
PERSPECTIVES IN PEDIATRIC PATHOLOGY

Neonatal Necrotizing Enterocolitis: Clinical Considerations and Pathogenetic Concepts

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ABSTRACT

Necrotizing enterocolitis (NEC), a disease affecting predominantly premature infants, is a leading cause of morbidity and mortality in neonatal intensive care units. Although several predisposing factors have been identified, such as prematurity, enteral feeding, and infection, its pathogenesis remains elusive. In the past 20 years, we have established several animal models of NEC in rats and found several endogenous mediators, especially platelet-activating factor (PAF), which may play a pivotal role in NEC. Injection of PAF induces intestinal necrosis, and PAF antagonists prevent the bowel injury induced by bacterial endotoxin, hypoxia, or challenge with tumor necrosis factor- α (TNF) plus endotoxin in adult rats. The same is true for lesions induced by hypoxia and enteral feeding in neonatal animals. Human patients with NEC show high levels of PAF and decreased plasma PAF-acetylhydrolase, the enzyme degrading PAF. The initial event in our experimental models of NEC is probably polymorphonuclear leukocyte (PMN) activation and adhesion to venules in the intestine, which initiates a local inflammatory reaction involving proinflammatory mediators including TNF, complement, prostaglandins, and leukotriene C₄. Subsequent norepinephrine release and mesenteric vasoconstriction result in splanchnic isch-

emia and reperfusion. Bacterial products (e.g., endotoxin) enter the intestinal tissue during local mucosal barrier breakdown, and endotoxin synergizes with PAF to amplify the inflammation. Reactive oxygen species produced by the activated leukocytes and by intestinal epithelial xanthine oxidase may be the final pathway for tissue injury. Protective mechanisms include nitric oxide produced by the constitutive (mainly neuronal) nitric oxide synthase, and indigenous probiotics such as *Bifidobacteria infantis*. The former maintains intestinal perfusion and the integrity of the mucosal barrier, and the latter keep virulent bacteria in check. The development of tissue injury depends on the balance between injurious and protective mechanisms.

Key words: bacteria, inflammation mediators, intestinal diseases, necrotizing enterocolitis, neonatal diseases, platelet-activating factor

DEFINITION AND GENERAL CONSIDERATIONS

Necrotizing enterocolitis (NEC) has a multifactorial etiology and an incompletely defined pathogenesis. This disease predominantly affects neonates and produces severe, necrotizing injury to the intestine. Because the underlying clinical cir-

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cumstances are not uniform, NEC may represent a syndrome, with common findings and a variety of etiologies. Intestinal necrosis, representing a late-stage response, is consistent with a common pathogenesis, but disparate etiologies are possible. Necrosis of the intestine can occur at any age following the sudden, complete occlusion of the blood supply to the bowel. In the newborn, thromboemboli secondary to intravascular catheters may cause bowel infarction. However, since neonatal NEC cannot be traced to thromboemboli, it is considered nosologically distinct from this kind of bowel infarction. Therefore, in the following discussion, NEC is understood to exclude cases of bowel infarction associated with thromboembolic lesions.

NEC remains a leading cause of morbidity and mortality in neonatal intensive care units, with a reported incidence of approximately 10% among very low birth weight infants (< 1500 g) [1], and a mortality of 26% [2]. A disease of serious prognosis, advanced cases of NEC may cause multisystem organ failure [3]. Of the 2500 cases occurring annually in the United States [4,5], 20–60% require surgical treatment [6]. At least 80% of patients are preterm, or have low, or very low birth weight, and the incidence of the disease is inversely proportional to the gestational age [4,7,8]. Advances in the supportive care of premature babies, such as the use of surfactant, improved technologies for mechanical ventilation, and wider availability of skilled personnel, enable the very premature to survive, and in so doing increase the population of patients susceptible to NEC. Thus, it may be that despite medical advances that would potentially reduce the incidence of the disease, the incidence of NEC remains unchanged over the last 20 years [1,9]. Infants of extremely low birth weight (< 1000 g) and those 28 wk or less of gestational age are at greater risk of NEC than infants born closer to term. The severity of the disease, risks of complications, and mortality are greater in infants of extremely low birth weight [10].

NEC is uncommon in term infants, in whom it usually appears within 2 to 3 days after birth, whereas in the preterm it begins at 10 to 15 days after birth [11]. Presumably, a postnatal insult is followed by the pathogenetic events that lead to the tissue devastation characteristic of NEC. The initi-

ating and pathogenetic factors may differ in patients of different age groups. In any case, the clinical consequences do not differ substantially in the various patient populations, including the infants of extremely low birth weight or extreme prematurity [10]. The symptoms have been staged according to widely used criteria [12,13]. The infant manifests abdominal distension (among the most common signs of NEC), vomiting, increased gastric residual, lethargy, apnea, bradycardia, or guaiac-positive stools. In stage I, there are no clear radiological signs, and these nonspecific manifestations suggest the disease but give no indication of the status of the bowel or the prognosis. In stage II, the diagnosis is clearly established, with the appearance of pneumatosis intestinalis or free air in the portal vein. Stage III indicates more advanced disease, as manifested by shock, disseminated intravascular coagulation, acidosis, thrombocytopenia, and sometimes intestinal perforation.

PATHOLOGIC ANATOMY OF NEC: CATALOGUE RAISONNÉ OF LESIONS

The predominant anatomic lesion of NEC is coagulative or ischemic necrosis [14–17] (Fig. 1A–C). The usual site is the ileocolic region. This may be because of remoteness of the ileocolic arterial branches from the main blood supply of the superior mesenteric artery, which also supplies the proximal intestine. In about half the cases, the necrosis involves both the small and large intestine; continuous or discontinuous involvement occurs in approximately equal proportions [16,17]. The affected bowel is grossly distended, lusterless, and gray or greenish-gray, but it may be dark purple or black in the areas containing extensive hemorrhage. The soft, fragile wall may perforate when the involvement is severe and transmural. Perforation tends to occur at the junction between normal and necrotic bowel, but it may appear in the midst of a devitalized region, and sometimes at more than one site. Gas bubbles, which may be grossly visible in the intestinal wall, involve the entire colon more commonly in the term infant than in the premature infant [16].

Ischemia in NEC accounts for the necrosis, but the mechanism remains unresolved. Nowicki [18] distinguished extrinsic and intrinsic mecha-

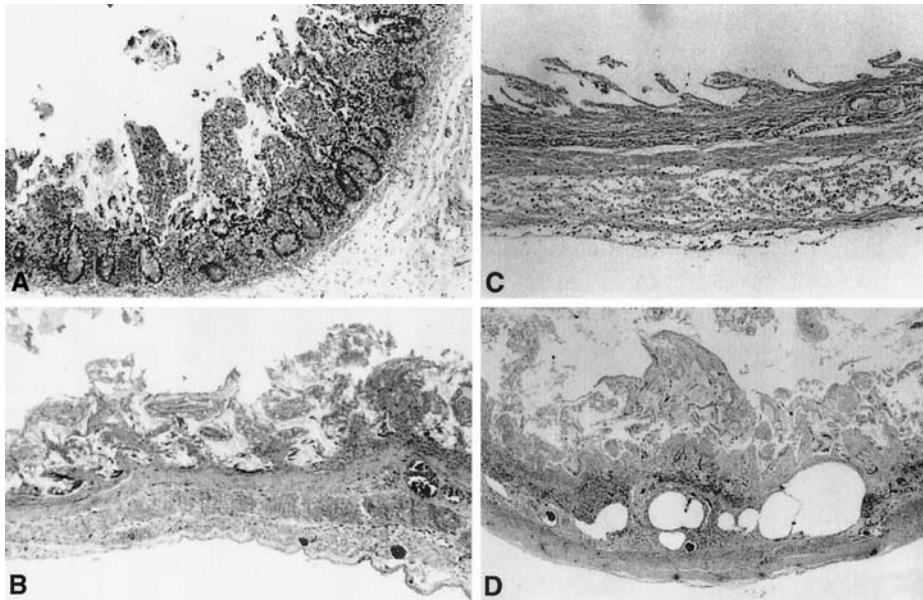


Figure 1. Microscopic appearance of the small intestine from an infant with necrotizing enterocolitis, showing areas of mild mucosal injury (A), extensive mucosal necrosis (B), transmural necrosis (C), and with pneumatosis intestinalis (D). (From Hsueh et al. [132], with permission.)

nisms of vascular regulation. Extrinsic vascular regulation integrates the circulation of the intestine with systemic cardiovascular reflexes. An atavistic “diving reflex” (so named after the physiological changes noted in seals upon diving) [18] has been hypothesized in neonates who experience severe anoxic episodes, during which blood is diverted preferentially to the heart and the brain, to the detriment of the abdominal organs. Although the diving reflex hypothesis is supported by much experimental evidence in animals [18], it cannot satisfactorily explain all the clinical observations in NEC. Presumably, the reflex takes place as a result of a postulated ischemic insult during parturition [19], whereas the manifestations of NEC usually start during the 2nd wk of postnatal life. Vascular reactivity in early postnatal life has been assumed to differ from that of older subjects. However, the intestinal vasculature of 2–3-day-old piglets manifests autoregulatory “escape” from sustained sympathetic stimulation, in the same manner as the intestine of older swine. Experimentally, sympathoadrenergic stimulation causes transient intestinal vasoconstriction, and normal oxygen uptake is restored after 3 to 5 min [20,21]. Moreover, prospective clinical studies do not always establish an association between neonatal hypoxia or asphyxia and the development of NEC: most patients with NEC have no clinically apparent hypoxemia at birth [7,18,20]. These discrepancies by no means exclude an important participa-

tion of autonomic neural influences in the development of NEC. Other extrinsic regulatory mechanisms, such as the participation of the renin-angiotensin axis in bowel ischemia deserve serious investigation. Angiotensin receptors are densely distributed in the bowel [22]. This may explain why ischemic colitis that develops from mesenteric vasoconstriction during experimental cardiogenic shock cannot be prevented by total adrenergic blockade but is completely abolished by drugs such as captopril, which ablate the renin-angiotensin axis [23].

The intrinsic vasoregulation of the intestine, defined as that “mediated by effector mechanisms produced and released within the intestine and its attendant circulation,” has been studied in denervated intestinal segments and other *in vivo* and *in vitro* models [18]. A “metabolic theory” stresses homeostatic control by local tissue need for oxygen, and a “myogenic reflex theory” proposes vasoconstriction in the intestinal circulation in response to changes in venous pressure. Presumably, labile, active myogenic vascular responses in the very young increase their susceptibility to intestinal ischemia [24].

Other “intrinsic” vasoregulatory influences leading to intestinal ischemia include the potent agents that are considered central to a theoretical pathogenesis of NEC (*vide infra*). The clinical situation is more complex than any hypothetical model centered upon experimental observations.

As Kosloske [25] observed, the chronology of clinical events is not always clear. In patients with congenital heart disease or cardiogenic shock, hemodynamic disturbances acquire a significant role in the causation of NEC, but this does not gainsay the utility of clarifying the basic steps by which the disease is initiated and maintained.

Necrosis of the bowel can develop secondary to mesenteric thromboembolism. In neonates, thrombosis is usually an untoward effect of the placement of an umbilical artery catheter. However, in most patients with NEC, no occlusion of large arteries can be identified. NEC and infarction are probably different clinicopathological entities, even though both manifest coagulative necrosis. An infarct is usually single and should follow the distribution of the arterial blood supply. In contrast, NEC is basically an inflammatory process, and a venule may be the initiating site of the pathophysiology. The affected areas are often multiple, are random, and are not necessarily related to the arterial supply. The early histological change of NEC is coagulative necrosis, but inflammatory cells infiltrate when the disease progresses [17]. Bacteria are important in NEC, since the disease does not occur before the colonization of the intestine by bacteria. In a fetus whose intestinal contents are sterile, compromise of the blood supply may result in intestinal injury. In the healing process, atresia or stenosis may develop. Anaerobic bacteria in the lumen of the bowel might be expected to proliferate in a segment of devitalized bowel. Bacterial overgrowth in NEC seems to exceed that in other diseases with ischemic bowel [17]. Intestinal pneumatosis, the peculiar and characteristic finding seen in many cases of NEC, is not observed in infarcts. The formation of gas bubbles within the wall of the intestine (Fig. 1D), develops largely as a result of the fermentation of intraluminal contents by bacteria, and is associated more with NEC than with any other necrotizing conditions affecting the intestine. Bacterial production of P-galactosidase, which reduces pH by fermentation of lactose, has been suggested to contribute to the development of NEC [26]. The ability of colonizing bacteria to ferment lactose is not correlated with the production of NEC [27]; moreover, the endemic cases of NEC are not consistently associated with a single infectious agent

or with a particularly virulent organism that produces highly damaging toxins or that displays great entero-invasiveness or entero-aggregative ability. Disparate microorganisms have been isolated from the stools of NEC patients, and in some cases from both blood and stools: *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Clostridium perfringens*, *Clostridium difficile*, *Clostridium butyricum* [28], coagulase-negative Staphylococci [29], coronavirus, rotavirus, and enteroviruses [30].

Intestinal inflammation affects about 90% of the patients with NEC and is considered an appropriate host response to necrosis and proliferating bacteria [17]. Inflammation tends to be less severe following sudden occlusion of the arterial circulation, as with thromboembolism, and much more conspicuous when devitalization of the bowel is gradual. According to Ballance et al. [17], the character of the inflammation in colitis of infectious origin differs from that in NEC. Microabscesses and crypt abscesses are common in infectious colitis, but they affect only 10% of patients with NEC. Moreover, extensive necrosis beyond the inflammation is a feature of NEC that is generally absent in cases of infectious enterocolitis.

Regenerative changes in NEC are usually marked by replacement of the mucosa by a cuboidal or tall epithelium displaying hyperchromatic nuclei, with mitotic activity and without mucin production. This layer covers granulation tissue or a partly reconstituted lamina propria with distorted, morphologically aberrant glands [15,31]. Regenerative changes may appear even in cases without a protracted history. Ballance et al. [17] found reparative activity of recent onset in 68% of the patients, all undergoing surgery for the first time. These findings suggest that the disease may have started earlier than could be inferred from the degree of severity and/or duration of clinical symptoms.

ANIMAL MODEL 1: BOWEL NECROSIS INDUCED BY PLATELET-ACTIVATING FACTOR, LIPOPOLYSACCHARIDE, AND TUMOR NECROSIS FACTOR- α

We developed a model of bowel necrosis in adult rats and mice by injecting endotoxin (lipopolysaccharide, LPS) [32], PAF (platelet-activating factor,

paf-acether) [33,34], tumor necrosis factor- α (TNF) [35], or a combination of these agents. The rationale for using these agents is as follows:

LPS: NEC is clearly associated with intestinal bacterial growth, since NEC usually develops following oral feeding, and oral feeding markedly increases the growth of *E. coli* in the intestinal tract [36]. No single infectious agent has been isolated consistently from patients with NEC. We hypothesized that resident intestinal flora such as *E. coli* and its toxic product, LPS, would be causative agents of NEC.

PAF: Injection of LPS induces endogenous production of PAF [37,38], systemic administration of PAF [39–41] to animals mimics signs of shock, and PAF antagonists prevent LPS-induced shock [41,42].

TNF: LPS induces endogenous TNF production [38,43,44] and administration of TNF causes shock [45,46], whereas pretreatment of the animal with anti-TNF [46] ameliorates endotoxin shock and increases survival.

PAF, an endogenous phospholipid with potent proinflammatory actions, causes small intestinal necrosis

PAF is an endogenous phospholipid mediator produced by inflammatory cells, endothelial cells, platelets [39,40,47], and bacteria of the intestinal flora, such as *E. coli* [48]. Systemic administration of PAF induces an immediate and sometimes transient hypotensive response. With large doses, the shock becomes profound, irreversible, and intestinal necrosis develops rapidly. Early injury is usually detectable within 15 min. PAF is probably the most potent systemically administered agent for inducing intestinal injury. In our experiments, as little as 2.5 $\mu\text{g}/\text{kg}$ often caused small intestinal necrosis of varying degree in the rat. Since rat platelets are refractory to PAF [33,49], the pathogenesis of necrosis cannot be due to the thromboembolic effect of PAF. The necrosis, usually focal, involved the jejunum, ileum, especially the distal ileum, and/or cecum. With high doses, the entire small bowel could be affected. The necrosis began at the villus tip (Fig. 2A) [33], often involved more than half of the villus (Fig. 2B), and sometimes extended to the submucosa or even the serosa (Fig. 2C).

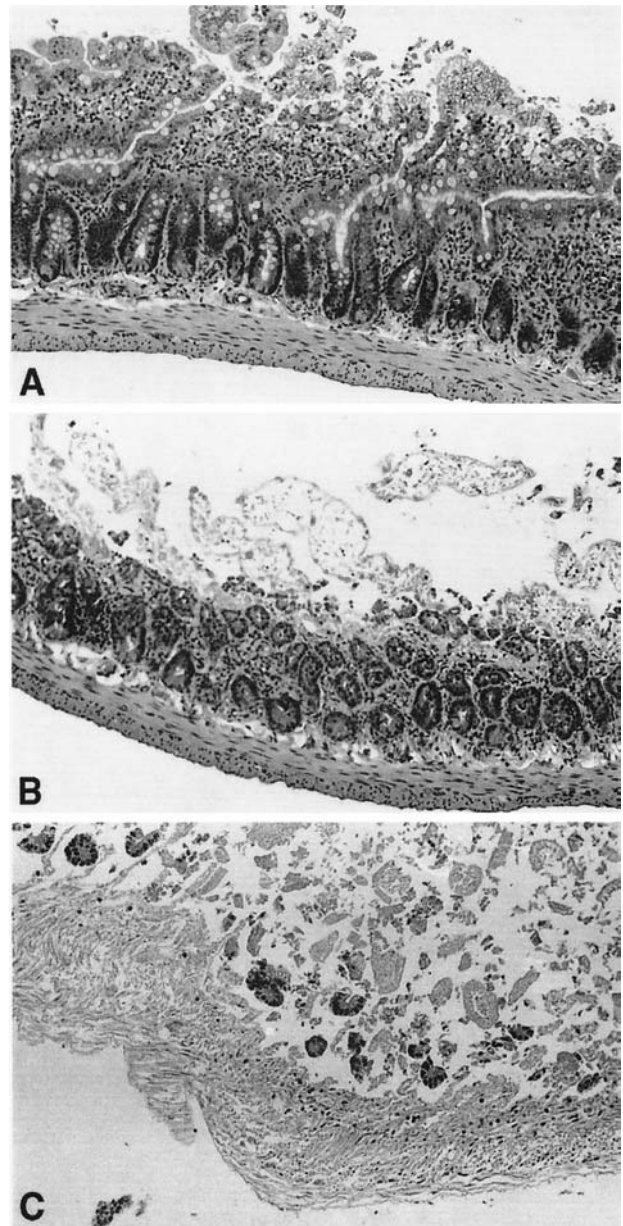


Figure 2. Microscopic appearance of the small intestine from a rat injected with platelet-activating factor (PAF; 2.5 $\mu\text{g}/\text{kg}$) showing early stage of intestinal injury with loss of epithelial cells at the villus tips (A), extensive mucosal necrosis with loss of villi (B), and transmural necrosis (C) (H&E stain). (From Hsueh et al. [132], with permission.)

Although LPS alone can cause hypotension and intestinal necrosis, the required dosage is often high ($> 5 \text{ mg}/\text{kg}$). LPS is a potent “priming” agent for PAF: a small dose of LPS (0.5 mg/kg) acts synergistically with a low dose of PAF (Table 1) [33,34,50]. LPS-induced intestinal injury is blocked by pretreatment with PAF antagonists [32], suggesting that this effect is mediated by endogenous PAF.

Table 1. Synergistic effects of PAF, LPS, and TNF on systemic blood pressure, hematocrit, and intestinal injury in rats^a

Agent (mg/kg)	End blood pressure (mm Hg)	Hematocrit	Gross necrosis (% rats affected)
PAF (0.007) ^a	40 ± 9	59 ± 2	80% mild, ^c 20% moderate ^d
LPS (2) ^a	119 ± 14	44 ± 2	50% mild
PAF (0.007) + LPS (2) ^a	20 ± 6	65 ± 2	100% moderate
LPS (0.2) ^b	95 ± 6	42 ± 1	None
TNF (0.5) ^b	88 ± 8	44 ± 1	None
LPS (0.2) + TNF (0.5) ^b	20 ± 5	46 ± 3	80% moderate, 20% mild

PAF, platelet-activating factor; LPS, lipopolysaccharide (bacterial endotoxin); TNF, tumor necrosis factor- α .

^aAll values were obtained 30 min after the injection of PAF [33]. (In later studies, the dose of PAF used to induce the same degree of bowel necrosis was reduced to 0.002–0.003 mg/kg, when pure C16-PAF was used.)

^bAll values were obtained 2 h after the injection of TNF [35].

^cMild necrosis: involving top third of villi.

^dModerate necrosis: involving more than top one-third of villi, but confined to the mucosa.

One probable reason why the small intestine, in particular the ileum, is especially sensitive to PAF action, is its high content of PAF receptors (PAF-R). Using quantitative polymerase chain reaction (PCR), we found that the ileum has the highest number of PAF-R transcripts: $(3.49 \pm 0.15) \times 10^7$ molecules/ μ g RNA [51]. The PAF-R content of jejunum was only 56% of that of the ileum, and the spleen was only 30%. Other organs, e.g., lung, kidney, heart, stomach, and liver, had less than 1% of that of ileum [51]. PAF, even at doses below those causing bowel necrosis, almost doubled PAF-R mRNA in the intestine [51]. The increase was biphasic; the second peak (at 6 h) seemed dependent on endogenous PAF and TNF [51]. In the small intestine, PAF receptor was localized mainly in epithelial cells and eosinophils of the lamina propria [52].

PAF has a short half-life in the blood, being rapidly degraded by serum acetylhydrolase into the biologically inactive lyso-PAF [53–55]. Paradoxically, the *in vivo* action of PAF is prolonged. One mechanism that may account for this prolonged action is that PAF induces its own production in tissues [56]. When PAF antagonists were given before PAF challenge, the production of PAF (and PAF-like phospholipids) was markedly reduced (Table 2) [32,56]. (Since in these studies we assessed the biological activity, rather than chemical analysis of PAF, we could not differentiate PAF

from PAF-like phospholipids; the latter bind to PAF receptor and have effects that are much like those of PAF [57]).

Polymorphonuclear leukocyte activation and polymorphonuclear leukocyte-endothelial cell adhesion: initial event

The initial event following PAF challenge is probably polymorphonuclear leukocyte (PMN) activation and PMN-endothelial adhesion. PMN-depletion markedly reduced PAF-induced bowel injury [58–60]. The major adhesion molecule involved in the PAF effect is leukocyte β 2-integrin, especially CD11b/CD18, since pretreatment with anti-CD11b or anti-CD18 antibody largely prevents PMN influx as well as PAF-induced bowel injury [60]. Anti-CD18 also prevents the PAF-induced increase in endothelial [61] and mucosal [62] permeability. P-selectin-deficient mice and fucoidin-treated intercellular adhesion molecule-1 (ICAM-1) deficient mice are also protected from the adverse effects of PAF [63], suggesting a possible role of P-selectin. Paradoxically, fucoidin, a potent inhibitor of selectins, shows no protective effect [62,63]. The marked increase in PMN influx (adhered to vessels) is reflected by the increased myeloperoxidase (MPO) content in the intestine [62]. Yet extravascular PMN infiltration is not found by histological examination, indicating that PMN transmigration into tissues is not required for necrosis.

Table 2. List of drugs and agents that prevent or ameliorate PAF-induced intestinal necrosis in rats

Agent	Dose (mg/kg)	Mechanism	Reference
FPL 55712	5	LTC4/D4 antagonist	34
ICI 198615	10–20	LTC4/D4 antagonist	93
Phenoxybenzamine	20	Alpha blocker	93
Superoxide dismutase + catalase	@10 ^a	Oxygen radical scavenger	66
Allopurinol	5	Xanthine oxidase inhibitor	58, 66
WEB 2086	1	PAF antagonist, also blocks endogenous PAF production	50, 56
PGE1	0.27 ^a	Vasodilation, cytoprotection, inhibits norepinephrine	93
Combined antibiotics ^b		Diminish gut flora	88
Anti-PMN serum ^c		PMN depletion	60
Anti-CD18	0.5	Blocks PMN adhesion	60
Anti-CD11b	1.5	Blocks PMN adhesion	60
+Anti-CD11a	0.67		
SIN-1 ^d	1	NO donor	96

LTC4/D4, leukotriene C4/D4; PMN, polymorphonuclear leukocytes; NO, nitric oxide; PG, prostaglandin.

^a@10, total dose of superoxide dismutase and catalase was 10 mg/kg each, slow iv infusion, beginning 30 min before PAF and continuous for 150 min.

^bCombination of neomycin, 250 mg/kg/d, polymyxin B, 9 mg/kg/d, and metronidazole, 50 mg/kg/d in drinking water for 4–5 d.

^c5 ml/kg/d, ip, for 2 d.

^d3-morpholinisydonimine, 30 min before PAF, iv.

Reactive oxygen species produced by intestinal xanthine dehydrogenase/oxidase: final pathway?

The final effector of PAF causing cell injury is most likely reactive oxygen species (ROS). One of the major endogenous sources of ROS in the intestine is the xanthine dehydrogenase/xanthine oxidase system (XD/XO) [64]. XD, the precursor of XO, is constitutively and abundantly expressed in the intestinal villus epithelium [58,65], which catalyzes the conversion of hypoxanthine to xanthine, coupled with the reduction of NAD⁺ to NADPH. Because XO uses molecular oxygen rather than NAD⁺ as an electron receptor and thereby generates superoxide, XD to XO conversion (during ischemia) has been suggested to play the central role in intestinal reperfusion injury [64]. In normal rat intestine, the total XD+XO content (XD/XO ratio approximately 80:20) is higher in the jejunum than in the ileum (the colon has low XD+XO) [58]. Interestingly, following PAF challenge, it is the ileum that shows the most dramatic XD to XO conversion (more than twofold increase in XO) [58]. This change is rapid, detected at 15 min, and by 60 min, more than 60% of the total XD+XO activity has converted to XO [58]. The conversion takes

place mainly in the villus epithelial cells, but not in the crypt epithelium, and the major pathway is probably via activated protease [58]. How this activation is related to PMN activation and adhesion to endothelial cells, remains enigmatic. The central role of XO and ROS in causing the injury is supported by pretreatment with allopurinol [58,66], a xanthine oxidase inhibitor, which largely prevents PAF-induced bowel necrosis (Table 2). Infusion of superoxide dismutase plus catalase also alleviates the injury [66] (Table 2).

TNF induces intestinal injury and endogenous PAF production

TNF has many proinflammatory actions [67–69], such as inducing leukocyte and endothelial adhesion molecules, activating PMNs and endothelial cells, and causing production of other cytokines [67–69], including TNF itself [67–69], eicosanoids [67,68], and PAF [70,71]. Intravenous injection of TNF (1 mg/kg) also induces hypotension and mild intestinal injury in rats [35]. The effects of TNF and LPS are synergistic: TNF (0.5 mg/kg), when combined with LPS (200 mg/kg), causes profound shock and severe intestinal necrosis in rats [35] and mice [72]. PAF is probably the endogenous

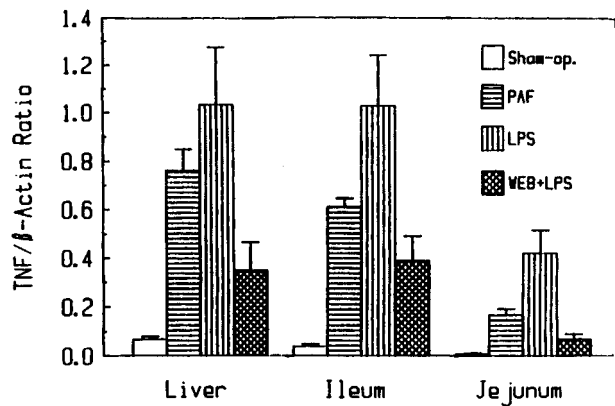


Figure 3. PAF induces tumor necrosis factor (TNF) mRNA production in the rat liver and ileum. mRNA from the liver and the small intestine was extracted 30 min after injection of a low dose (1 μ g/kg, iv) of PAF, LPS (2 mg/kg, iv), or WEB-2086 (1 mg/kg) plus LPS. Results calculated from Northern blot analysis [62]. β -Actin, a housekeeping gene, is used as the common denominator for calculation, and quantity of TNF mRNA is expressed as ratio of TNF mRNA/ β -actin mRNA. Sham-op., sham operation; LPS, lipopolysaccharide (bacterial endotoxin); WEB, WEB-2086. (From Hsueh et al. [132], with permission.)

mediator for TNF/LPS, since PAF was detected after administration of TNF/LPS [35], and pretreatment with a PAF receptor antagonist protects mice from shock induced by TNF/LPS, intestinal injury, and death (Table 1) [35].

PAF induces TNF expression and activates transcription factor nuclear factor κ B in the intestine

The splanchnic bed is considered a major source of TNF production in vivo [73]. We have shown that LPS (2 mg/kg) and PAF (1 μ g/kg), at doses below those causing shock and intestinal injury, stimulate TNF gene expression and protein production in the rat's liver and small intestine, predominantly in the ileum [74] (Fig. 3). WEB-2086, a PAF antagonist, only partially blocked LPS-induced TNF mRNA formation (Fig. 3), suggesting that LPS induces TNF formation via both PAF-dependent and PAF-independent pathways. In normal intestine, TNF is constitutively expressed at very low levels within Paneth cells [75]. During the acute stage of NEC, TNF gene transcripts markedly increase not only within Paneth cells, but also in lamina propria eosinophils, and infiltrating (but not resident) macrophages [76]. Paneth cells are also rich in

group IIA phospholipase A₂ (PLA₂-IIA) [77], an acute phase protein, which is also upregulated by PAF [78].

Production of many proinflammatory cytokines, including TNF, is upregulated by transcription factors, such as nuclear factor κ B (NF- κ B) [79]. TNF activates NF- κ B in vitro [79,80], a pathway that may be involved in TNF self-activation. Low doses of TNF (1 mg/kg) or PAF (1 μ g/kg), which are below those causing shock and intestinal injury, increase the mRNA of NF- κ B precursor, p50/p105, in the small intestine [81]. The action of PAF is as potent as, but more rapid than, that of LPS [81]. PAF also rapidly induces NF- κ B nuclear translocation and activation in the intestine, mainly as p50 homodimers [82]. LPS also activates NF- κ B, but as p50-p65 dimer, and its effect is partly mediated via endogenous PAF and TNF [83]. The role of this transcription factor is unclear, but preliminary experiments show that blocking NF- κ B with decoy [84] or with NF- κ B essential modulator (NEMO) (IKK γ) binding peptide [85] attenuates PAF-induced injury.

PAF increases intestinal mucosal permeability and enhances participation of bacterial products (e.g., LPS) in the pathogenesis of bowel necrosis

A PAF challenge increases gut mucosal permeability [86]. This event precedes cell necrosis, and occurs at doses below that causing necrosis [86]. PAF alters the cytoskeletal structure of the intestinal epithelium and induces tyrosine phosphorylation of E-cadherin, an epithelial membrane component of the zona adherens [86]. This may be physiologic, since glucose-induced increased mucosal permeability is blocked by PAF antagonists [86]. In NEC, this action of PAF may facilitate the entry of bacterial products, e.g., LPS from the gut lumen into the tissues, triggering the inflammatory cascade. Indeed, our data suggest that endogenous bacterial toxins from the intestinal lumen play a central role in PAF-induced shock and bowel injury: (1) endotoxin-resistant mice are protected from PAF-induced intestinal injury [87]; (2) germ-free rats are protected from PAF-induced prolonged shock and bowel injury, and the protection is lost when these animals are primed with exogenous LPS [88]; and (3) conventional rats, treated with combined anti-

biotics which markedly decrease intestinal bacteria, are protected to a large extent from the injurious effects of PAF [88] (Table 2). PAF probably causes intestinal injury and deleterious systemic changes via a synergistic action with endogenous bacterial toxins from intestinal bacteria [34,89]. LPS may not be the only bacterial product that synergizes with PAF to produce tissue damage, since polymyxin B (which inhibits LPS) alone was without protective effects [88].

Other mechanisms of intestinal injury: leukotrienes, catecholamines, complement system, and group II phospholipase A₂

PAF has a prolonged *in vivo* action despite its short half-life in the circulation. Furthermore, PAF is a vasodilator *in vitro* [90], but high doses cause sustained vasoconstriction of the splanchnic bed *in vivo* [90,91]. These apparently paradoxical effects could be reconciled by the observations that leukotriene C₄ (LTC₄) [92] and norepinephrine [93], which cause splanchnic vasoconstriction, are released after PAF injection. Moreover, *in vivo* administration of antagonists to peptide leukotrienes [34,91], or alpha blockers [91], do not reverse shock, but prevent PAF-induced intestinal injury (Table 2).

The complement system, especially C5, may also participate in producing NEC, since the injection of PAF activates the complement system *in vivo* [59], and C5 deficient mice are protected from TNF/LPS- or PAF-induced injury [59,70].

Endogenous protective mechanisms in intestine: nitric oxide and neuronal nitric oxide synthase

Nitric oxide (NO) is produced endogenously by three nitric oxide synthase (NOS) isoforms: the constitutive neuronal (type I) nNOS, the inducible (type II) iNOS, and the endothelial (type III) eNOS [94]. More than 90% of the total NOS in the small intestine is nNOS [95] (Fig. 4A,B). Although iNOS is constitutively present (mainly in the epithelial cells), it accounts for less than 10% of the total NOS activity [95,96], and eNOS is barely detectable in the intestine. PAF rapidly decreases intestinal nNOS protein, mRNA, and enzyme activity [95] (Fig. 4C), but has little effect on iNOS [96]. Interestingly, the degree of injury is inversely re-

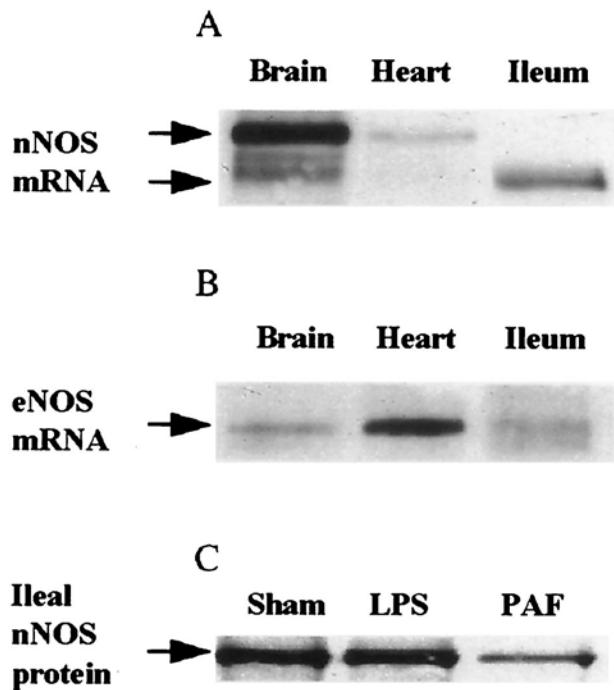


Figure 4. Gene expression and protein content of neuronal nitric oxide synthase (nNOS) in the ileum. **A, B.** Gene expression of nNOS and endothelial nitric oxide synthase (eNOS) in various organs by semiquantitative polymerase chain reaction. The predominant NOS in the brain and ileum is nNOS (A). In contrast, the predominant NOS in the heart is eNOS (B). **C.** Western blot showing rapid down-regulation of nNOS in the ileum by PAF (2.5 g/kg, *iv*).

lated to the nNOS activity, suggesting a protective role of nNOS. The protective role of NO is supported by the following observation: (1) NOS inhibitor L-NAME aggravates PAF-induced necrosis [97]; (2) iNOS inhibitors are protective only when there is "sufficient" nNOS activity [96]; and (3) NO donors significantly reduce PAF-induced bowel injury [96]. NO may help to maintain the integrity of the mucosal barrier and the microvasculature, to increase blood flow, and to inhibit leukocyte adhesion [98].

ANIMAL MODEL 2: HYPOXIA AND LPS/HYPOXIA IN EXPERIMENTAL NEC

Several conditions that involve decreased oxygen delivery to the mesenteric circulation are associated with an increased risk of NEC in human infants. These include asphyxia [99], cyanotic congenital heart disease [100], decreased mesenteric blood flow as reported in intrauterine growth re-

tardation [101], and maternal cocaine use [102]. In animal models of NEC, hypoxia is associated with the development of ischemic bowel necrosis [103] but does not define the mechanism of bowel injury.

We first explored the role of hypoxia in ischemic bowel necrosis using young (25–30-day-old) adult male Sprague-Dawley rats [104]. The animals were exposed to acute severe hypoxia by placing them in 100% nitrogen for 2 min, or to subacute moderate hypoxia by placing them in a 10% oxygen atmosphere for 15 or 30 min. Plasma levels of PAF were markedly elevated in the animals treated with 30 min of moderate hypoxia when compared with controls, and were also elevated in animals treated with only 2 min of severe hypoxia [104]. Thirty minutes of moderate hypoxia produced mild to moderate ischemic bowel necrosis, with no evidence of necrosis in any other organs. (Two minutes of severe hypoxia were not sufficient to induce bowel injury.) The bowel injury was prevented by two structurally unrelated PAF antagonists, WEB 2086 and SRI 63-441. We concluded that hypoxia results in a rapid increase in endogenous PAF levels and that PAF is a mediator of hypoxic intestinal injury.

In addition to decreased mesenteric oxygen delivery, bacterial colonization of the gastrointestinal tract is generally held to be an important prerequisite for the development of NEC [105]. The importance of bacteria can be inferred from the observations that full-blown ischemic bowel necrosis cannot be reproduced in a sterile animal model [106], and, although bowel infarction occurs in the fetuses, typical NEC has never been reported as present at birth or in a stillborn infant [15]. Because hypoxia alone produced relatively mild bowel injury in our model, we hypothesized that hypoxia and bacterial endotoxin (LPS) might act synergistically to produce more severe bowel injury.

We treated young adult male Sprague-Dawley rats with either hypoxia alone (5% oxygen for 90 min), LPS alone (2 mg/kg *Salmonella typhosa* endotoxin, i.v.), or LPS + hypoxia (LPS given at 0 min followed 90 min later by hypoxia for 90 min) [107]. Both LPS alone and hypoxia alone caused little or no intestinal injury, whereas combined treatment with LPS and hypoxia resulted in significantly worse gross and microscopic intestinal in-

jury. This injury was ameliorated by treatment with the PAF antagonists, either WEB 2086 or SRI 63-441. Animals treated with LPS + hypoxia tended to have higher plasma PAF levels than animals in the other groups, but the difference did not reach statistical significance in this study. Both LPS and LPS + hypoxia caused a significant increase in plasma TNF levels. We concluded that LPS and hypoxia act synergistically to produce bowel necrosis and that PAF is an important mediator in this process [107].

We explored the role of endogenous NO in the pathogenesis of hypoxia-induced intestinal injury [108,109]. Inhibition of endogenous NO production with L-arginine analogs significantly worsened the bowel injury produced by 90 min of 10% oxygen exposure, suggesting that endogenous NO production constitutes an important defense mechanism against hypoxia-induced intestinal injury. PAF levels were significantly elevated in the intestines of animals treated with hypoxia and a NO synthase inhibitor, and the intestinal injury seen in these animals was prevented with the PAF antagonist WEB 2086. In the vascular endothelium, NO synthesized from L-arginine by the constitutive form of nitric oxide synthase (cNOS) limits neutrophil adhesion, promotes microvascular integrity, and maintains basal vasodilator tone [110]. In a related study, inhibition of endogenous NO production markedly worsened the bowel injury and intestinal neutrophil accumulation caused by PAF [109].

ANIMAL MODEL 3: NEONATAL NEC— ROLE OF HYPOXIA, ENTERAL FEEDING, AND ENDOGENOUS PAF

A major challenge in understanding NEC in human infants is the absence of a perfect experimental animal model. Although several animal models have been used, most lack some or all of the cardinal features of the human condition. The adult rat model characterizes PAF and other mediators in acute ischemic bowel necrosis, but it lacks the critical predisposing feature of prematurity. The role of PAF in human NEC remains speculative.

In published experiments on neonatal animals, the model of Barlow et al. [111], first described in 1972, most closely resembles human NEC [111–113]. In this model, newborn rat pups

Table 3. Effect of experimental protocol on neonatal NEC and mortality

No. of neonatal rats	Treatment			NEC (%)	Death (%)
	Bacteria	Hypoxia	Formula		
22	+	+	+	77	86
8	+	-	+	0	12
13	+	+	-	0	0
14	-	+	+	57	57
8 ^a	+	+	+	75	100
8 ^a	-	-	+	38	75

NEC, necrotizing enterocolitis.

^aPreterm rat pups.

were removed from their mothers, exposed to maternal milk, stressed briefly with asphyxia, colonized with gram-negative enteric bacteria, and fed with artificial formula. By the 3rd day of life, most animals developed abdominal distention and discoloration, bloody stools, respiratory distress, cyanosis, hemorrhagic intestinal necrosis, and microscopic evidence of severe necrosis identical to the pathology observed in neonatal NEC. Maternal milk, milk leukocytes, immunoglobulin, and oral antibiotics were identified as important for the prevention of NEC [114,115].

We set out to reproduce the findings of Barlow et al. and to better characterize the pathologic findings [116]. Neonatal rats delivered via abdominal incision were maintained in a neonatal incubator and received the following stresses: (1) artificial formula feedings (0.1 ml every 3 h via orogastric tube, 200 cal/kg/d, advanced as tolerated); (2) asphyxia (100% N₂ for 50 s twice daily); and (3) *E. coli* inoculation (1 × 10⁹ organisms/day via orogastric tube). Our data (Table 3) confirm that asphyxia and formula feeding together are necessary to produce NEC in this model. Enteral bacterial inoculation was not a critical factor in our model, since more than half of the animals treated with asphyxia and formula alone developed disease compared to those treated with asphyxia, formula, and bacteria (*P* = NS, not significant). Pathologic findings were similar to human NEC. Grossly, the intestine was hemorrhagic, with friable, occasionally segmental lesions, but often involving most of the intestinal length. In most ani-

mals, necrosis extended from villus to the submucosa (Fig. 5A,B) and often transmurally (Fig. 5C,D).

To evaluate the role of PAF, animals stressed with asphyxia, formula feeding, and bacterial inoculation were compared with those pretreated with the PAF receptor antagonists WEB 2170 and WEB 2086 [117]. WEB 2170 in appropriate enteral dosing (10 mg/kg q am/30 mg/kg q pm) significantly reduced the incidence of NEC and death compared with controls (Table 4). A four-fold higher WEB 2170 dosing regimen did not alter the incidence of NEC, presumably because of an agonist effect on the PAF receptor at very high doses [118]. In contrast, WEB 2086 did not reduce the incidence of NEC in stressed animals, presumably because WEB 2086 has a much shorter half-life than WEB 2170. Intestinal PAF concentrations were elevated (270 ± 80 pg/g) in animals stressed with asphyxia, formula feeding, and bacterial inoculation compared with age-matched, healthy, maternally fed controls (70 ± 50 pg/g, *P* < 0.05). To further clarify the role of endogenous PAF in NEC, neonatal rats were treated with the PAF degrading enzyme, PAF-acetylhydrolase (PAF-AH), as enteral supplementation in doses approximately 10-fold higher than found in human breast milk. This intervention markedly reduced the incidence of NEC from 19/26 in controls to 6/26 (*P* < 0.05) [119]. In addition, PAF-AH (human, recombinant protein) was identified by immunohistochemistry throughout the intestinal tract and remained functionally active for greater than 24 h after dosing [119]. Interestingly, there was no measurable human PAF-AH in the circulation of animals using a sensitive monoclonal antibody/enzyme linked immunosorbent assay (ELISA) technique [119]. Taken together, the data support the hypothesis that endogenous PAF acts as a critical mediator in this neonatal rat model of NEC.

Experimental studies on phospholipase further support the role of PAF in the neonatal rat model. Phospholipase A₂ (PLA₂) consists of a diverse family of enzymes with potent biological activity [120]. Group IIA PLA₂, a secretory form of PLA₂, appears to be important in the inflammatory cascade and may regulate PAF production [121]. The regulation of group IIA PLA₂ mRNA in intestine from animals stressed with asphyxia, formula

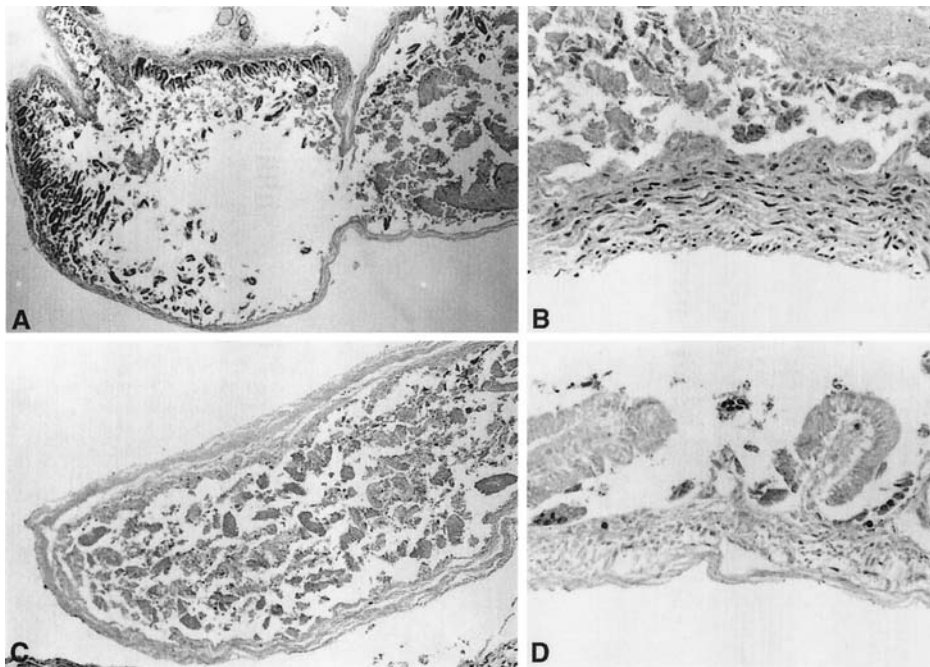


Figure 5. Necrotizing enterocolitis in neonatal rats subjected to asphyxia, formula feeding, and bacteria ingestion. **A,B.** Small intestinal loop showing necrosis with loss of villi. **C,D.** Areas of transmurial necrosis (H&E stain). A and C, low magnification; B and D, high magnification. (From Hsueh et al. [132], with permission.)

Table 4. Effect of PAF receptor antagonists^a on death and NEC in neonatal rats

	WEB 2170 (10/30 mg/kg)	WEB 2170 ×4 (30/120 mg/kg)	WEB 2086 (10/30 mg/kg)
NEC			
Control	14/18 (78%)	10/12 (83%)	9/12 (75%)
WEB treatment	3/17 (18%)*	9/11 (82%)	7/13 (54%)
Death			
Control	17/18 (94%)	11/12 (92%)	9/12 (75%)
WEB treatment	6/17 (35%)*	10/11 (91%)	9/13 (69%)

^aWEB dosing regimen represents a.m./p.m. dosing schedule. WEB 2086 and WEB 2170: PAF antagonists (gifts from Boehringer Ingelheim, Mainz, Germany).

* $P < 0.001$ using Fisher's exact test.

feeding, and bacterial inoculation was compared with control, maternally fed animals. Northern blot analysis using a cDNA probe for group II PLA₂ showed an almost 3.9-fold increase in mRNA in the stressed animals compared with controls ($n = 6$ in each group; Caplan et al., unpublished observations), further supporting the role of PAF activation in the development of NEC.

Protective role of probiotics and polyunsaturated fatty acids in neonatal rat model?

Additional studies were performed to understand the role of bacterial flora and long chain polyunsaturated fatty acids (PUFA) on the pathophysiol-

ogy of NEC. Since healthy breast-milk fed neonates are colonized with multiple flora including a predominance of *Bifidobacteria* and *Lactobacilli*, neonatal animals were treated with 10^9 *Bifidobacteria infantis* organisms/day and evaluated for the development of NEC, endotoxin translocation, mucosal permeability, and PLA₂-II mRNA expression. *Bifidobacteria infantis* supplementation reduced the incidence of NEC (7/24 vs. 19/27 control, $P < 0.05$) but did not alter the colonization pattern of gram negative organisms [122]. *Bifidobacteria infantis* were identified in the stool and intestinal lumen of treated animals but absent in controls. In addition, *Bifidobacteria infantis* treatment markedly reduced PLA₂-II gene expression in intestinal

tissue (42 ± 29 mol/ μ g tissue vs. 802 ± 320 control, $P < 0.01$) but had no effect on mucosal permeability [122]. The results suggest that *Bifidobacteria infantis* reduces the incidence of NEC by altering PAF metabolism and bacterial translocation.

The role of polyunsaturated fatty acids on the inflammatory cascade, especially the omega-3 fish oil preparations, have been well recognized. Since these compounds seem to reduce the incidence of NEC in a human trial, we evaluated them in the neonatal rat model. Animals were treated with arachidonic acid (34 mg/100 ml) and docosahexanoic acid (23 mg/100 ml) and studied for the development of NEC, PLA₂ gene expression, apoptosis, and endotoxin translocation. PUFA supplementation did not alter the semiquantitative assessment of intestinal epithelial apoptosis, but it reduced the incidence of NEC and death compared to controls, and decreased endotoxemia at 24 and 48 h. Furthermore, PUFA decreased PLA₂ mRNA synthesis but had no effect on iNOS gene expression in intestinal homogenate [123].

CORRELATION OF HUMAN NEC WITH EXPERIMENTAL NEC

Experimental evidence strongly supports the role of PAF, LPS, and TNF in acute ischemic bowel necrosis and in the neonatal rat model of NEC. Some data from human studies suggest a similar pathophysiology in neonatal NEC. Local and systemic PAF concentrations are elevated in neonates with NEC, and feeding alone promotes PAF production. We and other investigators found higher circulating plasma levels of PAF and/or PAF-like phospholipid in NEC patients compared with age-matched, illness-matched controls [124,125]. These NEC patients also had higher circulating TNF- α levels [124] and lower plasma acetylhydrolase activity (PAF-degrading enzyme) than control babies. Enteral feeding itself caused elevations of circulating PAF levels in a significant percentage of preterm infants [126], although the circulating acetylhydrolase activity was not affected by the feeding regimen. Circulating PAF may not adequately reflect the activity in the local environment (intestinal lumen/mucosa), but stool PAF concentrations also increased with feedings [127]. Fourteen days after feedings were begun, the PAF levels were approximately threefold higher than prefeed-

ing values (1028 ± 244 pg/g vs. 357 ± 76 pg/g, $P < 0.05$) [126]. Stool samples from seven patients with NEC (stage II or III) had the highest levels, with a mean PAF concentration eightfold higher than controls (2484 ± 154 pg/g) [127].

The apparent increased PAF production in experimental and human NEC fails to explain why NEC exclusively afflicts newborn infants. Several factors may predispose newborns and especially premature infants to NEC, e.g., immature gastrointestinal host defense, dysfunctional mesenteric blood flow autoregulation, and low PAF-degrading enzyme acetylhydrolase (PAF-AH) [55,128]. Although plasma PAF-AH activity is lower in NEC patients than in controls [124], PAF-AH activity is low in newborns as a group, reaching normal adult values at 6 wk of life [129]. Infants fed with breast milk (containing significant PAF-AH activity) have a much lower risk of NEC than infants fed with formula (without measurable PAF-AH activity) [130]. In animal experiments, upregulation of PAF-AH can prevent ischemic bowel necrosis following exogenous PAF infusion [131]. These data strongly support the role of PAF in neonatal NEC and suggest that low neonatal PAF-AH activity may in part explain the neonates' predilection of NEC.

CONCLUSIONS: PROPOSED MECHANISM FOR PATHOGENESIS OF NEC

We hypothesize that the initial insult in the chain of events leading to NEC could be perinatal hypoxia or a mild postnatal infection, either of which results in mild mucosal damage (Fig. 6). Following formula feeding and the proliferation of the intestinal flora, bacteria may attach to the damaged intestinal epithelium because of immaturity of the "mucosal barrier," thus eliciting endogenous production of PAF (and PAF-like phospholipids) and TNF. The major source of PAF may be epithelial cells, lamina propria cells, or endothelial cells. Although gut bacteria may themselves form PAF, the normal mucosal barrier probably prevents any deleterious action on the epithelium. However, in immature or mildly damaged mucosa, the close proximity of bacteria and intestinal epithelial cells may facilitate transcellular permeation of PAF into the mucosa. If the acetylhydrolase is low (as in the case of premature infants), PAF, which increases the intestinal epithelial permeability in vivo, may ac-

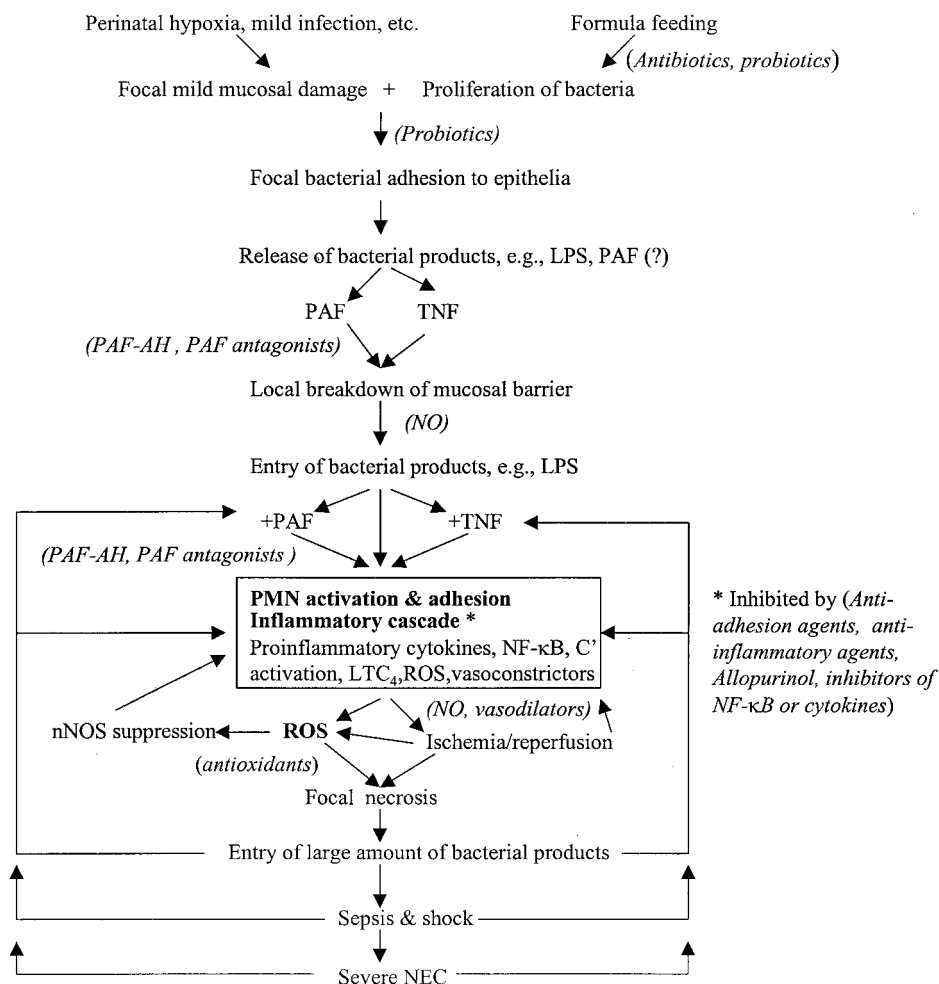


Figure 6. Flow diagram of the proposed pathogenesis of NEC (inhibitors are in *italics* and within parentheses). PAF-AH, PAF acetylhydrolase; NO, nitric oxide; C', complement; NF-κB, nuclear factor-κB; LTC₄, leukotriene C₄; ROS, reactive oxygen species; NEC, necrotizing enterocolitis; PMN, polymorphonuclear leukocyte.

accumulate locally, leading to focal mucosal “leak” and local entry of bacteria or bacterial products. PAF may then synergize with LPS and/or TNF, reaching the threshold necessary to trigger a cascade of inflammatory events: PMN activation and adhesion to venular endothelium, increase in vascular permeability, complement activation, NF-κB translocation, induction of proinflammatory cytokines and adhesion molecules, and release of ROS and inflammatory mediators (including LTC₄, prostaglandins, and PAF). Eventually, vasoconstriction occurs leading to ischemia and subsequent reperfusion. Activation of xanthine oxidase with massive reactive oxygen species production occurs as a consequence of ischemia and/or protease activation. The final result depends on the balance between the injurious mechanisms (inflammatory mediators, cytokines, ischemia) and the protective mechanisms (mainly nNOS). An imbalance favoring the former will result in serious breakdown of the mucosal barrier and bacterial

entry, thereby launching a self-perpetuating vicious cycle, leading to shock, sepsis and, sometimes, death.

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REFERENCES

1. Uauy R, Fanaroff AA, Korones S, et al. Necrotizing enterocolitis in very low birth weight infants: biodemographic and clinical correlates. *J Pediatr* 1991;119:630-638.
2. Hack M, Horbar J, Malloy M, et al. Very low birth weight outcomes of the National Institute of Child Health and Human Development neonatal network. *Pediatrics* 1991; 87:587-597.
3. Morecroft JA, Hamilton PA, Holmes SJK. Necrotizing enterocolitis-multisystem organ failure of the newborn? *Acta Paediatr* 1994;Suppl 396:21-23.
4. Kliegman RM, Fanaroff AA. Medical progress: necrotizing enterocolitis. *N Engl J Med* 1984;310:1093-1103.
5. Neu J, Weiss MD. Necrotizing enterocolitis: pathophysi-

- ology and prevention. *J Parenter Enter Nutr* 1999;23:5 Suppl:S13-S17.
6. Gupta SK, Burke G, Herson VC. Necrotizing enterocolitis: laboratory indicators of surgical disease. *J Pediatr Surg* 1994;29:1472-1475.
 7. Stoll J, Kanto WP Jr, Glass R, et al. Epidemiology of necrotizing enterocolitis: a case control study. *J Pediatr* 1980;96:447-451.
 8. De Curtis M, Paone C, Vetrano G, et al. A case control study of necrotizing enterocolitis occurring over 8 years in a neonatal intensive care unit. *Eur J Pediatr* 1987;146:398-400.
 9. Ryder RW, Shelton JD, Guinan ME. Necrotizing enterocolitis: a prospective multicenter investigation. *Am J Epidemiol* 1980;112:113-123.
 10. Rowe MI, Reblock KK, Kurkchubasche AG, et al. Necrotizing enterocolitis in the extremely low birth weight infant. *J Pediatr Surg* 1994;29:987-991.
 11. Hollwarth ME. Necrotizing enterocolitis: an editorial. *Acta Paediatr* 1994;Suppl 396:1.
 12. Bell MJ, Ternberg JL, Feigin RD, et al. Neonatal necrotizing enterocolitis. *Ann Surg* 1978;187:1-7.
 13. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. *Pediatr Clin North Am* 1986;33:179-201.
 14. Benirschke K. Pathology of neonatal enterocolitis. In: Moore TD, ed. *Necrotizing Enterocolitis in the Newborn Infant: Report of the 68th Ross Conference on Pediatric Research*. Columbus, OH: Ross Laboratories, 1974;29-30.
 15. deSa DJ. The spectrum of ischemic bowel disease in the newborn. *Perspect Pediatr Pathol* 1976;3:273-309.
 16. Polin RA, Pollack PF, Barlow B, et al. Necrotizing enterocolitis in full-term infants: a case-control study. *J Pediatr* 1976;89:460-462.
 17. Ballance WA, Dahms BB, Shenker N, et al. Pathology of neonatal necrotizing enterocolitis: a ten-year experience. *J Pediatr* 1990;117:S6-S13.
 18. Nowicki P. Intestinal ischemia and necrotizing enterocolitis. *J Pediatr* 1990;117:S14-S19.
 19. Lloyd JR. The etiology of gastrointestinal perforations in the newborn. *J Pediatr Surg* 1969;4:77-84.
 20. Kliegman RM, Hack M, Jones P, Fanaroff AA. Epidemiologic study of necrotizing enterocolitis among low-birth-weight infants. *J Pediatr* 1982;100:440-444.
 21. Ross G. Escape of mesenteric vessels from adrenergic and nonadrenergic vasoconstriction. *Am J Physiol* 1971;221:1217-1222.
 22. Sechi LA, Valentin JP, Griffin CA, Schambelan M. Autoradiographic characterization of angiotensin II receptor subtypes in rat intestine. *Am J Physiol* 1993;265:1 Pt 1:G21-G27.
 23. Bailey RW, Hamilton SR, Morris JB, et al. Pathogenesis of nonocclusive ischemic colitis. *Ann Surg* 1986;203:590-599.
 24. Crissinger KD, Kviety PR, Granger DN. Developmental intestinal vascular responses to venous pressure elevation. *Am J Physiol* 1981;130:537-542.
 25. Kosloske AM. A unifying hypothesis for pathogenesis of necrotizing enterocolitis. *J Pediatr* 1990;117:S68-S74.
 26. Carbonaro CA, Clark DA, Elseviers D. A bacterial pathogenicity determinant associated with necrotizing enterocolitis. *Microb Pathog* 1988;5:427-436.
 27. Gupta S, Morris JG, Panigrahi P, et al. Endemic enterocolitis: lack of association with a specific infectious agent. *Pediatr Infect Dis J* 1994;13:728-734.
 28. Kliegman RM, Fanaroff AA, Izant R. Clostridia as pathogens in necrotizing enterocolitis. *J Pediatr* 1979;95:287-289.
 29. Mollitt DL, Tepas JJ, Talbert JL. The role of coagulase negative staphylococcus in neonatal necrotizing enterocolitis. *J Pediatr Surg* 1988;23:60-63.
 30. Rotbart HA, Nelson WL, Glode MP, et al. Neonatal rotavirus-associated necrotizing enterocolitis: case control study and prospective surveillance during an outbreak. *J Pediatr* 1988;112:87-93.
 31. Joshi VV, Winston YE, Kay S. Neonatal necrotizing enterocolitis: histologic evidence of healing. *Am J Dis Child* 1973;126:113-116.
 32. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Platelet-activating factor: an endogenous mediator for bowel necrosis in endotoxemia. *FASEB J* 1987;1:403-405.
 33. Gonzalez-Crussi F, Hsueh W. Experimental model of ischemic bowel necrosis. The role of platelet-activating factor and endotoxin. *Am J Pathol* 1983;112:127-135.
 34. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Platelet-activating factor-induced ischemic bowel necrosis. An investigation of secondary mediators in its pathogenesis. *Am J Pathol* 1986;122:231-239.
 35. Sun XM, Hsueh W. Bowel necrosis induced by tumor necrosis factor in rats is mediated by platelet-activating factor. *J Clin Invest* 1988;81:1328-1331.
 36. Brown EG, Ainbender E, Henley WL, Hodes HL. Etiologic role of bacteria and intestinal function. In: Brown EG, Sweet AY, eds. *Neonatal Necrotizing Enterocolitis*. New York: Grune & Stratton, 1980;69-100.
 37. Feuerstein G, Hallenbeck JM. Prostaglandins, leukotrienes, and platelet-activating factor in shock. *Annu Rev Pharmacol Toxicol* 1987;27:301-313.
 38. Bone RC. The pathogenesis of sepsis. *Ann Intern Med* 1991;115:457-469.
 39. Benveniste J. Paf-acether, an ether phospholipid with biological activity. *Prog Clin Biol Res* 1988;282:73-85.
 40. Hanahan DJ. Platelet activating factor: a biologically active phosphoglyceride. *Annu Rev Biochem* 1986;55:483-509.
 41. Handley DA. Platelet-activating factor as a mediator of endotoxin-related diseases. In: Handley DA, Saunders RN, Houlihan WJ, Tornesch JC, eds. *Platelet-Activating Factor in Endotoxin and Immune Diseases*. New York: Marcel Dekker, 1990;451-495.
 42. Toth PD. The biological effects of PAF antagonists on endotoxemia. In: Handley DA, Saunders RN, Houlihan WJ, Tornesch JC, eds. *Platelet-Activating Factor in Endotoxin and Immune Diseases*. New York: Marcel Dekker, 1990;589-608.
 43. Beutler B, Cerami A. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. *Annu Rev Biochem* 1988;57:505-518.
 44. Zanetti G, Heumann D, Gerain J, et al. Cytokine production after intravenous or peritoneal gram-negative bacterial challenge in mice. Comparative protective efficacy of antibodies to tumor necrosis factor-alpha and to lipopolysaccharide. *J Immunol* 1992;148:1890-1897.
 45. Tracey KJ, Beutler B, Lowry SF, et al. Shock and tissue injury induced by recombinant human cachectin. *Science* 1986;234:470-474.
 46. Tracey KJ, Fong Y, Hesse DG, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* 1987;330:662-664.
 47. Snyder F. Platelet-activating factor and related acetylated lipids as potent biologically active cellular mediators. *Am J Physiol* 1990;259:C697-C708.
 48. Denizot Y, Dassa E, Benveniste J, et al. Paf-acether production by *Escherichia coli*. *Biochem Biophys Res Commun* 1989;161:939-943.
 49. Sanchez-Crespo M, Alonso F, Inarrea P, et al. Vascular

- actions of synthetic PAF-acether (a synthetic platelet-activating factor) in the rat: evidence for a platelet independent mechanism. *Immunopharmacology* 1982;4:173-185.
50. Hsueh W, Gonzalez Crussi F, Arroyave JL, et al. Platelet activating factor-induced ischemic bowel necrosis: the effect of PAF antagonists. *Eur J Pharmacol* 1986;123:79-83.
51. Wang H, Tan X, Chang H, Gonzalez-Crussi F, Remick DG, Hsueh W. Regulation of platelet-activating factor receptor gene expression in vivo by endotoxin, platelet-activating factor and endogenous tumour necrosis factor. *Biochem J* 1997;322:603-608.
52. Wang H, Tan XD, Chang H, Huang W, Gonzalez-Crussi F, Hsueh W. Platelet-activating factor receptor mRNA is localized in eosinophils and epithelial cells in rat small intestine: regulation by dexamethasone and gut flora. *Immunology* 1999;97:447-454.
53. Stafforini DM, Elstad MR, McIntyre TM, et al. Human macrophages secrete platelet-activating factor acetylhydrolase. *J Biol Chem* 1990;265:9682-9687.
54. Tarbet EB, Stafforini DM, Elstad MR, et al. Liver cells secrete the plasma form of platelet-activating factor acetylhydrolase. *J Biol Chem* 1991;266:16667-16673.
55. Tjoelker LW, Wilder C, Eberhardt C, et al. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature* 1995;374:549-553.
56. Zhang C, Hsueh W, Caplan MS, Kelly A. Platelet activating factor-induced shock and intestinal necrosis in the rat: role of endogenous platelet-activating factor and effect of saline infusion. *Crit Care Med* 1991;19:1067-1072.
57. Lloberas N, Torras J, Herreroi-Fresneda I, et al. Postischemic renal oxidative stress induces inflammatory response through PAF and oxidized phospholipids. Prevention by antioxidant treatment. *FASEB J* 2002;16:908-910.
58. Qu X-W, Rozenfeld RA, Huang W, Bulkley GB, Hsueh W. The role of xanthine oxidase in platelet-activating factor induced intestinal injury in the rat. *Gut* 1999;44:203-211.
59. Sun X, Hsueh W. Platelet-activating factor produces shock, in vivo complement activation, and tissue injury in mice. *J Immunol* 1991;147:509-514.
60. Sun XM, Qu XW, Huang W, Granger DN, Bree M, Hsueh W. Role of leukocyte beta 2-integrin in PAF-induced shock and intestinal injury. *Am J Physiol* 1996;270:G184-G190.
61. Kubes P, Suzuki M, Granger DN. Platelet-activating factor-induced microvascular dysfunction: role of adherent leukocytes. *Am J Physiol* 1990;258:G158-G163.
62. Kubes P, Arfors KE, Granger DN. Platelet-activating factor-induced mucosal dysfunction: role of oxidants and granulocytes. *Am J Physiol* 1991;260:G965-G971.
63. Sun X, Rozenfeld RA, Qu X, Huang W, Gonzalez-Crussi F, Hsueh W. P-selectin-deficient mice are protected from PAF-induced shock, intestinal injury, and lethality. *Am J Physiol* 1997;273:G56-G61.
64. Granger DN. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am J Physiol* 1988;255:6 Pt 2:H1269-H1275.
65. Kooij A, Bosch KS, Frederiks WM, Van Noorden CJ. High levels of xanthine oxidoreductase in rat endothelial, epithelial and connective tissue cells. A relation between localization and function? *Virchows Arch [B]* 1992;62:143-150.
66. Cueva JP, Hsueh W. Role of oxygen derived free radicals in platelet activating factor induced bowel necrosis. *Gut* 1988;29:1207-1212.
67. Jaattela M. Biologic activities and mechanisms of action of tumor necrosis factor-alpha/cachectin. *Lab Invest* 1991;64:724-742.
68. Vilcek J, Lee TH. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J Biol Chem* 1991;266:7313-7316.
69. Strieter RM, Kunkel SL, Bone RC. Role of tumor necrosis factor-alpha in disease states and inflammation. *Crit Care Med* 1993;21 (10 Suppl):S447-463.
70. Camussi G, Bussolino F, Salvidio G, Baglioni C. Tumor necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *J Exp Med* 1987;166:1390-1404.
71. Valone FH, Epstein LB. Biphasic platelet-activating factor synthesis by human monocytes stimulated with IL-1-beta, tumor necrosis factor, or IFN-gamma. *J Immunol* 1988;141:3945-3950.
72. Hsueh W, Sun X, Rioja LN, Gonzalez-Crussi F. The role of the complement system in shock and tissue injury induced by tumour necrosis factor and endotoxin. *Immunology* 1990;70:309-314.
73. Fong YM, Marano MA, Moldawer LL, et al. The acute splanchnic and peripheral tissue metabolic response to endotoxin in humans. *J Clin Invest* 1990;85:1896-1904.
74. Huang L, Tan X, Crawford SE, Hsueh W. Platelet-activating factor and endotoxin induce tumour necrosis factor gene expression in rat intestine and liver. *Immunology* 1994;83:65-69.
75. Keshav S, Lawson L, Chung LP, et al. Tumor necrosis factor mRNA localized to Paneth cells of normal murine intestinal epithelium by in situ hybridization. *J Exp Med* 1990;171:327-332.
76. Tan XD, Hsueh W, Gonzalez-Crussi F. Cellular localization of TNF-alpha transcripts in normal bowel and in necrotizing enterocolitis. TNF gene expression in Paneth cells, intestinal eosinophils and macrophages. *Am J Pathol* 1993;142:1858-1865.
77. Nevalainen TJ, Gronroos MJ, Kallajoki M. Expression of group II phospholipase A2 in the human gastrointestinal tract. *Lab Invest* 1995;72:201-208.
78. Tan XD, Wang H, Gonzalez-Crussi FX, Chang H, Gonzalez-Crussi F, Hsueh W. Platelet-activating factor and endotoxin increase the enzyme activity and gene expression of type II phospholipase A2 in the rat intestine. *J Immunol* 1996;156:2985-2990.
79. Collart MA, Baeuerle P, Vassalli P. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κ B-like motifs and of constitutive and inducible forms of NF- κ B. *Mol Cell Biol* 1990;10:1489-1506.
80. Baeuerle PA. The inducible transcription activator NF- κ B: regulation by distinct protein subunits. *Biochim Biophys Acta* 1991;1072:63-80.
81. Tan X, Sun X, Gonzalez-Crussi FX, Gonzalez-Crussi F, Hsueh W. PAF and TNF increase the precursor of NF-kappa B p50 mRNA in mouse intestine: quantitative analysis by competitive PCR. *Biochim Biophys Acta* 1994;1215:157-162.
82. De Plaen IG, Tan XD, Chang H, Qu X-W, Liu Q-P, Hsueh W. Intestinal NF- κ B is activated, mainly as p50 homodimers, by platelet-activating factor. *Biochem Biophys Acta* 1998;1392:185-192.
83. De Plaen IG, Tan XD, Chang H, Liu Q, Remick DG, Hsueh W. Lipopolysaccharide activates nuclear factor κ B in rat intestine: role of endogenous platelet-activating factor and tumor necrosis factor. *Br J Pharmacol* 2000;129:307-314.
84. Wang H, Qu XW, De Plaen I, Hsueh W. Pretreatment

- with NF- κ B decoy ODN reduces PAF-induced bowel necrosis in the rat (Abstract). *FASEB J* 2001;15:A241.
85. De Plaen IG, Wang L, May MJ, Ghosh S, Qu XW, Hsueh W (2002) Selective inhibition of nuclear factor- κ B blocks the bowel injury induced by platelet-activating factor (Abstract 809). *Pediatr Res* 51:139A.
 86. Tan XD, Chang H, Qu XW, Caplan M, Gonzalez-Crussi F, Hsueh W. PAF increases mucosal permeability in rat intestine via tyrosine phosphorylation of E-cadherin. *Br J Pharmacol* 2000;129:1522–1529.
 87. Sun X, Caplan MS, Liu Y, Hsueh W. Endotoxin resistant mice are protected from PAF-induced shock, tissue injury and death. Roles of TNF, complement activation and endogenous PAF production. *Dig Dis Sci* 1995;40:495–502.
 88. Sun X, MacKendrick W, Tien J, et al. Endogenous bacterial toxin is required for the injurious effect of PAF. *Gastroenterology* 1995;109:83–88.
 89. Deitch EA, Ma L, Ma WJ, et al. Inhibition of endotoxin-induced bacterial translocation in mice. *J Clin Invest* 1989;84:36–42.
 90. Siren AL, Feuerstein G. Effects of PAF and BN 52021 on cardiac function and regional blood flow in conscious rats. *Am J Physiol* 1989;257:H25–H32.
 91. Zhang C, Hsueh W. PAF-induced bowel necrosis. Effects of vasodilators. *Dig Dis Sci* 1991;36:634–640.
 92. Hsueh W, Gonzalez-Crussi F, Arroyave SL. Release of leukotriene C4 by isolated, perfused rat small intestine in response to platelet-activating factor. *J Clin Invest* 1986; 78:108–114.
 93. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Sequential release of leukotrienes and norepinephrine in rat bowel after platelet-activating factor. A mechanistic study of platelet-activating factor-induced bowel necrosis. *Gastroenterology* 1988;94:1412–1418.
 94. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001;357:593–615.
 95. Qu XW, Wang H, Rozenfeld RA, Huang W, Hsueh W. Type I nitric oxide synthase (NOS) is the predominant NOS in rat small intestine: regulation by PAF. *Biochim Biophys Acta* 1999;1451:211–217.
 96. Qu XW, Rozenfeld RA, Huang W, Sun X-m, Tan X-d, Hsueh W. Roles of nitric oxide synthases in platelet-activating factor-induced intestinal necrosis in rats. *Crit Care Med* 1999;27:356–364.
 97. MacKendrick W, Caplan M, Hsueh W. Endogenous nitric oxide protects against platelet-activating factor-induced bowel injury in the rat. *Pediatr Res* 1993;34:222–228.
 98. Kubes P, McCafferty DM. Nitric oxide and intestinal inflammation. *Am J Med* 2000;109:150–158.
 99. Wiswell T, Robertson C, Jones T, et al. Necrotizing enterocolitis in full-term infants: a case-control study. *Am J Dis Child* 1988;142:532–535.
 100. Leung M, Chau K, Hui P, et al. Necrotizing enterocolitis in neonates with symptomatic congenital heart disease. *J Pediatr* 1988;113:1044–1046.
 101. Hackett GA, Campbell S, Gamsu H, et al. Doppler studies in the growth retarded fetus and prediction of neonatal necrotizing enterocolitis, haemorrhage, and neonatal morbidity. *Br Med J* 1987;294:13–16.
 102. Czyrko C, Del Pin CA, O'Neill JA, et al. Maternal cocaine abuse and necrotizing enterocolitis: outcome and survival. *J Pediatr Surg* 1991;26:414–421.
 103. Hansbrough F, Priebe CJ, Falterman KW, et al. Pathogenesis of early necrotizing enterocolitis in the hypoxic neonatal dog. *Am J Surg* 1983;145:169–175.
 104. Caplan MS, Sun X-M, Hsueh W. Hypoxia causes ischemic bowel necrosis in rats: the role of platelet-activating factor (PAF-acether). *Gastroenterology* 1990;99:979–986.
 105. MacKendrick W, Caplan M. Necrotizing enterocolitis: new thoughts about pathogenesis and potential treatments. *Pediatr Clin North Am* 1993;40:1047–1059.
 106. Musemeche CA, Kosloske AM, Bartow SA, et al. Comparative effects of ischemia, bacteria and substrate on the pathogenesis of intestinal necrosis. *J Pediatr Surg* 1986; 21:536–537.
 107. Caplan M, Kelly A, Hsueh W. Endotoxin and hypoxia-induced intestinal necrosis in rats: the role of platelet activating factor. *Pediatr Res* 1992;31:428–434.
 108. Caplan M, Hedlund E, Hill N, MacKendrick W. The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. *Gastroenterology* 1994;106:346–352.
 109. MacKendrick W, Caplan M, Hsueh W. Endogenous nitric oxide protects against platelet-activating factor-induced bowel injury in the rat. *Pediatr Res* 1993;34:222–228.
 110. Moncada S, Higgs EA. The L-arginine-nitric oxide pathway. *New Engl J Med* 1993;329:2002–2012.
 111. Barlow B, Santulli TV, Heird WC, et al. An experimental study of acute neonatal enterocolitis—the importance of breast milk. *J Pediatr Surg* 1974;9:587–594.
 112. Topalian SL, Ziegler MM. Necrotizing enterocolitis: a review of animal models. *J Surg Res* 1984;37:320–366.
 113. Crissinger KD. Animal models of necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 1995;20:17–22.
 114. Pitt J, Barlow B, Heird WC. Protection against experimental necrotizing enterocolitis by maternal milk. 1. Role of milk leukocytes. *Pediatr Res* 1977;11:906–909.
 115. Barlow B, Santulli TV. Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. *Surgery* 1975;77:687–690.
 116. Caplan MS, Hedlund E, Adler L, Hsueh W. Role of asphyxia and feeding in a neonatal rat model of necrotizing enterocolitis. *Pediatr Pathol* 1994;14:1017–1028.
 117. Caplan MS, Hedlund E, Adler L, Lickerman M, Hsueh W. The platelet-activating factor receptor antagonists WEB 2170 prevents neonatal necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 1997;24:296–301.
 118. Hu W, McNicholl IK, Choy PC, Man RYK. Partial agonist effect of the platelet-activating factor receptor antagonists, WEB 2086 and WEB 2170, in the rat perfused heart. *Br J Pharmacol* 1993;110:645–650.
 119. Caplan MS, Lickerman M, Adler L, Dietsch GN, Yu A. The role of recombinant platelet-activating factor acetylhydrolase in a neonatal rat model of necrotizing enterocolitis. *Pediatr Res* 1997;42:779–883.
 120. Mukherjee AB, Miele L, Pattabiraman N. Phospholipase A2 enzymes: regulation and physiological role. *Biochem Pharmacol* 1994;48:1–10.
 121. Vadas P, Pruzanski W. Induction of group II phospholipase A2 expression and pathogenesis of the sepsis syndrome. *Circ Shock* 1993;39:160–167.
 122. Caplan MS, Miller-Catchpole R, Kaup S, et al. Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Gastroenterol* 1999;117:577–583.
 123. Caplan MS, Russell T, Xiao Y, Amer M, Kaup S, Jilling T. Effect of polyunsaturated fatty acid supplementation on intestinal inflammation and necrotizing enterocolitis in a neonatal rat model. *Pediatr Res* 2001;49:647–652.
 124. Caplan MS, Sun X-M, Hsueh W, Hageman JR. Role of platelet activating factor and tumor necrosis factor-alpha in neonatal necrotizing enterocolitis. *J Pediatr* 1990;116: 960–964.
 125. Rabinowitz SS, Dzakpasu P, Piecuch S, Leblanc P, Va-

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- lencia G, Kornecki E. Platelet-activating factor in infants at risk for necrotizing enterocolitis. *J Pediatr* 2001;138:81–86.
126. MacKendrick W, Hill N, Hsueh W, Caplan M. Increase in plasma platelet-activating factor levels in enterally fed preterm infants. *Biol Neonate* 1993;64:89–95.
127. Amer MD, Caplan MS. Neonatal necrotizing enterocolitis increases platelet activating factor levels in the stool of newborn infants. *Clin Res* 1994;42:372A.
128. Farr RS, Wardlow ML, Cox CP, Meng KE, Greene DE. Human serum acid-labile factor is an acetylhydrolase that inactivates platelet-activating factor. *Fed Proc* 1983;42:3120–3122.
129. Caplan M, Hsueh W, Kelly A, Donovan M. Serum PAF acetylhydrolase increases during neonatal maturation. *Prostaglandins* 1990;39:705–714.
130. Lucas A, Cole TJ. Breast milk and neonatal necrotizing enterocolitis. *Lancet* 1990;336:1519–1523.
131. Furukawa M, Lee EL, Johnston JM. Platelet-activating factor-induced ischemic bowel necrosis: the effect of platelet-activating factor acetylhydrolase. *Pediatr Res* 1993;34:237–241.
132. Hsueh W, Caplan MS, Tan X, MacKendrick W, Gonzalez-Crussi F. Necrotizing enterocolitis of the newborn: pathogenetic concepts in perspective. *Pediatr Dev Pathol* 1998;1:2–16.