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Endotoxin and Hypoxia-Induced Intestinal Necrosis in Rats: The Role of Platelet Activating Factor

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ABSTRACT. We have previously shown that intravascular platelet activating factor (PAF) causes ischemic bowel necrosis in rats morphologically similar to neonatal necrotizing enterocolitis (NEC). Because endotoxin (LPS) and hypoxia are risk factors for NEC, we studied their effect on PAF metabolism and the development of intestinal injury. Young male Sprague-Dawley rats were anesthetized with pentobarbital and divided into six experimental groups: *1*) control, *2*) LPS alone (2 mg/kg), *3*) hypoxia alone (5% O_2), *4*) LPS + hypoxia, *5*) WEB 2086 (PAF antagonist) + LPS + hypoxia, and *6*) SRI 63-441 (PAF antagonist) + LPS + hypoxia. Evaluations included blood pressure recording, superior mesenteric artery blood flow, arterial blood gas, white blood cell count, hematocrit, plasma PAF, plasma acetylhydrolase, plasma tumor necrosis factor, intestinal perfusion, and intestinal injury at 3 h. We found that LPS + hypoxia synergistically contributed to hypotension (mean blood pressure 27 ± 5.6% baseline *versus* 101 ± 3.9% control), metabolic acidosis (pH 7.05, base deficit 24 mEq/L), hemoconcentration, decreased superior mesenteric artery blood flow (2.2 ± 0.3 mL/min *versus* 5.8 ± 0.2 mL/min control), and intestinal injury. The morbidities resulting from LPS + hypoxia were partially or completely prevented by PAF antagonists. In addition, animals treated with LPS + hypoxia had neutropenia, elevated plasma acetylhydrolase, and elevated plasma TNF. These results suggest that endogenous PAF may contribute to LPS + hypoxia-induced intestinal hypoperfusion and necrosis. (*Pediatr Res* 31: 428-434, 1992)

Abbreviations

PAF, platelet activating factor
LPS, lipopolysaccharide, endotoxin
WBC, white blood cell count
TNF, tumor necrosis factor
NEC, necrotizing enterocolitis
SMA, superior mesenteric artery

NEC is a common gastrointestinal disease of premature infants that has a high incidence of morbidity and mortality (1). Although the pathophysiology is poorly understood, the accepted "multifactorial theory" suggests that the combination of prematurity, intestinal ischemia, and bacterial colonization predispose to the development of the disease (2, 3). We have previously shown that intravascular administration of PAF (PAF-acether), an endogenous phospholipid inflammatory mediator, produces intestinal injury in an animal model (4). In addition, it is known that endotoxin (LPS)-induced shock and intestinal injury can be prevented by PAF receptor antagonists (5, 6). Because we have recently reported increased plasma PAF concentrations in newborns afflicted with NEC (7), it is possible that, in addition to causing experimental intestinal injury, PAF is an important endogenous mediator of the human disease.

Additional factors related to PAF metabolism may play a role in the development of intestinal injury. PAF is rapidly degraded to the inactive metabolite lysoPAF by acetylhydrolase (8), a 43-kD acid-labile enzyme critical to PAF regulation. We have shown that serum acetylhydrolase is decreased in NEC patients compared with controls (7) and that newborn infants have lower acetylhydrolase activity than older children and adults (9). Because the majority of NEC cases occur in the neonatal period, suppressed PAF degradation may contribute to the disease process. TNF-α is a 17-kD protein synthesized by macrophages and monocytes after endotoxin stimulation (10). TNF causes shock and hemorrhagic intestinal necrosis in animal models (11, 12), and TNF-induced intestinal lesions can be prevented by PAF receptor antagonists (11). Furthermore, our recent evidence suggests that plasma TNF levels are increased in NEC patients compared with controls (7). These findings suggest that PAF, acetylhydrolase, and TNF all may contribute to the development of intestinal injury and NEC.

Hypoxia and bacterial factors (LPS), important risk factors in neonatal NEC, have been used to study intestinal injury in animal models. We have shown that acute, severe hypoxia and prolonged, moderate hypoxia result in intestinal ischemia and mild microscopic intestinal necrosis (13). Barlow *et al.* (14) showed that bacterial colonization and hypoxia together in neonatal rats resulted in severe intestinal necrosis similar to human NEC. We designed the current study to investigate the pathophysiology of intestinal ischemia and injury caused by bacterial endotoxin (LPS) and hypoxia and to evaluate the role of PAF, acetylhydrolase, and TNF in this new experimental model of intestinal necrosis.

MATERIALS AND METHODS

Animals. Young male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 60-100 g were used for most experiments. Animals were anesthetized with pentobarbital (50 mg/kg, intraperitoneal injection) and tracheotomized to prevent upper airway obstruction and for assisted ventilation. A carotid artery catheter (PE 50; Beckton Dickinson and Co., Parsippany, NJ) was inserted for blood pressure monitoring and arterial blood sampling, and a jugular venous catheter

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(PE 10) was used for drug injections. Animal experiments were conducted using the highest standards of humane care.

Experimental Protocol. To evaluate the combined effects of LPS and hypoxia on intestinal perfusion and necrosis, we assigned five animals to each of the following experimental groups (power calculations using previous data for animals treated with hypoxia alone specified that for an $\alpha = 0.05$ and $\beta = 0.20$ five animals would adequately rule out a type II error): *1*) control; *2*) LPS (*Salmonella typhi*; Sigma Chemical Co., St. Louis, MO) alone, 2 mg/kg i.v. at time 0; *3*) hypoxia alone using a gas mixture consisting of 5% O_2 and 95% N_2 with a ventilator rate of 60 breaths/min (small animal ventilator; Harvard Apparatus, South Natick, MA), starting at time 90 min; *4*) LPS (2 mg/kg i.v.) at time 0 + hypoxia at 90 min (LPS + hypoxia); *5*) WEB 2086 (1 mg/kg i.v.) 10 min before time 0, followed by LPS + hypoxia; and *6*) SRI 63–441 (5 mg/kg i.v.) 10 min before to time 0, followed by LPS + hypoxia. SRI 63–441 (a generous gift from Dr. Dean Handley, Sandoz Research Institute, Hanover, NY) and WEB 2086 (a generous gift from Dr. H. O. Heuer, Boehringer Ingelheim, Mainz, Germany) are two structurally unrelated PAF receptor antagonists that effectively inhibit PAF-induced shock and intestinal injury (15, 16). Both antagonists were tested in control animals and had no effect on blood pressure, intestinal blood flow, or intestinal injury.

Mean arterial blood pressure was recorded throughout the experiment, and at 180 min the following studies were performed: *1*) hematocrit, WBC, and arterial blood gas analysis from peripheral arterial blood; *2*) plasma PAF, acetylhydrolase activity, and TNF determinations; *3*) qualitative intestinal perfusion using Evans blue dye; and *4*) intestinal necrosis by gross and histologic assessment. In a separate group of animals, continuous mesenteric blood flow measurements were performed using ultrasonic transit-time technology.

The Evans blue dye technique is a qualitative method of measuring intestinal perfusion that highly correlates with the microscopic assessment of intestinal necrosis (17). The dye (2 g/L, 0.5 mL intraarterial at 180 min) distributes through the circulation and stains tissues with intact blood flow. With this method, markedly hypoperfused intestine does not stain blue, and these sections can be identified and the length quantified. By dividing the length of normally perfused intestine by the total intestinal length, we can estimate the percentage of intestinal perfusion (18).

To substantiate the qualitative estimations of blood flow from the Evans blue dye method, we repeated the same experiment in adult rats (225–275 g) to study mesenteric blood flow quantitatively. These measurements could not be performed in young rats because of limitations in flow probe size. After the initial surgery, the abdomen was opened and the SMA exposed. A 2-mm blood flow probe (Transonic Systems, Inc., Ithaca, NY) was placed around the artery and attached to a blood flow meter (Transonic Systems, Inc.) for continuous SMA blood flow recordings. This technology uses ultrasonic transit-time principles such that blood flow is sensed independent of vessel size, vessel to flowprobe alignment, flow velocity profile, turbulence, hematocrit, or particulate content of liquid. With use of this methodology, we can obtain valid and reliable measurements of blood flow and not blood flow velocity. Continuous mesenteric blood flow measurements were performed on five animals in each of the experimental groups outlined above.

Gross intestinal necrosis was scored as none, mild, moderate, or severe based on the stereomicroscopic assessment of hemorrhage and vasoconstriction by an observer unaware of the study group. Intestinal specimens were prepared for routine histologic evaluation using formaldehyde fixation and hematoxylin and eosin staining. Sections were read by a pathologist unaware of the experimental protocol, and necrosis assessed as none, minimal (epithelial cell separation from the lamina propria), mild (epithelial cell necrosis confined to the tips of the villi), and moderate (partial loss of intestinal villi).

Assays. PAF assay. Bioactive plasma PAF was measured using the rabbit platelet serotonin release assay as previously described (5). Phospholipids were extracted from plasma using Folch's method (19), dried under a stream of nitrogen, and separated by thin-layer chromatography using a solvent system of 65/35/6 chloroform/methanol/water (vol/vol/vol). Portions that comigrated with authentic PAF standard were scraped, eluted with 1/2/0.8 chloroform/methanol/water (vol/vol/vol), dried in nitrogen, and dissolved in PBS with albumin (5 mg/mL). Samples were added to rabbit platelets that were previously incubated with [^{3}H]serotonin, and amount of serotonin released was compared with that elicited by standard dilutions of PAF. The presence of PAF in this bioassay was validated in some samples using *1*) further phospholipid separation by HPLC [Varian HPLC equipped with Ultrasphere-Si, 5-μm column using solvent system isopropanol-hexane (1:1) and a flow rate of 2 mL/min; a linear gradient was used starting with 96% isopropanol-hexane:4% water, equilibrating to 8% water by 15 min] (20) and *2*) hydrolysis of PAF with phospholipase A_2 (enzyme excess confirmed by incubation of [^{3}H]PAF with 10 μg/mL pancreatic phospholipase A_2 and documenting conversion to [^{3}H]lysoPAF by thin-layer chromatography) before bioassay and inhibiting serotonin release, and in all samples by *3*) PAF antagonist SRI 63–441 (Sandoz Research Institute, Hanover, NY) prevention of sample-induced stimulation of platelet serotonin release. Extraction efficiency was calculated by adding [^{3}H]PAF (10 000 cpm) to each sample before the extraction procedure.

Acetylhydrolase activity. Plasma acetylhydrolase activity was quantified by measuring the effect of sample on the degradation of radiolabeled PAF (9). The incubation mixture included *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.4, 0.1 mol/L), plasma (20 μL, 1:1 dilution with saline), 1-[^{3}H]-alkyl-2-acetylglycerophosphorylcholine (10 000 cpm) and 80 μmol/L unlabeled PAF. The reaction was stopped after 30 min with glacial acetic acid (10 N) and phospholipids extracted using Folch's method (19). The organic phase was plated on silica gel G plates for thin-layer chromatography using the solvent system 65/35/6 chloroform/methanol/water (vol/vol/vol). Zones comigrating with PAF and lysoPAF standards were scraped and counted in a scintillation counter (Beckman Instruments, Fullerton, CA). Acetylhydrolase activity was expressed as nmol lysoPAF formed/mL/min.

TNF assay. Plasma TNF was measured with a bioassay using the fibrosarcoma cell line, 1591 RE 3.5 as previously described (7). Plasma samples were diluted 1:20 in minimal essential media and incubated with fibrosarcoma cells (5×10^3 cells/well) in 96-well flat bottom plates for 48 h at 37°C in 5% CO_2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 25 μL; Sigma Chemical Co., St. Louis, MO] dye was added to each well to stain viable fibrosarcoma cells. After 4 h, SDS (Sigma Chemical Co.; 10% SDS with 0.01 N HCl) was added to each well to solubilize the cells. The following morning cell lysis was quantified spectrophotometrically by measuring the change in absorbance at 610 nm using a multiscan photometer (Flo Labs, McLean, VT). Results were compared with a standard curve obtained using recombinant murine TNF (a generous gift from Genentech, South San Francisco, CA) and expressed as U/mL, where 1 U/mL equalled 50% cell killing. Antimouse TNF antibody (gift from Dr. Steven Kunkel, Ann Arbor, MI) was used in some samples to confirm that killing was secondary to TNF and not some other serum constituents.

Statistics. Data are expressed as the mean ± SEM. Statistical analyses were done using analysis of variance with repeated measures to compare multiple groups, with the method of Bonferroni to identify specific intergroup differences where appropriate. To compare histologic and gross intestinal necrosis, proportions of injury severity for each group were calculated and these proportions compared using χ^2 analysis. For all evaluations, p values less than 0.05 were considered significant.

RESULTS

All experimental groups included five animals for analysis except for the group treated with LPS + hypoxia. In this protocol, three animals died during the study (one each at 120, 150, and 175 min), and, to ensure enough samples for valid statistical comparisons, a total of eight animals were studied in this group. Blood gas analysis and intestinal histologic evaluations included all eight animals, but the remainder of measurements included only those surviving the entire study period.

The mean arterial blood gas values at 3 h are seen in Table 1. Acidosis occurred with LPS alone (pH 7.24) and hypoxia alone (pH 7.17), was most severe with both LPS + hypoxia (pH 7.05, $p = 0.01$), and improved with SRI ($p = 0.05$ compared with LPS + hypoxia). Although LPS alone and hypoxia alone developed significant base deficits, LPS + hypoxia had the most severe metabolic acidosis ($p < 0.001$ compared with control, $p < 0.05$ compared with LPS or hypoxia alone). Furthermore, the PAF receptor antagonists significantly reduced the metabolic component of the acidosis ($p < 0.05$ compared with LPS + hypoxia). Arterial hypoxemia (Po_2 approximately 5.9–6.3 kPa *versus* 16 kPa control, $p < 0.001$) occurred after the hypoxia protocol and was similar in hypoxia alone, LPS + hypoxia, WEB, and SRI groups. Finally, the groups treated with hypoxia tended to hyperventilate, as indicated by lower Pco_2 values (Table 1).

Because of small differences in initial blood pressure between groups, mean arterial pressure is expressed as a percentage of initial baseline blood pressure. As represented in Figure 1, hypotension occurred at 90 min in the LPS group (mean arterial pressure: 54 ± 44% baseline *versus* 97 ± 3.8% control, $p < 0.01$) and in animals treated with LPS + hypoxia (50 ± 4.4%) The hypotensive effect induced by LPS administration at 90 min was prevented by PAF antagonists (blood pressure: 99 ± 5.4% WEB, $p < 0.01$, and 77 ± 7.3% SRI, $p < 0.05$). By 180 min, hypoxia alone (blood pressure: 46 ± 8%) and LPS alone (61 + 10%) resulted in significant hypotension. In addition, LPS + hypoxia produced severe hypotension (27 ± 5.6%, $p < 0.01$). The groups treated with PAF receptor antagonists (49 ± 9.1% WEB, 49 ± 7.0% SRI, $p = 0.06$) showed a trend toward improved blood pressure compared with LPS + hypoxia.

As assessed by the Evans blue dye method, intestinal perfusion was normal in the LPS alone group (96% normal intestinal length), mildly abnormal in the hypoxia alone group (76% normal, $p = 0.03$), and significantly decreased in the group treated with LPS + hypoxia (28% normal intestinal length, $p < 0.001$, figure 2*A*). In addition, WEB and SRI prevented the LPS + hypoxia-induced hypoperfusion.

In a different group of animals, regional blood flow measurements of the SMA were performed and at 180 min were consistent with the Evans blue dye determinations (Fig. 2*B*). After surgery at time 0, blood flow was similar between groups and ranged from 9 to 11 mL/min. By 90 min, blood flow decreased slightly in all groups, but remained similar between groups (control: 7.6 ± 1.0 mL/min; LPS: 6.7 ± 1.5 mL/min; hypoxia: 8.5 ± 2.1 mL/min; LPS + hypoxia: 6.0 ± 0.7 mL/min; and WEB: 6.6 ± 1.1 mL/min; p = NS, data not shown). At 180 min, SMA blood flow was 5.8 ± 0.2 mL/min for controls, 3.9 ± 0.6 mL/min for LPS alone, and 4.8 ± 0.6 mL/min for hypoxia alone (p = NS, Fig. 2*B*). LPS + hypoxia decreased SMA flow to 2.2 ± 0.3 mL/min by 180 min ($p < 0.05$). The LPS + hypoxia-induced decrease in blood flow was prevented by WEB pretreatment (4.9 ± 0.9 mL/min).

As seen in Table 2, control animals all had normal intestine by gross inspection and microscopic evaluation. Animals treated with LPS alone had mostly normal intestine both grossly and histologically. The hypoxia protocol led to minimal gross and minimal to mild microscopic bowel necrosis. However, LPS + hypoxia resulted in mild to moderate gross (100% of animals) and microscopic (87% of animals) intestinal pathology (Fig. 3). Consistent with the intestinal blood flow data, the LPS + hypoxia-induced intestinal injury was markedly improved by PAF receptor antagonists (χ^2: $p < 0.001$).

At the end of the experimental period, the group treated with LPS + hypoxia had a higher hematocrit (0.482 ± 0.028, $p < 0.05$; Fig. 4) compared with the other groups (control 0.398 ± 0.009); this hemoconcentration was prevented by both WEB and SRI (0.400 ± 0.032 and 0.438 ± 0.014, respectively, $p < 0.05$).

As seen in Figure 5, LPS alone produced neutropenia at 3 h (3.41 ± 0.52 cells × 10^9/L *versus* 5.70 ± 0.23 cells × 10^9/L control, $p < 0.003$), but hypoxia alone had no effect on the WBC count (6.21 ± 1.75 × 10^9/L). In addition, LPS + hypoxia produced neutropenia (2.38 ± 0.40 cells × 10^9/L), which was not reversed with WEB or SRI pretreatment (2.42 ± 0.29 *versus* 3.26 ± 0.50 cells × 10^9/L, respectively).

Plasma PAF levels (Fig. 6) tended to increase with LPS, hypoxia, and LPS + hypoxia (8.2 ± 2.1 ng/mL control, 18.8 ± 4.6 ng/mL LPS, 17.9 ± 6.5 ng/mL hypoxia, 23.1 ± 6.8 ng/mL LPS + hypoxia); however, using analysis of variance, statistical significance was not achieved.

TNF activity was assayed at the end of the experimental protocol. Increased plasma TNF concentrations were found after LPS alone (120 ± 70 IU/mL *versus* 1 IU/mL for controls, $p < 0.01$; Fig. 7) and LPS + hypoxia (320 ± 170 IU/mL); however, hypoxia alone did not increase circulating TNF activity. The difference in TNF concentrations between groups treated with LPS alone and LPS + hypoxia was not statistically significant.

Finally, plasma acetylhydrolase activity was increased 3 h after LPS alone (124.8 ± 5.7 *versus* 75.8 ± 10.0 nmol/mL/min control, $p < 0.003$; Fig. 8) and LPS + hypoxia (115.2 ± 10.1 nmol/mL/min), but not in response to hypoxia alone. Of interest, the triazolam PAF receptor blocker WEB 2086 did not prevent the LPS + hypoxia-induced increase in acetylhydrolase activity (115.2 ± 10.1 nmol/mL/min).

DISCUSSION

NEC, a gastrointestinal disease of premature infants, may be the third leading cause of death in infants less than 1 y of age (1,

Table 1. *Mean arterial blood gas values at 3 h**

Group	pH (units)	Pco_2 (kPa)	Po_2 (kPa)	BD (mmol/L)	*n*
Control	7.34 ± 0.03	6.7 ± 0.5	16.5 ± 0.5	0 ± 0.5	5
LPS	7.24 ± 0.06	6.7 ± 1.1	15.6 ± 1.6	7.2 ± 1.0†	5
Hypoxia	7.17 ± 0.03†	3.2 ± 0.4†	6.0 ± 0.7‡	19.0 ± 1.2‡	5
LPS + hypoxia	7.05 ± 0.02‡	4.8 ± 0.8	6.3 ± 0.5‡	24.0 ± 1.2§	8
WEB	7.11 ± 0.02	4.1 ± 0.7	5.9 ± 0.2‡	19.0 ± 1.4‖	5
SRI	7.15 ± 0.03‖	2.9 ± 0.6†	6.3 ± 0.4‡	17.6 ± 2.9‖	5

* Data represent the mean ± SEM. See Materials and Methods for protocol, which describes experimental groups. BD, base deficit.
† $p < 0.05$ compared with control groups.
‡ $p < 0.01$ compared with control groups.
§ $p < 0.001$ compared with control.
‖ $p < 0.05$ compared with LPS + hypoxia.

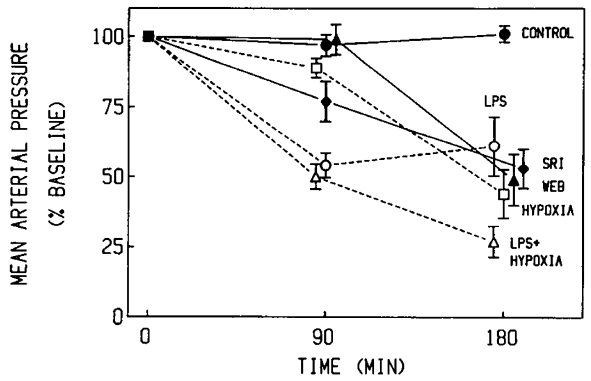

Fig. 1. Mean arterial blood pressure for experimental groups over time. Data points represent mean ± SEM. Because of variability in initial blood pressure, blood pressure is expressed as % of baseline before treatment. At 90 min, LPS and LPS + hypoxia had significantly lower blood pressure (54 ± 4.4% and 50 ± 4.5%, respectively) than the other groups (control 97 ± 3.8%, $p < 0.01$). By 180 min, LPS + hypoxia produced severe hypotension (27 ± 5.6% baseline).

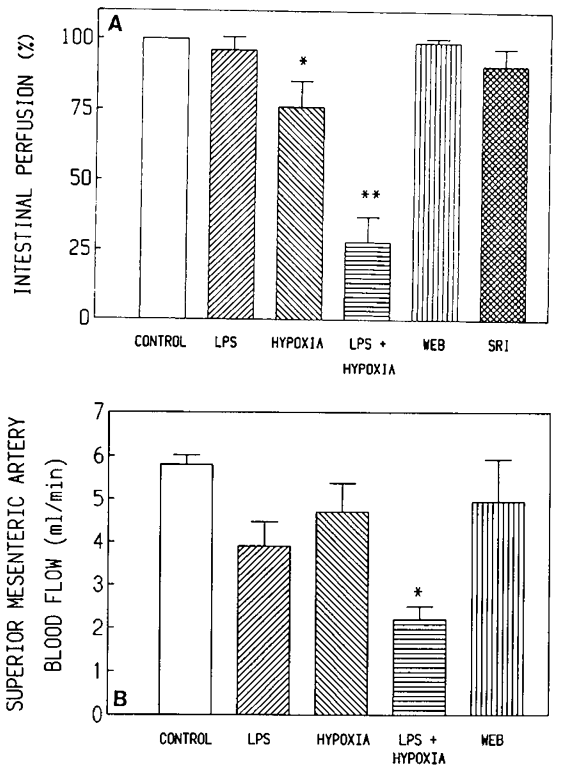

Fig. 2. *A*, Effect of experimental protocol on intestinal perfusion as measured at 3 h by Evans blue dye technique. Perfusion is expressed as % of total intestinal length (see Materials and Methods). Data represent mean % ± SEM. *, $p < 0.05$; **, $p < 0.001$. *B*, Effect of experimental protocol on transit-time ultrasound SMA blood flow measurements. SMA flow is expressed as mean ± SEM. *, $p < 0.05$.

2). Although many risk factors have been identified from epidemiologic studies, the pathophysiology is poorly understood. Perinatal hypoxia is associated with an increased incidence of NEC in some reports (1, 21). In other studies, bacterial infections have been identified in a large percentage of patients with intestinal necrosis (22) and are presumed to play an important role in the pathogenesis. Infection and hypoxia-induced intestinal ischemia are important risk factors for NEC but fail to fully explain its pathophysiology.

PAF is an endogenous phospholipid inflammatory mediator that causes capillary leak, shock, thrombocytopenia, neutropenia, pulmonary hypertension, and bronchoconstriction after intravascular administration into experimental animals (23). In addition, we have previously shown that exogenous intravascular PAF in rats causes intestinal necrosis morphologically similar to neonatal NEC (4). In this model, PAF alone or low dose PAF plus low dose LPS produce intestinal lesions. It is known that LPS increases systemic (24) and intestinal PAF concentrations (5) and that these may contribute to intestinal injury (4). Furthermore, we have shown that severe hypoxia increases plasma PAF concentrations and leads to intestinal hypoperfusion and intestinal injury, which can be prevented by PAF receptor antagonists (13). However, either severe hypoxia or endotoxin alone (even at high doses) produces only superficial mucosal injury in animals, whereas the degree of bowel injury observed in patients afflicted with NEC is usually more severe. In the present study, we have established an experimental model by exposing the animals to both LPS and hypoxia, and this model more closely resembles the intestinal pathology of NEC.

A low dose of LPS was used in the present study. At this dose, LPS alone did not cause intestinal hypoperfusion or significant tissue injury. Other hemodynamic effects were also mild: small decreases in blood pressure and SMA blood flow were observed; hemoconcentration and acidosis were minimal. LPS, however, does cause neutropenia, increased plasma levels of TNF and PAF, and elevated plasma acetylhydrolase activity. Hypoxia alone in this protocol also exerted mild adverse hemodynamic effects: mild hypotension with minimal decrease in SMA flow. The acidosis is slightly more pronounced. There were no changes in WBC count, TNF level, or acetylhydrolase activity. However, hypoxia alone was able to induce intestinal hypoperfusion and tissue injury. Thus, leukopenia (presumably due to neutrophil adherence to the vasculature), TNF production, and a significant drop of SMA flow are not necessary prerequisites for the development of intestinal necrosis in hypoxia. We have previously shown that pretreatment with PAF antagonists prevents hypoxia-induced intestinal injury, suggesting PAF to be the pivotal mediator in this model (13). We also hypothesized that the injurious effect of anoxia itself may contribute to the cell necrosis (13).

When the animal was exposed to LPS (low dose) + hypoxia (Po_2 approximately 6.0 kPa), profound shock with severe drop in SMA flow and marked intestinal hypoperfusion developed. Most animals treated by this combined exposure had significant intestinal injury, compared with none in the LPS group and less than half in the hypoxia group; furthermore, moderate intestinal necrosis was found. These animals also showed significant hemoconcentration (an indicator of vascular leakage). Curiously, there appeared to be no synergy of LPS + hypoxia upon leukopenia, TNF or PAF levels, or plasma acetylhydrolase activity. Because infection and hypoxia are contributing factors in neonatal NEC, this model may more closely approximate the pathophysiology of the clinical disease.

It is of particular interest that the hemodynamic effects of LPS + hypoxia, *e.g.* hypotension, reduced SMA flow, and hemoconcentration, are largely blocked by two structurally different PAF antagonists. Although these agents have no independent effect on resting blood pressure or mesenteric blood flow, they completely prevented LPS + hypoxia-induced intestinal hypoperfusion and necrosis. Whereas these compounds may have other effects on cells independent of their ability to block PAF receptors, these observations indicate that although LPS and hypoxia may each induce release of multiple mediators PAF is a contributing mediator for intestinal injury.

The mechanism by which PAF injures bowel tissue is unclear. Previous experiments have shown that, in addition to causing direct intestinal vasoconstriction, PAF acts via other inflammatory and vasoactive mediators such as leukotrienes (17), norepinephrine (25), activated complement fragments (26), and toxic oxygen radicals (27). Furthermore, PAF is probably also an important mediator in the systemic pathophysiologic changes induced by hypoxia and LPS. This is suggested by our observation that PAF antagonists partially prevent hypotension, hemo-

Table 2. *Gross and microscopic assessment of intestinal necrosis**

Group	Gross necrosis				Histologic score			
	0	Min	Mild	Mod	0	Min	Mild	Mod
Control	100	0	0	0	100	0	0	0
LPS	60	20	20	0	80	20	0	0
Hypoxia	20	80	0	0	0	40	20	20
LPS + hypoxia	0	0	62	38	0	12	12	75
WEB	80	20	0	0	20	80	0	0
SRI	20	80	0	0	60	40	0	0

* Numbers represent the percentage of animals in each group with the appropriate score. Gross and histologic scoring systems are explained in Materials and Methods. For both gross assessment and histology, χ^2 analysis revealed $p < 0.001$. Min, minimal; Mod, moderate.

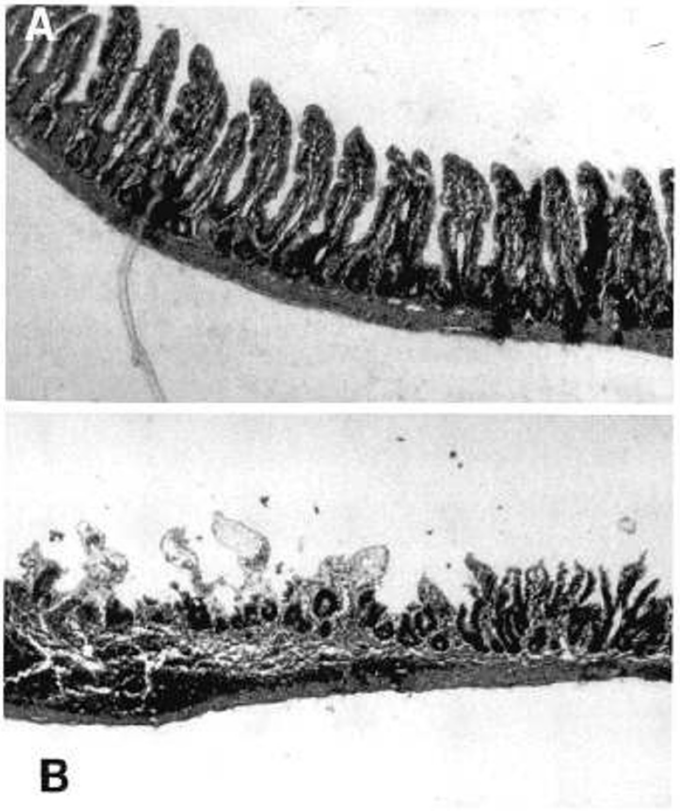

Fig. 3. *A*, Intestinal histology of representative animal pretreated with WEB 2086, then treated with LPS + hypoxia. The villus architecture is normal, and there is minimal mucosal hemorrhage (original magnification ×120). *B*, Intestinal histology from animal treated with LPS + hypoxia. There is almost complete loss of intestinal villi with mucosal hemorrhage (original magnification ×120).

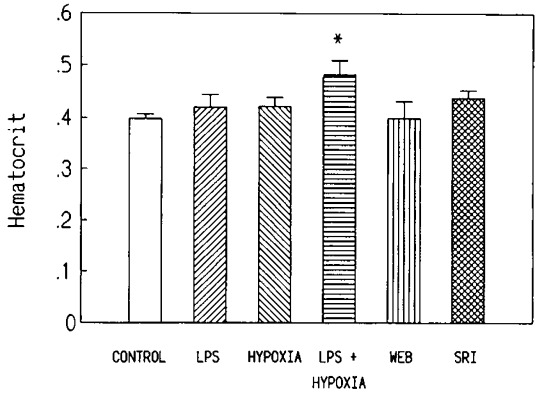

Fig. 4. Effect of experimental protocol on hematocrit at 3 h. Data represent mean ± SEM. Animals treated with LPS + hypoxia had higher hematocrit (0.482 ± 0.028, $p < 0.05$) than all other groups. *, $p < 0.05$.

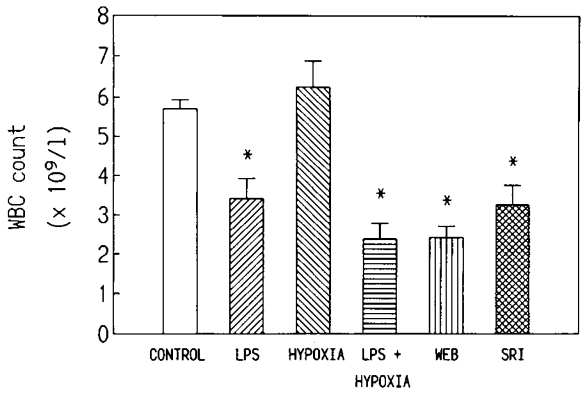

Fig. 5. Effect of experimental protocol on WBC count (cells × 10^9/L) at 3 h. Data represent mean ± SEM. Groups treated with LPS (3.41 ± 0.52 × 10^9/L), LPS + hypoxia (2.38 ± 0.40 × 10^9/L), WEB (2.42 ± 0.29 × 10^9/L), and SRI (3.26 ± 0.50 × 10^9/L) had lower WBC counts than control (5.70 ± 0.23 × 10^9/L) and hypoxia alone (6.25 ± 0.62 × 10^9/L). *, $p < 0.05$.

concentration, and metabolic acidosis. Our hypothesis for the mechanism of shock and tissue injury is as follows. It is known that both LPS and hypoxia can cause endothelial injury (28), thus resulting in capillary leakage and vasodilation. Vasodilation and capillary leakage further lead to hypovolemia, hypotension, hemoconcentration, tissue ischemia, and metabolic acidosis. Finally, shock itself increases circulating plasma PAF levels (29). The exact reason why intestinal tissue is selectively damaged remains unclear.

TNF is a potent inflammatory mediator with cytotoxic actions (12). It is known that LPS administration *in vivo* causes TNF release (30), and that the splanchnic circulation is a significant source of TNF production (31). In addition, experiments suggest that TNF-induced intestinal injury occurs and can be prevented by PAF antagonists (11). Finally, *in vitro*, PAF can stimulate TNF production (32) and TNF can stimulate PAF production (33), suggesting a potential vicious cycle for intestinal injury. In our study, TNF release was noted after LPS injection, but there was no significant difference between animals treated with LPS and those treated with hypoxia + LPS. However, recent studies suggest that a membrane-bound, 26-kD TNF molecule is associated with monocytes and may have biologic activity independent of the 17-kD TNF (34). Further study of TNF in hypoxia-induced intestinal injury is warranted.

There was an increase in PAF-specific acetylhydrolase activity after LPS administration, suggesting that increased PAF levels are due to increased PAF production and not diminished PAF degradation. It is possible that acetylhydrolase may be up-regulated as a defense response, to counteract PAF-induced tissue injury. This response is different from what we found in newborn infants with NEC (7), inasmuch as these patients had lower acetylhydrolase activity than age-matched controls. Interestingly, PAF antagonists had no effect on the LPS-induced acetylhydro-

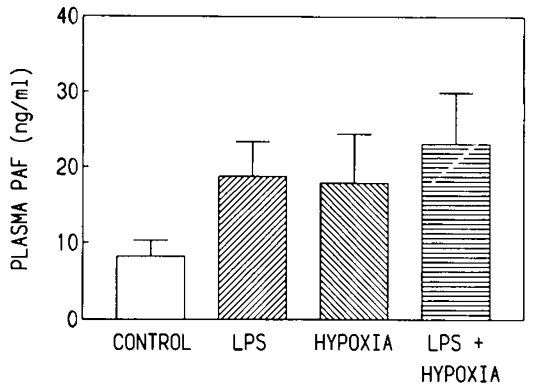

Fig. 6. Plasma PAF concentrations for experimental groups at 3 h as measured by rabbit platelet serotonin release assay (p = NS).

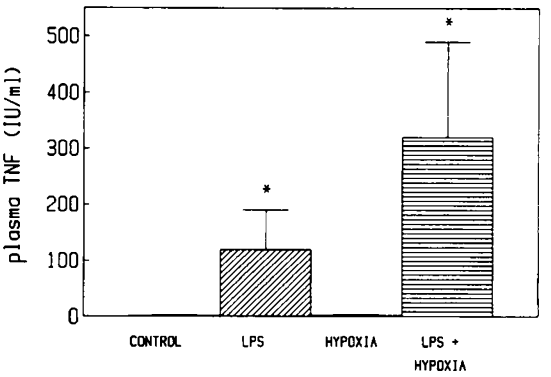

Fig. 7. Plasma TNF concentrations (IU/mL) for experimental groups at 3 h. Data represent mean ± SEM. LPS alone increased circulating TNF (120 ± 70 IU/mL), as did LPS ± hypoxia (320 ± 170 IU/mL). *, $p < 0.01$.

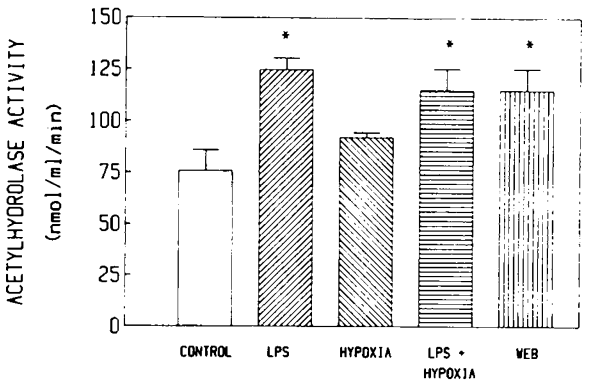

Fig. 8. Plasma acetylhydrolase activity (nmol/mL/min) for experimental groups at 3 h. Data represent mean ± SEM. *, $p < 0.05$.

lase up-regulation, suggesting that agents other than PAF are involved in the regulation of this enzyme.

In summary, we have developed a new model of experimental intestinal necrosis using i.v. endotoxin and alveolar hypoxia together, and have shown that these factors act synergistically to reduce intestinal blood flow and promote intestinal injury. In addition, because PAF receptor antagonists prevent LPS + hypoxia-induced bowel necrosis, it is plausible that PAF is an endogenous mediator in this disease process.

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Announcement

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