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 αvβ3-integrin expression on monocytes and increase α2β1-integrin-dependent migration of monocytes (Ni jhuis 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...
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 are reported to 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 monocoyte 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 humified atmosphere of 5% CO2. 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<0.01 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<0.01 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. (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.

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
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 monocyctic 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 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<0.01 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<0.01 vs. control. ***p<0.001 vs. PG.

**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<0.01 vs. control. ***p<0.001 vs. PG.

**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-
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 CXCL8 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 attenuate 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 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 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 names 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.

**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. Data are expressed as mean±SD (n=3 replicates/group). **p<0.01 vs. control.

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 (Asehnouve 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.

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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 CXCL8 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 Akt 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++. 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 (Asehanoune 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 and DPI, PKC activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. The authors do not have any conflicts of interest.

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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.