Deficiency of Phospholipase A₂ Group 7 Decreases Intestinal Polyposis and Colon Tumorigenesis in Apc\textsuperscript{Min\textsuperscript{+\textsuperscript{+}}} Mice

Changxin Xu\textsuperscript{1,}, Ethan C. Reichert\textsuperscript{1,}, Tomoyuki Nakano\textsuperscript{1,}, Mariah Lohse\textsuperscript{1,}, Alison A. Gardner\textsuperscript{1,}, Mónica P. Revelo\textsuperscript{2,}, Matthew K. Topham\textsuperscript{1,\textsuperscript{*}\textsuperscript{,\textsuperscript{4}}}, and Diana M. Stafforini\textsuperscript{1,\textsuperscript{3}}

Abstract

Platelet-activating factor (PAF) is a naturally occurring phospholipid that mediates diverse effects such as physiological and pathological inflammation, immunosuppression, and cancer. Several lines of evidence support both positive and negative roles for PAF in carcinogenesis. PAF stimulates cell growth, oncogenic transformation, and metastasis, but can also limit proliferation and induce apoptosis. The biological context and microenvironment seem to define whether PAF has pro- or anticarcinogenic effects. To investigate the role of exacerbated PAF signaling in colon cancer, we conducted cell-based and in vivo studies using genetically engineered mice lacking expression of phospholipase A₂ group 7 (PLA₂G7), an enzyme that specifically metabolizes PAF and structurally related glycerophospholipids. Absence of PLA₂G7 robustly decreased intestinal polyposis and colon tumor formation in Apc\textsuperscript{Min\textsuperscript{+\textsuperscript{+}}} mice, suggesting an antitumorigenic role for PAF in settings characterized by aberrant function of the tumor suppressor Adenomatous polyposis coli (Apc). In colonic epithelial cells, exposure to a PAF analog led to dephosphorylation of Akt at serine-473 and induction of apoptosis. The mechanism of this response involved formation of a complex between β-arrestin 1 and the Akt phosphatase PHLPP2, and activation of the intrinsic pathway of apoptosis. Our results suggest that strategies based on inhibiting PLA₂G7 activity or increasing PAF-mediated signaling hold promise for the treatment of intestinal malignancies that harbor mutations in APC. Cancer Res; 73(9): 2806–16. ©2013 AACR.

Introduction

Colorectal cancer is one of the leading causes of cancer death in the Western world. More than 80% of colorectal cancers harbor mutations in the Adenomatous polyposis coli (APC) gene (1). A number of studies suggest that the generation of an inflammatory microenvironment promotes tumor development (2). In addition, the risk of developing colorectal cancer is related to the duration, extent, and severity of inflammatory disease (3). Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a proinflammatory phospholipid that plays important roles in the control of immune cell functions (4). PAF evokes growth factor-like signals in many cell types (5) and mediates its effects primarily through a G-protein-coupled receptor (PAFR; ref. 6). Overexpression of PAFR results in development of melanocytic tumors (7) and promotes ovarian cancer (8). PAF is metabolized to lyso-PAF by specific intracellular and secreted, calcium-independent, phospholipase A₂ (PLA₂) activities known as PAF acetylhydrolases. The secreted, plasma form of this family of enzymes is also known as phospholipase A₂ group 7 (PLA₂G7).

Numerous studies have shown that in settings of intestinal inflammation, PAF plays a detrimental role. Excess PAF generated during acute bowel injury actively contributes to the development of ischemic colitis and inflammatory bowel diseases (9) by increasing vascular permeability, inflammation, and chronic epithelial damage (10, 11). Enhanced PAF signaling by deletion of PLA₂G7 increased the severity of inflammation in a neonatal model of necrotizing enterocolitis (12). Similarly, in models of melanoma and breast cancer, PAF accumulation is thought to enhance tumorigenesis owing to its role in oncogenic transformation (5), metastasis (13), immuno-suppression (14), and angiogenesis (15).

Although most studies suggest a tumor-promoting role for PAF, the biological consequences associated with enhanced signaling via PAF/PAFR are highly dependent on the biological context and the levels of PAF and/or other PAFR ligands. PAF is synthesized in small intestinal tissues (16) and has homeostatic functions in the normal bowel, such as regulation of vectorial ion transport and mucosal permeability (17, 18). Thus, disruption of homeostatic PAF signaling may have untoward effects in defined settings. Although deletion of PLA₂G7 increased inflammation in a neonatal model of necrotizing enterocolitis, mortality rates before 24 hours of life were significantly lower in PLA₂G7\textsuperscript{−\textsuperscript{−}} pups compared with wild-type controls (12). Thus, both PLA₂G7 and PAF seem to play...
multiple roles depending on developmental stage, degree of intestinal damage, and extent of inflammation. Clearly, signaling events mediated by the PAF/PAFR/PLA2G7 axis are highly dependent on the environment and ligand concentration, and need to be defined in specific biological settings.

In the intestine, exposure to micromolar levels of PAF has antitumorigenic roles as judged by its ability to inhibit proliferation, induce differentiation, and suppress the malignant phenotype of human colon carcinoma cells (4). PAF has been reported to mediate apoptosis through both the extrinsic (i.e., Fas/FasL) and intrinsic (mitochondrial) apoptotic pathways (19, 20). Interestingly, lower levels of PAF were detected in tumor tissues from patients with advanced forms of colon cancer (Duke stages 3 and 4) compared with tumors evaluated at earlier disease stages (21). Plasma and tumor tissues from patients diagnosed with colon cancer have been reported to express 50% higher levels of PLA2G7 activity compared with samples from apparently healthy subjects (21, 22). Similar trends were observed in tissue extracts from liver metastases of colorectal cancers (23). Expression of PLA2G7 mRNA is upregulated by combined mutation of Ras and p53 in young adult murine colon cells, and silencing PLA2G7 expression reduces the growth of these cells in vivo (24). PLA2G7 has been reported to be a cooperation response gene that plays a role in malignant cell transformation (24).

The ApcMin+/− mouse model of multiple intestinal neoplasia is commonly used to identify mediators and characterize mechanisms that govern initiation, establishment, and progression of intestinal tumors (25). ApcMin+/− mice are heterozygous for a mutation that results in a truncated form of Apc; these animals develop spontaneous adenomas in the small intestine and fewer adenomas in the colon (25). Their propensity to form adenomas is similar to that of familial adenomatous polyposis patients who harbor mutations in the tumor suppressor APC. These subjects develop multiple intestinal adenomas that progress into malignant adenocarcinomas (26). Most human colorectal cancers also harbor mutations in APC that are thought to impact key processes affecting tumorigenesis, such as differentiation, Wnt/β-catenin signaling, and retinoic acid biosynthesis (26). In this study, we investigated whether altered signaling via the PAF/PAFR axis affects events relevant to colon carcinogenesis in settings characterized by deregulated APC function. To accomplish this, we conducted studies in ApcMin+/− mice crossed to animals that lack expression of PLA2G7, one of the key enzymes involved in PAF metabolism. To address mechanistic issues, we investigated responses to PAF treatment in human colon cancer cells. Our data show that genetic deletion of Pla2g7 decreases intestinal polyposis through PAFR-mediated dephosphorylation of Akt by the phosphatase PHLPP2, and induction of apoptosis via the intrinsic pathway.

Materials and Methods

Please see Supplementary Material for additional information.

Cell culture and treatment

Human colorectal adenocarcinoma cell lines HT-29, Caco-2, Lovo, SW-480 cells, and colorectal carcinoma HCT-116 cells were obtained from American Type Culture Collection, where they are regularly tested for viability, recovery, growth, morphology, and isoenzymology. Human colonic adenocarcinoma HCA-7 cells were from Life Technologies Corporation Collection. All lines were maintained at 37 °C in a 5% CO2 atmosphere, in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Phospholipids (i.e., PAF, cPAF, lyso-PAF) used to stimulate cells were dried under a stream of nitrogen and were resuspended in 1 mg/mL fatty-acid free bovine serum albumin before addition to the cells.

Histology and immunohistochemistry

Segments of the small intestines of ApcMin+/− and ApcMin+/−/Pla2g7−/− mice were fixed in 4% formaldehyde overnight, embedded in paraffin, and cut in 4-μm sections. After deparaffinization and rehydration, sections were stained with hematoxylin and eosin according to standard procedures. Immunohistochemical examination was done using a rabbit anti-PCNA antibody diluted 100 fold. Biotinylated anti-rabbit IgG (200-fold dilution, Vector Laboratories, Inc.) was used as the secondary antibody. Staining was done using avidin-biotin reagents (Vectastain ABC reagent), 3,3′-diaminobenzidine, and hydrogen peroxide. As negative controls, consecutive sections were immunostained without exposure to the primary antibody. These sections showed virtually no signal and are, therefore, not shown. TUNEL assays were conducted using ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, S7101), following the manufacturer’s recommendations. Counterstaining was done using a 50:50 mixture of 2% Alcian blue and 2% methyl green.

Assessment of cellular proliferation

HT-29 cells seeded in 96-well plates were allowed to grow in the presence of 10% FBS, and were then placed in 0% to 0.1% FBS-containing medium, as indicated in the figure legends. The cells were exposed to fresh media containing cPAF and BNG52021 (where indicated) every 24 hours. After a total of 72 hours at 37 °C, we assessed cellular proliferation using the XTT assay, following the manufacturer’s specifications. Briefly, each well was supplemented with XTT labeling mixture and the plates then were incubated for 4 hours at 37 °C. Absorbance at 450 and 650 nm was measured using a plate reader.

Gene silencing and overexpression

siRNAs (including negative controls) were purchased from Dharmacon, RNAi Technologies. siRNAs (10 μg of annealed oligos) were transfected using Dharmacon transfection reagents, as recommended by the manufacturer. The target sequences for PHLPP1 and PHLPP2 siRNAs are listed below:

PHLPP1 5′-GGAATCAACTGGTCACATT-3′

PHLPP2 5′-CCTAAGTGGCAACAAGCTT-3′

The cells were harvested 24–48 hours following transfection. For overexpression studies, we seeded the cells evenly in 6-well plates and, after overnight incubation, transfected 2 μg of each plasmid DNA using Fugene Transfection Reagent (Promega Corporation).
Animals
C57BL/6J Apc<sup>Min+/–</sup> mice were bred with either C57BL/6J or C57BL/6J Pla2g7<sup>+/–</sup> mice (12) to generate animals that carried a mutant Apc allele and 2 or 0 functional Pla2g7 alleles (Apc<sup>Min+/–</sup> and Apc<sup>Min+/–</sup>/Pla2g7<sup>−/−</sup>, respectively). The animals were observed daily for clinical signs and morbidity, and were sacrificed using CO2 asphyxiation when they reached approximately 100 days of age. We dissected and washed the colons as previously described (27), stained them using methylene blue, and then assessed the number of polyps and tumors using a dissecting microscope. Most polyps were adenomas, identified by their appearance as grossly visible, preinvasive lesions that were common in the small intestine of Apc<sup>Min+/–</sup> mice. Colonic lesions that displayed a focally invasive phenotype were categorized as tumors. Edelfosine studies were conducted on mice aged 34 days (range, 31–36 days) by supplementing the drinking water with 12.5 μg/mL edelfosine in 0.1% ethanol for an average of 65 days (range, 62–69 days). All experimentation was conducted following approval by the University of Utah's Animal Care and Use Committee.

Statistical analyses
Immunoblot data are representative of at least two independent analyses conducted using similar experimental conditions. Protein determination assays were conducted in duplicate and the results typically varied by no more than 5%. In our animal studies, we report average numbers of polyps or tumors per mouse ± SD. The significance of differences in polyp and tumor incidence was analyzed using Student t test. Results were deemed to be statistically significant when P values were lower than 0.05.

Results
Deletion of Pla2g7 decreases small intestinal polypl and colon tumor incidence in Apc<sup>Min+/–</sup> mice
To evaluate the impact of Pla2g7 on intestinal polyposis, we crossed Pla2g7<sup>−/−</sup> mice with Apc<sup>Min+/–</sup> animals; the characteristics of the cohorts are summarized in Table 1. The median age of the mice at the time of analysis was 98 days and the proportion of male and female animals was comparable in the 2 groups. We found that deletion of Pla2g7 was associated with decreased polyposis in the proximal, mid, and distal regions of the small intestine (Table 1). In addition, the number of colonic tumors was significantly lower in Apc<sup>Min+/–</sup>/Pla2g7<sup>−/−</sup> compared with Apc<sup>Min+/–</sup> mice (Table 1). All differences remained statistically significant when female and male mice were analyzed separately. Within each genotype, gender did not affect the number of intestinal polyps.

Table 1. Deletion of Pla2g7 decreases polypl number in the Apc<sup>Min+/–</sup> model of intestinal polyposis

<table>
<thead>
<tr>
<th></th>
<th>Apc&lt;sup&gt;Min+/–&lt;/sup&gt;</th>
<th>Apc&lt;sup&gt;Min+/–&lt;/sup&gt;/Pla2g7&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>P</th>
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<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>21</td>
<td>–</td>
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<tr>
<td>Number of males (%)</td>
<td>7 (41%)</td>
<td>12 (57%)</td>
<td>–</td>
</tr>
<tr>
<td>Number of females (%)</td>
<td>10 (59%)</td>
<td>9 (43%)</td>
<td>–</td>
</tr>
<tr>
<td>Median age (days)</td>
<td>98</td>
<td>98</td>
<td>–</td>
</tr>
<tr>
<td>Age range (days)</td>
<td>84–100</td>
<td>78–102</td>
<td>–</td>
</tr>
<tr>
<td>Proximal intestinal polyps&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 2.6</td>
<td>3.0 ± 2.2</td>
<td>0.0010</td>
</tr>
<tr>
<td>Mid intestinal polyps&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.1 ± 12.3</td>
<td>12.0 ± 7.6</td>
<td>5.2 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Distal intestinal polyps&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.0 ± 17.4</td>
<td>22.1 ± 16.0</td>
<td>0.0004</td>
</tr>
<tr>
<td>Colon polyps&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6 ± 1.7</td>
<td>0.3 ± 0.6</td>
<td>0.2458</td>
</tr>
<tr>
<td>Colon tumors&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6 ± 1.3</td>
<td>0.5 ± 0.9</td>
<td>0.0020</td>
</tr>
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<sup>a</sup>Data reported represent the average number of polyps per mouse ± SD.
<sup>b</sup>Data reported represent the average number of tumors per mouse ± SD.

cPAF inhibits proliferation and induces apoptosis of colonic epithelial cells through the PAF receptor
To elucidate the mechanism whereby absence of PLA2G7 decreased intestinal polyposis, we evaluated responses elicited after exposure of colon cancer cells to PAF. Previous studies showed that PAF mediates differentiation, proliferation, and growth inhibitory responses, depending on the biological context (4, 8). To characterize effects in the intestinal epithelium, we exposed HT-29 colonic epithelial cells to PAF (0–1 μmol/L) in medium containing relatively low (0.5%) serum levels to limit PAF degradation by PLA2G7. PAF modestly inhibited cellular growth (not shown), but stronger growth inhibitory effects were observed with methylcarbamoyl PAF (cPAF), a metabolically stable analog that is resistant to hydrolysis by PLA2G7 (Fig. 1A). The concentrations used to assess effects on cellular growth were below the CMC of PAF (reported to be 2.5–3.0 μmol/L; ref. 28), suggesting that the observed effects were the consequence of lipid signaling events and did not represent nonspecific cellular lysis. We next assessed whether participation of PAFR was required for growth inhibitory effects. Supplementation with the PAFR antagonist BN-52051 rescued cPAF-induced inhibition of cellular growth (Fig. 1B), establishing that in colonic epithelial cells cPAF inhibits cellular proliferation via PAFR.

We next investigated the mechanisms accounting for cPAF-mediated effects on cellular survival. Lu and colleagues previously showed that in rat small intestinal epithelial cells PAF induces apoptosis by increasing caspase-3 activity and DNA
fragmentation (29). Overnight treatment of serum-starved HT-29, Caco-2, HCA-7, and LoVo cells with cPAF increased caspase-3 activation and PARP-1 cleavage (Fig. 2A). Although apoptosis in response to cPAF was observed in most colon cancer cells, we detected differences in the magnitude of individual responses; SW-480 cells were the most resistant (not shown). These variations may be because of differential susceptibility to PAF or altered function/expression of downstream effectors required for apoptosis in each cell line, or both. cPAF-induced apoptosis required PAFR expression as the specific PAFR antagonist WEB2086 inhibited this response (Fig. 2B) and lyso-PAF failed to induce PARP-1 cleavage (not shown). The proapoptotic agent anisomycin induced apoptosis, as expected, and low levels of cPAF potentiated this effect (Fig. 2C). These results suggest that cPAF can singly initiate the apoptotic cascade and also augment programmed cell death induced by other agents.

**Basal and stimulated PAFR activation decrease pAkt473 through a G-protein-independent mechanism**

To identify downstream events after challenge with cPAF, we evaluated the state of activation of signaling pathways known to affect cellular growth, survival, and apoptosis. Akt has been implicated in the regulation of multiple biological processes ranging from glycogenesis to embryonic development, inflammation, apoptosis, and cell proliferation. Moreover, activation of the PI3K/Akt pathway plays a central role in tumorigenesis across a variety of malignancies. We found that cPAF consistently decreased the level of basal pAkt473 in HT-29 cells for relatively prolonged time periods (Fig. 3A). This response was not limited to a single colon cancer cell line because HCT-116, LoVo, and HCA-7 (not shown) cells responded in similar fashions (Fig. 3B). Slight variations in the state of basal cellular activation likely accounted for variability in the extent of pAkt473 dephosphorylation among experiments. However, the response was consistently observed throughout the study (average decrease in pAkt473 = 65%, n = 17). Although cPAF affected the extent of Akt phosphorylation at serine-473, we observed modest effects on phosphorylation at serine-308 (64% vs. 35% decrease in pAkt473 and pAkt308, respectively; Fig. 3C).

Consistent with our observations in cell growth assays, cPAF-mediated decrease in pAkt473 levels required PAFR, as the specific antagonists BN-52051 and CV-3988 partially prevented this effect (Fig. 4A). In addition, we detected increased levels of pAkt473 in HT-29 cells treated for 30 minutes with the same antagonists (Fig. 4B). These combined results indicate that in intestinal epithelial cells PAFR attenuates basal and stimulated Akt phosphorylation.

PAFR can interact with multiple G-proteins and lead to activation of diverse signaling pathways (7). PAFR ligands have been shown to activate Gαi/Gqαi, leading to decreased cAMP levels and protein kinase A (PKA) activity (7). Signaling through cAMP/PKA was previously reported to modulate the PI3K/Akt signaling axis in coronary microvessels (30). We tested the effect of pertussis toxin, which ribosylates Gαi/Gqαi in a Gαiγ heterotrimeric state-dependent fashion (7), and found that the inhibitor did not significantly affect cPAF-mediated dephosphorylation of pAkt473 (Supplementary Fig. S1A). These results indicate that PAFR expressed on colon epithelial cells does not couple to Gαi/Gqαi. In addition, we found that inhibition and activation of PKA with KT-5720 and forskolin, respectively, did not affect cPAF-induced dephosphorylation of pAkt473 in a
manner consistent with a role of PKA in this response (Supplementary Fig. S1B and S1C). Our studies suggest that PAFR negatively regulates pAkt473 levels through mechanisms that are largely independent of PKA and that do not require coupling to Ga\textsubscript{i}/Ga\textsubscript{0}.

In additional studies we tested whether Ga\textsubscript{q} and/or βγ subunits released from heterotrimeric G-proteins were involved in dephosphorylation of pAkt\textsuperscript{473}. Functional coupling to Ga\textsubscript{q} activates PLC-β, increases production of inositol (1,4,5)-trisphosphate (6) and activates PI3K/Akt signaling. Our results (Fig. 3) showing that cPAF treatment decreases pAkt\textsuperscript{473} levels combined with the observation that PLC inhibitors had no effect on cPAF-induced effects on pAkt\textsuperscript{473} levels (not shown) suggest that PAFR coupling to Ga\textsubscript{q} is not required for reduced pAkt\textsuperscript{473}. We next considered whether PKC activation, which usually occurs after coupling of PAFR to Gβγ subunits (7), was required for dephosphorylation of pAkt\textsuperscript{473}. We examined the effect of the PKC inhibitors bisindolylmaleimide-I (BIM-I, an inhibitor of classical and novel PKC subfamilies), Gö-6983 (a broad-spectrum PKC inhibitor), and Gö-6976 (an inhibitor of PKC-α and PKC-β). We found that these agents failed to block cPAF-mediated effects on pAkt\textsuperscript{473} does not require PAFR coupling to βγ subunits or PKC activation. Our combined observations suggest that mechanisms other than canonical signaling through G-proteins are involved in cPAF-mediated dephosphorylation of pAkt\textsuperscript{473}. These results are somewhat reminiscent of studies showing that in certain cells, agonist stimulation of PAFR activates signaling pathways through G-protein-independent mechanisms (31).

The Akt phosphatase PHLPP2 and β-arrestin mediate cPAF-induced dephosphorylation of pAkt\textsuperscript{473}

Dephosphorylation of pAkt\textsuperscript{473} is catalyzed by okadaic acid-sensitive protein phosphatases (PP; ref. 32) and by PHLPP enzymes (PH domain and leucine-rich repeat protein

Figure 2. cPAF specifically induces apoptosis and promotes anisomycin-induced apoptosis in colon cancer cells. A, HT-29, Caco-2, HCA-7, and LoVo cells were starved overnight in serum-free medium (HT-29 and Caco-2) or medium containing 0.1% serum (HCA-7 and LoVo). The next day, we subjected the cells to overnight (16 hours) treatment with vehicle or the indicated concentrations of cPAF. Solubilized extracts then were subjected to electrophoresis and immunoblot analyses to assess caspase-3 activation and PARP-1 cleavage. β-Actin levels were determined for normalization purposes. B, starved HT-29 cells were preincubated with the PAFR antagonist WEB2086 or with vehicle for 1 hour at 37°C. We then added cPAF and incubated the cells overnight at 37°C. Protein extracts were subjected to electrophoresis and immunoblot analyses to assess PARP-1 cleavage and β-actin content. Band intensity was determined using GelQuant.NET, as described in Materials and Methods. cPARP-1, cleaved PARP-1. C, HT-29 cells were starved for 48 hours and then were pretreated with vehicle or cPAF (0.5–100 nmol/L) for 30 minutes before addition of anisomycin (25 μg/mL). The cells then were incubated for 6 hours in the presence of anisomycin and cPAF. PARP-1 cleavage and β-actin content were determined as described earlier.
cells were treated with cPAF or vehicle for 30 minutes at 37°C. The levels of pAkt473, Akt, and β-actin then were determined in protein lysates, using immunoblot analyses. A, starved HT-29 cells were treated with 1.5 μmol/L cPAF or vehicle for 10, 30, and 240 minutes at 37°C. The levels of pAkt473, Akt, and β-actin then were determined in protein lysates, using immunoblot analyses. B, starved HCT-116 and LoVo cells were treated with 1.5 μmol/L cPAF or vehicle for 30 minutes at 37°C. The levels of pAkt473, Akt, and β-actin then were determined using immunoblot analyses. C, starved HT-29 cells were treated with cPAF or vehicle for 30 minutes at 37°C. The levels of pAkt473, pAkt308, Akt, and β-actin then were determined as earlier.

One of the mechanisms whereby receptor signaling events are blocked involves β-arrestin, a molecule long known to associate with activated receptors and limit further signaling (35). β-Arrestin recruitment to GPCRs is thought to terminate signaling by providing a scaffold for the assembly of complexes involving the PI3K, Ras, and ERK1/2 axes, among others (31, 36, 37). Previous studies have shown that PAFR agonists induce translocation of β-arrestins from the cytoplasm to the cell membrane and that PAFR binds β-arrestins (38). To investigate whether β-arrestins participate in cPAF/PHLPP2-induced dephosphorylation of pAkt473, we expressed PHLPP2 and FLAG-tagged β-arrestin 1 in HT-29 cells, and then treated the cells with cPAF or vehicle. Immunoprecipitation of β-arrestin 1 revealed that cPAF specifically increased association between PHLPP2 and β-arrestin 1, and promoted dephosphorylation of Akt at serine-473 (Fig. 5B), suggesting that cPAF decreases pAkt473 levels via β-arrestin 1 and PHLPP2.

**Effects of Pla2g7 deletion and pharmacological activation of PAF/PAFR signaling in vivo**

Our next goal was to assess whether functional changes resulting from stimulation with cPAF in cultured cells were recapitulated in vivo. First, we evaluated the consequences of deletion of Pla2g7 in ApcMin/−/− mice, which is expected to result in substrate accumulation. We conducted histological and immunohistochemical analyses in distal small intestinal tissues from ApcMin/−/− mice harboring 0 or 2 wild-type Pla2g7 alleles. H&E staining revealed similar intestinal architecture in ApcMin/−/− intestinal tissues, regardless of Pla2g7 expression (Fig. 6A). However, tissues from ApcMin/−/− mice were characterized by the presence of several islands of proliferating cells that were absent in mice lacking expression of functional Pla2g7 alleles (Fig. 6B). In addition, ApcMin/−/−/Pla2g7−/− mice expressed PCNA-positive epithelial cells primarily in the base region of the crypt, suggesting that deletion of Pla2g7 decreases epithelial cell proliferation along the villus axis. Staining for TUNEL showed that intestinal apoptosis increased in the absence of Pla2g7 (Fig. 6C). The positive effects of Pla2g7 deletion on apoptosis occur at relatively early stages of polyph positivity as ApcMin/−/−/Pla2g7−/− mice showed clear evidence of intestinal apoptosis at 5 weeks of life (Fig. 6D). Finally, in vivo activation of PAF signaling by oral administration of edelfosine, a metabolically stable analog of PAF, significantly decreased the incidence of colon tumors in ApcMin/−/− mice (Fig. 6E).
PLA2G7 expression is elevated in colon tumors compared with normal tissues

Previous studies reported increased PLA2G7 activity (22, 23) and mRNA levels (24) in tumor tissues and plasma from colon cancer patients compared with samples from healthy donors. To investigate whether expression of PLA2G7 protein correlates with colon tumorigenesis in humans, we used publicly available information provided by The Swedish Human Protein Atlas project (39). These analyses revealed increased levels of PLA2G7 in colon adenocarcinomas compared with normal colonic tissues (Supplementary Table S1). This observation, combined with functional analyses in animal and cellular models (this study and ref. 24) suggest that PLA2G7 actively contributes to the pathogenesis of colorectal cancer.

Discussion

Our results showed that in all regions of the small intestine analyzed, ApcMin+/+ mice developed substantially less intestinal polyps and tumors in the absence of functional Pla2g7 alleles. Intestinal tissues from Pla2g7-null mice showed decreased proliferation when compared with tissues from control ApcMin+/+ mice. These results were complemented with studies in cultured colonic epithelial cells exposed to a nonhydrolyzable analog of PAF. We found evidence for cPAF-mediated inhibition of cellular proliferation, caspase-3 activation, and PARP-1 cleavage in a variety of colonic epithelial cells. These responses were mediated by PAFR and were associated with the formation of a complex between β-arrestin 1 and the Akt phosphatase PHLP2. Taken together, our data suggest that cPAF-mediated dephosphorylation of pAkt473 enhances apoptosis and attenuates activation of cancer-relevant downstream effector molecules, thus limiting proliferation and cell survival in vivo.

PLA2 activities play dual roles in carcinogenesis, and these functions are tissue- and context-dependent. These enzymes modulate the levels of biologically active lipids whose functions are strongly affected by the environment. For example, Pla2g2a encodes a secretory PLA2 (sPLA2-IIA) that reduces intestinal tumor development in ApcMin+/+ mice (40) but enhances colon tumor cell growth and infiltration in a tumor explant model (41). Interestingly, the Pla2g2a gene is naturally disrupted in a number of mouse strains, including C57BL/6, the strain used in our studies (42). The physiological function of sPLA2-IIA likely differs from that of PLA2G7 as the enzymes metabolize different phospholipid substrates (43). Our studies in mice indicate that deletion of Pla2g2a attenuates intestinal polyposis, suggesting that the enzyme actively contributes to colon tumorigenesis. Consistently, PLA2G7 has been proposed to play a causal role in colon tumor formation downstream of oncogenic mutations (24). In addition, decreasing PLA2G7 expression in human prostate cancer cells limited migration and proliferation, and induced apoptosis (44), suggesting similar functions for PLA2G7 in prostate tumors.

Highly controlled, limited PAF production is likely to have multiple beneficial effects in the intestine. Normal mucosal epithelial cells are rapidly proliferating and completely turn over every 24 to 96 hours and low-level activation of PAF signaling may contribute to the maintenance of a normal intestinal barrier, enhancing protection and facilitating repair from injury (45). Kelly and colleagues showed that bacterial translocation across the intestinal epithelial barrier requires PAFR and suggested that this may represent a physiological mechanism to clear bacteria (46). Our studies, combined with those of others (4), showed that activation of the PAF/PAFR axis may have beneficial effects in colon cancer by mediating growth-inhibitory, proapoptotic functions. Indeed, supplementation of the drinking water with edelfosine, a stable PAF analog, reduced the incidence of colon tumors in ApcMin+/+ mice, in agreement with our studies in cultured cells.

It is well recognized that chronic bowel inflammation predisposes to colon cancer. Exacerbated signaling through the PAF axis is thought to negatively impact intestinal diseases such as necrotizing enterocolitis, ulcerative colitis, and Crohn’s disease. Increased apoptosis can be highly detrimental in these settings, which are characterized by exacerbated inflammation and chronic tissue damage. PAF

Figure 4. PAFR is required for cPAF-mediated dephosphorylation of pAkt473. A, HT-29 cells were starved overnight and then were incubated with 10 μmol/L BN-52051 or CV-3988 for 3 hours at 37°C. We then exposed the cells to 1.5 μmol/L cPAF for 30 minutes and assessed the levels of pAkt473 and β-actin, as earlier. B, HT-29 cells were starved overnight and then were incubated with 2 μmol/L BN-52051 or CV-3988 for 30 minutes at 37°C. We then exposed the cells to 1.5 μmol/L cPAF for 10 minutes and assessed the levels of pAkt473 and β-actin.
The cells with cPAF for 45 minutes at 37°C. The cells were starved overnight in serum-free media. We then stimulated PHLPP2, as described in Materials and Methods. After 24 hours at 37°C, cells were transfected with control oligos, or with siRNAs for PHLPP1 or PHLPP2, as described in Materials and Methods. We determined the extent of cPAF-induced recruitment of β-arr estin 1 in this response, lead us to speculate that cPAF induces phosphorylation of PAFR, β-arr estin recruitment, formation of stable β-arr estin–PAFR complexes, and sustained dephosphorylation of pAkt473 through a G-protein–independent pathway. Similarly, other GPCRs [i.e., dopamine receptor (D2R) and protease-activated receptor-2] respond to ligand stimulation in this fashion (49).

Our findings that PAFR activation enhances dephosphorylation of pAkt473 by PHLPP2, and that pAkt308 levels are modestly decreased are also novel. PHLPPs are phosphatases that specifically dephosphorylate serine-473 in the hydrophobic phosphorylation motif. The expression of PHLPP1 and PHLPP2 is either lost or decreased in 78% and 86% of colon tumor tissues, respectively, suggesting that PHLPP is a colon cancer tumor suppressor (50). Dephosphorylation of Akt at both serine-473 and threonine-308 leads to a reduction of kinase activity and can result in activation of substrates such as glycogen synthase kinases 3α and 3β (GSK3α and GSK3β) that are negatively regulated by Akt. We found no evidence for cPAF-induced effects on GSK3β phosphorylation, which is consistent with modest cPAF-mediated effects on threonine-308. Instead, we found that cPAF-induced dephosphorylation of pAkt473 inhibited the survival-promoting effects of Akt and induced apoptosis by activating caspase-3 and stimulating PARP-1 cleavage.

In summary, we have shown that constitutive deletion of Pla2g7 decreases intestinal polyposis in the ApcMin/+ model. Our studies in mouse tissues and in intestinal epithelial cells suggest that this effect is the consequence of ligand-mediated activation of PAFR, decreased Akt activation through PHLPP2, and enhanced apoptosis. The study points at inhibition of Pla2g7 activity as a possible novel strategy for the treatment of inherited forms of colon cancer in which the function of APC is compromised. Our observations are potentially generalizable to sporadic forms of the disease, as mutations in APC are common to most colon cancers.
Figure 6. Effect of Pla2g7 deletion and pharmacological activation of PAF/PAFR signaling in vivo. A, H&E-stained sections from small intestine show well-preserved villi in ApcMin/+ mice and atrophic-appearing villi in ApcMin/+ /Pla2g7−/− mice. Original magnification, ×20. B, immunohistochemistry of PCNA shows higher labeling in epithelial cell nuclei from ApcMin/+ mice and scant labeling in ApcMin/+ /Pla2g7−/− animals. Original magnification, ×20. C, TUNEL assays show higher labeling in epithelial cells from ApcMin/+ /Pla2g7−/− mice compared with ApcMin/+ mice. Original magnification, ×20. D, expression of caspase-3, cleaved caspase-3, and β-actin in distant intestinal tissue extracts from 5-week-old wild-type (WT), ApcMin/+, and ApcMin/+ /Pla2g7−/− mice. E, effect of oral administration of edelfosine, a metabolically stable PAF analog, on the incidence of colon tumors in ApcMin/+ mice.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C. Xu, M. K. Topham, D. M. Stafforini
Development of methodology: C. Xu, T. Nakano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Xu, T. Nakano, M. Lohse, A. A. Gardner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Xu, M. P. Revelo, M. K. Topham, D. M. Stafforini
Writing, review, and/or revision of the manuscript: C. Xu, M. P. Revelo, M. K. Topham, D. M. Stafforini

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Changxin Xu, Ethan C. Reichert, Tomoyuki Nakano, et al.


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