine substrate (0.5 mM) was composed of 0.5 μM L-[2,3,4,5-^{3}H]-arginine (62.0 Ci/mM; Amersham, Little Chalfont, UK) and 499.5 μM L-arginine (Sigma). Reactions were stopped after 30 min by adding 450 μl of stop buffer composed as 50 mM N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 5.5 and 5 mM EDTA. After incubation, a fraction (540 μl) of the final volume (reaction mixture, homogenate, and stop buffer) was transferred to microspin nylon-filter tubes (Alltech, Deerfield, IL) containing 160 mg of AG 50W-X8 Resin, 100–200 mesh, and sodium form (Biorad, Hercules, CA). Samples were briefly stirred in the spin cups in order to maximize the exposure to the cation exchange resin and then centrifuged at 3000g for 3 min at room temperature. The cation exchange resin traps ^{3}H-arginine, whereas ^{3}H-citrulline, stoichiometrically (1:1) formed during the production of nitric oxide, is collected in the flowthrough after centrifugation. A fraction (450 μl) collected from column holders was transferred to scintillation vials (RPI, Mt Prospect, IL) and 1.5 ml of scintillation fluid (Ecolume, ICN, Costa Mesa, CA) was added. After 24 h of dark adaptation, sample radioactivity was quantified in a liquid scintillation counter (LS 5801 Beckman, Fullerton, CA). Counter error was preset at 2σ or 0.5%.

Statistical Analysis

Viability and hatchability. The number of viable embryos on E16 (approximately 24 h postinjection) and the number of hatchlings were analyzed using χ^2 analyses.

Blood vessel diameter. The apparent blood vessel diameters obtained during and after infusions were expressed and analyzed as percents of their individual baseline values. A two-factor repeated measures ANOVA and factorial ANOVAs for each of the four times after infusions were started, followed by preplanned contrasts were used for group comparisons, where appropriate. Based on previous studies (Mendoza-Baumgart et al, 2004; Schrott et al, 2002; Zhang et al, 1998), showing the vasoconstrictive effect of cocaine upon the chicks' extraembryonic vasculature, a unidirectional Dunnett's test was used as the preplanned contrast to compare blood vessel diameters of each treated group against the control group. Because of the possibility that the baseline diameters were partially influenced by constitutive synthase activity and nitric oxide production, we also compared vessel diameters in the L-NAME + NaCl group with other groups using the protected least significant difference (PLSD) test.

Nitric oxide synthase activity. Citrulline formation, expressed as pmol/100 mg brain, was analyzed as a measure of synthase activity. One-factor ANOVA followed by the Dunnett's test were used for statistical comparisons. We hypothesized an enhanced effect of cocaine upon blood vessel diameters because we used a synthase inhibitor (ie L-NAME). However, absent previous experimental evidence as to L-NAME's capacity to inhibit the enzyme, at the dose injected, a two-tailed contrast was used.

RESULTS

Viability and Hatchability (Toxicity)

An overall χ^2 analysis found a significant effect of treatment upon E16 viability ($\chi^2_3 = 31.52$, $p < 0.0001$). Pretreatment

Table 1 Viability and Hatchability

E15 treatment	Number viable/injected (%)	Number hatched/injected (%)
NaCl+NaCl	34/35 (97)	33/35 (94)
LNAME+NaCl	32/35 (91)	32/35 (91)
NaCl+coc	25/35 (71)[+]	23/35 (66)[+]
LNAME+coc	16/35 (46)*	14/35 (40)*

[+]A significant decrease in viability and hatchability in the NaCl+cocaine group (pretreated eggs) compared to the NaCl+NaCl group, $p < 0.005$.
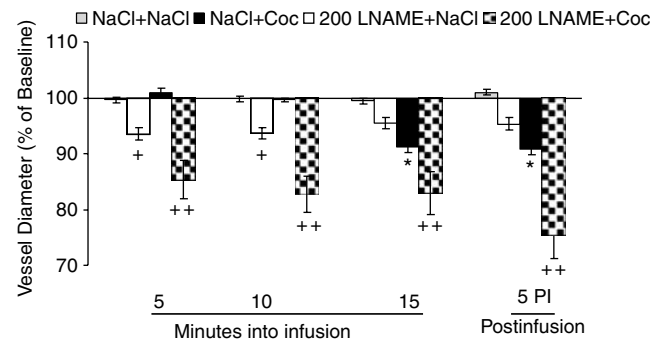*A significant decrease in viability and hatchability compared to all other groups when pretreated with LNAME followed by cocaine, $p < 0.03$, or better.

Figure 2 Blood vessel diameter. [+]A significantly decreased (BV) diameter in the L-NAME + NaCl-treated group compared to the NaCl + NaCl- and NaCl + cocaine-treated groups 5 and 10 min into the infusion. *A significantly decreased (BV) diameter in the NaCl + cocaine-treated group after at least 15 min into the cocaine infusion. [++]A significantly decreased (BV) diameter in the L-NAME + cocaine-treated group as soon as 5 min into the cocaine infusion when compared to all other groups.

Figure 3 Citrulline formation. *A significantly decreased concentration of citrulline from enzymatic conversion of arginine in the L-NAME + cocaine-treated group, indicative of reduced nitric oxide synthase (NOS) activity; $p < 0.02$. Brains were taken from embryos after a dose of cocaine comparable to an infusion dose of 22.5–45 mg cocaine/kg egg.

with L-NAME followed by cocaine caused a significant decrease (54%) in viability compared with cocaine (29%) alone ($\chi_1^2 = 4.77$, $p < 0.03$). There was also an overall significant effect of treatment upon hatchability ($\chi_3^2 = 34.24$, $p < 0.0001$). Pretreatment with L-NAME followed by cocaine did not substantially reduce viability after E16, measured as hatchability on E20–22. Of the 34 viable embryos in the NaCl + NaCl group on E16, 33 eventually hatched. The remaining groups likewise were little affected after E16 (Table 1).
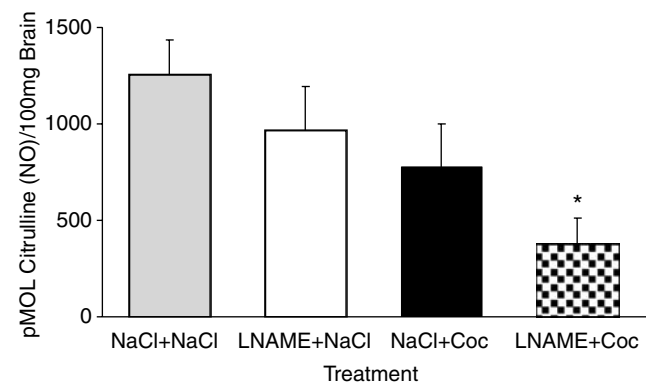
Blood Vessel Diameter

Baseline blood vessel diameters of the four treatment groups, when quite variable, were nevertheless not significantly different from one another. The mean absolute value for baseline diameters was 250 μm, ranging in size from 182 to 319 μm. Examples of images of the blood vessels before and after exposure to the vasoconstrictive action of salicylate plus cocaine at a time when eggs pretreated with NaCl, followed by cocaine, did not yet show a vasoconstrictive effect of cocaine are shown elsewhere (Mendoza-Baumgart et al, 2004).

Repeated-measures ANOVA showed a significant main effect of treatment (F 3,28 = 19.24; $p < 0.001$) and a repeated measures effect (F 3,84 = 13.77; $p < 0.001$). There was also a significant treatment by repeated measures interaction (F 9,84 = 11.59; $p < 0.001$). Blood vessel diameters, relative to their respective baseline diameters, varied between 99.4 and 101.1% for the NaCl + NaCl group, with an average of 100% for the four times after initiating infusions. The L-NAME + NaCl-treated group showed a slight and transient but significant 6% reduction in vessel diameter compared to NaCl + NaCl- and NaCl + cocaine-treated groups observed at 5 and 10 min into the cocaine infusion. The vessel diameters from the NaCl + cocaine-treated group, which were not significantly affected at 5 and 10 min, took 15 min of cocaine infusion to cause a 9% reduction in diameter (ie vasoconstriction), which was significant compared with the NaCl + NaCl-treated group at this time, as well as at 5 min postinfusion. Pretreatment with L-NAME sensitized the vasculature to the vasoconstrictive effect of cocaine, causing a significant reduction (15%) in vascular size immediately (ie within 5 min), showing progressively greater vasoconstriction over the 20 min recording period, with an even greater 24% decrease in diameter at this time. Because

cocaine by itself caused a significant reduction in diameters at 15 and 5 min after the infusion was terminated, we took the liberty of comparing the diameters of both cocaine groups at these times with the use of the PLSD test. The outcomes indicated that the 17% reduction in vessel diameter in the L-NAME + cocaine group was significantly greater than the 9% reduction in the NaCl + cocaine group at 15 min. Furthermore, 5 min after the infusion (ie postinfusion), the NaCl + cocaine group's vessel diameters remained at 91% of baseline, whereas the vessel diameters in the L-NAME + cocaine group was reduced to almost 25%; likewise to a significant degree (Figure 2).

Nitric Oxide Synthase Activity

Only the brains from the L-NAME + cocaine-treated group showed a significant decrease of 70% in ^{3}H-citrulline (ie nitric oxide) formation, when compared with the NaCl + NaCl-treated group (Figure 3). The smaller reductions in enzyme activity in brains from the L-NAME + NaCl and NaCl + cocaine groups were not statistically reliable at the time we harvested brains, which was the experimental equivalent to doses and times when we saw enhanced

vasoconstriction of extraembryonic vessels in eggs pretreated with L-NAME and infused with cocaine.

DISCUSSION

The slight (6%) transient (ie 5 and 10 min) but significant decrease in vessel diameter caused by injection of L-NAME suggests that nitric oxide may be constitutively produced by the endothelial cells for the maintenance of normal vascular tone and integrity (Nakamura et al, 2004; Wang et al, 2004; White et al, 2004). The lack of, or reduced nitric oxide production may then have been compensated for by other vasodilatory mechanisms, such as prostacyclin production (Bobadilla et al, 2005; Harrison and Cai, 2003). Although it has been reported that the lack of nitric oxide can decrease the biosynthesis of prostacyclin (Sautebin and Di Rosa, 1994), the dose of L-NAME we used may not have been sufficient enough to cause a substantial decrease in prostanoid synthesis, which could explain the return of vessel diameter sizes to normal, in the L-NAME + NaCl group, by 15 min into the infusion.

Cocaine by itself caused significant vasoconstriction compared to controls (ie NaCl + NaCl) and the L-NAME + NaCl group, but not until 15 min into the infusion and we have previously reported this vasoactive property of cocaine in this model (Mendoza-Baumgart et al, 2004; Schrott et al, 2002; Zhang et al, 1998). In addition, cocaine's vasoconstrictor action has been known for decades. It has been used as an experimental tool to block reuptake of released norepinehrine (NE) or effects of NE or other drugs that are put into isolated organ baths or injected into experimental animals (Zimmerman and Gisslen, 1968; Allen et al, 1965; Marsh and Van Liere, 1950). When pretreated with L-NAME, there was an enhancement of the vasoconstriction (ie the blood vessel diameters decreased significantly as soon as 5 min into the infusion, the earliest time taken for analyses). This synergistic vasoconstrictive effect of the combination of L-NAME + cocaine may be owing to the inability of the blood vessel to mount a compensatory vasodilation/relaxation in response to the vasoactive effect of cocaine and is possibly secondary to the lack of, or reduced nitric oxide, which has been reported to modulate or enhance production of a prostanoid vasodilator, prostacyclin (Sautebin and Di Rosa, 1994). This effect of L-NAME in combination with cocaine upon the extraembryonic vasculature is similar to our previous study with salicylate in combination with cocaine, which resulted in an increase in brain inducible nitric oxide synthase mRNA concentration. We postulated that the increased synthase mRNA might be owing to a compensatory response to a decrease in prostacyclin and/or nitric oxide production caused by salicylate + cocaine. These experiments were undertaken to test that hypothesis more directly. The significant decrease in synthase activity caused only by the combination of L-NAME + cocaine in the present study supports our hypothesis alluded to above. These developmental insults may have caused toxic and lethal effects on the developing embryo, leading to the greater decrease in both viability and hatchability demonstrated in the first experiment.

Cocaine using pregnant women presenting to a physician with symptoms of headache, abdominal pain, blurred vision, and increased blood pressure may be misdiagnosed as suffering from pre-eclampsia (Towers et al, 1993) and been given aspirin as part of their treatment. Moreover, aspirin, or more potent antiplatelet aggregation agents, has been suggested as a prophylactic treatment of cocaine abusers at risk for ischemic strokes, or with cerebral perfusion deficits, but the risk of gastrointestinal bleeding was a concern (Kosten et al, 2003, 2004). However, cocaine abuse is also associated with various hemorrhagic cerebrovascular accidents (Boco and Macdonald, 2004; Howington et al, 2003) and cocaine abusers experiencing headache caused by vasoconstriction and/or a CVA may self-medicate with more than one 325 mg dose of aspirin (Wagener et al, 1994), which has typically been used in hospital-based diagnostic or treatment studies. We have reported that salicylate, the major metabolite of aspirin, caused a strikingly similar enhanced toxicity we report with L-NAME when used in combination with cocaine (Mendoza-Baumgart et al, 2004). This toxic interactive effect of cocaine upon a developing organism or in adult cocaine abusers, when used with various substances, is the basis for this study.

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