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Platelet-activating factor-induced NF- κ B activation and IL-8 production in intestinal epithelial cells are Bcl10 dependent

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Abstract

Background—Platelet-activating factor (PAF), a potent pro-inflammatory phospholipid mediator, has been implicated in inducing intestinal inflammation in diseases such as inflammatory bowel disease (IBD) and necrotizing enterocolitis (NEC). However, its mechanisms of inducing inflammatory responses are not fully understood. Therefore, studies were designed to explore the mechanisms of PAF-induced inflammatory cascade in intestinal epithelial cells.

Methods—NF- κ B activation was measured by luciferase assay and ELISA, and IL-8 production was determined by ELISA. Bcl10, CARMA3 and MALT1 mRNA and protein levels were assessed by real-time RT-PCR and Western blot, respectively. siRNA silencing of Bcl10 was used to examine its role in PAF-induced NF- κ B activation and IL-8 production. Promoter region of Bcl10 gene was cloned by PCR method and promoter activity measured by luciferase assay.

Results—The adaptor protein Bcl10 appeared to play an important role in PAF-induced inflammatory pathway in human intestinal epithelial cells. Bcl10 was required for PAF-induced I κ B α phosphorylation, NF- κ B activation, and IL-8 production in NCM460, a cell line derived from normal human colon, and Caco-2, a transformed human intestinal cell line. PAF also stimulated Bcl10 interactions with CARMA3 and MALT1, and up-regulated Bcl10 expression in these cells via transcriptional regulation.

Conclusion—These findings highlight a novel PAF-induced inflammatory pathway in intestinal epithelial cells, requiring Bcl10 as a critical mediator and involving CARMA3/Bcl10/MALT1 interactions. The proinflammatory effects of PAF play prominent roles in the pathogenesis of IBD and this pathway may present important targets for intervention in chronic inflammatory diseases of the intestine.

Keywords

IBD; inflammation; Caco-2; NCM460

INTRODUCTION

Platelet-activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid mediator produced by most cells and tissues. PAF is involved in many biological processes like cellular activation, cytoskeletal reorganization, intracellular signaling, and is one of the most potent mediators in many inflammatory processes (1-4). It

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induces inflammatory reactions, and also mediates synthesis and release of other mediators to aggravate the degree of inflammation (5,6). PAF is also produced and degraded by the human intestinal epithelium where it mediates a range of pro-inflammatory and other biological effects (2), including modulation of ion transport, prostaglandin and eicosanoid synthesis, induction of apoptosis, and activation of NF- κ B (7-11). PAF levels are elevated in tissues and/or serum in response to pathogen infection, and in patients with Crohn's disease, ulcerative colitis, and neonatal necrotizing enterocolitis (NEC), and these increased levels appear to correlate with disease severity (12-15). PAF acts by binding to, and activating G-protein coupled PAF receptors (PAF-R), that are present in most tissues, but are found in highest concentrations on intestinal epithelium (2). Constitutive expression of PAF-R has been shown in various human intestinal cell lines as well as in human colonic and small intestinal epithelium (2).

PAF causes intestinal injury primarily via induction of an inflammatory cascade, however, the mechanisms of this inflammatory pathway are not fully understood (6). PAF induces cytokine and chemokine gene expression in a wide variety of cells (1,16,17), via activation of the transcription factor NF- κ B, yet the early receptor-mediated signaling events that initiate this response are not completely defined (1). The mammalian NF- κ B family contains five members: NF- κ B1 (p105 and p50), NF- κ B2 (p100 and p52), c-Rel, RelB, and RelA (p65). NF- κ B dimers are retained in an inactive form in the cytoplasm by interactions with inhibitory I κ B proteins. Most physiological and pathological signals for NF- κ B activation depend on I κ B kinase (IKK)-controlled phosphorylation of I κ B proteins on conserved serine residues, leading to their ubiquitination-mediated degradation and subsequent liberation of NF- κ B, which then enters the nucleus to regulate the transcription of target genes (18-21). In recent years, a similar pathway of NF- κ B activation has been described in lymphocytes that mediate antigen-induced lymphocyte proliferation by bridging T or B cell receptor-dependent activation of NF- κ B (22). The pathway in lymphocytes includes three major signaling molecules: (i) CARMA1 [caspase recruitment domain (CARD) containing membrane-associated guanylate kinase (MAGUK) protein 1], a scaffolding protein that serves to integrate the upstream signal to downstream factors (23-26) (ii) Bcl10, a CARD-containing intermediate bridging factor (27-30), and (iii) MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1), a paracaspase, that when oligomerized through interaction with Bcl10, either directly or indirectly stimulates the IKK complex (31-34). This pathway was originally thought to play exclusive role in the immune system because CARMA1 is chiefly expressed in lymphocytes (23,24). However, a second member of the CARMA family, CARMA3, was found to have a wider tissue distribution profile (25,26). Recent studies have shown that the CARMA3-Bcl10-MALT1 signalosome complex functions in nonimmune cells and mediate inflammatory signaling induced by lysophosphatidic acid (35) and angiotensin II (36). We have recently shown the role of Bcl10 in mediating carrageenan and lipopolysaccharide-induced inflammatory responses in human and murine intestinal epithelial cells (37-40). In this study, we demonstrate that Bcl10 plays an important role in mediating the inflammatory pathway in response to PAF stimulation of human intestinal epithelial cells, and also provide evidence for PAF-induction of CARMA3/Bcl10/MALT1 interactions.

MATERIALS AND METHODS

Reagents and Antibodies

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) was obtained from Sigma-Aldrich (St. Louis, MO). Antibodies specific for Bcl10 (Cat# sc-13153), CARMA3 (Cat# sc-47826), MALT1 (Cat# sc-46677) and I- κ B α (Cat# sc-847) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl10 siRNA and transfection reagents were obtained from Qiagen (Valencia, CA).

Cell lines, Cell culture and Treatments

The human colonic epithelial cell line NCM460, derived from normal colonic mucosa, was grown in M3:10 medium (INCELL, St. Antonio, TX) and maintained at 37°C in a humidified, 5% CO₂ environment. Caco-2 cells were maintained in DMEM with 4.5 g/L glucose, 50 kU/L penicillin, 5 mg/L streptomycin, and 20% fetal bovine serum. For experiments, confluent cells in cell culture flasks were trypsinized and seeded into either 6-well or 12-well plates at a cell density of 2×10^4 cells/ml. At 60-70% confluency, cells were used for treatments. Serum was reduced to 1% for overnight before treatments and also during the treatments.

RNA extraction and real-time RT-PCR

The total RNA from NCM460 and Caco-2 cells was prepared using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. An equal amount of RNA for each sample was reverse-transcribed and amplified in one-step reaction using Brilliant SYBR Green QRT-PCR master mix kit (Stratagene, La Jolla, CA). and using Mx 3000 (Stratagene). The gene specific primers for CARMA1, CARMA2, CARMA3 and MALT1 used for the RT-PCR reactions are shown in Table 1. The quantitation of the amplification was expressed as a ratio of $2^{\Delta\text{Ct-CARMA1,2,3}}$ or $\text{MALT1}/2^{\Delta\text{Ct-}\beta\text{-actin}}$, where $\Delta\text{Ct-CARMA}$ or MALT1 and $\Delta\text{Ct-}\beta\text{-actin}$ represents the difference between the threshold cycle of amplification of CARMA or MALT1 and β -actin.

Co-immunoprecipitation and Immunoblotting

Cells treated with vehicle or PAF at indicated concentrations were washed in cold PBS and lysed in a lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate and 1X protease inhibitor cocktail (Roche)]. The cell lysates were pre-cleared with protein A/G plus-agarose (Santa Cruz) and then incubated with anti-CARMA3 or anti-MALT1 antibodies at 4°C for 16 h followed by incubation with protein A/G plus-agarose for 5 h. Parallel control experiments were performed by incubating the pre-cleared cell lysate with normal rabbit IgG followed by incubation with protein A/G plus-agarose. The agarose beads were collected by centrifugation, washed 4 times with lysis buffer and heated to 95°C for 5 min after adding Laemmli buffer. The resulting immunoprecipitates were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with anti-Bcl10 antibody. Immunoblots were visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

NF- κ B activity

To study the NF- κ B activation by PAF, NF- κ B-Luciferase reporter assay was performed according to manufacturer's instructions (Clontech, CA). Briefly, NCM460 or Caco-2 cells were transfected with p-NF- κ B-Luc (Clontech) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). This plasmid contains NF- κ B binding consensus element upstream of luciferase reporter gene. Twenty four hours after transfection, cells were treated with PAF (10 μ M) or TNF- α (100 ng/ml) as positive control for another 24 h. Luciferase assays were performed as described previously (41) and results were expressed as RLU/mg protein.

siRNA silencing of Bcl10

siRNA for Bcl10 silencing, and control, scrambled siRNA labeled with rhodamine were obtained commercially (Qiagen, Valencia). The Bcl10 siRNA (150 ng; 0.6 μ l) in 100 μ l serum-free culture medium was mixed with 12 μ l of HighPerfect Transfection reagent (Qiagen) by vortex mixing, maintained at room temperature for 10 min, then added dropwise to NCM460 cells grown on 12-well plates to the density of approximately $2.5 \times$

10⁵ per well. Plates were swirled for uniform distribution of the transfection reagent and incubated at 37°C and 5% CO₂ for 24 h. Subsequently, cells were treated with PAF for another 24 h before the spent media were collected for IL-8 measurement and cells were harvested to measure phospho-I κ B in cytoplasmic extracts and NF- κ B in the nuclear extracts. Silencing of Bcl10 in the cells was assessed by Bcl10 ELISA.

ELISA for Bcl10

The levels of Bcl10 was determined by a solid-phase sandwich ELISA, as previously reported by us (42). Control or treated cells were lysed in RIPA buffer (50 mM Tris-HCl containing 0.15 M NaCl, 1% Nonidet P40, 0.5% deoxycholic acid and 0.1% SDS, pH 7.4) and the cell extracts were stored at -80°C until assayed. Bcl10 molecules in the samples or standards were captured in the wells of a microtiter plate precoated with rabbit polyclonal antibody to Bcl10 (QED Bioscience, San Diego, CA). Immobilized Bcl10 was detected by a mouse monoclonal antibody to Bcl10 (Novus Biologicals, Littleton, CO) and goat anti-mouse IgG-HRP complex (Santa Cruz). The peroxidase enzyme activity bound to Bcl10 was determined by chromogenic reaction with hydrogen peroxide-tetramethylbenzidine and measuring the intensity of the color at 450 nm in ELISA plate reader (SLT, Spectra). Bcl10 concentrations of the samples were extrapolated from a standard curve derived by using known concentrations of recombinant Bcl10 (Calbiochem, EMD Bioscience, San Diego, CA). Sample values were normalized with the total cell protein concentrations determined by BCA protein assay kit (Pierce, Rockford, IL).

ELISA for phospho-I κ B

Phospho-I κ B was measured by the PathScan Sandwich ELISA (Cell Signaling Technology, Danvers, MA) according to manufacturer's instructions. Control or PAF-treated cells were lysed in ice-cold lysis buffer (Cell Signaling), containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sn-glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1 mM PMSF, sonicated twice for 20 s and centrifuged at 13,500 *g* for 10 min at 4°C. The supernatant (cell lysate) was collected and stored at -80°C until assayed. Phospho-I κ B α in the samples was captured in microtiter wells coated with monoclonal antibody to I κ B and detected by an anti-phospho-I κ B α antibody specific to phosphorylated Ser32 and an HRP-conjugated secondary antibody. The color intensity of the HRP reaction product, which was proportional to the quantity of phospho-I κ B α , was measured at 450 nm and the values were normalized with the total cellular protein.

ELISA for IL-8

The secretion of IL-8 in the spent media of control and PAF treated cells was measured by DuoSet ELISA kit for human IL-8 (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. IL-8 was captured into the wells of a microtiter plate precoated with anti-IL-8 monoclonal antibody. Immobilized IL-8 was then detected by biotin-conjugated secondary IL-8 antibody and streptavidin-horseradish peroxidase (HRP). Hydrogen peroxide-tetramethylbenzidine chromogenic substrate was used to develop the color, which was measured at 450 nm with a reference filter of 570 nm in an ELISA plate reader (SLT, Spectra). The IL-8 concentrations were extrapolated from a standard curve plotted by using known concentrations of IL-8. The sample values were normalized with total protein content (BCA Protein assay kit; Pierce) and expressed as picograms per milligram cellular protein.

Cloning of 5'-untranslated region of Bcl10 gene and measurement of promoter activity

A 1310 bp fragment of the 5'-untranslated region of Bcl10 gene was cloned into the pGL2 reporter plasmid (Promega, Madison, WI) between *Xho*I and *Hind*III sites upstream of the luciferase reporter gene. We used Elongase Amplification System (Invitrogen) and PCR method to clone this fragment using human genomic DNA as the template and the gene specific primer pairs shown in Table 1. A touch-down long PCR method was used with the following amplification conditions: heating at 94°C for 30 s, followed by 40 cycles, 30 s each, of varying annealing temperatures (5×83°C, 5×80°C, 5×75°C, 5×70°C, 20×6°C) and then elongation at 68°C for 10 min. The PCR product was purified using the gel extraction kit (Qiagen), digested with *Xho*I and *Hind*III, and ligated to the corresponding sites in pGL2. The plasmid construct was designated as pBcl1310. In order to determine the promoter activity of this fragment, NCM460 and Caco-2 cells were transfected with p-Bcl1310 using Lipofectamine 2000 (Invitrogen). Twenty four h after transfection, cells were treated with PAF for additional 24 h. Subsequently, promoter activity was determined by measuring luciferase activity according to the procedure described previously (41). Promoter activity was expressed as relative luciferase units(RLU)/mg protein. The potential transcription factor binding elements in the cloned promoter region were determined by using the programs TFSEARCH and Motif Search (<http://motif.genome.jp/>).

Statistical analyses

The data presented are mean $\pm$ SEM of 3-4 independent experiments. Difference between control versus various treatments was analyzed using one-way ANOVA, with Dunnett's multiple comparison test for repeated comparisons to the control. Differences were considered significant at $P < 0.05$.

RESULTS

PAF-triggered NF- κ B activation in intestinal epithelial cells

To confirm that PAF has a specific direct effect on the activation of NF- κ B in NCM460 and Caco-2 cells, we used the NF- κ B transcription reporter vector p-NF- κ B-Luc for transfecting the cells. This vector contains NF- κ B consensus sequence located upstream of the firefly luciferase reporter gene. TNF- α was used as a positive control for NF- κ B activation. After 24 h of stimulation, TNF- α (100 ng/ml) caused 10-fold activation of NF- κ B-dependent reporter gene while activation by PAF (1, 5 and 10 μ M) was dose dependent ranging between 2-4-fold in NCM460 (Figure 1A) and 2-5-fold in Caco-2 cells (Figure 1B).

Bcl10 expression increased in response to PAF

Our previous studies showed that carrageenan and LPS induced Bcl10-dependent NF- κ B activation in intestinal epithelial cells, and also enhanced Bcl10 protein expression (37,38). Therefore, we sought to investigate if PAF, that triggers NF- κ B activation in NCM460 and Caco-2 cells, also increases Bcl10 expression. The results of our initial experiments showed that there is a dose-dependent increase in Bcl10 levels in response to PAF treatments for 24 h in both the cell lines, as assessed by Western blot (Figure 2A and 2B).

PAF-induced NF- κ B activation and IL-8 production are Bcl10 dependent

Various recent reports have shown the role of Bcl10 in receptor-mediated inflammatory responses, more particularly those involving G-protein coupled receptors that lead to NF- κ B activation and cytokine production (35,36,43). Since PAF is known to act via G-protein coupled PAF-receptors, and the mechanisms of PAF-induced inflammatory pathway in the human intestine are not well understood, we examined if PAF-triggered NF- κ B activation in intestinal epithelial cells is Bcl10-dependent. During NF- κ B activation, inhibitory I κ B

proteins are phosphorylated by the IKK signalosome, thereby releasing NF- κ B for nuclear translocation to activate target genes (18,20,21). Therefore, we used ELISA to measure NF- κ B (p65) in the nuclear fraction and phospho-I κ B α in the total cell lysate to assess NF- κ B activation in response to PAF treatment. Additionally, to confirm the role of Bcl10 in mediating PAF-induced NF- κ B activation, both phospho-I κ B α and nuclear NF- κ B were measured in NCM460 cells after siRNA silencing of Bcl10. There was 2-3-fold increase in nuclear p65 (Figure 3A) and total phospho-I κ B α (Figure 3B) in response to 1-10 μ M PAF, compared to untreated controls. However, PAF had no effect on phospho-I κ B α or nuclear NF- κ B (p65) levels in NCM460 cells where Bcl10 was silenced by siRNA. Our previous studies showed that carrageenan and LPS-induced activation of NF- κ B in NCM460 cells was accompanied by Bcl10-dependent IL-8 production (37,38). Therefore, we next tested whether PAF could induce IL-8 secretion in NCM460 cells in a Bcl10-dependent manner. Whereas in wild-type NCM460 cells there was a dose-dependent increase in IL-8 in response to 1-10 μ M PAF, there was no effect of PAF on IL-8 secretion in Bcl10 deficient cells (Figure 3C). The extent of silencing as measured by Bcl10 ELISA is shown in Figure 3D. These results clearly indicate that PAF-induced NF- κ B activation and IL-8 production in NCM460 cells are Bcl10-dependent.

Time-course of PAF-induced increase in NF- κ B, IL-8 and Bcl10

We next sought to analyze the time-course of NF- κ B activation, IL-8 production and increase in Bcl10 protein levels in response to 1 μ M PAF treatments. Results presented in Figure 4A show that PAF triggered NF- κ B activation and IL-8 production as early as 3h after treatments. Fold increases for NF- κ B or IL-8, compared to untreated control, remained similar at all time points. PAF also enhanced Bcl10 expression at 3h; however, unlike NF- κ B or IL-8, maximum induction was observed at 12h after treatment. Determination of PAF-induced phosphorylation of I κ B α by Western blots showed that I κ B α decreased and phospho-I κ B α increased starting 30-60 min after PAF treatment (Figure 4B).

CARMA3 and MALT1 are highly expressed in NCM460 and Caco-2 cells

Various recent reports showed that Bcl10-dependent inflammatory pathways in nonimmune cells involve CARMA-3/Bcl10/MALT1 signalosome complex (35,36,43). Therefore, real-time quantitative RT-PCR was used to examine if members of the CARMA family (CARMA1,2,3) and MALT1 were expressed in intestinal epithelial cells. CARMA3 mRNA levels were very high in both NCM460 and Caco-2 cells, whereas CARMA1 and 2 expressions were negligible (Figure 5). MALT1 was also expressed in both cell lines, although 2-fold higher expression was observed in NCM460 compared to Caco-2 cells. These results are consistent with earlier reports that CARMA3 is expressed in nonimmune cells while expression of CARMA1 is exclusive to lymphocytes (36).

PAF enhanced Bcl10 interactions with CARMA3 and MALT1

We next used co-immunoprecipitation experiments to examine molecular interactions of Bcl10 with CARMA3 and MALT1 in NCM460 cells in response to PAF treatments. Higher amounts of Bcl10 were precipitated by CARMA3 (Figure 6A) or MALT1 antibodies (Figure 6B) in cells treated for 24 h with 5 and 10 μ M PAF, indicating that PAF increased interactions of Bcl10 with CARMA3 and MALT1. The molecular interaction was specific because incubation of the cell lysate with normal rabbit IgG, instead of anti-CARMA3 or anti-MALT1 antibodies, failed to immunoprecipitate Bcl10 (not shown). Similar interactions of Bcl10 with CARMA3 and MALT1 were also obtained in Caco-2 cells (results not shown).

Regulation of Bcl10 expression by PAF is transcriptional

Since PAF enhanced Bcl10 protein expression in NCM460 and Caco-2 cells, it was of interest to examine whether this increase involves transcriptional regulation. We cloned a 1310 bp fragment of 5'-untranslated region upstream of Bcl10 gene in pGL2 reporter plasmid, and searched for cis elements for binding important transcription factors, using computer program (Figure 7). The cloned fragment showed very high promoter activity in both the cell lines (10-fold in NCM460 and 50-fold in Caco-2 cells compared to pGL2-basic control) (Figure 8A and 8B), and comprised cis elements for various transcription factors, including HSF, Sp1, AP1 and NF- κ B (Figure 7). Interestingly, 10 and 20 μ M PAF treatment for 24 h showed a dose-dependent increase in promoter activity in both NCM460 (Figure 8A) and Caco-2 cells (Figure 8B). These results suggest that PAF enhances Bcl10 expression in intestinal epithelial cells via transcriptional regulation.

DISCUSSION

The studies described here elucidate a previously uncharacterized novel signaling pathway used by platelet-activating factor (PAF), for NF- κ B activation and IL-8 production in human intestinal epithelial cells. PAF, which is produced by a variety of cells and tissues, is a potent phospholipid mediator involved in multiple biological effects, such as intracellular signaling and apoptosis (44-47), modulating intestinal ion transport (6) and inducing diverse allergic and inflammatory reactions (48). The human colonic epithelial cells contribute to intestinal PAF production under normal and inflammatory conditions (2). PAF levels were increased within inflamed mucosa of patients with ulcerative colitis or Crohn's disease (13,49-51). Increased levels of PAF have been detected in animal models of colitis, and PAF receptor antagonists decreased mucosal inflammation in these models (52). Recently, it has also been shown that PAF can directly damage intestinal epithelial cells by activating chloride channels leading to intracellular acidosis and apoptosis (6).

Various studies have shown that PAF is a proximal inducer of the transcription factor NF- κ B, a pivotal regulator of the expression of proinflammatory cytokines and many immunoregulatory molecules in response to inflammatory stimuli (53,54) and microbial infection (55). PAF has been shown to activate NF- κ B and NF- κ B target genes such as IL-8 in a variety of cell types (16,17,56-58). In rat intestinal epithelium, NF- κ B was activated *in vivo* after intravenous injection with PAF, although it was not known if, in this case, NF- κ B activation was a direct effect of PAF (10). Therefore, despite several studies that demonstrated NF- κ B activation and cytokine production in response to PAF in various cell types, the receptor-mediated early signaling events that initiate these responses are not fully understood. In this report, we have shown direct *in vitro* activation of NF- κ B by PAF in intestinal epithelial cells. We also demonstrate that Bcl10 is an important mediator of this previously undescribed signaling pathway for PAF-induced inflammatory responses in human intestinal epithelial cells. Bcl10 was first shown as an adaptor protein that mediates antigen receptor-induced NF- κ B activation in lymphocytes (27,59,60). However, in recent years, role of Bcl10 in mediating the proinflammatory signaling cascade leading to NF- κ B activation has been reported in various nonimmune tissues (35,36,43). In this study, we have conclusively shown the role of Bcl10 in PAF-induced proinflammatory pathway in intestinal epithelial cells, as both NF- κ B activation and IL-8 production induced by PAF were significantly reduced in cells after silencing of Bcl10 by siRNA. Our previous studies demonstrated that Bcl10 also plays a pivotal role in TLR4-mediated inflammatory responses induced by the food additive carrageenan or by lipopolysaccharide (LPS) in colonic epithelial cells (39,40). However, the role of Bcl10 in mediating NF- κ B activation seemed to be specific to certain activators, because it has been shown earlier by us (37,38) and others (35) that Bcl10 deficiency did not affect TNF- α -induced NF- κ B activation.

Bcl10 was first identified as a mutated gene in mucosa-associated lymphoma tissue (MALT) lymphomas (29) and the protein was subsequently shown to be pivotal in a signaling pathway leading to NF- κ B activation (61-64). The hallmark of this pathway is the formation of a signalosome complex CARMA1-Bcl10-MALT1 (60,64), which directly or indirectly activates IKK complex through a phosphorylation and ubiquitination-dependent pathway, leading to NF- κ B activation (21,65). This signaling pathway originally described in lymphocytes, has also been reported in non-immune cells, where CARMA3, a second member of the CARMA family, participates in forming the signalosome. CARMA3 (CARD10) has a wider tissue distribution (25,26). Our quantitative real-time RT-PCR results clearly demonstrate that there is a high level of expression of CARMA3 and MALT1 in both NCM460 and Caco-2 cells. Therefore, based on our results of co-immunoprecipitation, showing increased interaction between Bcl10 with CARMA3 and MALT1 in response to PAF, we consider it likely that the PAF-induced NF- κ B pathway also involves the assembly of CARMA3/Bcl10/MALT1 signalosomes. Nevertheless, additional studies employing siRNA silencing of CARMA3 and MALT1, or using cells deficient in these proteins, will be needed to confirm their roles in PAF-induced inflammatory pathway in intestinal epithelial cells. However, in recent years, various studies have demonstrated the key role of CARMA3/Bcl10/MALT1 complex in mediating the inflammatory cascade leading to NF- κ B activation and cytokine/chemokine production in nonlymphoid cells (36,43). PAF is also an important inflammatory mediator in cells of the immune system. In view of the critical role of CARMA-Bcl10-MALT1 signalosome in NF- κ B activation in lymphocytes and macrophages, which are also active producers of PAF, investigation of the role of this signalosome complex in mediating the effects of PAF in cells of the immune system is of interest and might have relevance to IBD. Therefore, a detailed mechanistic analysis of the effects of PAF on immune cells is warranted in future studies, and should yield important information on the role of PAF in the pathophysiology of IBD.

In this report, we have also shown that PAF increases the expression of Bcl10 protein levels in NCM460 and Caco-2 cells. This increase appeared to be at the transcriptional level, because PAF also stimulated Bcl10 promoter activity in these cells. We found that PAF induces NF κ B activation in a Bcl10 dependent manner as early as 3h after exposure and this activation compared with untreated control remained similar at longer time points. PAF treatment also increased Bcl10 protein level even after 3h of exposure, however, the maximum fold increase over control was observed at 12h. These data suggest that PAF-induced NF κ B activation and induction of Bcl10 occur concurrently, although initial events of NF κ B activation might require endogenous Bcl10. Further, this NF κ B activation may lead to constitutive induction of Bcl10 by transcriptional activation of the Bcl10 promoter. Since the promoter region of Bcl10 has a potential binding site for NF κ B, it will be of interest to investigate in future, the transcription factors and signaling intermediates involved in modulating Bcl10 gene expression in response to PAF treatment.

In conclusion, we provide evidence for a novel proinflammatory signaling pathway which is induced by PAF in intestinal epithelial cells. The results of this study are significant because the CARMA/Bcl10/MALT1 signaling complex appears to have an important role in nonimmune cells, in this case, in intestinal epithelial cells. These findings shed light on the molecular link between PAF-stimulation of its receptor and NF- κ B activation in intestinal epithelial cells. The proinflammatory effect of PAF may have a significant role in the pathogenesis of IBD and NEC, and may represent an important target for intervention in inflammatory diseases of the intestine.

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List of abbreviations

PAF	platelet activating factor
LPS	lipopolysaccharide
Bcl10	B-cell lymphoma 10
CARMA1	caspase recruitment domain-containing membrane-associated guanylate kinase protein 1
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
IKK	I-kappaB kinase
IBD	inflammatory bowel disease
NEC	necrotizing enterocolitis

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Figure 1.

PAF causes NF- κ B activation in intestinal epithelial cells. NCM460 (A) or Caco-2 (B) cells were transfected with p-NF- κ B-luc reporter plasmid. After 24 h cells were treated with PAF or TNF- α at indicated concentrations for another 24 h. Cells were then harvested and luciferase activity was measured and expressed as relative luciferase units/mg protein. Values are mean $\pm$ SEM, $n = 3$, *Different from control, $P < 0.05$.

Figure 2.

PAF increases Bcl10 protein expression in intestinal epithelial cells. Lysates were prepared from control or PAF-treated cells after 24h. Equal amounts of proteins from NCM460 (A) or Caco-2 (B) cell lysates were subjected to SDS-PAGE and probed with anti-Bcl10 antibody in Western blots. After stripping with 0.2N NaOH, blots were re-probed with anti- β -actin antibody. Representative blots of three independent experiments are shown in both (A) and (B). Lower panels in both (A) and (B) show the densitometric analysis of band intensities plotted as ratio of band intensities (arbitrary units) of Bcl10 and β -actin.

Figure 3.

Bcl10 regulates PAF-induced NF- κ B activation and IL-8 production. NCM460 cells were transfected with control or Bcl10 siRNA for 24 h and then treated with PAF at indicated concentrations for another 24 h, after which, nuclear extracts, cell lysates, or the spent media were analyzed by ELISA for different parameters. (A): Nuclear extracts were prepared and NF- κ B (p65) levels were measured (B): Cell lysates were made and used for measuring phospho-I κ B levels (C): Spent cell culture media were used to measure the amounts IL-8 secreted (D): Bcl10 levels in the cell lysates were measured. Values are mean $\pm$ SEM, n = 3, *Different from control, $P < 0.05$.

Figure 4.

Time-course of PAF-induced NF- κ B activation, I- κ B α phosphorylation and increase in IL-8 and Bcl10: (A) NCM460 cells were treated with 1 μ M PAF for indicated time points, after which, nuclear extracts, cell lysates, or the spent media were analyzed by ELISA for NF- κ B (p65), Bcl10, and IL-8 respectively. Each parameter was also analyzed in untreated controls at each time point. Values represent percent increase over control and are mean $\pm$ SEM, n = 3, *Different from control, $P < 0.05$. (B) NCM460 cells were treated with 1 μ M PAF for indicated time points. Cell lysates were prepared, samples containing equal amounts of proteins were subjected to SDS-PAGE, and probed with anti-I- κ B α antibody. The blot was stripped with 0.2N NaOH, and re-probed with anti-phospho-I- κ B α or anti- β -actin antibody.

Figure 5.

CARMA3 and MALT1 are expressed in intestinal epithelial cells. Total RNA was extracted from NCM460 and Caco-2 cells and used for real-time quantitative RT-PCR. mRNA levels for CARMA1, 2, 3 and MALT1 were determined and normalized against β -actin mRNA. For comparing relative expressions of each gene between NCM460 and Caco-2 cells, an mRNA expression value was arbitrarily set at 1 for the gene with lowest normalized mRNA levels (in this case, CARMA1 in NCM460 cells). Results represent mean $\pm$ SEM, n = 3.

Figure 6.

PAF stimulates interaction of Bcl10 with CARMA3 and MALT1. Cell lysates of control or 24 h PAF-treated NCM460 cells, containing equal amounts of proteins, were used to immunoprecipitate (IP) Bcl10 with anti-CARMA3 (A) or anti-MALT1 (B) antibody. Immunoprecipitates were subjected to SDS-PAGE and probed with anti-Bcl10 antibody in Western blots (WB). After stripping with 0.2N NaOH, blots were re-probed with anti-CARMA3 or anti-MALT1 antibodies. Representative blots of three independent experiments are shown in both (A) and (B). Lower panels in both (A) and (B) show the densitometric analysis of band intensities plotted as ratio of band intensities (arbitrary units) of Bcl10 and CARMA3 (A) or MALT1 (B).

GGTCTGGGGATAGTCTCTCTTCATCAGTCTCCCCCTATAAAATGGAGGACTCATA
 HSF CdxA
 GTATAAGTTGCCTAGAACATATTAGTAAGGACTCAATAAAATGTTTGAGTAGGATGAG
 Ik-2/cEBP GATA-1, 2
 CTGGGTGGCTCATGCCTGTAGTCCCTGCACTTTGGGAAGCCGAGGCGGGTGGATG
 p300 ADR1
 GCTTGAGCCCAGGAGTTTGAGACCAGCCTGGGCAACATGGCGAAACTCCATCTCT
 TATA AP-4
 ATAAAAAATACAAAAAACTAGCTGGACTCCCAGCTGCTTGGTGGCTGAGGTGGAA
 HSF-2 GCR1
 GGATCGCCTGAGCCCTGGAGGTGGAAGCTGCAGTGAGCCGAGAATCGCACCCT
 CAP HFH-2
 GCACTCCAGCCTGGGCGACAGAGTGAGATCCTGTCTCAATAAATAAATAAATAATC
 c-Myb HSF
 AGTTGAGTGCATACTGTGTGCCAACTACCTTTAAGAACTTTACATATGATCCCGGC
 GATA-1, 2
 AGCCCTAAAAGGTAAGCATATCTATCTCCATTACACAGATTAATAAGGAAAAG
 SRY
 CTATGTTTATAATGAACAGTGACCAAAAAGTATGCAAACGTTAAATGAGGTGTTAA
 HSF HSF
 GTGGTGGGATTGTAGGTGGTTTTCTTTTCTTTTCAAAAATTTGTTTAGTATTGTAGT
 USF
 TAGGCTGTTTGTGTGTATGTCATGTGTATGTGTGTATGAGTGCGTTTGTGTGTGACCA
 c-Myb
 AAACAGTAAGTGGCAAACGTAACAACAGGAAATTGTAGGGCAGGGTCTGGGAAAG
 MZF1
 GGGAGGAACCCAAGGTACTGACAAGCCCAGACCGGACTCCCCGAAGTGAGCAG
 MyoD/AP4 ADR1
 GCAGCAAAGGCTGAGAGTGCCAGCTGCCTGGGCACGACGGACCCACCCACGGA
 AACGATGCCTTATGAGGAAGCTAACCCGGGCCACTTACCGGACCTGCGCAGGCGT
 NF-κB/c-Rel SP1 HSF
 GGCTTGAGGAAACGCCCGCTGTGGGCGGAGCCACCCGAAAGGCTCCGGTCCGGG
 Sp1 AP2 GATA-1
 GCGGGAACAGGATCGGCCCGCGGGCTGGCGTCGATAGGCTGGCGGGCTCTGCTA
 AP2
 AGCGACGCGCCTCGCCGTGGGGCGGTGCCTGCGCCTGAGCCTAGAGGGAAGGAA
 CGCTGCTCCGAGCTCCGCGTCGCGTCGCGTAGATTGCGCTCGCCGTCGACCTCAG
 AGGCGGGGCCGGAAGCGCTACGTTTTGACCCCCGAGTCCCTCTGTTCCCGAAGG
 GGCGGCCGTCTTTCTCCCGACCCGCTCCGCCTCCTCTCTTCTTCCCATGCCGAA
 GCCCCCAGCCAGGGCGGGGCGGCGCAGCCCCGAGCTCCCGGACCCGGAAGAAGC
 GCCATCTccgcgctccaccatg

Figure 7.

The 5'-flanking region of the Bcl10 gene cloned into pGL2. A 1310 bp fragment of the 5'-untranslated region of the Bcl10 gene was cloned into pGL2 vector using PCR method. The sequence downstream of the cloned region, till the initiation codon, is depicted in lower cases. Since the transcription initiation site is not determined, (+) or (-) signs to indicate bases upstream or downstream of transcription initiation are not used. Potential binding sites (cis elements) for the indicated transcription factors are underlined.

Figure 8.

PAF stimulates Bcl10 promoter activity in intestinal epithelial cells. The 1310 bp promoter region of Bcl10 gene cloned in pGL2 (p-Bcl1310) was used to examine its promoter activity by transient transfection. NCM 460 (A) or Caco-2 (B) cells were transiently transfected with p-Bcl10. After 24 h, cells were treated with indicated concentrations of PAF for another 24 h. Cells were then harvested, luciferase activity was measured and expressed as RLU/mg protein. Values are mean $\pm$ SEM, $n = 3$, *Different from control, $P < 0.05$.

Table 1

Primers used for real-time RT-PCR analysis and cloning experiments

Gene	Accession no.	Primer sequence
<u>Primers for real-time RT-PCR</u>		
CARMA1	NM_032415	(F): 5'-TTGTGGGAGAATGTGGAGTGT-3' (R): 5'-TGCCCCCTGGTATGTAGAATG-3'
CARMA2	NM_024110	(F): 5'-CCAACATGGTTTCCTCCTGT-3' (R): 5'-GCAGGGTCTGTTCTTCTCTT-3'
CARMA3	NM_014550	(F): 5'-CAAGAGAGCAAAGGGTGAAG-3' (R): 5'-GAGGTTGGCACGAATGTAGAA-3'
MALT1	NM_006785	(F): 5'-CATGTTTGAGACCTTCAACAC-3' (R): 5'-CCAGGAAGGAAGGCTGGAA-3'
<u>Primers for cloning promoter region of Bcl10</u>		
Bcl10	NM_003921	(F): 5'-ATCCTCGAGGGTCTGGGGATAGT CGTCTCT-3' (R): 5'-ATCAAGCTTAGATGGCGCTTCTTC CGGGTG-3'