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Polyunsaturated fatty acids block platelet-activating factor-induced phosphatidylinositol 3 kinase/Akt-mediated apoptosis in intestinal epithelial cells

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Abstract

We have shown earlier that platelet-activating factor (PAF) causes apoptosis in enterocytes via a mechanism that involves Bax translocation to mitochondria, followed by caspase activation and DNA fragmentation. Herein we report that, in rat small intestinal epithelial cells (IEC-6), these downstream apoptotic effects are mediated by a PAF-induced inhibition of the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (Akt) signaling pathway. Treatment with PAF results in rapid dephosphorylation of Akt, phosphoinositide-dependent kinase-1, and the YXXM p85 binding motif of several proteins and redistribution of Akt-pleckstrin homology domain-green fluorescent protein, i.e., an *in vivo* phosphatidylinositol (3,4,5)-trisphosphate sensor, from membrane to cytosol. The proapoptotic effects of PAF were inhibited by both n-3 and n-6 polyunsaturated fatty acids but not by a saturated fatty acid palmitate. Indomethacin, an inhibitor of prostaglandin biosynthesis, did not influence the baseline or PAF-induced apoptosis, but 2-bromopalmitate, an inhibitor of protein palmitoylation, inhibited all of the proapoptotic effects of PAF. Our data strongly suggest that an inhibition of the PI 3-kinase/Akt signaling pathway is the main mechanism of PAF-induced apoptosis in enterocytes and that polyunsaturated fatty acids block this mechanism very early in the signaling cascade independently of any effect on prostaglandin synthesis, and probably directly via an effect on protein palmitoylation.

Keywords

platelet-activating factor; protein kinase B

PLATELET-ACTIVATING FACTOR (PAF) has been implicated in inflammation and/or exaggerated apoptosis occurring in several disease states such as hypoxia-reoxygenation-induced brain injury (2), human immunodeficiency virus neuropathogenesis (23), *N*-methyl-D-aspartate-induced neuron apoptosis (21), asthma (27), inflammatory bowel disease (25), and necrotizing enterocolitis (NEC) (9). NEC is an inflammatory disease of the distal small intestine and proximal colon, primarily of preterm infants weighing <1,500 g. Studies in animal models and in humans indicate that PAF plays an important role in NEC pathogenesis (7) and that supplementation of polyunsaturated fatty acids (PUFA) to preterm formula reduces the incidence of both experimental and human NEC (8,10,19). We have shown that, in a neonatal rat model of NEC, where the pathogenic role of PAF has been well established, early

accelerated apoptosis of enterocytes plays an underlying role in subsequent massive tissue necrosis and that caspase inhibition prevents disease progression in this model (16). In an in vitro model, we further demonstrated that PAF induces apoptosis in rat small intestinal epithelial cells (IEC-6) by eliciting Bax translocation to mitochondria, which is followed by a collapse of mitochondrial membrane potential and subsequent activation of caspase 3 (18). Several relatives of the PAF receptor (PAFR) in the G protein-coupled receptor (GPCR) family regulate the phosphatidylinositol 3 kinase (PI 3-kinase)/protein kinase B (Akt) signaling pathway either positively (12) or negatively (29), and activation of the PI 3-kinase/Akt signaling pathway (reviewed in Ref. 11) by growth factors has been shown to be a critical determinant of cell survival in most cell types. Akt phosphorylation can be inhibited by saturated fatty acids (26) and has been shown to be either positively or negatively regulated by dietary PUFA (1,28).

One of the multiple mechanisms by which PUFA can modify cellular events is to interfere with palmitoylation (32). Palmitoylation is a posttranslational modification that is necessary for efficient signal transduction by many GPCRs. PUFA can modify GPCR signaling by directly interrupting palmitoylation (14) or decreasing ligand binding (6). Although palmitoylation has been implicated in GPCR signaling, whether this modification bears physiological or pathological significance in PAFR signaling has not yet been characterized.

The present study was designed to determine whether a modulation of the PI 3-kinase/Akt pathway plays a role in PAF-induced enterocytes apoptosis, whether PUFA can interfere with the effects of PAF on Akt signaling, and to evaluate the mechanisms by which PUFA act on this pathway. Our data indicate that PAF causes a dephosphorylation of several intermediates of the PI 3-kinase/Akt signaling pathway, that PI 3-kinase inhibition results in enterocyte apoptosis, and that heterologous overexpression of Bcl-2 can completely block both PAF-induced and PI 3-kinase inhibition-induced apoptosis. Our data also show that PUFA can block PAF-induced enterocyte apoptosis at a step proximal to Akt dephosphorylation via a mechanism that does not appear to involve an effect on prostaglandin biosynthesis, but rather via a mechanism involving an effect on PAFR palmitoylation.

MATERIALS AND METHODS

Fatty acids, arachidonic acid (AA) (20:4 n-6), docosahexaenoic acid (DHA, 22:6 n-3), and palmitic acid (PA, 16:0) were purchased from Sigma-Aldrich (St. Louis, MO).

PUFA were dissolved in 100% ethanol to make 100 mM stock solutions, and PA was dissolved in 70% ethanol to make 10 mM stock. After preparation, all fatty acid solutions were aliquoted, sealed under argon, and stored at -20°C until use. The PAF was purchased from Sigma-Aldrich and dissolved in 100% ethanol to reach a stock concentration of 20 mM. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR) and dissolved in dimethyl sulfoxide for a 1 mg/ml stock solution. YO-PRO-1 was purchased from Molecular Probes with a stock concentration of 1 mM. Antibodies for Akt, phospho-Akt (Ser⁴⁷³ or Thr³⁰⁸), phosphoinositide-dependent kinase-1 (PDK1), phospho-PDK1, phospho-p85 binding motif, and PI 3-kinase inhibitor LY-294002 were all purchased from Cell Signaling (Beverly, MA). [³H]palmitate was purchased from American Radiolabeled Chemical (St. Louis, MO).

Cell culture and fatty acid supplementation

Rat IEC-6 were maintained in DMEM (GIBCO-BRL, Rockville, MD) containing 5% FBS, 100 $\mu\text{g}/\text{ml}$ penicillin and streptomycin, 4 mM glutamine, and 0.1 mg/ml insulin. For caspase assays, cells were plated in a 100-mm plate and allowed to grow to 80% confluence. Cells were then treated with or without 100 μM AA, 67 μM DHA, or 50 μM PA for 24 h for caspase assays

or for 10 min for Akt phosphorylation assays followed by treatment with PAF or LY-294002. For DNA fragmentation enzyme-linked immunosorbent assay (ELISA) assays, 10,000 cells were plated in each well of a FALCON 96-well flat bottom plate (Becton-Dickinson Labware, Franklin Lakes, NJ) and allowed to recover for 24 h in a culture incubator. The cells were then enriched with fatty acids for 24 h followed by PAF treatment for 18 h.

Using the Akt-PH domain fusion protein and fluorescence digital live cell microscopy as in vivo phosphatidylinositol (3,4,5)-trisphosphate sensor

The Akt-pleckstrin homology (PH) domain-green fluorescent protein (GFP) expression construct was kindly provided by Dr. Tamas Balla (National Institutes of Health). IEC-6 cells were transfected with this construct using Lipofectamine 2000, according to the manufacturer's instructions. Stable cell lines were established by selection in G418 and colony isolation. Cells were plated on coverslips and mounted on the stage of an Olympus IMT-2 inverted microscope in an FCS2, temperature-regulated, perfused, closed microscopy chamber (Biopetechs, Butler, PA). Some cells were pretreated for 1 h with 100 μ M AA, 67 μ M DHA, or 10 μ M LY-294002. Cells were perfused with HEPES-buffered physiological buffer (in mM: 116 NaCl, 5.4 KCl, 0.4 MgCl₂, 1.8 CaCl₂, 5.5 glucose, 26 NaHCO₃, 0.9 NaH₂PO₄, and 10 HEPES 0.5 Na, pH 7.4), and treatments were applied as indicated in Figs. 1 and 4. Images were collected in 1-min intervals using a charge-coupled device camera (Photometrics, Tucson, AZ) and were analyzed using IPLab Spectrum software. For quantitative analysis, intensity profiles under 80 pixel long linear regions of interest were captured, and the delta peak was determined as shown in Fig. 4, *G* and *H*. Measurement was performed on at least two image sequences and at least three lines for the baseline and posttreatment image from each image sequence.

Assay for caspase activity

Cell lysates were collected as the following categories: control, PAF, PUFA or PA treated, and PAF or LY-294002 + PUFA or PA treated. Cells were pretreated with or without PUFA or PA for 24 h and then treated with or without 20 μ M PAF or 10 μ M LY-294002 for 6 h. Cells were harvested and spun down at 700 g and then the pellets were washed with PBS two times and lysed in cell lysis buffer containing 10 mM Tris•HCl (pH 7.5), 10 mM NaH₂PO₄/NaHPO₄ (pH 7.5), 130 mM NaCl, 1% Triton X-100, and 10 mM Na₄P₂O₇ on ice for 20 min. After centrifugation at 10,000 g for 10 min, supernatants were collected and were mixed with either substrate (Ac-DEVD-AFC; BioVision, Mountain View, CA), or substrate + inhibitor (Ac-DEVDCHO; BioVision).

Fluorescence was measured at 400 nm excitation and 505 nm emission following 2 h of incubation (the development of fluorescence signal was linear between 10 min and 4 h). Caspase 3 activity was measured by quantifying the cleavage of the fluorogenic peptide substrate Ac-DEVD-AFC. Specific caspase activity was calculated by subtracting the fluorescence intensity measured in the presence of substrate + inhibitor from the fluorescence observed by incubating with substrate alone. Sample fluorescence intensities were normalized to the intensity of untreated cells and were expressed as a percentage of control.

DNA fragmentation with ELISA

DNA fragmentation was analyzed using an ELISA kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Absorbance at 405 nm was measured intermittently in 20-min intervals until absorbance values obtained from the sample representing cells were at least 0.2 optical density (OD) units above the substrate blanks. Absorbance of blanks was subtracted, and OD values were normalized to the mean of OD values representing the samples of untreated cells and expressed as a percentage of controls.

Phosphorylation status of proteins in PI 3-kinase/Akt pathways

The Akt phosphorylation assay was performed using the PhosphoPlus Akt (Ser⁴⁷³) Antibody Kit from Cell Signaling Technology according to the manufacturer's protocol with minor modification as described in the following. The collected lysates were also used to test the p85 binding motif, PDK1, and phospho-PDK1. Briefly, cells were scraped in control or treated culture medium and spun down at 700 g. The pellets were washed with 1× PBS one time and transferred to 1.5-ml Eppendorf tubes. Cells were then immediately lysed by adding 100 µl of 1× SDS sample buffer and kept on ice for 10 min. The suspension was sonicated for 20 s to shear DNA and to reduce the sample viscosity. Twenty microliters of each sample were heated at 95–100°C for 5 min. After being cooled on ice, the samples were centrifuged for 2 min before being loaded on an 8% SDS-PAGE.

Western blotting

Each lane of an SDS-PAGE was loaded with extracts representing equal amounts of protein, and, following the separation, they were transferred to a polyvinylidene difluoride membrane. After being blocked with 5% nonfat milk in 0.1% of Tween 20 in TBS, the membrane was incubated with appropriate antibodies. Blots were visualized using enhanced chemiluminescence (ECL + Plus) detection solution (Amersham Pharmacia Biotech, Piscataway, NJ) and scanned with a Phosphor Imager (Molecular Dynamics, Piscataway, NJ).

Statistical analysis

All data were presented as means ± SE. Statistical analyses were performed using ANOVA (GraphPad Prism, San Diego, CA). Differences were considered significant at $P < 0.05$. In figures, where one group was different from all others, we used an asterisk to indicate this difference. In figures where multiple groups were different from each other, statistical significance is indicated by letters above columns. Columns with the same letters are statistically identical, whereas columns with distinct letters are different with at least $P < 0.05$.

RESULTS

Treatment with PAF induces rapid dephosphorylation of Akt, PDK1, and p85 binding motif in IEC-6

We have shown earlier that PAF induces caspase 3 activation and DNA fragmentation in IEC-6 (18). To examine whether the PI 3-kinase/Akt pathway is involved in PAF-induced apoptosis, we analyzed the effects of treatment with PAF on the phosphorylation states of Akt, p85 binding motif, and PDK1 in IEC-6 by Western blotting and on Akt translocation from membranes to cytosol using the Akt PH domain fused with GFP in live cell imaging. Treatment with 10 µM PAF for 20 min induced a dephosphorylation of Akt (Fig. 1A). To confirm that in IEC-6 cells Akt phosphorylation is dependent on PI 3-kinase activity, we performed a similar analysis following treatment with the specific PI 3-kinase inhibitor LY-294002. As depicted in Fig. 1B, the inhibition of PI 3-kinase completely abolished the phospho-Akt signal without affecting the total Akt levels in these cells. Phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) is the product of PI 3-kinase (13) and functions as a ligand to the PH domain of Akt. PI 3-kinase activation and subsequent PIP₃ accumulation in the plasma membrane initiate Akt translocation from cytosol to the plasma membrane. Therefore, the cellular distribution of Akt is a powerful indicator of PI 3-kinase activity (3). To monitor PI 3-kinase activity in live cells, we stably transfected an Akt-PH domain-GFP fusion protein expression construct (a generous gift from Tamas Balla, National Institutes of Health) in IEC-6 cells. Using this construct and live cell fluorescence digital imaging, we have found that, in resting state IEC-6 cells, a substantial portion of Akt-PH-GFP is localized at membranes in the vicinity of cell-to-cell junctions, indicating that there is a constitutively present PIP₃ pool mainly localized near

junctional complexes (Fig. 1, C-1). These data suggest that PI 3-kinase is constitutively active in these cells. Upon treatment with PAF, Akt-PH-GFP dissipates from junctional complexes and redistributes to the cytoplasm, indicating a depletion of PIP₃ and suggesting an inhibition of PI 3-kinase by PAF [Fig. 1, C-2 and Supplemental Movie 1 (Supplemental data for this article is available online at the American Journal of Physiology: Gastrointestinal and Liver Physiology website.)]. Similarly, LY-294002 caused Akt-PH-GFP translocation from membrane to cytosol (Fig. 1, C-3 and C-4), indicating the depletion of PIP₃ by inhibition of PI 3-kinase. To further investigate the site of action by PAF in the PI 3-kinase signaling pathway, we analyzed steps upstream of Akt phosphorylation and have found that treatment with PAF reduced phosphorylation levels of PDK1, i.e., another PH domain-containing kinase that is corecruited with Akt and mediates its phosphorylation. Treatment with PAF also reduced phosphorylation levels of multiple polypeptides containing the YXXM p85 binding motif (Fig. 1D).

Overexpression of Bcl-2 blocks PAF and PI 3-kinase inhibitor-induced caspase activation but does not affect PAF- or PI 3-kinase-induced Akt dephosphorylation

Earlier we have shown that PAF-induced apoptotic signaling involves a sequence of Bax translocation to mitochondria, collapse of the mitochondrial membrane potential, caspase 3 activation, and then DNA fragmentation and that heterologous overexpression of Bcl-2 blocks this entire cascade (18). To determine whether the PI 3-kinase inhibition-induced apoptosis follows the same signaling pathway and to determine whether Akt dephosphorylation is upstream or downstream of the Bcl-2-sensitive step, we tested the effect of Bcl-2 overexpression on PAF and PI 3-kinase inhibition-induced Akt dephosphorylation and on caspase 3 activation in IEC-6/Lac/Bcl-2 cells. In these cells (11), where heterologous Bcl-2 expression is under the control of a lactose-inducible promoter, treatment with isopropyl- β -D-thiogalactopyranoside (IPTG) results in a high level of Bcl-2 overexpression (Fig. 2A). IPTG-induced overexpression did not affect either PAF-induced or PI 3-kinase-induced Akt dephosphorylation (Fig. 2b), but Bcl-2 overexpression completely blocked both PAF-induced and LY-294002-induced caspase activation (Fig. 2C). These data indicate that Bcl-2 acts downstream of Akt in the apoptotic signaling cascade in IEC-6 and also suggest that PAF-induced and PI 3-kinase inhibition-induced apoptosis follow a common pathway.

PUFA block the PAF-induced p85 binding motif, PDK1, and Akt dephosphorylation but do not affect the PI 3-kinase-induced Akt dephosphorylation

Because PAF and epithelial apoptosis have been shown to play significant roles in NEC (9, 18), and PUFA supplementation to infant formula prevented NEC in a human clinical trial and in a neonatal rat model of NEC (9), we aimed to investigate the effects of PUFA on PAF-induced apoptotic signaling in enterocytes. We have found that supplementation of either AA or DHA resulted in an increased baseline level of Akt phosphorylation, that supplementation of either AA or DHA reduced the extent of PAF-induced Akt dephosphorylation (Fig. 3A), and, in samples pretreated with either n-3 or n-6 PUFA, Akt phosphorylation levels remained at or above the level of Akt phosphorylation in untreated samples. To determine whether the inhibitory effect of PUFA on the PAF-induced Akt dephosphorylation is proximal or distal to PI 3-kinase inhibition in the PAF-induced apoptotic signaling cascade and to determine whether the inhibitory effect of PUFA on PAF-induced Akt dephosphorylation is a specific effect, we analyzed the effect of PUFA on PI 3-kinase inhibition-induced Akt dephosphorylation. Supplementation of neither AA nor DHA had any effect on the LY-294002-induced Akt dephosphorylation (Fig. 3B). These findings suggest that PUFA act via a mechanism that is upstream of the PI 3-kinase activity in the regulation of this pathway by PAF and that the effect of PUFA to inhibit the PAF-induced Akt dephosphorylation is a specific effect on PAF-induced signaling that is not a general effect on the PI 3-kinase-Akt pathway. To further analyze the mechanism of PUFA effects, we evaluated the effects of PAF

and PUFA supplementation on other steps that are thought to be proximal to Akt phosphorylation in the PI 3-kinase-dependent regulation of cell survival. PAF caused a dephosphorylation of the p85 binding motif, which is known to be involved in PI 3-kinase activation, and caused dephosphorylation of PDK1, the intermediate signaling step between PI 3-kinase and Akt phosphorylation (Fig. 3C). Both of these effects of PAF were completely blocked by PUFA.

To analyze the effects of PUFA on PAF-induced PIP₃ depletion in vivo, we employed again our live cell PIP₃ sensor assay described above. In this series of experiments, we confirmed our earlier observations that there is a considerable pool of membrane-bound Akt-PH-GFP, indicative of constitutive PI 3-kinase activity in IEC-6, and that PAF treatment resulted in a redistribution of Akt-PH-GFP from junctional complexes to the cytoplasm, indicating a depletion of PIP₃ and therefore an inhibition of PI 3-kinase by PAF (Fig. 4, A, B, and G, and Supplemental Movie 2). However, following a pretreatment with, and in the continuous presence of 100 μ M AA, the PAF-induced Akt-PH domain redistribution from the membrane to cytoplasm was inhibited (Fig. 4, C, D, and H, and Supplemental Movie 2). On the other hand, pretreatment with AA did not prevent Akt-PH-GFP redistribution from the membrane to cytosol by the PI 3-kinase inhibitor LY-294002 (Fig. 4, E, F, and I, and Supplemental Movie 3). Similar observations were made using a DHA pretreatment (see Supplemental Movie 4).

The cyclooxygenase inhibitor indomethacin does not affect the baseline or PAF-induced Akt dephosphorylation

The best characterized mechanism by which PUFA have been shown to affect biological systems is their effect on prostaglandin and prostacyclin biosynthesis. When PUFA act on the prostaglandin and prostacyclin biosynthesis, the effects of n-3 and n-6 PUFA are typically antagonistic. Generally, n-6 PUFA is considered to be proinflammatory by providing a substrate for the production of the two and four series prostaglandins and leukotrienes; DHA is considered to be anti-inflammatory by providing substrate for the production of the three and five series prostaglandins and leukotrienes. Notably, with regard to the inhibition of the PAF-induced apoptotic pathway, both n-3 and n-6 PUFA appear to act interchangeably, suggesting the involvement of a mechanism that is independent of an effect on prostaglandin biosynthesis. To further investigate the role of prostaglandin biosynthesis in the inhibitor effects of PUFA on PAF-induced apoptosis, we evaluated the effects of the cyclooxygenase inhibitor indomethacin on PAF-induced Akt dephosphorylation. We have found that treatment of cells with indomethacin had no effect on the baseline, PAF-induced Akt dephosphorylation or on DNA fragmentation (Fig. 5, A and B). A lesser-studied mechanism by which PUFA can affect GPCR signaling is a direct effect of PUFA on protein palmitoylation (32). If the effects are mediated by palmitoylation-dependent mechanisms, n-3 and n-6 fatty acids should have similar effects, but the saturated fatty acid palmitate should have no effect or should be antagonistic to the effects by PUFA. When we tested the effects of PUFA and palmitate, we have found that n-3 and n-6 PUFA had identical effects in blocking the PAF-induced DNA fragmentation, but the saturated fatty acid PA had no inhibitory effect on the PAF-induced DNA fragmentation and raised the baseline DNA fragmentation (Fig. 5C). These data are all consistent with the notion that PUFA act through a mechanism other than an effect on prostaglandin synthesis.

Inhibition of protein palmitoylation as a potential mechanism of PUFA inhibiting PAF's effects

Because an effect on prostaglandin biosynthesis does not appear to play a role in the inhibitory effect of PUFA on the PAF-induced Akt dephosphorylation, we sought to determine whether another known effect of PUFA, i.e., an inhibitory effect on protein palmitoylation, might be the underlying mechanism. To address this question, we studied the effect of 2-bromopalmitate

(2-BP), a synthetic and specific inhibitor of protein palmitoylation, on PAF-induced and PI 3-kinase inhibitor LY-294002-induced Akt dephosphorylation. Using Western blotting, we have found that, similar to PUFA, 2-BP also inhibited the PAF-induced Akt dephosphorylation (Fig. 6A). Also similarly to our experiments with PUFA, 2-BP was unable to inhibit the LY-294002-induced Akt dephosphorylation (Fig. 6B). These data are consistent with the notion that inhibition of protein palmitoylation is a mechanism that can prevent PAF-induced apoptotic signaling, and this may be the main mechanism by which PUFA prevents PAF-induced apoptosis.

DISCUSSION

NEC is one of the leading causes of death or long-term complications following premature birth (7). PAF is one of the critical mediators of NEC pathogenesis (9), and profuse enterocyte apoptosis precedes and underlies experimental NEC in rodents (16). Because PAF is a potent inducer of enterocyte apoptosis (18), it is of interest to understand the mechanisms of PAF-induced apoptotic pathways and to identify means of pharmacological intervention that may prevent or stop these pathways. PUFA are naturally occurring, abundant, and inexpensive nutritional components that are known for their various health benefits. They have been shown to prevent both human (10) and experimental (8,19) NEC, and recent evidence indicates that they can modulate signaling via receptors in the GPCR family (20), of which PAFR is a member. Data presented in this manuscript provide some insight into the mechanisms by which the beneficial effects of PUFA may counteract the detrimental effects of PAFR signaling in enterocytes.

Our previous studies have shown that the PAF-induced apoptotic pathway in enterocytes is tightly under control of Bcl-2 expression levels, since heterologous inducible overexpression of Bcl-2 completely abrogated the activation of apoptosis by PAF in IEC-6 cells (18). We used this knowledge to analyze the sequence of events in the PAF-induced apoptotic cascade and found that Akt dephosphorylation precedes the Bcl-2-sensitive step in the cascade because Bcl-2 overexpression did not inhibit PAF- or LY-294002-induced Akt dephosphorylation but blocked PI 3-kinase inhibition-induced or PAF-induced caspase 3 activation, i.e., a step in PAF-induced apoptotic signaling that we have shown to be downstream of mitochondrial depolarization (see Fig. 7).

The mechanisms by which PAF participates in various diseases are complex and appear to be tissue and cell type specific. Several examples indicate that at least one of the mechanisms by which PAF enacts its biological role is via the regulation of programmed cell death. PAF promotes cell survival of several cell types that can actively promote inflammation such as polymorphonuclear neutrophils (5) and lymphocytes (30), but PAF has been shown to be proapoptotic for a number of cell types that undergo apoptosis under inflammatory conditions, including neurons (23), oligodendrocytes (15), and enterocytes (18). These opposing effects on cell survival endow PAF with very potent “double-edged” inflammatory and cytotoxic properties by promoting the survival of inflammatory mediator-producing cells and by promoting tissue destruction via directly causing cell death. The mechanisms underlying these contrasting effects are not yet known, but the differential responsiveness of the PI 3-kinase/ Akt signaling pathway to PAF in these various cell types might provide a clue regarding the dichotomy of pro- and antiapoptotic effects of PAF in these cells. In polymorphonuclear leukocytes, PAF activates PI 3-kinase, resulting in rapid Akt phosphorylation (17) and delayed apoptosis, whereas here we show that PAF induces a rapid dephosphorylation of the YXXM p85 binding motif of several proteins in IEC-6, as well as dephosphorylates PDK1 and Akt, resulting in a deregulation of this pathway and causing apoptosis. The molecular details underlying these cell type-specific differences in signaling are yet to be determined but may include differential G protein coupling, differential expression of PI 3-kinase isoforms, or

differential expression of arrestins, a family of proteins that was identified initially to be important in GPCR trafficking and lately emerged as important mediators of G protein-independent signaling via GPCRs (4,31).

Despite the well-established beneficial effects of long-chain PUFA supplementation on various diseases, our understanding of how PUFA exert their biological effects is incomplete. Our previous studies have shown that PUFA supplementation reduced the incidence of NEC, an intestinal injury associated with PAF and apoptosis, albeit without a detailed analysis of the mechanisms by which PUFA may act (8,19). The current study, although it was performed exclusively in tissue culture model systems, may shed some light on the mechanism by which PUFA elicit their beneficial effects in inflammatory conditions, such as NEC.

PAFR is a member of the large GPCR family, a family sharing many common features, including one or more acylation consensus cysteine residues in the COOH-terminus cytoplasmic tail (24). Other proteins that interact with G protein receptors, such as G proteins, kinases, and arrestins, have been shown to be palmitoylated. This common posttranslational modification targets these molecules to specialized cholesterol- and sphingolipid-rich, detergent-resistant microdomains of the plasma membrane, often referred to as lipid rafts. It has been thought that clustering of GPCRs with G proteins and kinases to rafts creates receptor/signal transduction complexes, enabling very efficient signaling (22). Incorporation of PUFA in place of palmitate has been shown to decrease the affinity of raft-targeted proteins to rafts (32). It has been demonstrated that arachidonate can be incorporated in G proteins in place of palmitate (14) and that PUFA can modulate G protein function directly (20).

Our data are consistent with the notion that the effects of PUFA on PAF-induced pathology are mediated at least in part by altered palmitoylation. It is conceivable that, in more complex *in vivo* systems, such as animal models, an effect on prostaglandin synthesis might play a role; however, in the isolated tissue culture model presented herein, such effects were not observed. AA and DHA appeared to be equally efficient in blocking the PAF-induced Akt dephosphorylation, and these two PUFA should have opposing effects on prostaglandin synthesis, suggesting that the observed inhibitory effect by these PUFA was not due to prostaglandin biosynthesis. Our experiments using the cyclooxygenase inhibitor indomethacin, which had no effect on Akt phosphorylation levels or on DNA fragmentation with or without PAF and/or PUFA, confirm this suggestion. On the other hand, 2-BP, a bone fide palmitoylation inhibitor, had very similar effects to PUFA on PAFR signaling. These data together strongly suggest that an effect of PUFA on protein acylation might play a dominant role in their effects on PAF-induced cellular effects. The ability of PUFA to modulate signaling via interfering with acylation is not likely to be specific for only the PAFR but might affect signaling via other GPCRs as well. Furthermore, it is likely that the effects of PUFA on palmitoylation are not restricted to only the PAFR but likely extend to other members of the receptor signal transduction complex such as G proteins, arrestins, and some kinases, and all of these effects may contribute to the overall inhibitory effect of PUFA on PAF-induced apoptosis. For simplicity, we only indicated potential effects on the PAFR itself in Fig. 7.

Finally, there is an apparent stimulatory effect of PUFA on the baseline survival signals in IEC that we have not addressed experimentally in this present study. Nevertheless, this effect is likely mediated by a protein palmitoylation-dependent baseline inhibitory activity as well, since 2-BP mimicked the effects of PUFA.

In summary, our studies indicate that PAF causes apoptosis in enterocytes by inhibiting the PI 3-kinase/Akt signaling pathway and that PUFA can completely block this effect by a mechanism that is likely mediated by an effect on protein acylation (Fig. 7). A clear description of the interactions between PI 3-kinase/Akt signaling, PAF, and PUFA will aid our

understanding of the mechanisms involved in diseases where inflammation-induced cell death plays a role. Furthermore, a better characterization of the mechanisms by which PUFA might affect GPCR signaling inflammation and apoptosis may give us ideas how to better utilize these inexpensive and highly accessible molecules to improve health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Platelet-activating factor (PAF) inhibits phosphatidylinositol 3-kinase (PI 3-kinase). **A—C:** intestinal epithelial cells (IEC-6) were grown to confluence in petri dishes and treated with or without 10 μM PAF or 10 μM LY-294002. **C:** IEC-6 cells stably transduced with a protein kinase B (Akt)-pleckstrin homology (PH) domain-green fluorescent protein (GFP) fusion protein were plated on glass coverslips and subjected to live cell fluorescence imaging microscopy. **A and B:** after treatment, cells were lysed, and lysates were separated on SDS-PAGE and then subjected to immunoblotting using either a polyclonal antibody to phosphor-Akt Ser⁴⁷³ (*top*) or a polyclonal antibody to Akt (*bottom*). Below the representative fluorescence images are graphs depicting quantification results from 12 independent

experiments in which phosphorylated (p) Akt levels were normalized to total Akt signal and to pAkt level in untreated samples. *Statistically significant difference from control at $P < 0.001$. *C*: IEC-6 cells stably transfected with an Akt-PH domain-GFP fusion protein were plated on coverslips and then mounted in a temperature-regulated, perfused chamber on the stage of an inverted microscope. Time-lapse fluorescence digital imaging was performed by collecting images in 30-s intervals. *C-1* and *C-3* depict fluorescence images collected before treatments and *C-2* and *C-4* after treatment with PAF (10 μ M, 10 min; *C-2*) or LY-294002 (10 μ M, 10 min; *C-4*). Arrows point to Akt-PH-GFP accumulation near cell-to-cell junctions before treatment (*C-1*, *C-3*), and * depicts gaps opening up between cells following treatment by PAF (*C-2*) or LY294002 (*C-4*) (also see Supplemental Movie 1). *D*: lysates from IEC-6 cells treated with and without 10 μ M PAF were analyzed on SDS-PAGE and then subjected to Western blotting either using polyclonal antibodies to phospho-p85 binding motif (*top*), phosphor-PDK1 Ser²⁴¹ (*middle*), or PDK1 (*bottom*). Both PAF and LY-294002 caused an inhibition of Akt phosphorylation within 20 min. Akt-PH domain was constitutively localized to surface membranes near junctional complexes. PAF and inhibition of PI 3-kinase by LY-294002 caused a redistribution of membrane-bound Akt-PH-GFP to the cytosol. Furthermore, PAF caused a dephosphorylation of PDK1 and P85 binding motif.

Fig. 2.

Bcl-2 overexpression differentially affects PAF-induced and PI 3-kinase inhibition-induced Akt dephosphorylation and caspase activation. IEC-6 cells were cultured in petri dishes and were treated with or without 3 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 24 h to induce Bcl-2 expression. Following treatments as indicated, cells were lysed and analyzed for IPTG-induced Bcl-2 expression (A) and LY-294002-induced and PAF-induced Akt dephosphorylation using Western blotting, or LY-294002-induced and PAF-induced caspase activation using fluorogenic caspase 3 substrate as described in MATERIALS AND METHODS (B). Graphs in B depict mean $\pm$ SE. quantification results from 6 independent experiments where pAkt levels were normalized to total Akt signal and to pAkt level in untreated samples. *Statistically significant difference from control at $P < 0.001$. IPTG induced a high level of heterologous Bcl-2 expression that resulted in a complete inhibition of LY-294002-induced and PAF-

induced caspase activation but did not have an effect on LY-294002-induced or PAF-induced Akt dephosphorylation. *Statistically significant increase at $P < 0.01$ compared with untreated control.

Fig. 3.

Effect of polyunsaturated fatty acid (PUFA) supplementation on PAF and PI 3-kinase inhibitor-induced Akt dephosphorylation in IEC-6. IEC-6 cells were grown in petri dishes and treated as indicated. Phosphorylation state of Akt was analyzed by Western blotting using phospho-Akt-specific antibodies as indicated. Cell lysates were also analyzed with Akt antibodies to ensure that the reduced signal with the phosphorspecific antibodies was not due to a loss of Akt. Graphs below the representative fluorescence images depict quantification results from 6 independent experiments where pAkt levels were normalized to total Akt signal and to pAkt level in untreated samples. Different letters indicate statistically significant differences between groups at $P < 0.05$. Columns with identical letters are statistically indistinguishable.

PAF induced a marked dephosphorylation of Akt. PUFA potently blocked PAF-induced dephosphorylation of Akt (A) but had no effect on LY-294002-induced dephosphorylation of Akt (B). In C, cells were grown in petri dishes and were treated as indicated under lanes. Row on *top* depicts blotting results using an antibody specific for phosphorylated p85 binding motif, row in *middle* depicts the blot using a phosphor-PDK1 antibody, and the row on *bottom* shows the loading control blotting using a PDK1 antibody. PAF inhibited the phosphorylation of p85 binding motif and PDK1. This experiment was repeated three times. PAF induced a marked dephosphorylation of p85 binding motif proteins and PDK1. PUFA potently blocked PAF-induced dephosphorylation of PDK1 and p85 binding motif in a dose-dependent manner.

Fig. 4.

PAF causes translocation of constitutively membrane-bound Akt-PH domain-GFP fusion protein to the cytoplasm, and this effect is inhibited by pretreatment and the presence of PUFA. IEC-6 cells stably transfected with an Akt-PH domain-GFP fusion protein (Akt-PH-GFP) were plated on coverslips and then mounted in a temperature-regulated, perfused chamber on the stage of an inverted microscope. A series of fluorescence images collected before (A) or after (C and E) pretreatment with arachidonic acid (AA; 100 μM , 1 h pretreatment and then 10 min in the chamber); after treatment with PAF (10 μM , 10 min; B); after treatment with PAF in the presence of AA following the pretreatment with AA (D) and after treatment with LY-294002 in the presence of AA following the pretreatment with AA (F) is shown. G—I: quantitative assessment of the visual information. G and H: representative, normalized intensity traces over 80 pixel length lines as marked in A—D with an indication of how Δ peak is determined. I: summary of results measured over several lines across multiple image sequences. *Statistical

significance from all other groups at $P < 0.05$ (PAF $n = 8$, all others $n = 6$). Akt-PH domain was constitutively localized to the membrane near junctional complexes (arrows). This membrane localization became somewhat more pronounced after pretreatment with AA (also see Supplemental Movies 2 and 3). PAF caused a redistribution of membrane-bound Akt-PH-GFP to the cytosol (also see Supplemental Movie 2). AA partially blocked PAF-induced Akt-PH-GFP redistribution (Supplemental Movie 2) but did not block Akt-PH-GFP redistribution induced by the PI 3-kinase inhibitor LY-294002 (Supplemental Movie 3). This experiment was repeated 3 times with AA and 3 times with 67 μM docosahexaenoic acid (DHA; Supplemental Movie 4) with similar results.

Fig. 5.

Indomethacin does not affect baseline or PAF-induced Akt phosphorylation and DNA fragmentation, AA and DHA inhibit PAF-induced DNA fragmentation equally, and palmitic acid (PA) causes an increased baseline DNA fragmentation. Cells were grown in petri dishes (A) or 96-well plates (B and C) and treated as indicated. Following 20 min of treatment, cells were lysed, and Akt phosphorylation state was probed using Western blotting (A), or cells were treated for 16 h and DNA fragmentation was evaluated using enzyme-linked immunosorbent assay as described in MATERIALS AND METHODS. Graphs below the representative fluorescence images depict quantification results from 4 independent experiments where pAkt levels were normalized to total Akt signal and to pAkt level in untreated samples. Different letters indicate statistically significant differences between groups at $P < 0.05$. Columns with identical letters are statistically indistinguishable. Treatment with indomethacin did not have an effect on baseline Akt phosphorylation, DNA fragmentation ($n = 20$), or PAF-induced Akt dephosphorylation and increased DNA fragmentation. Both AA ($n = 30$) and DHA ($n = 40$) inhibited PAF-induced DNA fragmentation, and PA ($n = 24$), a saturated fatty acid, caused an increased baseline DNA fragmentation; “n” denotes the no. of experiments.

Fig. 6.

2-Bromopalmitate (2-BP) inhibited PAF-induced Akt dephosphorylation. *A* and *B* depict results of experiments in which cells were plated in petri dishes and then treated as indicated, and Akt phosphorylation was probed by Western blotting. *Top*: blot probed with pAkt. *Bottom*: loading control probed with an antibody to Akt. Graphs below the representative fluorescence images depict quantification results from 9 independent experiments in which pAkt levels were normalized to total Akt signal and to pAkt level in untreated samples. Different letters indicate statistically significant differences between groups at $P < 0.05$. Columns with identical letters are statistically indistinguishable. Similar to PUFA, 2-BP blocked PAF-induced Akt dephosphorylation but did not block Akt dephosphorylation elicited by the PI 3-kinase inhibitor LY-294002.

Fig. 7.

Proposed mechanism of PAF-induced enterocyte apoptosis and its inhibition by PUFA. Survival signals maintain a baseline PI 3-kinase activity, resulting in Akt phosphorylation and a steady-state inhibitory effect on the mitochondrial death signaling. Activation of the palmitoylated PAF receptor (PAFR) relays a strong inhibitory signaling to PI 3-kinase, resulting in loss of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), dephosphorylation of Akt, and activation of the mitochondrial death pathway. PUFA can interfere with this mechanism by inhibiting palmitoylation of the PAFR and other members of the receptor signal transduction complex (not shown for simplicity). The effect of PAFR activation is mimicked by direct inhibition of PI 3-kinase with LY-294002, but this effect cannot be counteracted by PUFA because palmitoylation is not involved in the signaling downstream of PI 3-kinase. Heterologous overexpression of Bcl-2 can limit both PAF-induced and PI 3-kinase-inhibition-induced apoptosis at the level of mitochondrial protection.