Nitric Oxide and Prostaglandin Response to Group B Streptococcal Infection in the Lung

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Abstract. Group B streptococcus (GBS) causes a complex inflammatory process that involves prostaglandins (PG) and nitric oxide (NO). The goal of this study was to examine the inflammatory response to GBS in the lung and the co-regulation of the PG and NO pathways, if any, both in vitro and in vivo. Sprague Dawley rats were treated with various combinations of GBS, aminoguanidine (AG), a selective inducible nitric oxide synthase (iNOS) inhibitor, and L-arginine (LA), a NO donor. The mRNA expression of the COX 2 gene was studied by reverse transcriptase polymerase chain reaction (RT-PCR) in rat lung tissue. The studies were confirmed in vitro using human lung epithelial (A549) cells treated with GBS, AG, and LA (in combinations similar to the rats) for 3 and 24 hr, after which PG E2 levels in the media were measured by enzyme linked immunosorbent assay (ELISA). COX 2 mRNA in rat lung tissue was significantly induced by GBS (p = 0.04), an effect that was suppressed by AG (p = 0.02). In the A549 cell line, PG E2 levels increased with GBS treatment at 3 and 24 hr (p <0.001). When AG was added, PG E2 levels were suppressed (p = 0.03) after 24 hr; LA partly reversed the suppression of PG E2 levels (p = 0.039). These data indicate that GBS infection causes significant COX 2 induction and PG E2 synthesis in lung tissue, regulated at least partly by the NO pathway. The interaction between the 2 pathways may play a pathogenic role in GBS lung infections and could be a potential target for pharmacological manipulation.

Keywords: Group B streptococcus, nitric oxide, prostaglandins, aminoguanidine, COX gene expression

Introduction

Group B streptococcal (GBS) infection remains a leading cause of neonatal sepsis and pneumonia [1]. Early onset GBS infection is associated with pneumonia in a majority of cases and is characterized by severe lung injury, the pathological hallmarks of which are pulmonary edema, alveolar hemorrhage, and hyaline membrane formation. A complex inflammatory process involving biochemical mediators such as TNFα, interleukins, prostaglandins (PG), and nitric oxide (NO), has been demonstrated[1-5]. The microvascular endothelium, alveolar epithelial cells, and pulmonary endothelium are the major sites of inflammatory response [5,6].

Cyclooxygenase (COX) catalyses the first committed step in the synthesis of prostaglandins (ie, conversion of arachidonic acid to PGH₂). The COX gene exists in 2 isoforms—COX 1 and COX 2 [7]. COX 1 is a constitutive isoform present in many cell types and COX 2 is an inducible form expressed in monocytes, endothelial cells, and fibroblasts in response to inflammation and infection.

Nitric oxide is a biological signaling and effector molecule that regulates other pro-inflammatory mediators. It is derived from the conversion of L-arginine to L-citrulline by nitric oxide synthase. The nitric oxide synthase gene

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(NOS) also exists in constitutive and inducible forms. The inducible isoform (iNOS) is regulated by cytokines and bacterial lipopolysaccharides. The constitutive isoform exists in vascular endothelium (eNOS), neurons (bNOS), and renal epithelial cells and is involved in basal physiological functions such as vasodilation and neurotransmission [7,8].

GBS infection has been shown to induce iNOS and COX 2 gene expression in cerebral endothelial cells and human monocytes, possibly through a common pleotropic transcription factor, NF-κB, or through the mitogen-activated protein kinase (MAPK) system [1,9].

The interactions of the NO pathway and COX 2 gene expression are complex and not entirely clear. An inflammatory response in many cell types causes up-regulation of COX 2, often along with an increase in NOS [10,11]. The regulation of each enzyme may be cell specific and may depend on the inducing agent. It is unclear which response occurs first, how the two are interrelated, and at what level — gene transcription, post-transcription, or modulation of enzyme activity [7,12-14].

The aim of the present study was to examine the inflammatory response to GBS in the lung and the co-regulation of the PG and NO pathways, if any, both in vivo in rat lung tissue and in vitro in human lung epithelial cells. Our hypothesis was that COX mRNA expression and PG E2 synthesis are both induced in GBS lung infection and that these responses are regulated, at least in part, by the nitric oxide pathway.

Materials and Methods

The Animal Investigation Committee of Wayne State University approved this study protocol; the investigations were performed in accordance with NIH guidelines for the use of animals in research.

Preparation of animal tissue. Lung tissue was obtained from 3-wk-old male Sprague Dawley rats weighing 100 to 125 g. They were randomly assigned to 1 of 5 treatment groups (4 to 6 rats/group): (1) controls (normal saline); (2) aminoguanidine (AG), a selective iNOS inhibitor; (3) GBS; (4) GBS + AG, and (5) GBS + AG + L-arginine (LA). The animals were given ip injections of 0.5 ml of sterile normal saline, aminoguanidine (100 mg/kg), 0.5 ml of dry heat inactivated group B streptococcus (10^9 CFU/ml) resuspended in 0.5 ml of saline, and/or L-arginine (300 mg/kg), according to the treatment group. The rats were killed at 18 hr post-treatment.

RNA extraction. Lung tissue was extracted at 18 hr post treatment (based on previous studies that showed that the peak time of expression of COX genes is 18 to 24 hr). Total cellular RNA was extracted from lung tissue samples that had been kept frozen at -70°C. The extraction procedure involved homogenization with TRIzol reagent (Gibco BRL/Life Tech, Carlsbad, CA) to inactivate RNAses and dissociate tissue. After washing and drying, RNA was resolved by denaturing formaldehyde agarose gel electrophoresis. The gels were stained with ethidium bromide to verify the quality of the RNA and to confirm equal loading of RNA samples.

Serum L-lactic acid levels were measured by a standard blood gas analyzer (using an enzyme sensor) in all rats as a marker of systemic illness.

COX 2 mRNA analysis by RTPCR. mRNA expression of COX 1, COX 2, and β-actin (as internal control) were studied by a semi-quantitative 2-step RTPCR analysis. cDNA was prepared by reverse transcription of 1 µg RNA at 25°C for 10 min, 42°C for 1 hr, and then denaturation at 99°C for 5 min. Amplification was done by PCR according to the manufacturer’s protocol (BioSource International, Inc, Camarillo, CA) with 20 mM TRIS, 50 mM KCl, 2 mM magnesium chloride, 0.2 mM of each dNTP, 0.1 mM of each sense and antisense primer, and 1 U Taq polymerase for 35 cycles, each involving a hot start at 95°C for 1 min, 55-60°C for 45 sec, 72°C for 1 min, and finally 72°C for 10 min. All primers were chosen to span an intron so that genomic DNA contamination would not interfere with the analysis. The amplified cDNA fragments were subjected to 6% acrylamide gel electrophoresis and the autoradiographic signals were quantitated by laser densitometry. Data were expressed relative to β-actin, which served as the internal control.

GBS treatment of A549 cells in vitro and Western blot assay of iNOS. Human lung epithelial cells (A549) were treated with saline and heat-inactivated GBS (10^9 CFU/ml) for 3, 6, 24, 48, or 72 hr. To confirm in vitro responsiveness to GBS, iNOS expression was analyzed by Western blot assay.

Measurement of nitrite/nitrate levels. Isocritic high-performance anion-exchange liquid chromatography (HPLC) was used to measure nitric oxide metabolites, nitrate (NO_3^-) and nitrite (NO_2^-), in media after GBS treatment for 24 hr. The accuracy of the HPLC method was established by the spiked recovery approach. Random samples were spiked with standards to verify retention times and recovery. Intra-assay validation was calculated by the mean ± SD of triplicate samples of 6 different spiked concentrations for both anions. The HPLC method has a sensitivity of 10 μmol/L. Nitrite/nitrate concentrations were expressed as mmol/mg protein.

Assay of prostaglandin E2 levels. A549 cells were treated with (1) saline (controls); (2) AG at 100 μg/ml medium; (3) LA at 1 μl/ml medium; (4) GBS; (5) GBS + AG; and (6) GBS+AG+LA for 3 and 24 hr and PGE_2 levels were assayed in the cell culture media using an enzyme linked immunosorbent assay kit (BioDesigns, Inc, Minneapolis, MN).
Fig. 1. A representative RT-PCR autoradiogram showing the induction of COX 2 mRNA in the lung tissues of GBS-treated rats (Group 3), compared to controls treated with saline (Group 1) and AG alone (Group 2), its suppression by co-treatment with AG and GBS (Group 4), and a partial reversal of the AG effect after addition of LA in GBS infected rats (Group 5).

Fig. 2. The effect of GBS treatment on COX 2 mRNA expression in lung tissue and its co-regulation by AG. Rats were treated as follows: group 1 (saline), group 2 (AG), group 3 (GBS), group 4 (GBS+AG), and group 5 (GBS+AG+LA). The graph represents changes in densitometric units (DU) compared to controls (mean ± SD) *p < 0.04, **p < 0.02.

Fig. 3. GBS induction of iNOS expression in lung epithelial cells (A549) analyzed by Western blotting. Cells were treated with saline (negative control, sample 1), and heat-inactivated GBS (10^9 cfu/ml) for 3, 6, 24, 48, or 72 hr (samples 2-6 respectively). Sample 7 represents a positive control for the Western blot assay.
Statistical analyses. Data were expressed as mean ± SD. In cell culture the means were derived from 6 experiments. Comparison of means from multiple groups was done by ANOVA followed by t test; statistical significance was defined as p <0.05. The gel shown in Fig. 1 is representative of 4 separate experiments.

Results

Inflammatory response to GBS infection in rat lung. Serum lactic acid levels were measured in all rats to confirm that the GBS infection induced a systemic inflammatory response. In our study (as reported earlier), the serum lactic acid level in GBS-treated rats was 3.9 ± 0.7 mg/dl, compared to levels of 1.4 ± 0.2 mg/dl in the control group (p <0.04) [5]. COX 1 and β-actin gene expression, as measured by mRNA, were unaltered by GBS treatment. COX 2 mRNA was induced by GBS treatment (mean = 29,131 densitometric units in the GBS treated group compared to 16,774 in the controls, p = 0.04) (Fig. 2).

GBS inflammation in vitro in human lung epithelial cells (A549). GBS induced the maximum expression of iNOS protein at 24 and 48 hr, as measured by Western blot assay (Fig. 3). GBS treatment also caused increased levels of nitrite/nitrate in the medium at 24 hr, compared to controls (50 vs 17 nmol/mg; p = 0.02). GBS treatment increased prostaglandin levels compared to controls, AG alone, and LA alone at both 3 and 24 hr (p <0.001) (Table 1).

Nitric oxide and prostaglandin co-regulation in GBS infection in rat lung. Aminoguanidine is an iNOS specific inhibitor and acts as a competitive inhibitor by binding to heme iron at the catalytic site of the enzyme nitric oxide synthase. By adding aminoguanidine, we evaluated the effects of iNOS suppression on the inflammatory response induced by GBS. To confirm that the effects of aminoguanidine on COX gene expression are indeed due to the involvement of nitric oxide, L-arginine, an exogenous NO donor, was added in order to reverse the effects of aminoguanidine. The increase in serum lactic acid levels in response to GBS infection was partly prevented by pretreatment with aminoguanidine (2.6 ± 0.2 mg/dl compared to 3.9 ± 0.7 mg/dl). The induction of COX 2 mRNA expression by GBS treatment, as measured by RT PCR, was suppressed when aminoguanidine was added (29,131 DU in the GBS group vs 13,377 DU in the GBS+AG group, p = 0.02) (Fig. 2). COX 2 mRNA in the GBS + aminoguanidine group was comparable to the control group (13,377 DU vs 16,774 DU in controls, p = 0.78). The suppression of COX 2 caused by aminoguanidine treatment was partly reversed by L-arginine (19,455 DU in the GBS+AG+LA group compared to 13,377 DU in the GBS+AG group), but the effect did not reach statistical significance. By itself, the iNOS inhibitor, aminoguanidine, did not have any significant effect on COX gene expression.

Table 1. Measurements by ELISA of PG E2 levels (mean ± SD, pg/ml) in the cell culture media of human lung epithelial cells (A549) at 3 and 24 hr after initiation of the specified treatments.

<table>
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<tr>
<th>Treatment interval (hr)</th>
<th>Prostaglandin E2 levels in culture media (mean ± SD, pg/ml)</th>
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<tr>
<td></td>
<td>Controls</td>
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<tr>
<td>2</td>
<td>175.1 ± 73.2</td>
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<td>24</td>
<td>518 ± 343</td>
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† At 24 hr after initiation of treatment, PG E2 levels in the GBS and GBS+AG+LA groups were >1274 pg/ml, which is the upper limit of the ELISA assay; these data were taken as 1274 pg/ml for statistical analysis.
* p <0.001,
** p = 0.03,
# p = 0.039.
NO and PG co-regulation in vitro. The GBS-treated human lung epithelial (A549) cells had significantly higher levels of PG E2 in the cell culture medium compared to the control, AG-treated, and LA-treated cells at 3 and 24 hr. Treatment with AG and GBS significantly suppressed the GBS effect at 24 hr (p = 0.03) but not at 3 hr. Again, co-treatment with LA, GBS, and AG significantly increased the PG levels at 24 hr, compared to the GBS and AG group (p = 0.039) (Table 1).

Discussion

In the present study, ip treatment of rats with GBS induced a systemic inflammatory response, reflected by elevated serum lactic acid levels. The pulmonary inflammatory response was reflected by a significant induction of COX 2 mRNA expression in rat lung tissue. In vitro, in the human lung epithelial cell line (A549), there was significant induction of iNOS protein at 24 and 48 hr, increase in nitrite/nitrate levels at 24 hr, and PG E2 levels at 3 and 24 hr in the cell culture medium.

We previously reported the effect of GBS infection on serum lactic acid in rats and speculated that the mechanism is inadequate tissue perfusion and secondary lactic acidosis [5]. Other investigators have also used lactic acidosis as an objective marker of rat sepsis [15]. GBS inflammation has been previously studied in certain tissues. For example, GBS meningitis in the piglet model induces iNOS expression in the cerebral microvascular endothelial cells [1]. In vitro, human monocytes reveal significant induction of COX 2 mRNA expression and protein synthesis in a time- and concentration-dependent manner [9]. The putative mechanism for up-regulation of the inflammatory genes seems to be the activation of NF-kB or the protein kinase system [1,16-18].

We studied the GBS inflammatory response and the interaction of the PG and NO pathways in the lung because it is a common site of symptomatic GBS infection in neonates and serves as the portal of entry into the bloodstream and subsequent sepsis [4]. Our study was conducted in a rat model of systemic GBS infection. In an attempt to localize the cellular origin of the response and to validate our results in vitro, we selected the lung epithelial cell line. These cells are the site of surfactant production, the deficiency of which is established in GBS infection. The A549 cell line is readily available and the iNOS mRNA expression, NO secretion in response to inflammatory mediators, as well as release of IL-8 have been demonstrated by other investigators in response to GBS in this cell line [4,19]. In fact, GBS β-hemolysin expression correlates with A549 lung epithelial cell injury [20]. We therefore decided to perform similar experiments in the rat lung and the A549 cell line.

Data on the in vivo inflammatory response to GBS infection in the lung are sparse. A previous study from our group showed that in the rat lung, there was 6-fold induction of IL-6 and 3-fold induction of MIP-2 compared to controls [5]. The results of the present study show an induction of COX 2 mRNA that is similar to lung injury in other conditions. For example, in a rat model of meconium aspiration, up-regulation of COX 2 and NOS 2 were demonstrated [18,19]. Our in vitro experiments in the lung epithelial cell line suggest that the site of induction of iNOS and prostaglandin production in response to GBS lung infection may be the alveolar epithelial cell.

In rat lung tissue, COX 2 mRNA induction was dependent on the nitric oxide pathway. The co-regulation of the 2 pathways was again apparent in the lung epithelial cells; however, other cell types such as macrophages and endothelial cells were not tested and could also be involved. Previous studies on the co-regulation of nitric oxide and prostaglandin pathways in different tissues have yielded contradictory results. In vitro, NO stimulates COX activity in fibroblasts and COX induction in a LPS-activated mouse macrophage cell line (RAW 264.7) [21]. NO is known to enhance PG synthesis and/or COX 2 expression in human and bovine articular cartilage, human microvascular endothelial cells, rat renal cortical cells in response to volume depletion, and in vitro in retinal cells exposed to elevated glucose and in the retinas of diabetic animals [22-25]. However, an opposite effect of suppression of COX activity by NO has been demonstrated in rat hepatic macrophages, macula densa cells in mice, and lipopolysaccharide treated bovine pulmonary artery endothelial cells.
In certain other conditions, such as *Helicobacter pylori* gastritis, the COX 2 expression in gastric epithelium does not appear to be influenced by the iNOS pathway [30].

The present study has some limitations. We did not localize the cell of COX 2 mRNA expression or co-regulation with NO directly in the rat lung tissue. We did not examine the molecular mechanism of the interaction of the two pathways. In our rat model, GBS infection was acquired by the hematogenous route. We have previously reported on the systemic and intense pulmonary inflammation induced by this route; however, this differs from the inhalation route common to most neonatal GBS infections [4]. The animals were sacrificed at 18 hr post-treatment to extract lung tissue, based on previous studies that showed the peak time of expression of COX mRNA to be 18 to 24 hr. Therefore, it was not possible to assess the clinical effects of GBS infection or iNOS suppression on the rats. Also, a previous study demonstrated a biphasic increase in NO on direct continuous measurement of diffusible NO in the pulmonary artery of Wistar-Kyoto rats during lipopolysaccharide-induced endotoxemia [31]. The initial increase at about 9 min was associated with constitutive eNOS activation and the second phase at 45 min was secondary to iNOS activation. The later sampling times in our experiments may have missed an early NO response phase during GBS infection.

We speculate that the interaction between the PG and NO pathways may have a critical pathogenic role in GBS infection and might be a target for pharmacological manipulation in the future. However, the issue of NO inhibition in the lung may be particularly complex because of its critical role in the regulation of pulmonary vascular resistance in neonates. To evaluate the efficacy of AG in a clinically meaningful way, further studies using AG after GBS exposure are warranted.

In summary, our results show that GBS infection in the lung causes an induction of COX 2 mRNA in rat lung and an increase in PG E2 levels in human lung epithelial cell culture media. There appears to be co-regulation between the PG and NO pathways as demonstrated by the suppression of COX 2 mRNA expression by aminoguanidine in rat lung tissue and also of PG levels in cell culture media. We believe these data are important in understanding the mechanisms of GBS and indeed, other infections. Elucidating these complex interactions may allow more rational pharmacologic approaches to the treatment and prevention of the diseases in which they play a role.

**References**


