

# Intestinal Epithelial Cell Apoptosis, Immunoregulatory Molecules, and Necrotizing Enterocolitis

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## Abstract

Necrotizing enterocolitis is one of the most severe, life-threatening consequences of premature birth, affecting 5-15% of premature neonates with birth weights <1,500 grams. Many lines of evidence suggest a role for the dysregulation of enterocyte apoptosis in NEC pathogenesis. In addition to apoptosis, the roles of several inflammatory mediators such as platelet-activating factor, IL-8, TNF $\alpha$  and endotoxin have been shown to be pathogenic. Receptors for these ligands and downstream cellular signaling pathways, such as mitochondrial injury-induced caspase activation and NF $\kappa$ B-mediated transcriptional regulation are thought to be involved in the mechanisms of mucosal injury in NEC. In this review, we attempt to summarize the role of enterocyte apoptosis in NEC along with an analysis of the connection between inflammatory signaling and apoptosis in this disease.

## Introduction

The nineteenth century French physiologist Claude Bernard, who is considered to be the father of modern physiology, eloquently defined the concept of homeostasis:

“The living body, though it has need of the surrounding environment, is nevertheless relatively independent of it. This independence, which the organism has of its external environment, derives from the fact that in the living being, the tissues are in fact withdrawn from direct external influences and are protected by a veritable internal environment which is constituted, in particular, by the fluids circulating in the body.” [1]

One of the most important requirements to maintain this homeostasis is the integrity of epithelial tissues, which constitute the barrier and transport facility between the highly regulated internal milieu and the variable outside world. As it is detailed through various chapters of this book, the intestinal epithelium is a dynamic and complex structure and it is in charge of simultaneously forming a barrier and mediating interactions between the internal milieu of the body and the intestinal content, which is a direct extension of the external environment. This interface is extremely intricate due to the number of transport processes that are required for food digestion, absorption of nutrients and regulation of intestinal luminal environment; the presence of vast quantity and diversity of microbes that inhabit the intestinal lumen; and the diverse immune and immune regulatory processes that take place concurrently. This complexity is compounded by the fact that enterocytes have one of the highest turnover rates of all cell types in the human body, with the entire intestinal epithelial lining being renewed every few days. In the early neonatal period, this rapid enterocyte turnover coincides with fast growth and with the adaptation of the intestine to interactions with the extrauterine environment and to the stress of food intake.

In the early neonatal period, the adjustment from being exposed to the very stable amniotic fluid to processing and absorbing food involves large scale changes in gene expression of transporters, enzymes, and receptors, as well as an adaptation of splanchnic circulation to the rapidly increasing energy consumption of the active intestine and to the need for carrying the absorbed nutrients to the systemic circulation. Another part of this adaptation is the colonization of the lamina propria with cells of the adaptive immune system, which enables the host to mount proper immune reactions to invading pathogens. Yet another part of this process is the adjustment of the innate immune system's reactivity, allowing the host to accommodate

the colonization of probiotic bacteria and to reduce reactivity to low levels of bacterial cell wall components or other microbial constituents that may be present in food normally. All of these adaptation processes have important implications for intestinal diseases occurring in the perinatal period. To orchestrate the processing and absorption of nutrients and the aforementioned various dramatic changes, a number of mediators that are not present in the intestinal microenvironment prior to birth must be released in an orderly fashion after oral feeding begins. The colonization of the lamina propria with lymphocytes, granulocytes and monocytes increases the number of highly reactive cell types that are capable of producing cytokines and chemokines which may have profound effects on epithelial physiology and viability. The proper adjustment of innate immune signaling is essential to preempting any unnecessary inflammatory signaling by the epithelium in reaction to normal intestinal luminal content which, in turn, may trigger additional inflammatory signaling by inflammatory cells that are establishing their presence in the lamina propria.

While the aforementioned growth and adaptation processes progress with high fidelity and without major difficulties in mature neonates, in the premature infant these mechanisms appear to be less than perfect, as up to 15% of premature newborns weighing less than 1500 grams suffer from a catastrophic collapse of enteric integrity following the beginning of oral feeding and are affected by a disease termed necrotizing enterocolitis (NEC). Although our understanding of the exact mechanisms that are responsible for the collapse of mucosal integrity is just emerging, there are a number of mediators and cellular processes that have been identified as major players in pathology.

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## The risk factors and clinical features of NEC

NEC is a devastating gastrointestinal disease affecting premature infants, and despite recent advances in neonatology, it remains a leading cause of morbidity and mortality in this high-risk population [2]. The disease incidence varies worldwide, but estimates in babies born weighing less than 1500 grams range from 10% in the U.S. to 14% in Argentina and 28% in Hong Kong. In one report, the incidence of death from NEC was just below that from sudden infant death syndrome, a leading cause of death in infants [3]. The number one predictor of risk for NEC is prematurity. There is a very clear and inverse relationship between gestational age and NEC incidence as well as birth weight and NEC incidence [4]. Compromised circulation and oxygenation likely plays a role, since NEC is observed in full term infants only if disordered circulation occurs as a result of major cardiac malformations, open heart surgery, polycythemia, double-exchange transfusion or birth asphyxia and the incidence of NEC in premature neonates who had congenital heart disease is significantly higher than in infants without heart disease [5]. Breast feeding provides some protection from NEC; therefore, formula feeding is considered to be a risk factor. Aberrant bacterial colonization or exposure to pathogens is thought to predispose to NEC, but this area is not very well characterized in detail [6]. Typically, NEC presents several days after initiation of oral feeding with symptoms of distended abdomen, reduced peristalsis and bloody stool, and the disease often progresses to include systemic signs of inflammation. The definitive diagnosis of NEC is made based on the signs of pneumatosis intestinalis and/or gas in the portal circulation on abdominal x-ray. Currently, the mainstay of initial treatment is non-specific supportive care, and includes the management of systemic inflammation and sepsis. Surgical intervention is provided for signs of intestinal perforation and/or worsening local and systemic signs of disease, and might include a conservative approach with bedside drainage or an exploratory laparotomy with resection of necrotic bowel. There is no clear consensus regarding the benefits of conservative or more invasive surgical management as recent studies have identified similar morbidity and mortality rates amongst these two options. Notably, surgical intervention does not seem to influence mortality in this complex disorder.

## The role of intestinal ischemia

The newborn intestinal vasculature exhibits very low resistance, primarily due to an increased baseline and stimulus-induced production of endothelial-derived nitric oxide [7]. This low baseline vascular resistance limits the ability of the neonatal splanchnic vasculature to adapt to systemic decrease of blood supply or oxygen. Furthermore, due to the role of endothelium in maintaining this low vascular resistance, any endothelial dysfunction may lead to severe vasoconstriction. It has been shown that in human neonatal intestinal microvasculature there is severe vasoconstriction in areas of necrosis and that in these submucosal arterioles there is a defective endothelium-mediated autoregulation in response to pressure changes [8]. This is compounded by an exaggerated endothelin-1-dependent vasoconstrictive response [9]. Tissue ischemia is a potent inducer of inflammatory molecules, including platelet-activating factor [10,11].

## The role of platelet activating factor homeostasis

Studies from our lab and others have shown that PAF plays an important role in the pathophysiology of intestinal inflammation and NEC in adult rats; for example: 1) exogenous PAF given intravenously results in ischemic bowel necrosis [12], 2) endotoxin, hypoxia, or TNF-induced intestinal injury can be prevented by PAF receptor antagonists

[13-15], 3) endotoxin and hypoxia stress increases intestinal PAF content [13]. Additional experiments have evaluated the importance of PAF in neonatal rats using the typical risk factors of NEC, including asphyxia and formula feeding [16]. In this model, we have shown that PAF receptor blockade reduces the incidence of NEC, and that the PAF-degrading enzyme PAF-AH given with enteral feeding interferes with the initiation of NEC [17,18]. Breast milk, which is thought to reduce the risk for NEC, contains significant quantities of this enzyme. Importantly, both PAFR antagonist and PAF-AH protected animals from NEC when they were administered luminally, i.e.; mixed into the formula end without absorbing into the systemic circulation. These data strongly suggest that luminal PAF content and PAFR in the luminal plasma membranes of enterocytes might have important pathological significance.

## PAFR receptor in enterocytes

The PAFR was originally cloned from guinea pig lung [19], then subsequently from a number of other species. Based on its general architecture PAFR belongs to the seven transmembrane domain, G protein coupled receptors (GPCRs). PAFR is expressed on the surfaces of a broad range of cells, including various leukocytes [20,21], endothelial cells [22], neurons [23] and epithelial cells lining the airways [24] and the GI tract [25]. PAFR is expressed at the highest level in intestinal epithelial cells [26,27], yet its physiological function in these cells is poorly characterized. We have found that the PAFR is localized and functions exclusively in the apical plasma membrane in cultured colonic epithelial cells [25]. PAFR activation by mucosal PAF elicits Cl<sup>-</sup> transport in colonocytes [25], apoptosis in small intestinal epithelial cells [28] and intracellular acidification in both colonocytes and small intestinal epithelial cells [29]. Strikingly, PAFR can be activated on the mucosal surfaces of colonic epithelial cells only at three orders of magnitude higher PAF concentrations than in non-polarized cells [25]. These findings correlate with reports indicating a similar low affinity activation in airway epithelial cells [24]. The data suggest that the exclusive apical localization of PAFR has a discrete physiological significance, and that the investigation of PAFR targeting and function in epithelial cells has the potential to reveal details that cannot be obtained from non-polarized cells. It will be essential to characterize the molecular determinants of targeting, trafficking and function of the PAFR in polarized epithelial cells. The PAFR belongs to one of the largest receptor families known in biology, the seven transmembrane domain G protein coupled receptors (GPCR). Therefore, knowledge accumulated about the PAFR in intestinal epithelial biology will have relevance to signaling via many others in this large family.

## GPCRs in intestinal epithelial cells

Cultured intestinal epithelial cells express a number of GPCRs, such as the VIP receptor [30], secretin receptor [31],  $\beta$  adrenergic receptor [32], angiotensin II and lysophosphatidic acid receptors [33], muscarinic acetylcholine receptor [34], PAF receptor [26], proteinase-activated receptors 1 and 2 [35,36], purinergic receptors [37] and the thromboxane receptor (our unpublished data), to name a few. Activation of many of these receptors profoundly affects enterocyte proliferation, differentiation and cell death among their regulatory effects on many other cellular functions. The various GPCRs elicit their effects on enterocytes via multiple distinct signaling mechanisms. Many of these signaling mechanisms were characterized based on the pharmacology of agonist-induced epithelial transport properties. For instance, VIP acts primarily through G $\alpha_s$  and elicits a sustained Cl<sup>-</sup> secretory current via generation of cAMP [38], while purinergic

receptor activation results in an increase of intracellular free calcium via G(i/o) [39], which is a poor direct activator of Cl<sup>-</sup> currents, but results in a large potentiation of cAMP-induced secretory current. Furthermore, while we have overwhelming evidence that PAFR activation is a potent pro-apoptotic signal for enterocytes [28,29,40], activation of other GPCR-s, such as the lysophosphatidic acid receptor is antiapoptotic [41]. Given that both Gs and G(i/o)-dependent signaling has implications on epithelial proliferation and cell death, GPCR signaling is an important aspect of NEC pathogenesis. The best characterized GPCR in NEC pathogenesis is the PAFR, but many other epithelial GPCRs and their signaling mechanisms warrant further investigation in this matter.

### PAFR signaling

Activation of PAF-receptor leads to stimulation of several signal transduction pathways culminating in physiological or pathological regulation of vasoconstriction and/or vasodilatation, leukocyte stimulation and migration, synthesis and activation of cell adhesion molecules, increased capillary permeability, production of reactive oxygen and nitrogen species, and alterations in intestinal mucosal permeability [42,43]. To elicit signaling, the PAFR is capable of linking to both Gs or G(i/o) [44], but it is more commonly found to link to Gi [45,46]. Downstream from G protein activation, PAFR activation results in phosphatidylinositol turnover [47], phosphorylation of signaling proteins, such as  $\beta$ -catenin [48], VE-cadherin [49], ERK1/2 [50], Tyk2 [51], elevation of intracellular free [Ca<sup>++</sup>] [52], protein kinase C activation [53], and subsequent activation of signal transducers and activators of transcription (STATs) [54, 55] and NF $\kappa$ B [56-58]. We identified three major downstream consequences of these PAFR-activation-evoked signaling steps in intestinal epithelial cells that may have significance in NEC pathogenesis. These are 1) regulation of Cl<sup>-</sup> channels [25], 2) regulation of gene expression (our unpublished data) and 3) regulation of apoptosis [28].

### Regulation of epithelial ion transport by PAF

Since many GPCRs, including the the VIP receptor [38], secretin receptor [59],  $\beta$  adrenergic receptor [60], muscarinic acetylcholine receptor [61], protease-activated receptors [35], and purinergic receptors [37] regulate ion transport in enterocytes it is very feasible that PAF does the same. In order to verify this expectation we investigated PAF-induced transepithelial ion transport and have found that indeed PAF activates a transepithelial ion flux in HT29-Cl19A polarized colonic epithelial monolayers [25]. We also found that the PAFRs localize to the apical plasma membrane of polarized colonocytes, suggesting interesting implications for the luminal origin of PAF in NEC pathology. In our more recent studies we have found that in addition to regulating transepithelial ion flux, PAFR activation may lead to intracellular acidification due to release of HCO<sub>3</sub><sup>-</sup> through CLC-3 chloride channels [29]. These findings may be significant because several caspases and apoptosis-related nucleases have acidic pH optima [62] and cytoplasmic acidification has been shown to promote apoptosis [63]. To support this notion, we have found that over-expression of the Na<sup>+</sup>/H<sup>+</sup> exchanger NHE1, or knocking down CLC-3 using shRNA results in inhibition of PAF-induced apoptosis in enterocytes [29].

### Regulation of epithelial gene expression by PAF

Many GPCRs that are expressed in epithelial cells are known to regulate gene expression in various cell types. PAFR signaling specifically, is known to regulate gene expression as activation of

the PAFR leads to expression of immediate, early oncogenes in rat fibroblasts [64], HEC-1A endometrial carcinoma cells [53], and in A-431 human epithelial carcinoma cells [65]. More importantly from the point of view of enteral health, PAFR activation leads to intestinal TNF $\alpha$ , PLA2-II, PAFR gene expression in an *in vivo*, PAF-perfusion-induced bowel necrosis model [66-68]. As discussed above, signaling via the PAFR results in the activation of transcription factors such as STAT [55] and NF $\kappa$ B [58,59]. These transcription factors are likely to play major roles in PAF-induced gene expression regulation. We have found that PAF induces expression of PAFR, PLA2-II and toll-like receptor 4 (TLR4) and 2 (TLR2) in enterocytes (our unpublished data). While the upregulation of PAFR and PLA2-II is evidently important because it suggest that there is an autoregulatory positive feedback loop for PAF-induced cellular effects, the upregulation of TLR-s warrants further discussion.

### The role of TLR-s and bacterial colonization in NEC

Toll-like receptors have been identified as the class of pattern recognition receptors mediating signaling upon the host's encounter with microorganisms. There are now over 10 human TLR-s identified; the most prominent example being TLR4, which recognizes lipopolysaccharide, an endotoxin that is present in the cell wall of gram negative bacteria [69]. As one of the predominant pathways following the initial signaling steps of TLR4 activation is I $\kappa$ B ubiquitination and degradation, enabling NF $\kappa$ B translocation to the nucleus and activation of mRNA transcription, primarily of pro-inflammatory cytokines [70]. Data from neonatal rodents and human fetal tissue explants suggest that this pro-inflammatory pathway is prone to excessive activation in the neonatal period, even more so in premature rodents and humans [71,72]. The reason for this hypersensitivity is only partly understood but recent findings have shed some light on the underlying mechanisms. Unlike in the mature, healthy intestinal epithelial cell, where TLR4 is poorly expressed, in the neonate that has experienced asphyxia and formula feeding, TLR4 is up-regulated on the luminal side of the intestinal epithelium, thereby allowing for gram negative bacteria or their cell wall components to activate TLR4 signaling [73]. Another line of evidence suggests that I $\kappa$ B expression in epithelial cells is lower in fetal and premature neonatal intestine and activation of TLR signaling results in higher levels of I $\kappa$ B phosphorylation in these premature cells than in more mature enterocytes [72]. These findings together suggest that a combination of higher receptor expression, diminished inhibitory capacity by I $\kappa$ B and more active signaling contribute to the exaggerated NF $\kappa$ B activation and inflammatory mediator production in the premature gut. The pathological significance of TLR4-dependent and NF $\kappa$ B-mediated pro-inflammatory signaling in NEC is underscored by the observation in two independent studies that TLR4 mutant mice are protected from experimental NEC and additional findings that NF $\kappa$ B inhibitors reduce the risk for experimental NEC [71,73].

### Enterocyte apoptosis in NEC

The layer of enterocytes forms a dynamic barrier between the balanced milieu intérieur [1] and the intestinal luminal content. It has been postulated that a collapse of this barrier is an important step in NEC pathogenesis [74]. One obvious mechanism that may damage the integrity of this barrier is the death of enterocytes on a massive scale. Investigation of human intestinal samples that was obtained during surgery for NEC revealed that there is an abundance of apoptotic nuclei in the epithelium of intestines affected by NEC [75]. To determine whether this observation is a mere coincidence, or whether apoptosis may indeed play a pathological role in NEC, we investigated

the role of apoptosis in a well established animal model of NEC. In addition to confirming the findings in the human specimens that NEC is accompanied by profuse enterocyte apoptosis, these animal studies have shown that apoptosis occurs prior to gross histological damage and, more importantly, inhibition of apoptosis by chemical caspase inhibitors preempted the development of experimental NEC [76]. Since this early observation, several studies have shown that the development of experimental NEC is halted by various growth factors that cause enterocyte cell survival, such as EGF [77], IGF-I [78] and hb-EGF [79], or by a blocking antibody to TNF $\alpha$  [80] which is a proapoptotic molecule for enterocytes *in vitro* [81].

### The role of defective repair mechanisms in NEC

Given that there are repair mechanisms to correct the defects in the epithelial lining that are caused by apoptosis, it has been postulated that these repair mechanisms are likely to be deficient in premature neonates, otherwise the damage caused by apoptotic death could be bypassed. An evidence for such defective repair mechanisms was found when the role of TLR4 was evaluated in a neonatal murine model of NEC. It was found that in experimental NEC, in addition to increased apoptosis as was shown earlier, there was a reduced epithelial cell proliferation and defective migration along the crypt villus axis; these defects required intact TLR4 signaling and were accompanied with an increased phosphorylation of focal adhesion kinase (FAK) on S722, i.e., a phosphorylation state of FAK that inhibits epithelial cell migration [82]. *In vitro*, TLR4 activation resulted in FAK phosphorylation and inhibited enterocyte migration.

### Mechanisms leading to large scale enterocyte apoptosis

We are only beginning to understand the mechanisms of NEC but there is a convergence of evidence indicating a role for premature and exaggerated enterocyte apoptosis in the disease. To understand the underlying mechanisms that may be responsible for the large scale apoptosis of enterocytes, multiple studies have investigated the mediators and mechanisms regulating enterocyte apoptosis. Cell survival is regulated by a balance of survival and death signals. Cell death is initiated by either a loss of survival signal or by the activation of an active cell death signal. Survival signal for enterocytes comes from three principal sources: 1) cell to extracellular matrix attachment [Strater, 1996 #5668], 2) homotypic cell adhesion [Ireland, 2004 #5669] and 3) growth factor receptor activation [Clark, 2005 #5413]. Cell death signals may come from either the loss of any of the above 3 survival signals or by evoking a number of cell death signals, including: 1) nutrient deprivation [Lemasters, 2005 #5670], 2) DNA damage [Okudela, 1999 #5671], 3) activation of a death receptor pathway [Tang, 2004 #5672], 4) mitochondrial damage [Lu, 2004 #5013] or 5) signaling that may uncouple any of the survival signals.

### Growth factors and NEC

In the neonatal period, an important source of survival signals for enterocytes derives from human milk. There is evidence to indicate that preterm infants who are fed maternal milk are significantly less likely to develop NEC than those infants fed commercial infant formula [83]. Breast milk contains several growth factors that are known to promote epithelial survival such as epidermal growth factor (EGF) [84], heparin-binding EGF-like growth factor (hb-EGF) [85], hepatocyte growth factor (HGF) [86], insulin-like growth factor (IGF) [87], transforming growth factor-beta (TGF- $\beta$ ) [88], erythropoietin [89] and vascular endothelial growth factor (VEGF) [90]. Several of these factors have recently been found to be potentially protective against development

of NEC when given as formula supplementation in animal studies [79,91] and all of these growth factors have been shown to promote enterocyte survival either *in vivo* or *in vitro* [78,86,92-95]. Commercial formulas are devoid of these growth factors and, therefore, enterocytes of formula-fed infants are deprived of the exogenous supplementation of trophic and survival signals imparted by these molecules. However, NEC is extremely rare in full term infants even if they receive 100% of their nutrition from formula instead of breast milk. This is not necessarily surprising, since there are endogenous sources for all of these growth factors and there is evidence that several pro-apoptotic mechanisms are exaggerated in premature newborns. Nevertheless, a better understanding of endogenous sources for these growth factors and the developmental regulation of their expression in endogenous sources may be important. For instance, EGF and IGF is produced in the salivary gland [96,97] and a recent study has shown a gestational-age dependent increase in salivary EGF output while revealing a correlation between salivary EGF release kinetics and the incidence of NEC [98]. Similar information regarding developmental regulation of endogenous sources of other milk-derived epithelial-protective growth factors is not yet available, but should be investigated. However, it is well established that there are several mechanisms in addition to growth factor withdrawal that may actively signal cell death and several of these appear to play roles in NEC pathogenesis.

### Pro-apoptotic signaling in NEC

Some GPCRs have been shown to signal cell survival in enterocytes, such as cholinergic receptors [99] and the lysophosphatidic acid (LPA) receptor [41], and some have shown to induce apoptosis, such as proteinase-activated receptor [100] and PAFR [28]. Since PAF has been shown to have pathogenic significance in NEC, we investigated the mechanisms of PAF induced apoptosis in IEC-6 cells. We have found that PAF induces apoptosis in enterocytes via a sequence of events that involves Bax translocation to mitochondria and the collapse of mitochondrial membrane potential within 30 minutes of exposure to PAF, followed by caspase activation that maximizes by 6 hrs of exposure, which is followed by DNA fragmentation plateauing at 12-16 hrs after PAF treatment [28]. In the same study we have found that heterologous over-expression of Bcl-2, a molecular antagonist of Bax, prevented PAF-induced collapse of mitochondrial membrane potential and apoptosis in these cells, indicating that mitochondrial damage is important in the mechanism of PAF-induced apoptosis, and suggesting that understanding the expression profiles of the Bcl family of apoptosis regulators during intestinal development may be important for our understanding of NEC pathogenesis. In light of our earlier observation that wide-spread enterocyte apoptosis precedes and accompanies gross tissue necrosis in an animal model of NEC and that caspase inhibition prevents the development of experimental NEC [76], our *in vitro* evidence for PAF-induced enterocyte apoptosis is a significant observation to aid our mechanistic understanding of NEC pathogenesis. Additionally, we have shown that PAF does not directly regulate apoptosis, but that signaling via the PAFR induces TLR4 expression in enterocytes (unpublished data). TLR4, the receptor for LPS, has been shown to take part in NEC pathogenesis [73,82], causes enterocyte apoptosis [101] and defective enterocyte migration [82]. Furthermore, TLR4 activation leads to induction of inflammatory molecules such as TNF $\alpha$  [102] and nitric oxide (NO) [103]; both of these molecules have been shown to be involved in NEC pathogenesis [15,81,104] and have been shown to be pro-apoptotic for enterocytes [81,105]. It is notable that other GPCRs that are closely related to PAFR may impart either pro or anti-apoptotic signals on enterocytes as discussed above [41,99,100] and the PAFR can cause anti-apoptotic

signaling in other cell types that can actively promote inflammation such as PMN [106] and lymphocytes [108]. These findings suggest that we are only beginning to skim the surface of the intricate mechanisms of GPCR-mediated cell death and survival and that other GPCRs may play significant roles in NEC.

### Nutrition, PAFR signaling, apoptosis and NEC

A seemingly unlikely convergence of two parallel research areas may offer valuable insights into NEC pathogenesis and potentially into clinically useful NEC prevention strategies. Several years ago, a clinical study that was geared towards evaluating the efficacy of polyunsaturated fatty acid (PUFA) supplementation to premature neonatal formula to improve long term neurodevelopmental outcomes has found, as an incidental finding, that PUFA supplementation reduced the incidence of NEC [108]. In order to verify these findings and to further understand the mechanisms that may be involved in this unexpected beneficial effect of PUFA, in two subsequent studies we have shown that PUFA supplementation to formula dramatically reduced the incidence of NEC in rodent models of NEC [109,110]. Importantly, both in the human study and in our animal model experiments, both n-3 and n-6 PUFA appeared to be protective, which is quite different from some other models where n-3 PUFA is beneficial via antagonizing undesirable pro-inflammatory prostaglandin and leukotriene production from n-6 PUFA precursors [111]. In the mean time, our laboratory was heavily involved in investigating pro-apoptotic signaling mechanisms via PAFR activation. We have found that the earliest step that we can identify in this signaling cascade is a PAFR activation-induced inhibition of the phosphatidylinositol 3 kinase (PI3K) [40]. Due to accumulating evidence indicating that PUFA can inhibit signaling via GPCR-s via a unique mechanism based on an effect on protein acylation and due to the role of PUFA in both human and experimental NEC, in the same study we investigated the effect of polyunsaturated fatty acids on PAFR signaling. We have found that, paralleling our *in vivo* data, both n-3 and n-6 PUFA antagonized PAFR activation-induced signaling and apoptosis, this effect was independent of prostaglandin synthesis and was mimicked by a synthetic inhibitor of palmitoylation 2Br-palmitate [40]. Palmitoylation is a posttranslational modification on many proteins and it is a covalent attachment of a fatty acyl chain to cysteine residues that are surrounded by basic and aromatic amino acids [112]. Typically, the fatty acyl chain that is involved in such a reaction is palmitate, as the most common saturated fatty acid in cells. The reaction takes place between fatty acyl coenzyme A (CoA) and appropriate cysteine residues in peptide chains even in the absence of enzymes, but there are enzymes known to facilitate both incorporation and hydrolysis of this bond and their expression levels have implications on apoptotic signaling [113,114]. The attachment of saturated fatty acyl chains endow proteins with new characteristics,

such as converting cytoplasmic proteins to membrane-bound entities and targeting proteins to cholesterol-rich membrane microdomains, commonly referred to as lipid rafts [115,116]. Palmitoylation is excessively common in the family of GPCR-s and has been thought to be important in the formation of receptor-signal-transduction complexes, by targeting GPCR-s, G proteins and kinases that execute G-protein-dependent signaling to lipid rafts while enhancing signaling efficiency, by creating proximity between molecules that need to interact for signaling to take place [116,117]. It has been shown that PUFA can displace palmitate in normally palmitoylated proteins in a competitive manner and that this displacement of palmitate results in diminished signaling [115,118]. In our most recent study, we were able to document that both n-3 and n-6 PUFA can displace palmitate in C317 of the C terminus cytoplasmic tail of the PAFR (unpublished observation). These data indicate that PUFA are potent modulators of PAFR signaling via a mechanism that is independent of their effect on prostaglandin and leukotriene biosynthesis, and that they may be effective in the prevention of NEC where signaling via the PAFR plays a major role.

### Summary

Inflammatory signaling and enterocyte apoptosis play important roles in NEC pathophysiology. Based on results from animal models, human sample analysis and based on available data from *in vitro* experimentation, we hypothesize (Figure 1) that the premature infant, when exposed to risk factors for NEC: 1) exhibits a propensity to produce and react to platelet-activating factor, 2) consequently, PAFR activation on enterocytes leads to abnormally increased TLR4 expression, which in turn, together with bacterial colonization 3) results in a hyperactive innate immune system that is skewed toward a pro-inflammatory response in the intestinal epithelium; these factors lead to intestinal epithelial cell apoptosis, mucosal barrier dysfunction, and necrosis, ultimately resulting in NEC in a subset of patients. There may be several other contributing factors to these alterations, including a dysregulation of splanchnic microcirculation, decreased input of exogenous growth factors due to formula feeding, a deficient production of endogenous growth factors due to prematurity and potential signaling via other pro-apoptotic mechanisms. A better understanding of these mechanisms by future studies and elucidation of the mechanisms of enterocyte apoptosis may lead to new preventive or therapeutic approaches for NEC. Nevertheless, the findings discussed throughout this chapter reaffirm the genius of Claude Bernard, who made his seminal discoveries on the importance of the regulated internal milieu of the human body and they show that understanding the miracles and wonders of a single cell layer that is 15-30  $\mu\text{m}$  thick and is responsible to separate and connect our regulated internal fluids with the widely changing external environment is one of the keys that we need to find to further human health.

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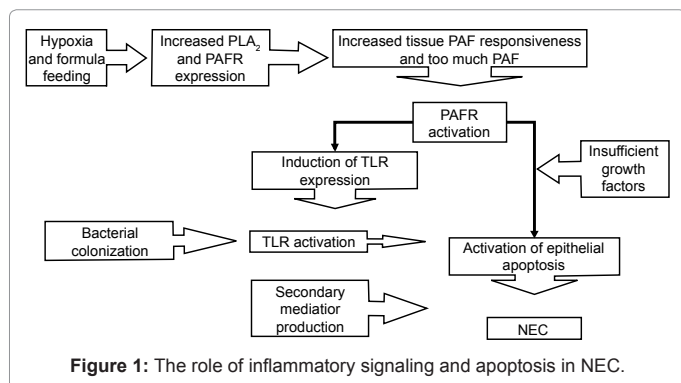


Figure 1: The role of inflammatory signaling and apoptosis in NEC.

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