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Involvement of Multiple Signaling Molecules in Peptidoglycan-induced Expression of Interleukin-1α in THP-1 Monocytes/Macrophages

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The expression of interleukin-1α (IL-1α) is elevated in monocytic cells, such as monocytes and macrophages, within atherosclerotic arteries, yet the cellular molecules involved in cytokine upregulation remain unclear. Because peptidoglycan (PG), a major component of gram-positive bacterial cell walls, is detected within the inflammatory cell-rich regions of atheromatous plaques, it was investigated if PG contributes to IL-1α expression in monocytes/macrophages. Exposure of THP-1 monocytic cells to PG resulted in elevated levels of *IL-1α* gene transcripts and increased secretion of IL-1α protein. The transcription and secretion of IL-1α were abrogated by OxPAPC, an inhibitor of TLR2/4, but not by polymyxin B that inhibits lipopolysaccharide-induced TLR4 activation. To understand the molecular mechanisms of the inflammatory responses due to bacterial pathogen-associated molecular patterns (PAMPs) in diseased arteries, we attempted to determine the cellular factors involved in the PG-induced upregulation of IL-1α expression. Pharmacological inhibition of cell signaling pathways with LY294002 (a PI3K inhibitor), Akti IV (an inhibitor of Akt activation), rapamycin (an mTOR inhibitor), U0126 (a MEK inhibitor), SB202190 (a p38 MAPK inhibitor), SP6001250 (a JNK inhibitor), and DPI (a NOX inhibitor) also significantly attenuated the PG-mediated expression of IL-1α. These results suggest that PG induces the monocytic or macrophagic expression of IL-1α, thereby contributing to vascular inflammation, via multiple signaling molecules, including TLR2, PI3K/Akt/mTOR, and MAPKs.

Key words : Interleukin-1α, monocytes/macrophages, peptidoglycan, signaling pathways

Introduction

Peptidoglycan (PG), a major cell-wall component of gram-positive bacteria, is abundantly present in the flora of the normal human gut and other mucosa, where it functions as a signaling molecule [9]. PG is recognized by the innate immune system as a bacterial pathogen-associated molecular pattern (PAMP) and promotes inflammation via toll-like receptors (TLRs) [26, 29]. PG induces $\alpha_m\beta_2$-integrin expression, thereby increasing β_2-integrin-dependent monocyte migration [19]. PG upregulates the expression of cell adhesion molecules in endothelial cells [7] and enhances the production of proinflammatory cytokines and chemokines by monocytes/macrophages [12, 13]. As a higher proportion of PG is observed within human atherosclerotic lesions, mainly in the macrophage-rich atheromatous regions [12], it is presumed to be an additional pro-inflammatory factor in the lesion. Therefore, the elucidation of whether and how PG induces the expression of inflammatory cytokines will enhance the understanding of the role of bacterial PAMPs in atherogenesis.

Owing to their strong proinflammatory action, IL-1 cytokines are considered instrumental in the propagation of inflammation in vascular walls during atherosclerosis [3]. Animal and clinical studies have shown that the expression of IL-1α or IL-1R1 receptor is linked to atherosclerosis. Cells present in human atherosclerotic lesions, macrophages, smooth muscle cells, and endothelial cells, produce IL-1α [18, 25]. Blocking IL-1R signaling in IL-1R1$^{-/-}$ mice reduces the progression of atherosclerosis under experimental conditions known to favor the aggravation of vascular lesions, even with multiple active predisposing factors, including genetics, diet, and infection exposure (i.e., ApoE$^{-/-}$ mice fed with high-fat diet and *P. gingivalis* injection) [5]. Therefore, understanding the regulation of IL-1α expression is important owing to its

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close association with the vascular disease.

In the present study, we investigated the effects of PG on monocyte/macrophage expression using a human acute monocytic cell line and found that PG significantly induced IL-1α expression at transcript and protein levels. We also sought to identify cellular molecules that play roles in PG-induced expression of IL-1α by using pharmacological inhibitors that inhibit signaling pathways.

Materials and Methods

Cell culture and reagents

The THP-1 cell line was purchased from and maintained as suggested by the American Type Culture Collection (ATCC; Manassas, VA, USA). PG was isolated from *Staphylococcus aureus*, polymyxin B, and oxidized 1-palmitoyl-2-arachidonosyl-sn-phosphatidylcholine (OxPAPC) was purchased from InvivoGen (San Diego, CA, USA). Endotoxin-free bovine serum albumin (BSA), LY294002, diphenyleneiodonium chloride (DPI), N-acetylcysteine (NAC), rapamycin, and SP600 125 were purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126, SB202190, and Akt inhibitor IV (Akti IV) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell treatment

For inhibition experiments, THP-1 cells were treated for 1 hr with the indicated chemicals before stimulation for 9 hr with PG (1 μg/ml). *IL-1α* transcript levels were amplified by reverse transcription (RT)-polymerase chain reaction (PCR) and quantitative real-time PCR. The amount of IL-1α released into the medium was measured by enzyme-linked immunosorbent assay (ELISA).

RT-PCR

THP-1 cells were incubated with 0.1% BSA in RPMI 1640 medium overnight and then exposed to PG. Total RNA was extracted from cells and reverse-transcribed for an hour at 42℃ with Moloney murine leukemia virus reverse transcriptase. The PCR amplification was performed for 25 cycles (94 ℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s) in the presence of primers. The primers for IL-1α were 5′-AATGACGCCCT CAATCAAAG-3′ (forward) and 5′-TGGGTATCTCAGGCA TCTCC-3′ (reverse), and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-AAGCTCTGCGTG ACTGTCCT-3′ (forward) and 5′-GCTTGCTTCTTTTGGTT TGG-3′ (reverse). The PCR products were visualized using 2% agarose gel electrophoresis with ethidium bromide staining.

Real-time PCR

Quantitative real-time PCR was performed in triplicate using the LightCycler® 96 Real-Time PCR System (Roche Life Science, Germany) following a previously reported protocol [14]; each 20 μl reaction consisted of 4 μl of cDNA template, 10 μl of SYBR Green Master Mix, and 2 μl of 10 pM forward and reverse primers of IL-1α. The thermal cycling conditions were as follows: 95℃ for 10 min, followed by 45 cycles at 95℃ for 10 s, 50℃ for 10 s, and 72℃ elongation period for 10 s. The relative expression of each gene was calculated as the ratio to the housekeeping gene, GAPDH, using the LightCycler® 96 software (Version 1.1.0.1320, Roche, Germany). IL-1α mRNA levels were normalized to those of GAPDH. The real-time PCR primers used for IL-1α were 5′-GAATGACGCCCTCAATCAAAGT-3′ (forward) and 5′-TCATCTTGGGCAGTCACATACA-3′ (reverse); GAPDH, 5′-GAAGGTGAAGGTCGGAGT-3′ (forward) and 5′-GAAGAT GGTGATGGGATTTC-3′ (reverse).

ELISA

The amount of secreted IL-1α was determined using a commercially available ELISA kit as per the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). THP-1 cells were incubated with 0.1% BSA in RPMI 1640 medium overnight, and cell culture media were collected after exposure to PG. Culture media and standards for IL-1α were added to a microtiter plate pre-coated with a monoclonal antibody against IL-1α. After incubation for 2 hr, wells were washed and incubated with an enzyme-conjugated polyclonal antibody specific for IL-1α. The substrate was added after several washes, and the color intensity was measured. The amount of IL-1α in the medium was determined using a standard curve.

Statistics

Data were statistically analyzed by one-way ANOVA followed by Dunnett multiple comparison tests using PRISM (version 5.0; GraphPad Software Inc., San Diego, CA, USA), and $p<0.05$ was considered statistically significant.

Results

Upregulation of IL-1α expression at transcript and protein levels

We determined concentration and time-course effects of

PG on transcription and secretion of IL-1α. Levels of *IL-1α* transcripts were examined by RT-PCR following the treatment of THP-1 monocytic cells with PG. *IL-1α* mRNA was barely detectable in THP-1 cells in the absence of PG. Transcription of IL-1α was induced in the presence of 100 ng/ml PG, and the induction was more evident at 1,000 ng/ml PG (Fig. 1A). An increased transcription was observed as early as 3 hr post-treatment, which was enhanced and persisted for up to 9 hr post-treatment with PG (Fig. 1B). Effects of PG on IL-1α release were investigated. THP-1 cells constitutively secreted a small quantity of IL-1α, and the secretion increased by 2.9 and 3.8 folds in the presence of 100 and 1,000 ng/ml PG, respectively, as compared to the control (Fig. 1C). PG started to promote secretion of this cytokine 3 hr post-treatment, which was further enhanced in proportion to the treatment duration with PG (Fig. 1D). These results indicated that PG increased transcription and protein expression of IL-1α.

Effects of TLR2/4 inhibition on PG-induced expression of IL-1α

PG preparations can be contaminated with lipopolysaccharide (LPS) which increases the secretion of pro-inflammatory cytokines and chemokines, including IL-1α. Therefore, we examined whether LPS, if any, contributed to PG-mediated upregulation of IL-1α using polymyxin B, a potent inhibitor of LPS. Polymyxin B did not reduce the transcript levels and secretion of IL-1α induced PG (Fig. 2A, Fig. 2B). An involvement of TLR2 was examined using OxPAPC, a TLR2/4 inhibitor. OxPAPC almost completely blocked the PG-induced elevation of *IL-1α* transcripts and profoundly attenuated the secretion of IL-1α protein (Fig. 2A, Fig. 2B).

Roles of Akt and mTOR (mammalian target of rapamycin) in IL-1α expression

PG activates the Akt pathway by enhancing phosphorylation [14]. To investigate whether Akt participated in PG-induced expression, two inhibitors, LY294002 and Akti IV, were employed (Fig. 3A, Fig. 3B). LY294002 is a reversible inhibitor of phosphoinositide 3-kinase (PI3K), an Akt activator, whereas Akti IV inhibits Akt activation. Both LY294002 and Akti IV remarkably attenuated PG-induced gene transcription of IL-1α. They also blocked protein secretion. The levels of secreted IL-1α were reduced to that of the control in the presence of Akti IV or LY294002.

Akt exerts its biological effects by activating mTOR, a serine/threonine kinase [8]. Therefore, we investigated whether mTOR participated in PG-induced expression using an inhibitor of mTOR, rapamycin (Fig. 4A, Fig. 4B). Rapamycin affected the IL-1α expression at transcript and protein levels. PG induced transcription and secretion of IL-1α, but treatment with rapamycin resulted in a significant reduction in *IL-1α* transcript levels, which was accompanied by the attenu-

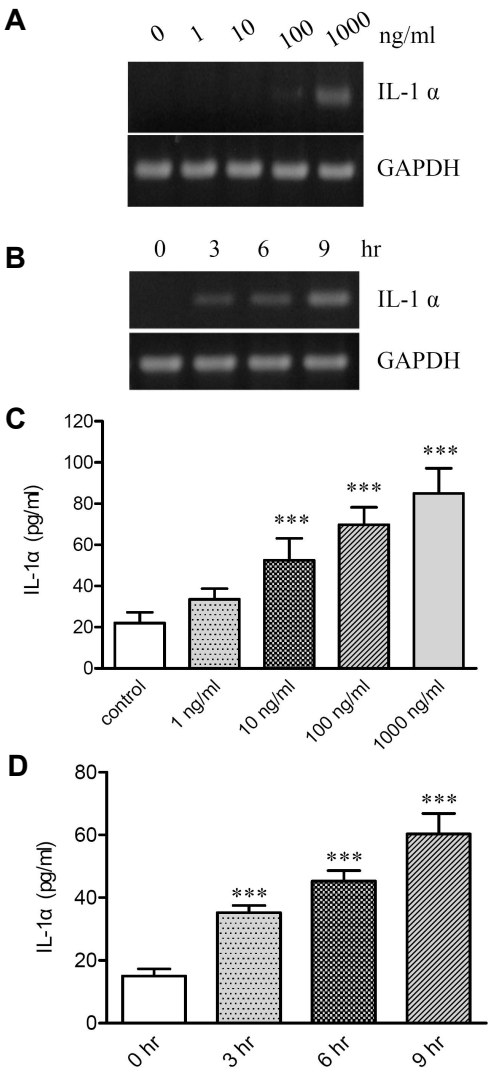

Fig. 1. Expression of IL-1α in the presence of PG. THP-1 cells (1×10^6 cells/ml) were treated for 9 hr with the indicated amount of PG (A) or incubated for the indicated time periods with 1 μg/ml PG (B), after which *IL-1α* transcripts were amplified by RT-PCR. Cells (1×10^6 cells/ml) were stimulated for 9 hr with or without (control) the indicated amount of PG (C) or incubated for the indicated time periods with 1 μg/ml PG (D). The levels of IL-1α protein released into the culture media were measured by ELISA. Data are expressed as the mean ± SD (n=3 replicates/group). $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control.

ated secretion of the corresponding protein.

Involvement of MAPKs in IL-1α expression induced by PG

PG enhanced phosphorylation of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK), suggesting activation of the kinases [14]. To assess the role these kinases in PG-induced IL-1α upregulation, the following inhibitors were employed: U0126 (an inhibitor of ERK), SB202190 (an inhibitor of p38 MAPK), and SP600125 (an inhibitor of JNK). All three inhibitors affected IL-1α expression at mRNA and protein levels (Fig. 5A, Fig. 5B). They not only attenuated PG-induced gene transcription but also significantly inhibited secretion of IL-1α.

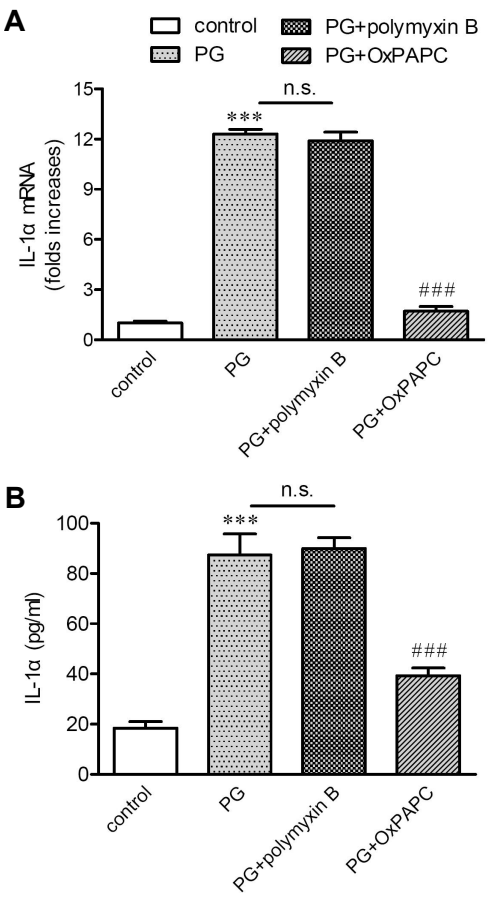

Fig. 2. Distinct effects of polymyxin B and OxPAPC on IL-1α expression induced by of PG. THP-1 monocytic cells were stimulated for 9 hr with or without PG (1 μg/ml) after treatment for 1 hr with polymyxin B (10 mg/ml) or OxPAPC (30 μg/ml). The *IL-1α* transcripts were quantified by real-time PCR (A), and IL-1α protein released into the medium was determined (B). Data are expressed as the mean ± SD (n=3 replicates/group). ***p<0.001 vs. control. ### p<0.001 vs. PG. n.s.: not significant.

Roles of ROS in PG-induced expression of IL-1α

We also investigated whether ROS played a role using NAC and DPI (Fig. 6A, Fig. 6B). DPI, an inhibitor of NADPH oxidase, significantly inhibited PG-induced transcription and attenuated secretion of IL-1α. However, NAC, a direct scavenger of ROS, did not affect expression of IL-1α induced by PG.

Discussion

IL-1α is translated as a 31 kDa precursor, and the release of mature IL-1α requires extracellular calpain-mediated cleavage of the precursor [28]. Both precursor and mature forms of IL-1α are biologically active. Upon binding of IL-1α to IL-1 receptor type 1 (IL-1R1), the IL-1R accessory protein

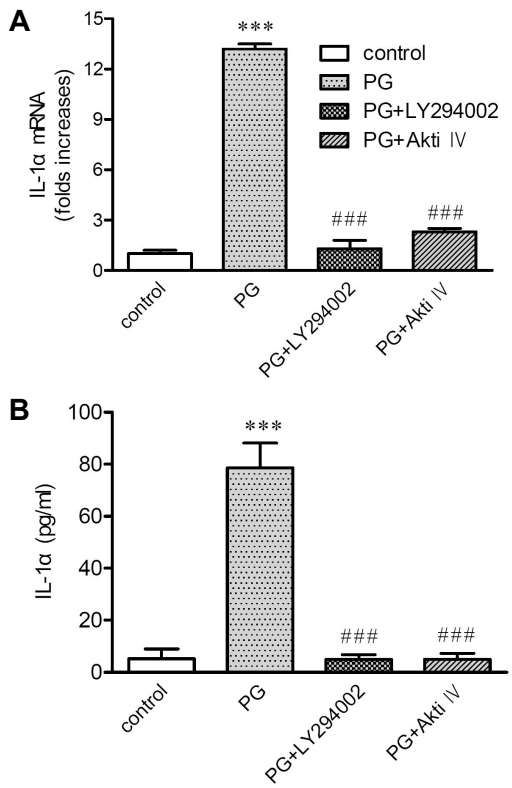

Fig. 3. Inhibitory effects of LY294002 and Akti IV on IL-1α upregulation. Following treatment with LY294002 and Akti IV (10 μM each), monocytic cells were stimulated for 9 hr with or without PG. The *IL-1α* transcripts were quantified by real-time PCR (A), and the amount of IL-1α released into the medium was assessed by ELISA (B). Data are expressed as the mean ± SD (n=3 replicates/group). ***p<0.001 vs. control. ###p<0.001 vs. PG.

is recruited by the receptor complex, and intracellular signaling transduction is triggered through a p38 mitogen-activated protein kinase (MAPK)-activated phosphorylation cascade [16, 28]. The signaling cascade culminates in the activation of the transcription factor nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1), and the ensuing expression of proinflammatory genes [2].

The present study demonstrated that PG, a bacterial component that is present in atherosclerotic lesions, upregulated IL-1α expression both at the mRNA and protein levels in the human macrophage THP-1 cell line. This finding is consistent with the results of a previous study by Wang et al. [27], who reported the induction of IL-1α and IL-1β expression by PG and LPS in human blood monocytes by using a ribonuclease protection assay. PG induced *IL-1α* gene transcripts, which was not detected in the absence of PG, enhancing the IL-1α secretion. PG-mediated IL-1α secretion, although significant, was not impressive when the extent of IL-1α transcription induction was considered. It is assumed that if THP-1 cells are exposed to a second stimulus in addition to PG, they may secrete more active IL-1α [6].

The LPS contamination during PG preparation contributes to or maybe responsible to produce cytokines through TLR4 activation. To investigate whether LPS contributed to PG-mediated IL-1α expression, we used polymyxin B, which prevents activities of LPS by binding to it [4]. It was found that polymyxin B did not affect PG-mediated IL-1α expression, indicating that the upregulation of IL-1α observed in this study was induced by PG.

Although PG induces IL-1α expression, the cellular molecules involved have not yet been identified. The present study

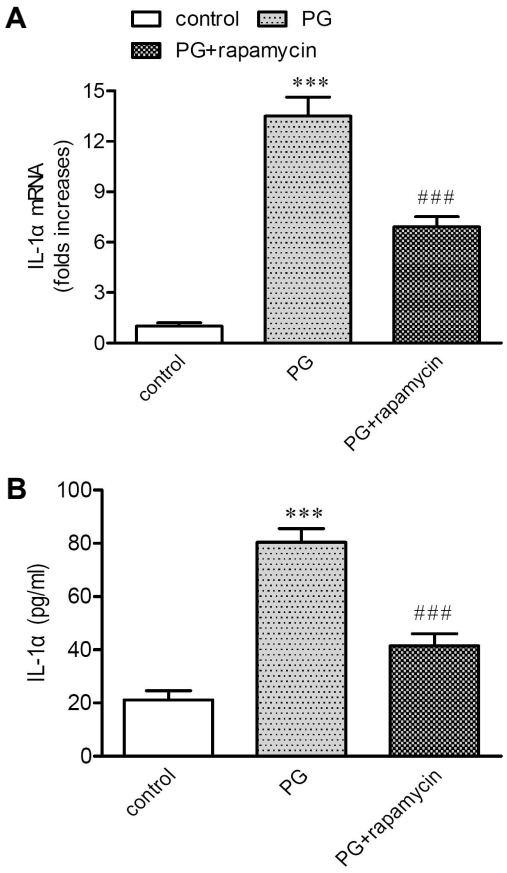

Fig. 4. Inhibitory activity of rapamycin on IL-1α expression. Monocytic cells were stimulated for 9 hr with or without PG after pre-treatment with rapamycin (100 nM). Levels of *IL-1α* gene transcripts were assessed by real-time PCR (A), and the amount of released IL-1α protein was measured (B). Data are expressed as the mean ± SD (n=3 replicates/group). ***p<0.001 vs. control. ###p<0.001 vs. PG.

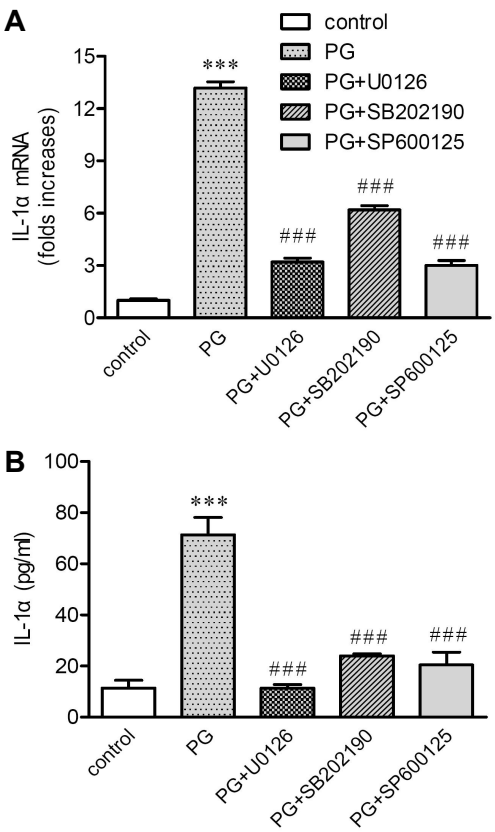

Fig. 5. Suppressive effects of inhibitors of MAPKs on the upregulation of IL-1α. After treatment for 1 hr with the indicated MAPK inhibitors (10 μM each), monocytic cells were stimulated for 9 hr with or without PG. Levels of *IL-1α* transcripts were quantified by real-time PCR (A), and the amount of IL-1α released into the medium was measured by ELISA (B). Data are expressed as the mean ± SD (n=3 replicates/group). ***p< 0.001 vs. control. ###p<0.001 vs. PG.

aimed to identify the cellular factors that play a role in PG-mediated IL-1α expression. Because PG is a bacterial PAMP recognized by TLR2 that triggers the activation of signaling pathways leading to inflammatory responses [1, 11, 26], we investigated receptor-mediated IL-1α expression using OxPAPC, an inhibitor of TLR2/4. OxPAPC completely blocked cytokine secretion and transcription. The complete inhibition by OxPAPC, but not by polymyxin B, indicated that TLR2 is responsible for PG-induced IL-1α expression.

PG enhances Akt phosphorylation [14], indicating that PG activates Akt kinase. Therefore, we investigated whether Akt and PI3K [17] are involved in PG-mediated IL-1α expression. Akt inhibition completely blocked the secretion of IL-1α and inhibited the transcription of its gene. PI3K inhibition also resulted in blockage of IL-1α expression. These results indicated that both PI3K and Akt activities are required for PG-mediated IL-1α expression. Akt exerts its effects through protein targets, including mTOR, a kinase protein that is predominantly found in the cytoplasm [15]. We investigated whether mTOR was involved. Rapamycin inhibition of mTOR resulted in a significant reduction in transcription as well as secretion of IL-1α. These results indicate that rapamycin inhibits PG-mediated IL-1α expression at both the mRNA and protein levels. This confirms that mTOR is responsible for protein synthesis, as it exerts its effects by turning the translational machinery of the cell on and off [8, 21]. These results indicate that the PI3K-dependent Akt/mTOR pathway plays a crucial role in PG-mediated IL-1α upregulation.

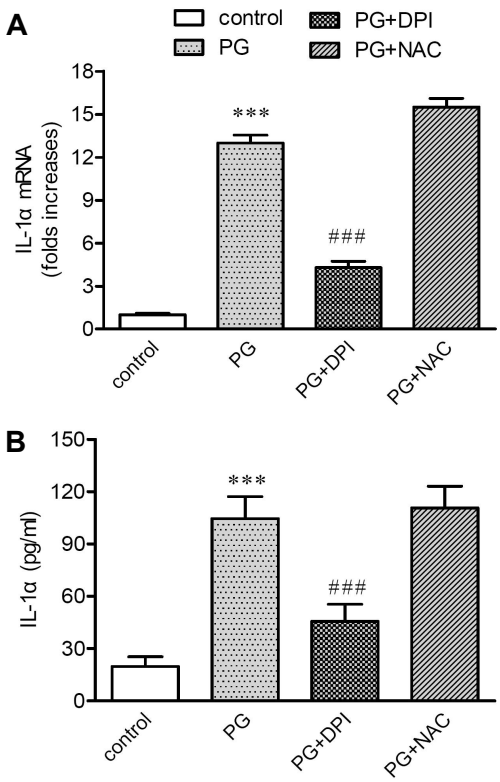

Fig. 6. Effects of ROS quenchers on IL-1α expression. Monocytic cells were pretreated with DPI (10 μM) or NAC (5 mM) prior to stimulation for 9 hr with or without PG. Levels of *IL-1α* transcripts were assessed by quantitative PCR (A), and the amount of released IL-1α protein was determined (B). Data are expressed as the mean ± SD (n=3 replicates/group). ***p<0.001 vs. control. ###p<0.001 vs. PG.

MAPKs are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities, are activated by PG [14], and MAPKs mediate cytokine and chemokine production in response to TLR-2, -4, and -9 activation [24]. These reports indicate that kinases are involved in IL-1α expression. Therefore, we investigated the role of MAPKs in cytokine production. Selective inhibition of ERK, p38 MAPK, or JNK resulted in a significant attenuation of IL-1α expression, indicating the active participation of these kinases in IL-1α expression. These results indicate that the activation of ERK, p38 MAPK, and JNK is required for PG-mediated IL-1α expression.

PG increases ROS production in human blood leukocytes [20], and TLR2 is involved in ROS production [10]. Thus, ROS is one of the signaling molecules below TLR2. Since PG induced IL-1α expression in a TLR2 dependent manner, the present study used DPI and NAC to investigate whether ROS were involved in IL-1α expression. DPI is an inhibitor of NADPH oxidase that produces ROS and inhibits ROS formation [22]. NAC, a thiol compound that acts as a cysteine source for the repletion of intracellular glutathione, is a direct scavenger of ROS [23]. DPI significantly attenuated PG-mediated upregulation of IL-1α. These results indicate that ROS originating from NADPH oxidase actively participates in PG-mediated IL-1α upregulation.

Exposure of monocytes/macrophages to PG would result in increased transcription and secretion of IL-1α, and results of this study suggest that multiple pathways including TLR2, Akt, mTOR, ERK, JNK and NADPH oxidase, are involved in the IL-1α expression, and through which PG will contribute to vascular inflammation (Fig. 7). We think roles of these molecules are specific because cell viability was unaffected by their individual inhibitors (Fig. 8). This study, however, did not determine whether the molecules act independently or in a cooperative manner to upregulate IL-1α expression. Further investigation is necessary to elucidate the types of connections or crosstalk that may occur in the context of a

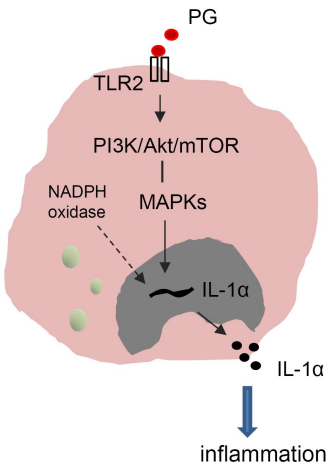

Fig. 7. Putative roles of signaling molecules involved in PG-induced expression of IL-1α. Upon binding of PG to its receptor, TLR2, on surface, monocytes/macrophages are activated and cell signaling is transmitted via PI3K-Akt-mTOR and MAPKs to induce transcription of the *IL-1α* gene. The transcription results in increased secretion of the IL-1α which promotes inflammation.

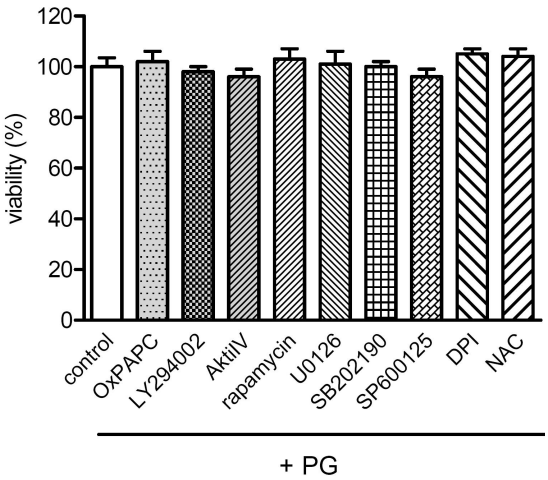

Fig. 8. Effects of inhibitors on cell viability. THP-1 cells were treated for 9 hr with indicated inhibitors at the concentrations employed in this study in the presence of PG. Cell viability was determined by using a Vi-Cell XR cell counter. Viability of cells cultured in medium with PG alone was considered 100%. Data are expressed as the mean ± SD (n=3 replicates for each group).

possible signaling cascade.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : THP-1 단핵구의 펩티도글리칸 유래 인터루킨-1 알파 발현에서 TLR2, PI3K/Akt/mTOR, MAPKs의 역할

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본 연구에서는 죽상경화 플락에서 발견되는 펩티도글리칸이 혈관염증에서 어떠한 역할을 하는지 알아보기 위하여 염증성 사이토카인의 한 종류인 인터루킨-1 알파의 발현에 대한 영향을 조사하였다. 실험방법으로는 혈관염증을 주도하는 단핵구/대식세포인 THP-1 세포주에 펩티도글리칸을 처리하고 인터루킨-1 알파의 발현을 RT-PCR, real-time PCR, ELISA 방법으로 분석하였다. 펩티도글리칸의 처리 시간과 농도에 비례하여 단핵구/대식세포에서 인터루킨-1 알파의 전사체와 단백질 분비가 증가함을 관찰하였다. 또한 펩티도글리칸의 작용기전을 규명하기 위하여 신호전달을 차단하는 억제제를 세포에 처리하고 인터루킨-1 알파의 발현을 조사하였다. TLR2/4의 억제제인 OxPAPC 그리고 세포 kinase의 작용을 억제하는 LY294002 (PI3 kinase 억제), Akti IV (Akt 억제), rapamycin (mTOR 억제), U0126 (MEK 억제), SB202190 (p38 MAPK 억제), SP6001250 (JNK 억제), DPI (NOX 억제)를 처리하는 경우 인터루킨-1 알파 전사체의 발현 그리고 단백질의 분비가 감소되었다. 반면에 LPS의 작용을 억제하는 polymyxin B는 인터루킨-1 알파의 발현에 영향을 주지 않았다. 이상의 결과는, 펩티도글리칸이 TLR2, PI3K, Akt, mTOR, MAPKs를 통하여 단핵구/대식세포의 인터루킨-1 알파 발현을 증가시키고 혈관염증에 기여한다는 것을 나타낸다.