Ibuprofen Intervention in Canine Septic Shock: Reduction of Pathophysiology without Decreased Cytokines

ARNOLD G. CORAN, M.D.,' ROBERT A. DRONGOWSKI, M.A., JANE J. PAIK, B.S., AND DANIEL G. REMICK, M.D.

Section of Pediatric Surgery, C. S. Mott Children's Hospital and University of Michigan Medical School, Ann Arbor, Michigan 48109-0245

Submitted for publication February 6, 1991

This study was undertaken to evaluate the effect of a cyclooxygenase inhibitor, ibuprofen, at various time intervals in a live Escherichia coli model of canine septic shock. Group I (control) animals (n = 5) received a LD100 dose of 10⁸ live E. coli per kilogram were given no further treatment. Group II animals (n = 5) received a 10 mg/kg bolus of ibuprofen 10 min prior to bacterial infusion. Group III animals (n = 5) received ibuprofen 15 min after the bacterial infusion. Statistical analysis revealed the following: Group II animals had significantly higher MABP and significantly lower levels of serum fluorescent products (superoxide radical activity), plasma thromboxane B2, prostaglandin E2, and endotoxin levels compared to Group I animals (P < 0.05). Plasma levels of tumor necrosis factor (TNF) and interleukin-6 (IL-6) were significantly elevated (P < 0.05) from baseline in all animals (Groups I, II, and III), but ibuprofen treatment failed to either increase or decrease these levels. This study demonstrates that ibuprofen treatment can significantly reverse the deleterious hemodynamic and metabolic effects commonly seen in live E. coli septic shock without depressing the endogenous production of TNF or IL-6. These data support the hypothesis that sepsis initiates a cascade of mediators with the cytokines TNF and IL-6 being proximal events which in turn stimulate the next level, with ibuprofen probably exerting its inhibitory effect distal to this point in the cascade.

INTRODUCTION

Gram-negative septic shock remains a major clinical problem with significant associated morbidity and mortality. Oxygen-derived free radicals, which are generated during tissue ischemia via the enzyme xanthine oxidase or from activated neutrophils, have been implicated as mediators of tissue injury via peroxidation of lipid membranes [1] in a wide variety of disease processes including shock [2, 3]. Prostaglandins, which are derived from the arachidonic acid cascade and which possess a variety of vasoactive properties, have been demonstrated to be elevated in experimental models of endotoxin shock [4-15]. In addition, several investigators have suggested that toxic oxygen metabolites and peroxides are formed during arachidonic acid metabolism [16-19]. Recent attention has focused on the role of the cytokine tumor necrosis factor (TNF) in septic shock [20, 21]. Levels of TNF are elevated during septic episodes, and elevated serum levels are predictive of fatal outcome [22]. Furthermore, TNF can directly stimulate neutrophils [23] and arachidonic acid metabolism [24, 25].

Ibuprofen, a nonsteroidal, anti-inflammatory agent, which inhibits cyclooxygenase, has been shown to have beneficial effects in hemorrhagic and endotoxin shock by stabilizing blood pressure, preventing acidosis, and increasing survival [4, 5, 26-29]. It has been demonstrated that administration of cyclooxygenase inhibitors such as aspirin, indomethacin, or ibuprofen eliminates the increase in plasma levels of arachidonic acid derivatives seen in shock and improves survival [27, 30, 31].

The precise biochemical mechanisms involved in these protective effects of ibuprofen are likely multifactorial, involving both interference with oxidant injury and inhibition of arachidonic acid metabolite formation. This study was designed to document the metabolic and hemodynamic efficacy of ibuprofen treatment instituted both pre- and postlive Escherichia coli administration in young mongrel puppies and to investigate the role of tumor necrosis factor as a mediator in septic shock.

MATERIALS AND METHODS

Fifteen mongrel puppies weighing between 2 and 8 kg were randomly divided into three experimental groups as follows: Group I (n = 5), E. coli infusion with no further treatment; Group II (n = 5), bolus intravenous injection of ibuprofen³ (10 mg/kg) 10 min prior to the bacterial infusion; and Group III (n = 5), the same ibuprofen dose 15 min after completion of the bacterial infusion. On the experimental day, each puppy received

¹ To whom reprint requests should be addressed.

³ Donated by the Upjohn Company, Kalamazoo, Michigan.
were added to the plates and allowed to incubate over-
actinomycin D (Calbiochem, La Jolla, CA). The cells
were resuspended at 5 x 10⁶ cells/ml in RPMI 1640 with
10% fetal calf serum, 2 mM L-glutamine, and 0.5 µg/ml
actinomycin D (Calbiochem, La Jolla, CA). The cells
were added to the plates and allowed to incubate over-
night at 37°C. Cell lysis was detected by adding MTT-
tetrazolium and incubating the plates an additional 4 hr.
The dark purple tetrazolium salts were then dissolved in
acidified isopropanol. Units of TNF were calculated
based on a recombinant standard run in the same assay.

Catecholamines. Norepinephrine, epinephrine, and
dopamine were measured by high pressure liquid chromo-
tagraphy (HPLC) with electrochemical detection. An
internal standard (1250 pg/ml dihydroxybenzylamine)
was added to each plasma sample. The catecholamines
were extracted from the plasma by adsorption to alu-
mina at neutral pH and subsequently eluted with 0.1 M
perchloric acid. The catecholamines were then eluted
with aqueous acetonitrile/chloroacetic acid/sodium oc-
tyl sulfate (5%/0.1 M/300 µg/liter) by reversed phase
HPLC on a heated (35°C) C-18 column (0.5 µ Biophase, 25 x 0.4 cm, Bioanalytical Systems, West Lafayette,
IN). They were detected amperometrically with a glassy
carbon electrode (LC-4B, Bioanalytical Systems) at 0.65
V. The concentration of each catechol was measured by
comparison of the resultant peak heights with those pro-
duced by the pure catecholamines and corrected for rec-
covery by reference to the internal standard [35].

Prostaglandin E₂. C-18 Sep-Pak cartridges (Waters
Associates, Milford, MA) were prewashed with 5.0 ml of
methanol followed by 5.0 ml of deionized water. A 1.0-ml
plasma sample, to which [3H]thromboxane B₂ (0.01 µCi;
NET-603, New England Nuclear, Boston, MA) was
added, was then applied to a C-18 Sep-Pak cartridge.
The cartridge was then washed with 20% methanol (5.0
ml) followed by deionized water (5.0 ml). The prostaglan-
dins were subsequently eluted by two sequential 1.0-ml
washes with 80% methanol. The solvent was evaporated
from the extract under a stream of dry air at ambient
temperature and the residue dissolved in 0.1 M
phosphate-buffered saline containing 0.1% pigskin gel (pH
7.4) (PBS-Gel) prior to radioimmunoassay analysis. The
recovery of [3H]thromboxane B₂, monitored by counting
a 100-µl aliquot of the reconstituted sample, averages
89.6 ± 4.5 (SD)%. Levels of prostaglandin E₂ ([3H]PgE₂)
were then measured by radioimmunoassay using com-
mercially available antibody (Catalog Number 114010,
Cayman Chemical Co., Ann Arbor, MI) and [3H]PgE₂
tracer (NET-428, New England Nuclear). The tracer,
test sample, antibody, and 400 µl PBS-Gel were incu-
bated for 18 hr at 4°C. The unbound prostaglandin was
subsequently absorbed by addition of 1.0 ml of a suspesi-
on consisting of 0.25% activated charcoal (Norit A,
Sigma Chemical Co., Kansas City, MO) and 0.025%
Dextran T70 (Pharmacia Fine Chemicals, Uppsala,
Sweden) in 0.1 M phosphate-buffered saline (PBS), pH
7.4. The supernatant was then mixed with 5.0 ml of scin-
tillation fluid (Ecolume, ICN Biochemicals, Irvine, CA).
The β-radioactivity due to antibody-bound tracer was
measured in a liquid scintillation counter (Model 6893,
Tm Analytic, Elk Grove Village, IL). The concentration
FIG. 1. The cardiac output is expressed as a percentage of the baseline. Each value is the mean of five animals. Although pretreatment with ibuprofen improved cardiac output, this was not significant.

of PgE₂ in each sample was determined using a four-parameter logistic curve fit option of an MS-DOS program (Arbor Immunalysis, Ann Arbor, MI) on a Zenith Z-158 PC (Zenith Data Systems, St. Joseph, MI). This assay has a sensitivity of 60 pg/ml and an interassay coefficient of variation of 14% [36].

Thromboxane B₂. Thromboxane B₂ was measured by radioimmunoassay method using commercially available [3H]thromboxane B₂ ([3H]TxB₂) tracer (NET-603, New England Nuclear, Boston, MA), antibody (Fitzpatrick) and a procedure similar to that used for measurement of PgE₂. The thromboxane B₂ assay has a sensitivity of 18 pg/ml and an interassay coefficient of variation of 9% [37].

Endotoxin assay. Endotoxin levels were measured by using the Limulus Amebocyte Lysate Test (Whittaker Bioproducts, Inc.). The plasma samples were diluted 1:10 with pyrogen-free water, then heated to 75°C for 15 min. Fifty microliters of sample or standard was placed in standard microtiter ELISA plates. Following a fifteen-min incubation at 37°C, 50 μl of limulus amebocyte lysate was added to each well, followed by an additional 30-min incubation. One hundred microliters of chromogenic substrate solution was added to each well, incubated for 6 min, and the reaction stopped by the addition of 50 μl of 25% acetic acid. The microtiter plates were then analyzed at 405 nm with a Bio-Tek EL-312 BioKinetics Reader (Winooski, VT).

Interleukin-6 assay. The IL-6 assay was performed using the B9 cell line, which is very sensitive to IL-6. This assay was performed by placing serial dilutions of the samples into 96-well microtiter plates and then adding 5000 B9 cells in RPMI 1640 with 2 mM L-glutamine, 25 mM Hepes, 1% pen/strep, 5 × 10⁻⁶ M mercaptoethanol, and 10% fetal calf serum. The cells were incubated for 68 hr at 37°C and pulsed for the final 6 hr with MTT-tetrazolium. The crystals were dissolved with actified isopropanol, and the units were calculated based upon a standard curve run in the same assay [38].

Data were subjected to statistical analyses as follows: Group I (control, n = 5), Group II (pre-bacterial ibuprofen-treated animals, n = 5), and Group III (postbacterial infusion ibuprofen-treated animals, n = 5), Figs. 1–8. The Michigan Interactive Data Analysis System (MIDAS) was used to analyze data by univariate and repeated measures analysis of variance, with P values less than 0.05 considered significant.

RESULTS

Mean cardiac output. Mean cardiac outputs, expressed as a percentage of baseline levels, are shown in Fig. 1. In Group I (control animals) mean cardiac output dropped to below 60% of baseline levels and continued to decrease throughout the study period. In contrast, in Group II (prebacteria ibuprofen-treated animals), mean cardiac output dropped to 50% of baseline levels by 60 min postbacterial infusion and then continued to rise to approximately 70% of baseline. In Group III (postbacteria ibuprofen-treated animals), mean cardiac output dropped significantly after the bacterial infusion, followed by a gradual increase after administration of the ibuprofen, however, these increases were not statistically significant.

Mean arterial pressure. Mean systemic arterial blood pressure, expressed as a percentage of baseline levels, is depicted in Fig. 2. In the Group I (control) animals, mean arterial pressure decreased throughout the
FIG. 3. The plasma levels of PGE$_2$ represent the mean values of five animals. Pretreatment with ibuprofen prevented the rise in plasma PGE$_2$.

FIG. 4. The plasma levels of TXB$_2$ represent the mean values of five animals. Pretreatment with ibuprofen prevented the rise in plasma TXB$_2$.

-course of the experiment. The mean arterial pressure in the Group II (pretreated) animals was significantly higher than in the control animals, remaining at about 90% of baseline levels throughout the study period. In the Group III (post-treated) animals, mean arterial pressure decreased to 50% of baseline following the bacterial infusion and then gradually rose to about 70% of initial levels following the administration of the ibuprofen.

Prostaglandin. Mean blood prostaglandin E$_2$ (PGE$_2$) levels are shown in Fig. 3. The Group II (pretreated) animals experienced significantly decreased levels of PGE$_2$ in comparison with either the Group I (control) or Group III (post-treated) animals, consistent with its potent activity as a cyclooxygenase inhibitor.

Thromboxane. Mean blood Thromboxane B$_2$ (TBX$_2$) levels are shown in Fig. 4. The Group II (pretreated) animals have significantly decreased levels of TBX$_2$, compared with the Group I (control) animals. Mean blood Thromboxane B$_2$ levels in the Group III (post-treated) animals were intermediate between the Group I and Group II dogs.

Fluorescent products. Serum levels of fluorescent products of lipid peroxidation were significantly lower in both the pretreated (Group II) and the posttreated (Group III) dogs in comparison with the control (Group I) animals (Fig. 5).

Catecholamines. Plasma catecholamine levels (epinephrine, norepinephrine, and dopamine) are depicted in Table 1. Mean plasma catecholamine levels began to increase significantly 90 min following the *E. coli* infusion in all three experimental groups. There were no significant between-group differences in mean catecholamine levels during the experiment.

Interleukin-6. Mean plasma levels of interleukin-6 increased significantly from baseline levels 90 min after the bacterial infusion; however, there are no significant between-group differences (Fig. 6). Neither prior or post-treatment with ibuprofen altered the rise in IL-6.

Tumor necrosis factor. Mean levels of tumor necrosis factor increased significantly from baseline 60 minutes after the bacterial infusion and peaked at 2–3 hr in all three groups. There were no significant between-group differences (Fig. 7). Ibuprofen treatment appeared to prevent the characteristic rapid decline in plasma TNF.

FIG. 5. The plasma levels of fluorescent products represent the mean values of five animals. Pretreatment or post-treatment with ibuprofen prevented the rise in plasma fluorescent products.
TABLE 1
Mean Plasma Catecholamine Levels (Epinephrine, Norepinephrine, and Dopamine) during Live E. coli Septic Shock in Dogs

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (I)</th>
<th>Pre-ibupr (II)</th>
<th>Post-ibupr (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>82</td>
<td>89</td>
<td>75</td>
</tr>
<tr>
<td>15</td>
<td>251</td>
<td>133</td>
<td>476</td>
</tr>
<tr>
<td>30</td>
<td>1458</td>
<td>245</td>
<td>984</td>
</tr>
<tr>
<td>60</td>
<td>2713</td>
<td>412</td>
<td>2517</td>
</tr>
<tr>
<td>90</td>
<td>14606</td>
<td>6066</td>
<td>29120</td>
</tr>
<tr>
<td>120</td>
<td>24819</td>
<td>10927</td>
<td>34475</td>
</tr>
<tr>
<td>180</td>
<td>21755</td>
<td>31569</td>
<td>58380</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>479</td>
<td>303</td>
<td>517</td>
</tr>
<tr>
<td>15</td>
<td>358</td>
<td>355</td>
<td>800</td>
</tr>
<tr>
<td>30</td>
<td>733</td>
<td>350</td>
<td>896</td>
</tr>
<tr>
<td>60</td>
<td>846</td>
<td>863</td>
<td>1276</td>
</tr>
<tr>
<td>90</td>
<td>5778</td>
<td>2064</td>
<td>4523</td>
</tr>
<tr>
<td>120</td>
<td>7710</td>
<td>9809</td>
<td>13750</td>
</tr>
<tr>
<td>180</td>
<td>9815</td>
<td>18575</td>
<td>35476</td>
</tr>
<tr>
<td>Dopamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>13</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>30</td>
<td>21</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>60</td>
<td>26</td>
<td>40</td>
<td>18</td>
</tr>
<tr>
<td>90</td>
<td>54</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>120</td>
<td>164</td>
<td>61</td>
<td>60</td>
</tr>
<tr>
<td>180</td>
<td>173</td>
<td>112</td>
<td>224</td>
</tr>
</tbody>
</table>

Endotoxin. Mean plasma levels of endotoxin are shown in Fig. 8. The Group II (pretreated) animals experienced significantly decreased levels of endotoxin in comparison with the Group I (control) animals.

DISCUSSION
Several experimental models of septic shock have been used to demonstrate an increase in the circulating levels of the end products of the cyclooxygenase pathway, including PGE₂ [6,39] and TBX₂ [40]. Given their documented increase during episodes of sepsis, it is reasonable to test the hypothesis that the formation of these potent metabolites may be playing a critical role in the pathogenesis of septic shock. Indeed, previous investigators have also shown that the cyclooxygenase inhibitor, ibuprofen, offers protection against the deleterious hemodynamic effects of septic shock [5, 7, 28]. Our data confirm these previous reports and show that ibuprofen will significantly improve hemodynamic function in animals given a lethal bacterial challenge of E. coli. Both cardiac output and mean arterial blood pressure were better maintained with ibuprofen therapy, and prior administration of the drug provided greater protection compared to giving the drug after the bacterial challenge. Pretreatment with ibuprofen prevented the rise in both PGE₂ and TBX₉ induced by the septic challenge.

Recent studies have focused attention on the role of endogenously produced cytokines on the altered pathophysiology observed in septic shock [20, 21]. The link between the generation of tumor necrosis factor and septic shock is very strong, and TNF is considered pivotal in the development of septic shock. Data supporting
the role of TNF come from several independent investigations. First, plasma levels of TNF are elevated in patients in septic shock [22] or patients in intensive care units [41, 42], and the plasma level of TNF is an independent predictor of outcome. Second, administration of purified, recombinant TNF to experimental animals will induce organ injury [43], even when given to mice which are not sensitive to the effects of endotoxin [44]. Finally, antibodies to TNF will prevent the lethality observed in septic shock in response to either lipopolysaccharide [45], or live E. coli [46, 47]. Elevated levels of interleukin-6 have also been reported in septic shock [48, 49].

Given the pivotal role of TNF in septic shock and the ability of ibuprofen to block the altered pathophysiology, it would be tempting to speculate that TNF production may be inhibited. However, since in vitro studies have shown that PGE inhibits the production of TNF [25], blocking the cyclooxygenase pathway could potentially augment cytokine production. Our data indicate that treatment with ibuprofen has virtually no effect on the production of either TNF or IL-6, although there is slightly increased production at the 3-hr time point. Thus, the beneficial effects of cyclooxygenase inhibition are not mediated through decreased levels of TNF.

Other investigators have shown that administration of purified, recombinant TNF will activate the cyclooxygenase pathway in vivo. Kettlehut et al. [50] showed that the lethality of exogenous administered TNF could be blocked with ibuprofen, or indomethacin. Similarly, many of the pathophysiologic derangements induced with recombinant TNF could be blocked by ibuprofen [51]. The most likely explanation for these observations is that sepsis initiates a cascade of interactions which culminate in multiorgan failure and death. In this scenario, the endotoxin from the bacteria causes TNF production in the host. This burst of TNF activity stimulates the next wave of the cascade, including products of the cyclooxygenase pathway, the prostaglandins and thromboxanes. Therefore, although neither bacteria nor TNF by themselves are directly injurious, together they synergistically perpetuate the cascade of events which leads to injury and multiorgan failure [52]. Our data support this hypothesis, since there is no diminution in the levels of TNF or IL-6, while there is an improvement in the overall status of the animals in Groups II and III.

The data regarding the endotoxin measurements are intriguing, since blocking the cyclooxygenase pathway apparently allowed for more efficient clearance of the bacteria. It is possible that inhibiting production of prostaglandins, which are immunosuppressive agents, resulted in more efficient functioning of the reticuloendothelial system, so that the bacteria were cleared more efficiently. Further studies of this area are warranted.

These data show that inhibition of the cyclooxygenase pathway results in markedly improved hemodynamics and a decrease in serum flourescent products during E. coli-induced septic shock. The improvements occurred without any decrease in the production of TNF or IL-6 and lends further support to the hypothesis of a cytokine-induced cascade of interactions which lead to injury during the development of septic shock. Ibuprofen apparently exerts its inhibitory effects at a more distal point in this cascade.

ACKNOWLEDGMENTS

Supported in part by a grant from the American Heart Association of Michigan and National Institute of Health Grants HL39339 and GM44918.

REFERENCES


