Selective inhibition of COX-2 improves early survival in murine endotoxemia but not in bacterial peritonitis

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Reddy, Raju C., Gina H. Chen, Kazuhiro Tateda, Wan C. Tsai, Susan M. Phare, Peter Mancuso, Marc Peters-Golden, and Theodore J. Standiford. Selective inhibition of COX-2 improves early survival in murine endotoxemia but not in bacterial peritonitis. Am J Physiol Lung Cell Mol Physiol 281: L537–L543, 2001.—Prostaglandins of the E series are believed to act as important mediators of several pathophysiological events that occur in sepsis. Studies were performed to evaluate the effect of cyclooxygenase (COX)-2-specific inhibition on the outcome in murine endotoxemia and cecal ligation and puncture (CLP). We observed a significant time-dependent upregulation of PGE2 production in both blood and lung homogenates of mice administered lipopolysaccharide intraperitoneally, which was nearly completely suppressed by the administration of the COX-2 inhibitor NS-398. Treatment with NS-398 significantly improved early but not late survival in lipopolysaccharide-challenged mice. On the contrary, elevated PGE2 levels were found in bronchoalveolar lavage fluid but not in plasma of mice subjected to CLP (21 gauge). Pretreatment with NS-398 failed to significantly improve survival in CLP mice. No significant differences were noted in plasma or lung homogenate proinflammatory cytokine levels or lung neutrophil sequestration between the NS-398-treated and control groups. These results demonstrate that selective COX-2 inhibition confers early but not long-term benefits without affecting the expression of proinflammatory cytokines or the development of lung inflammation.

prostaglandin E2; cyclooxygenase-2; cecal ligation and puncture; NS-398; sepsis

SEPSIS IS A COMPLEX SYSTEMIC ILLNESS that is manifest by varying degrees of hypotension, coagulopathy, and multiorgan dysfunction (7). Despite advances in supportive care, the mortality rate in patients with severe sepsis continues to exceed 30%. The sepsis syndrome is associated with the unabated release of inflammatory mediators, including cytokines, chemokines, and eicosanoids, which often results in detrimental effects to the host (7, 14). Previous animal and human studies (2, 15) have demonstrated elevated levels of prostanooids in both experimental and clinical sepsis syndrome. PGE2 is one of the most potent and inducible of the prostanooids produced in states of inflammation. PGE2, produced by the metabolism of arachidonic acid by the enzyme cyclooxygenase (COX), is believed to be an important modulator of several of the observed events in sepsis. Specifically, there is evidence to support roles for PGE2 as a mediator of sepsis-induced immunosuppression, an inhibitor of proinflammatory cytokine expression from monocytes, and an inducer of interleukin (IL)-10 production (24, 25). Conversely, PGE2 has been shown to mediate detrimental effects in sepsis, including vasodilation and increased vascular permeability (19). In addition, its role as a mediator in fever induction and augmentation of pain is well established (16). Several reports (1, 5, 11, 13, 27) utilizing endotoxin-challenged animal studies have shown beneficial effects with nonselective COX inhibitors. These beneficial effects were felt to be mediated, in part, by the mitigation of the pathophysiological events in sepsis induced by prostaglandins.

COX exists as two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed, whereas COX-2 is expressed at low levels in most normal resting cells. Marked upregulation of COX-2 occurs in synovioocytes, macrophages, and endothelial cells during stress and in inflammatory conditions such as sepsis. COX-2 expression is induced by a number of cytokines including tumor necrosis factor (TNF)-α and IL-1, mitogens or growth factors, lipopolysaccharide (LPS), and other inflammatory stimuli (9). Recent studies (16, 17, 22) have provided evidence pointing to significant advantages in the use of selective COX-2 inhibitors over their nonselective counterparts. The specific benefits of COX-2 inhibitors include decreased gastrointestinal toxicity and bleeding (17, 23).

The purpose of this study was to determine the role of COX-2 in the pathogenesis of sepsis by employing two murine models of sepsis: intraperitoneal LPS administration and cecal ligation and puncture (CLP). The effects of selective COX-2 inhibition on prostaglan-
Materials and Methods

Reagents. NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methyleneisulfonamide; Cayman Chemical, Ann Arbor, MI) was prepared by dissolving the compound in DMSO. CD-1 mice were administered NS-398 intraperitoneally in 40 μl of DMSO at a dose of 15 mg/kg at designated time intervals. LPS (Escherichia coli type 0111:B4, Sigma) was diluted in sterile normal saline and sonicated before each administration. Polyclonal anti-murine TNF, IL-10, IL-12, KC, and macrophage inflammatory protein (MIP)-2 antibodies used in the ELISAs were produced by immunization of rabbits with murine recombinant cytokines with complete Freund’s adjuvant at multiple intradermal sites. Carrier-free murine recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). Purified antibodies for ELISA were obtained by purification over an endotoxin-free protein A column.

Animals. Specific pathogen-free CD-1 mice (6- to 12-wk females; Charles River Breeding Laboratories) were used in all experiments. CD-1 mice were chosen because the CLP and endotoxinemia models have been well characterized in this outbred strain. All mice were housed in specific pathogen-free conditions within the animal care facility at the University of Michigan (Ann Arbor, MI) until the day of death.

Animal model of abdominal sepsis. CLP with either a 21- or 25-gauge needle was used as model of systemic sepsis syndrome as previously described (26). In distinct contrast to CLP models with larger-gauge cecal punctures (19 gauge and larger), in which most animals rapidly develop bacteremia due to enteric organisms and death occurs as a result of polymicrobial sepsis, CLP with a 21- or 25-gauge needle results in the development of bacteremia in a minority of the animals (11). However, this insult induces a marked septic response, with death occurring in 20–30% of animals with a 25-gauge needle and 40–60% mortality with a 21-gauge needle compared with a mortality rate of >90% in mice undergoing 18-gauge CLP (26). To perform this procedure, pathogen-free female CD-1 mice were anesthetized with pentobarbital sodium (50 μg/kg ip; Butler, Columbus, OH) followed by inhaled methoxyflurane (Metafane, Pitman-Moore, Mundelein, IL) as needed. In these mice, a 1- to 2-cm longitudinal incision to the lower right quadrant of the abdomen was performed, and the cecum was exposed. The distal one-third of the cecum was ligated with a 3-0 silk suture and punctured through and through with either a 21- or 25-gauge needle. A small amount of the bowel contents was then extruded through the puncture site. The cecum was replaced in the peritoneal cavity, and the incision was closed with surgical staples. In sham control animals, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. All mice were administered 1 ml of sterile saline subcutaneously for fluid resuscitation during the postoperative period.

PGE2 and thromboxane B2 extraction and analysis. PGE2 and thromboxane (TX) B2 were extracted from bronchoalveolar lavage (BAL) fluid, whole lung homogenates, and plasma with C18 Sep-Pak cartridges (Waters Associates, Milford, MA) as previously described (18). The extracts were evaporated to dryness under nitrogen and stored at −80°C. Before analysis, the extracts were resuspended in cell culture medium and assayed for PGE2 and TXB2 with an enzyme immunoassay kit (Cayman Chemical).

Core body temperature determination. Rectal temperature (as a measure of core body temperature) was determined at baseline and at predetermined time points post-LPS administration with a model 49 TA digital thermometer equipped with a YSI series 400 probe (2-mm diameter; Yellow Springs Instruments, Yellow Springs, OH). Before temperature measurement, the probe was coated with Surgilube lubricant (Division of Atlanta, Melville, NY) and inserted ~1.5–2 cm into the rectum.

Murine cytokine ELISAs. Murine TNF, IL-10, IL-12, KC, and MIP-2 were quantitated with a modification of a double-ligand method as previously described (26). Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with 50 μl/well of rabbit antibody against the various cytokines (1 μg/ml in 0.6 M NaCl, 0.26 M H3BO4, and 0.08 M NaOH, pH 9.6) for 16 h at 4°C and then washed with PBS, pH 7.5, and 0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. The plates were washed four times with wash buffer, 1:10 diluted (neat and 1:10) cell-free supernatants (50 μl) were added in duplicate followed by incubation for 1 h at 37°C. The plates were washed four times followed by the addition of 50 μl/well of biotinylated rabbit antibodies against the specific cytokines (3.5 μg/ml in PBS, pH 7.5, 0.05% Tween 20, and 2% FCS), and the plates were incubated for 30 min at 37°C. The plates were washed again four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and the plates were incubated for 30 min at 37°C. The plates were washed again four times, and chromogen substrate (Bio-Rad Laboratories) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl/well of a 3 M H2SO4 solution. The plates were read at 490 nm in an ELISA reader. The standards were 1:2 log dilutions of recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine cytokine concentrations >25 pg/ml. The ELISAs did not cross-react with IL-1, IL-2, IL-4, or IL-6. In addition, the ELISAs did not cross-react with other members of the murine chemokine family, including murine JE/monocyte chemoattractant protein-1, regulated on activation normal T cell expressed and secreted, growth-related gene-a, or epithelial cell-derived neutrophil-activating protein-78.

Lung myeloperoxidase assay. Lung myeloperoxidase (MPO) activity (as an assessment of neutrophil influx) was quantitated by a method previously described (21). Briefly, whole lungs were homogenized in 2 ml of a solution containing 50 mM potassium phosphate, pH 6.0, 5% hexadecyltrimethylammonium bromide, and 5 mM EDTA. One hundred microliters of the resulting homogenate were sonicated and centrifuged at 12,000 rpm for 15 min. The supernatant was mixed 1:15 with assay buffer and read at 490 nm. MPO units were calculated as the change in absorbance over time.

Statistical analysis. Data were analyzed with the Prism 3.0 statistical program (GraphPad Software, San Diego, CA). Survival data were compared with Fisher’s exact test. All data are expressed as means ± SE. Comparisons between two experimental groups of data were performed with Student’s unpaired t-test. Comparisons among three or more experimental groups were performed with ANOVA followed by Dunnett’s test. Data were considered significant if P values were <0.05.

Results

Production of PGE2 in murine endotoxemia. Experiments were first performed to assess the time-dependent production of PGE2 in murine endotoxemia. CD-1
mice were challenged with 250 μg of LPS intraperitoneally, and then the PGE₂ levels were determined in plasma and lungs at multiple time points after LPS administration. As shown in Figs. 1 and 2, LPS administration resulted in a rapid increase in PGE₂ levels, peaking at 6 h in plasma (Fig. 1) and lung homogenates (Fig. 2) and returning to baseline levels by 12 h. Similarly, LPS administration resulted in the induction of TXB₂ (the stable metabolite of TXA₂) in lung homogenates, which was maximal 6 h post-LPS challenge (26,800 ± 4,812 pg/ml in LPS-treated animals compared with 4,678 ± 501 pg/ml in vehicle-treated animals; P < 0.01).

**Effect of NS-398 on PGE₂ production after LPS administration.** After demonstrating elevated levels of PGE₂ in plasma and lung homogenates after LPS administration, CD-1 mice were administered NS-398 intraperitoneally at varying doses, and PGE₂ levels were assessed in these same compartments. NS-398 at a dose of 15 mg/kg administered 2 h before LPS administration, 4 h later, and every 12 h thereafter completely suppressed production of PGE₂ in plasma (Fig. 1) and lungs (Fig. 2) to baseline levels. The suppressive effects of NS-398 were maintained at a dose of 10 mg/kg (data not shown). In addition, we observed equivalent suppression of PGE₂ whether NS-398 was administered at the same time as or 2 h before LPS administration (data not shown). Furthermore, treatment with NS-398 resulted in a 45% reduction in maximal TXB₂ levels in lung homogenates after LPS administration (data not shown).

COX-2 inhibition significantly reduces early but not late endotoxin-induced mortality. Experiments were performed to determine the effects of COX-2 inhibition on the mortality in mice administered LPS. For mortality studies, a dose of 700 μg LPS/animal was used, a dose that represented an ~80% lethal dose in control animals. As shown in Fig. 3, pretreatment of animals with NS-398 2 h before LPS administration, 4 h later, and every 12 h thereafter resulted in 95% survival at 24 h, whereas only 65% survival was observed in animals administered vehicle alone (P < 0.05). Survival after 24 h, however, was not significantly different between the two groups, although a trend toward improved survival was noted in the NS-398-treated animals. No effect of vehicle (DMSO) was observed because survival in animals challenged with LPS alone was identical to that observed in mice administered vehicle plus LPS (data not shown).

**Effect of COX-2 inhibition on gross motor activity and temperature regulation.** We next assessed the effect of NS-398 administration on gross motor activity and rectal temperature in LPS-treated mice. CD-1 mice were administered either vehicle or NS-398 15 mg/kg 2 h before LPS administration, 4 h later, and every 12 h thereafter. The mice administered vehicle were more lethargic and displayed decreased oral in-
take and substantially more piloerection compared with NS-398-treated mice. As shown in Fig. 4, mice receiving vehicle alone had significant elevations in body temperature 1 and 2 h post-LPS administration followed by a drop in core temperature thereafter. In contrast, no early increase in core temperature was observed in mice treated with NS-398 before LPS administration, although the decrease in temperature by 6 h was similar to that observed in control animals.

**PGE2 inhibition does not alter cytokine production after LPS administration.** Inflammatory cytokines and chemokines have previously been shown to modulate many of the observed events in endotoxemia (20, 28). Moreover, PGE2 is known to suppress the inflammatory cytokines IL-12, TNF-α, and IL-1 and several of the chemokines in vitro. We performed experiments examining the role of COX-2 inhibition on the expression of proinflammatory cytokines during endotoxia. CD-1 mice were pretreated with NS-398 (15 mg/kg) or vehicle 2 h before and 4 h after the intraperitoneal administration of LPS (500 μg), and then plasma was collected at designated time points post-LPS. As shown in Fig. 5, endotoxin challenge resulted in peak increases in plasma TNF-α levels in control animals at 2 h, with levels decreasing to baseline by 6 h after LPS. Likewise, endotoxin challenge resulted in peak increases in plasma levels of the C-X-C chemokine KC in both groups between 4 and 6 h after LPS. Importantly, there were no significant differences in TNF-α and KC between NS-398-treated mice and control mice at any time point. In addition, there was no significant change in the levels of other relevant cytokines, including IL-12 and IL-10, and the C-X-C chemokine MIP-2 (data not shown).

**Pretreatment with NS-398 does not alter lung polymorphonuclear neutrophil sequestration after LPS administration.** Experiments were next performed to assess the effects of COX-2 inhibition on lung polymorphonuclear neutrophil sequestration after LPS challenge. Mice were treated with either vehicle or NS-398 2 h before endotoxin challenge, and then lung MPO activity (as a measure of neutrophil sequestration) was determined 2 and 6 h after LPS. As shown in Fig. 6, challenge with LPS (500 μg) resulted in a significant increase in lung MPO activity 2 and 6 h after LPS compared with that in saline-challenged animals. Treatment with NS-398 did not alter the LPS-induced increase in lung MPO activity at either time point after LPS, indicating that the early protective effects of COX-2 inhibition in endotoxemia were not a result of attenuation of lung polymorphonuclear neutrophil sequestration.

**Production of PGE2 in bacterial peritonitis.** Experiments were next performed to define the production of PGE2 in a more clinically relevant model of sepsis, namely the CLP model. CD-1 mice underwent 21-gauge CLP, and PGE2 levels were then determined in plasma and lungs at multiple time points after CLP. CLP resulted in no significant change in PGE2 in plasma (Fig. 7) or lung homogenates (data not shown). However, a small but significant increase in PGE2 in
Inhibition of COX-2 does not improve survival in CLP. Our initial studies provided evidence that COX-2 inhibition in animals challenged with LPS resulted in early survival benefits. Additional studies were performed to examine the effects of NS-398 administration on the survival of animals with abdominal sepsis. In these studies, CD-1 mice were subjected to either 21- or 25-gauge CLP. Mice were pretreated with NS-398 (15 mg/kg) 2 h before CLP, 4 h after CLP, and every 12 h thereafter. As shown in Fig. 8, COX-2 inhibition resulted in a trend toward decreased mortality in 21-gauge CLP compared with animals receiving vehicle (P = 0.2). In animals undergoing 25-gauge CLP, mortality rates between the two groups were nearly identical. Additionally, treatment of 21-gauge CLP mice with NS-398 had no effect on peripheral blood leukocyte counts, the number of peritoneal fluid leukocytes, and peritoneal fluid bacterial colony-forming units compared with those in CLP animals receiving vehicle (data not shown).

DISCUSSION

A number of studies have examined the role of non-selective COX inhibitors both in animal models of sepsis and in patients with and sepsis syndrome. Several
Existing evidence supports both anti- and pro-inflammatory effects of NS-398 on the generation of pulmonary inflammation in endotoxin-treated mice or peritoneal inflammation in CLP mice. Peritoneal inflammation was assessed by quantitating total peritoneal leukocyte cell numbers and specific cell types 12 and 24 h post-CLP and were found not to be significantly different between the two groups (data not shown). The lack of effects on inflammatory cell influx was not due to incomplete inhibition of COX-2 activity because we observed a rather profound inhibition of PGE2 in NS-398-treated mice at all time points examined. These observations validate the use of NS-398 as an inhibitor of inducible PGE2 production. We also found that selective COX-2 inhibitors result in decreases in TXB2 production in endotoxin-challenged animals.

Cytokine profiles from murine models of sepsis, LPS administration and CLP, have been well characterized previously (20). The effects of selective COX-2 inhibition on cytokine production during experimental sepsis syndrome has not been examined previously. It would be predicted based on previous predominantly in vitro studies (24, 25) that inhibition of PGE2 would result in increased production of various proinflammatory cytokines such as TNF-α and IL-12. Contrary to these observations, our results are similar to a previous report (8) that failed to observe a significant change in systemic cytokine and chemokine levels during nonsteroidal anti-inflammatory drug administration in LPS-induced sepsis.

Treatment of mice with NS-398 resulted in early benefits in murine endotoxemia but not in CLP. There are several possible explanations for the disparity in the effects observed. First, we detected substantial induction of PGE2 in endotoxin-challenged mice but rather limited production of PGE2 in animals undergoing CLP. This is likely attributable to differences in the magnitude of systemic exposure to endotoxin and inflammatory cytokines, which is much more pronounced in animals challenged with bolus LPS than the more delayed and limited release in animals undergoing CLP. Also, the cause of mortality in CLP is multifactorial, and the degree to which bacteria are contained within the abdominal cavity partially dictates survival in these animals. In contrast to the findings made in a rat burn infection model, we did not observe any differences in bacterial clearance as assessed by culturing peritoneal fluid 12 and 24 h post-CLP and determining bacterial colony-forming units from the peritoneal cavity in NS-398-treated mice undergoing CLP compared with those in control animals. Likewise, we found no alteration in the clearance of Klebsiella pneumoniae from the lungs of NS-398-treated mice using a murine gram-negative pneumonia model (data not shown). Thus, as opposed to regulation of the 5-lipoxygenase pathway, our data suggest that regulation of the COX-2 pathway has little impact on localized antibacterial host responses (4).

In summary, selective inhibition of COX-2 results in improvement in early survival in murine endotoxemia but not in a more physiologically relevant model of abdominal sepsis (CLP). The early improvement in survival in endotoxin-challenged animals was not at-
tributable to changes in inflammatory cytokine expression or organ-specific neutrophil sequestration. Selective inhibition of COX-2 in sepsis requires further study. However, the findings reported here are disappointing, particularly given the lack of benefit observed in animals with abdominal sepsis.

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REFERENCES