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ORIGINAL ARTICLE

Role of platelet-activating factor in the pathogenesis of 5-fluorouracil-induced intestinal mucositis in mice

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Abstract
Purpose Gastrointestinal mucositis is a common side effect of cancer chemotherapy. Platelet-activating factor (PAF) is produced during gut inflammation. There is no evidence that PAF participates in antineoplastic-induced intestinal mucositis. This study evaluated the role of PAF in 5-fluorouracil (5-FU)-induced intestinal mucositis using a pharmacological approach and PAF receptor knockout mice ($PAFR^{-/-}$).
Methods Wild-type mice or $PAFR^{-/-}$ mice were treated with 5-FU (450 mg/kg, i.p.). Other mice were treated with saline or BN52021 (20 mg/kg, s.c.), an antagonist of the PAF receptor, once daily followed by 5-FU administration. After the third day of treatment, animals were sacrificed and tissue samples from the duodenum were removed for morphologic evaluation. In addition, myeloperoxidase activity and the cytokine concentration were measured.
Results 5-FU treatment decreased the duodenal villus height/crypt depth ratio, increased MPO activity, and increased the concentration of TNF-α, IL-1β and KC in comparison with saline-treated animals. In $PAFR^{-/-}$ mice and PAFR antagonist-treated mice, 5-FU-dependent intestinal damage was reduced and a decrease in duodenal villus height/crypt depth ratio was attenuated. However, the 5-FU-dependent increase in duodenum MPO activity was not affected. Without PAFR activation, 5-FU treatment did not increase the TNF-α, IL-1β and KC concentration.
Conclusions In conclusion, our study establishes the role of PAFR activation in 5-FU-induced intestinal mucositis. This study implicates treatment with PAFR antagonists as novel therapeutic strategy for this condition.

Keywords PAF · Intestinal mucositis · 5-fluorouracil · Cytokines

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Introduction

The antimetabolite agent 5-fluorouracil (5-FU) is used to treat various cancers, including colorectal and breast cancers [22, 35], and can induce intestinal damage, referred to as intestinal mucositis [1]. Gastrointestinal mucositis is a common side effect of cancer chemotherapy for which there is no efficient treatment. To date, it is the most significant dose-limiting toxicity of 5-FU treatment [3]. Previous studies have demonstrated that gastrointestinal mucositis is a consequence of various processes, such as apoptosis, hypoproliferation, inflammatory response, altered absorptive capacity, bacteria proliferation and colonization, and contributes to intestinal barrier dysfunction [3, 39]. In addition, cancer chemotherapy-induced intestinal mucositis increases the expression of cytokines, such as TNF-α, IL-1β, and IL-6 [21, 25].

Platelet-activating factor (PAF) is a phospholipid mediator that is produced by most cells and tissues and is a

potent mediator of many inflammatory processes, prostaglandin and eicosanoid synthesis, induction of apoptosis, and nuclear factor kappa B activation [2, 7, 23, 26, 41]. Platelet-activating factor (PAF) is produced endogenously in the gut during inflammatory bowel disease [40]. PAF participates in the pathogenesis of necrotizing enterocolitis [15] and intestinal damage associated with endotoxic shock [16]. Studies have demonstrated that systemic administration of PAF to rats results in intestinal necrosis in a dose-dependent manner [16]. Furthermore, treatment with PAF antagonists reduces mucosal inflammation in an animal model of colitis [5, 24]. In addition, the intestinal epithelium produces PAF during inflammatory events [2, 6, 26]. PAF activates receptors that are constitutively expressed in various intestinal cell lines [26].

Considering the lack of information concerning the pathophysiological role of PAF in cancer chemotherapy-induced intestinal mucositis, we determined the role of PAF in 5-fluorouracil (5-FU)-induced intestinal mucositis using knockout animals and an antagonist of PAF receptors (PAFR).

Materials and methods

Animals

BALB/c (8–10-wk-old) mice were obtained from the School of Medicine of Ribeirão Preto. Animals were housed in cages in temperature-controlled rooms and received food and water ad libitum. $PAFR^{-/-}$ mice were generated as previously described and backcrossed for a minimum of 10 generations into the BALB/c background [18]. Animal treatment and surgical procedures were performed in accordance with the Brazilian College of Animal Experimentation (COBEA) and were approved by an ethics committee.

Role of PAF in 5-FU-induced intestinal mucositis in mice

Intestinal mucositis was induced in $PAFR^{-/-}$ mice or wild-type BALB/c mice by intraperitoneal administration of 5-FU (450 mg/kg). In addition, wild-type mice were treated with a PAFR antagonist (BN52021, 20 mg/kg, s.c., once daily) or vehicle (saline) followed by 5-FU (450 mg/kg, i.p) administration. After the third day of treatment, animals were sacrificed and tissue samples from the duodenum were removed for histologic analysis (evaluated by a pathologist using a blind method) or stored at −70°C for evaluation of cytokine (TNF-α, IL-1β, KC) and myeloperoxidase (MPO) activity. Blood samples were also collected for counting leukocytes.

Leukocyte count [32]

Mice were anesthetized with an ether overdose, and blood samples were collected by heart puncture. The total number of white cells was determined using a Neubauer chamber in blood samples diluted in Turk's solution (1:10). Data were expressed as the number of leukocytes per mm^3 of blood.

Intestinal morphology and histopathology

Segments of duodenum were collected. Samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. Measurement of the villus height (from the top of the villus to the villus–crypt junction) and the crypt depth (defined as the depth of invagination between adjacent villi) was performed by light microscopy using a calibrated micrometer (10X). Ten intact and well-oriented villi and crypts were measured and averaged for each sample [34].

Intestinal MPO activity

MPO is an enzyme that is primarily expressed in azurophilic neutrophil granules. MPO is commonly used as a biochemical marker of granulocyte infiltration into various tissues, including the gastrointestinal tract. The extent of neutrophil accumulation in the intestinal mucosa was measured by the MPO activity assay [4]. Briefly, 50 mg of intestinal tissues were homogenized in HTAB buffer (Sigma). The homogenates were centrifuged at 4,500 rpm for 7 min at 4°C. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride (Sigma) and 1% hydrogen peroxide (Merck). Results were reported as MPO units/mg of tissue. A unit of MPO activity was defined as the amount necessary to convert 1 μmol of hydrogen peroxide into water at 22°C in 1 min.

Detection of cytokines (TNF-α, IL-1β and KC) in duodenum

Tissue samples were stored at −70°C until experimentation. The tissue was homogenized and processed as described previously [33]. The TNF-α, IL-1β, and KC concentrations were determined by ELISA as described previously [8]. Briefly, microtiter plates were coated with antibodies against mouse TNF-α, IL-1β, and KC (2 μg ml^{-1}) overnight at 4°C. After blocking the plates, the experimental samples and positive controls were added in duplicate and incubated at 4°C for 24 h. Plates were washed three times with buffer and then incubated with biotinylated sheep polyclonal antibodies against TNF-α,

IL-1β, or KC (diluted 1/1,000 with assay buffer 1% BSA). After an additional incubation at room temperature for 1 h, the plates were washed and 50 µl of an avidin-HRP solution (diluted 1:5,000) was added. The colored reagent o-phenylenediamine (OPD; 50 µl) was added 15 min later and plates were incubated in the dark at 37°C for 15–20 min. The reaction was quenched with H_2SO_4, and absorbance was measured at 490 nm. Data represent the mean ± SEM.

Statistical analysis

Data represent the mean ± standard error of the mean (SEM) for each group. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni's test. Observed differences with a P value < 0.05 were considered significant.

Results

As shown in Table 1, 5-FU treatment significantly induced leukopenia in both wild-type and PAFR$^{-/-}$ mice. In agreement, PAFR inhibition with BN52021 did not prevent 5-FU-induced leukopenia.

Loss of PAFR in mice attenuated, at least partially, 5-FU-induced intestinal mucositis. As shown in Fig. 1, 5-FU treatment caused a significant shortening of villi height (VH), increase in crypt depth (CD), and decrease in the VH/CD ratio in wild-type mice. PAFR$^{-/-}$ mice experienced less 5-FU-induced intestinal damage and the duodenal VH/CD ratio was significantly reduced, primarily due to the prevention of CD enhancement. However, inhibition of PAFR did not prevent the 5-FU-dependent decrease in VH.

BN52021 treatment, as shown in Fig. 2, also attenuated 5-FU-induced intestinal mucositis. In addition, BN52021 treatment prevented the 5-FU-induced reduction in the

Table 1 Effect of 5-FU treatment upon leukocyte count in mice pretreated with BN 52021 and PAFR$^{-/-}$ animals

Groups ($n = 8$)	No cells/mm^3
PAF$^{+/+}$ (control/wild-type)	7,173.00 ± 1,032.50
PAF$^{+/+}$ + 5-FU	1,746.00 ± 371.00*
PAF$^{+/+}$ + BN 52021 + 5-FU	2,250.00 ± 484.90*
PAFR$^{-/-}$	6,133.00 ± 510.20
PAFR$^{-/-}$ + 5-FU	1,788.00 ± 456.20*

Values were reported as mean ± SEM

* $P < 0.05$ compared to PAF (+/+) (control/wild-type). ANOVA and Bonferroni's test

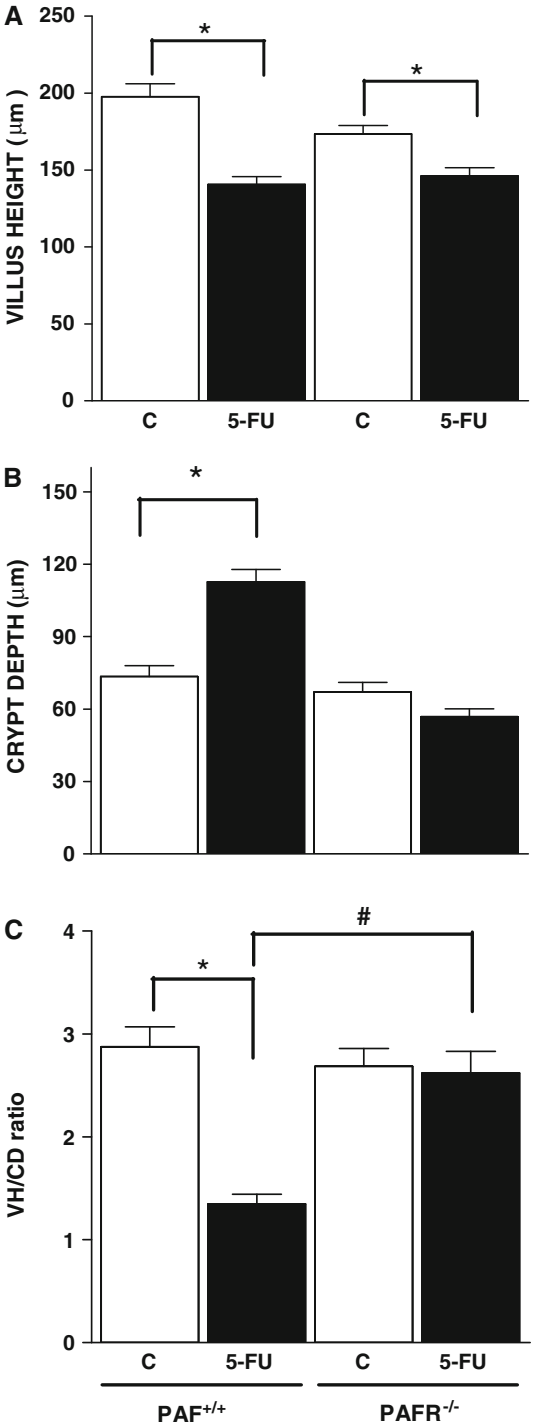

Fig. 1 PAFR$^{-/-}$ mice presented less 5-fluorouracil-induced duodenal damage than wild-type animals. 5-FU (450 mg/kg) reduced villus high (VH) and increased crypts depth (CD) and VH/CD ratio in wild-type animals. In PAFR$^{-/-}$ animals, 5-FU reduced the VH (**a**) but did not increase CD (**b**) or reduced VH/CD ratio (**c**). Values were reported as mean ± SEM, $n = 6$. *$P < 0.05$ compared to control (**c**), $^{\#}P < 0.05$ compared to 5-FU + PAF$^{+/+}$. ANOVA and Bonferroni's test

duodenal VH/CD ratio, primarily by attenuating the decrease in VH. However, 5-FU-induced deepening of the crypt depth was not affected.

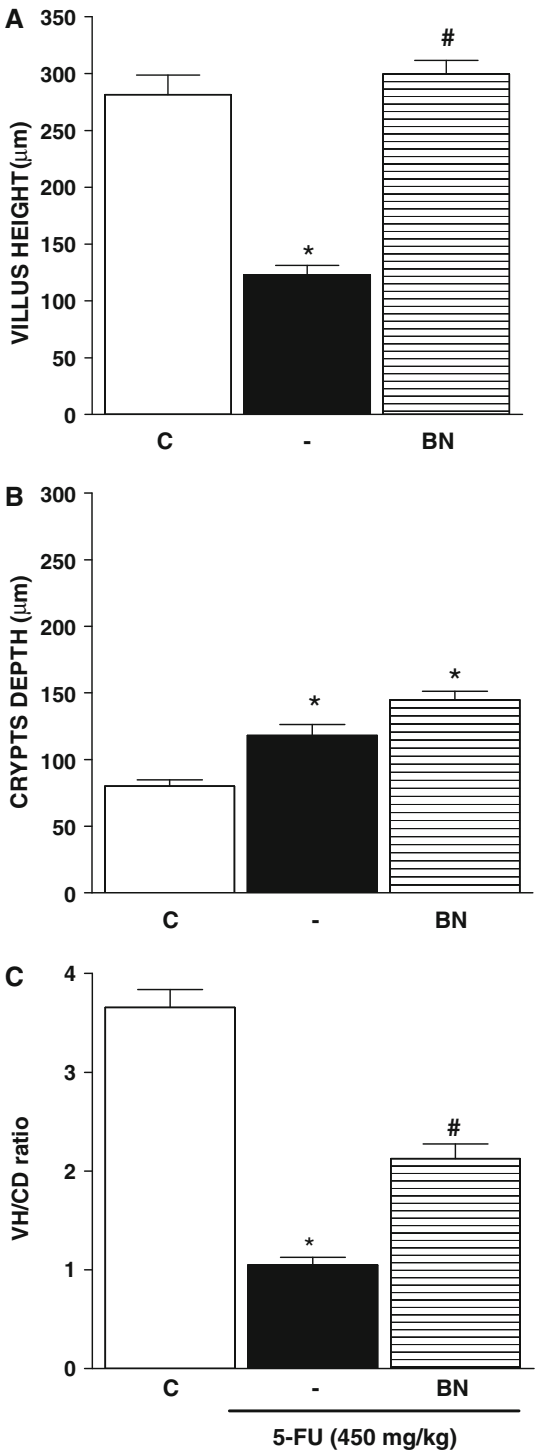

Fig. 2 PAFR antagonist, BN 52021, reduced 5-fluorouracil-induced duodenal damage. BN 52021 prevented the 5-FU-induced reduction in villus height (**a**) and in VH/CD ratio (**c**). Values were reported as mean ± SEM, $n = 6$. $^{*}P < 0.05$ compared to control (**c**), $^{\#}P < 0.05$ compared to 5-FU + saline (−). ANOVA and Bonferroni's test

Figure 3 presents duodenum photomicrographs, which demonstrate 5-FU-induced intestinal mucositis in wild-type animals. This is characterized by the loss of normal crypt architecture, shortened villi recovered with fattened and vacuolated cells (panel C), and inflammatory cell infiltration into the lamina propria (panel D). This morphology is apparent when compared to control mice (panel A and B). In addition, $PAFR^{-/-}$ (panel E, F) or pre-treated with BN 52021 (panel G, H) showed preservation of the villi and crypts, when compared with 5-FU treatment mice (panel C and D).

In addition, as shown in Fig. 4, 5-FU treatment increased MPO activity in the duodenum in BN52021-treated wild-type mice and $PAFR^{-/-}$ mice. Genetic (knockout mice) and pharmacological (BN52021) inhibitions of PAFR did not affect MPO activity in the duodenum.

As shown in Fig. 5, 5-FU treatment increased TNF-α, IL-1β, and KC concentrations in the duodenum. In contrast, 5-FU treatment did not increase cytokine expression in PAF knockout mice.

Discussion

The mechanism of intestinal mucositis development during anticancer treatment is unknown. In this study, we demonstrate for the first time the role of platelet-activating factor (PAF) in the pathogenesis of 5-FU-induced intestinal mucositis using $PAFR^{-/-}$ mice and pharmacological inhibition of the PAF receptor with BN52021 [11]. We demonstrated that $PAFR^{-/-}$ mice were protected against microscopic intestinal damage caused by 5-FU treatment. In addition, the beneficial effect of BN52021 treatment on gut injury suggests that PAF detrimentally affects 5-FU-dependent mucositis. These data are in agreement with other studies that suggest that PAF causes gut inflammatory disease, such as endotoxemia [16, 31], necrotizing enterocolitis [28], hemorrhagic shock [9], and intestinal ischemia and reperfusion injury [38]. Merendino and co-workers [26] demonstrated the constitutive expression of the PAF receptor in human intestinal epithelial cells in vitro and in vivo. These studies clearly indicate that PAF is an important signaling mediator in intestinal cells during normal and inflammatory conditions.

Numerous studies from other laboratories and our own have demonstrated that 5-FU treatment induces neutrophil infiltration and decreases the villus/crypt ratio in rodents [12, 17, 29, 34]. According to Pritchard et al. [30], damage results from the combined effects of apoptosis and inhibition of cell proliferation, which, in turn, reduces the cell count in both intestinal crypts and villi.

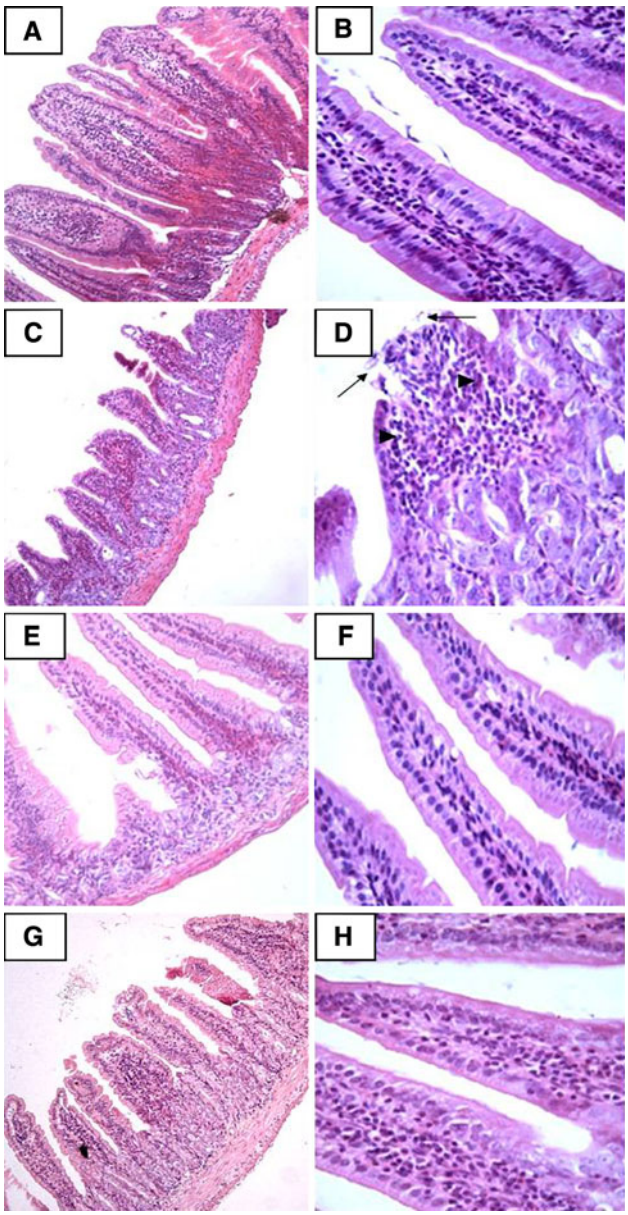

Fig. 3 Histologic analysis of representative duodenum. Control mice showing normal duodenal tissue (**a**, **b**). 5-FU-induced intestinal mucositis in wild-type animals showing loss of normal crypts architecture, shortened villi recovered with fattened and vacuolated cells (*arrow*), and inflammatory cells infiltration in the lamina propria (*arrowhead*) (**c**, **d**). PAFR$^{-/-}$ (**e**, **f**) or pre-treated with BN 52021 (**g**, **h**) submitted to 5-FU treatment (450 mg/kg), revealing villi and crypts preservation. H&E staining (**a**, **c**, **e**, **g** 100×/**b**, **d**, **f**, **h** 400×)

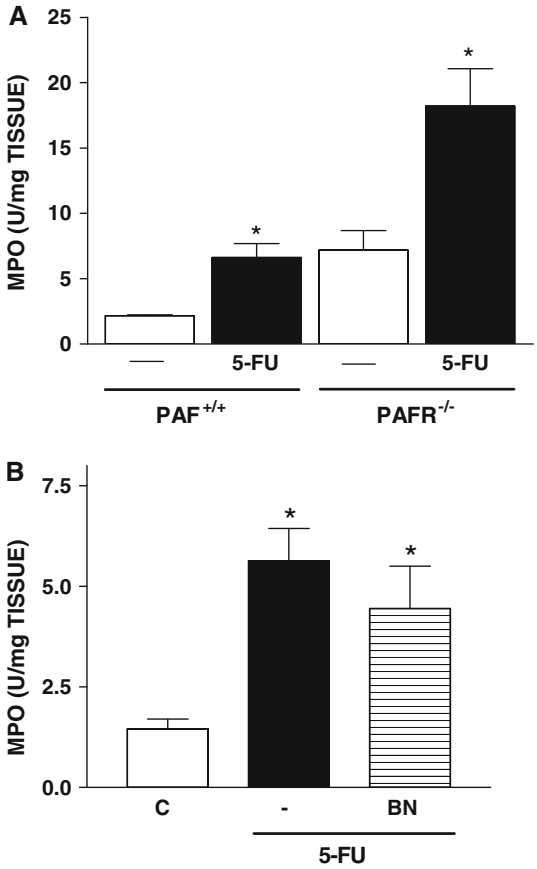

Fig. 4 Absence of PAF signaling (**a**) or PAFR blockade (**b**) did not reduce 5-fluorouracil-induced increase on duodenal MPO activity in mice. 5-FU induced an increase in duodenal MPO activity in wild-type, in PAFR$^{-/-}$, and in mice that were pretreated with the PAF antagonist BN 52021. Values are reported as the mean ± SEM expressed as units of MPO per mg of tissue, $n = 6$. $^{*}P < 0.05$ compared to control. ANOVA and Bonferroni's test

Numerous barriers limit pathogen and bacterial product access to the intestinal enterocyte surface and to the lamina propria. The pathogenesis of mucositis involves bacterial colonization through intestinal barriers and sites of ulceration in the gut [36]. 5-FU-dependent intestinal injury is associated with inflammatory infiltration into the intestine [34]. In our study, neutrophil infiltration to the site of intestinal lesion was assessed by the myeloperoxidase (MPO) activity assay. We showed that epithelial injury and neutrophil infiltration into the wall of the gut correspond to the inflammatory phase of mucositis as described previously [10, 36]. Our data suggest that the protective effects of PAF receptor inhibition may be independent of neutrophil modulation; a significant improvement in the morphological parameters was observed in PAFR$^{-/-}$ and BN52021-treated mice in spite of the elevated MPO activity. However, two opposing conclusions about this data could be made.

First, it has been demonstrated in an animal model of severe sepsis that PAF inhibits neutrophil migration toward the infected area [27]. PAFR$^{-/-}$ and PAFR antagonist-treated mice that underwent cecal ligation and puncture, which induces sepsis, have a low bacterial count and increased neutrophil count in the peritoneal cavity. These effects increased the survival rate. Therefore, the protective effects of PAFR inhibition may be associated with augmented MPO activity (indirect neutrophil infiltration) in

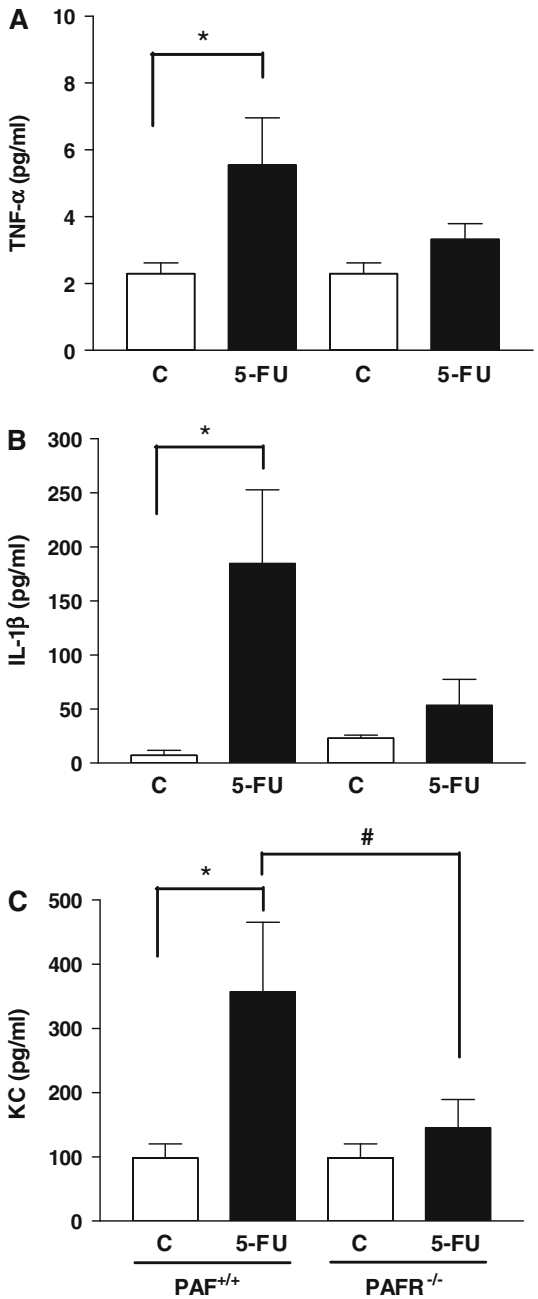

Fig. 5 Duodenal concentration of TNF-α (**a**), IL-1β (**b**), and KC (**c**) are attenuated in PAFR$^{-/-}$ mice treated with 5-FU in comparison with wild-type animals. 5-FU induced an increase in duodenal concentrations of TNF-α (**a**), IL-1β (**b**), and KC (**c**) in wild-type, but not in PAFR$^{-/-}$ mice. Values are reported as the mean ± SEM expressed as concentration in pg/ml, $n = 6$. $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to 5-FU + PAF$^{+/+}$. ANOVA and Bonferroni's test

the intestinal mucosa through the modulation of neutrophil migration.

Second, the increase in viable neutrophils in the mucosa may enhance the regulation of bacterial colonization. Therefore, it is likely that PAF inhibits neutrophil apoptosis. Infiltrating neutrophils exert antimicrobial protection until they undergo apoptosis. Hotchkiss et al. [14] demonstrated the prevention of lymphocyte apoptosis during sepsis significantly improves animal survival, suggesting that immune depression from a loss of lymphocytes may affect survival during sepsis. Furthermore, Kuijpers et al. [19] showed that recombinant human plasma-derived PAF-acetylhydrolase, an enzyme that degrades PAF, prevents spontaneous neutrophil apoptosis. In addition, these authors demonstrated that WEB-2086, a PAFR antagonist, rescued neutrophils from apoptosis. These observations may have important implications for 5-FU-induced intestinal toxicity.

In a study published by Ferreira et al. [13], it was assessed the tumor growth, angiogenesis, and inflammation in normal (WT) mice and those lacking the receptor for PAF, through gene deletion (PAFR$^{-/-}$). These authors found an increase in the number of neutrophils in the ascites fluid from the PAFR$^{-/-}$ mice bearing the Ehrlich tumor in its ascites form, but a decrease in neutrophil accumulation in the peritoneum. In our standpoint, the inflammatory context found in our experiments differs completely from that presented by Ferreira et al. [13]. However, we believe that PAFR$^{-/-}$ mice may present some phenotypical variation that could account to the higher migration of neutrophils to the intestinal sites during mucositis which was not observed in WT mice treated with the PAF antagonist. Such hypothesis merits further investigation.

The pathophysiology of the intestinal mucositis involves the excessive production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and KC. KC is a homolog of human IL-8 [21]. To determine the effects of PAF on 5-FU-induced inflammatory cytokine production, duodenal cytokine production was measured in PAFR$^{-/-}$ mice. Liu et al. [20] demonstrated that PAF increases the proteolytic processing of NF-kappaB and that expression of proinflammatory cytokines, which results in PAF-induced systemic inflammation and acute bowel injury. Our results demonstrate that PAFR$^{-/-}$ mice had significantly less proinflammatory cytokine production in duodenal tissue. This result strongly suggests that PAF activates a cytokine cascade that leads to the intestinal damage during 5-FU administration. This is in contrast to the effect of PAF receptor inhibition to increase the neutrophil infiltration and reduce the pro-inflammatory cytokine concentration. However, control of bacterial colonization in the intestine may account for the reduction in cytokine release. Similar to our findings, Souza et al. [37] demonstrated that PAFR$^{-/-}$ and PAFR antagonist-treated mice are protected from severe dengue infection and have low cytokine levels in the blood and spleen. This suggests that PAFR activates cytokines during dengue virus infection.

It is important to mention that the modulation of PAFR does not affect 5-FU-induced cytotoxicity because neither BN52021 treatment nor genetic ablation of PAFR reversed 5-FU-dependent leucopenia.

In conclusion, our study establishes the role of PAFR activation in 5-FU-induced intestinal mucositis. This study implicates treatment with PAFR antagonists as novel therapeutic strategy for this condition.

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