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Peptidoglycan enhances secretion of monocyte chemoattractants via multiple signaling pathways

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ABSTRACT

Peptidoglycan (PG) is detected in a high proportion in inflammatory cell-rich regions of human atheromatous plaques. In the present study, we determined the cellular factors involved in PG-mediated chemokine expression in mononuclear cells in order to understand the molecular mechanisms of inflammatory responses to bacterial pathogen-associated molecular patterns in the diseased artery. Exposure of human monocytic leukemia THP-1 cells to PG resulted in not only enhanced secretion of CCL2 and CCL4 but also profound induction of their gene transcripts, which were abrogated by oxidized 1-palmitoyl-2-arachidonosyl-sn-phosphatidylcholine, an inhibitor of Toll-like receptors (TLRs)-2/4, but not by polymyxin B. PG enhanced phosphorylation of Akt and mitogen-activated protein kinases and activated protein kinase C. Pharmacological inhibitors such as SB202190, SP6001250, U0126, Akt inhibitor IV, rapamycin, and RO318220 significantly attenuated PG-mediated up-regulation of CCL2 and CCL4. We propose that PG contributes to vascular inflammation in atherosclerotic plaques by upregulating expression of mononuclear cell chemoattractants via TLR-2, protein kinase C, Akt, mTOR, and mitogen-activated protein kinases.

1. Introduction

Chemokines play important roles in the initiation and progression of atherosclerosis. They promote recruitment and migration of inflammatory cells into the atherosclerotic lesion and induce activation of endothelial cells and leukocyte subsets, leading to release of inflammatory cytokines and chemokines. These in turn further promote recruitment and activation of leukocytes in the atherosclerotic lesion [1]. The CC chemokine family, to which CCL2 (monocyte chemotactic protein-1) and CCL4 (human macrophage inflammatory protein-1β) belong, plays a key role in the recruitment of mononuclear cells into atherosclerotic lesions. Enhanced levels of CCL4 are associated with carotid atherosclerosis [2]. CCL2 strongly induces mononuclear cells, predominantly monocytes and lymphocytes, to migrate and infiltrate atherosclerotic lesions [3], and its expression is linked to atherosclerosis. The higher the expression of CCL2, the greater the chance of developing atherosclerosis [4]. Conversely, the deletion of the CCL2 gene or the gene for its receptor is followed by a significant reduction in the development of atherosclerotic plaques [5]. Therefore, it is important to understand the regulation of CCL2 and CCL4 expression because of their close association with atherosclerosis.

Peptidoglycan (PG), the major cell wall component of Gram-positive bacteria, is abundantly present in the flora of the normal human gut and other mucosa. At nonmucosal sites, PG is recognized by the innate immune system as a bacterial pathogen-associated molecular pattern (PAMP) and promotes inflammation via Toll-like receptors (TLR) [6]. PG is reported to induce αmβ2-integrin expression by monocytes and increase the β2-integrin-dependent migration of monocytes [7]. PG can upregulate the expression of adhesion molecules by endothelial cells [8] and proinflammatory cytokines [9] and chemokines [10] by monocytes/macrophages. Since PG is detected in human atherosclerotic lesions, mainly in macrophage-rich atheromatous regions [11], it is presumed to be an additional pro-inflammatory factor in the lesion. Therefore, elucidation of signaling pathways through which PG induces inflammatory responses will contribute to the current knowledge of the role of bacterial PAMPs in atherogenesis. However, the molecular mechanisms through which PG induces chemokine expression have not yet been defined.

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In the present study, we investigated whether PG affected secretion of mononuclear cell chemoattractants by monocytes/macrophages. We found that PG significantly enhanced secretion of CCL2 and CCL4 by THP-1 cells. Moreover, we identified cellular molecules involved in PG-mediated chemokine expression, and found that TLR2, Akt, mTOR, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) were involved in PG-mediated CCL2 and CCL4 upregulation.

2. Materials and methods

2.1. Cell culture and reagents

The THP-1 cell line, a human acute monocytic leukemia cell line, was purchased from and maintained as suggested by the American Type Culture Collection (ATCC, Manassas, VA, USA). PG isolated from *Staphylococcus aureus* (endotoxin free), polymyxin B, and oxidized 1-palmitoyl-2-arachidonosyl-sn-phosphatidylcholine (OxPAPC) were purchased from InvivoGen (San Diego, CA, USA). Endotoxin-free bovine serum albumin (BSA), RO318220, GF109203X, LY294002, diphenyleneiodonium (DPI), N-acetylcysteine (NAC), rapamycin, and SP600125 were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). U0126, SB202190, and Akt inhibitor IV (Akti IV) were purchased from Cell Signaling Technology (Danvers, MA, USA). For inhibition experiments, THP-1 cells were stimulated for 9 h with PG (1 μg/ml) after treatment for 1 h with indicated chemicals, and amounts of CCL2 and CCL4 released into the medium were measured by ELISA, and transcripts of CCL2 and CCL4 genes were amplified by RT-PCR.

2.2. Western blot analysis

THP-1 cells were lysed using a lysis buffer (1% sodium dodecyl sulfate, 1 mM $NaVO_3$, 10 mM Tris–HCl [pH 7.4]) containing protease inhibitors. After removal of cell debris by centrifugation, the cell lysate was separated by SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After the membranes were blocked by 1 h incubation in 5% skim milk/0.1% Tween 20 in phosphate buffered saline (PBS), they were incubated overnight at 4 °C with appropriate primary antibodies. The antibodies used in this study were purchased from various vendors as previously described [12]. After three washes using 0.1% Tween 20 in PBS, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using a chemiluminescent reagent.

2.3. CCL2 and CCL4 enzyme linked immunosorbent assays (ELISAs)

The amounts of secreted CCL2 and CCL4 were determined using commercially available ELISA kits, according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). THP-1 cells were exposed to PG. And cell culture media were collected. Cell culture media and standards for CCL2 or CCL4 were added to a microtiter plate precoated with monoclonal antibody against CCL2 or CCL4. After incubation for 2 h, the plate was washed and incubated with an enzyme-conjugated polyclonal antibody specific for CCL2 or CCL4. The substrate was added after several plate washes, and color intensity was measured. The amount of CCL2 or CCL4 present in the medium was determined from a standard curve. Data are expressed as mean ± SD.

2.4. Reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNAs were extracted from cells and reverse-transcribed for an hour at 42 °C with Moloney murine leukemia virus reverse transcriptase. PCR amplification was performed for 25 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) in the presence of primers. The primers for CCL2 were 5-TCTGTGCCTGCTGCTCATAG-3 (forward) and 5-CAGATCTCCTTGGCCACAAT-3 (reverse), and the primers for CCL4 were 5-AAGCTCTGCGTGACTGTCCT-3 (forward) and 5-GCTTGCTTCTTTTGGTTTGG-3 (reverse). Primers for the PKC subtypes were prepared as previously described [13]. PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

2.5. PKC activity assay

PKC activity was determined using a PKC activity assay kit (Assay Designs Inc., Ann Arbor, MI, USA) according to the manufacturer's instructions. Cells were collected and lysed in ice-cold lysis buffer containing 20 mM 3-morpholinopropanesulfonic acid, 50 mM β-glyceraldehyde phosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA 1% NP40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethanesulphonyl fluoride, and 10 μg/ml each aprotinin and leupeptin. The cell lysate was centrifuged to obtain supernatant. Purified active PKC provided in the kit and the supernatants were added to wells in a 96-well plate, and PKC reactions were initiated by adding ATP. After incubation for 90 min, the reactions were stopped by aspirating the wells. Phospho-specific substrate antibody was added to each well and incubated for 50 min at room temperature. HRP-conjugated secondary antibody was added and incubated for 30 min at room temperature. After the plate was washed, substrate was added, and the plate was incubated at room temperature. Absorbance was measured at 450 nm.

2.6. Statistics

Statistical analyses were performed using GraphPad PRISM, version 5.0 (GraphPad Software Inc., San Diego, CA, USA), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Upregulation of CCL2 and CCL4 expression by PG at messenger and protein levels

We investigated the effects of PG on CCL2 and CCL4 secretion by THP-1 cells using ELISA. THP-1 cells constitutively secreted a small quantity of CCL2, and PG significantly enhanced secretion of the chemokine. In comparison with control cells cultured in the absence of PG, the amount of secreted CCL2 was increased by 8.3- and 14.1-fold in the presence of 100 and 1000 ng/ml of PG, respectively. PG also enhanced CCL4 secretion in a concentration-dependent manner. In comparison with control cells, the amount of secreted CCL4 was increased by 1.9-, 5.6-, and 7.7-fold in the presence of 10, 100, and 1000 ng/ml of PG, respectively (Fig. 1A). We also investigated if PG influenced CCL2 and CCL4 expression at the messenger level. CCL2 and CCL4 gene transcripts were barely detected in THP-1 cells in the absence of PG. PG induced expression of CCL2 and CCL4 transcripts at 10 ng/ml of PG, and the induction was more evident at 100 ng/ml of PG (Fig. 1B). We examined if TLRs mediated CCL2 and CCL4 expression in response to PG using OxPAPC, a TLR2/4 inhibitor. Treatment with OxPAPC abolished PG-mediated expression of CCL2 and CCL4 at the messenger and protein levels. PG-mediated secretion of CCL2 and CCL4 was almost completely blocked and reduced to that of the control cells in the presence of OxPAPC (Fig. 1C), and PG-induced expression of CCL2 and CCL4 transcripts was also abrogated in the presence of OxPAPC (Fig. 1D). Since PG preparations can be contaminated with lipo-

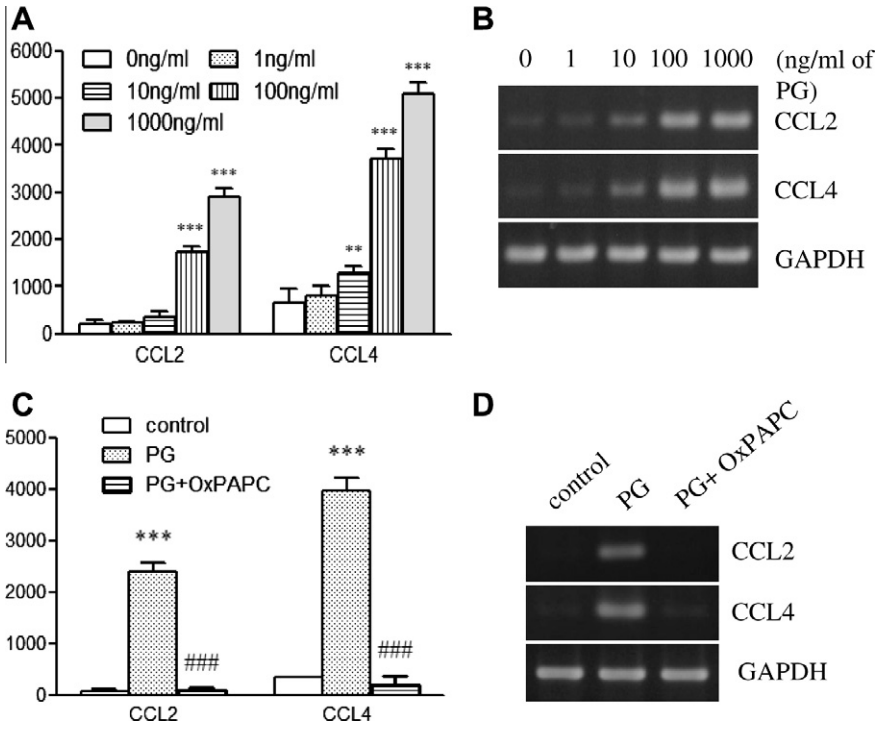

Fig. 1. Effect of PG on secretion and gene transcription of CCL2 and CCL4. (A and B) THP-1 cells (1×10^6 cells/ml) were incubated for 9 h in the absence or presence of the indicated concentrations of PG. Amounts of CCL2 and CCL4 released into the medium were measured by ELISA, and CCL2 and CCL4 gene transcripts were amplified by RT-PCR (B). Data are expressed as mean ± SD. $^{**}P < 0.01$ vs. 0 ng/ml. $^{***}P < 0.001$ vs. 0 ng/ml. (C and D) THP-1 cells were stimulated for 9 h with or without PG (1 μg/ml) after treatment for 1 h with OxPAPC (30 μg/ml). Amounts of CCL2 and CCL4 released into the medium were measured (C), and transcripts of CCL2 and CCL4 genes were amplified (D). $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. PG. Results shown are representative of three separate experiments.

polysaccharide (LPS), which increases secretion of pro-inflammatory cytokines and chemokines by monocytes [14], we examined whether LPS contributed to PG-mediated up-regulation of CCL2 and CCL4 using polymyxin B, a potent inhibitor of LPS. Polymyxin B did not attenuate PG-mediated secretion of CCL2 and CCL4 or expression of their transcripts (see Supplemental data).

3.2. Roles of phosphoinositide 3-kinase (PI3K)/Akt and mTOR in PG-induced CCL2 and CCL4 expression

We examined if PG affected Akt activity using western blot analysis to detect the phosphorylated form of the kinase. PG enhanced Akt phosphorylation. Maximum Akt phosphorylation occurred 20 min post-treatment with PG and was sustained up to 40 min post-treatment (Fig. 2A). To investigate the roles of Akt in PG-mediated CCL2 and CCL4 expression, we used two inhibitors, LY294002 and Akti IV. Both inhibitors significantly affected CCL2 expression at the messenger and protein levels. PG-induced CCL2 gene expression was abrogated by LY294002 and Akti IV. PG-mediated CCL2 secretion was significantly inhibited or completely blocked by LY294002 and Akti IV, respectively. CCL4 expression was also influenced by Akt inhibition. Akti IV remarkably attenuated PG-mediated induction of CCL4 gene expression and completely blocked CCL4 secretion. LY294002 slightly attenuated PG-induced CCL4 gene transcription without influencing CCL4 secretion. Since Akt exerts its biological effects through activation of the mammalian target of rapamycin (mTOR) [15], we investigated whether mTOR participated in PG-mediated CCL2 and CCL4 expression using rapamycin, an inhibitor of mTOR. It appeared that rapamycin affected CCL2 and CCL4 expression mainly at the protein level. PG-mediated CCL2 and CCL4 secretion was significantly reduced in the presence of rapamycin (Fig. 2D) with slight attenuation of PG-induced CCL2 and CCL4 gene transcription (Fig. 2E).

3.3. Roles of PKC in PG-induced CCL2 and CCL4 expression

We investigated whether PKC is important in PG-mediated CCL2 and CCL4 expression using RT-PCR. We found that THP-1 cells expressed PKC-βI, −βII, −λ, −η, and −θ subtypes. Because we were able to detect their transcripts, but transcripts of PKC-α, −δ, −ε, −γ, −ξ, and −μ subtypes, were not seen (Fig. 3A). Next, we examined the effects of PG on PKC activity. PG activated PKC (Fig. 3B). PKC activity was enhanced 3.4-, 4.8-, and 3.7-fold at 10, 20, and 30 min, respectively, post-treatment with PG. To assess involvement of PKC in PG-mediated up-regulation of CCL2 and CCL4, we used two different PKC inhibitors, GF109203X and RO318220. Of the two inhibitors, RO318220 significantly inhibited PG-mediated secretion of CCL2 and CCL4, as well as remarkably attenuated PG-induced CCL2 and CCL4 gene transcription. GF109203X, however, did not affect PG-mediated CCL2 and CCL4 up-regulation (Fig. 3C and D).

3.4. Roles of MAPKs and reactive oxygen species (ROS) in PG-induced CCL2 and CCL4 expression

We investigated whether PG affected the activities of MAPKs by examining the phosphorylated forms of the kinases on western blots. PG enhanced phosphorylation of extracellular signal-regulated kinase (ERK) and p38 MAPK. Maximum phosphorylation of ERK and p38 MAPK occurred 20 min post-treatment with PG and was sustained up to 30 min post-treatment (Fig. 4A). PG also weakly affected phosphorylation of c-jun N-terminal kinase (JNK). To assess the roles of MAPKs in PG-induced up-regulation of CCL2 and CCL4, we used the following MAPK inhibitors: SB202190 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), and U0126 (ERK inhibitor). Those inhibitors significantly blocked PG-mediated CCL2 secretion as well as profoundly inhibited PG-induced CCL2 gene transcription (Fig. 4B and C). Among the inhibi-

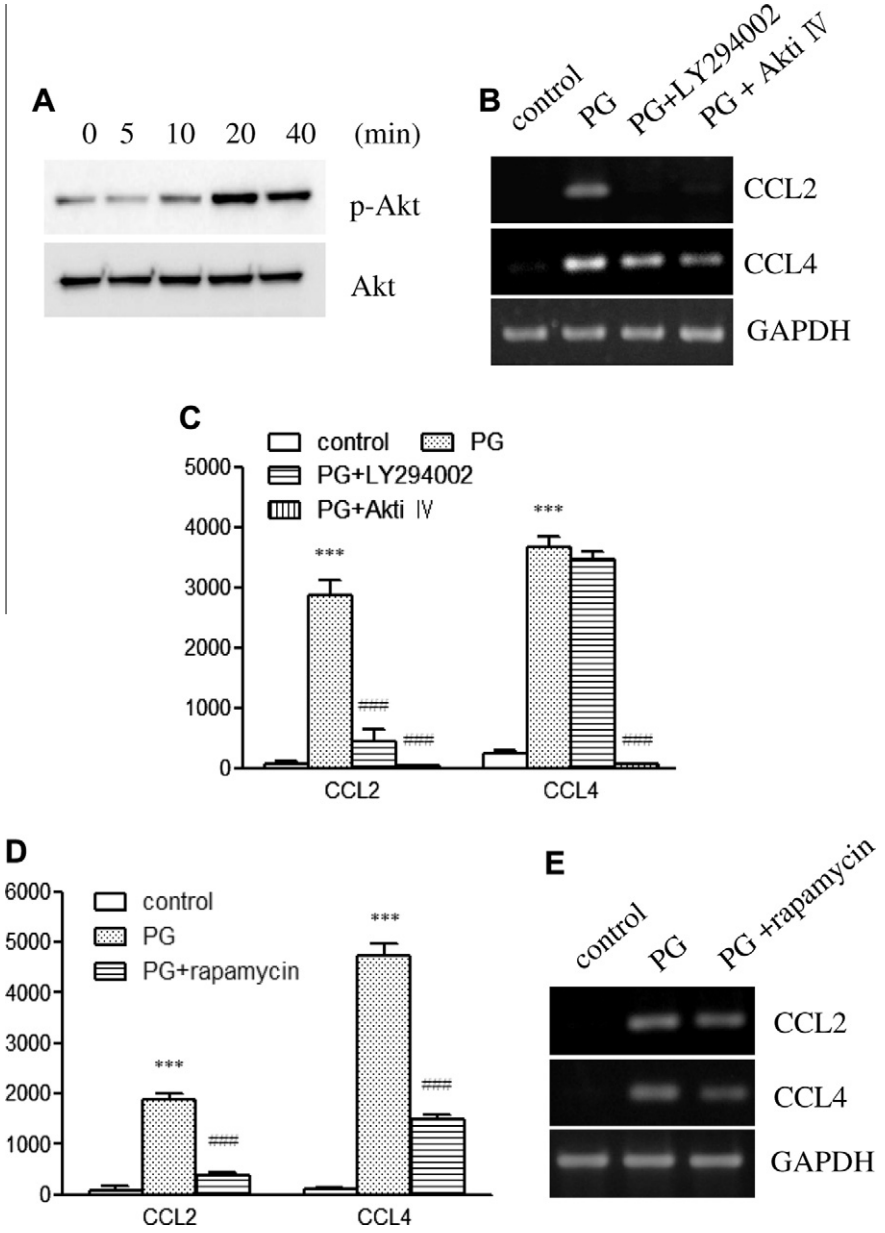

Fig. 2. The effects of LY294002, Akti IV, and rapamycin on PG-mediated upregulation of CCL2 and CCL4. (A) THP-1 cells were exposed to PG for the indicated time periods, after which an equal amount of protein was analyzed by western blotting using antibodies against Akt and phosphorylated Akt. (B–E) THP-1 cells were stimulated for 9 h with or without PG (1 μg/ml) after pretreatment with LY294002, Akti IV (10 μM each) (B and C), or rapamycin (100 nM) (D and E). Amounts of CCL2 and CCL4 released into the medium were measured (C and D), and transcripts of CCL2 and CCL4 genes were amplified (B and E). $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. PG. Results shown are representative of three separate experiments.

tors, U0126 completely blocked CCL2 secretion by THP-1 cells. The MAPK inhibitors also affected CCL4 expression. U0126, SB202190, and SP600125 significantly inhibited PG-mediated secretion of CCL4 as well as attenuated PG-induced CCL4 gene transcription (Fig. 4B and C).

We also investigated whether NADPH oxidase, which produces ROS, played a role in PG-mediated CCL2 and CCL4 expression using DPI. DPI significantly inhibited PG-mediated secretion of CCL2 and CCL4 as well as attenuated PG-induced CCL2 and CCL4 gene transcription (see Supplemental data).

4. Discussion

We demonstrated that PG, a bacterial component present in atherosclerotic lesions, upregulated CCL2 and CCL4 expression both at the messenger and protein levels in the human monocyte/macrophage THP-1 cell line. This finding is consistent with results of a previous study by Wang et al. [10]. Using an RNAse protection assay, they reported that PG and LPS induced expression of CCL2, CCL3, CCL4, and CXCL8 in human blood monocytes. However, the PG preparation used in that study was contaminated with LPS. It is possible that LPS contamination occurring during PG preparation contributes to or may be responsible for the production of inflammatory cytokines and chemokines through activation of TLR4. To investigate whether contaminating LPS contributed to PG-mediated CCL2 and CCL4 expression, we used polymyxin B. Polymyxin B binds LPS, thereby preventing its biological effects [16]. We found that polymyxin B had no influence on PG-mediated CCL2 and CCL4 expression at both the protein and messenger levels (see Supplemental data), which indicates that the upregulation of CCL2 and CCL4 observed in this study was induced by PG alone.

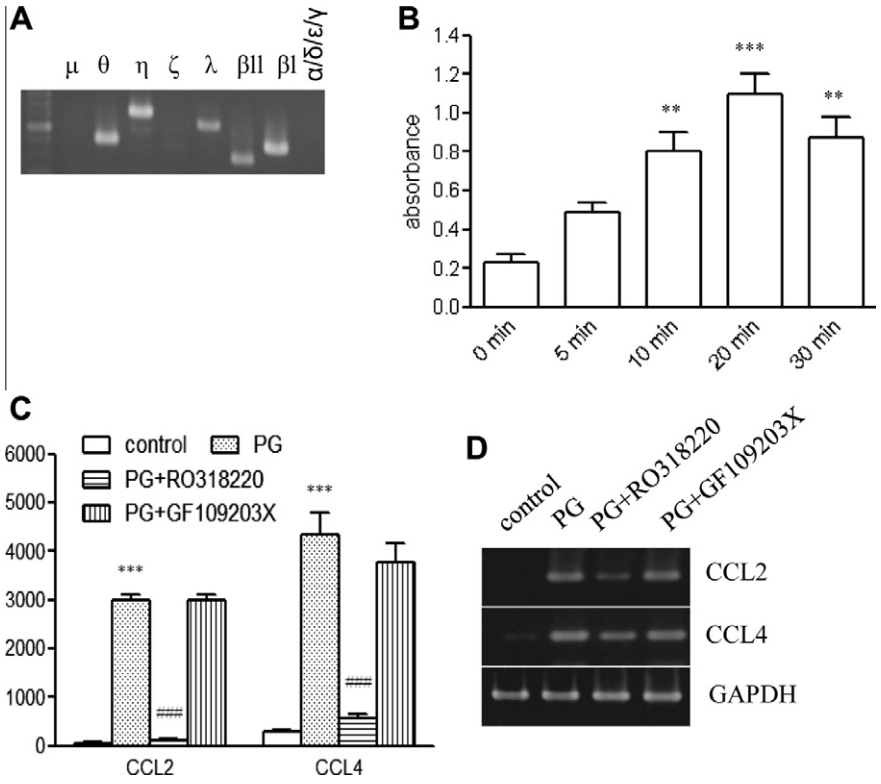

Fig. 3. Effects of PKC inhibitors on PG-mediated upregulation of CCL2 and CCL4. (A) Total RNA was isolated from THP-1 cells, and transcripts of PKC subtypes were amplified by RT-PCR. (B) THP-1 cells were exposed to PG for the indicated time periods, after which PKC activity was determined using the PKC kinase activity assay kit. Data are expressed as mean ± SD. $^{**}P < 0.01$ vs. 0 min. $^{***}P < 0.001$ vs. 0 min. (C and D) THP-1 cells were stimulated for 9 h with or without PG (1 μg/ml) after pretreatment with RO318220 (1 μM) and GF109203X (3 μM). Amounts of CCL2 and CCL4 released into the medium were measured (C), and transcripts of CCL2 and CCL4 genes were amplified (D). $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. PG. Results shown are representative of three separate experiments.

It is evident that PG induces expression of chemokines, but which cellular molecules are involved in chemokine expression in response to PG has not yet been clarified. In the present study, we tried to identify the cellular factors involved in PG-mediated CCL2 and CCL4 expression. Because PG is a bacterial PAMP recognized by TLR2, we investigated receptor-mediated CCL2 and CCL4 expression using OxPAPC, an inhibitor of TLR2/4. OxPAPC completely blocked secretion of the chemokines and transcription of their genes. The complete inhibition of PG-mediated CCL2 and CCL4 expression by OxPAPC indicates that TLR2 is responsible for PG-induced CCL2 and CCL4 expression.

We found that PG enhanced Akt phosphorylation, which indicates PG activates the kinase. We investigated if Akt and PI3K, an Akt activator [17], were involved in PG-mediated CCL2 and CCL4 expression. Akt inhibition completely blocked the secretion of CCL2 and CCL4 and also inhibited transcription of their genes, whereas PI3K inhibition resulted in significant attenuation of CCL2 expression, but not of CCL4 expression. These results indicate that both PI3K and Akt are necessary for PG-mediated CCL2 expression, whereas Akt is sufficient for CCL4 expression in response to PG. Akt achieves its biological effects through protein targets. One downstream target of Akt includes mTOR, a kinase protein predominantly found in cell cytoplasm [18]. We investigated if mTOR was involved in PG-mediated CCL2 and CCL4 expression. Rapamycin inhibition of mTOR resulted in significant reduction of CCL2 and CCL4 secretion, whereas rapamycin slightly attenuated transcription of their genes. These results indicate that rapamycin inhibits PG-mediated CCL2 and CCL4 expression at the protein level but not at the messenger level. We think that these results conform with the fact that mTOR is responsible for protein synthesis, as it exerts its effects primarily by turning on and off the translational machinery of the cell [19].

MAPKs, the serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities, mediate chemokine production in TLR-2, -4, and -9 signaling [20]. Since we found that TLR-2 is responsible for PG-mediated CCL2 and CCL4 expression, we investigated if MAPKs played roles in the expression of these chemokines. We demonstrated that PG elevated phosphorylation of MAPKs, and that selective inhibition of ERK, p38 MAPK, and JNK resulted in significant attenuation of CCL2 and CCL4 expression. These results indicate that activation of MAPKs is required for PG-mediated CCL2 and CCL4 expression. The selective inhibitors of ERK, p38 MAPK, and JNK more efficiently inhibited expression of CCL2 than of CCL4. The results indicate that CCL2 expression is more dependent than CCL4 expression on activation of MAPKs. Since PG causes increased ROS production in human blood leukocytes [21], we used DPI to investigate whether ROS was also involved in CCL2 and CCL4 expression. DPI is an inhibitor of NADPH oxidase, which produces ROS; thereby DPI inhibits ROS formation [22]. DPI significantly attenuated PG-mediated secretion of CCL2 and CCL4 by THP-1 cells. Of the two chemokines, expression of CCL2 was more significantly affected by DPI, which suggests that ROS more actively participates in CCL2 expression.

PKC is reported to mediate gene expression in response to PG. PG enhanced cyclooxygenase-2 gene expression in mouse macrophages in a PKC-dependent manner [23]. We found that THP-1 cells expressed functional PKC in response to PG, which is consistent with the finding by Asehnoune et al. who reported that PKC was able to activate Akt in a TLR2-dependent fashion [24]. Using PKC inhibitors, we investigated if PKC played roles in PG-mediated CCL2 and CCL4 expression. RO318220 significantly attenuated CCL2 and CCL4 expression, but GF109203X did not. The two inhibitors are bisindolylmaleimide derivatives of staurosporine, and in-

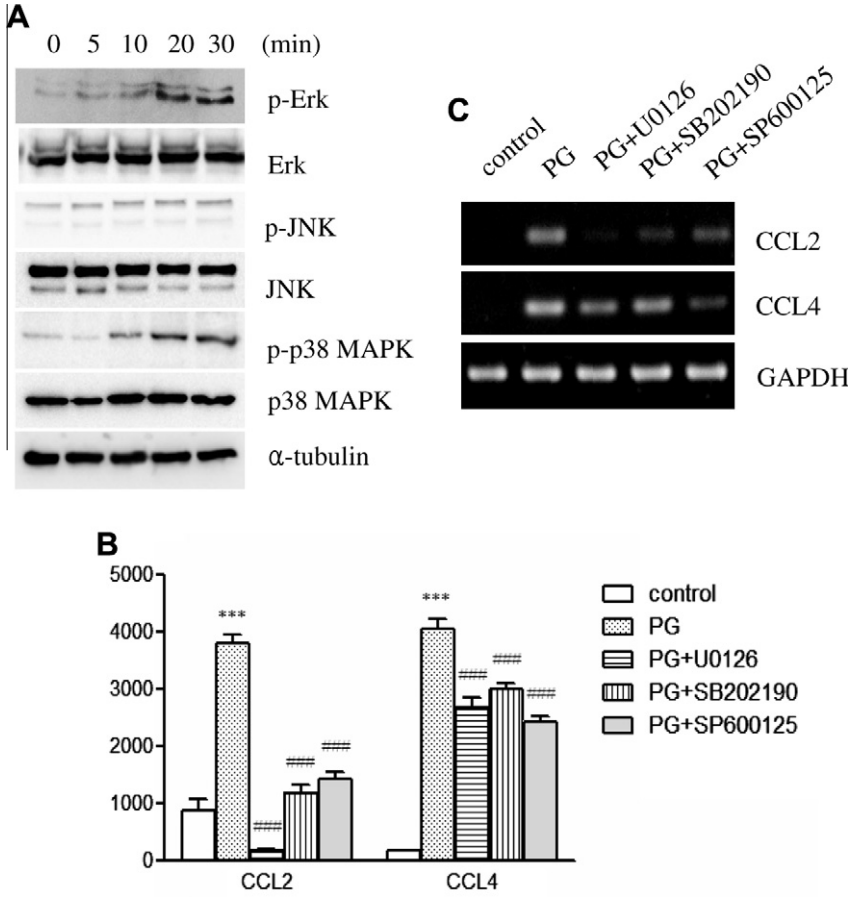

Fig. 4. Effects of inhibitors of MAPKs on PG-mediated upregulation of CCL2 and CCL4. (A) THP-1 cells were exposed to PG for the indicated time periods, after which an equal amount of protein was analyzed by western blotting using antibodies against phosphorylated and unphosphorylated forms of ERK, p38 MAPK, and JNK. (B and C) THP-1 cells were stimulated for 9 h with or without PG (1 μg/ml) after pretreatment with indicated MAPKs inhibitors (10 μM each). Amounts of CCL2 and CCL4 released into the medium were measured (B), and transcripts of CCL2 and CCL4 genes were amplified (C). $^{***}P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs. PG. Results shown are representative of three separate experiments.

hibit mixed isoforms of PKC [25]. We think that the difference between the two inhibitors in their effects on CCL2 and CCL4 expression may result from additional inhibitory activity of other kinases by RO318220. RO318220 inhibits Akt, c-Raf, MAPKK-1, and p42 MAPK, but GF109203X does not [25]. We found that activation of Akt and p44/42 MAPK is required for CCL2 and CCL4 expression. It is possible that attenuation of PG-mediated CCL2 and CCL4 expression by RO318220 may have been caused by its inhibition of other enzymes such as Akt and p42 MAPK. Further investigation is necessary to clarify the roles of PKC in PG-mediated CCL2 and CCL4 expression.

We showed that incubation of THP-1 cells with PG enhanced secretion of CCL2 and CCL4 and induced transcription of their genes, and found that TLR2, Akt, mTOR, PKC, MAPKs, and ROS participated in that process. This study, however, did not specifically determine if these factors acted in an independent or cooperative manner to upregulate CCL2 and/or CCL4 expression. Further investigation is needed to elucidate the types of connections or crosstalk that may be occurring in the context of a possible signaling cascade.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.136.

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