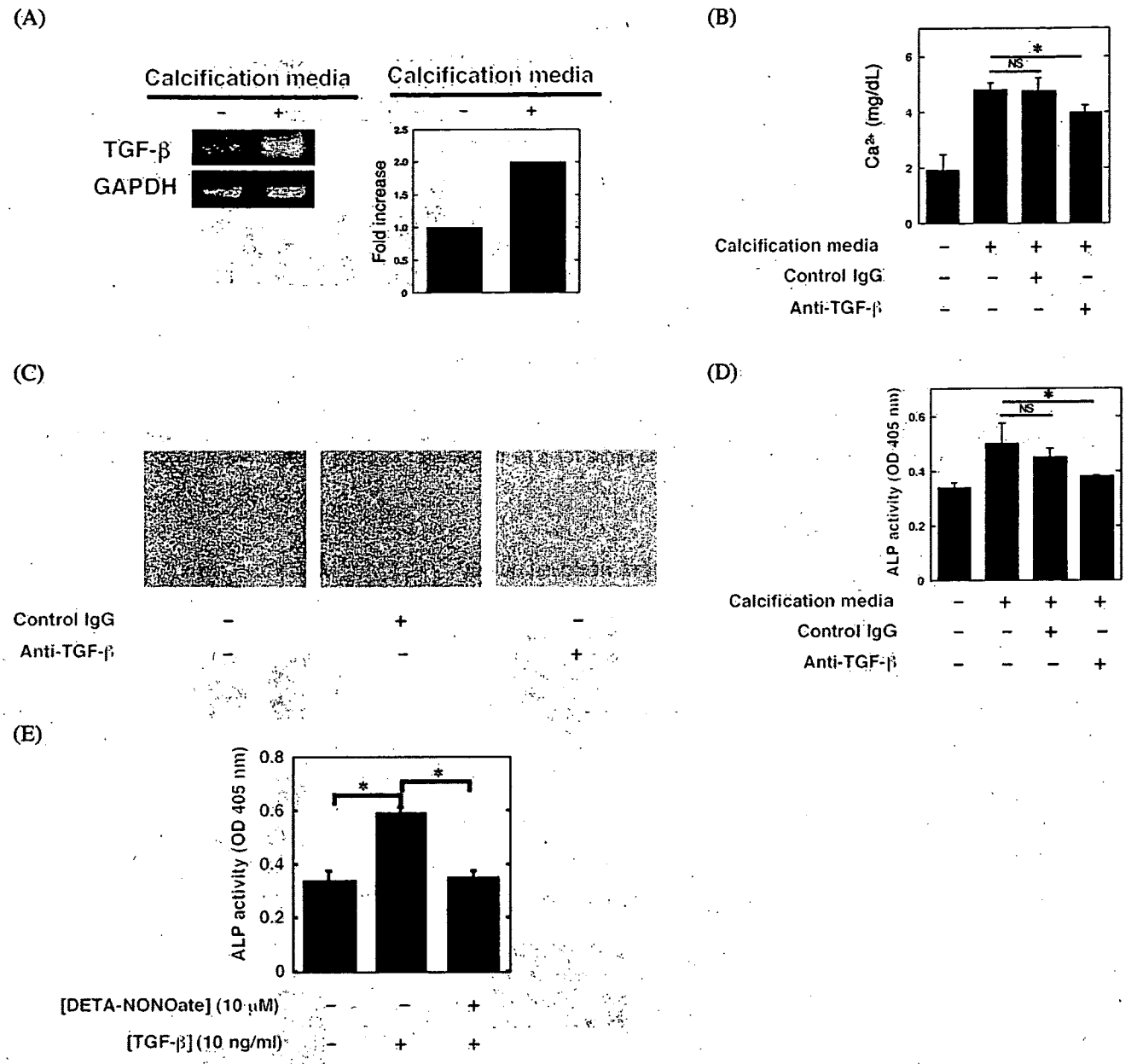

Figure 4 Transforming growth factor-β (TGF-β) induces vascular smooth muscle cell (VSMC) calcification. (*A*) TGF-β expression increases in calcifying VSMCs. VSMCs were grown for 14 days in the presence or absence of calcification medium. Then TGF-β gene expression in the cells was measured by RT-PCR. (*B*) Antibody to TGF-β inhibits VSMC calcification. VSMCs were grown in calcification media with various concentration of anti-TGF-β antibody or control IgG for 14 days. Then, calcium concentration in the cells was measured as described in Methods section ($n = 4$–$6 \pm$ SEM, $^*P < 0.05$). (*C*) Antibody to TGF-β inhibits VSMC calcification. VSMCs were grown in calcification media with various concentration of anti-TGF-β antibody or control IgG for 14 days. Then, cells were then stained with Alizarin red. (*D*) Antibody to TGF-β inhibits VSMC calcification. VSMCs were grown in calcification media with various concentration of anti-TGF-β antibody or control IgG for 14 days, and ALP activity at OD 405 nm was measured ($n = 4$–$6 \pm$ SEM, $^*P < 0.01$). (*E*) NO inhibits TGF-β induction of ALP activity. VSMCs were treated with DETA-NONOate for 2 days in the presence or absence of TGF-β. Then ALP activity in the cells was measured ($n = 5$–$6 \pm$ SEM, $^*P < 0.01$).

calcification medium induced NOS in VSMCs, where NOS is acting in a negative feedback loop. The degree of an increase in the expression of NOS isoforms was different respectively. NOS isoforms may play a different role in the negative feedback. In addition, other studies show that calcifying vascular cells, a subpopulation of cells from the artery wall and cardiac valves, have the ability to undergo osteoblastic differentiation and mineralization, and these cells have the potential for multiple lineages similar to mesenchymal stem cells.[25] Primary VSMCs might contain these cells. These cells may also have the ability to differentiate into the other type cells. Therefore, nNOS may be expressed in calcifying vascular cells though nNOS was absent in VSMCs. Further investigation would be required to clarify the details.

How does nitric oxide inhibit vascular calcification?

NO activates soluble guanylyl cyclase to produce cGMP that is involved in the relaxant response of VSMCs. Thus, we examined the effect of the guanylate cyclase inhibitor (ODQ) and PKG inhibitor (KT5823) on calcification and osteoblastic differentiation of VSMCs following NO treatment.

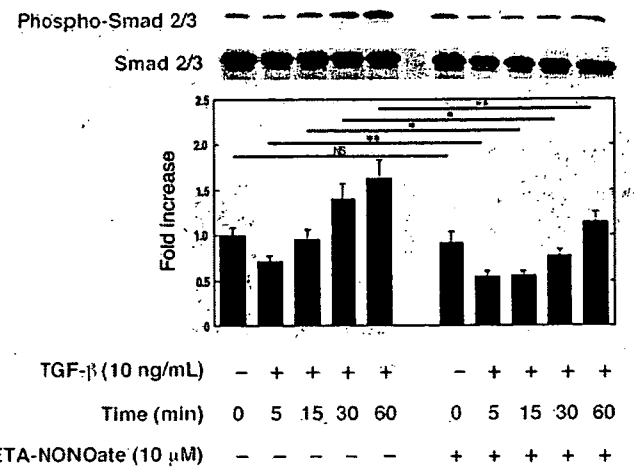

Figure 5 Nitric oxide (NO) regulates transforming growth factor-β (TGF-β) signalling in vascular smooth muscle cells (VSMCs). VSMCs were pretreated with DETA-NONOate for 60 min and then stimulated with 10 ng/mL TGF-β for the indicated periods. Phosphorylation of Smad2/3 was measured by western blot

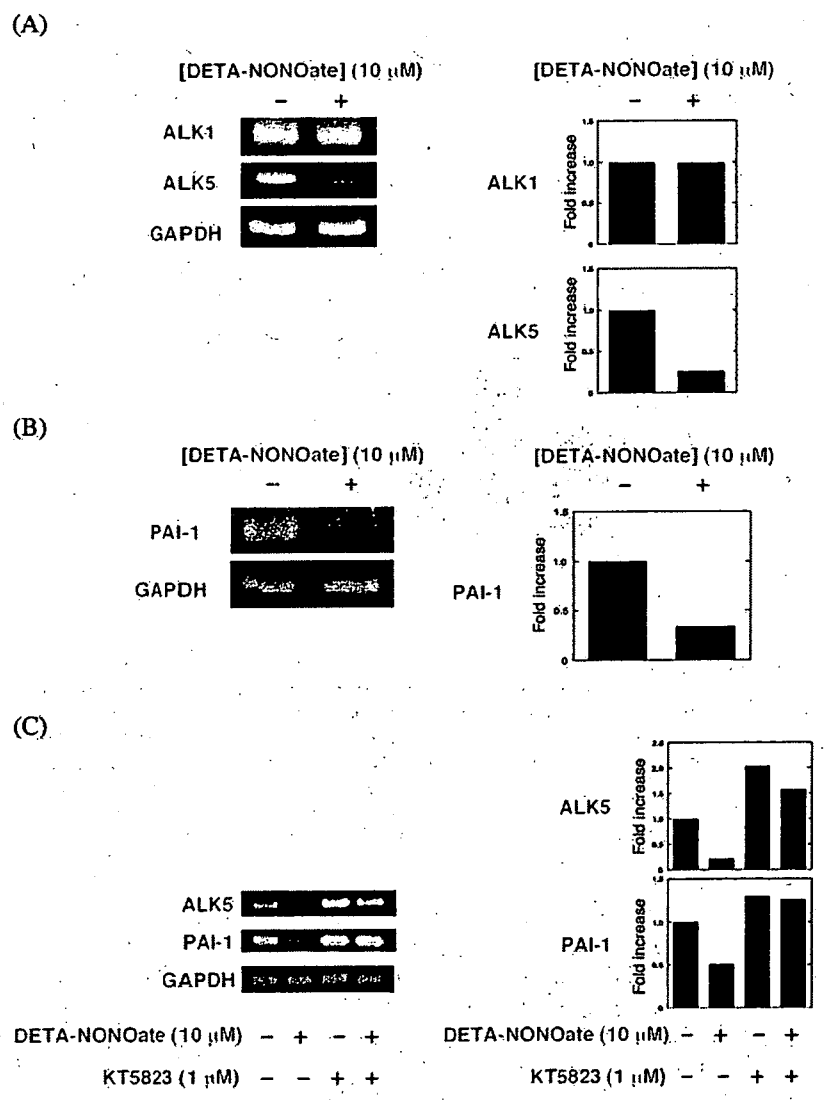

Figure 6 Nitric oxide (NO) inhibits transforming growth factor-β (TGF-β)-induced gene expression. (*A*) Effect of NO on ALK1 and ALK5 gene expression. Vascular smooth muscle cells (VSMCs) were grown in calcification media with 10 μM DETA-NONOate for 14 days. Then ALK1 and ALK5 gene expression in the cells was measured by RT–PCR. (*B*) Effect of NO on PAI-1 gene expression. Calcifying VSMCs were grown with 10 μM DETA-NONOate for 14 days. Then PAI-1 gene expression in the cells was measured by RT–PCR. (*C*) KT5823 blocks NO inhibition of ALK5 and PAI-1 gene expression. VSMCs were grown in calcification media with DETA-NONOate for 14 days in the presence or absence of KT5823, then ALK5 and PAI-1 gene expression in the cells was measured by RT–PCR. Similar results were obtained with three additional and different samples.

Inhibition of guanylate cyclase and PKG reversed the inhibitory effect of NO on vascular calcification and osteoblastic differentiation of VSMCs. Treatment of calcifying VSMCs with cGMP analogue inhibited vascular calcification and osteoblastic differentiation. These results suggest that NO regulates vascular calcification in part through the action of cGMP. However, ODQ and KT5823 did not increase VSMC calcification in the absence of NO donor. On the other hand, ODQ and KT5823 increased osteoblastic differentiation in the absence of NO donor. These data show that another possibility remains that is additional cGMP independent pathways such as S-nitrosylation of proteins by NO may also regulate calcification. We speculate as follows. First, osteoblastic differentiation is increased in VSMCs. Second, calcium accumulates in osteoblastic VSMCs. Finally, VSMC calcification is increased. cGMP/PKG signalling pathway may inhibit osteoblastic differentiation and NO may inhibit both VSMC calcification and osteoblastic differentiation. Further investigations would be required to clarify the details.

TGF-β can act as an anti-inflammatory and anti-atherogenic cytokine with a protective role in the complications of atherosclerosis. However, TGF-β also regulates vascular smooth muscle differentiation and vascular calcification.[6,15] We showed that NO reduced TGF-β signalling by decreasing expression of a TGF-β receptor ALK5, resulting in a down-regulation of TGF-β signal that induces phosphorylation of Smad2/3. TGF-β transduce signals via two distinct type I receptors, ALK1 and ALK5.[26] ALK5 induces phosphorylation of Smad2/3, while ALK1 induces phosphorylation of Smad1/5. Our results suggest that TGF-β signal via ALK5/Smad2/3 in VSMC is important for inducing vascular calcification. In addition, KT5823 reversed the inhibitory effect of NO on the ALK5 gene expression. Recently, Saura *et al.*[27] have shown that NO regulates the transcriptional responses to TGF-β by inhibiting Smad nuclear accumulation via PKG activation in ECs. This important study suggests a molecular mechanism by which NO regulates TGF-β signalling in calcification. We also found that NO regulates the TGF-β/ ALK5/Smad2/3 signalling, inhibiting TGF-β-induced gene expression of PAI-1. In addition, KT5823 reversed the inhibitory effect of NO on the PAI-1 gene expression. The fibrinolytic system plays an important role in vascular and tissue housekeeping. PAI-1 plays a key role in regulating the fibrinolytic system by serving as the primary inhibitor of t-PA and u-PA. Several groups have reported excess PAI-1 in atherosclerotic plaques in humans,[28–30] a finding that is exaggerated in type 2 diabetics.[31] These studies suggest that PAI-1 plays an important role in atherosclerosis. PAI-1 may also play an important role in vascular calcification. Inhibition of PAI-1 gene expression by NO may have an important role of calcification in VSMCs. Further investigations would be required to clarify the details.

Clinical aspects of nitric oxide and vascular calcification

NO inhibits vascular inflammation: vascular injury and atherosclerosis are more severe in knockout mice lacking eNOS or iNOS; conversely, gene therapy with NOS ameliorates arteriosclerosis.[32–35] Patients with endothelial dysfunction and defective NO synthesis is at increased risk for cardiovascular events. Our data suggest that NO and compounds that induce NO synthesis may be useful not only in inhibiting vascular inflammation, but also in preventing vascular calcification.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: All authors have no conflict of interest.

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Original article

Arginine-specific gingipain A from *Porphyromonas gingivalis* induces Weibel-Palade body exocytosis and enhanced activation of vascular endothelial cells through protease-activated receptors

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Abstract

Gingipains, cysteine proteases derived from *Porphyromonas gingivalis*, are important virulence factors in periodontal diseases. We found that arginine-specific gingipain A (RgpA) increased the responsiveness of vascular endothelial cells to *P. gingivalis* lipopolysaccharides (LPS) and *P. gingivalis* whole cells to induce enhanced IL-8 production through protease-activated receptors (PARs) and phospholipase C (PLC) γ. We therefore investigated whether RgpA-induced enhanced cell activation is mediated through exocytosis of Weibel-Palade bodies (WPBs) because they store vasoactive substances. RgpA rapidly activated PAR- and PLCγ-dependent WPB exocytosis. In addition, angiopoietin (Ang)-2, a substance of WPB, enhanced IL-8 production by *P. gingivalis* LPS, suggesting that Ang-2 mediates the RgpA-induced enhanced cell responses. Thus, we propose a novel role for RgpA in induction of a proinflammatory event through PAR-mediated WPB exocytosis, which may be an important step for enhanced endothelial responses to *P. gingivalis*.

Keywords: Gingipain; *Porphyromonas gingivalis*; Vascular endothelial cells; Weibel-Palade body; Exocytosis; Protease-activated receptors; Periodontitis

1. Introduction

Porphyromonas gingivalis is a principal periodontopathic pathogen [1,2]. Although *P. gingivalis* has a number of virulence factors, including lipopolysaccharide (LPS) and fimbriae, the most notable factors are the cysteine proteases termed gingipains. Two kinds of arginine residue-specific gingipains, RgpA and RgpB, and another type of lysine residue-specific gingipain have so far been identified [3–5]. Gingipains associated with released vesicles from *P. gingivalis* cells especially exert various pathophysiological effects through cleavage or degradation of in-host proteins, such as tissue proteins, coagulation factors and cytokines.

Microvessels are thought to be one of the first lines of defense against *P. gingivalis* in periodontal tissue. *P. gingivalis* promotes transmigration of neutrophils and monocytes from blood vessels into periodontal tissue and increase vascular permeability. Proinflammatory effects of *P. gingivalis* are thought to be dependent on gingipain-induced activation of vascular endothelial cells to induce an increase in vascular permeability, cytokine production and adhesion molecule expression [6,7].

Abbreviations: Ang, angiopoietin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HKPG, heat-killed whole cells of *P. gingivalis*; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharide; PLC, phospholipase C; PAR, protease-activated receptor; RgpA, arginine-specific gingipain A; siRNA, short interfering RNA; TLR, Toll-like receptor; VWF, von Willebrand factor; WPB, Weibel-Palade body.

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In this study, we explored gingipain-induced responses of vascular endothelial cells in detail. We found that RgpA induces degranulation and modulates *P. gingivalis*-induced proinflammatory responses of endothelial cells through the release of storage substances, in which protease-activated receptor (PAR)-mediated signaling plays an important role.

2. Materials and methods

2.1. Reagents, chemicals and cell culture

A23187, U73122, 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid–acetoxymethyl ester (BAPTA–AM) and highly purified LPS from *Escherichia coli* were purchased from Sigma–Aldrich (St Louis, MO). Leupeptin was purchased from Peptide Institute (Osaka, Japan). Preparation of vesicle-associated RgpA from *P. gingivalis* HG66 in culture media was described previously [8]. Purified LPS from *P. gingivalis* ATCC33277, heat-killed whole cells of *P. gingivalis* (HKPG) ATCC33277 and the synthetic bacterial lipopeptide Pam_3CSK_4 were obtained from InvivoGen (San Diego, CA). Recombinant human interleukin (IL)-1β and angiopoietin-2 (Ang-2) were obtained from R&D systems (Minneapolis, MN). Human umbilical vein endothelial cells (HUVECs) were grown as described previously [9]. Cells were used for experiments from passages 4 to 8.

2.2. RNA interference in HUVECs

Gene-specific short interfering RNAs (siRNAs) for human PARs, human Ang-2 and a control oligonucleotide were purchased from Dharmacon (Chicago, IL). RNA interference in HUVEC was performed according to the method described previously [9].

2.3. Determination of von Willebrand factor (VWF) by ELISA

Confluent HUVECs seeded on 24-well plates were prepared in serum-free EGM-2 media. Cells were stimulated for 1 h with RgpA. The culture media were collected and clarified for analyses of the amounts of VWF as described previously [9].

2.4. Determination of interleukin IL-8 by ELISA

Confluent HUVECs seeded on 24-well plates were stimulated for 6 h with *P. gingivalis* LPS or HKPG in the presence or absence of 200 nM RgpA. Then the culture media were collected and clarified for analyses of the amounts of IL-8 using a human IL-8 ELISA kit (Invitrogen) according to the manufacturer's instructions. Results are representative of three separate experiments and are expressed as means ± standard deviation (SD) of triplicate wells.

2.5. Quantitative RT-PCR

Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Valencia, CA). Transcripts were quantified by real-time quantitative PCR on a LightCycler ST300 system (Roche). All values were normalized to the level of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. The primer sets were as follows: human PAR_1: sense, 5′-cccgctgttgtctgccc-3′ and antisense, 5′-ggttcctgagaagaaatgaccg-3′; human PAR_2: sense, 5′-gaggtattgggtcatcg-3′ and antisense, 5′-ggctgggaacagaaag-3′; human PAR_3: sense, 5′-ggacaggagccacgat-3′ and antisense, 5′-ccacagggtcacagca-3′; human PAR_4: sense, 5′-agcacgccctcaatcct-3′ and antisense, 5′-cagccatgcagagtccaa-3′; human GAPDH: sense, 5′-gaaggtgaaggtcggagtc-3′ and antisense, 5′-gaagatggtgatgggatttc-3′; human Ang-1: sense, 5′-gaaggg aaccgagcc-3′ and antisense, 5′-gggcacatttgcaca-3′; human Ang -2: sense, 5′-ccacaaatggcatctacacg-3′ and antisense, 5′-cccagccaatattctcctga-3′; and human IL-8: sense, 5′-cttggcag ccttcctgatttc-3′ and antisense, 5′-tcagccctcttcaaaaacttc-3′. The PCR amplicons were visualized on 1.5% agarose gels stained with ethidium bromide and photographed under UV light. Results are representative of three separate experiments.

2.6. Immunofluorescence of Ang-2 and VWF

Confluent HUVECs were prepared and fixed at −20 °C with methanol for 60 min. Immunostaining was carried out using an anti-Ang-2 rabbit polyclonal antibody (Santa Cruz Biotechnology) and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Invitrogen). Images were obtained by a fluorescent microscope IX71 (magnification ×40) with DP70 image capture (Olympus).

2.7. Statistical analysis

All values were evaluated by statistical analyses using Student's *t*-test. Differences were considered to be statistically significant at the level of $P < 0.01$.

3. Results

3.1. Effect of RgpA on the early-phase inflammatory response in HUVECs

We first investigated the effect of RgpA on *P. gingivalis*-induced inflammatory responses of vascular endothelial cells. HUVECs responded to RgpA, *P. gingivalis* LPS and HKPG to induce IL-8 production (Fig. 1A). RgpA synergically or synergistically enhanced IL-8 production by *P. gingivalis* LPS and HKPG (Fig. 1A). We also found that RgpA could upregulate IL-8 production by *E. coli* LPS, Pam_3CSK_4 and IL-1β (Fig. 1B). It has been reported that RgpA directly induce proinflammatory responses through PAR activation [10]. PARs are a family of G protein-coupled receptors that require cleavage at the N-terminus to be activated [11,12]. Among four members of PARs, HUVECs expressed mRNAs of PAR_1, PAR_2 and PAR_3 but not that of PAR_4 (Fig. 1C and

data not shown). We utilized siRNAs to investigate the role of PARs, which could effectively reduce each PAR mRNA expression (Fig. 1C). We confirmed that transfection of the mixture of siRNAs for PAR_{1-3} could simultaneously reduce the expression of PAR_{1-3} (Fig. 1C). The upregulatory effect of RgpA on IL-8 production was reduced by knockdown of PAR_{1-3} (Fig. 1D). We also found that inhibition of PLCγ, a common mediator of PAR signaling, by U73122 abolished RgpA-induced enhanced IL-8 production (Fig. 1E). Thus, the PAR–PLCγ pathway plays a crucial role in RgpA-induced enhanced activation of vascular endothelial cells.

3.2. RgpA activates Weibel-Palade body exocytosis

Because early endothelial activation involves degranulation of the endothelial cell-specific granules Weibel-Palade bodies

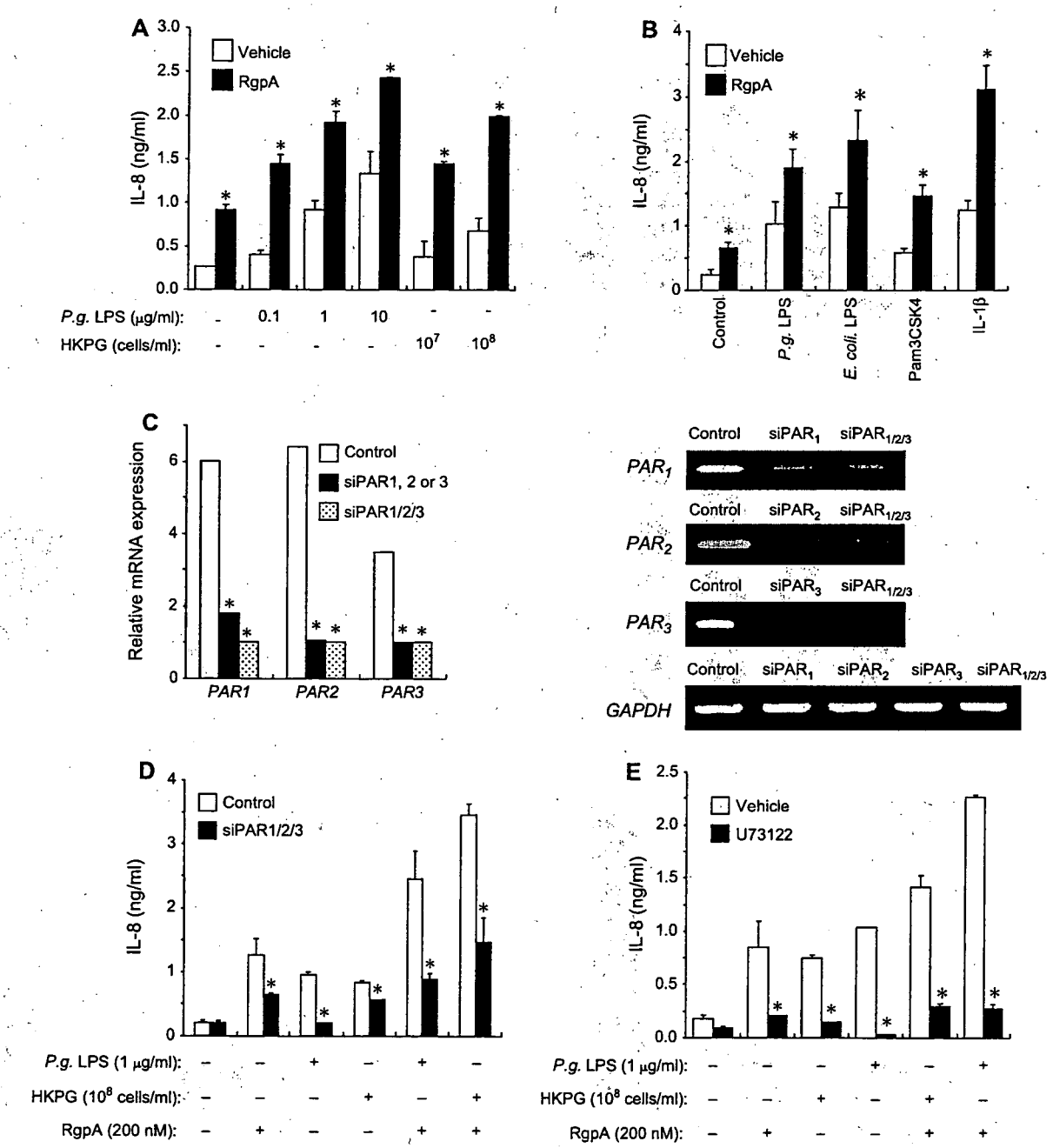

Fig. 1. Effect of RgpA on IL-8 production induced by *P. gingivalis* LPS or HKPG. (A, B) HUVECs were stimulated for 6 h with the indicated concentrations of LPS or HKPG (A) or with *E. coli* LPS (10 ng/ml), *P. gingivalis* LPS (1 μg/ml), Pam_3CSK_4 (0.5 μg/ml) or IL-1β (1 ng/ml) (B) in the presence or absence of 200 nM RgpA. The amounts of IL-8 released into the media were measured by ELISA. Each value is the mean ± SD ($n = 3$) *vs control group, $P < 0.01$. (C) HUVECs were transfected with siRNA for PAR_1, PAR_2 or PAR_3 or together with these siRNAs. The expression levels of mRNAs of PAR_1, PAR_2 and PAR_3 were determined by quantitative RT-PCR *vs control group, $P < 0.01$. (D, E) HUVECs were transfected together with siRNAs for PAR_1, PAR_2 and PAR_3 (D) or pretreated for 30 min with 10 μM U73122 (E). Cells were then stimulated for 6 h with 1 μg/ml *P. gingivalis* LPS or 10^8 cells/ml HKPG in the presence or absence of 200 nM RgpA. The amounts of IL-8 released into the media were measured by ELISA. Each value is the mean ± SD ($n = 3$) *vs control group, $P < 0.01$.

(WPBs) [13–15], we thought to determine whether RgpA-induced activation of vascular endothelial cells is affected by WPB exocytosis. We first examined whether RgpA could activate Weibel-Palade body exocytosis in HUVEC. Quantification of exocytosis was performed by measuring the amount of VWF, an essential constituent of WPBs. RgpA-induced VWF release in a similar manner to the calcium ionophore A23187 (Fig. 2A). The release of VWF occurred within 5 min and continued for at least 60 min after stimulation (Fig. 2B). Presence of the cysteine protease inhibitor leupeptin greatly reduced induction of VWF release by RgpA but not that by A23187 (Fig. 2C), suggesting that RgpA-induced exocytosis depends on its protease activity.

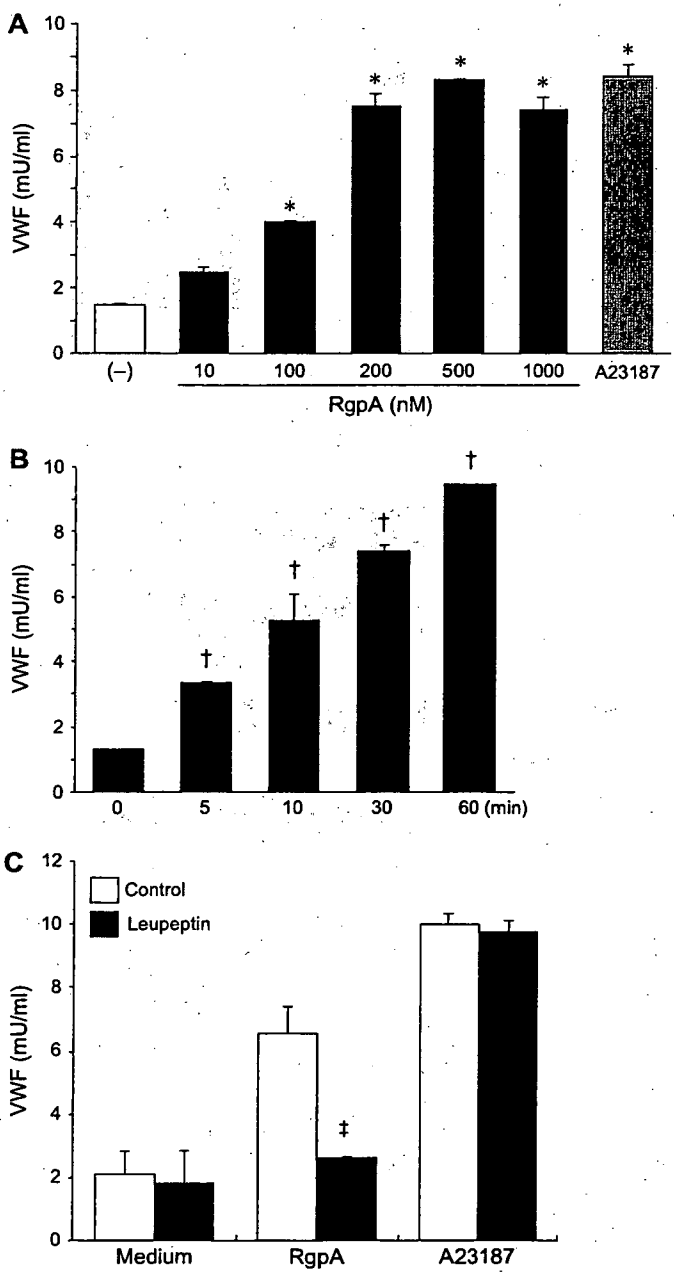

Fig. 2. Induction of WPB exocytosis by RgpA. (A) HUVECs were stimulated for 1 h with RgpA at the indicated concentrations or with 10 μM A23187. (B) HUVECs were stimulated with 200 nM RgpA for the indicated periods. (C) HUVECs were stimulated for 1 h with 200 nM RgpA or 10 μM A23187 in the presence or absence of 2 μM leupeptin. The amounts of VWF released into the media were measured by ELISA. Each value is the mean ± SD ($n = 3$) *vs vehicle group, $P < 0.01$; †vs '0 min', $P < 0.01$; and ‡vs control group, $P < 0.01$.

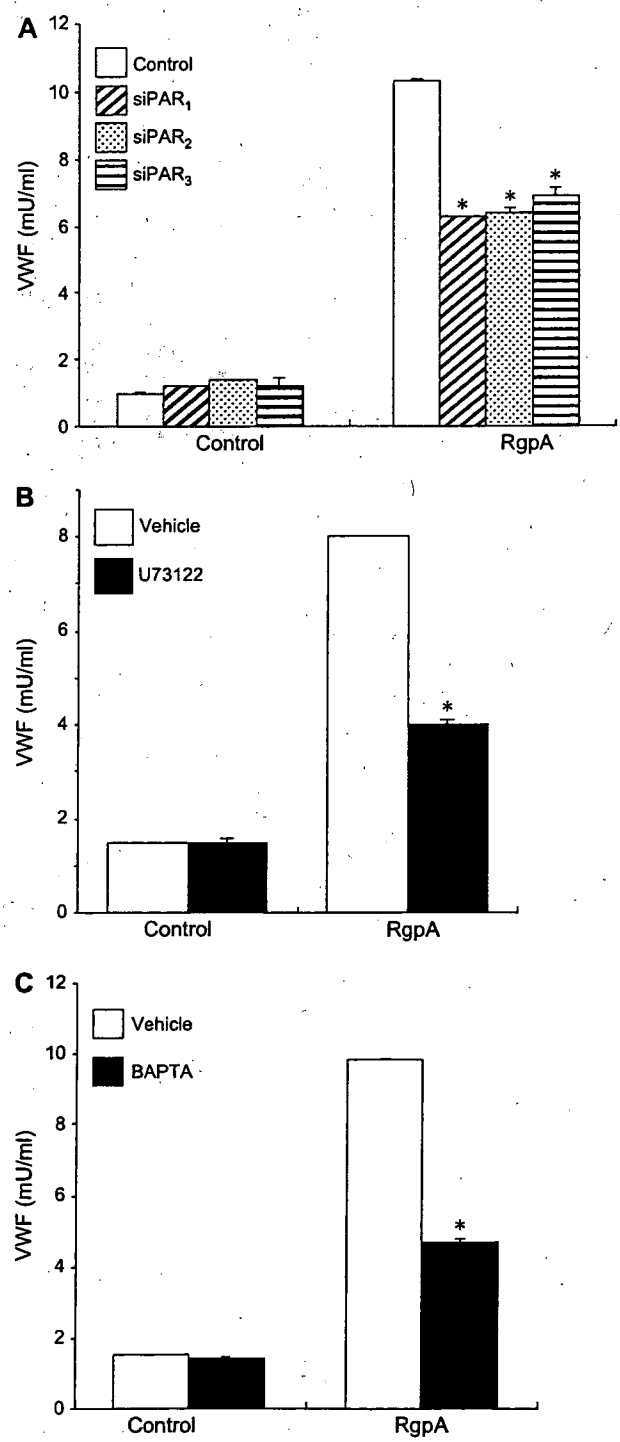

Fig. 3. Regulatory mechanisms of RgpA-activated WPB exocytosis. (A) HUVECs transfected with siRNAs for PAR_1, PAR_2 or PAR_3 were stimulated for 1 h with 200 nM RgpA or 10 μM A23187. (B) HUVECs pretreated for 30 min with 10 μM U73122 were stimulated for 1 h with 200 nM RgpA. (C) HUVECs pretreated for 30 min with 20 μM BAPTA–AM were stimulated for 1 h with 200 nM RgpA. The amounts of VWF released into the media were measured by ELISA. Each value is the mean ± SD ($n = 3$) *vs control group, $P < 0.01$.

3.3. RgpA activates WPB exocytosis through the PAR–PLCγ pathway

We next examined whether RgpA-induced WPB exocytosis was mediated by PARs and PLCγ. As shown in Fig. 3A, transfection of respective PAR siRNA could significantly suppress RgpA-induced VWF release. Common regulated WPB exocytosis is activated through an increase in intracellular Ca^{2+} level after stimulation with various secretagogues [13,14]. In addition, PLCγ is known as a regulator of intracellular calcium release by the generation of inositol (1,4,5) triphosphate. We found that the PLCγ inhibitor U73122 decreased RgpA-induced VWF release (Fig. 3B). Furthermore, RgpA-induced VWF release was significantly suppressed by the cell-permeable Ca^{2+} chelator BAPTA–AM (Fig. 3C). Thus, RgpA activates intracellular Ca^{2+}-dependent WPB exocytosis through the PAR–PLCγ pathway.

3.4. Effect of WPB components on P. gingivalis-induced inflammatory responses

We finally investigated whether released substances from WPB affect the RgpA-induced enhanced cell activation. Among known substances of WPB, we focused on the Tie-2 receptor ligand and Ang-2 because Ang-2 is known to enhance TNF-α-induced proinflammatory responses in HUVECs [16]. HUVECs expressed mRNA of Ang-2, but not that of another Tie-2 ligand Ang-1 (Fig. 4A). Stimulation with *P. gingivalis* LPS did not alter these expressions (Fig. 4A). Ang-2 protein was localized in granules (Fig. 4B). We found that recombinant Ang-2 added to the culture increased IL-8 production by *P. gingivalis* LPS (Fig. 4C). Furthermore, knockdown of Ang-2 suppressed IL-8 expression induced by *P. gingivalis* LPS (Fig. 4D). Thus, Ang-2 has a role to mediate the enhanced activation of endothelial cells.

4. Discussion

PARs play important roles in the regulation of several physiological and pathological effects, including coagulation, inflammation and vascular homeostasis [11,12,17]. We found that endothelial PARs were involved in RgpA induction of WPB exocytosis and regulation of endothelial cell activation. PAR-mediated responses in endothelial cells are known to be activated by thrombin. Thrombin stimulation elicits a capillary leak in vivo and increased endothelial monolayer permeability in vitro [18,19]. In addition, thrombin activates WPB exocytosis via PAR_1 or PAR_2 [20]. Thus, in a way similar to thrombin,

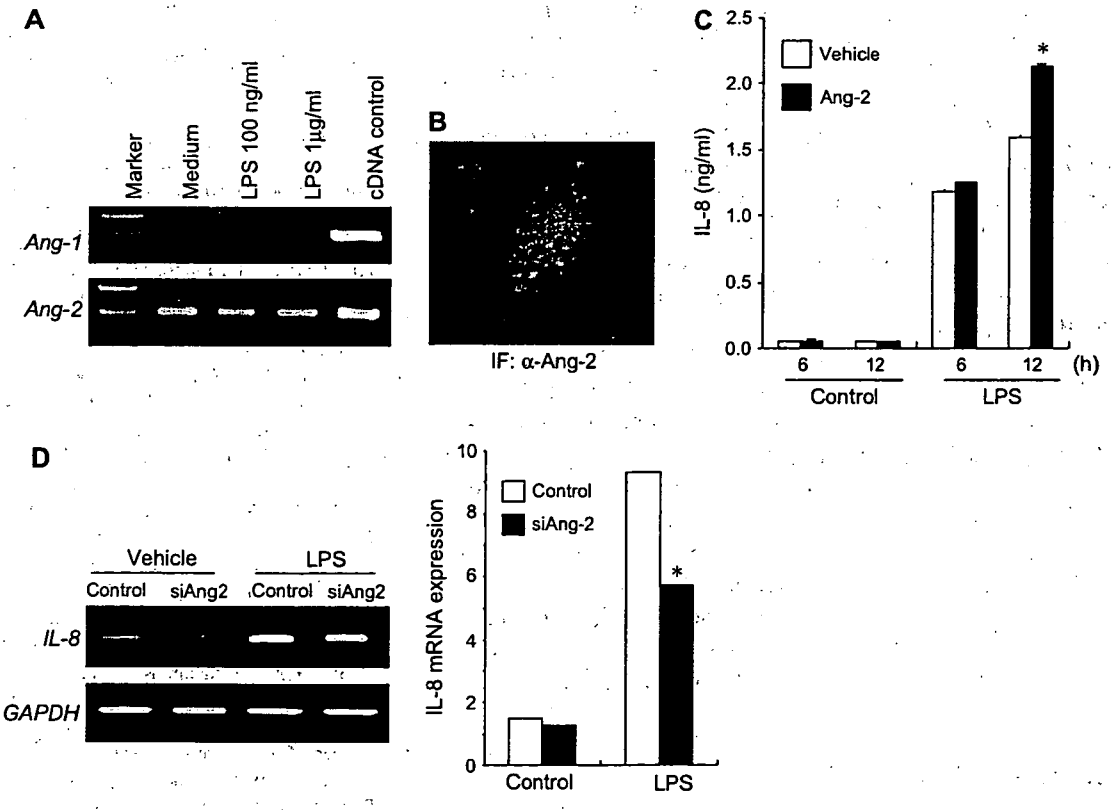

Fig. 4. Effect of Ang-2 on IL-8 production by *P. gingivalis* LPS. (A) Total RNA of HUVECs stimulated for 6 h with or without given concentrations of LPS was extracted for RT-PCR using specific primer sets for Ang-1 or Ang-2. The cDNA of Ang-1 and Ang-2 were used as templates for positive controls. (B) HUVECs were stained immunofluorescently with anti-Ang-2 antibody. Cell nuclei were stained with Höechst33342. (C) HUVECs were stimulated for 6 h or 12 h with 1 μg/ml *P. gingivalis* LPS in the presence or absence of 200 ng/ml recombinant Ang-2. The amounts of IL-8 released into the media were measured by ELISA. Each value is the mean ± SD ($n = 3$) *vs control group, $P < 0.01$. (D) HUVECs transfected with siRNA for Ang-2 were stimulated for 6 h with 1 μg/ml *P. gingivalis* LPS. The expression level of IL-8 mRNA was determined by quantitative RT-PCR *vs control group, $P < 0.01$.

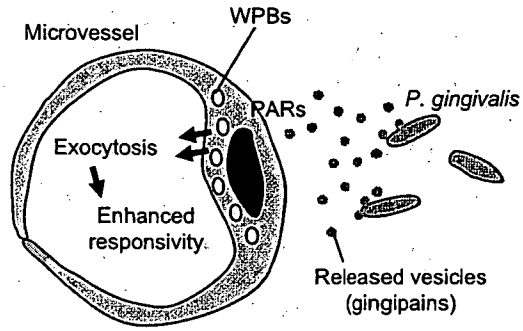

Fig. 5. Schematic of the effect of vesicle-associated RgpA on vascular endothelial cells.

RgpA may activate PAR signaling, leading to activation and promotion of leukocyte adhesion to the vascular endothelium, which links inflammation and coagulation in a variety of pathological settings.

P. gingivalis has a broad array of virulence factors as immunostimulatory compounds [21]. Several factors function as agonists for Toll-like receptor (TLR) 2 and TLR4 [22–25]. We found that IL-8 production not only by *P. gingivalis* LPS and HKPG but also by TLR4-agonistic *E. coli* LPS, TLR2-agonistic Pam_3CSK_4 and IL-1β was upregulated by RgpA, suggesting the synergism of TLRs/IL-1R and PARs. Indeed, several recent studies have suggested that TLRs and PARs synergistically function in induction of proinflammatory responses [26,27]. The presence of gingipains at the site of *P. gingivalis* infection may affect responsiveness of the vascular endothelium to virulence factors from *P. gingivalis*.

WPB exocytosis induces release of storage compounds and may control local or systemic physiological and pathological effects, including leukocyte rolling, thrombus formation, vascular inflammation and angiogenesis. Storage components of WPBs have various vasoregulatory activities. We demonstrated that Ang-2 could regulate endothelial response to *P. gingivalis* (Fig. 4C). Ang-2 has been identified as a functional antagonist of Ang-1. Ang-2 sensitizes endothelial cells to TNF-α, thereby acting as a switch of vascular responsiveness towards inflammatory stimuli [16]. It has been suggested that diabetes, an important risk factor for periodontal disease, induces increase in Ang-2 transcription and expression [28]. Thus, Ang-2 released from WPBs may be an important determinant of the severity of periodontal diseases caused by *P. gingivalis* infection.

Our study proposes that *P. gingivalis* infection can modulate inflammatory responses of vascular endothelial cells through release of gingipains (Fig. 5). Such an effect may have a crucial role in the initiation and regulation of periodontitis.

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Synthesis and Characterization of a Dipalmitoylated Lipopeptide Derived from Paralogous Lipoproteins of *Mycoplasma pneumoniae*▽†

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Genomic analysis of *Mycoplasma pneumoniae* revealed the existence of a large number of putative lipoprotein genes compared with the numbers in other bacteria. However, the pathogenic roles of *M. pneumoniae* lipoproteins are still obscure. In this study, we synthesized a lipopeptide (designated *M. pneumoniae* paralogous lipoprotein 1 [MPPL-1]) in which an *S*-dipalmitoylglyceryl cysteine was coupled to a peptide with a consensus sequence of a putative paralogous lipoprotein group characteristic of *M. pneumoniae*. The cytokine-inducing activity of MPPL-1 in human monocytic cells was much weaker (~700-fold weaker) than that of the known mycoplasmal *S*-dipalmitoylated lipopeptide FSL-1 or MALP-2. MPPL-1 required Toll-like receptor (TLR2) to activate NF-κB-dependent gene transcription in HEK293 cells, although a 1,000-fold-larger amount of MPPL-1 was needed to exert activity similar to that of FSL-1 in the cells. TLR2-mediated recognition of MPPL-1 was synergistically upregulated by TLR6 but not by TLR1 or TLR10, although the activity was still weak. In addition, MPPL-1 did not antagonize FSL-1 recognition in human monocytic cells and TLR2/TLR6-expressing HEK293 cells. Thus, these results suggest that there is preferential selective recognition of diacylated lipopeptides due to the magnitude of an affinity with TLR2 and TLR6 and the roles of increased paralogous lipoprotein genes of *M. pneumoniae* in evasion of TLR2 recognition.

Membrane-bound lipoproteins are thought to play important roles in the survival of bacteria through four main functions: a structural function, a transport function, an adhesion function, and an enzymatic function (7). Many lipoproteins have been identified in various species of bacteria and have been shown to comprise a framework structure containing a lipidated N-terminal cysteine residue coupled to distinct polypeptides. The maturation of bacterial lipoproteins generally comprises three steps; the first step involves diacylglyceryl modification of a cysteine residue by diacylglycerol transferase, the second step involves cleavage of the leader peptide by signal peptidase II, and the final step involves N acylation of the N-terminal diacylglyceryl cysteinyl residue, with which lipoproteins are synthesized as triacylated lipoproteins (7). It has also been shown that lipoproteins derived from *Rhodopseudomonas viridis* and several mycoplasmal species do not undergo modification in the final step and are synthesized as diacylated lipoproteins (7).

In contrast to their crucial functions in the survival of bacteria, bacterial lipoproteins act as pathogenic substances to stimulate the immune systems of humans and animals through the recognition receptors that monitor exogenous pathogens (3). Toll-like receptors (TLRs) are central pattern recognition receptors of the innate immune system that recognize a wide range of invading microorganisms through conserved chemical structures in their cells (34). TLR2 is essential for mediation of immune responses to the most diverse set of molecular structures of microbes, including peptidoglycans, lipoteichoic acids, porins, lipoarabinomannans, and lipoproteins/lipopeptides (21, 34). TLR2 forms heteromers with either TLR1 or TLR6, probably to discriminate the structures of molecular patterns, especially the N-terminal lipidated cysteinyl portions of bacterial lipoproteins as active sites (4, 29). TLR1 and TLR6 have been reported to be involved in simple discrimination of the difference between triacylated and diacylated lipoproteins/lipopeptides (36, 37). However, recent arduous work by several study groups has shown that such diverse potentials of TLR1 and TLR6 are largely dependent on more subtle structures of lipoproteins/lipopeptides, such as the length of an N-terminal fatty acid chain, the chirality of the central carbon of the diacylglycerol, and the charge of the C-terminal amino acids (5, 6, 28). It has been suggested that in addition to TLR1 and TLR6, TLR10, which is not encoded in the murine genome, is related to TLR2 recognition because of its sequence similarity and the possibility that it forms a heteromer with TLR2 (8, 12).

Mycoplasmas are microbes in regressive evolution and differ from other microbes in many respects. For example, they completely lack a cell wall, and their bilipid membrane is therefore the only structure that regulates interactions with the external environment (31). Some mycoplasmas cause severe respira-

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tory, arthritic, and urogenital diseases in humans and animals. *Mycoplasma pneumoniae* is a human pathogen that causes "atypical pneumonia," particularly in older children and young adults (38). The genome size of *M. pneumoniae* is ~820 kb, and the genomic sequence has been completely analyzed (13, 14). Interestingly, a large number of putative lipoprotein-encoding genes have been identified in the genome (46 of 689 genes; 6.68%) compared with the numbers of such genes in the genomes of other microbes, such as *Escherichia coli* K-12 (22 of 4,243 genes; 0.52%) and *Bacillus subtilis* (26 of 4,105 genes; 0.63%) (7). Even in the closely related sister species *Mycoplasma genitalium*, only 21 putative lipoproteins (encoded by 477 genes; 4.4%) could be found. Despite the existence of such genetic data, little is known about the roles of lipoproteins in *M. pneumoniae* pathogenicity, although there has been much interest in the pathogenic roles of membrane lipoproteins of other mycoplasmal species during infection because of their diverse functions, including adherence to host cells, antigenic variation, and TLR2- and TLR6-mediated immunostimulation (30).

In this study, we attempted to synthesize a lipopeptide having an *S*-(2,3-bispalmitoyloxypropyl)-cysteine residue coupled to an N-terminal consensus peptide of *M. pneumoniae*-specific lipoproteins encoded by paralogous genes. Interestingly, the level of immunostimulatory activity of this lipopeptide was much lower than that of the known mycoplasmal lipopeptide MALP-2 or FSL-1 despite the structural uniformity. We also investigated the recognition of this lipopeptide by TLRs.

MATERIALS AND METHODS

Preparation of synthetic lipopeptides. The synthetic lipopeptides FSL-1 and MALP-2 were prepared as described previously (17). *S*-(2,3-bisacyloxypropyl)-cysteinyl TGIQADLRNLIK, designated *M. pneumoniae* paralogous lipoprotein 1 (MPPL-1), was synthesized using a method similar to the method used for synthesis of FSL-1 and MALP-2. Briefly, the side chain-protected sequence TGIQADLRNLIK was constructed with an automated peptide synthesizer (model 433; Applied Biosystems). (9-Fluorenylmethoxy carbonyl)-*S*-(2,3-bispalmitoyloxypropyl)-cysteine (Novabiochem) was manually coupled to the peptide resin by using a 1-hydroxy-7-azabenzotriazole-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/CH_2Cl_2-dimethylformamide solvent system. The 9-fluorenylmethoxy carbonyl and resin were removed from the lipopeptide by using trifluoroacetic acid. The lipopeptide was extracted into 90% acetic acid, lyophilized, and purified by preparative high-pressure liquid chromatography with a reversed-phase C_{18} column (30 by 250 mm). The level of purity of the lipopeptide was confirmed by analytical high-pressure liquid chromatography with a reversed-phase C_{18} column (4.6 by 150 mm) to be 96%. All of the lipopeptides were used without separation of the *S*-form and *R*-form stereoisomers. The lipopeptides were dissolved in phosphate-buffered saline containing 10 mM *n*-octyl-β-glucopyranoside at a concentration 0.5 mM and stored at −80°C until they were used.

Cell culture. Dulbecco modified Eagle medium, RPMI 1640 medium, penicillin G, streptomycin, and trypsin-EDTA were obtained from Sigma. Human monocytic cell line THP-1 was cultured in RPMI 1640 medium as described previously (19). Human embryonic kidney HEK293 cells were grown in Dulbecco modified Eagle medium as described previously (18).

Determination of IL-6 and IL-8 by enzyme-linked immunosorbent assays (ELISA). A total of 1×10^5 THP-1 cells were stimulated for 12 h with various concentrations of mycoplasmal lipopeptides, and the amounts of interleukin-6 (IL-6) and IL-8 released into the media were determined by using human IL-6 Cytoset and human IL-8 Cytoset (Invitrogen), respectively, according to the instructions of the manufacturer. The results described below are representative of three separate experiments, and the data are expressed as means and standard deviations.

DNA cloning. Plasmids encoding human TLR1, TLR2, and TLR6 have been described previously (18). Human TLR10 cDNA was obtained by reverse transcription-PCR of RNA isolated from human umbilical vein endothelial cells and then cloned into a pEF6 vector (Invitrogen). The DNA sequences were confirmed by the dideoxy chain termination method by using an ABI Prism 3100 genetic analyzer.

Luciferase reporter gene assay. HEK293 cells were plated at a concentration of 0.5×10^5 cells per well in 24-well plates before transfection. The cells were transiently transfected with an NF-κB-driven firefly luciferase reporter plasmid (pNF-κB-Luc; Stratagene) and a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK; Promega) together with TLR-encoding plasmids. After 24 h of incubation, the cells were stimulated for 6 h with MPPL-1 or FSL-1 in media containing 1% fetal bovine serum. Then the cells were lysed, and the luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) according to instructions of the manufacturer. The results below, expressed as the means and standard deviations of values for triplicate wells, are representative of three separate experiments. The experiment using HEK293 cells stably expressing TLR2 has been described previously (20).

Statistics. All values were evaluated by statistical analysis using Student-Newman-Keul's test. Differences were considered to be statistically significant at a P value of <0.05.

RESULTS

Preparation of MPPL-1. Himmelreich et al. reported that 46 protein genes were identified as genes encoding putative lipoproteins in the *M. pneumoniae* M129 (=ATCC 29342) genome based on the following characteristic lipoprotein-specific features: (i) the presence of one or more basic amino acids among the first five to seven amino acids of the N terminus, (ii) the presence of a hydrophobic signal peptide, and (iii) the presence of a cysteine residue immediately downstream of the signal peptide (13). However, we found that 48 proteins had these lipoprotein signatures. The N-terminal lipoprotein moieties of all putative lipoproteins are shown in Table S1 in the supplemental material. The amino acid sequences of these lipoproteins are included in the data at a website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=genome), and the protein designations were based on the MPN numbering scheme described by Himmelreich et al. (13). Importantly, many of these putative lipoproteins have recently been confirmed to be functionally expressed in the microorganism (11, 33, 39). In addition to 48 putative lipoproteins, there are several proteins with high levels of similarity to the lipoproteins without the lipoprotein signature at the N terminus (13), but we did not include these proteins in the list.

Comparison of 30 amino acids of N-terminal lipoprotein moieties revealed that the *M. pneumoniae* lipoproteins include members of seven subgroups, which are probably groups of paralogous lipoproteins (see Table S1 in the supplemental material). We focused on group 1 composed of MPN011, MPN054, MPN271, MPN369, MPN411, MPN467, MPN650, and MPN654 because the N-terminal sequences of these putative lipoproteins could not be identified by a BLAST search in other known organisms, even the sister species *M. genitalium*, suggesting that the lipoprotein genes were propagated uniquely in the evolution of this microorganism. The sequence of MPN505 is also very similar to the sequences of these lipoproteins, but MPN505 lacks the lipoprotein signature. Importantly, the study of Hallamaa et al. showed that there was expression of mRNAs for all group 1 lipoproteins and the detectable proteins MPN271, MPN411, and MPN650 (11). Comparison of N-terminal sequences of these lipoproteins revealed that the levels of similarity of MPN271, MPN369,

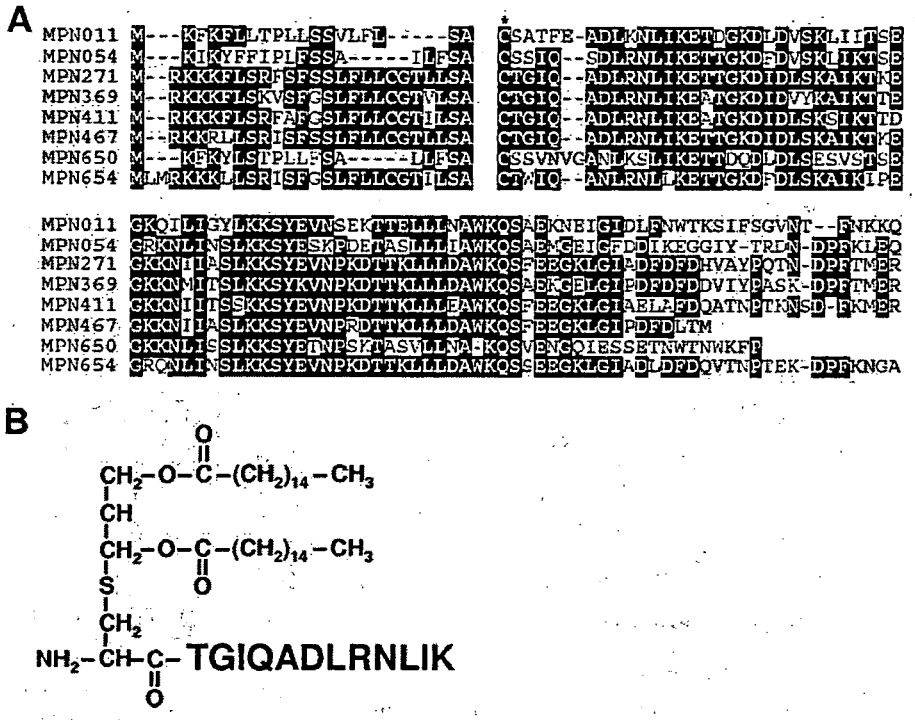

FIG. 1. Synthesis of MPPL-1. (A) Alignment of putative paralogous lipoproteins. The N-terminal sequences of MPN011, MPN054, MPN271, MPN369, MPN411, MPN467, MPN650, and MPN654 were compared. The cysteine residue immediately downstream of the signal peptide is indicated by an asterisk. (B) Structure of MPPL-1.

MPN411, MPN467, and MPN654 are particularly high (Fig. 1A). These putative lipoproteins have a characteristic feature; namely, the C-terminal amino acid residue flanking the cysteine residue immediately downstream of the signal peptide is threonine, although the corresponding amino acid of common bacterial lipoproteins is glycine, alanine, or serine.

To analyze the pathological roles of this paralogous lipoprotein group, we attempted to synthesize a lipopeptide having an N-terminal sequence common to these lipoproteins. The results of previous work suggested that synthetic lipopeptides with original peptide sequences with more than 10 amino acids could mimic the immunostimulatory activity of natural lipoproteins (24, 27, 32). Therefore, we determined that the partial consensus sequence of MPN271, MPN369, MPN411, MPN467, and MPN654 is TGIQADLRNLIK, which should couple to an *S*-dipalmitoylglyceryl cysteine. The structure was chemically synthesized using a method similar to the method used for synthesis of known mycoplasmal lipopeptides described previously (17, 32), and the protein was designated MPPL-1 (Fig. 1B). All of our preparations of mycoplasmal lipopeptides were synthesized as mixtures of the *S*-form and *R*-form stereoisomers.

Immunostimulatory activity of MPPL-1. To investigate the immunostimulatory activity of MPPL-1, we examined the induction of cytokine production in human monocytic THP-1 cells, comparing the activity of MPPL-1 with the activities of two synthetic mycoplasmal lipopeptides. MALP-2 (*S*-dipalmitoylglyceryl CGNNDESNISFKEK) derived from *Mycoplasma fermentans* was first identified and characterized by Mühlradt's group as a compound that can activate macrophages even at picomolar concentrations (24). FSL-1 (*S*-dipalmitoylglyceryl CGDPKHPKSF) derived from *Mycoplasma salivarium* has recently been characterized by our group as a potent immunostimulatory compound, whose activity has been proposed to be stronger than that of MALP-2 (16, 17, 27). MPPL-1 could induce production of IL-8 in a dose-dependent manner at a concentration of ≥10 nM, whereas FSL-1 and MALP-2 could induce the production of IL-8 at picomolar concentrations (Fig. 2A). To induce a level of IL-8 production similar to the level induced by 1 nM FSL-1, a 300-fold-higher concentration of MPPL-1 was required (Fig. 2A). Moreover, similar weak activity of MPPL-1 was also observed when IL-6 production in THP-1 cells was examined (Fig. 2B). In this case, the concentration of FSL-1 needed to induce a level of IL-6 production similar to that induced by 1 μM MPPL-1 was 700-fold lower (Fig. 2B). Thus, the immunostimulatory activity of MPPL-1 is much weaker than the activities of structurally similar lipopeptides.

TLR recognition of MPPL-1. It has been shown that FSL-1 and MALP-2 stimulate human cells via recognition by TLR2 and TLR6 (26, 27, 35). We first examined whether MPPL-1 was recognized by TLR2 using HEK293 cells intrinsically lacking expression of TLR2 and responsiveness to TLR2 ligands (1). MPPL-1 could not stimulate parental HEK293 cells at concentrations ranging from 100 fM to ~10 μM (data not shown) but could stimulate the cells stably transfected with TLR2, leading to induction of NF-κB activation, in a dose-dependent manner (Fig. 3A). Therefore, MPPL-1 recognition was completely dependent on TLR2 in the same way that FSL-1 and MALP-2 recognition was. However, an approximately 1,000-fold-higher concentration of MPPL-1 was re-

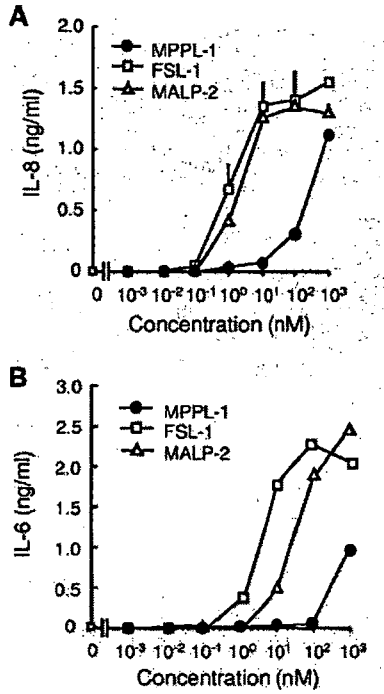

FIG. 2. Cytokine-inducing activity of MPPL-1. A total of 1×10^5 THP-1 cells were stimulated for 12 h with the concentrations of MPPL-1, FSL-1, and MALP-2 indicated. Then the amounts of IL-8 (A) and IL-6 (B) released into the media were determined by ELISA. The results are representative of three separate experiments, and the data are means and standard deviations.

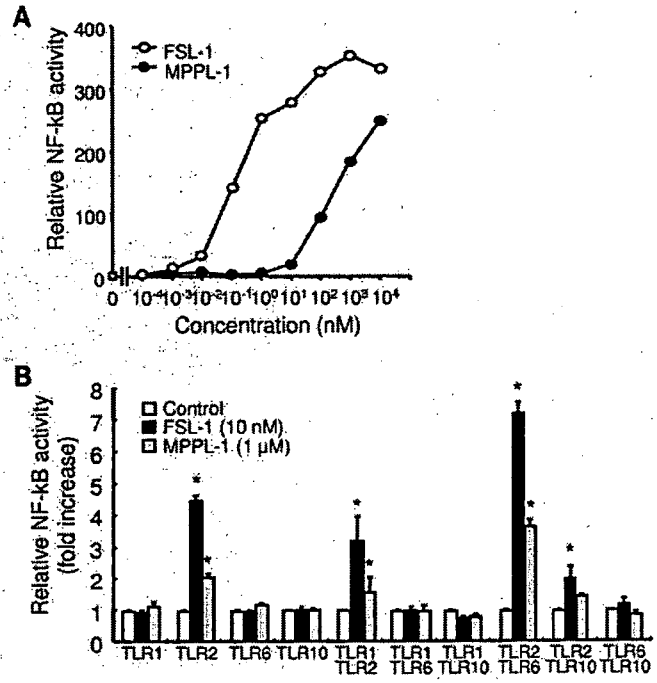

FIG. 3. TLR usage of MPPL-1. (A) HEK293 cells stably transfected with TLR2 were prepared and transiently transfected with an NF-κB-driven firefly luciferase reporter plasmid. The cells were stimulated for 6 h with the concentrations of MPPL-1 and FSL-1 indicated. Then the cells were lysed, and the luciferase activity was measured. The results, expressed as the means of values for triplicate wells, are representative of three separate experiments. (B) HEK293 cells were transiently transfected with an NF-κB-driven firefly luciferase reporter plasmid together with the TLR-encoding plasmids indicated. The cells were stimulated for 6 h with 1 μM MPPL-1 or 10 nM FSL-1. Then the cells were lysed, and the luciferase activity was measured. The results, expressed as means and standard deviations of values for triplicate wells, are representative of three separate experiments. An asterisk indicates that the P value was <0.05 for a comparison with the control group.

quired for activity similar to that of FSL-1 in TLR2-expressing HEK293 cells (Fig. 3A).

We further investigated the requirement for TLR1, TLR6, and TLR10 for recognition of MPPL-1, since TLR2 has been shown to form not only a homomer but also heteromers with these TLRs (29). MPPL-1 could not activate HEK293 cells transfected with TLR1, TLR6, or TLR10 alone (Fig. 3B). Similarly, MPPL-1 could not activate cells transfected with a combination of TLR1 and TLR6, TLR1 and TLR10, or TLR6 and TLR10 (Fig. 3B). Compared with the MPPL-1 activity in the cells transfected with TLR2 alone, cotransfection of TLR6 with TLR2 synergically augmented the activity of MPPL-1 in a way similar to way observed with FSL-1, whereas cotransfection of TLR1 or TLR10 with TLR2 did not (Fig. 3B). Thus, MPPL-1 is preferentially recognized by TLR2/TLR6 in human cells in a manner similar to the recognition of FSL-1 and MALP-2.

Possibility of an antagonistic effect of MPPL-1 on TLR2 recognition. TLR4 recognition of *E. coli* lipopolysaccharide can be antagonized by structurally similar compounds that have weak TLR4-stimulating activities (9, 10, 23, 25). However, it is still not clear whether TLR2 recognition of lipopeptides can be antagonized by structurally similar compounds. The results described above raise the possibility that MPPL-1 has an antagonistic effect on FSL-1 recognition by TLR2/TLR6, because MPPL-1 exhibits a much lower level of activity than FSL-1 exhibits through recognition by TLR2/TLR6. We therefore examined the IL-6-producing activity of FSL-1 in the presence and absence of a higher concentration of MPPL-1. IL-6 production induced by 1 or 10 nM FSL-1 was not altered by the presence of 1 μM MPPL-1 (Fig. 4A). Moreover, the presence of MPPL-1 was found to slightly increase the activity of FSL-1 as determined by analysis of NF-κB activation in HEK293 cells (Fig. 4B), and this analysis was more sensitive than an IL-6 ELISA with THP-1 cells. In addition, the MPPL-1 effect on FSL-1 recognition was not altered in the presence or absence of TLR1, TLR6, or TLR10 cotransfection (Fig. 4B). Similar results were obtained in experiments using MALP-2 (data not shown).

DISCUSSION

We have been interested in the immunostimulatory activity of mycoplasmal diacylated lipoproteins/lipopeptides and the pathological roles of these proteins in mycoplasmal infections. So far, lipopeptides FSL-1 and MALP-2 have been identified as potent immunostimulatory compounds (22, 24). In this study, we synthesized lipopeptide MPPL-1 having a structure common in mycoplasmal lipopeptides, an *S*-dipalmitoylglyceryl cysteine residue coupled to a distinct peptide, which was determined on the basis of paralogous lipoproteins charac-

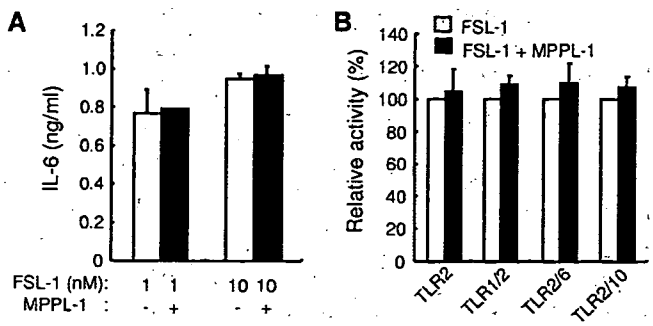

FIG. 4. Antagonistic effect of MPPL-1. (A) A total of 1×10^5 THP-1 cells were stimulated for 12 h with 1 or 10 nM FSL-1 in the presence or absence of 1 μM MPPL-1. Then the amounts of IL-6 released into the media were determined by ELISA. The results, expressed as means and standard deviations, are representative of three separate experiments. (B) HEK293 cells were transiently transfected with an NF-κB-driven firefly luciferase reporter plasmid together with the TLR-encoding plasmids indicated. The cells were stimulated for 6 h with 1 nM FSL-1 in the presence or absence of 100 nM MPPL-1. Then the cells were lysed, and the luciferase activity was measured. The results, expressed as means and standard deviations of values for triplicate wells, are representative of three separate experiments.

teristic of *M. pneumoniae*. The cytokine-inducing activity of MPPL-1 in human cells was very weak compared with that of FSL-1 or MALP-2. At a higher concentration, MPPL-1 could weakly stimulate cells via TLR2/TLR6 recognition. However, MPPL-1 could not antagonize FSL-1 recognition by TLR2. These findings raised several important possibilities for biological activities of mycopalsmal lipopeptides, as discussed below.

Recent studies have revealed that the immunostimulatory activity of bacterial lipoproteins is completely dependent on the recognition and signal transduction by TLR2 that functions together with several associated molecules. TLR6 has been considered to be an essential participant in the discrimination of mycoplasmal diacylated lipoproteins/lipopeptides by TLR2, because MALP-2 recognition was impaired in macrophages from TLR6-deficient mice (36) and was reduced by a blocking antibody to TLR6 in human cells (26). However, Buwitt-Beckmann et al. found that C-terminal addition of SKKKK to the peptide moiety of MALP-2 converted the MALP-2 recognition by TLR2/TLR6 into recognition by a TLR6-independent mechanism (6). In addition, we previously reported that substitution of the C-terminal amino acid of FSL-1 (F to R) greatly impaired the immunostimulatory activity (27). Therefore, discrimination of diacylated lipopeptides by TLR2 and TLR6 has been suggested to be dependent on the amino acid sequence or structure of the peptide portion, although recognition of the lipolyated cysteine residue may be dependent on other molecules, such as CD36 (15). Furthermore, a recent report suggested that TLR1 participates in the recognition of a dipalmitoylated lipoprotein derived from *M. pneumoniae* (MPN602) (33). In this study, MPPL-1 was shown to be recognized by TLR2 and TLR6 but not by TLR1 or TLR10, as observed for MALP-2 and FSL-1. We could not discern a role for TLR10 in the recognition of mycoplasmal lipopeptides, although it is possible that TLR10 participates in accurate discrimination of bacterial lipoproteins/lipopeptides in human cells.

It is possible that studies of TLR antagonists may lead to the development of efficient therapeutic regulators of microbial infection or excess inflammation. In this study, however, MPPL-1 could not antagonize TLR2 recognition of FSL-1 (Fig. 4). The weak TLR2-stimulating activity of MPPL-1 raises the possibility that the peptide moiety of MPPL-1 has a low affinity for TLR6 but does not have an affinity for either TLR1 or TLR10. This possibility may be supported by our results showing that a small amount of FSL-1, which may have a stronger affinity than MPPL-1 has, could be preferentially recognized by TLR2 and TLR6 more than a larger amount of MPPL-1 could be recognized (Fig. 4). Moreover, our results may provide strong evidence for different ligand recognition mechanisms of TLR2 and TLR4, because TLR4 recognition of lipopolysaccharide is known to be antagonized by structurally similar compounds that have weak TLR4-stimulating activities (9, 10, 23, 25). Further study is needed to determine the detailed recognition machinery of mycoplasmal lipoproteins/lipopeptides.

The magnitude of the immunostimulatory activity of bacterial lipoproteins has been thought to be one of the crucial factors for pathogenicity of bacteria (3) which may be involved in the severity of host immune responses after bacterial infection. However, the presence of immunostimulatory compounds on the surface of bacterial cells leads to efficient clearance of bacteria through activation of immune cells, resulting in great reductions in efficient propagation and colonization on the host cell surface. To avoid activation of immune responses, several pathogenic bacteria have been shown to modify their surface molecules so they do not stimulate the TLR recognition system. For example, α- and ε-*Proteobacteria*, including *Campylobacter jejuni*, *Helicobacter pylori*, and *Bartonella bacilliformis*, modify the N-terminal D1 domain of flagellin, leading to evasion of TLR5 recognition (2). Therefore, structural modification of pathogen-activated molecular patterns may be important for bacterial pathogenicity. However, it has not been determined whether *M. pneumoniae* has the ability to evade immune systems. So far, mycoplasmal lipoproteins/lipopeptides have been identified to determine strong activators of immune cells in crude mixtures of lipoproteins obtained using methods such as Triton X-114 phase separation (24, 32, 33). In a recent study performed by Shimizu et al. (33), lipoprotein MPN602, which may have the strongest activities in *M. pneumoniae* lipoprotein mixtures, was identified by using a method to separate the fraction that strongly stimulates 293T cells transfected with TLR2 to activate NF-κB (33). MPN602 does not belong to a paralogous lipoprotein family, as shown in Table S1 in the supplemental material. Interestingly, it was also found that only a few lipoproteins possessed strong immunostimulatory activities and that the majority of lipoproteins had weak or no immunostimulatory activity (24, 32, 33). Consistent with this possibility, only a few lipoproteins with potent immunostimulatory activity have been identified so far, although there are many lipoproteins in mycoplasmal species. These observations suggest that the majority of lipoproteins of *M. pneumoniae*, including paralogous lipoprotein family members, have weak immunostimulatory activities. Moreover, our results suggest that propagation of genes encoding lipoproteins with

weak immunostimulatory activity may be an important factor for the pathogenicity of *M. pneumoniae* through which the microorganism may evade TLR2 recognition. Further detailed investigations of the functions and immunostimulatory activities of lipoproteins found in *M. pneumoniae* are needed to address this possibility.

The bacterial lipoprotein structure has been found to be a lipidated (commonly palmitoylated) triacylated or diacylated *S*-glyceryl cysteine residue coupled to distinct polypeptides. However, the coupled peptide sequence has been shown to have a great effect on the immunostimulatory activity of the whole molecule. Therefore, synthesis and characterization of lipopeptides based on the known lipoprotein sequences of the N terminus may be an effective method for determining unknown biological activities of bacterial lipoproteins. Moreover, exhaustive screening of synthetic lipopeptides can lead to the identification of novel bacterial pathogenicities and to the development of biologically beneficial compounds or immune regulators. Also, it is possible that a cognate ligand for TLR10 will be identified by screening of these lipopeptides.

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Pathogen Recognition by Toll-like Receptor 2 Activates Weibel-Palade Body Exocytosis in Human Aortic Endothelial Cells*

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The endothelial cell-specific granule Weibel-Palade body releases vasoactive substances capable of modulating vascular inflammation. Although innate recognition of pathogens by Toll-like receptors (TLRs) is thought to play a crucial role in promotion of inflammatory responses, the molecular basis for early-phase responses of endothelial cells to bacterial pathogens has not fully been understood. We here report that human aortic endothelial cells respond to bacterial lipoteichoic acid (LTA) and synthetic bacterial lipopeptides, but not lipopolysaccharide or peptidoglycan, to induce Weibel-Palade body exocytosis, accompanied by release or externalization of the storage components von Willebrand factor and P-selectin. LTA could activate rapid Weibel-Palade body exocytosis through a TLR2- and MyD88-dependent mechanism without *de novo* protein synthesis. This process was at least mediated through MyD88-dependent phosphorylation and activation of phospholipase Cγ. Moreover, LTA activated interleukin-1 receptor-associated kinase-1-dependent delayed exocytosis with *de novo* protein synthesis and phospholipase Cγ-dependent activation of the NF-κB pathway. Increased TLR2 expression by transfection or interferon-γ treatment increased TLR2-mediated Weibel-Palade body exocytosis, whereas reduced TLR2 expression under laminar flow decreased the response. Thus, we propose a novel role for TLR2 in induction of a primary proinflammatory event in aortic endothelial cells through Weibel-Palade body exocytosis, which may be an important step for linking innate recognition of bacterial pathogens to vascular inflammation.

The onset of inflammatory responses of vascular endothelial cells plays crucial roles in recruitment of immune cells, thrombus formation, and development of vascular inflammation or atherosclerosis. Early endothelial activation involves dual phases: rapid translocation of P-selectin to the endothelial surface and slower synthesis and expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1).[2] The former process is accompanied by rapid exocytosis of Weibel-Palade bodies, which are endothelial cell-specific storage granules that contain vascular modulators, including von Willebrand factor (VWF), P-selectin, IL-8, eotaxin-3, endothelin-1, CD63/lamp3, osteoprotegerin, and angiopoietin-2 (1, 2). During Weibel-Palade body exocytosis, these proteins are transported to the outside of the cell upon stimulation or vascular damage and may control local or systemic pathobiological effects, including thrombosis and atherogenesis. Regulated Weibel-Palade body exocytosis is known to be initiated through an increase of intracellular calcium level after stimulation with various secretagogues, including calcium ionophores, thrombin, histamine, TNF-α, and extracellular ATP (1, 2).

Recently, excess innate immune responses of vessel walls or endothelium to invading pathogens have been suggested to be linked to atherogenesis. Several common bacterial infectious agents or invasive pathogens, such as *Chlamydia pneumoniae*, *Helicobacter pylori*, *Porphyromonas gingivalis*, and oral commensal bacteria, have so far been detected in vessel walls or atherosclerotic lesions in humans (3, 4). However, the linkage between artery endothelial innate recognition of such pathogens and inflammatory responses has not been fully elucidated.

For the detection of invasive bacteria in host defense, several Toll-like receptors (TLRs) are employed to identify molecular motifs that usually compose bacterial bodies (5). Among TLR members in humans, TLR2 detects the widest range of common bacterial constituents, such as lipoteichoic acids (LTA),

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[2] The abbreviations used are: ICAM-1, intercellular adhesion molecule 1; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; FSL-1, synthetic *S*-dipalmitoylglyceryl-CGDPKHPKSF derived from *Mycoplasma salivarium*; HAEC, human aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; IRAK, IL-1R-associated kinase; LTA, lipoteichoic acid; MALP-2, synthetic *S*-dipalmitoylglyceryl-CGNNDESNISFKEK derived from *Mycoplasma fermentans*; Pam_3CSK_4, synthetic *N*-palmitoyl-*S*-dipalmitoylglyceryl-CSKKKK derived from *E. coli*; PGN, peptidoglycan; PLC, phospholipase C; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR-associated factor; VWF, von Willebrand factor; LPS, lipopolysaccharide; IL-1R, interleukin-1 receptor; siRNA, small interference RNA; ELISA, enzyme-linked immunosorbent assay; PI3K, phosphatidylinositol 3-kinase; IFN, interferon.

peptidoglycans (PGN), bacterial di- or triacylated lipoproteins or lipopeptides, lipoarabinomannans, porins, and fimbriae (5–8). TLR4 and TLR5 contribute to the recognition of only a few bacterial components, *i.e.* LPS and flagellin (9, 10). Because TLR1 and TLR6 participate in the accurate discrimination of molecular structures by TLR2 as coreceptors, several molecules, including CD14, CD36, and LOX-1, further facilitate the interactions of TLR2 with bacterial pathogens (5, 11, 12). After recognition of cognate agonists, endothelial TLRs activate the classic Toll/IL-1R signaling pathway utilizing MyD88 and IL-1R-associated kinase (IRAK)-1, which ultimately activate a TNFR-associated factor (TRAF) 6 complex and IκBs and the release and translocation of active NF-κB to the nucleus. The artery endothelial NF-κB signaling pathways downstream of TLRs are thought to participate in the development of artery inflammatory diseases or atherogenesis through the promotion of the expression of a large number of proinflammatory mediators and adhesion molecules (13–15). However, it is still not known whether artery endothelial TLRs are primary initiators or modulators of the diseases.

In this study, we investigated the early-phase proinflammatory responses of human aortic endothelial cells (HAECs) to bacterial cell wall constituents. We found that recognition of bacterial constituents by TLRs, especially by TLR2 but not TLR4, could activate Weibel-Palade body exocytosis. We further investigated the involvement of MyD88 in regulation of the cell response.

EXPERIMENTAL PROCEDURES

Reagents, Chemicals, and Antibodies—LTA and PGN from *Staphylococcus aureus* and LPS from *Escherichia coli* O26:B6 were obtained from Sigma-Aldrich. Rough-form LPS from *Salmonella minnesota* R595 and flagellin from *Salmonella typhimurium* strain 14028 were obtained from Alexis Biochemicals. Pam_3CSK_4 (16) was obtained from InvivoGen. Preparation of FSL-1 and MALP-2 was described previously (17–19). A23187, the cell-permeable calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), cycloheximide, and the phospholipase C (PLC) γ inhibitor U-73122 were purchased from Sigma. LY294002 was purchased from Calbiochem. Monoclonal antibodies to human TLR2, TL2.1 (BD Biosciences), TL2.3 (eBioscience), and IMG-319 (Immugenex), were purchased for a TLR2 blocking study and flow cytometry. Antibodies to PLCγ1 and phosphorylated PLCγ1 (Y783) were obtained from Cell Signaling Biotechnology. All other reagents were obtained from Sigma-Aldrich unless otherwise indicated.

DNA Cloning—A human TLR2-encoding plasmid was prepared as described previously (17). The dominant negative TLR2 (P681H) was constructed using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Cell Culture and Transfection of siRNA—HEK293 cells and human monocytic THP-1 cells were grown as described previously (20). HAECs and HUVECs were grown in endothelial growth medium-2 (Camblex) as described previously (21). These endothelial cells were used for experiments from passages 4 to 8. All of the gene-specific siRNA oligonucleotides for human TLR1, TLR2, TLR6, MyD88, and IRAK-1 and a control oligonucleotide were purchased from Dharmacon. Although the sequences were not provided by the manufacturer, significant suppressive effects on the respective gene expression could be confirmed by reverse transcription-PCR compared with the control transfection (data not shown). For the transfection of siRNA, confluent HAECs or HUVECs seeded on 6- or 24-well plates were prepared and washed once with Opti-MEM I medium (Invitrogen). Transfection of siRNAs (100 nM) was performed with Lipofectin reagent (Invitrogen) as instructed by the manufacturer. Toxi-Blocker transfection supplement (TOYOBO) was used to prevent cytotoxicity of lipofection reagents. After 12 h of incubation, culture media were changed to endothelial growth medium-2 media, and incubation was continued for 24 h.

Luciferase Reporter Gene Assay—HEK293 cells stably transfected with human TLR2 gene (or mock control vector) were plated at 5×10^4 cells/well in 24-well plates before DNA transfection. The cells were transiently transfected with 50 ng of an NF-κB-driven firefly luciferase reporter plasmid (pNF-κB-Luc, Stratagene) and 5 ng of a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK, Promega). After 12 h of incubation, the cells were transfected with 100 nM siRNA oligonucleotide for MyD88 (or glyceraldehyde-3-phosphate dehydrogenase control). Toxi-Blocker transfection supplement was used to prevent cytotoxicity of lipofection reagents. After a further 24 h of incubation, the cells were stimulated with TLR2 agonists in media containing 1% fetal bovine serum for 6 h. Then the cells were lysed, and luciferase activity was measured as described previously (17, 20).

Determination of VWF, IL-8, and TNF-α by ELISA—HAECs were grown on 24-well plates, then washed and placed in 200 μl of Opti-Mem I (Invitrogen) containing 1% fetal bovine serum without growth factors, and stimulated with various concentrations of TLR2 agonists for 60 min. The amount of VWF released into the medium was measured by a VWF ELISA kit (American Diagnostica) according to the manufacturer's instructions. Results are representative of three separate experiments and expressed as means ± S.D. To clarify the mechanism by which TLR2 induces VWF exocytosis, HAECs were pretreated for 30 min with 10 μM U-73211 and then stimulated with LTA for 60 min. For other experiments, HAECs were pretreated with 10 μM BAPTA-AM for 30 min or 10 ng/ml IFN-γ for 12 h or precultured with $CaCl_2$-free DMEM for 1 h. To determine the amounts of IL-8 released, HAECs were grown on 96-well plates and then washed and placed in 200 μl of Opti-Mem I (Invitrogen) containing 1% fetal bovine serum and stimulated for 4 h with various concentrations of TLR2 agonists. The amounts of IL-8 released into the media were measured by human IL-8 Cytoset (Invitrogen) according to the manufacturer's instructions. THP-1 cells (1×10^5) were stimulated for 6 h with various concentrations of TLR2 agonists. The amounts of TNF-α released into the media were measured by human TNF-α Cytoset (Invitrogen) according to the manufacturer's instructions. Results are representative of three separate experiments and expressed as means ± S.D.