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Vasoconstriction Caused by Cocaine is Enhanced by Sodium Salicylate: Is Inducible Nitric Oxide Synthase mRNA Related?

Mary Irene Mendoza-Baumgart[1], Marco Pravetoni[1] and Sheldon B Sparber*[,1,2]

[1]Department of Pharmacology, University of Minnesota, Minneapolis, MN, USA; [2]Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA

We have previously found that sodium salicylate (NaSal), injected into chicken eggs at nontoxic doses used for quantifying hydroxyl free radicals in hearts and brains of embryos, caused or exacerbated hemorrhages and dramatically reduced hatchability when combined with cocaine (Coc). It has also been reported that inducible nitric oxide synthase (iNOS) gene expression is altered in brain in response to vascular damage and inflammation. In this study we measured diameters of membrane-bound blood vessels (BV) before and after pretreatment with saline (NaCl) or NaSal (100 mg/kg egg), followed by infusion of either NaCl or Coc HCl (total of 67.5 mg/kg egg) during 15 min. Brains and hearts of the embryos were then analyzed for iNOS messenger RNA (mRNA) concentrations. Coc caused vasoconstriction that was significant 5 min postinfusion (5 min PI) of the entire dose (ie after 67.5 mg/kg egg). Significant vasoconstriction was evident within 5 min in the group injected with NaSal followed by infusion with Coc (ie after 22.5 mg Coc/kg egg). Expression of iNOS mRNA was significantly increased only in the brains of the group exposed to NaSal plus Coc, and the increase was inversely related to BV diameter. These data are discussed in relation to effects of salicylate upon prostanoid synthesis and/or nitric oxide synthesis via iNOS inhibition and their possible relationship to Coc-associated cerebral vascular and/or cardiovascular events in abusing humans. *Neuropsychopharmacology* (2004) **29,** 1294–1300, advance online publication, 3 March 2004; doi:10.1038/sj.npp.1300421

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INTRODUCTION

Cocaine (Coc) use has been linked with cerebrovascular accidents (ie hemorrhage and/or ischemia) in adults (Levine *et al*, 1991) and in offspring of Coc-using mothers (Frank *et al*, 1999; Kapur *et al*, 1991). In addition, rat (Church *et al*, 1988) and lamb (Akoka *et al*, 1999) fetuses showed hemorrhages in their brains after Coc exposure during gestation. Coc's effects, however, are not limited to cerebrovascular impairments.

Human and animal studies have shown that Coc use during pregnancy can have detrimental effects to both the mother and the developing organism (Young *et al*, 1992; Plessinger and Woods, 1993). Headache (Towers *et al*, 1993), increased blood pressure (Covert *et al*, 1994), abruptio placentae, preterm labor, and spontaneous abortion (Little *et al*, 1989; Ryan *et al*, 1989) are some of the many maternal consequences associated with Coc use. Teratogenic effects (eg cardiac and skeletal defects) and neurobehavioral changes (eg seizures, learning, and/or attention deficits) are associated with exposure to Coc *in utero* (Young *et al*, 1992; Plessinger and Woods, 1993; Vorhees *et al*, 1995).

Coc-using pregnant women suffering from headache may self-medicate with the over-the-counter drug aspirin. If brought to a hospital with symptoms of headache, abdominal pain, blurred vision, and increased blood pressure, these women may be misdiagnosed as suffering from pre-eclampsia (Towers *et al*, 1993) for which aspirin is often used as part of the treatment. Despite conflicting reports as to the benefits of aspirin in treating pre-eclampsia (Crandon and Isherwood, 1979; Dekker and Sibai, 2001; Mattar and Sibai, 1999), it is still widely used by clinicians (Bower, 1998).

Aspirin, which has a half-life of approximately 15 min in humans, is hydrolyzed to salicylate (Sal). Depending on the dose administered, Sal has a half-life of between 2 and 15 h or more (Furst *et al*, 1979; Roberts *et al*, 1983). The present study used sodium salicylate (NaSal) because of its longer half-life and to eliminate the anticoagulant effect from the acetate moiety of aspirin. Aspirin and its metabolite salicylate have also been reported to have teratogenic potential in animals (Kotwani *et al*, 1994; Mcgarrity *et al*, 1981; Kimmel *et al*, 1971). An epidemiological study has suggested that maternal aspirin ingestion during the first half of pregnancy was associated with attention deficits and

*Correspondence: SB Sparber, Department of Pharmacology, University of Minnesota, 6-120 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455-0217, USA, Tel: +1 612 625 5657, Fax: +1 612 625 8408, E-mail: sparb001@umn.edu
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low IQ scores in 4-year-old children (Streissguth *et al*, 1987). In addition, it has been reported that offspring of pregnant rats administered aspirin may have impairment in learning abilities (Butcher *et al*, 1972).

We have previously shown that Coc caused vasoconstriction of extra-embryonic blood vessels (BV) and caused herniated umbilici in chick hatchlings (Zhang *et al*, 1998). Little is known, however, about the effects of combined use of, or exposure to Coc and Sal, other than what appears to be substantial interactive toxicity, manifest as hemorrhages and associated death of chicken embryos exposed to the combination (Venturini and Sparber, 2001; Castelli *et al*, 2001). One possible mechanism for the interactive toxicity of the combination of Coc and NaSal is greatly augmented effects of Coc (eg greater vasoconstriction) leading to greater ischemia and possibly unabated increases in intraluminal pressure, causing hemorrhages and/or inflammation secondary to vasospasm (Zuccarello *et al*, 1998; Conway and Tamargo, 2002).

It has been reported that there is elevated brain nitric oxide synthase (iNOS) expression following subarachnoid hemorrhage associated with cerebral vasospasm (Sayama *et al*, 1999) as well as enhanced iNOS gene expression in brain in response to ischemia and inflammation (del Zoppo *et al*, 2000; Wong *et al*, 1996; Moro *et al*, 1998). Furthermore, it has been demonstrated that uteroplacental ischemia produced an increased iNOS messenger RNA (mRNA) in fetal rat cerebral cortex (Gonzalez-Barrios *et al*, 2002). The vitelline vessels associated with the chorioallantoic membrane, which we have used to study vascular effects, is considered to be the avian homolog of the mammalian placenta (Metcalfe and Stock, 1993).

To further characterize the potential effects of Coc and NaSal on the developing embryo, this study was undertaken to determine the effect of this combination on the extra-embryonic BV diameter as a marker for altered vascular function and to determine if there is an association between vasoconstriction and the concentration of iNOS mRNA in hearts or brains of exposed embryos.

METHODS

Subjects

Fertilized chicken (*Gallus domesticus*) eggs (White Leghorn × White Leghorn) were obtained from the University of Minnesota Poultry Nutrition Research facility (St Paul, MN). The eggs were refrigerated at 14–16°C for 24–48 h to synchronize embryogenesis, and were then set in a rotating, forced-air incubator (Humidaire Hatchette, New Madison, OH) at 37.5°C and 56–58% relative humidity. The day the eggs were set was regarded as day zero of embryogenesis (E0).

Eggs were candled on E14 for embryo viability and to locate and mark the aircell and an injection/infusion site about 2 cm below the aircell, avoiding membrane-bound BV. The injection site was disinfected with a drop of 2% iodine tincture and washed immediately with cotton gauze dipped in 70% ethanol. A 1.2-mm dental burr attached to a small drill was used to make a hole through the shell, taking care not to puncture the underlying membrane. The holes were covered with plastic tape (3M, St Paul, MN) and replaced in the incubator.

Drugs and Treatment

Coc HCl was kindly provided by the National Institute on Drug Abuse. NaSal was purchased from Aldrich Chemicals (Milwaukee, WI). Coc and NaSal were dissolved in filtered (Acrodisc 0.2 μm filter, Pall Corp, Ann Arbor, MI) distilled water just prior to their use. The dose of Coc was based on previous experiments in our laboratory, within the range that produces moderate to severe effects, including reduced hatchability of chick embryos. The NaSal dose used was devoid of toxicity (ie reduced hatchability) when injected by itself (Castelli *et al*, 2001). Avian isotonic NaCl (0.85% w/v) was used for control injections and infusions. In all, 16 eggs with viable embryos were randomly assigned to one of four groups ($n = 4$/group). On E15, the eggs were injected with NaSal (100 mg/kg egg) or 20 μl NaCl 1 h prior to the start of a 15-min infusion (min Inf) of either Coc HCl (0.23 mg/egg/min) or NaCl. Coc or NaCl was infused at a volume of 10 μl/min. Thus, the four groups were designated NaCl + NaCl, NaSal + NaCl, NaCl + Coc, and NaSal + Coc.

Experiment 1: Blood Vessel Visualization, Recording, and Quantifying Vascular Diameter

The procedure and equipment used are described in detail elsewhere (Zhang *et al*, 1998). Briefly, a circular 3–4 cm diameter hole was made over the marked air cell of each egg. Mineral oil (0.5 ml) was dropped onto the exposed membrane to make it transparent and reveal the membrane-bound vasculature. A 1 cm long, 250-μm-diameter, 3.0 silk suture, previously soaked in mineral oil, was placed near the desired BV as a reference. The egg was placed upright on a sponge cradle directly under an endoscope (Olympus, model A5257) in an incubator maintained at 37°C. The injection hole was facing toward the incubator door to facilitate subsequent infusion. The endoscope, which was connected to a color camera (Toshiba)–digital AV mixer (Panasonic)–VCR (Panasonic) system, was lowered until an image of BV and the suture were in focus on a video monitor. A PE-20 polyethylene catheter, with a bulbous expansion as a stop (de Balbian Verster *et al*, 1971), was inserted into the injection hole extending 3.5 mm into the egg with the other end connected to a syringe mounted on an infusion pump (Harvard Apparatus, Millis, MA). The egg was allowed to acclimate for 5 min before a baseline (BL) video recording was made. After BL recording, an infusion of either Coc or NaCl was started. Recordings were made at 5, 10, and 15 min into the infusion (5, 10, or 15 min Inf) and at 5 min after infusion was stopped (5 min postinfusion; 5 min PI). All recordings were stored on VHS tape for subsequent measurement of the BV and suture diameters. A Macintosh computer equipped with a videospigot frame grabber card (SuperMac Technology, Inc., CA) was used to capture the images from the VHS tapes and convert them to digital format for subsequent image analysis, using NIH Image software (Division of Computer Research and Technology, NIH, Bethesda, MD). The BV and suture widths were measured, in pixels, and were converted to μm by comparison with the suture as a reference. During

acquisition (ie video recording) and measurement of treatment effects upon BV diameters, the experimenter was blind as to pretreatment and infusions in order to eliminate potential bias.

Experiment 2: Brain and Heart iNOS mRNA Quantification

RNA isolation. Immediately after BV visualization and recording, brains and hearts of the embryos were removed and placed in Trizol reagent (Invitrogen, Carlsbad, CA), a phenol and guanidine isothiocyanate solution, in a volume of 1 ml/100 mg of tissue. The tissues were homogenized immediately. The RNA extraction procedure performed was derived from the Trizol reagent protocol based on the single-step method of RNA isolation developed by Chomczynski and Sacchi (1987). Chloroform, isopropanol, and diethyl pyrocarbonate (DEPC) were purchased from Sigma (St Louis, MO). The extracted RNA was placed in 300 μl of DEPC-water (1% v/v DEPC) and incubated at 58°C for 10 min to dissolve the RNA completely. The concentration of total RNA in the samples was assessed by spectrophotometry using a wavelength of 260 nm for RNA, and wavelengths of 280 and 320 nm, respectively, for DNA and protein contamination. Analysis was made using a Soft-Pac module for a DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA)

Reverse Transcription: Production of Complementary DNA from mRNA

Reverse transcription was performed with 5 μg of total RNA from each organ.

RNA and 1.0 μl Oligo $d(T)_{12-18}$ (Invitrogen) were incubated at 70°C for 10 min in 0.2 ml nuclease-free tubes (RPI Corp., Mt Prospect, IL). Following completion of this first heating cycle, 7 μl of reaction mixture composed of 1 × first-strand buffer, 10 mM dithiothreitol (DTT), and 0.5 mM deoxynucleotide-3-phosphate mixture (dNTP) (Invitrogen) was added. The tubes were then incubated at 42°C for 2 min, after which 200 U of Superscript RNase H− Reverse Transcriptase (Invitrogen) was added. Samples were again incubated at 42°C for 50 min, followed by a cycle of 70°C for 15 min. The reverse transcription products were stored at −80°C until further use.

Polymerase Chain Reaction for Amplification of cDNA

A measure of 2 μl of the reverse transcription solution containing cDNA was amplified through polymerase chain reaction (PCR) technology. The PCR reaction mixture was composed of 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM 5′-primer, 0.5 μM 3′-primer, and 2.5 U of *Taq* DNA polymerase (Invitrogen) dissolved in DEPC-water.

Initial incubation, at 94°C for 10 min, was followed by 30 cycles: 94°C for 30 s, 56°C for 1 min, and 72°C for 1.5 min. After 30 cycles, samples were incubated at 72°C for 10 min. All the incubations were conducted on a GeneMate Genius 120 Thermal Cycler (ISC Bioexpress, Kaysville, UT).

Samples were separately amplified with two different sets of primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and iNOS. Amplification of GAPDH message was conducted as a qualitative internal control, since we had not previously included iNOS gene products in experiments of this type and to confirm if RT-PCR procedure was working. Although GAPDH is one of the most commonly used housekeeping gene products, GAPDH message was not used as an endogenous quantitative standard in our study, since other studies have shown that the expression of this gene and other housekeeping genes is not, as most experimenters assume, constant. Expression of the so-called housekeeping genes, including GAPDH gene products, can vary as a consequence of drug treatment in hens brain (Damodaran *et al*, 2002) or during stages of development of bovine preimplantation embryos (Robert *et al*, 2002). Therefore, only iNOS PCR products from the different groups were quantified via densitometry as described below.

The following primers were used for GAPDH amplification: (5′-primer) 5′-TGTGACTTCAATGGTGACA and (3′-primer) 5′-CAGATCAGTTTCTATCAGC.

The iNOS primers were: (5′-primer) 5′-TTCAAACCTCATGCTGTTAA and (3′-primer) 5′-ACTGTAGTACTGCTTGAGAA. All primers were custom-made and purchased from Invitrogen. The primers were based upon the sequence retrieved from NCBI GenBank database from the coding sequence of chicken (*G. domesticus*): GAPDH (accession M11213) and iNOS (accession U46504).

Polyacrylamide Gel Electrophoresis to Separate PCR Products for Densitometry

The PCR products from GAPDH and iNOS mRNA were separated on a vertical electrophoresis unit (Owl Separation Systems, Portsmouth, NH) using a polyacrylamide gel. Each gel was composed of 2.6 mM ammonium persulfate, TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) (Invitrogen), 3.8% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide (BioRad, Hercules, CA), 0.1% (v/v) TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) (Sigma), and ultrapure deionized water.

The PCR products were pipetted onto each well of the gel in a total loading volume of 18 μl, composed of 15 μl of PCR reaction solution combined with loading buffer (0.25% w/v bromphenol blue, 30% v/v glycerol (Sigma) in ultrapure deionized water). In each gel, three lanes were loaded with a 50 bp DNA Molecular Weight Ladder (Invitrogen). GAPDH and iNOS PCR products from samples from the control and the treated groups were loaded on each gel.

The gels were run at 50 mA for about 1 h or when the bromphenol blue dye of the loading buffer reached the bottom of the vertical gel, and then stained for 10 min in 0.01% (w/v) ethidium bromide (Sigma) solution. PCR products were visualized using Gel Doc 1000 apparatus (Biorad) and the images transferred to a Macintosh computer. Densitometric quantification of the bands obtained from the iNOS PCR products was carried out using Molecular Analyst software (Biorad). Although cDNAs were the products amplified, separated, and quantified, we refer to them as the starting nucleic acid (ie mRNA).

Statistical Analysis

BV and suture values obtained during and after infusions were expressed and analyzed as percent of their individual BL values because of the large variability between subjects. The values for iNOS message were obtained from densitometric analysis and expressed as pixels/band.

Repeated-measures ANOVA followed by factorial ANOVAs was used for statistical comparisons where appropriate. Based on a previous study (Zhang *et al*) showing the vasoconstrictive effect of Coc upon the chick's extra-embryonic vasculature, unidirectional Dunnett's test was used as the preplanned contrast to compare BV diameters of each treated group against the control group. Least square linear regression analysis between brain or heart mRNA and BV diameters was also carried out.

RESULTS

Experiment 1: Blood Vessel Experiment

Examples of images of the BV from eggs with various treatments are shown in Figure 1. Baseline BV diameters of the four treatment groups, while quite variable, were nevertheless not significantly different from one another. The mean absolute value for BL diameters was 231 μm, ranging in size from 189 to 273 μm ($n = 16$).

Repeated-measures ANOVA showed a significant main effect of treatment ($F_{3,12} = 7.37$; $p < 0.005$) and a repeated-measures effect ($F_{3,12} = 4.485$; $p < 0.01$). There was also a significant treatment effect by repeated-measures interaction ($F_{9,36} = 3.29$; $p = 0.005$). Examination of Figure 2 shows that BV diameters, relative to their respective BL diameters, did not vary by more than a few percent for the NaCl + NaCl group and the NaSal + NaCl group at all times. The NaCl + Coc BV diameters were not significantly affected for the first three time points (ie 5, 10, and 15 min Inf). Unidirectional Dunnett's test showed that BV diameters from the Coc-only-treated group took 20 min (ie 15 min of Coc Inf + 5 min PI) to cause a significant (18%) reduction in diameter (ie vasoconstriction) compared to the NaCl + NaCl-treated group, while pretreatment with NaSal sensitized the vasculature to the vasoconstrictive effect of Coc, causing a significant (12%) reduction in vascular size immediately (ie within 5 min Inf), showing progressively greater vasoconstriction over the 20 min recording period with a 17% decrease in BV diameter at this time.

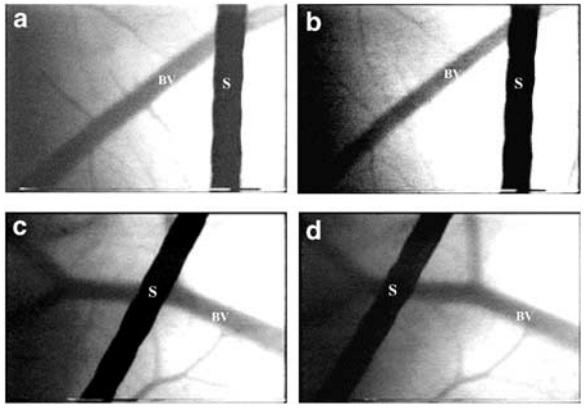

Figure 1 Sample BV image. Panels a and c are images from recordings made at baseline while panels b and d are images from recordings made after 5 min into infusion of Coc. Panels a and b show an obvious reduction in BV diameter, relative to baseline diameter, measured where BV is superimposed upon the images, from a subject in the NaSal + Coc group. Panels c and d show no apparent effect in a subject from the NaCl + Coc group. S is superimposed upon the images where measurements were taken for diameters of the sutures.

Experiment 2: Brain iNOS mRNA

Figure 3 shows an example of a gel containing samples from each treated group, along with the molecular weight ladder. Dunnett's test indicated that only the NaSal + Coc-treated group showed a significant increase in iNOS mRNA in their brains compared with the NaCl + NaCl group's brains (Figure 4). The concentration of iNOS mRNA in the hearts of the treated groups was not different from controls (data

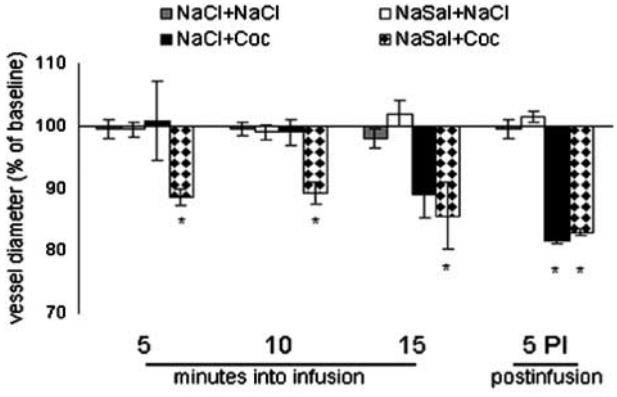

Figure 2 BV diameter. Combined NaSal + Coc decreased BV diameter as soon as 5 min into the infusion, while Coc alone (ie NaCl + Coc) did not have an effect until 5 min PI. *$p < 0.05$ or better *vs* controls (NaCl + NaCl) using unidirectional Dunnett's test. Values are expressed as % of baseline BV diameter. Vertical lines represent ± SEM.

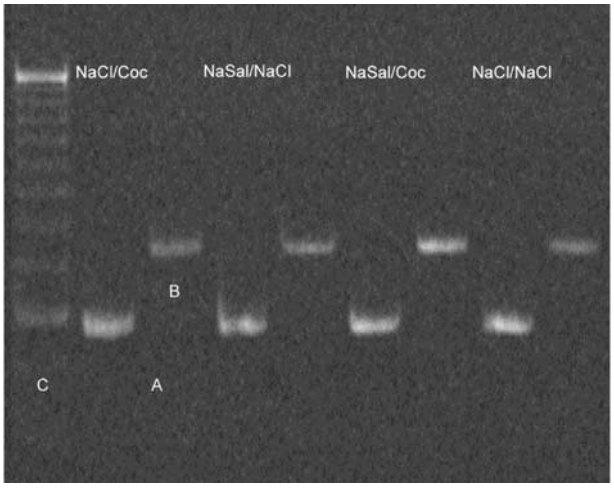

Figure 3 Sample of a gel showing cDNA bands derived from extracted mRNA for the 'housekeeping' GAPDH gene (A) and iNOS gene (B). (C) The 50 bp cDNA ladder.

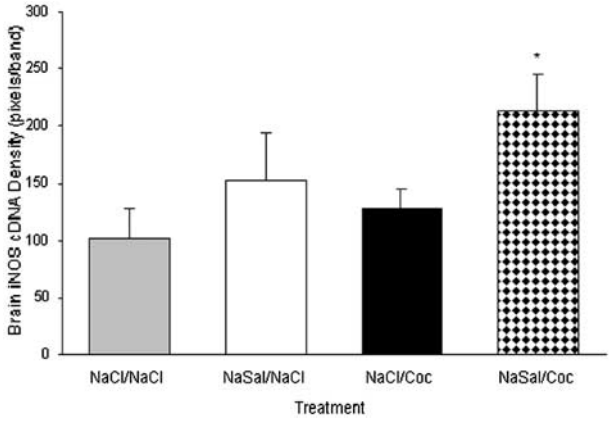

Figure 4 Brain iNOS mRNA expression. Only the NaSal + Coc-treated group demonstrated an increase in iNOS mRNA expression. $*p < 0.05$, Dunnett's test. Vertical lines represent SEM.

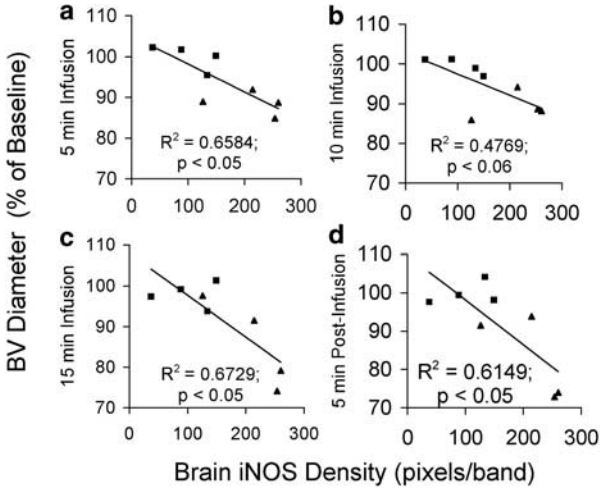

Figure 5 Regression analysis of brain iNOS *vs* BV size. There are significant inverse relationships between BV size and brain iNOS mRNA expression at three of the four times during and after infusions. Values used are from the NaCl + NaCl and NaSal + Coc groups. All other treatment combinations did not show significant relationships. ■ NaCl + NaCl data; ▲ NaSal + Coc data.

not shown). Regression analyses were carried out on data derived from the NaCl + NaCl and NaSal + Coc ($n = 4 + 4$) since only the NaSal + Coc group showed a significant increase in expression of iNOS message. The outcome of the regression analysis is displayed in Figure 5, which shows results for all four times (ie 5, 10, 15 min inf, and 5 min PI).

DISCUSSION

NaSal by itself did not have an effect upon BV size. However, NaSal greatly enhanced the vasoconstriction caused by Coc on the extra-embryonic vasculature.

Coc by itself caused vasoconstriction, as expected, but not until 5 min after the infusion was terminated. When pretreated with NaSal, the BV diameters decreased significantly as soon as 5 min into the infusion, the earliest time taken for analysis. The mechanisms responsible for the interactive effect of the combination of NaSal + Coc upon the vasculature of the homolog of the mammalian placenta are most likely due to several actions. For example, inhibition of synthesis of the potent vasodilator prostacyclin by NaSal (Shimokawa *et al*, 1988; Vane and Botting, 1998) may have contributed to the inability of the BV to counteract the effect of Coc, resulting in enhanced vasoconstriction. Another possibility is that, as NaSal blocks the cyclooxygenase pathway, arachidonic acid metabolism may shift toward increased production of leukotrienes, which have been demonstrated to have vasoactive effects in human placenta (Thorp *et al*, 1988), ovine fetoplacental circulation (Meyer *et al*, 1990), and in guinea pig cerebral arteries (Uski and Hogestatt, 1992), and consequently exacerbate the vasoconstriction caused by Coc. The enhanced vasoconstriction caused by combined NaSal + Coc may decrease blood supply to the embryo, leading to organ (eg brain) hypoperfusion and hypoxia (Covert *et al*, 1994). Alternatively, the NaSal + Coc-induced reduction in the diameter of the BV can dramatically increase resistance, based on Poiseuille equation ($R = 8nL/\pi r^4$) and most likely cause an increase in blood pressure (hypertension) similar to that observed in fetal sheep following an increase in uterine vascular resistance caused by Coc (Covert *et al*, 1994). Hypertension increases transmural BV pressure, which may compromise BV wall integrity and lead to vascular leakage or rupture evidenced by edema and/or hemorrhages (Lou, 1980). In another study, Mangiardi *et al* (1988) reported that hypertension (eg Coc induced) is associated with development of subarachnoid or intracerebral hemorrhages and is related to increase blood pressure.

The enhanced vasoconstriction observed in this study and/or the hemorrhages reported previously (Venturini and Sparber, 2001; Castelli *et al*, 2001) by NaSal + Coc can activate vasospastic-related inflammatory processes in the brain (Zuccarello *et al*, 1998; Conway and Tamargo, 2002). For example, angiographic inflammatory changes (ie poor vascularization and vessel wall irregularities) in rabbits are presumed to occur due to a vasospastic mechanism (Martinez *et al*, 1996). In addition, the enhanced vasoconstriction seen with the combination of NaSal + Coc may lead to ischemia and induce inflammatory reactions, such as microglial cell activation (Garcia *et al*, 1993) and/or intravascular leukocyte adhesion to the cerebral endothelium (Garcia *et al*, 1994). An increase in leukotriene production, as mentioned earlier, may also induce inflammatory changes (Henderson, 1994). The inflammatory processes may stimulate microglial cells to produce inflammatory cytokines, like interleukin-1-beta (Il-1β), which can lead to iNOS mRNA expression (Mizushima *et al*, 2002; del Zoppo *et al*, 2000).

While it has been reported that Sal blocks induction of iNOS expression, possibly by inhibiting iNOS at the level of transcription, through an NF-Kβ-independent mechanism (Farivar and Brecher, 1996), several other studies suggest that NaSal is interfering with iNOS at a post-transcriptional level, rather than at the level of mRNA transcription. For example, NaSal inhibited iNOS protein synthesis and consequently NO production but did not affect iNOS mRNA expression in RAW 264.7 macrophages exposed to

lipopolysaccharide (LPS) plus interferon-gamma (INF-γ) (Ryu *et al*, 2000). The same authors found no effect of NaSal on iNOS mRNA stability (ie at the post-transcriptional level) similar to a report that NaSal does not affect iNOS mRNA half-life, therefore not altering iNOS mRNA stability (Farivar and Brecher, 1996). In another study, NaSal inhibited IL-1β-induced NO formation by blocking iNOS protein synthesis by either NF-Kβ activation or iNOS mRNA expression in rat hepatocytes (Sakitani *et al*, 1997). Thus, it can be argued that Sal is not a transcriptional inhibitor of iNOS and does not alter mRNA stability (such as mRNA half-life), but it interferes with translational and post-translational processing of iNOS (Amin *et al*, 1995).

If Sal interferes with translational and/or post-translational events, this may result in decreased iNOS enzyme synthesis with a consequent decrease in NO production. Such an action could partially explain why the vasoconstriction is enhanced while the vascular bed is exposed to the Coc insult after injection of NaSal into eggs with embryos. If Sal is interfering with compensatory mechanisms, such as inhibiting the synthesis of vasodilatory prostaglandins, which relax vascular smooth muscle, and, at the same time, is blocking iNOS protein production and/or decreasing its catalytic activity, the synergy of these actions may be great enough to augment the developmental toxicity of Coc, as previously reported by this laboratory (Venturini and Sparber, 2001; Castelli *et al*, 2001).

In the absence of sufficient compensatory synthesis and release of NO in response to Coc-induced vasoconstriction, the insult may cause a compensatory increase in iNOS mRNA production. In the present study, we show that increased iNOS mRNA is significantly correlated with the degree of vasoconstriction when Coc is combined with Sal and we interpret these findings thusly.

It has recently been reported that compromised relative cerebral blood flow (rCBF) in abstinent Coc abusers could be significantly reversed by treatment with the proton-pump inhibitor ameloride; aspirin was essentially devoid of this effect (Kosten *et al*, 2003). While it had previously been suggested to use these compounds to treat ischemia-related hypoperfusion of brain in Coc abusers, the potential for aspirin-related bleeding was mentioned as a cautionary factor (Kosten, 1998). Since, the combination of aspirin or Sal plus Coc was not studied with regard to rCBF, the possibility of a vasospastic or hemorrhagic event cannot be ruled out.

We can therefore conclude that the combination Na-Sal + Coc may be detrimental to a developing embryo if exposed to combinations of both drugs when, for example, Coc-using pregnant women medicate (self or prescribed) with aspirin. It remains to be determined if other nonsteroid anti-inflammatory agents also enhance the developmental toxicity or vasoconstrictive actions of Coc. We have recently carried out experiments with the NOS inhibitor L-nitro-arginine-methyl-ester or the selective iNOS inhibitor aminoguanidine (Misko *et al*, 1993), and have likewise found enhanced effects of Coc upon hatchability and/or vasoconstriction (unpublished observations), supporting the tentative interpretation of our results, as discussed above.

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