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Short communication

Inhibition of nitric oxide synthase partially attenuates alterations in the blood-cerebrospinal fluid barrier during experimental meningitis in the rat

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Abstract

The permeability of the blood-cerebrospinal fluid (blood-CSF) barrier is increased upon exposure to lipopolysaccharides during bacterial meningitis. Lipopolysaccharides induce nitric oxide (NO) synthase in a variety of cells. Increased meningeal NO production and blood-CSF barrier permeability were observed in a rat model of meningitis. Administration of aminoguanidine, an inhibitor of NO synthase, blocked meningeal NO production and significantly attenuated permeability changes in the blood-CSF barrier. It is hypothesized that pathological production of NO may contribute to the disruption of the blood-CSF barrier during meningitis.

Keywords: Nitric oxide (NO); Meningitis; Blood-cerebrospinal fluid barrier; Aminoguanidine

1. Introduction

Despite antibiotic therapy, bacterial meningitis continues to inflict a relatively high morbidity and mortality rate of 5–26%, depending on the age of the patient population group and the type of Gram-negative organism. An inflammatory response to bacterial lipopolysaccharides promotes an increased permeability of the blood-brain and blood-cerebrospinal fluid (blood-CSF) barriers, leading to vasogenic cerebral edema, intracranial hypertension due to fluid accumulation, changes in regional cerebral perfusion, loss of cerebrovascular autoregulation, depressed cerebral metabolism, and in severe cases, neuronal dysfunction and neurotoxicity. These pathophysiologic alterations in meningitis are thought to initiate or contribute to long-term neurological problems (i.e. cranial nerve damage, cerebral infarction, recurrent seizures, learning disabilities and/or mental retardation) in as many as 30% of bacterial meningitis survivors (Quagliarello and Scheld, 1992).

Bacterial meningitis alters the blood-brain and blood-CSF barriers in vivo (Quagliarello and Scheld, 1992). The cellular mechanisms through which lipopolysaccharides disrupt the integrity of the blood-brain and blood-CSF barriers are poorly understood. Current evidence suggests a complex interplay involving complement factors, polymorphonuclear leukocyte recruitment and elaboration of various inflammatory mediators (e.g. oxygen free radicals, complement factors, prostaglandins, cytokines) from a diversity of cell types.

Two recent reports speculated that increased nitric oxide (NO) synthesis may occur during meningitis in humans (Visser et al., 1994; Milstien et al., 1994). NO synthesis can be immunoinduced in endothelial cells cultured from murine brains, as well as from fibroblasts cultured from neonatal rat pup meninges by lipopolysaccharides and inflammatory cytokines (Kilborn and Belloni, 1990; Boje and Arora, 1992). Moreover, it is generally recognized that pathological production of NO has proinflammatory effects (reviewed in Schmidt and Walter, 1994). These observations suggest the hy-

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pothesis that pathological production of NO during meningitis mediates disruption of the blood-CSF barrier.

2. Materials and methods

2.1. Induction of experimental meningitis and administration of aminoguanidine

One day before the study, male Sprague-Dawley rats (250–300 g) were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) for surgical catheterization of the right jugular vein. Following an overnight recovery, animals were briefly anesthetized with diethyl ether and placed in a stereotaxic frame for intracisternal injection of 0 or 25 μg lipopolysaccharides (*Escherichia coli* serotype 0127:B8) in 10 μl sterile artificial cerebrospinal fluid (consisting of 120 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.67 mM NaH_2PO_4, 0.3 mM Na_2HPO_4, pH 7.4). Rats were allowed to recover in their cages with free access to food and water.

Immediately after intracisternal injection of lipopolysaccharides or artificial CSF, aminoguanidine hemisulfate (Sigma Chemical Co.) was administered as a bolus loading dose (180 mg/kg) followed by constant rate infusion (1.04 mg/kg/h) through the jugular vein catheter. The design of this empirical dosing regimen was based on the pharmacokinetic parameters estimated from the aminoguanidine concentration-time data of Beaven et al. (1969), with the intention of maintaining relatively constant concentrations of aminoguanidine over the 6.5 h experimental treatment period.

2.2. Determination of the blood-CSF barrier permeability

Assessment of the permeability of the blood-CSF barrier was determined using Evans blue dye. At 5.5 h after intracisternal injection, a bolus dose of Evans blue (0.5 ml of a 2% solution in saline) was administered through the jugular vein catheter. One hour later, a blood sample for Evans blue analysis was obtained (6.5 h after lipopolysaccharides administration). Rats were then rapidly anesthetized with intravenous thiamylal (40 mg/kg) and killed by intravenous injection of isotonic KCl (2 ml). Animals were placed in a small animal stereotaxic frame for collection of CSF by intracisternal puncture. Evans blue concentrations in serum (1 μl in 50 μl water) and CSF (50 μl) were assayed by spectrophotometric analysis at 590 nm using a Fisher Biotech BT2000 absorbance plate reader.

2.3. Analytical detection of NO from meningeal tissues

Meninges and the choroid plexi were dissected from the brain surfaces and various ventricles for determination of NO synthase activity. Meningeal tissues were dissociated into a single cell suspension by incubation with collagenase solution (150 units/ml) at 37°C for 30 min, followed by extrusion through a 22 gauge hypodermic needle. Since hemoglobin inhibits the detection of gaseous NO, hemoglobin removal from the cell suspension was accomplished by a 90 s incubation with ammonium chloride-potassium chloride lysing solution to rupture contaminating red blood cells. The cell preparation was centrifuged and resuspended in 0.55 ml of culture media (DMEM) containing 150 units/ml of superoxide dismutase. The meningeal cell preparation (0.5 ml) was incubated for 30 min at 37°C in a 5.0

Table 1
Detection of nitric oxide produced from lipopolysaccharides-immunostimulated meninges

Intracisternal dose	Added stabilizer or inhibitor	Headspace NO (pmol/million cells/60 min) (mean ± S.E.M.) (*n*)
Artificial cerebrospinal fluid	None	0.3 ± 0.3 [a] (4)
25 μg lipopolysaccharides	None	86.1 ± 5.5 (18)
25 μg lipopolysaccharides	Superoxide dismutase (150 units/ml)	293 ± 55 [a] (4)
25 μg lipopolysaccharides	Oxyhemoglobin	0 ± 0 [a] (4)
25 μg lipopolysaccharides	Methemoglobin	56.2 ± 26.6 (4)
25 μg lipopolysaccharides	L-N^G-Nitroarginine (1 mM)	35.2 ± 4.5 [a] (3)
25 μg lipopolysaccharides	L-N^G-Methylarginine (1 mM)	8.2 ± 4.9 [a] (3)
25 μg lipopolysaccharides	L-N^G-Aminoarginine (1 mM)	1.2 ± 1.2 [a] (3)
25 μg lipopolysaccharides	Nitroguanidine (1 mM)	87.6 ± 14.6 (4)
25 μg lipopolysaccharides	Methylguanidine (1 mM)	60.4 ± 20.5 (4)
25 μg lipopolysaccharides	Aminoguanidine (1 mM)	10.9 ± 6.2 [a] (4)

Rats were dosed with intracisternal lipopolysaccharides (0 or 25 μg) in artificial CSF. Six and a half hours later the meninges and choroid plexi were harvested and analyzed in vitro for the production of NO. Pharmacologic agents were added to the in vitro preparation.
[a] $P < 0.01$ by Student's *t*-test versus 25 μg lipopolysaccharides without added agents.

ml gas-tight vial with constant stirring. All of the headspace gas of the vial was vacuum aspirated into a Nitric Oxide Chemiluminescent Detector (Sievers Instruments, Boulder, CO, USA) for NO analysis. The NO detector was calibrated using authentic NO gas (5.2 ppm in nitrogen).

In another set of experiments the pharmacological and analytical specificity for NO production from meningeal cells was assessed using various stabilizers and inhibitors of NO or NO synthase. Dissociated meningeal cells were obtained from rats previously injected with 25 μg lipopolysaccharides. The preparations were incubated with or without various additives in a sealed vial, and the headspace gaseous phase of the sealed vial was assayed for NO at 60 min.

2.4. Statistical analysis

Data were analyzed for statistical differences by Student's *t*-test or by one-way analysis of variance followed by Neuman-Keuls post-hoc analysis.

3. Results

Significant amounts of NO were detected in the headspace gas from the meninges of lipopolysaccharide-treated rats compared to control rats (Table 1). The analytical specificity for NO production from meningeal cells was confirmed using various stabilizers and inhibitors of NO or NO synthase (Table 1). The amount of detected NO was significantly increased in the presence of added superoxide dismutase, an agent which prolongs the stability of NO (Boje and Skolnick, 1992). Oxyhemoglobin significantly decreased NO detection by chelating NO (Boje and Skolnick, 1992). Methemoglobin was used as a control for oxyhemoglobin, and as expected, did not alter the detection of NO. Known inhibitors of NO synthase (L-N^G-nitroarginine, L-N^G-methylarginine, L-N^G-aminoarginine and aminoguanidine) effectively blocked the production of NO from immunostimulated meningeal preparations. Nitroguanidine and methylguanidine were used as controls for aminoguanidine, and as expected, did not inhibit NO synthase. These results demonstrate the specificity of the analytical assay for the detection of authentic NO produced from immunostimulated meninges.

Fig. 1A provides evidence for the hypothesis that NO mediates permeability changes in the blood-CSF barrier during experimental meningitis. The administration of aminoguanidine did not alter the integrity of the blood-CSF barrier in control rats, as indicated by negligible CSF concentrations of Evans blue dye in the control group. Remarkably, CSF dye concentrations in lipopolysaccharide-treated rats were partially attenuated by aminoguanidine ($P < 0.05$). The aminoguanidine dosing regimen also effectively inhibited meningeal NO production (Fig. 1A) induced by prior treatment with lipopolysaccharides. Serum Evans blue concentrations were statistically similar in the three groups of rats (Fig. 1B), thus ruling out major changes in the systemic pharmacokinetics of Evans blue as a cause for the different CSF concentrations among the groups. Moreover, normalization of the CSF dye concentrations by the serum concentrations statistically reflected

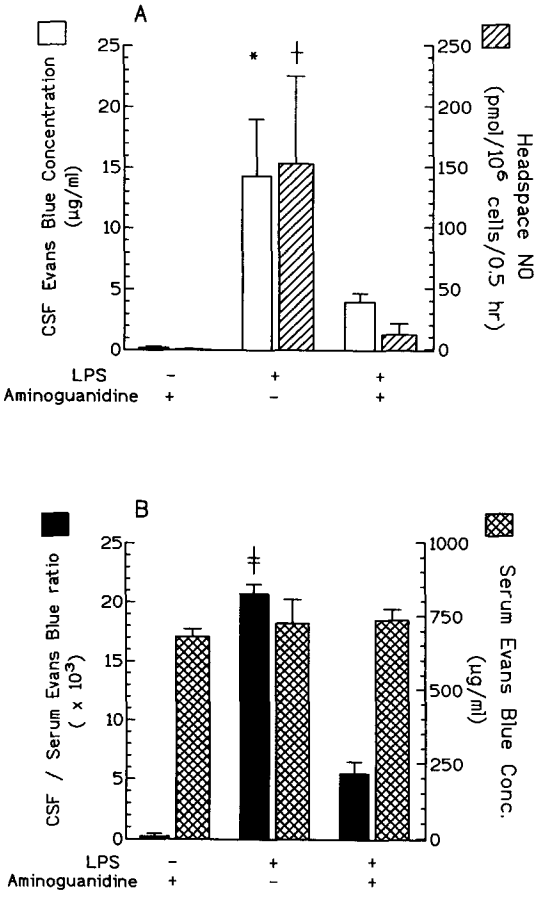

Fig. 1. (A) Cerebrospinal fluid Evans blue concentration (open bars) and meningeal NO production (diagonal bars) in rats treated with intracisternal lipopolysaccharides (0 or 25 μg) and intravenous saline or aminoguanidine. Results are the mean ± S.E.M. of six rats per treatment group. CSF Evans blue concentration: * $P < 0.05$ from the other two treatment groups by analysis of variance followed by Neuman-Keuls post-hoc analysis. Headspace NO: † $P < 0.05$ from the other two treatment groups by ANOVA followed by Neuman-Keuls post-hoc analysis. The lipopolysaccharides (+)/aminoguanidine (+) treatment group was not statistically different from the lipopolysaccharides (−)/aminoguanidine (−) control group for either NO production or CSF Evans blue concentration. (B) The ratio of cerebrospinal fluid/serum Evans blue concentration (filled bars) and serum Evans blue concentration (hashed bars) in rats treated with intracisternal lipopolysaccharides (0 or 25 μg) and intravenous saline or aminoguanidine. CSF/serum Evans blue ratio: ‡ $P < 0.05$ from the other two treatment groups by ANOVA followed by Neuman-Keuls post-hoc analysis.

the same trends observed for the untransformed CSF concentration data (Fig. 1B).

4. Discussion

The hypothesis that NO acts as an inflammatory mediator in experimental meningitis was supported by the analytical detection of NO produced by immunostimulated meninges obtained from lipopolysaccharide-treated rats. NO synthase inhibitors blocked the production of NO from lipopolysaccharide-immunostimulated meningeal preparations with a pharmacological rank order similar to that previously described for immunostimulated macrophages (Lambert et al., 1991). Although aminoguanidine was previously reported to inhibit immunoinduced NO synthase (Misko et al., 1993), no such effect was observed for either nitro- or methylguanidine. These results confirm that authentic NO is produced from the meningeal tissues of lipopolysaccharide-treated rats.

It was necessary to derive a rational dosing regimen for in vivo NO synthase inhibitor administration in order to effectively block meningeal NO synthesis for assessment of blood-CSF permeability changes. Aminoguanidine was the NO synthase inhibitor of choice for two important reasons: (1) limited literature information on the pharmacokinetics of aminoguanidine (Beaven et al., 1969) permitted the empirical design of a reasonable dosing regimen; and (2) aminoguanidine is relatively specific for the lipopolysaccharide-immunoinduced NO synthase (Misko et al., 1993). Aminoguanidine has relatively low potency and efficacy for the constitutive form of NO synthase, which contributes to the regulation of blood pressure (Misko et al., 1993). Sustained experimental hypertension is known to disrupt the blood-CSF and the blood-brain barriers presumably through a filtration mechanism (Rapoport, 1976). Additionally, one study reported that high infusion rates of N^G-nitroarginine methyl ester (a relatively specific inhibitor of constitutive NO synthase) produced significant increases in mean arterial blood pressure and blood-brain barrier disruption to proteins (Prado et al., 1992).

The aminoguanidine dosing regimen effectively blocked meningeal NO production in rats treated with intracisternal lipopolysaccharides. More importantly, a significant decrease in the blood-CSF barrier permeability to Evans blue dye was observed in those lipopolysaccharide-treated rats which received aminoguanidine. Complete inhibition of meningeal NO production did not fully prevent inflammatory opening of the blood-CSF barrier, as the Evans blue permeability in rats treated with lipopolysaccharides and aminoguanidine was ~ 70% less than that observed in the group treated with lipopolysaccharides and saline. The partial attenuation of blood-CSF barrier permeability changes by a NO synthase inhibitor suggests that NO may be an important mediator in the meningeal inflammatory process.

In conclusion, the findings of this study argue for a major, but not exclusive role for NO-mediated permeability changes in the blood-CSF barrier during experimental meningitis. Further studies are needed to better define the mechanisms by which NO disrupts the blood-CSF barrier. Additionally, these results suggest that NO synthase inhibitors may be *potentially* useful as an adjunct therapy in clinical meningitis, but additional rigorous animal studies are obligatory before clinical studies are initiated.

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