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COMMON PATHWAYS FOR ACTIVATION OF PROINFLAMMATORY GENE EXPRESSION BY G PROTEIN-COUPLED RECEPTORS IN PRIMARY LUNG EPITHELIAL AND ENDOTHELIAL CELLS

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Abstract

Acute lung injury is associated with an inflammatory response resulting from the action of multiple mediators. Many proinflammatory mediators released during lung injury exert effects by binding to G protein-coupled receptors (GPCRs). Our earlier studies showed that substance P (SP), a ligand for the tachykinin 1 receptor, induced NF- κ B activation and IL-8 upregulation through a G_q-dependent pathway. Here we extend these findings by examining effects of multiple ligands for G_q-coupled GPCRs in primary human small airway epithelial cells (SAECs) and rat lung microvessel endothelial cells (RLMVECs). SP, bradykinin, protease activated receptor 2 agonist, and platelet activating factor (PAF) stimulated IL-8 production in SAECs, whereas only SP and PAF upregulated CINC-1 (a rat IL-8 homolog) in RLMVECs. Using signaling inhibitors, we investigated PAF-induced IL-8 expression and SP-induced CINC-1 expression in primary cells. Signaling cascades were similar in SAECs and RLMVECs and involved phospholipase C/calcium/PKC and Ras/Raf/Erk pathways. In addition, the tyrosine kinase inhibitor AG 17 and the proteasome inhibitor MG132 significantly reduced IL-8 and CINC-1 expression induced by GPCR ligands. The results demonstrate a common signaling pathway in primary lung epithelial and endothelial cells, suggesting a generalized mechanism for the induction of proinflammatory gene expression by G_q-coupled GPCRs following lung injury.

INTRODUCTION

Acute lung injury (ALI) can result from exposure to a variety of chemical [1], physical [2], and biological agents [3]. ALI is characterized by lung cell death, alveolar/capillary barrier disruption, pulmonary edema, inflammation, and impaired gas exchange [4]. Inflammatory responses associated with ALI typically result in the influx of neutrophils into the lung. These inflammatory cells through their capacity to release toxic substances may sustain or amplify lung injury. Pulmonary injury results in the production or release of multiple mediators that promote neutrophilic inflammation. Injury to the lung causes the formation or release of many types of mediators, including neuropeptides, kinins, chemokines, chemotactic factors, hormones, eicosanoids, proteinases, and nucleotides, that produce their effects by binding to G protein-coupled receptors (GPCRs). GPCRs are central regulators of cellular homeostasis and responses to environmental stimuli. Signaling initiated by GPCRs has been implicated in

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regulating cell survival, inflammation, and tissue repair. GPCR-activating ligands, including substance P (SP) [5], CXC chemokines [2], bradykinin (BK) [6], and platelet activating factor (PAF) [7], have been shown to promote inflammatory responses in the injured lung. However, the signal transduction pathways by which activation of GPCRs leads to stimulation of proinflammatory gene expression are not fully understood.

We showed previously that SP, a ligand for the tachykinin 1 receptor (Tacr1; also known as the neurokinin 1 receptor), stimulates proinflammatory gene expression in A549 human lung epithelial cells, and we identified a signal transduction pathway by which Tacr1 stimulation leads to the activation of the proinflammatory transcription factor NF- κ B [8]. In the present study, we extend these findings by examining the effects of multiple GPCR ligands in primary lung epithelial and endothelial cells. Our results suggest that the signaling pathway we have identified is a general mechanism for promoting lung inflammation, as it is activated by multiple GPCR ligands in lung epithelial and endothelial cells.

MATERIALS AND METHODS

Materials

Human small airway epithelial cells (SAECs), human lung microvascular endothelial cells (HLMVECs), SAGM medium, and EGM-2-MV medium were purchased from Lonza (Walkersville, MD). Rat lung microvessel endothelial cells (RLMVECs) and MCDB-131 medium were purchased from VEC Technologies (Rensselaer, NY). The interleukin 8 (IL-8) ELISA kit was purchased from Pierce Biotechnology (Rockford, IL). DuoSet CINC-1 ELISA reagents were purchased from R&D Systems (Minneapolis, MN). SP and BK were purchased from Tocris Cookson (Ellisville, MO). Protease activated receptor 2 agonist II (PAR-2A), PAF, U73122, BAPTA-AM, Go6850, manumycin A, GW 5074, U0126, AG 17, MG132, and Erk inhibitor III were purchased from EMD Biosciences (San Diego, CA). TNF was obtained from PeproTech (Rocky Hill, NJ). MTT cell viability assay reagents were purchased from Roche Applied Science (Indianapolis, IN).

Cell culture and treatments

SAECs were cultured in SAGM, and HLMVECs were grown in EGM-2-MV according to the supplier's instructions. Passage number was limited to seven or less from original stocks. The RLMVECs were cultured in MCDB-131 media according to the supplier's instruction. Passage number was limited to five or less from original stocks. For treatment with GPCR ligands, SAECs were seeded into 24-well plates in SAGM. The following day cells were changed into SABM (basal medium without growth factors) for an additional 1-2 days before treatment. SAECs were treated with 10^{-5} M SP, BK, PAR-2A, or PAF in SABM for 4 h, after which cell culture media was collected and frozen for subsequent analysis of IL-8 levels. When present, signaling inhibitors were added to cultures 30 min before PAF treatment. Cells not receiving ligand or inhibitors were treated with corresponding vehicle solutions. Four to six replicate wells per group were analyzed. RLMVECs or HLMVECs were seeded into 24-well plates in MCDB-131 or EGM-2-MV media, respectively, and cultured overnight before use. The following day, endothelial cells were treated with 10^{-5} M SP, BK, PAR-2A, or PAF in DMEM without serum for 4 h. When present, signaling inhibitors were added to cultures 20 min before SP treatment. Four replicate wells per group were analyzed. All reported findings were verified in multiple independent experiments. All inhibitors were examined for effects on cell viability by MTT assay. None of the inhibitors caused a significant decrease in cell viability in either SAECs or RLMVECs except AG 17, which resulted in a 22% decrease in viability in RLMVECs at the 10^{-5} M dose (not shown).

IL-8 and CINC-1 assays

IL-8 and CINC-1 were measured in tissue culture supernatants by ELISA according to the manufacturers' instructions.

Data analysis

Data are presented as group means $\pm$ standard error of the mean (SEM). Group means were compared using analysis of variance (ANOVA) and further analyzed by post hoc tests (Dunnett's or Tukey's multiple comparison tests; GraphPad Prism). Differences were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

Treatment of SAECs, RLMVECs, and HLMVECs with GPCR ligands induces the proinflammatory cytokines IL-8 and CINC-1

IL-8 and CINC-1 belong to the CXC chemokine family, which plays a critical role in the attraction and activation of neutrophils. To evaluate the impact of GPCR ligands on chemokine expression, we examined the effect of SP, BK, PAR-2A, and PAF on IL-8 secretion by SAECs and HLMVECs and CINC-1 production by RLMVECs. These ligands were chosen because they are formed or released following lung injury and they bind to receptors known to have the capacity to signal through the G_q family of G proteins. We therefore hypothesized that these ligands may stimulate proinflammatory signaling pathways related to those we previously identified in A549 cells. Stimulation of SAECs with SP, BK, PAR-2A, or PAF elicited significant increases in IL-8 production (Figure 1A). In HLMVECs, treatment with PAF, but not any of the other three ligands resulted in elevated IL-8 production (Figure 1B). In RLMVECs, SP and PAF, but not BK, or PAR-2A, significantly increased CINC-1 levels in the culture media (Figure 1C). The results demonstrate that these three types of primary cells all respond to at least one of the GPCR ligands tested, but the spectrum of responses in each type of cell was unique.

Characterization of signaling pathways underlying PAF-induced IL-8 production and SP-induced CINC-1 production: effects of phospholipase C, calcium, and protein kinase C inhibitors

We previously identified a signaling pathway by which stimulation of a G_q -coupled GPCR results in NF- κ B activation and IL-8 production in the human lung epithelial A549 cell line [8]. We sought to determine whether a similar pathway was operative in primary lung epithelial and endothelial cells. To examine whether this was a generalized pathway that was present in multiple species, we chose to examine signaling in rat lung endothelial cells. Activation of G_q is known to stimulate phospholipase C; thus we examined the effect of the phospholipase C inhibitor U73122 on IL-8 or CINC-1 expression in response to PAF in SAECs (Figure 2A) and SP in RLMVECs (Figure 2B). IL-8 production stimulated by PAF treatment was inhibited to below basal levels by 10 μ M U73122. CINC-1 production stimulated by SP treatment was significantly attenuated at both 1 μ M (77%) and 10 μ M (94%) U73122. These results show that PAF-induced IL-8 expression and SP-induced CINC-1 expression involve the activation of phospholipase C.

Phospholipase C catalyzes the formation of inositol triphosphate and diacylglycerol, signaling molecules that trigger intracellular calcium release and protein kinase C (PKC) activation, respectively. Pharmacological inhibitors of calcium signaling and PKC were used to examine the role of these molecules in chemokine production induced by PAF in SAECs and SP in RLMVECs. PAF-induced IL-8 production was inhibited 83% by treatment with the calcium chelator BAPTA-AM at 1 μ M (Figure 3A). SP-induced CINC-1 production was significantly

reduced below basal levels following treatment with 10 μ M BAPTA-AM (Figure 3B). The protein kinase C inhibitor Go6850 also significantly inhibited PAF- and SP-induced IL-8 and CINC-1 expression by 33-73% (Figure 4). This suggests a possible involvement of PKC in mediating IL-8 and CINC-1 production, with the caveat that Go6850 may inhibit other kinases under some conditions [9].

Effect of Ras/Raf/Erk inhibitors

A well characterized signaling cascade that is commonly activated downstream of phospholipase C involves the GTPases Ras and Raf and the MAP kinase Erk. A role for Ras in mediating PAF-induced IL-8 production and SP-induced CINC-1 production was examined by using the farnesyltransferase inhibitor manumycin A. PAF-stimulated IL-8 production was significantly attenuated at 1 μ M (74%) and 10 μ M (below basal levels) manumycin A (Figure 5A). SP-stimulated CINC-1 production was reduced significantly below basal levels by 10 μ M manumycin A (Figure 5B). Treatment with a lower dose of manumycin A (1 μ M) in conjunction with SP treatment consistently resulted in a significant increase in CINC-1 compared with SP treatment alone by a mechanism that is unclear. Treatment of SAECS with PAF and 10 μ M GW5074, a compound that has been used as a Raf inhibitor but is now known to inhibit other kinases such as HIPK2, MST2, and GAK [10], resulted in a decrease in IL-8 production to below basal levels (Figure 6). GW5074 had no consistent effect on CINC-1 production in SP-treated RLMVECs (not shown). The contribution of Erk activity to PAF- and SP-induced chemokine production was examined using inhibitors of MEK (the enzyme responsible for phosphorylation and activation of Erk) and Erk inhibitor III. The MEK inhibitor U0126 significantly inhibited IL-8 expression in response to PAF in SAECS (Figure 7A) and CINC-1 expression in response to SP in RLMVECs (Figure 7B). PAF-stimulated IL-8 production was significantly attenuated by 90% at 1 μ M U0126 and reduced below basal levels at 10 μ M U0126. SP-stimulated CINC-1 production was attenuated to approximately basal levels by 10 μ M U0126. The increase in IL-8 production induced by PAF was also significantly attenuated by 51% at 10 μ M Erk inhibitor III, and treatment with 100 μ M Erk inhibitor III reduced PAF-induced IL-8 production below basal levels (Figure 8A). In RLMVECs, SP-stimulated CINC-1 production was attenuated by 91% at 100 μ M Erk inhibitor III (Figure 8B).

Effect of Pyk2 and proteasome inhibitors

In many cell types, the phospholipase C/calcium/PKC signaling pathway is coupled to the Ras/Raf/Erk pathway through PKC activity. An additional mechanism by which these pathways may be connected is through the action of the calcium-dependent tyrosine kinase Pyk2 [11]. To examine the role of tyrosine kinases in GPCR-mediated chemokine expression, the tyrosine kinase inhibitor AG 17 (tyrphostin A9) was used. AG 17 is a potent inhibitor of Pyk2 activation [12], but also inhibits other kinases such as the platelet-derived growth factor β -receptor [13]. PAF-stimulated IL-8 production was significantly attenuated at 1 μ M (90%) and 10 μ M (99%) AG 17 (Figure 9A). Treatment with AG 17 also inhibited the release of CINC-1 by 58-70% after SP stimulation in RLMVECs (Figure 9B).

The expression of IL-8 and CINC-1 is regulated by the proinflammatory transcription factor NF- κ B. The classical NF- κ B activation pathway involves proteasomal degradation of the inhibitory subunit I κ B. To determine whether this pathway may be involved in GPCR-induced chemokine expression, cells were treated with the proteasome inhibitor MG132. PAF-stimulated IL-8 production was significantly inhibited by 10 μ M (59%) and 100 μ M (80%) MG132 (Figure 10A). SP-stimulated CINC-1 production was 96% attenuated by 100 μ M MG132. These results are consistent with PAF- and SP-induced IL-8 and CINC-1 expression involving activation of NF- κ B via the classical I κ B degradation pathway.

DISCUSSION

GPCRs regulate multiple aspects of responses to injury in the lung, including inflammation, vascular permeability, pulmonary edema, airway smooth muscle tone, cell survival, proliferation, and repair. Stimulation of some GPCRs is known to upregulate the expression of proinflammatory genes, such as cytokines and chemokines [14]. Acute lung injury triggers an inflammatory response driven by cytokines and chemokines that results in a rapid influx of neutrophils into lung tissue. Activation of neutrophils within lung tissue causes the release of toxic mediators that can worsen and perpetuate lung injury. Signaling pathways by which GPCRs stimulate cytokine production represent potential therapeutic targets for treating lung inflammatory diseases, but these signaling mechanisms are not completely understood.

GPCRs transduce intracellular signals by activating heterotrimeric G proteins, which have been classified into families based on structural and functional properties of the $G\alpha$ subunit. GPCRs coupled to the G_q family of $G\alpha$ proteins have the capability in some cell types to stimulate cytokine gene expression through the activation of the proinflammatory transcription factor NF- κ B. Examples of this include Tacr1 [8], PAF receptor [15], PAR-2 [16], and the BK B2 receptor [17]. Because most such studies were performed in transformed cells lines and/or with overexpressed receptors, we examined in the current experiments the ability of these GPCRs to induce chemokine expression in primary lung cells.

Our results indicated that IL-8 levels were significantly increased in human primary alveolar epithelial cells in response to SP, BK, PAR-2A, and PAF, four ligands known to stimulate G_q -coupled GPCRs. In contrast, human lung endothelial cells responded with increased cytokine production only to PAF, and rat lung endothelial cells responded to PAF and SP but not BK or PAR-2A. Thus, compared with epithelial cells, endothelial cells showed a more restricted response in the capacity to secrete IL-8 or CINC-1 in response to the panel of GPCR ligands we used. The magnitude of chemokine expression was also more pronounced in epithelial cells as compared with endothelial cells. These results are consistent with the concept that epithelial cells are a major source of cytokine production in vivo following lung injury, with high concentrations of chemokines secreted by epithelial cells serving to attract inflammatory cells to sites of injury. The failure of endothelial cells to respond to some of the GPCR ligands tested may occur because they do not express the appropriate receptors or because they lack the intracellular signaling pathways necessary for the response. For example, we have observed that in A549 cells transfected with the tachykinin 1 receptor, receptor stimulation with SP results in G_q -coupled calcium release ([8], our unpublished observations). In contrast, in 293 cells transfected with Tacr1, SP treatment does not elicit calcium release [18]. The mechanisms underlying differential responses such as these to GPCR stimulation are unknown and represent an interesting avenue of future investigation.

PAF has been well established as a mediator of lung injury in studies using in vivo models [19,20]. Because PAF is a potent inducer of pulmonary edema, much attention has been focused on this aspect of its function. Our results demonstrate that PAF also induces a robust increase in proinflammatory gene expression in lung epithelial cells. PAF has been shown previously to activate NF- κ B, which is a key regulator of chemokines such as IL-8 [15,21]. PAF has been demonstrated to upregulate cytokine expression in lung cells, including lung fibroblasts [22], airway smooth muscle cells [23], and transformed bronchial epithelial cells [24,25]. The results in the present study are to our knowledge the first to demonstrate IL-8 induction by PAF in primary lung epithelial cells.

Endothelial cells participate in inflammatory responses by regulating vascular permeability and the egress of inflammatory cells from the bloodstream. These processes are controlled by inflammatory mediators that induce acute changes in endothelial cell gene expression.

Although adhesion molecules are considered to be the most important proteins in this regard, previous studies have shown that endothelial cells also respond to inflammatory mediators, including those acting through GPCRs, by upregulating cytokine gene expression. For example, PAF induced the upregulation of tumor necrosis factor- α in human umbilical vein endothelial cells [26]. PAR-2 agonists caused increases in IL-8 in human umbilical vein endothelial cells [27] and dermal microvascular endothelial cells [28]. The present study is the first to report increased CINC-1 or IL-8 expression by SP treatment in endothelial cells, although there is precedence for SP inducing upregulation of COX-2, an enzyme responsible for the production of inflammatory lipid mediators [29], and for SP causing the activation of NF- κ B, an important factor mediating chemokine gene transcription [30].

We previously characterized a signal transduction pathway by which Tacr1 receptor stimulation results in NF- κ B activation and IL-8 production [8]. The results documented in the present work extend these studies in several important ways. The previous study was performed in A549 cells, a transformed lung cancer cell line. Here we demonstrate that an equivalent pathway is operative in primary cells, which increases the potential relevance to normal physiological processes. In our previous study, we studied intracellular signaling transduced by receptors that were overexpressed by transfection of Tacr1 cDNA; treatment of untransfected A549 cells with SP did not result in NF- κ B activation. In the experiments described here, signaling triggered by endogenous tachykinin and PAF receptors was investigated. These responses by endogenous receptors on primary lung cells are likely to more closely reflect signaling mechanisms in the intact lung. Whereas we previously examined only one GPCR ligand in epithelial cells, here we investigated multiple ligands in both epithelial and endothelial cells. As we obtained similar responses in the different model systems, our results provide evidence for a general signal transduction pathway responsible for promoting proinflammatory gene expression by GPCRs in multiple cell types.

The overall effects of signaling inhibitors were generally similar between human SAECs and RLMVECs, but some minor differences were observed that deserve comment. First, the phospholipase C inhibitor U73122 inhibited PAF-induced IL-8 production in SAECs at 10 μ M but not at 1 μ M. In contrast, SP-induced CINC-1 production was significantly inhibited by 1 μ M U73122 (Figure 2). Although the reason for this difference is not understood, it is consistent with previous results suggesting that higher concentrations of U73122 are necessary to inhibit phospholipase C mediated effects in lung epithelial cells compared with endothelial cells. For example, we previously observed similar effects in A549 cells [8], and other studies in lung epithelial cells have typically employed U73122 concentrations of at least 10 μ M [31,32,33,34]. In endothelial cells, a variety of events mediated by phospholipase C have been shown to be inhibited by 1 μ M U73122 [35,36,37,38]. A second difference in the response of the different cell types was the increase in CINC-1 expression following treatment of endothelial cells with 1 μ M manumycin A that was not observed in SAECs (Figure 5). This differential response to different doses of manumycin appears to be unusual but not unprecedented. Low doses of ras inhibitors, including manumycin, stimulated the growth of ras-transformed cells at low concentrations and inhibited growth at higher doses [39]. No information is available regarding the mechanisms underlying these effects. A third difference was the lack of response to the Raf-1 inhibitor GW5074 in the endothelial cells. One possibility to explain this result is that in epithelial cells Raf-1 is required, but in endothelial cells this pathway depends on other forms of Raf, such as B-Raf, which are not as readily inhibited by GW5074 [40].

Human IL-8 and rat CINC-1 are both acutely upregulated in response to a variety of injurious or proinflammatory stimuli and share common features by which gene expression is controlled. The IL-8 promoter contains an NF- κ B binding site, and this factor is the primary regulator of IL-8 at the transcriptional level. In addition, the proximal promoter contains binding sites for

NF-IL6 (C/EBP) and AP-1, which in some circumstances are required for maximal increases in gene expression. IL-8 expression may also be controlled at the level of message stability mediated by sequences in the 3' untranslated region of the mRNA [41]. Likewise, NF- κ B binding to a site in the proximal promoter region of CINC-1 is the most important mechanism controlling its upregulation [42]. Similar to IL-8, the CINC-1 gene contains consensus sequences for NF-IL6 binding motif and message instability, but the functional activity of these elements has not been demonstrated. The presence of an AP-1 site in the CINC-1 gene has not been reported. The results of the present study are consistent with a major role for NF- κ B in mediating the upregulation of IL-8 and CINC-1 in response to GPCR ligands, although additional roles for these other factors cannot be excluded.

Figure 11 depicts a proposed signal transduction pathway activated by ligands for G_q-coupled GPCRs based on the results shown here for SAECs and RLMVECs and in our earlier study in A549 cells [8]. In the present study, signaling pathways in primary cells were investigated using chemical inhibitors. In the cells used here, transfection methods that were effective in introducing DNA constructs into the primary cells also induced significant cell death (not shown). As we are examining signaling pathways activated during injury, this problem precluded the use of potentially more specific genetic inhibitors such as dominant-negative mutants. However, because we have examined the effects of inhibiting signaling molecules that are often closely linked (e.g. Ras/Raf/Erk), the overall evidence for involvement of these molecules is enhanced over the use of single signaling inhibitors in isolation. Although minor differences in the responses to some inhibitors were observed, the overall results implicating the signaling molecules shown in the model were consistent among the different cell types. The activation of phospholipase C by GPCRs through G_q leading to calcium release and PKC activation has been well characterized in many cell types. This proximal phospholipase C/calcium/PKC cascade is commonly linked to downstream activation of the Ras/Raf/Erk pathway. In some cells these pathways are linked by the calcium-activated tyrosine kinase Pyk2 [11]. Our results with the AG 17 inhibitor are consistent with the involvement of Pyk2 in the primary cells we used, but the limited specificity of this tyrosine kinase inhibitor does not allow us to definitively rule out the potential requirement for other kinases. Erk activation is well known to stimulate the activation of NF- κ B in response to a variety of external stimuli, but the exact mechanism by which this occurs and the nature of any potential intermediates remains unclear. One potential mechanism involves ribosomal S6 kinase 1, an Erk substrate which has been implicated in activating NF- κ B through both I κ B-dependent and -independent mechanisms [43,44].

The signaling mechanisms we have identified for PAF- and SP-induced upregulation of chemokine expression are generally consistent with previous experiments with G_q-coupled GPCRs. For example, treatment of A549 cells with BK led to NF- κ B activation mediated by the BK B2 receptor through a Ras/Raf/Erk-dependent pathway [17]. PAR-2-induced upregulation of IL-8 production in A549 cells was dependent on PKC, MEK, and tyrosine kinase activity [16]. SP-induced upregulation of NF- κ B in human endothelial cells was dependent on intracellular calcium release [30].

In summary, we have demonstrated increased cytokine production in primary lung epithelial and endothelial cells in response to multiple GPCR ligands. The intracellular signaling mechanisms responsible for the upregulation of IL-8 by PAF in primary epithelial cells and the increased expression of CINC-1 by SP in primary endothelial cells appeared similar based on responses to signaling inhibitors. The inhibitor studies were consistent with a model involving phospholipase C/calcium/PKC and Ras/Raf/Erk pathways. These results provide potential molecular targets for inhibition of GPCR-induced proinflammatory events. Furthermore, these findings suggest that the signaling pathway we have identified is a general mechanism for activating proinflammatory gene expression by GPCRs.

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Figure 1. Effect of the GPCR ligands SP, BK, PAR-2A, and PAF on IL-8 and CINC-1 production
 A. SAECs were treated with the indicated ligands for 4 h, and IL-8 was measured in cell culture supernatants by ELISA. B and C. HLMVECs (B) or RLMVECs (C) were treated with the indicated ligands for 4 h, and CINC-1 was measured in cell culture supernatants by ELISA. A. *, $P < 0.01$ vs. untreated. B. *, $P < 0.001$ vs. untreated. C. *, $P < 0.05$ and **, $P < 0.01$ vs. untreated.

Figure 2. Effect of the phospholipase C inhibitor U73122 on IL-8 and CINC-1 production
 SAECS (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without U73122 pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. PAF-treated without inhibitor. B. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. SP-treated without inhibitor.

Figure 3. Effect of the calcium chelator BAPTA-AM on IL-8 and CINC-1 production
 SAEs (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without BAPTA-AM pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.01$ vs. PAF-treated without inhibitor. B. *, $P < 0.01$ vs. untreated; †, $P < 0.001$ vs. SP-treated without inhibitor.

Figure 4. Effect of the protein kinase C inhibitor Go6850 on IL-8 and CINC-1 production
 SAECs (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without Go6850 pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.01$ vs. PAF-treated without inhibitor. B. *, $P < 0.001$ vs. untreated; †, $P < 0.05$; ††, $P < 0.01$ vs. SP-treated without inhibitor.

Figure 5. Effect of the Ras inhibitor manumycin A on IL-8 and CINC-1 production
 SAEs (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without manumycin A pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. PAF-treated without inhibitor. B. *, $P < 0.05$ vs. untreated; †, $P < 0.001$ vs. SP-treated without inhibitor.

Figure 6. Effect of the Raf-1 inhibitor GW5074 on IL-8 production

SAECs were untreated or exposed to PAF for 4 h, either with or without GW7074 pretreatment.

*, $P < 0.001$ vs. untreated; †, $P < 0.01$ vs. PAF-treated without inhibitor.

Figure 7. Effect of the MEK inhibitor U0126 on IL-8 and CINC-1 production

SAECs (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without U0126 pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. PAF treated without inhibitor. B. *, $P < 0.05$ vs. untreated; †, $P < 0.05$ vs. SP treated without inhibitor.

Figure 8. Effect of Erk inhibitor III on IL-8 and CINC-1 production

SAECs (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without Erk inhibitor III pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. PAF-treated without inhibitor. B. *, $P < 0.01$ vs. untreated; †, $P < 0.01$ vs. SP-treated without inhibitor.

Figure 9. Effect of the protein tyrosine kinase inhibitor AG 17 on IL-8 and CINC-1 production SAECS (A) or RLMVECS (B) were untreated or exposed to PAF or SP for 4 h, either with or without AG 17 pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. PAF-treated without inhibitor. B. *, $P < 0.001$ vs. untreated; †, $P < 0.01$ vs. SP-treated without inhibitor.

Figure 10. Effect of the proteasome inhibitor MG132 on IL-8 and CINC-1 production
SAECs (A) or RLMVECs (B) were untreated or exposed to PAF or SP for 4 h, either with or without MG132 pretreatment. A. *, $P < 0.001$ vs. untreated; †, $P < 0.001$ vs. PAF-treated without inhibitor. B. *, $P < 0.01$ vs. untreated; †, $P < 0.01$ vs. SP-treated without inhibitor.

Figure 11. Signaling model for activation of cytokine gene expression by ligands for G_q-coupled GPCRs

A proposed signaling pathway based on the results of the present study and that of Williams et al. [8] is shown. See text for details. Abbreviations: DAG, diacylglycerol; Erk, extracellular regulated kinase; IKK, IκB kinase; IP₃, inositol triphosphate; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C; PLC, phospholipase C.