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Effect of Nitric Oxide Donors and Nitric Oxide Synthase Inhibitors in Neonatal Rat Endotoxic Shock

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ABSTRACT. Previous studies have shown an increased mortality in response to endotoxin in 24-hr-old neonatal rats compared with older neonates and adults. This increased susceptibility may be related to increased nitric oxide (NO) and thromboxane (TxB_2) production. Twenty-four-hour-old neonatal rat pups were given either N^G-nitro-L-arginine methyl ester (L-NAME; a nonspecific NO synthase inhibitor), *S*-methylthioisourea (SMT; a specific NO synthase inhibitor), or molsidomine (a NO donor) subcutaneously prior to or after an LD_{50} of intracardiac endotoxin. Mortality was followed for 72 hr. There was no statistically significant difference in mortality between control animals and those pretreated with L-NAME, SMT, or molsidomine. A trend toward increased mortality with nonspecific NO synthase inhibition and decreased mortality with the NO donor was noted. Splenic cells were obtained for *in vitro* cytokine stimulation studies. *In vitro* adherent splenic cell stimulation studies confirmed an increase in NO production with NO donor pretreatment and decreased production of NO with NO synthase inhibition pretreatment. There was no difference in TxB_2 production with either the NO synthase inhibitor or the NO donor. In conclusion, at the several doses employed, neither nonselective or selective NO synthase inhibitors nor NO donors prevented endotoxin-induced mortality in rat neonatal shock. Although these findings do not preclude possible involvement of NO in neonatal pathophysiology, increased NO production thus does not appear to be the primary determinant of the increased susceptibility of the neonatal rat to endotoxic shock. BIOCHEM PHARMACOL **58**;4:687–691, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. neonate; endotoxin; nitric oxide; thromboxane

Sepsis continues to be a major cause of morbidity and mortality in the neonatal population. The incidence of neonatal sepsis has been estimated to be as high as one to ten per 1000 live births [1]. Despite medical advances, the mortality remains as high as 20–75% [2–4]. Neonatal sepsis can involve both Gram-negative and Gram-positive infections. Gram-negative infections and the associated endotoxemia remain the most significant cause of neonatal morbidity and mortality.

Septic neonates are in a hyperdynamic state characterized by tachycardia and increased cardiac output [5], decreased systemic vascular resistance [6], and increased alveolar-arterial oxygen tension gradients [7]. Many of these symptoms of shock can be explained on the basis of increased NO¶ production, as this compound is a potent vasodilator [8] and an important regulator of microvascular perfusion [9].

NO is derived from the amino acid L-arginine by a redox reaction catalyzed by the enzyme NO synthase. Three different types of NO synthases have been identified. Types I and III are constitutively expressed, are calcium/calmodulin-dependent, and are found primarily in neuronal tissues and endothelial cells. Type II or inducible NO is not constitutively expressed, is calcium-independent, and can be induced by cytokines and endotoxin in a wide variety of cell types [8].

There continues to be controversy regarding the beneficial and/or detrimental roles of NO in sepsis. There have been multiple studies looking at this in adult animal models. Evans *et al.* [10] demonstrated that NO synthase inhibition can reverse some of the hemodynamic instability of murine sepsis, but does not improve overall mortality. Zurovsky and Eligal [11] demonstrated in a rat sepsis model that NO inhibition does not change mortality compared with rats given endotoxin alone. A canine sepsis model demonstrated that NO synthase inhibition improves regional hemodynamics, but decreases oxygen transport to the gut [12]. Kilbourn *et al.* [13] demonstrated that the use

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¶ *Abbreviations*: NO, nitric oxide; L-NAME, N^G-nitro-L-arginine methyl ester; SMT, *S*-methylthioisourea; L-NMA, N^G-methyl-L-arginine; LD_{50}, lethal dose for 50% of the animals tested; LPS, lipopolysaccharide; SNAP, *S*-nitroso-*N*-acetylpenicillamine; and TxB_2, thromboxane.

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of NO synthase inhibitors significantly improves the hemodynamic instability in canine endotoxic shock. In a septic canine model, NO synthase inhibition increased vascular resistance, but also increased mortality [14]. There also have been a few case reports of NO synthase inhibitor use in humans. The three patients reported each demonstrated improvement in hemodynamics after a NO synthase inhibitor was given; however, two of the three patients died [15, 16].

There remains a paucity of data on the role of NO in neonatal sepsis. Our previous observations have demonstrated increased endotoxin-stimulated NO production by splenic macrophages in neonatal versus adult rats [17]. Therefore, in the present study we sought to examine the effects of a nonspecific NO synthase inhibitor (L-NAME), a specific NO synthase inhibitor (SMT), and a NO donor (molsidomine) on mortality and cytokine production in our already established model of sepsis in neonatal rats. It was hypothesized that NO was involved in the increased sensitivity to endotoxin in neonatal rats and that inhibition of NO synthesis therefore may improve survival and/or modulate eicosanoid metabolism.

MATERIALS AND METHODS

Animals

All rats used in these experiments were housed in the facilities for laboratory animals provided by the Department of Laboratory Animal Resources. The Medical University of South Carolina laboratory animal care is in accordance with the National Institutes of Health guidelines. The Medical University of South Carolina has full accreditation from the American Association for the Accreditation of Laboratory Animal Care, effective November 5, 1987. Pregnant Sprague–Dawley rats were purchased from Charles River and were received on approximately day 14 of gestation. Each maternal rat produced a litter of approximately 10–12 pups. During the mortality studies, the pups were returned to their mothers. The maternal rats were allowed food and water *ad lib*.

Mortality Studies

Twenty-four-hour-old Sprague–Dawley neonatal pups were used in all the studies. Each litter of neonatal rat pups served as its own control. The approximate LD_{50} in 1-day-old rats given intracardiac (i.c.) injections of *Salmonella enteritidis* endotoxin, Boivin (Difco Laboratories), was determined to be 0.024 mg/kg. All i.c. injections were made with 30-gauge needles via the sub-xiphoid approach. There was a 0–9% mortality rate in all control neonatal groups as a consequence of i.c. injection. Pups that succumbed to this procedure within 1 hr after i.c. injection in either control or treatment groups were not included in the results. According to our previous procedure [17], one group of rats were injected subcutaneously (s.c.) with 50 μL of different doses of L-NAME 1 hr before a 50-μL LD_{50} of i.c. endotoxin. A second group of rats were injected s.c. with 50 μL of 5 mg/kg of SMT at the time of the LD_{50} of i.c. endotoxin or 2 hr after the LD_{50} of endotoxin. SMT (5 mg/kg) then was given every 8 hr for 3 days. A third group of rats were injected s.c. with 50 μL of different doses of molsidomine 1 hr before and 4 hr after a similar dose of i.c. endotoxin was given. All s.c. injections were made with 30-gauge needles at the base of the tail. Mortality was followed for 72 hr, as previous studies showed no further deaths after 72 hr [17, 18].

Preparation of Splenic Cells

Spleens were used as a source of adherent cell mediator production. Since eicosanoids and NO have been implicated in septic shock, these are the mediators we chose to study. Neonatal pups were euthanized by CO_2, followed immediately by abdominal incisions for harvesting spleens. Spleens then were placed in a solution of RPMI 1640 with streptomycin (50 μg/mL) and penicillin (50 U/mL) (Life Technologies). The spleens were dissected manually and aspirated into a 24-gauge needle to disperse free cells. The cell suspension obtained was centrifuged in a Beckman CS-15R three times for 7 min at 1000 rpm with RPMI (10 mL). Then the remaining cell pellet was diluted with RPMI (10 mL) prior to counting. Cell viability was determined by trypan blue (0.4%) exclusion. Viability of the cells was greater than 95%.

Stimulation of Adherent Spleen Cells from Neonatal Rats

Adherent splenic cells were isolated from 24-hr-old neonatal rats. Cells were incubated at 10^6/mL in flat-bottom, 24-well plates (Becton Dickinson Labware) for 2 hr and then washed to remove nonadherent cells. L-NMA, a nonspecific NO inhibitor, or SNAP was added to the adherent cells 30 min before LPS stimulation (1 μg for 24 hr). SNAP was used in the *in vitro* experiments instead of molsidomine because the latter must be metabolized by hepatic enzymes to the active form. Medium with and without endotoxin was added to the adherent cells. After 24 hr of incubation, cell-free medium was collected for assay of TxB_2 or nitrite, an indirect measure of NO. The mediators were expressed as activity per 100 μg of adherent protein.

NO Assay

NO production by adherent splenic cells was assessed by measuring the amount of nitrite, a metabolic product of NO, in cell culture supernatants. Briefly, 100 μL of splenic cell culture supernatant was mixed with 100 μL of Griess reagent (1:1, v/v, of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in H_2O/1% sulfanilamide in 5% H_2PO_4) in 96-well microtiter immunoassay plates; absorbance at 570 nm was measured with a Bio-Tek EL 312 microplate reader.

TABLE 1 Effects of L-NAME and molsidomine on endotoxin-induced lethality in the neonatal rat

Treatment	Dead/Total	Mortality (%)
Control	75/134	56
L-NAME		
1 mg/kg	13/23	57
10 mg/kg	11/21	52
30 mg/kg	7/11	84
60 mg/kg	5/7	71
Total	36/52	58
Molsidomine		
10 mg/kg	10/22	45
20 mg/kg	8/15	53
30 mg/kg	6/15	40
60 mg/kg	8/20	40
90 mg/kg	8/14	54
Total	40/86	47

Control and esxperimental animals were given 0.024 mg/kg of LPS (LD_{50}) by intracardiac injection. L-NAME was given s.c. 1 hr before LPS. Molsidomine was given 1 hr before and 4 hr after LPS. Mortality was followed for 72 hr.

The nitrite amount was expressed in nanomoles per milliliter and calculated from a $NaNO_2$ standard curve.

TxB_2 Assay

Samples were thawed and diluted in buffer containing 0.1% polyvinylpyrrolidine, 0.9% NaCl, 50 mM Tris base, 1.7 mM $MgSO_4$, and 0.16 mM $CaCl_2$, pH 7.4, prior to radioimmunoassay. TxB_2 was quantified by radioimmunoassay as described previously [19, 20].

Protein Assay

Protein determinations were made on parallel wells for each treatment. After cells were washed, they were dissolved in 1 N NaOH. Protein determinations were performed using the Bio-Rad protein assay dye reagent concentrate. The amount of protein was measured at absorbance 595 nm with a Bio-Tek EL 312 spectrophotometer.

Statistical Analysis

Mortality data were analyzed by the Chi-square method. Splenic cell mediator data were determined by ANOVA with intercomparison analysis using Fisher's least significant difference. Data are expressed as means ± SEM. A value of $P < 0.05$ was considered significant.

RESULTS

Mortality Studies

The percent of mortality of the neonatal pups in response to the various doses of L-NAME and molsidomine was determined (Table 1). The LD_{50} of endotoxin in 24-hr-old neonatal rat pups was determined previously to be 0.024 mg/kg [17]. Increasing doses of L-NAME given prior to administration of an LD_{50} of endotoxin did not cause a statistically significant change in mortality. However, the trend was toward a higher mortality with increasing doses of L-NAME pretreatment. Increasing doses of molsidomine given before and after administration of an LD_{50} of endotoxin also did not change mortality significantly. There was a trend toward protection with the higher doses of molsidomine. The percent of mortality of the neonatal pups to the two dosing regimens of SMT also was determined (Table 2). There was no statistically significant difference in mortality between the control group and the two SMT groups.

TABLE 2. Effects of SMT on endotoxin-induced lethality in the neonatal rat

Treatment	Dead/Total	Mortality (%)
Control	22/36	61
Simultaneous SMT*	21/38	55
Post-treatment SMT†	23/39	59

*Simultaneous SMT: rats were given 5 mg/kg of SMT at the same time as a LD_{50} of endotoxin 2nd then every 8 hr for 3 days.

†Post-treatment SMT: rats were given 5 mg/kg of SMT 2 hr after a LD_{50} of endotoxin and then every 8 hr for 3 days.

Splenic Cell Mediator Production

There was no statistically significant difference in TxB_2 production in the control adherent splenic cells stimulated with endotoxin compared with those given various doses of L-NMA or SNAP 30 min before 24 hr of endotoxin (1 μg/mL) stimulation (data not shown). When L-NMA was added to the adherent splenic cells 30 min before 24 hr of endotoxin (1 μg/mL) stimulation, there was a statistically significant decrease in NO production compared with endotoxin given alone (Fig. 1). When SNAP was added to the adherent splenic cells 30 min before 24 hr of endotoxin (1 μg/mL) stimulation, there was a statistically significant increase in production compared with endotoxin stimulation alone (Fig. 2).

DISCUSSION

Previous work from our group demonstrated a profound sensitivity of 24-hr-old rats to endotoxic shock compared with adult rats [17]. Zeller *et al.* [21] also demonstrated an increased susceptibility of 10-day-old rats to endotoxin compared with adult rats. The increased susceptibility of neonates to infection is well established. However, the exact pathophysiologic mechanisms of this increased susceptibility to endotoxin and infection remain unclear. Klein *et al.* [22] demonstrated decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and children. The increased susceptibility of neonatal rats to Group B streptococcal infection has been demonstrated to be due to decreased myeloid pools, a lag time in response to infection, and an inability to maintain myeloid

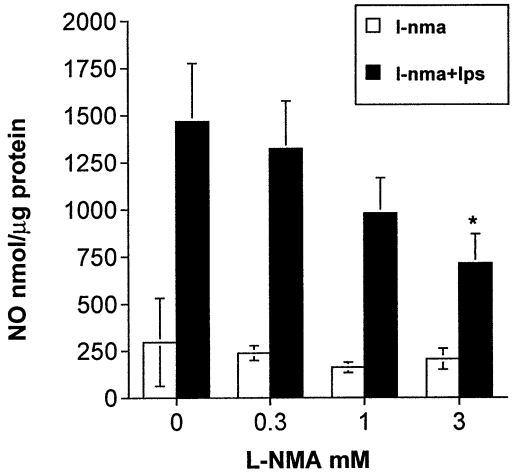

FIG. 1. Effect of L-NMA on NO production by adherent splenic cells from neonatal rats. The cells were pretreated with L-NMA (0, 0.3, 1, and 3 mM) for 30 min, and then were stimulated with endotoxin (LPS, 1 μg/mL). The supernatants were collected after 24 hr of endotoxin stimulation and were analyzed for NO. Values are means ± SEM, N = 3. Key: (*) $P < 0.05$.

pools [23]. A decreased production of cytokines by neonatal monocytes in comparison to their adult counterparts has been demonstrated [24, 25].

Previous studies from our group demonstrated an increased production of NO and TxB_2 in the adherent splenic cells of 24-hr-old rat pups compared with older neonatal and adult rats after *in vitro* endotoxin stimulation [17]. A deleterious role of TxB_2 in endotoxic shock has been shown [26], but the role of NO is less clear. Salvemini *et al.* [27–29] have shown that NO activates cyclooxygenase enzymes, therefore increasing the production of prostaglandins. This group further demonstrated in an *in vivo* rat model that inhibiting NO led to a decrease in prostaglandin production. However, our *in vitro* studies with endotoxin-stimulated adherent splenic cells showed that L-NMA and SNAP did not change TxB_2 levels significantly. Our present results suggest a lack of association between increased NO production and mortality. Doses of NO synthase inhibitors and NO donors were used within the range shown to affect adult rat mortality from endotoxic shock [30]. Although there was a trend toward protection with molsidomine and a trend toward worsening survival with L-NAME, our data demonstrate that pretreatment with L-NAME or molsidomine did not statistically change the mortality rates in a neonatal shock model. A study by Aranow *et al.* [31] demonstrated protection in a rat model of bacterial sepsis with the specific NO synthase inhibitor SMT. Our results did not show any protection with SMT at a similar dose in our neonatal rat model. The reason for the lack of an effect of these compounds is uncertain, but could reflect an immaturity of the vascular system toward modulation with NO. The role of NO in sepsis and shock, in general, remains controversial [32]. NO may contribute to the vascular hyporeactivity of endotoxic or septic shock [33]. Numerous studies have shown that inhibition of NO improves the hemodynamic instability caused by sepsis, but that survival is not improved [8, 9, 11, 34]. These latter studies however, used nonselective NO synthase inhibitors, whereas improved survival with selective inhibitors of the inducible form of NO may be a result of sparing inhibition of endothelial NO production [31]. Indeed, NO may provide some anti-inflammatory protection by reducing neutrophil and platelet adhesion, and by maintaining tissue perfusion [35, 36]. Molsidomine has been shown to reduce endotoxin-induced cytokine production and improve survival to endotoxic shock [30, 37].

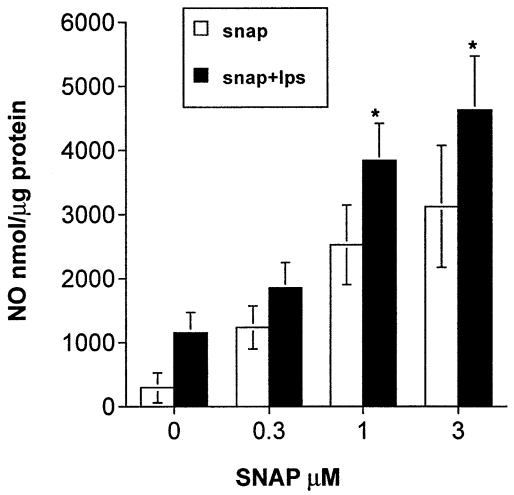

FIG. 2. Effect of SNAP on NO production by adherent splenic cells from neonatal rats. Treatment groups, data expression, and statistical analysis are as in Fig. 1. Values are means ± SEM, N = 3. Key: (*) $P < 0.05$.

In conclusion, neither nonselective and selective NO synthase inhibitors nor NO donors appear to prevent endotoxin-induced mortality in neonatal shock under the conditions tested. Thus, despite age-related differences in NO production [17], excessive NO production in neonates does not appear to contribute to their enhanced susceptibility to endotoxin.

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