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Peptidoglycan Up-Regulates CXCL8 Expression via Multiple Pathways in Monocytes/Macrophages

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Abstract

Peptidoglycan (PG), the gram positive bacterial pathogen-associated molecular patterns (PAMP), is detected in a high proportion in macrophage-rich atheromatous regions, and expression of chemokine CXCL8, which triggers monocyte arrest on early atherosclerotic endothelium, is elevated in monocytes/ macrophages in human atherosclerotic lesion. The aim of this study was to investigate whether PG induced CXCL8 expression in the cell type and to determine cellular signaling pathways involved in that process. Exposure of THP-1 cell, human monocyte/macrophage cell line, to PG not only enhanced CXCL8 release but also profoundly induced *il8* gene transcription. PG-induced release of CXCL8 and induction of *il8* gene transcription were blocked by OxPAPC, an inhibitor of TLR-2/4 and TLR4, but not by polymyxin B, an inhibitor of LPS. PG-mediated CXCL8 release was significantly attenuated by inhibitors of PI3K-Akt-mTOR pathways. PKC inhibitors, MAPK inhibitors, and ROS quenchers also significantly attenuated expression of CXCL8. The present study proposes that PG contributes to inflammatory reaction and progression of atherosclerosis by inducing CXCL8 expression in monocytes/macrophages, and that TLR-2, PI3K-Akt-mTOR, PKC, ROS, and MAPK are actively involved in the process.

Key Words: CXCL8, Monocytes/Macrophages, Peptidoglycan, PI3K

INTRODUCTION

Atherosclerosis is a chronic inflammatory disorder characterized by accumulation of monocytes/macrophages and lymphocytes within the arterial wall. At the early stage of atherosclerosis monocytes and lymphocytes circulating in the blood attach to the endothelium in atherosclerosis-predisposed areas of the arteries and migrate into the subendothelial layer of the intima where monocytes differentiate into macrophages (Libby, 2002). The attachment and migration is guided by endothelial leukocyte adhesion molecules and chemokines. In addition to the function of messengers directing leukocytes to sites of inflammation, chemokines can control homeostasis and other activities of emigrated cells. Chemokines activate macrophages and enhance release of inflammatory cytokines and can increase production of tissue factor, matrix metalloproteinases, and reactive oxygen species (ROS). These events will transform macrophages into an inflammatory, matrix degrading, procoagulant, and apoptosis-inducing phenotype (Libby, 2002; Zernecke *et al*., 2008). These findings indicate that chemokines are one of key factors contributing to the initiation and progression of atherosclerosis. Therefore, regulation of chemokines expression is important in vascular biology because of their diverse roles in atherogenesis.

Peptidoglycan (PG), the major constituent of Gram-positive bacteria, is a bacterial pathogen-associated molecular pattern (PAMP) detected in human atherosclerotic lesions. PG is mainly localized in macrophage-rich atheromatous regions in a high proportion (Laman *et al*., 2002). PG is abundantly present in the normal human gut flora and in other mucosa, but promotes inflammation at nonmucosal sites through engagement of Toll-like receptor (TLR2) (Yoshimura *et al*., 1999). PG can induce $\alpha_m\beta_2$-integrin expression on monocytes and increase β_2-integrin-dependent migration of monocytes (Nijhuis *et al*., 2007). PG is able to up-regulate the expression of adhesion molecules on endothelial cells (Ecs) (Dobrina *et al*., 1995) and induce expression of proinflammatory cytokines (Schrijver *et al*., 1999; Langer *et al*., 2008) and chemokines (Wang *et al*., 2000) by monocytes/macrophages. Therefore, clarification of mechanism of action of PG leading to inflamma-

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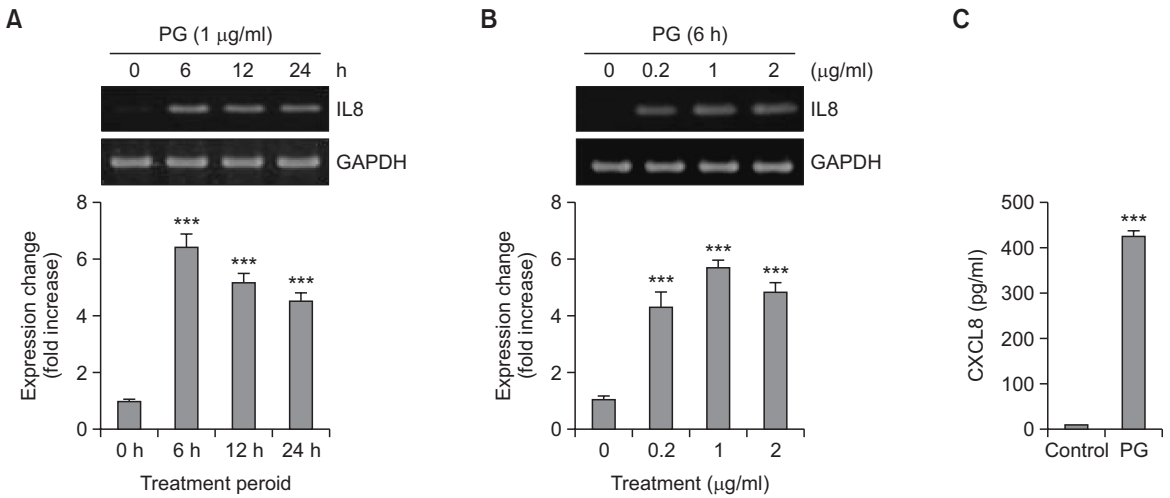

Fig. 1. The effect of PG on expression of CXCL8. (A, B) THP-1 cells (1×10^6 cells/ml) were treated for the indicated time periods with 1 μg/ml of PG (A) or were treated for 6 hr with indicated concentration of PG (B). Transcript of *il8* gene was amplified and quantified by RT-PCR and realtime PCR, respectively. The y-axis values represent fold increases of IL-8 mRNA levels normalized to GAPDH levels relative to those of control THP-1 cells incubated in medium alone. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. (C) THP-1 cells (1×10^6 cells/ml) cultured in growth media were incubated for 9 hr in the absence (control) or presence of 1 μg/ml of PG. CXCL8 secreted into the culture medium was measured using ELISA. Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control.

tory response will broaden current understanding on the role of bacterial PAMPs in atherogenesis.

CXCL8, a member of CXC subfamily of chemokines and encoded by the *il8* gene, was originally discovered and purified as a neutrophil chemotactic and activating factor. Clinical and animal studies have shown that CXCL8 is closely involved in the vascular disease. A clinical study has shown that CXCL8 is the most powerful predictor of future cardiovascular events independent of other cytokines as well as high sensitive C reactive protein (Inoue *et al*., 2008). The knock-out mice of IL8 homologue exhibit reduced levels of atherosclerotic lesions in animal experiments (Boisvert *et al*., 1998). This chemokine is believed to exacerbate atherosclerotic process by modifying cellular events during atherogenesis. CXCL8 mediates proliferation of vascular smooth muscle cells (VSMCs) (Yue *et al*., 1994), triggers monocyte arrest on early atherosclerotic endothelium (Huo *et al*., 2001), and recruits neutrophils and T cells to the site of tissue injury (Peveri *et al*., 1988; Schroder and Christophers, 1989). In addition, CXCL8 can affect stability of atherosclerotic plaque by down-regulating tissue inhibitor of metalloproteinase-1 expression in cholesterol-laden macrophages (Moreau *et al*., 1999) . Thus, understandings of *il8* gene expression in vascular cells appear to be important in therapeutic strategies for vascular diseases.

In this study we investigated the effects of PG on CXCL8 expression. Furthermore, it was determined which cellular factors were involved in PG-mediated regulation of the chemokine in order to clarify signal pathways responsible for inflammatory response to PG.

MATERIALS AND METHODS

Cell culture and reagents

THP-1 cell line, the human acute monocytic leukemia cell line, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in humidified atmosphere of 5% CO_2. Penicillin (50 units/ml) and streptomycin (50 μg/ml) were added to prevent bacterial contamination. Cells were maintained between 1,000 to a million cells per ml in the culture medium.

PG isolated from *S. aureus*, endotoxin-free bovine serum albumin (BSA), RO318220, GF109203X, LY294002, diphenyleneiodonium (DPI), N-acetylcystein (NAC), 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). Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) was purchased from Cell Sciences (Canton, MA, USA).

Enzyme linked immunosorbent assay (ELISA) of CXCL8

The amount of CXCL8 released from THP-1 cells into the culture medium was determined using a commercially available ELISA kit according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). THP-1 cells were incubated with 0.1% BSA in DMEM overnight and exposed to PG. Culture medium was isolated by centrifugation (2,000×*g* for 10 min) and kept -70°C until usage. The isolated culture medium and the recombinant CXCL8 standards provided in the kit were added to a plate pre-coated with a monoclonal antibody against CXCL8. After incubation for an hour, the plate was washed and incubated with an enzyme-linked polyclonal antibody specific for CXCL8. After several washes, the substrate solution was added, and the color intensity was determined. The amount of CXCL8 in the medium was calculated by using a recombinant CXCL8 standard curve.

Reverse transcription (RT) - real-time polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) and real-time PCR were

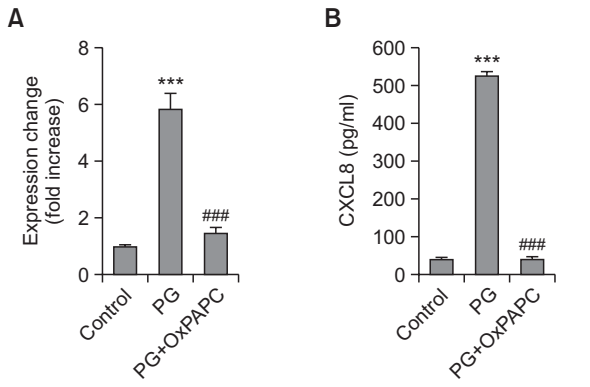

Fig. 2. The effects of OxPAPC on PG-induced CXCL8 expression. (A) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr in the absence or presence of OxPAPC (30 μM) and stimulated for 6 hr with PG (1 μg/ml). Transcript of *il8* gene was quantified by realtime PCR. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. ###p<0.001 vs. PG. (B) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr with or without OxPAPC and stimulated for 9 hr with PG. The amount of CXCL8 secreted into the culture medium was measured by ELISA (B). Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control. ###p<0.001 vs. PG.

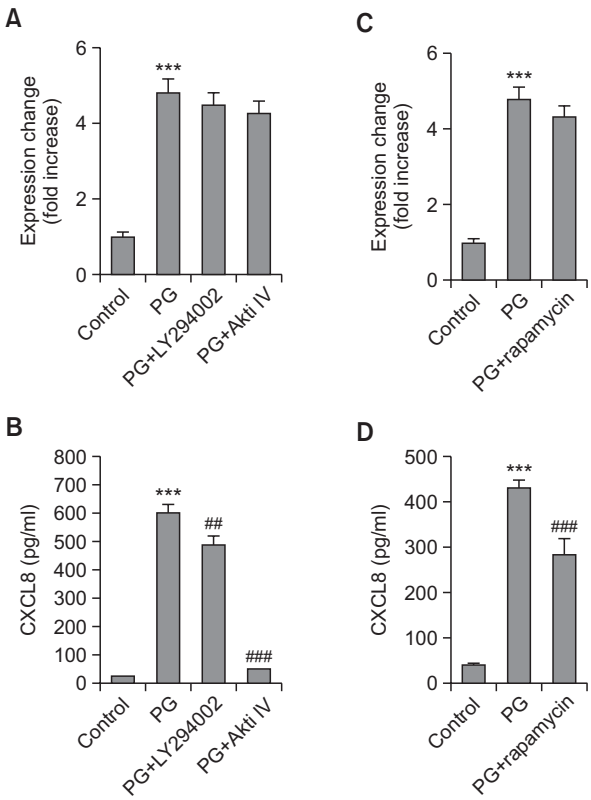

Fig. 3. The effects of PI3K/Akt inhibitors and rapamycin on PG-induced CXCL8 expression. (A) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr in the absence or presence of LY294002 or Akti IV (10 μM each) and stimulated for 6 hr with PG (1 μg/ml). Transcript of *il8* gene was quantified by realtime PCR. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. (B) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr with or without LY294002 or Akti IV (10 μM each) and stimulated for 9 hr with PG. The amount of CXCL8 secreted into the culture medium was measured by ELISA (B). Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control. ##p<0.01 vs. PG. ###p<0.001 vs. PG. (C) THP-1 cells were treated with medium alone (control) or pre-incubated with or without rapamycin (100 nM) for 2 hr and stimulated for 6 hr with PG (1 μg/ml). Transcript of *il8* gene was quantified by realtime PCR. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. (D) THP-1 cells were treated with medium alone (control) or PG for 9 hr in the absence and presence of rapamycin, and the amount of CXCL8 secreted into the culture medium was measured by ELISA. Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control. ###p<0.01 vs. PG.

performed using primers after reverse-transcription of total RNAs, as previously described (Heo *et al*., 2014) PCR was performed using *Taq* PCR Kit (New England Biolabs Ltd., Ipswich, MA). The cDNA was denatured at 90°C for 5 min followed by 25 cycles of PCR (95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec). In the analysis, transcript of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Real-time quantitative PCR was performed in triplicate using the LightCycler 96 Real-Time PCR System (Roche, Germany); each 20-μl reaction consisted of 10 μl of SYBR Green Master Mix, 2 μl of forward and reverse primers (10 pM each) of genes to be analyzed, and cDNA template. Thermal cycling conditions were as follows: 95°C for 10 min, and 45 cycles at 95°C for 10 sec, 50°C for 10 sec, and an elongation period for 10 sec at 72°C. The relative expression of each gene was then calculated as a ratio to GAPDH using LightCycler 96 software (Version 1.1.0.1320). Primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were 5-GAGTCAACGG ATTTGGTCCT-3 (forward) and 5-TGTGGTCATGAGTCCTTCCA-3 (reverse). Primers for IL8 were 5-TCTGCAGCTCTGTGTGAAGG-3 (forward) and 5-AATTTCTGTGTTGGCGCAGT-3 (reverse).

Statistics

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

RESULTS

Up-regulation of CXCL8 expression by PG at the messenger and protein levels

In order to investigate the effects of PG on CXCL8 expression in monocytes/macrophages, the levels of *il8* gene transcripts were examined by realtime-PCR after exposure of THP-1 cells to PG. Gene transcription of IL-8 was hardly detected from THP-1 cells in the absence of PG. PG, however, significantly induced transcription of *il8* gene. The induction of *il8* transcript was observed as early as 6 hr post-treatment and persisted up to 24 hr post-treatment (Fig. 1A). The induction of *il8* gene transcript occurred as low as 0.2 μg/ml of PG (Fig. 1B). Next, it was investigated whether PG also affected CXCL8 release. THP-1 cells secreted very low amount of CXCL8 in the absence of PG. CXCL8 release, however, significantly increased in the presence of PG. The amount of CXCL8 in the medium increased from 10 pg/ml to 420 pg/ml in

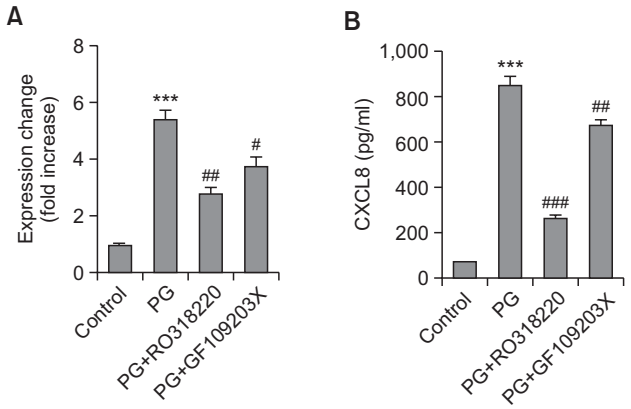

Fig. 4. The effects of PKC inhibitors on PG-induced CXCL8 expression. (A) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr in the absence or presence of RO318220 (3 μM) or GF109203X (1 μM) and stimulated for 6 hr with PG (1 μg/ml). Transcript of *il8* gene was quantified by realtime PCR. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. $^{\#\#}p$<0.01 vs. PG. $^{\#\#\#}p$<0.001 vs. PG. (B) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr with or without RO318220 (3 μM) or GF109203X (1 μM) and stimulated for 9 hr with PG. The amount of CXCL8 secreted into the culture medium was measured by ELISA (B). Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control. $^{\#\#}p$<0.01 vs. PG. $^{\#\#\#}p$<0.001 vs. PG.

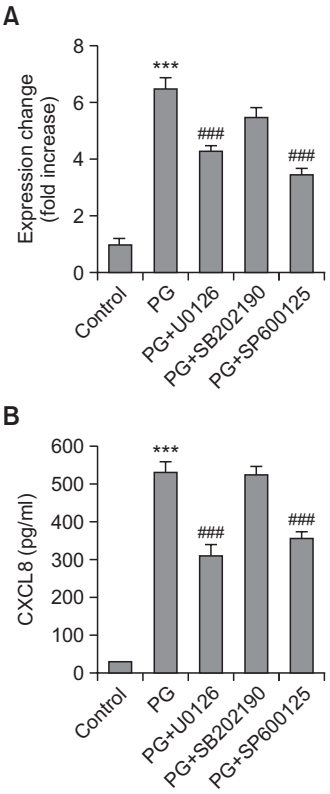

Fig. 5. The effects of MAPK inhibitors on PG-induced CXCL8 expression. (A) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr in the absence or presence of U0126, SB202190 or SP600125 (10 μM each) and stimulated for 6 hr with PG (1 μg/ml). Transcript of *il8* gene was quantified by realtime PCR. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. $^{\#\#\#}p$<0.01 vs. PG. (B) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr with or without U0126, SB202190 or SP600125 and stimulated for 9 hr with PG. The amount of CXCL8 secreted into the culture medium was measured by ELISA (B). Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control. $^{\#\#\#}p$<0.01 vs. PG.

response to PG, as determined by ELISA (Fig. 1C).

The effects of putative lipopolysaccharide (LPS) contaminant on PG-mediated CXCL8 expression

PG preparations can be contaminated with LPS, and LPS increases pro-inflammatory cytokines by monocytic cells (Song and Phelps, 2000) . Therefore, it was examined whether contaminating LPS contributed to PG-induced up-regulation of CXCL8 using polymyxin B, a potent inhibitor of LPS. PG induced *il8* gene transcript, and polymyxin B did not attenuate induction of *il8* gene transcript by PG (Supplementary Fig. 1A). In addition, it was determined whether polymyxin B affected CXCL8 secretion. PG significantly increased CXCL8 secretion. Polymyxin B did not influence release of CXCL8 from THP-1 cells in response to PG (Supplementary Fig. 1B). CXCL8 secretion increased from 10 pg/ml to 371 pg/ml and 352 pg/ml in the presence of PG and PG plus polymyxin B, respectively.

Involvement of TLR2 in PG-induced CXCL8 expression

PG is a ligand for TLR2. Therefore, it was investigated whether PG-induced CXCL8 expression was mediated by TLR2. THP-1 cells were pre-treated with a TLR2/4 inhibitor of OxPAPC, prior to incubation with PG, and the effects of OxPAPC on PG-mediated up-regulation of CXCL8 were investigated. At the transcriptional level, OxPAPC profoundly inhibited expression of *il8* gene transcript induced by PG (Fig. 2A). It was also examined whether OxPAPC affected secretion of CXCL8 by ELISA. OxPAPC almost completely blocked secretion of CXCL8 (Fig. 2B). CXCL8 secreted from THP-1 cells increased from 39 pg/ml to 520 pg/ml in response to PG, and it was reduced to that of control by pre-treatment with OxPAPC.

Roles of phosphoinositide 3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) in PG-induced CXCL8 expression

PG increases levels of phospho-Akt and association of the p85 subunit of PI3K with TLR2 (Ha *et al*., 2010) . Therefore, it was investigated whether Akt played roles in PG-induced CXCL8 expression using pharmacological inhibitors. THP-1 cells were pre-treated with LY294002, which inhibits PI3 kinase, an Akt activator, or Akti IV (an Akt inhibitor) prior to incubation with PG. When the effects of the inhibitors on CXCL8 expression were determined at the messenger, LY294002 and Akti IV did not decrease induction of PG-mediated *il8* gene transcript (Fig. 3A). The inhibitors, however, affected CXCL8 expression at the protein level. PG increased CXCL8 release from 25 pg/ml to 590 pg/ml, and LY294002 reduced the CXCL8 release to 488 pg/ml. Furthermore, Akti IV almost completely blocked PG-mediated CXCL8 release (Fig. 3B).

Since Akt exerts its biological effects through mTOR (Hahn-Windgassen *et al*., 2005), it was investigated whether mTOR was involved in PG-mediated CXCL8 expression using ra-

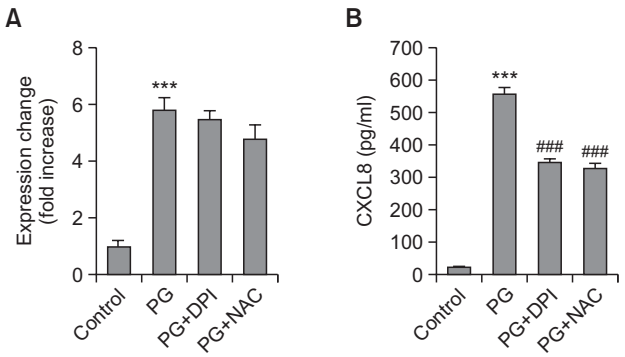

Fig. 6. The effects of ROS quenchers on PG-induced CXCL8 expression. (A) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr in the absence or presence of NAC (5 mM) or DPI (10 μM) and stimulated for 6 hr with PG (1 μg/ml). Transcript of *il8* gene was quantified by realtime PCR. Data are expressed as mean±SD (n=3 replicates for each group). ***p<0.001 vs. control. (B) THP-1 cells were treated with medium alone (control) or pre-incubated for 2 hr with or without NAC or DPI and stimulated for 9 hr with PG. The amount of CXCL8 secreted into the culture medium was measured by ELISA (B). Data are expressed as mean±SD (n=3 replicates/group). ***p<0.001 vs. control. ###p<0.01 vs. PG

pamycin, the inhibitor of mTOR. Rapamycin slightly attenuated induction of *il8* gene transcript by PG (Fig. 3C) and also significantly reduced CXCL8 release from THP-1 cells in response to PG (Fig. 3D). PG increased CXCL8 release from 41 pg/ml to 428 pg/ml, and rapamycin attenuated the CXCL8 release to 284 pg/ml.

Roles of protein kinase C (PKC) in PG-induced CXCL8 expression

PKC is able to activate Akt and mediates activation of transcription element nuclear factor-kappa B (NF-κB) in a TLR2 dependent fashion (Asehnoune *et al*., 2005). Therefore, it was investigated whether PKC is important in PG-mediated CXCL8 expression. To assess involvement of PKC in PG-mediated up-regulation of CXCL8, two PKC inhibitors, GF109203X and RO318220 were used. Both inhibitors inhibited PG-mediated expression of *il8* gene transcript (Fig. 4A). It was examined whether the PKC inhibitors affected release of CXCL8 by ELISA. GF109203X and RO318220 also significantly inhibited PG-mediated CXCL8 release. Of the two chemicals, RO318220 inhibited more remarkably than GF109203X did (Fig. 4B). PG increased CXCL8 release from 70 pg/ml to 840 pg/ml, which was reduced to 260 pg/ml and 668 pg/ml in the presence of RO318220 and GF109203X, respectively.

Roles of mitogen-activated protein kinases (MAPK) in PG-induced CXCL8 expression

Since MAPK are key elements in the TLR signaling (Kawai and Akira, 2006), it was investigated whether MAPK activity is required for PG-mediated CXCL8 expression. To assess roles of MAPK on PG-induced up-regulation of CXCL8, MAPK inhibitors - SP600125 (a JNK inhibitor), U0126 (an ERK inhibitor) and SB202190 (a p38 MAPK inhibitor) - were used. All of the three inhibitors attenuated induction of *il8* gene transcript in response to PG (Fig. 5A). It was examined whether the inhibitors affected release of CXCL8 by ELISA. U0126 and SP600125 significantly inhibited PG-mediated CXCL8 release. SB202190, however, did not affect release of CXCL8 (Fig. 5B). CXCL8 release increased from 26 pg/ml to 528 pg/ml, which was attenuated to 397 pg/ml, 521 pg/ml, and 423 pg/ml in the presence of U0126, SB202190, and SP600125, respectively.

Roles of reactive oxygen species (ROS) in PG-induced CXCL8 expression

Stimulation of TLR2 with *Mycobacterium tuberculosis* 19 kDa lipoprotein, a TLR2/1 agonist, results in production of ROS which contributes to pro-inflammatory response (Shin *et al*., 2008). Thus, it was investigated whether ROS played role in PG-mediated CXCL8 expression. To understand the role of ROS in expression of CXCL8, it was examined whether two ROS quenchers, DPI and NAC, influenced *il8* gene transcription and CXCL8 secretion. NAC appeared to attenuate induction of *il8* gene transcript, as determined by RT-PCR (Fig. 6A). When the effects of the inhibitors on CXCL8 release were determined, both DPI and NAC significantly suppressed PG-mediated CXCL8 secretion (Fig. 6B). PG increased CXCL8 release from 19 pg/ml to 556 pg/ml, which was attenuated to 342 pg/ml and 329 pg/ml in the presence of DPI and NAC, respectively.

DISCUSSION

Expression of cytokine or chemokine in vasculature contributes to vascular diseases. It is believed that cytokine- and chemokine-mediated inflammatory and immune process including immigration and infiltration of leukocytes, macrophages and T cells into and activation of them within the artery leads to progression of atherosclerosis and plaque instability. The specialized roles of cytokines or chemokines in atherosclerosis are well documented (Libby, 2002; Zernecke *et al*., 2008). Owing to active participation of chemokines in atherogenesis, the identification and characterization of the factors regulating chemokine expression are important and will lead us to new insights into pathogenesis and strategies for therapy of atherosclerosis.

The present study focused on expression of CXCL8 in response to PG, which is a bacterial PAMP present in the atherosclerotic lesion, and cellular molecules involved in PG-mediated CXCL8 expression using THP-1 cells. When THP-1 cells, the human monocytes/macrophages, were exposed to PG, CXCL8 transcript was significantly induced and release of CXCL8 was remarkably elevated. Not a few reports, however, indicated that LPS can be contaminated during PG preparation and the LPS might be responsible for production of inflammatory cytokines and chemokines. To make certain that PG-mediated CXCL8 expression was not attained by contaminating LPS, it was investigated if polymyxin B affected PG-mediated CXC8 expression. Polymyxin B binds LPS, thereby prevents biological effects of LPS such as production of cytokine and chemokines by LPS. It was found that polymyxin B did not attenuated PG-mediated CXCL8 expression at the messenger and protein levels. These data indicated that PG, but not contaminating LPS, up-regulated CXCL8 at transcriptional and post-translational levels. In addition, it was investigated whether TLR2 was responsible for the CXCL8 expression using OxPAPC, an inhibitor of TLR2/4. OxPAPC

completely inhibited CXCL8 expression. Taken together, the observation that PG-mediated CXCL8 expression was inhibited by OxPAPC, but not by polymyxin B, indicated that CXCL8 expression was induced by PG via TLR2.

Akt, a serine/threonine phosphoryl transferase and a downstream effector of the PI3K, is an important protagonist in cell fate determination with innumerable regulatory circuits operating in cell migration, survival, differentiation, as well as apoptosis. PI3K modifies the inner leaflet of the plasma membrane to provide attachment sites for signaling proteins and can activate Akt (Vivanco and Sawyers, 2002; Manning and Cantley, 2007). As PG was reported to modify activity of PI3K and Akt, it was investigated whether they were involved in CXCL8 expression. PG-mediated CXCL8 secretion was significantly attenuated by the PI3K inhibitor of LY294002 and completely blocked by the Akt inhibitor of Akti IV. The Akt inhibitor, however, did not block induction of CXCL8 transcript. Therefore, it seemed that Akt was more likely to participate in PG-mediated CXCL8 expression at the translational level. Akt achieves its biological effects through protein targets. One of the downstream targets of Akt includes mTOR. mTOR is a kinase protein predominantly found in the cytoplasm of the cell. It exerts its effects primarily by turning on and off cell's translational machinery, and is responsible for protein synthesis (Corradetti and Guan, 2006; Shaw and Cantley, 2006). The role of mTOR in the CXC8 expression was investigated using rapamycin. Rapamycin appeared to slightly attenuated induction of *il8* gene transcript and significantly inhibited CXCL8 release. The inhibition of CXCL8 release by rapamycin was not as impressive as that by Akti IV. Taken together, the data indicated that Akt played a major role in PG-mediated CXCL8 expression at the translational level.

PKC is a family of enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol or Ca^{2+}. Hence PKC enzymes play important roles in several signal transduction cascades (Parekh *et al*., 2000). It is probable that PKC play a role in CXCL8 expression. PKC plays a role in TLR2 signaling. PKCα/β were involved in TLR2/4 dependent activation of NF-κB (Asehnoune *et al*., 2005). PKC also can mediate gene expression in response to PG. PG activated PKC and induced cyclooxygenase-2 expression in mouse macrophages (Tzeng *et al*., 2010). Involvement of PKC in PG-mediated CXCL8 expression was investigated using two different PKC inhibitors, and both inhibitors attenuated CXCL8 expression at the messenger and the protein levels. These results indicate that certain type of G protein and its signaling molecules were actively involved in PG-mediated CXCL8 expression.

PG increases ROS production in human blood leukocytes (Saetre *et al*., 2000). Therefore, it was investigated whether PG induced CXCL8 expression by way of ROS using two different ROS quenchers, NAC and DPI. NAC is a thiol compound that can act as a cysteine source for the repletion of intracellular glutathione and act as a direct scavenger of ROS. DPI is an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces ROS, thereby inhibits ROS formation. Since NAC and DPI significantly attenuated CXCL8 release, which indicated that ROS was involved in CXCL8 expression at the protein level. ROS can mediate activation of MAPK (Torres, 2003), the serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities (Pearson *et al*., 2001). Therefore, it was investigated whether MAPK were involved in PG-mediated CXCL8 expression. The inhibitors of ERK, p38 MAPK, and JNK attenuated induction of *il8* gene transcript. PG-mediated CXCL8 release was also significantly inhibited in the presence of inhibitors of ERK and JNK. The data indicated that MAPK were involved in PG-mediated CXCL8 expression at the messenger and protein levels.

The present study demonstrated that PG induced CXCL8 expression in THP-1 cells and determined cellular factors which participated in that process. The data in this study suggest that PG, which is present in the human atherosclerotic lesion rich in macrophages, induce secretion of chemokines like CXCL8 and will contribute to progression of atherosclerosis. We propose that further investigation is necessary to confirm roles of individual inhibitors used in the current study in CXCL8 production by using an animal model of atherosclerosis promoted by infection with Gram-positive bacteria.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest.

REFERENCES

Asehnoune, K., Strassheim, D., Mitra, S., Kim, J. Y. and Abraham, E. (2005) Involvement of PKCα/β in TLR4 and TLR2 dependent activation of NF-κB. *Cell. Signal*. **17**, 385-394.

Boisvert, W. A., Santiago, R., Curtiss, L. K. and Terkeltaub, R. A. (1998) A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J. Clin. Invest*. **101**, 353-363.

Corradetti, M. and Guan, K. (2006) Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene* **25**, 6347-6360.

Dobrina, A., Nardon, E., Vecile, E., Cinco, M. and Patriarca, P. (1995) Leptospira icterohemorrhagiae and leptospire peptidolgycans induce endothelial cell adhesiveness for polymorphonuclear leukocytes. *Infect. Immun.* **63**, 2995-2999.

Ha, T., Hu, Y., Liu, L., Lu, C., McMullen, J. R., Kelley, J., Kao, R. L., Williams, D. L., Gao, X. and Li, C. (2010) TLR2 ligands induce cardioprotection against ischaemia/reperfusion injury through a PI3K/Akt-dependent mechanism. *Cardiovasc. Res*. **87**, 694-703.

Hahn-Windgassen, A., Nogueira, V., Chen, C. C., Skeen, J. E., Sonenberg, N. and Hay, N. (2005) Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J. Biol. Chem.* **280**, 32081-32089.

Heo, W., Kim, S., Eo, S., Rhim, B. and Kim, K. (2014) FSL-1, a toll-like receptor 2/6 agonist, induces expression of interleukin-1α in the presence of 27-hydroxycholesterol. *Korea J. Physiol. Pharmacol.* **18**, 475-480.

Huo, Y., Weber, C., Forlow, S. B., Sperandio, M., Thatte, J., Mack, M., Jung, S., Littman, D. R. and Ley, K. (2001) The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J. Clin. Invest.* **108**, 1307-1314.

Inoue, T., Komoda, H., Nonaka, M., Kameda, M., Uchida, T. and Node, K. (2008) Interleukin-8 as an independent predictor of long-term clinical outcome in patients with coronary artery disease. *Int. J. Cardiol.* **124**, 319-325.

Kawai, T. and Akira, S. (2006) TLR signaling. *Cell Death Differ.* **13**, 816-825.

Laman, J. D., Schoneveld, A. H., Moll, F. L., van Meurs, M. and Pasterkamp, G. (2002) Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. *Am. J. Cardiol.* **90**, 119-123.

Langer, M., Malykhin, A., Maeda, K., Chakrabarty, K., Williamson, K. S., Feasley, C. L., West, C. M., Metcalf, J. P. and Coggeshall, K. M. (2008) *Bacillus anthracis* peptidoglycan stimulates an inflammatory response in monocytes through the p38 mitogen-activated protein kinase pathway. *PLoS One*. **3**, e3706.

Libby, P. (2002) Inflammation in atherosclerosis. *Nature* **420**, 868-874.

Manning, B. D. and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274.

Moreau, M., Brocheriou, I., Petit, L., Ninio, E., Chapman, M. J. and Rouis, M. (1999) Interleukin-8 mediates downregulation of tissue inhibitor of metalloproteinase-1 expression in cholesterol-loaded human macrophages: relevance to stability of atherosclerotic plaque. *Circulation* **99**, 420-426.

Nijhuis, M. M., Pasterkamp, G., Sluis, N. I., de Kleijn, D. P., Laman, J. D. and Ulfman, L. H. (2007) Peptidoglycan increases firm adhesion of monocytes under flow conditions and primes monocyte chemotaxis. *J. Vasc. Res.* **44**, 214-222.

Parekh, D. B., Ziegler, W. and Parker, P. J. (2000) Multiple pathways control protein kinase C phosphorylation. *EMBO J.* **19**, 496-503.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K. and Cobb, M. H. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22**, 153-183.

Peveri, P., Walz, A., Dewald, B. and Baggiolini, M. (1988) A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J. Exp. Med.* **167**, 1547-1559.

Saetre, T., Kähler, H., Foster, S. and Lyberg, T. (2000) Peptidoglycan and lipoteichoic acid, components of the streptococcal cell wall, have marked and differential effects on adhesion molecule expression and the production of reactive oxygen species in human whole blood leukocytes. *Scand. J. Clin. Lab. Invest.* **60**, 311-322.

Schrijver, I. A., Melief, M. J., Eulderink, F., Hazenberg, M. P. and Laman, J. D. (1999) Bacterial peptidoglycan polysaccharides in sterile human spleen induce proinflammatory cytokine production by human blood cells. *J. Infect. Dis.* **179**, 1459-1468.

Schroder, J. M. and Christophers, E. (1989) Secretion of novel and homologous neutrophil-activating peptides by LPS-stimulated human endothelial cells. *J. Immunol*. **142**, 244-251.

Shaw, R. J. and Cantley, L. C. (2006) Ras, PI (3) K and mTOR signalling controls tumour cell growth. *Nature* **441**, 424-430.

Shin, D., Yang, C., Lee, J., Lee, S. J., Choi, H., Lee, H., Yuk, J., Harding, C. V. and Jo, E. (2008) Mycobacterium tuberculosis lipoprotein-induced association of TLR2 with protein kinase Cζ in lipid rafts contributes to reactive oxygen species-dependent inflammatory signalling in macrophages. *Cell. Microbiol.* **10**, 1893-1905.

Song, M. and Phelps, D. S. (2000) Comparison of SP-A and LPS effects on the THP-1 monocytic cell line. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279**, L110-117.

Torres, M. (2003) Mitogen-activated protein kinase pathways in redox signaling. *Front. Biosci.* **8**, d369-391.

Tzeng, J., Chen, B., Chang, H., Wang, J., Sureshbabu, M., Chien, M., Hsu, M., Bien, M., Chiu, W. and Hong, C. (2010) Involvement of phosphatidylcholine-phospholipase C and protein kinase C in peptidoglycan-induced nuclear factor-κB activation and cyclooxygenase-2 expression in RAW 264.7 macrophages. *Pharmacol. Res.* **61**, 162-166.

Vivanco, I. and Sawyers, C. L. (2002) The phosphatidylinositol 3-kinase-AKT pathway in human cancer. *Nat. Rev. Cancer* **2**, 489-501.

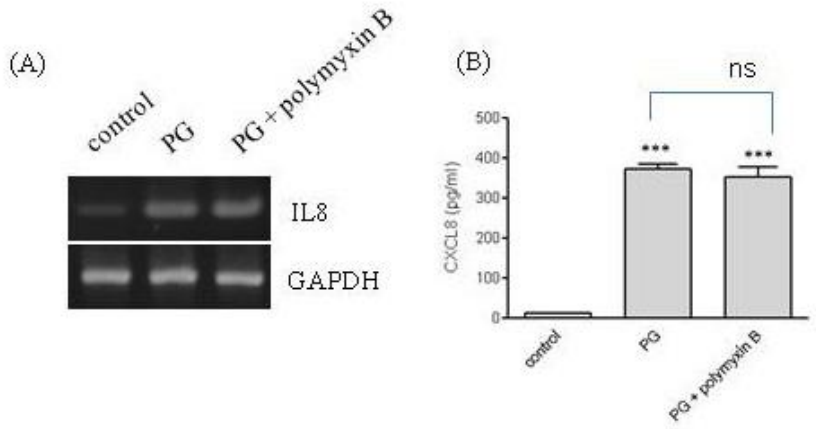
Wang, Z. M., Liu, C. and Dziarski, R. (2000) Chemokines are the main proinflammatory mediators in human monocytes activated by Staphylococcus aureus, peptidoglycan, and endotoxin. *J. Biol. Chem.* **275**, 20260-20267.

Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R. and Golenbock, D. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol.* **163**, 1-5.

Yue, T. L., Wang, X., Sung, C. P., Olson, B., McKenna, P. J., Gu, J. L. and Feuerstein, G. Z. (1994) Interleukin-8. A mitogen and chemoattractant for vascular smooth muscle cells. *Circ. Res.* **75**, 1-7.

Zernecke, A., Shagdarsuren, E. and Weber, C. (2008) Chemokines in atherosclerosis: an update. *Arterioscler. Thromb. Vasc. Biol.* **28**, 1897-1908.

Supplementary figure



Supplementary figure 1. The effects of polymyxin B on PG-induced CXCL8 expression.

(A) THP-1 cells were treated with medium alone (control) or PG (1 μg/ml) for 6 hr in the absence and presence of polymyxin B (10 μg/ml). Transcript of IL8 gene was amplified by RT-PCR.

(B) THP-1 cells were treated with medium alone (control) or with PG for 9 hr in the absence and presence of polymyxin B, and the amount of CXCL8 secreted into the culture medium was measured by ELISA (B). Data are expressed as mean±SD (n=3 replicates/group). *** $P<0.01$ vs. control. ns: not significant.