Dual Roles of Endogenous Platelet-Activating Factor Acetylhydrolase in a Murine Model of Necrotizing Enterocolitis

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ABSTRACT: Human preterm infants with necrotizing enterocolitis (NEC) have increased circulating and luminal levels of platelet-activating factor (PAF) and decreased serum PAF-acetylhydrolase (PAF-AH), the enzyme that inactivates PAF. Formula supplemented with recombinant PAF-AH decreases NEC in a neonatal rat model. We hypothesized that endogenous PAF-AH contributes to neonatal intestinal homeostasis and therefore developed PAF-AH$^{-/-}$ mice using standard approaches to study the role of this enzyme in the neonatal NEC model. After exposure to a well-established NEC model, intestinal tissues were evaluated for histology, proinflammatory cytokine mRNA synthesis, and death using standard techniques. We found that mortality rates were significantly lower in PAF-AH$^{-/-}$ pups compared with wild-type controls before 24 h of life but surviving PAF-AH$^{-/-}$ animals were more susceptible to NEC development compared with wild-type controls. Increased NEC incidence was associated with prominent inflammation characterized by elevated intestinal mRNA expression of sPLA$_2$, inducible NOS, and CXCL1. In conclusion, the data support a protective role for endogenous PAF-AH in the development of NEC, and because preterm neonates have endogenous PAF-AH deficiency, this may place them at increased risk for disease. (*Pediatr Res* 68: 225–230, 2010)

Neonatal necrotizing enterocolitis (NEC) is a common and devastating condition that afflicts premature newborn infants and is characterized by acute onset of intestinal inflammatory necrosis. The cause of NEC has not been elucidated, and mortality rates remain unacceptably high (1). Prematurity, formula feeding, intestinal ischemia, and bacterial colonization may contribute to the pathogenesis of NEC, because these stresses initiate proinflammatory signaling and intestinal injury in susceptible hosts. Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent proinflammatory mediator that contributes to the pathogenesis of NEC (2,3). PAF initiates signaling after binding to a G-protein-coupled receptor [PAF receptor, PAFR (4)] and activation of downstream events that result in epithelial apoptosis, leukocyte activation, and the initiation of NEC in experimental systems (5–7). Studies in human infants with NEC revealed increased luminal and systemic accumulation of PAF and decreased expression of PAF acetylhydrolase [PAF-AH, also known as PLA$_2$G7, LpPLA$_2$ (8,9)]. PAF-AH catalyzes the hydrolysis of the acetyl group esterifying the

sn-2 position of PAF, thereby abolishing its biologic activity (10). PAF-AHs exist in secreted (11) and intracellular (12) forms. The secreted form is found in biologic fluids, including plasma (11) and breast milk (13), but is absent in infant formula. Human neonates are deficient in PAF-AH expression and enteral administration of the recombinant enzyme markedly reduced the incidence of NEC in a rat model of the disease (14). To assess the importance of endogenous PAF-AH activity in neonatal NEC, intestinal inflammatory responses, and survival, we developed genetically engineered mice that lack expression of the plasma form of this enzyme. We found that deletion of PAF-AH reduced early mortality associated with bacterial exposure and asphyxia, but that survivors had significantly higher incidence of NEC and increased expression of proinflammatory mediators compared with wild-type mice. These findings suggest that endogenous PAF-AH protects against NEC and that deficiency of this protein, a feature characteristic of preterm infants, increases the risk of developing this disease.

METHODS

Animal model of NEC. All animal protocols were reviewed and approved by the NorthShore University Health System Institutional Animal Care and Use Committee. To investigate the impact of PAF-AH in neonatal NEC, we used a previously described rodent model of the disease (15,16). Neonatal mice were obtained from timed-pregnant animals *via* cesarean section at postconception days E20–21. Wild-type controls were C57BL/6J and the PAF-AH knockouts were backcrossed eight times onto the C57BL/6J parental strain before initiating experiments. Neonatal pups were gavaged using a 1.9F silicone catheter (PICC-NATE, UT Medical Products) containing Esbilac formula (PetAg, Inc.; 1.8 kcal/mL) every 3 h and supplemented with 1×10^7 or 1×10^4 bacterial loads of *Serratia marcescens* and *Streptococcus viridans* in their first feeding after birth and on each succeeding day. Pups were exposed to a brief episode of asphyxia (60 s in 100% N$_2$) followed by cold stress (4°C for 10 min) twice daily. Neonatal mice were monitored for ~24–48 h, a time during which NEC is known to develop. The pups were euthanized when signs of distress were apparent, or at 24–48 h, depending on the experimental design. We then isolated intestinal sections for histologic analyses. Portions of the tissues were snap-frozen in liquid N$_2$ and kept at −80°C for RNA extraction.

Histology. The intestine was removed and linearized in ice-cold normal saline solution. Duodenum, jejunum, ileum, and colon were embedded in OCT, sectioned, and stained with hematoxylin and eosin (H&E). A score was assigned by a blinded reviewer, using the NEC scoring system that we published previously (17). Briefly, intact villi received a score of 0, sloughing of epithelial cells on villous tips was scored 1, mid-villous damage was scored

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; **NEC,** necrotizing enterocolitis; **PAFR,** platelet-activating factor receptor; **PAF-AH,** PAF-acetylhydrolase; **PLA$_2$,** phospholipase A$_2$

2, a score of 3 represented complete villous necrosis, and 4 indicated transmural necrosis. Scores ≥ 2 were considered indicative of NEC.

PAF-AH activity assays. Intestinal PAF-AH activity was assayed according to Narahara *et al.* (18) and Miwa *et al.* (19), with minor modifications. Intestinal lysates were diluted to a concentration of 0.5 mg/mL using 0.25 M sucrose, before the assays. The mixtures included 50 μL of diluted samples, 300 μL of 50 mM Tris-HCl (pH 7.5) containing 0.2% BSA, 25 nmol of cold PAF, and 1 μL of 1-*O*-hexadecyl-2-[[3H]-acetyl] PAF (0.1 mCi/mL, Perkin-Elmer Life and Analytical Sciences, Inc., Boston, MA) in fatty acid-free BSA (0.2% final concentration). The volume was adjusted to 0.5 mL, and the reactions were terminated after 20 min with trichloroacetic acid (14%). One-tenth of the supernatant was added to 5 mL of scintillation fluid (Perkin-Elmer Life and Analytical Sciences), and the released [3H]-acetate was quantified by scintillation counting. Plasma PAF-AH activity was determined as previously described (20).

Quantitative RT-PCR. RNA samples were extracted from the ileum using RNeasy mini kits and Qiacube (Qiagen), following instructions provided by the manufacturer. RNA concentrations were measured by staining using SYBR Green II (Molecular Probes, Inc.). Blanks, standards, and samples were stained in DNAse and RNAse-free TE buffer (1:10,000-fold dilution). We determined the fluorescence intensity of RNA/SYBR Green II complexes and then calculated RNA concentrations based on a standard curve. Each PCR reaction (total volume = 10 μL) contained 0.5 μL of cDNA, 0.5 μL of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (final concentration = 0.5 μM), 0.5 μL of primer mix of gene of interest (final concentration = 0.5 μM), 5 μL of PerfeCTa qPCR SuperMix, UNG (Quanta BioSciences, Inc.), and 3.5 μL of DNAse-free, RNAse-free water. Reactions used cycling conditions that were optimized for these experiments. mRNA levels are expressed relative to those of GAPDH. The sequences of the primers used were previously described (16,21).

Statistics. Parametric data are presented as mean $\pm$ SEM. Comparisons between two groups were carried out using *t* test for unpaired data. Comparisons between three or more groups were performed using ANOVA and differences between groups were assessed using Tukey's post hoc test. Analysis of two independent variables was performed with a two-way ANOVA test. Ordinate data to compare mortality rates and NEC incidence rates were analyzed using a χ^2 test. Unless otherwise stated, a *p* value < 0.05 was considered significant,

RESULTS

Targeted disruption of the mouse PAF-AH gene. A 15.3-kb clone isolated from an adult 129/SvJ genomic DNA library spanned exons 2–7 of the mouse PAF-AH gene and was used to generate a targeting vector in which exons 3 and 4 were replaced by a self-excision cassette, ACN (22) (Fig. 1*A*). The resulting DNA (PAF-AH^{ACN}) was cloned into a plasmid that contained two copies of the TK gene in pBluescript, to generate a targeting vector. Screening was performed by long-range PCR using a primer located 218-bp upstream of the region of homology (p1, Fig. 1*B*) combined with control (p2) and diagnostic (p3) primers (Fig. 1*B*). A positive ES clone was microinjected into 126 blastocysts at Xenogen, and a chimeric male (χ) was bred to C57BL/6J females. Genomic DNA samples from positive and negative ES cells, from a chimeric mouse (χ), and from positive and negative F$_1$ progeny were analyzed in three independent PCR reactions (Fig. 1*C*). The mRNA generated in PAF-AH$^{-/-}$ animals lacked exons 3 and 4, as expected (not shown). We determined the levels of PAF-AH activity in the plasma of wild-type and PAF-AH$^{-/-}$ adult mice. The expression levels were consistent with our predictions based on genetic analyses (Fig. 1*D*). PAF-AH-deficient mice were generated at the University of Utah under the direction of DMS.
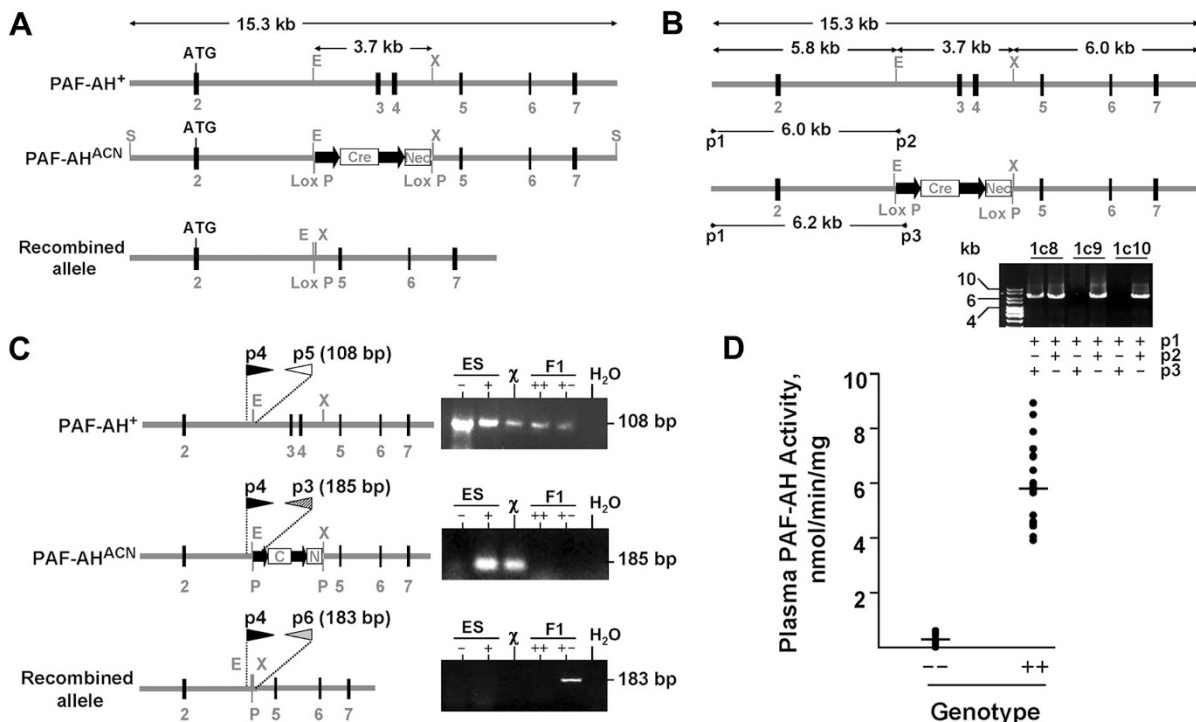
Figure 1. Targeted disruption of the mouse PAF-AH gene. (*A*) Mouse PAF-AH genomic locus and targeting strategy. E, *Eco*RI site; P, loxP site; *S*, *Sal*I site; X, *Xho*I site. (*B*) Long-range PCR analysis used to detect correct targeting in ES cell clones. Each clone was evaluated using control PCR primers (p1 and p2) and diagnostic primers (p1 and p3). The *bottom panel* shows examples of positive (1c8) and negative (1c9, 1c10) ES clones. (*C*) Germline transmission of the targeted allele to the F$_1$ generation was determined by PCR using a variety of primer combinations. The *right panels* show analyses of wild-type (−) and targeted (+) ES cell DNA, tail DNA from a chimeric mouse (χ), and DNA from F1$^{+/+}$ and F1$^{-/+}$ mice. (*D*) Levels of serum PAF-AH activity in wild-type ($n = 20$) and PAF-AH deficient mice ($n = 14$).
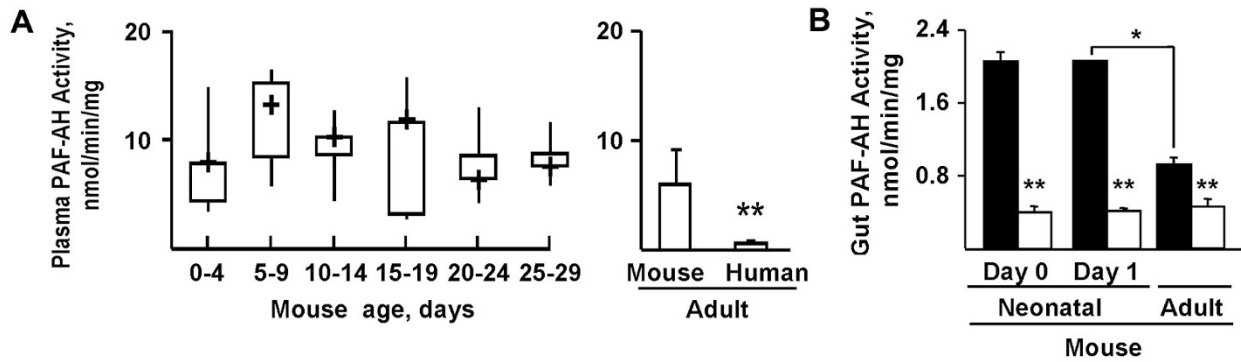
Figure 2. PAF-AH activity in plasma and intestinal tissues of wild-type and PAF-AH$^{-/-}$ mice. PAF-AH activity was determined as the rate of [^{3}H] acetate released from 1-*O*-hexadecyl-[^{3}H-*acetyl*]-PAF and expressed as nmol/min/mg. (*A*) *Box* and *Whisker* plot showing median serum PAF-AH activity in wild-type mice during development. The levels of PAF-AH activity in adult mouse and human serum samples are shown for comparison. *represents $p < 0.01$. (*B*) Gut PAF-AH activity in adult and neonatal wild-type (■) and PAF-AH$^{-/-}$ mice (□). *$p < 0.0001$, age effect, **$p < 0.0001$, genotype effect.
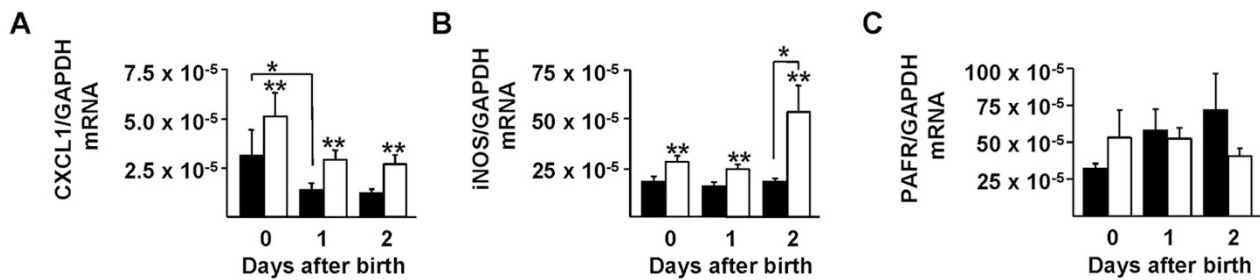
Figure 3. Expression of CXCL1 (*A*), iNOS (*B*), and PAFR (*C*) mRNA in the ileum of wild-type and PAF-AH$^{-/-}$ mice during development. mRNA levels were quantified by RT-PCR in the ileum of mother-fed wild-type (■) and PAF-AH$^{-/-}$ (□) animals at 0, 1, and 2 d after birth. For CXCL1, *$p < 0.01$, age effect and **$p < 0.01$, genotype effect. Two-day old pups displayed statistically higher iNOS levels compared with d 0 and 1 (*$p < 0.01$). In contrast, we detected no genotype- or age-dependent differences in PAFR mRNA levels.

Developmental control of PAF-AH expression in mice. Human infants express low levels of circulating PAF-AH activity and expression levels progressively reach adult values by 6 wk of life (9). We measured serum PAF-AH activity in pups of various ages and compared these values to those found in adult mouse serum samples. We found that neonatal and adult mice expressed comparable levels of PAF-AH activity in the plasma (Fig. 2*A*). All mice expressed higher levels of PAF-AH activity compared with humans (Fig. 2*A*), as previously reported (23).

Expression of PAF-AH in intestinal tissues. We next determined the levels of PAF-AH activity in intestinal tissues of neonatal and adult mice (Fig. 2*B*). We found that wild-type neonatal mice expressed higher intestinal PAF-AH activity compared with adult animals (Fig. 2*B*, two-way ANOVA, $p < 0.0001$, Bonferroni post hoc test). PAF-AH$^{-/-}$ mice expressed significantly lower activity compared with wild-type animals, regardless of age (Fig. 2*B*, two-way ANOVA, $p < 0.0001$, Bonferroni post hoc test). The most robust differences in expression were observed by comparing neonatal PAF-AH$^{-/-}$ *versus* wild-type mice (Fig. 2*B*). These results confirm that deletion of the plasma form of PAF-AH abolishes expression of serum and intestinal activities.

Deletion of PAF-AH increases proinflammatory gene expression. We next sought to characterize the consequences of PAF-AH deletion on the expression of several key proin-

flammatory mediators in healthy, mother-fed neonatal mice. We measured CXCL1 and inducible NOS (iNOS) mRNA expression because these mediators regulate key pathways in intestinal inflammation (24,25) and repair (26). In addition, we evaluated PAFR mRNA levels because this receptor regulates PAF-induced signaling. We found that PAF-AH$^{-/-}$ pups expressed higher levels of CXCL1 compared with wild-type controls (Fig. 3*A*). In addition, we observed that CXCL1 expression decreased with age in mice of both genotypes (Fig. 3*A*, $p < 0.05$; two-way ANOVA). Deletion of PAF-AH also resulted in higher intestinal iNOS expression (Fig. 3*B*, $p < 0.01$, two-way ANOVA). Although the levels of iNOS remained constant in wild-type mice over the period studied, iNOS expression increased significantly by 2 d of life in PAF-AH$^{-/-}$ animals compared with wild-type controls (Fig. 3*B*). In contrast, PAFR expression was similar between wild-type and knockout mice (Fig. 3*C*).

Survival of wild-type and PAF-AH$^{-/-}$ mice after bacterial challenge. To evaluate whether deletion of PAF-AH affected survival and/or NEC incidence, we used our model of NEC that includes asphyxia, formula feeding, and bacterial supplementation (16). When we subjected C57BL/6J wild-type and PAF-AH knockout mice to our model of NEC, we observed high early mortality rates in wild-type cohorts and lower incidence of early death in the PAF-AH knockout strain (Fig. 4). The etiology of early death was initially unclear, but
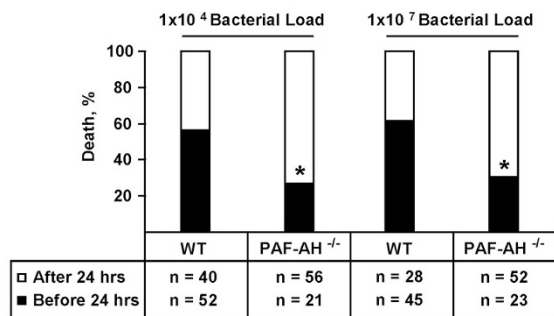
Figure 4. Mortality rates of wild-type and PAF-AH$^{-/-}$ mice before and after 24 h of life. Pups retrieved from timed-pregnant wild-type and PAF-AH$^{-/-}$ mice by cesarean section between E20 and E21 were subjected to our NEC protocol using a 1×10^4 or 1×10^7 bacterial load of *Serratia marcescens* and *Streptococcus viridans* in their first feeding after birth and on each succeeding day. Mortality incidence was significantly higher in wild-type compared with PAF-AH$^{-/-}$ mice before 24 h of life (Fisher's exact test, *p < 0.001).
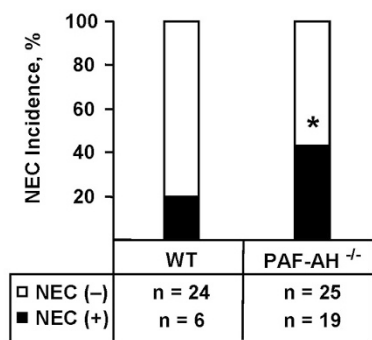
Figure 5. NEC incidence in wild-type and PAF-AH$^{-/-}$ mice after 24 h of life. Intestinal samples were scored on a scale of 0–4 to evaluate NEC severity. Animals that survived longer than 24 h with a score ≥ 2 were considered to have NEC. Data were analyzed using a χ^2 test. The incidence of NEC in PAF-AH$^{-/-}$ pups (43%) was significantly higher than that observed in wild-type controls (20%, *p < 0.05).

further studies revealed the presence of *Serratia marscesens* and *Streptococcal viridans* in the bloodstream of a high percentage of animals. To reduce the risk for sepsis and early death associated with exogenous bacterial supplementation, we lowered the bacterial load to 1×10^4 organisms/animal/d. However, this alteration did not significantly decrease the death rate (Fig. 4). Thus, deletion of PAF-AH decreased the incidence of early death after bacterial supplementation (Fisher's exact test, $p < 0.001$). These results suggest that accumulation of PAF and perhaps other PAF-AH substrates is protective against neonatal bacterial infection.

Susceptibility of wild-type and PAF-AH$^{-/-}$ mice to NEC. We next compared the incidence of NEC in wild-type and PAF-AH$^{-/-}$ animals that survived after 24 h. In these studies, we used a bacterial load of 1×10^4 organisms/animal/d and found higher incidence of NEC in PAF-AH$^{-/-}$ animals (43%, 19 NEC-positive *versus* 25 NEC-negative animals) compared with wild-type pups (20%, 6 NEC positive *versus* 24 NEC-negative animals, χ^2 test, $p < 0.05$) (Fig. 5). Similar to our previous studies, the severity of histologic injury was quite variable in both wild-type and PAF-AH$^{-/-}$ animals, and anatomic location was most prominent in ileum and proximal colon.

Proinflammatory gene expression in cold- and asphyxia-stressed wild-type and PAF-AH$^{-/-}$ mice. We found that PAF-AH$^{-/-}$ pups expressed significantly higher levels of CXCL1 and iNOS compared with wild-type animals (Fig. 6, *A* and *B*, $p < 0.05$; unpaired *t* test with Welch's correction). These differences were observed regardless of whether the animals died before or after 24 h of life (Fig. 6, *A* and *B*) and surpassed the increases in expression observed between healthy, mother-fed, wild-type and PAF-AH$^{-/-}$ mice (Fig. 3). We detected no differences in ileal PAFR mRNA levels in wild-type *versus* PAF-AH$^{-/-}$ pups subjected to our NEC model (Fig. 6*C*), suggesting that the capacity for PAFR-mediated signaling was not affected by deletion of PAF-AH. To investigate whether the potential for PAF production was differentially affected in wild-type and PAF-AH$^{-/-}$ pups, we assessed the levels of ileal sPLA$_2$ in both cohorts. We found that PAF-AH deficient mice subjected to our NEC model
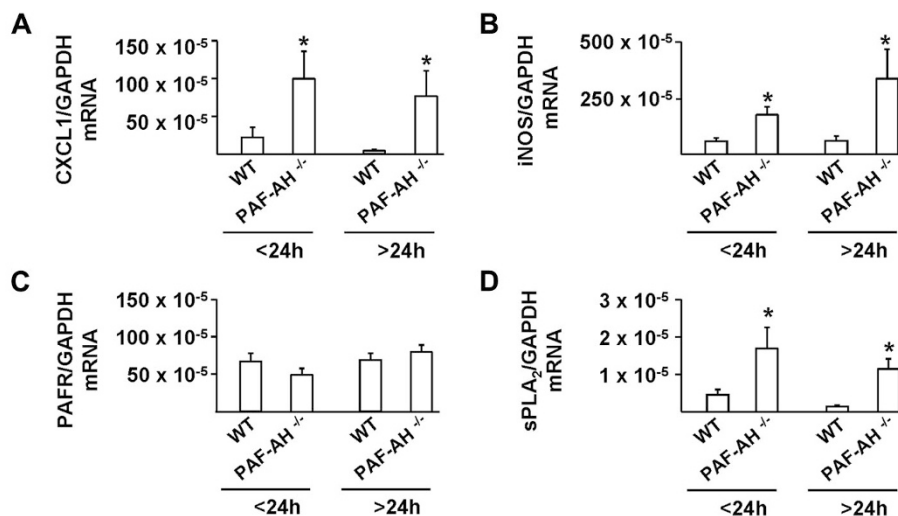
Figure 6. Expression of CXCL1 (*A*), iNOS (*B*), PAFR (*C*), and sPLA$_2$ (*D*) mRNA in mice subjected to a model of NEC. mRNA levels were quantified by RT-PCR in the ileum of wild-type and PAF-AH$^{-/-}$ mice subjected to our model of NEC using 1×10^4 bacterial load of *Serratia marcescens* and *Streptococcus viridans*. PAF-AH$^{-/-}$ expressed higher levels of mRNA for CXCL1 (*p < 0.05; *t* test), iNOS (*p < 0.05; *t* test), and sPLA$_2$ (*p < 0.05; *t* test). In contrast, we detected no genotype- or time-of-death-dependent differences in PAFR expression.

expressed elevated levels of sPLA$_2$ mRNA compared with equally treated wild-type animals, and that the extent of up-regulation varied according to survival. PAF-AH$^{-/-}$ animals that perished before 24 h displayed 3.7-fold higher levels of sPLA$_2$ mRNA compared with wild-type controls (Fig. 6D). PAF-AH$^{-/-}$ pups that survived longer than 24 h displayed 8-fold higher ileal sPLA$_2$ compared with wild-type pups ($p <$ 0.05; unpaired t test with Welch's correction, Fig. 6D). Increased expression of one of the rate-limiting enzymes in the PAF biosynthetic pathway suggests the possibility that a positive feedback loop leading to additional PAF synthesis is operative in PAF-AH$^{-/-}$ pups.

DISCUSSION

In this study, we demonstrate that neonatal mice deficient in endogenous PAF-AH are more susceptible to NEC in response to formula feeding, bacterial colonization, and asphyxia/cold stress, compared with wild-type pups. Because neonatal human infants have very low endogenous PAF-AH activity, this new model provides significant insight into the pathophysiology of neonatal NEC. PAF-AH$^{-/-}$ mice exposed to the NEC protocol expressed significantly higher sPLA$_2$, CXCL1, and iNOS compared with wild-type animals. These findings suggest that PAF-AH deficiency increases sPLA$_2$ and subsequent PAF accumulation, resulting in robust up-regulation of proinflammatory gene expression, intestinal inflammation, and NEC.

Several studies have investigated the importance of PAF and PAF-AH in the pathogenesis of NEC. In animal models, intra-aortic injection of PAF into adult rats caused ischemic intestinal necrosis similar to that observed in patients with NEC (27); pretreatment with dexamethasone or medroxyprogesterone significantly increased plasma PAF-AH activity and prevented the gross and histologic features of NEC (28). These observations support a role for endogenous PAF and PAF-AH activity as opposite players in the control of acute intestinal necrosis. We previously developed a neonatal rat model of NEC that incorporates key features of the human disease, including asphyxia, formula feeding, and bacterial colonization (14). In this model, enteral administration of recombinant PAF-AH significantly reduced the incidence of NEC (14). Our observation that neonatal rodents express high levels of endogenous plasma and intestinal PAF-AH activity (this study) while neonatal humans are deficient (9), led us to hypothesize that neonatal mice deficient in the endogenous enzyme might be a better model of human NEC. This study demonstrates that deficiency of intestinal and circulating PAF-AH markedly increases the severity of inflammation and the incidence of NEC, and due to adequate PAF-degrading capacity, wild-type control mice may not be a reliable model for human, neonatal disease.

We previously demonstrated a significant role for PAF-induced intestinal injury in experimental NEC (2,5). The mechanism whereby deletion of PAF-AH increases NEC severity is likely to involve exacerbated PAF signaling. PAF homeostasis is defined at the biosynthetic and catabolic levels (4). PAF biosynthesis through the remodeling pathway requires participation of specific PLA$_2$ isoforms and recently described lyso-PAF acetyltransferases (29,30). PAF-AH activities down-regulate PAF signaling in several biologic compartments. The observation that human neonates are deficient in circulating PAF-AH activity, combined with the presence of active enzyme in breast milk, led to the hypothesis that milk PAF-AH may confer protection against NEC development in preterm newborns (13). Recent work by Chen *et al.* (31) showed that inhibition of PAF-AH induces PAF accumulation equivalent to that observed after agonist stimulation in quiescent neutrophils and monocytes. These combined observations point to increased PAF signaling as a feature associated with PAF-AH deletion. The functional consequences associated with exacerbated PAF signaling in the intestine remain to be completely characterized, but previous work demonstrated that PAF up-regulates expression of sPLA$_2$ in this organ (32). In this study, we found that deletion of PAF-AH was associated with higher levels of ileal sPLA$_2$ mRNA in pups challenged with our NEC protocol. Thus, a feed forward mechanism of increased sPLA$_2$ and subsequent PAF production may account for enhanced NEC severity in PAF-AH$^{-/-}$ neonatal mice.

Increased inflammation is a feature of neonatal NEC in humans and in animal models of the disease (33–35). The proinflammatory functions of PAF suggest key roles in the final common pathway leading to NEC (2). We found that deficiency of PAF-AH was associated with increased levels of iNOS and CXCL1 (IL-8 in humans), two mediators whose expression has been shown to contribute to intestinal inflammation (36,37). Interestingly, the expression of these proinflammatory genes was elevated in unstressed, PAF-AH deficient animals. However, stress from bacterial exposure, formula feeding, and asphyxia further increased CXCL1 and iNOS mRNA levels in PAF-AH$^{-/-}$ pups compared with wild-type animals. These observations underscore that endogenous PAF-AH has important anti-inflammatory functions in neonatal animals.

Our studies revealed interesting and unexpected findings related to the role of endogenous PAF-AH in the intestine. We found that early mortality was lower (20–30%) in PAF-AH knockout compared with wild-type mice, regardless of bacterial load. Our ability to observe these differences was likely facilitated by the unexpected finding that the C57BL/6J background conferred neonatal mice with exquisite sensitivity to bacterial exposure. Blood culture results from these animals confirmed that most pups died from septic shock. The observation that PAF-AH$^{-/-}$ animals survived significantly longer than wild-type pups when subjected to identical stress suggests that increased local and systemic PAF and/or other PAF-AH substrates may be protective. PAF has been shown to be required for the ability of neutrophils to kill bacteria (38), and this property may attenuate early bacterial translocation, thus limiting events that lead to septic shock in newborns. Our combined results are consistent with a model whereby suboptimal PAF levels predispose an organism to infection, whereas excessive production of the mediator contributes to inflammation and bowel necrosis.

In conclusion, we have demonstrated that genetically deficient PAF-AH mice are protected from early mortality in

response to bacterial exposure, formula feeding, and asphyxia but develop significantly more NEC after 24 h compared with wild-type controls. The incidence of NEC correlates with increased intestinal proinflammatory gene expression in PAF-AH knockout animals. These novel findings identify two different roles for PAF-AH in the regulation of key responses during neonatal development. Our results highlight the potential utility of exogenous PAF-AH supplementation for the prevention of NEC in premature infants.

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