Application of prostaglandin E₂ improves ileal blood flow in NEC☆

Sarah K. Walker b, Paul J. Matheson a,b, Laura A. Galganski b, R. Neal Garrison a,b, Cynthia D. Downard c,*

a Robley Rex Veterans Affairs Medical Center at Louisville, KY, Louisville, KY
b Hiram C. Polk, Jr., M.D. Department of Surgery, University of Louisville, Louisville, KY
c Division of Pediatric Surgery, Hiram C. Polk, Jr., M.D. Department of Surgery, University of Louisville, Louisville, KY

ARTICLE INFO

Article history:
Received 21 January 2014
Accepted 27 January 2014

Key words:
Necrotizing enterocolitis
Prostaglandins E₂
Ileal blood flow regulation
Indomethacin

ABSTRACT

Purpose: Indomethacin, a nonselective prostaglandin inhibitor used to treat patent ductus arteriosus, is associated with intestinal perfusion inducing an NEC-like illness. We sought to define the contribution of prostaglandin E₂ (PG E₂) and its receptor EP4 to intestinal blood flow regulation in premature neonates with NEC.

Methods: Newborn Sprague-Dawley rats were randomized by litter to undergo experimental NEC induction or to serve as a CONTROL. At 48 hours of age, intestinal laser Doppler blood flow was assessed at baseline and after intraperitoneal administration of indomethacin, PG E₂, EP4 antagonist, or EP4 agonist. Data were analyzed using a 2-way ANOVA with post hoc Tukey-Kramer correction.

Results: At baseline, NEC animals had lower intestinal blood flow than controls. Indomethacin, PG E₂ and EP4 agonist all increased ileal blood flow, but PG E₂ and EP4 agonist increased blood flow the most in NEC pups. EP4 antagonist decreased intestinal perfusion in both groups.

Conclusion: The above evidence suggests the importance of PG E₂ and EP4 in regulation of neonatal intestinal blood flow. Since indomethacin treatment of patent ductus arteriosus in the premature infant is associated with an increased risk of intestinal perforation owing to compromised blood flow, PG E₂ supplementation might provide intestinal protection if administered simultaneously with indomethacin.

© 2014 Elsevier Inc. All rights reserved.

Necrotizing enterocolitis (NEC) is a disease of the premature intestine which is multifactorial. The pathogenesis of NEC includes decreased intestinal mucosal blood flow and subsequent tissue injury. Infants with patent ductus arteriosus (PDA) who undergo medical treatment for closure with nonsteroidal antiinflammatory (NSAID) medications such as indomethacin are at increased risk for intestinal perforation and an NEC-like illness [1].

Nonselective NSAIDs exert their effect by inhibiting both cyclooxygenase (COX) enzyme subtypes, prohibiting both vasodilatory and vasoconstricting arachidonic acid metabolite production. This imbalance presumably leads to alterations in blood flow which can decrease intestinal perfusion, which is primarily mediated by the vasodilatory prostaglandin E₂ (PG E₂) through action at its receptor EP4. This interaction is at least partly responsible for intestinal homeostasis and mucosal maintenance [2].

We hypothesized that topical application to the intestine of nonselective prostaglandin inhibitors such as indomethacin would decrease intestinal blood flow, and that topical PG E₂ application would increase intestinal blood flow. We also hypothesized that blockade of EP4, the receptor for PG E₂, would cause decreased intestinal blood flow, and that application of an EP4 agonist would cause increased intestinal blood flow. As intestinal vasoconstriction is thought to be a critical step in the pathogenesis of NEC, further elucidation of the pathway which affects intestinal blood flow, particularly in relation to common clinical scenarios such as medical treatment of PDA, will offer better direction toward control of vascular mechanisms and novel treatments in NEC [3].

1. Materials and methods

These studies were approved by the Institutional Animal Care and Use Committee, the Biohazard Safety Committee, and the Research and Development Committee of the Robley Rex Veterans Affairs Medical Center in Louisville, KY. Ten timed-pregnant Sprague-Dawley dams (Harlan, Indianapolis, IN) were housed in an AAALAC-approved Veterinary Medical Unit for at least 7 days prior to study. The dams were acclimated to a 12 hour light-dark cycle and fed standard rat chow and water ad libitum. The dams were then randomized to the CONTROL or experimental NEC protocol and their pups were assigned to groups by litter.

The CONTROL groups were vaginally delivered and kept with the dam until the day of the experiments. CONTROL animals were fed on demand by the dam and their hour of birth was recorded so that the pups could be time matched to the protocol of the NEC groups. The...
NEC groups were delivered by cesarean section 12 hours prematurely and then entered into the experimental NEC protocol. On the day of the cesarean section, the dam was weighed and anesthetized with carbon dioxide, the pups were delivered quickly after the dam underwent decerebration, and the pups were immediately revived and warmed in an incubator at 37.0 °C and the humidity recorded every 4 hours. The NEC groups were gavage fed via orogastric tube every 4–5 hours using a silastic catheter. NEC pups underwent intermittent episodes of hypoxia (100% nitrogen gas for 60 seconds) followed by hypothermia (4 °C for 10 minutes), and they were also given a single oral dose of lipopolysaccharide (2 mg/kg, Sigma-Aldrich, St. Louis, MO) at 10 hours of life. The NEC animals were fed a formula that contained Similac 60/40 (Ross Pediatrics, Columbus, OH) added to Esbilac (Pet-Ag, New Hampshire, IL) that was calculated to deliver 836.8 kJ/kg per day.

At 48 hours of life, all animals were weighed and then anesthetized with isoflurane (induction 3.5% and maintenance 1.0% in 2 L oxygen per minute). Body temperature was maintained at 37.0 ± 0.5 °C by heating pad and feedback controller. A laparotomy was performed for laser Doppler flowmetry. A Periflux laser Doppler flowmeter system (Perimed AB, Järfälla, Sweden) with a 7-site integrating flow probe was used to evaluate ileal perfusion. After laparotomy, warm sterile saline irrigation solution was placed in the peritoneum and the animals equilibrated for 20–30 minutes. Gross appearance of the intestines was recorded for signs of necrosis, hemorrhage, gaseous distension, or perforation. Any gross blood or stool that was present was flushed from the peritoneum with warm saline. At the completion of the equilibration period, baseline blood flow was recorded over 20 minutes and if stable (i.e., variation in flow <10%), the animal was entered into the laser Doppler flow protocol.

The CONTROL litters were randomized into the following groups: 1) CONTROL + indomethacin (n = 12); 2) CONTROL + PG E2 (n = 12); 3) CONTROL + EP4 receptor agonist (n = 10); 4) CONTROL + EP4 receptor antagonist (N = 10); or 5) CONTROL + PG E2 + EP4 receptor antagonist (n = 12). For the NEC group litters, they were randomized similarly on the day of the experiments: 6) NEC + indomethacin (n = 15); 7) NEC + PG E2 (n = 12); 8) NEC + EP4 receptor agonist (n = 12); 9) NEC + EP4 receptor antagonist (n = 12); or 10) NEC + PG E2 + EP4 receptor antagonist (n = 12).

In the groups that received prostaglandin agonists or antagonists, the drugs were dissolved in warm sterile saline to the final concentration and then placed on the washed peritoneum. Fig. 1 shows the experimental timeline. The drugs were allowed to dwell in the peritoneum and blood flow was recorded at 3, 10, and 30 minutes after the dwell was initiated. All experiment drugs were used in accordance with the manufacturer’s instructions and were applied topically in warmed sterile saline to the peritoneum. Indomethacin (Sigma-Aldrich, St. Louis, MO) was used at a final topical concentration of 10 uM. AH23848, an EP4 receptor antagonist, (Sigma-Aldrich) was used at a final concentration of 10 uM. CAY10598, an EP4 receptor agonist, (Cayman Chemical Co., Ann Arbor, MI) was used at a final topical concentration of 1 uM. Dinoprostone (prostaglandin E2, Sigma-Aldrich) was delivered at a final topical concentration of 50 uM.

All data are expressed as mean ± standard error of the mean (SEM). Differences between groups and time points were evaluated by two-way analysis of variance (ANOVA) for repeated measures (REMANOVA). Differences in body weight and bladder distension scores were measured by one-way ANOVA. The null hypothesis was rejected a priori at P < 0.05. When differences were found by ANOVA, the post hoc Tukey-Kramer honestly significant difference test was applied, which minimizes the risk of Type II error.

2. Results

The body weights in the NEC groups were significantly lower than the body weights in the CONTROL groups in spite of the aggressive
feeding protocol followed for the NEC groups, demonstrated in Table 1. These findings are in keeping with our prior studies that characterized this model [3–6]. In addition, Bladder Distension Score was also significantly lower in the NEC groups compared to the CONTROL groups, suggesting dehydration. There was improved hydration in the NEC + indomethacin and NEC + PGE2 + EP4 receptor antagonist groups compared to the other NEC groups. Baseline blood flow in the NEC groups was significantly lower compared to the CONTROL groups at 48 hours of life. These findings are also in keeping with the prior observations that we have published in this model [3].

Fig. 2 shows the ileal blood flow in the CONTROL groups at 48 hours of life. The average baseline ileal blood flow in all CONTROL groups was 65.4 ± 0.8 PU, denoted by the upper black line on the figure. The lower baseline flow of the NEC groups (51.1 ± 0.5 PU) is shown by the lower black line for comparison. The addition of indomethacin to the CONTROL + indomethacin group increased blood flow at 3, 10, and 30 minutes after topical application of the drug. Similarly, topical application of PG E2 also increased ileal blood flow at 3, 10, and 30 minutes after topical application of the drug. However, the topical application of EP4 receptors with topical application of CAY10598 also improved ileal blood flow compared to baseline levels. Topical instillation of AH23848, an EP4 receptor antagonist, decreased ileal blood flow for the entire 30 minute observation period. Finally, topical application with both PG E2 and AH23848, the EP4 receptor antagonist, prevented the observed increase in ileal blood that was seen in the CONTROL + PG E2 group alone.

Fig. 3 shows the ileal blood flow in the NEC groups at 48 hours of life. The average baseline ileal blood flow observed in all NEC groups was 51.1 ± 0.5 PU, denoted by the lower black line on the figure. The CONTROL baseline average blood flow is again included for comparison. Thus, as we have found previously, average baseline ileal blood flow in the NEC groups is much lower than the average baseline ileal blood flow observed in the CONTROL groups (51.1 ± 0.5 versus 65.4 ± 0.8 PU, *P* < 0.05). With topical application of indomethacin, ileal blood flow is significantly increased to about 65 PU and flow remains elevated throughout the observation period. Topical PG E2 has a more pronounced effect on ileal blood flow in the NEC + PG E2 group than it did on the CONTROL + PG E2 group, and flow remains elevated at the 30 minute time point. These levels are similar to those observed in the CONTROL groups and these data points are above the line denoting the 48-hour CONTROL levels (65.4 ± 0.8). The topical application of CAY10598 to activate EP4 receptors results in similar increases in blood flow to that observed with PG E2. However, the application of the EP4 receptor antagonist, AH23848, significantly lowered the blood flow in the NEC + EP4 antagonist group. The final group, NEC + PG E2 + EP4 antagonist, also has decreased blood flow during the observation period compared to baseline or to NEC + PG E2, suggesting that the effects of PG E2 are mediated through the EP4 receptor in these studies.

### 3. Discussion

In the neonatal intensive care unit, drastic improvements in critical care have resulted in survival of increasingly premature infants. With this enlarging population of preterm infants, the number of patients with patent ductus arteriosus has also increased. NSAIDs such as indomethacin and ibuprofen are routinely used to medically treat PDA as first-line treatment instead of the invasive surgical procedure of PDA ligation. NSAID administration has been associated with intestinal perforation and NEC as often as 12–30% of the time, depending on inclusion criteria [1,7]. The difficulty of predicting which infants will develop perforation or NEC in response to NSAIDs is puzzling. A retrospective study noted similar rates of NEC and perforation in infants with PDAs that were treated either with indomethacin, surgery, or a combination of the two, which points toward decreased intestinal blood flow as an etiology [7]. Bolus dosing of indomethacin, rather than slow infusion of indomethacin, has been associated with more adverse intestinal events, suggesting a severe sudden vasocstriction may occur with bolus administration and lead to perforation [8].

In this study, we sought to evaluate intestinal blood flow in response to topical treatment with PGE2, nonspecific inhibition of prostaglandins with indomethacin, and a specific EP4 agonist and antagonist to understand the roles of these molecules in neonatal intestinal perfusion, particularly in the face of NEC. PGE2 and its receptor EP4 have previously been identified as having key roles in the development of necrotizing enterocolitis [9]. COX-2 is a crucial enzyme for prostaglandin synthesis catalyzing the rate-limiting step of arachidonic acid metabolism into prostaglandins [10]. Endotoxin induces production of COX-2, and a subsequent increase in prostaglandins [11]. Prostaglandin E2 is the predominant gut prostaglandin, and its EP4 receptor is the primary mediator of prostaglandin-induced vasodilation in the intestine [12,13]. EP4 knockout mice have been shown to be more susceptible to colitis with disruption of mucosal barrier function and immune down-regulation [2]. Blocking of COX-2 and subsequently prostaglandins with NSAIDS may contribute to intestinal injury in this manner.

In our experiments, PGE2 and EP4 agonist administration caused a significant increase in intestinal blood flow, which supports our theory that both of these enzymes are crucial in control of neonatal intestinal blood flow. There was a significant decrease in intestinal blood flow with EP4 antagonist administration in our study, indicating that blood flow increases were mediated through the EP4 receptor. Previous studies have demonstrated that administration of PGE2 (dinoprostone) protects against vasocostriction when administered in concert with NSAIDS [14]. In our final experiment, the addition of an EP4 antagonist to the milieu abrogated the effects we had seen of PGE2, indicating that the mesenteric vascular effects of PGE2 are via EP4.

Research focusing on the local reaction of intestinal mesenteric blood flow to intraluminal administration of indomethacin in adult rats led to mesenteric longitudinal ulcer of the small intestine via ischemia-reperfusion injury [15]. The study went on to further note the increase in mesenteric blood flow from 1–6 hours after administration. In our model, the topical application of indomethacin had a slightly increased effect on blood flow in the NEC intestine, but perhaps the global effect of ischemia along the mesenteric side is more difficult to capture in our much smaller model (5 g vs. 250 g). Another explanation for our failure to capture an initial decrease in blood flow in the neonatal rat model is that the decreased perfusion

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Body weight, g</th>
<th>Bladder distension score</th>
<th>Baseline ileal blood flow, PU</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL + indomethacin</td>
<td>12</td>
<td>7.5 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>66.8 ± 0.8</td>
</tr>
<tr>
<td>CONTROL + PG E2</td>
<td>12</td>
<td>7.4 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>66.1 ± 0.8</td>
</tr>
<tr>
<td>CONTROL + EP4 agonist</td>
<td>10</td>
<td>8.0 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>63.1 ± 0.7</td>
</tr>
<tr>
<td>CONTROL + EP4 antagonist</td>
<td>10</td>
<td>8.3 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>63.7 ± 0.7</td>
</tr>
<tr>
<td>CONTROL + PG E2 + EP4</td>
<td>12</td>
<td>8.4 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>68.0 ± 0.8</td>
</tr>
<tr>
<td>NEC + indomethacin</td>
<td>15</td>
<td>5.1 ± 0.1*</td>
<td>1.7 ± 0.1*</td>
<td>52.6 ± 0.5*</td>
</tr>
<tr>
<td>NEC + PG E2</td>
<td>12</td>
<td>4.9 ± 0.1*</td>
<td>1.0 ± 0.1*</td>
<td>53.4 ± 0.5*</td>
</tr>
<tr>
<td>NEC + EP4 agonist</td>
<td>12</td>
<td>5.0 ± 0.1</td>
<td>0.8 ± 0.1*</td>
<td>52.4 ± 0.7*</td>
</tr>
<tr>
<td>NEC + EP4 antagonist</td>
<td>12</td>
<td>5.0 ± 0.1*</td>
<td>1.1 ± 0.1*</td>
<td>49.4 ± 0.7*</td>
</tr>
<tr>
<td>NEC + PG E2 + EP4 antagonist</td>
<td>12</td>
<td>5.0 ± 0.1*</td>
<td>1.8 ± 0.1*</td>
<td>49.0 ± 0.6*</td>
</tr>
</tbody>
</table>

*P* < 0.05 vs. corresponding CONTROL group (i.e., NEC + indomethacin vs. CONTROL + indomethacin, NEC + PG E2 vs. CONTROL + PG E2, etc.) by ANOVA and Tukey-Kramer HSD test.
Fig. 2. Ileal blood flow in the CONTROL groups. Indomethacin, PG E₂, and EP4 receptor agonist all increased ileal blood flow above the baseline levels for CONTROLS during the observation period. EP4 receptor antagonist decreased blood flow compared to baseline which persisted throughout the observation period. * \( P < 0.05 \) vs. Baseline Level, † \( P < 0.05 \) vs. CTRL + Indomethacin, ‡ \( P < 0.05 \) vs. CTRL + PG E₂, § \( P < 0.05 \) vs. CTRL + EP4 receptor agonist, and # \( P < 0.05 \) vs. CTRL + PG E₂ + EP4 receptor antagonist by 2-way ANOVA and Tukey-Kramer honestly significant difference test.

Fig. 3. Ileal blood flow in the NEC groups. Indomethacin, PG E₂, and EP4 receptor agonist all increased ileal blood flow above the baseline levels for NEC groups during the observation period, but only PG E₂ and EP4 receptor agonist increased flow above the baseline levels for 48-hour CONTROL groups. EP4 receptor antagonist decreased blood flow compared to baseline which persisted throughout the observation period. In addition, EP4 receptor antagonist prevented the increased flow observed with topical PG E₂ application. * \( P < 0.05 \) vs. baseline level, † \( P < 0.05 \) vs. NEC + indomethacin, ‡ \( P < 0.05 \) vs. NEC + PG E₂, § \( P < 0.05 \) vs. NEC + EP4 receptor agonist, and # \( P < 0.05 \) vs. NEC + PG E₂ + EP4 receptor antagonist by 2-way ANOVA and Tukey-Kramer honestly significant difference test.
caused by indomethacin takes place over a shorter period of time and the volatile neonatal circulatory system experiences a rapid swing.

We have previously found that this model of NEC produces Grade 1–4 NEC histologic changes at 48 hours of life in the groups undergoing the NEC protocol, and no evidence of histopathologic injury in the CONTROLS. In the current study we examined in histopathology in sentinel animals from each group and found that the NEC rats consistently score between Grade 1 and Grade 4 NEC, while the CONTROLS do not have any sign of injury. There was not thought to be adequate length of exposure of the intestine to the topically applied vasoactive mediators to affect histologic findings, therefore further investigation into this was not carried out.

Limitations to this study include the use of an animal model to simulate a human condition and the necessary artificial nature of our animal model. Our CONTROLS are time-matched to the NEC groups but are not weight or sex matched. We feel that the time-matched CONTROLS provide the best physiologic comparison for the NEC animals as many neonatal vascular processes occur in a time dependent, rather than gender or weight dependent, manner. Experiments using older animals might allow better matching of controls based on size or gender, but the view into the complex development of the neonatal intestinal vascular control mechanisms may be lost.

Further studies of the role of prostaglandins in the neonatal intestinal vasculature will involve evaluation of human blood specimens before and after indomethacin treatment for PDA, and correlating prostaglandin and metabolite levels prospectively to development of intestinal complications. This will allow better delineation of which infants are at risk for hypoperfusion of the intestine after NSAID treatment and may provide a target group for intervention in this disease process.

Acknowledgments

We would like to acknowledge the technical assistance of Amy J. Matheson and Samuel A. Matheson.

References


Discussion

Discussant Dr. Gail Besner, Columbus, OH: Congratulations on a lovely study again. I have a quick question. In your model of experimental NEC, not all of the animals get NEC obviously. I’m wondering what percent of your animals get NEC, and can you use your blood flow studies to try to differentiate why a certain percentage of animals who are exposed to the same degree of stress as their litter mates do not develop NEC whereas the litter mates do develop NEC. Can you tease that out based on blood flow?

Response Dr. Walker: We have confirmed that approximately 60% of our animals do get NEC and we need to go back and delineate which ones get NEC versus which ones don’t by comparing prediction of blood flow and then histopathology.