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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*.

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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

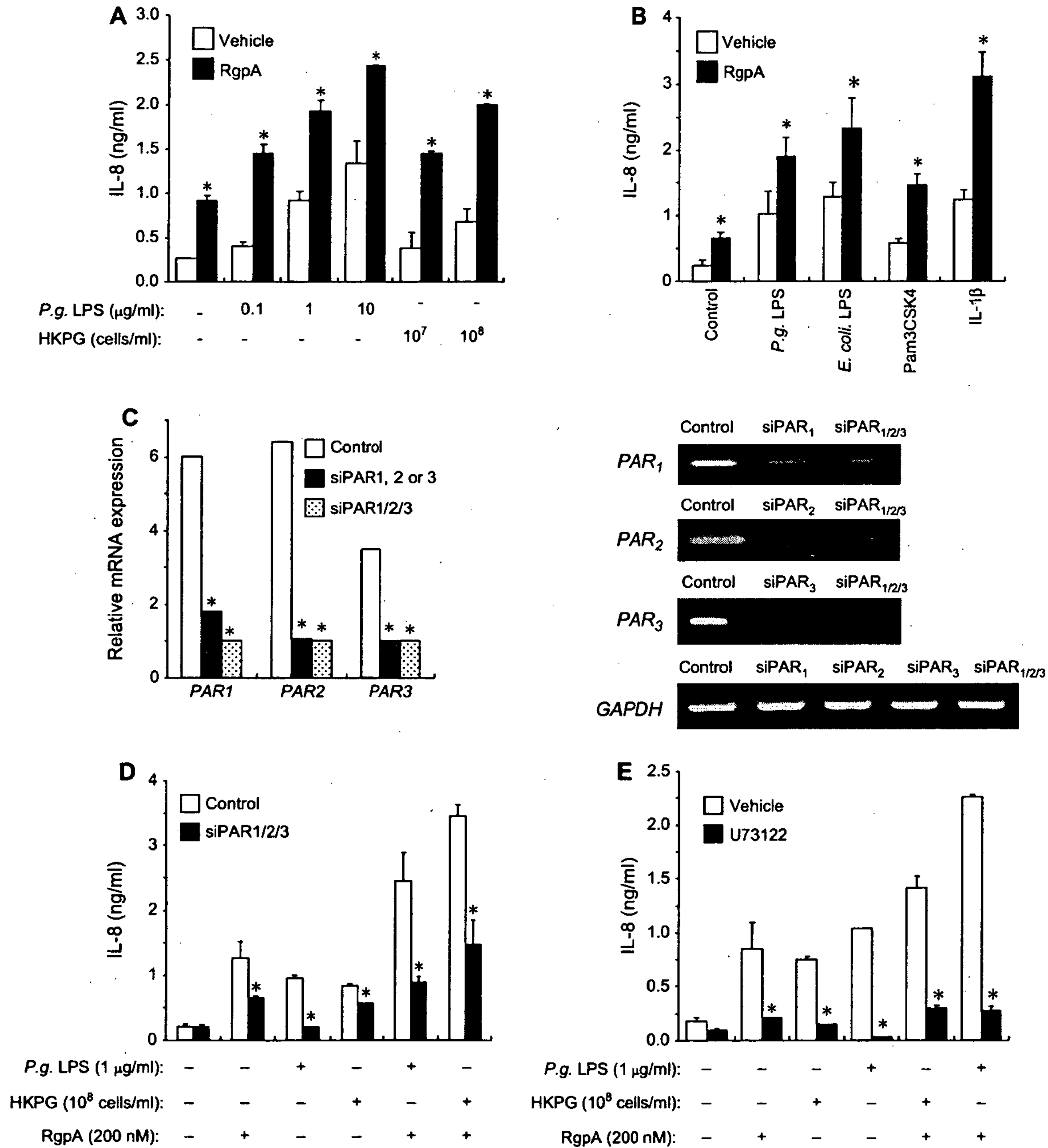

Fig. 1. Effect of RgpA on IL-8 production induced by *P. gingivalis* LPS or HKPG. (A, B) HUVECs were stimulated for 6 h with the indicated concentrations of LPS or HKPG (A) or with *E. coli* LPS (10 ng/ml), *P. gingivalis* LPS (1 μg/ml), Pam_3CSK_4 (0.5 μg/ml) or IL-1β (1 ng/ml) (B) in the presence or 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.

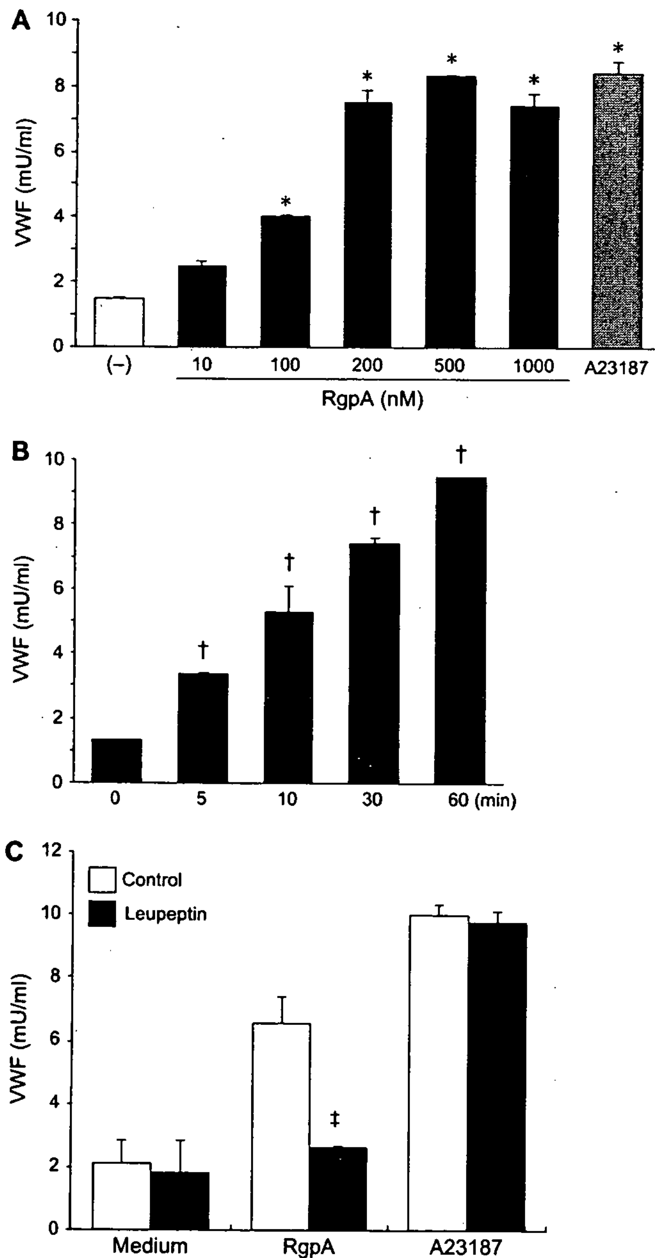

Fig. 2. Induction of WPB exocytosis by RgpA. (A) HUVECs were stimulated for 1 h with RgpA at the indicated concentrations or with 10 μM A23187. (B) HUVECs were stimulated with 200 nM RgpA for the indicated periods. (C) HUVECs were stimulated for 1 h with 200 nM RgpA or 10 μM A23187 in the presence or 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, $P < 0.01$.

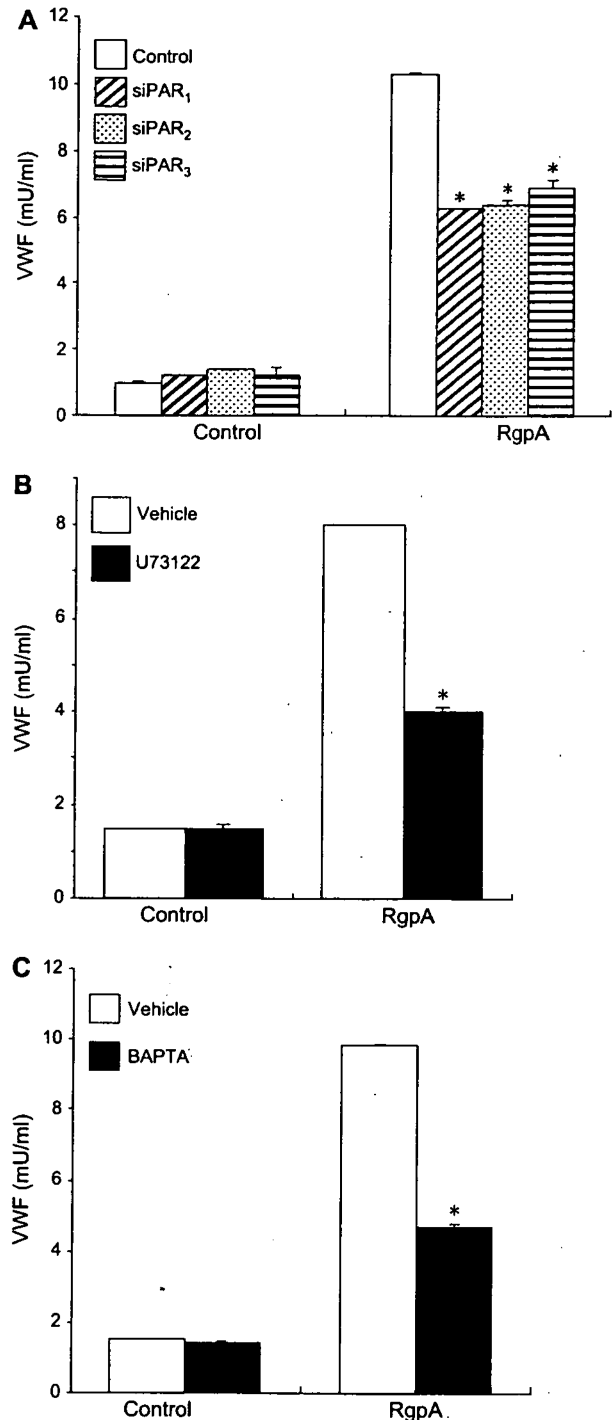

Fig. 3. Regulatory mechanisms of RgpA-activated WPB exocytosis. (A) HUVECs transfected with siRNAs for PAR_1, PAR_2 or PAR_3 were stimulated for 1 h with 200 nM RgpA or 10 μM A23187. (B) HUVECs pretreated for 30 min with 10 μM U73122 were stimulated for 1 h with 200 nM RgpA. (C) HUVECs pretreated for 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,

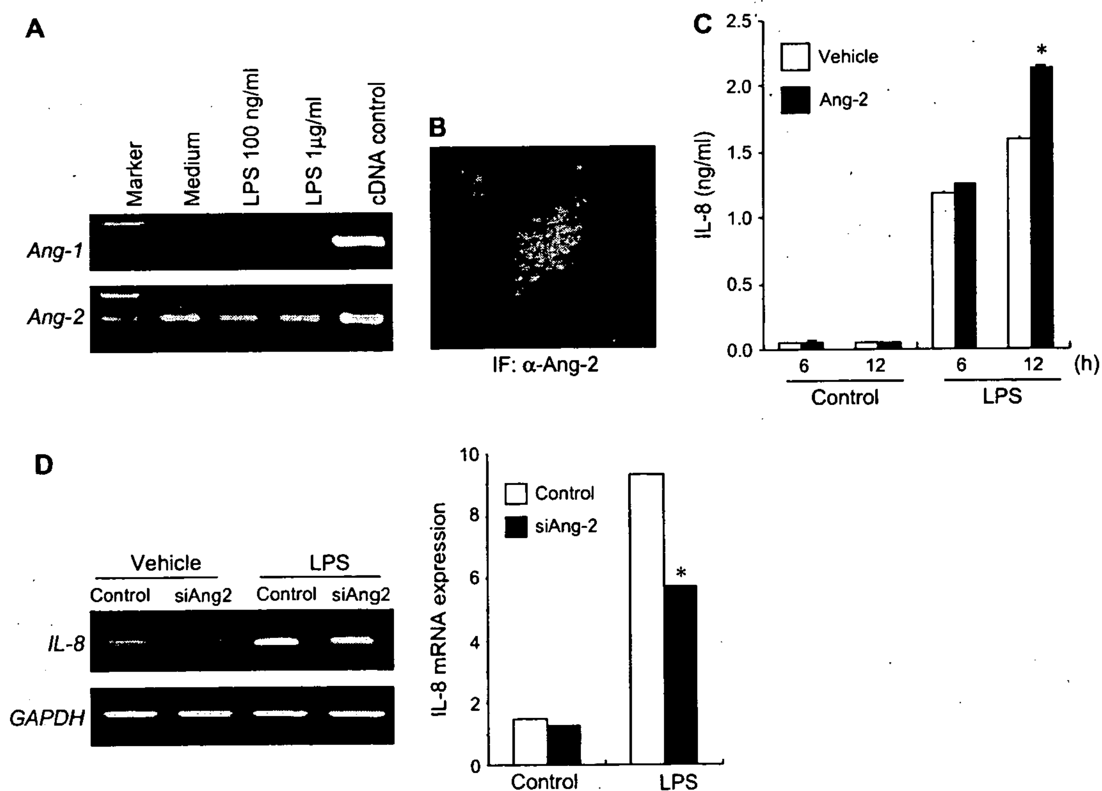

Fig. 4. Effect of Ang-2 on IL-8 production by *P. gingivalis* LPS. (A) Total RNA of HUVECs stimulated for 6 h with or without given concentrations of LPS was extracted for RT-PCR using specific primer sets for Ang-1 or Ang-2. The cDNA of Ang-1 and Ang-2 were used as templates for positive controls. (B) 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, $P < 0.01$.

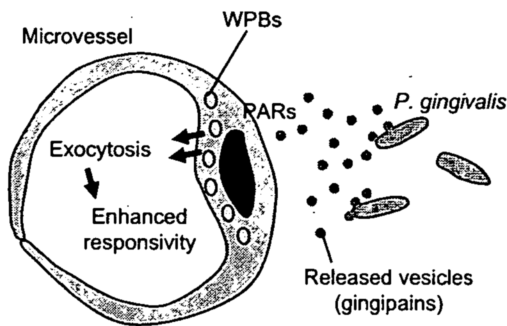

Fig. 5. Schematic of the effect of vesicle-associated RgpA on vascular endothelial cells.

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-a gonistic 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,

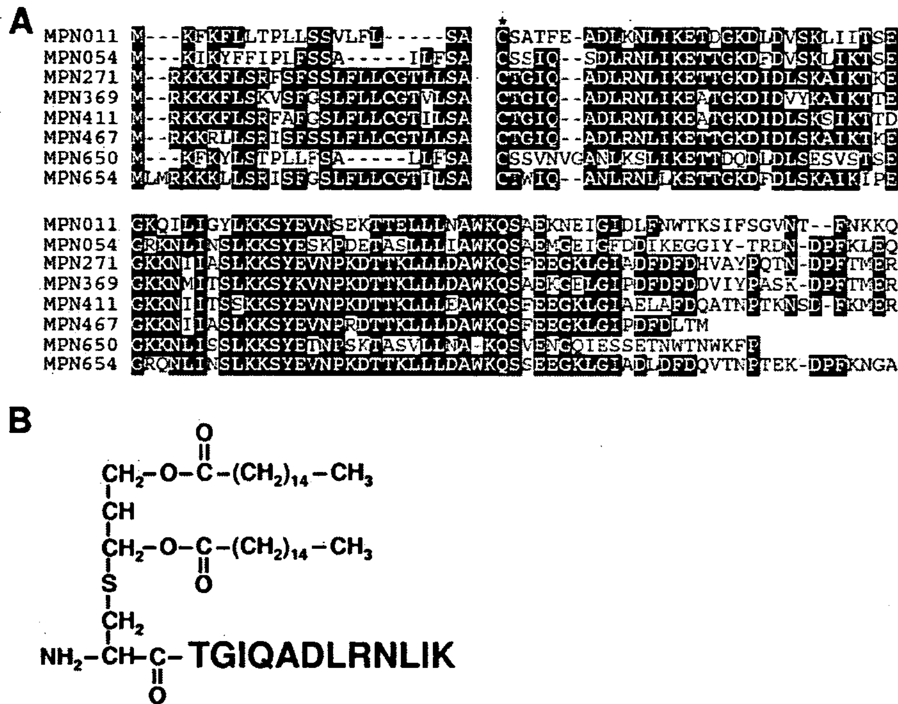

FIG. 1. Synthesis of MPPL-1. (A) Alignment of putative paralogous lipoproteins. The N-terminal sequences of MPN011, MPN054, MPN271, MPN369, MPN411, MPN467, MPN650, and MPN654 were compared. The cysteine residue immediately downstream of the signal peptide is indicated by an asterisk. (B) Structure of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

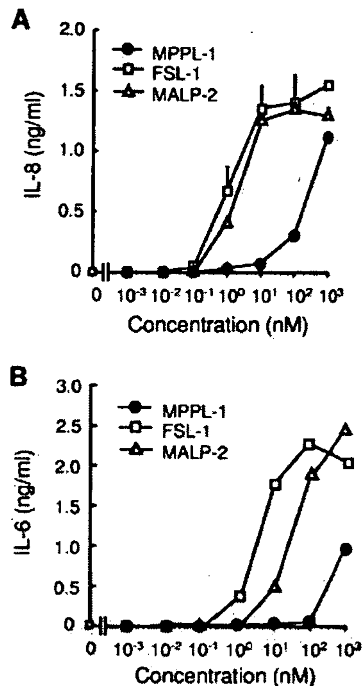

FIG. 2. Cytokine-inducing activity of MPPL-1. A total of 1 × 10^{5} THP-1 cells were stimulated for 12 h with the concentrations of MPPL-1, FSL-1, and MALP-2 indicated. Then the amounts of IL-8 (A) and IL-6 (B) released into the media were determined by ELISA. The results are representative of three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

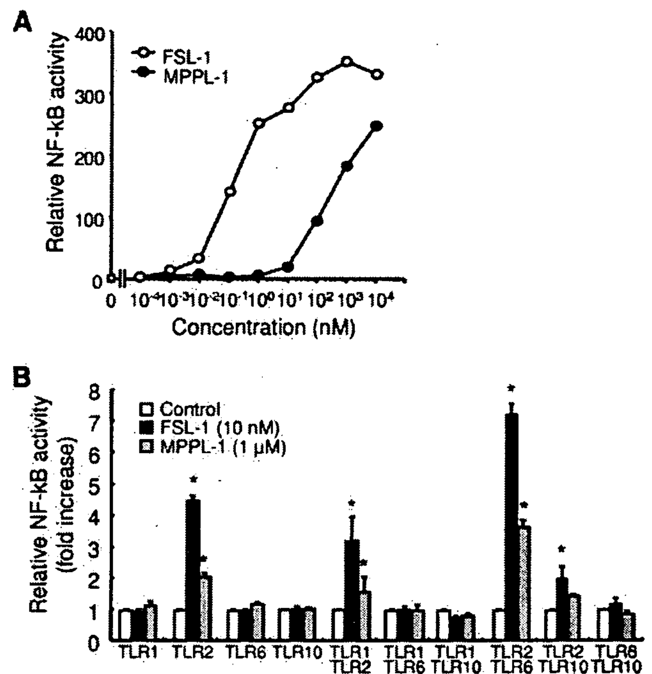

FIG. 3. TLR usage of MPPL-1. (A) HEK293 cells stably transfected with TLR2 were prepared and transiently transfected with an NF-κB-driven firefly luciferase reporter plasmid. The cells were stimulated for 6 h with the concentrations of MPPL-1 and FSL-1 indicated. Then the cells were lysed, and the luciferase 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-

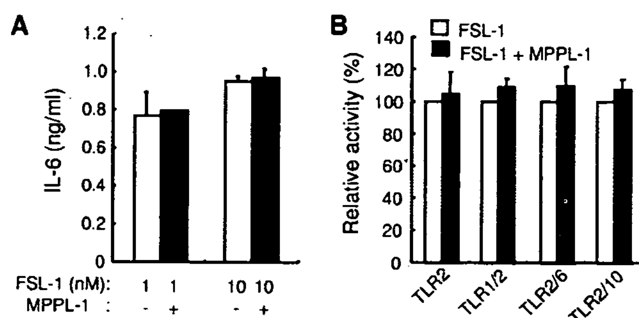

FIG. 4. Antagonistic effect of MPPL-1. (A) A total of 1×10^5 THP-1 cells were stimulated for 12 h with 1 or 10 nM FSL-1 in the presence or absence of 1 μM MPPL-1. Then the amounts of IL-6 released into the media were determined by ELISA. The results, expressed as means and standard deviations, are representative 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 atheroscrelotic 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.

Adhesion Assay—Confluent HAECs seeded on 24-well plates were treated with 10 μg/ml LTA for 60 min. The culture medium was then removed, and monocytic THP-1 cells (2.5 × 10^5) prelabeled with Alexa564-conjugated concanavalin A were added to the culture. Cells were then allowed to adhere for 30 min on a rocking platform. After two washes with phosphate-buffered saline, fluorescent images were immediately obtained by a fluorescent microscope IX71 with DP70 image capture (Olympus) and processed using Adobe Photoshop, version 7.0. Adhesion of red fluorescent cells was quantified in three fields per well. Results are representative of three separate experiments and expressed as means ± S.D.

Immunofluorescence of VWF—Confluent HAECs were treated with 10 μg/ml LTA or 10 μM A23187 for 60 min. The culture media were removed, and the cells were immediately fixed at −20 °C with methanol for 60 min. Immunostaining was carried out using an anti-VWF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa488-conjugated secondary antibody (Invitrogen). Cell nuclei were also stained with 2.5 μg/ml Höechst 33342 for 30 min. Images were obtained by a fluorescent microscope IX71 (magnification: ×40) with DP70 image capture (Olympus) in the presence of the Prolong Gold Antifade reagent (Invitrogen) and processed using Adobe Photoshop, version 7.0 (Adobe). Results are representative of three separate experiments.

Immunoblot Analysis—Confluent HAECs seeded on 60-mm plates were transfected with gene-specific siRNA and incubated in Opti-Mem I media containing 5% fetal bovine serum for 4–6 h. The cells were stimulated with 1 μg/ml LTA for 0–60 min and lysed with a buffer consisting of 20 mM Tris-hydrochloride (pH 7.2), 150 mM sodium chloride, 5 mM EDTA, and 1% Triton X-100 in the presence of protease inhibitors (Roche Applied Science) at 4 °C for 15 min followed by clarification by centrifugation at 12,000 × *g* for 10 min. SDS-PAGE and immunoblot analyses were performed as described previously (17, 20). Results are representative of three separate experiments.

Flow Cytometry—To assess the surface expression of P-selectin, confluent HAECs were treated with 10 μg/ml LTA for 30 min. To assess the surface expression of TLR2, confluent HAECs or HUVECs were treated with 10 ng/ml IFN-γ or they were incubated for 12 h under laminar flow. Cell culture under laminar flow was performed with a cone and plate apparatus as described previously (22). Magnitude of the flow was controlled at ~15 dyn/cm^2. The cells were then removed with phosphate-buffered saline containing 20 mM EDTA and fixed with phosphate-buffered saline containing 4% paraformaldehyde at 4 °C for 60 min. The cells were then incubated at 4 °C for 60 min with anti-TLR2 monoclonal antibody (IMG-319), anti-P-selectin monoclonal antibody (BD Biosciences), or isotype-matched mouse IgG and then with fluorescein isothiocyanate-conjugated anti-mouse IgG. Fluorescence was measured using a FACSCalibur (BD Biosciences).

Statistics—All values were evaluated by statistical analysis using one-way analysis of variance and Student-Newman-Keul's test. Differences were considered to be statistically significant at the level of $p < 0.05$.

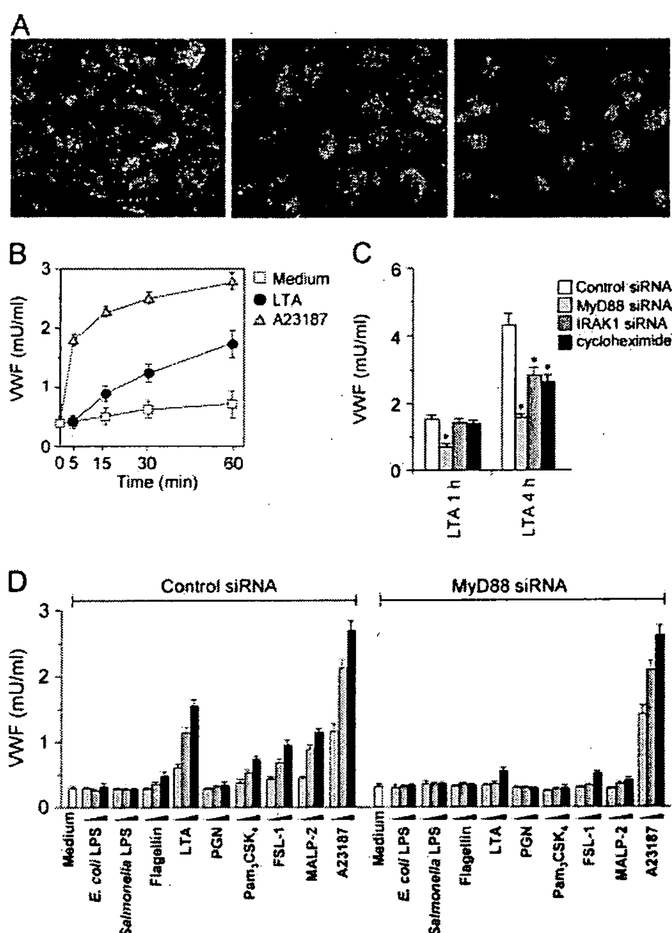

FIGURE 1. **MyD88-dependent Weibel-Palade body exocytosis by bacterial constituents.** *A*, HAECs stimulated with 10 μg/ml LTA or 1 μM A23187 for 60 min were fixed and stained immunofluorescently with anti-VWF antibody (*green*) and with Höechst33342 (*blue*). *Left*, unstimulated; *middle*, stimulated with LTA; *right*, stimulated with A23187. *B*, HAECs were stimulated with 1 μg/ml LTA or 1 μM A23187 for the indicated periods. The amounts of VWF released into the media were measured by ELISA. Each value is the mean ± S.D. (n = 3). *C*, HAECs transfected with MyD88 or IRAK1-specific or control siRNA were prepared. Cells were pretreated with 10 μg/ml cycloheximide for 30 min and then washed and stimulated with 10 μg/ml LTA for 60 min or 4 h. The amounts of VWF released into the media were measured by ELISA. Each value is the mean ± S.D. (n = 3). *, *versus* control group, $p < 0.01$. *D*, HAECs transfected with MyD88-specific or control siRNA were stimulated with *E. coli* LPS O26:B6 (0.01–1 μg/ml), LPS from *S. minnesota* (0.01–1 μg/ml), flagellin from *S. typhimurium* (0.1–10 μg/ml), LTA from *S. aureus* (0.1–10 μg/ml), PGN from *S. aureus* (0.1–10 μg/ml), Pam_3CSK_4 (0.1–10 μg/ml), FSL-1 (0.01–1 μg/ml), MALP-2 (0.01–1 μg/ml), and A23187 (0.1–10 μM) for 60 min, and then the amounts of VWF released into the media were measured. Each value is the mean ± S.D. (n = 3).

RESULTS

Induction of Weibel-Palade Body Exocytosis by Bacterial Constituents—We first examined whether bacterial LTA activated degranulation of Weibel-Palade bodies, because LTA has been reported to stimulate vascular endothelial cells, leading to induction of production of proinflammatory mediators, dysfunction, or cell death (23–25). After stimulation of HAECs for 30 min, LTA clearly decreased the amount of Weibel-Palade bodies, stained with an antibody to VWF, in the cells (Fig. 1*A*). Compared with the calcium ionophore (A23187)-induced response, we found that LTA gradually activated Weibel-Palade body exocytosis, quantification of which was performed by measuring the amount of VWF released into the media (Fig. 1*B*). VWF release by stimulation with

LTA for 60 min was not suppressed by treatment with the protein synthesis inhibitor cycloheximide, whereas the release by stimulation with LTA for 4 h was significantly suppressed by the treatment (Fig. 1C). We further investigated whether LTA induction of exocytosis was mediated through MyD88 and IRAK-1, common signaling molecules downstream of TLRs, because LTA is known as a TLR2 agonist. Interestingly, VWF release by stimulation with LTA for 60 min was suppressed by knockdown of the expression of MyD88 but not that of IRAK-1, whereas the release by stimulation with LTA for 4 h was significantly suppressed by each knockdown of MyD88 and IRAK-1 (Fig. 1C). Thus, these results suggest that LTA can induce Weibel-Palade body exocytosis through a MyD88-dependent rapid mechanism without *de novo* protein synthesis and an IRAK-1-dependent slower mechanism with *de novo* protein synthesis.

We also examined whether other bacterial cell wall constituents, as shown in Table 1, activated induction of VWF release after stimulation of HAECs for 60 min. Among the compounds that we tested, the synthetic analogs of bacterial lipoproteins Pam_3CSK_4, FSL-1, and MALP-2 and, to a lesser extent, flagellin induced VWF release in a dose-dependent manner (Fig. 1D, *left*). Interestingly, LPS from different bacterial species and PGN did not activate Weibel-Palade body exocytosis (Fig. 1D, *left*). In addition, we found that induction of exocytosis by bacterial compounds was also mediated by MyD88 as well as that by LTA (Fig. 1D, *right*). These results suggest that several types of, but not all, bacterial cell wall constituents can activate induction of TLR-MyD88-mediated exocytosis.

TABLE 1
Bacterial cell wall constituents used in this study

Substance	Origin (Ref.)	TLR recognition in human cells (Ref.)
LTA	*S. aureus*	TLR2 (7)
LPS	*E. coli*	TLR4 (10)
LPS	*S. minnesota*	TLR4 (10)
Flagellin	*S. typhimurium*	TLR5 (9)
PGN	*S. aureus*	TLR2 (8)
Pam_3CSK_4	Synthesis (*E. coli*) (16)	TLR1/TLR2 (41)
FSL-1	Synthesis (*M. salivarium*) (18)	TLR2/TLR6 (17)
MALP-2	Synthesis (*M. fermentans*) (19)	TLR2/TLR6 (55)

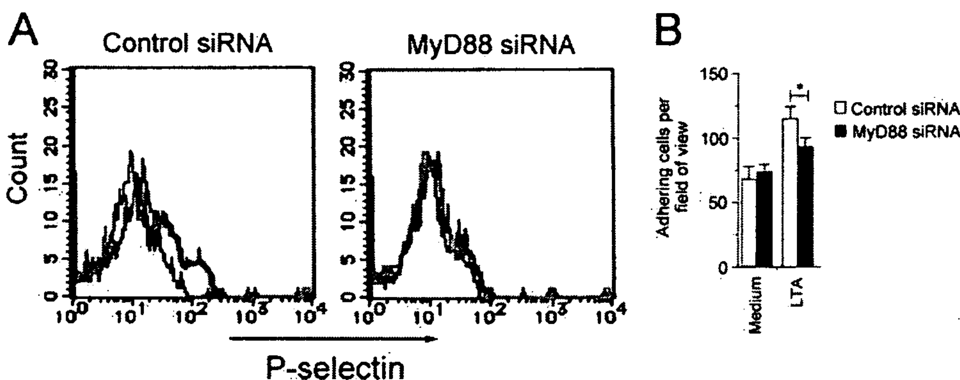

FIGURE 2. **MyD88-dependent P-selectin externalization by LTA.** *A*, HAECs transfected with MyD88-specific or control siRNA were stimulated with 10 μg/ml LTA for 60 min, and then surface P-selectin was detected by flow cytometry. *Shaded histogram*, not stimulated; *gray*, stimulated with LTA. *B*, HAECs transfected with MyD88-specific or control siRNA were stimulated with 10 μg/ml LTA for 60 min, and monocytes stained with conA-Alexa594 were then allowed to adhere for 20 min. Adhesion of red fluorescent cells was quantified in three fields per well by using an image analysis system. Each value is the mean ± S.D. ($n = 3$). *, $p < 0.01$.

Regarding the process of Weibel-Palade body exocytosis, we found that MyD88-dependent externalization of P-selectin was induced after stimulation of HAECs with LTA for 30 min (Fig. 2A). In addition, monocyte adhesion to HAECs was modestly increased in a MyD88-dependent fashion after LTA stimulation for 60 min (Fig. 2B).

Stimulatory Activities of LPS and PGN in HAECs—As stated above, LPS did not activate Weibel-Palade body exocytosis (Fig. 1D). However, LPS potently activated induction of MyD88-dependent IL-8 production in HAECs after stimulation for 4 h (Fig. 3A). Thus, the results shown in Figs. 1D and 3A suggest that endothelial TLR4 lacks the ability to induce rapid Weibel-Palade body exocytosis without *de novo* protein synthesis. Similarly to LPS, PGN did not activate Weibel-Palade body exocytosis (Fig. 1D). Also, PGN did not induce IL-8 production after stimulation for 4 h in HAECs, whereas LTA did (Fig. 3A). However, our preparation of PGN had activities to induce TNF-α production in THP-1 monocytes (Fig. 3B) and TLR2- and MyD88-dependent activation of NF-κB in HEK293 cells (Fig. 3C) in a way similar to that in the case of other TLR2 agonists. These results suggest that HAECs lack the ability to respond to PGN.

Induction of Weibel-Palade Body Exocytosis through TLR2—We then focused on LTA- and bacterial lipopeptide-induced Weibel-Palade body exocytosis. It has been reported that LTA and bacterial lipopeptides are TLR2 agonists (Table 1). In HUVECs, the lipopeptide FSL-1 induced VWF release (Fig. 4A). We found that this response was enhanced by increased expression of TLR2 by gene transfection (Fig. 4A). This result suggests that TLR2 recognition of bacterial constituents directly activates Weibel-Palade body exocytosis. Moreover, transfection of mutated TLR2 (P681H), which lacks the ability to interact with MyD88 (26), suppressed the release (Fig. 4B), consistent with the results presented in Figs. 1D and 2A showing that MyD88 was involved in the induction of Weibel-Palade exocytosis. In HAECs, knockdown of TLR2 expression resulted in almost complete suppression of VWF release by Pam_3CSK_4, FSL-1, MALP-2, and LTA (Fig. 4B). Moreover, knockdown of TLR6 expression resulted in a decrease in the activities of LTA, FSL-1, and MALP-2 and even that of Pam_3CSK_4 (Fig. 4B). In contrast to this, TLR1 interference did not affect VWF release (Fig. 4B), consistent with our observation that HAECs express very low levels of TLR1 mRNA compared with the levels of TLR2 mRNA (data not shown). These results suggest that endothelial recognition of pathogens by TLR2, or to a lesser extent by TLR6, contributes to induction of Weibel-Palade body exocytosis.

Involvement of PLCγ Activation in Weibel-Palade Body Exocytosis—Recent studies have shown that TLR2 signal transduction results in an increase of intracellular calcium level (27, 28). Indeed, we found that the intracellular calcium chelator BAPTA-AM suppressed LTA-induced exocytosis (Fig. 5A). We

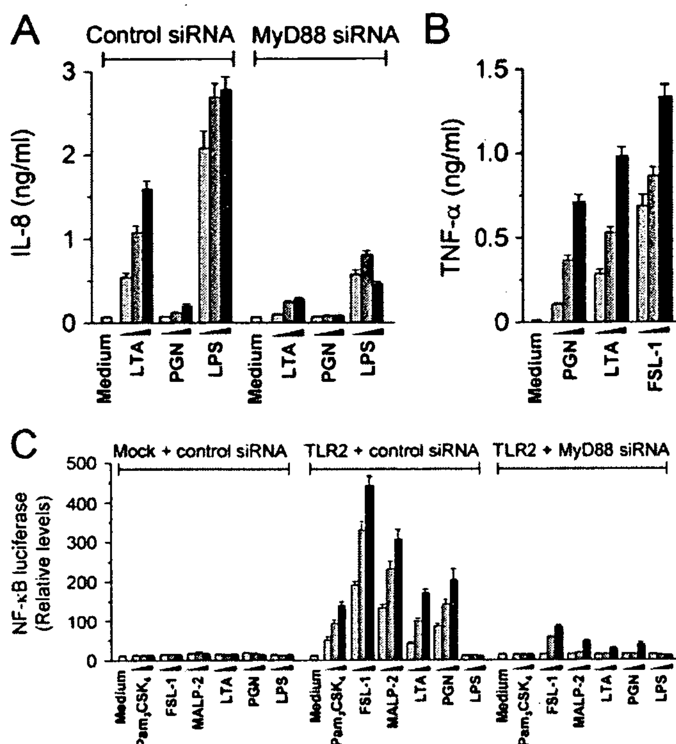

FIGURE 3. **Stimulatory activities of LPS and PGN in HAECs.** *A*, HAECs transfected with MyD88-specific or control siRNA were stimulated with LTA (0.1–10 μg/ml), PGN (0.1–10 μg/ml), and LPS (1–100 ng/ml) for 4 h, and then the amounts of IL-8 released into the media were measured. Each value is the mean ± S.D. ($n = 3$). *B*, THP-1 cells were stimulated with PGN (0.1–10 μg/ml), LTA (0.1–10 μg/ml), and FSL-1 (10–1 μg/ml) for 6 h, and then the amounts of TNF-α released into the media were measured. Each value is the mean ± S.D. ($n = 3$). *C*, HEK293 cells stably expressing TLR2, and control cells were prepared and then transfected with MyD88-specific siRNA and NF-κB-driven luciferase gene. The cells were stimulated with Pam_3CSK_4 (0.1–10 μg/ml), FSL-1 (0.01–1 μg/ml), MALP-2 (0.01–1 μg/ml), LTA (0.1–10 μg/ml), PGN (0.1–10 μg/ml), and LPS (1–100 ng/ml) for 6 h, and then luciferase activity was measured. Each value is the mean ± S.D. ($n = 3$).

therefore examined the role of PLCγ, a common regulator of intracellular calcium release by generating inositol 1,4,5-triphosphate (29), during TLR2-mediated Weibel-Palade body exocytosis. We found that the PLCγ inhibitor U-73122 significantly suppressed TLR2 agonist-induced VWF release (Fig. 5*B*). Because PLCγ isoforms are thought to be activated by phosphatidylinositol 3,4,5-trisphosphate, the product of phosphatidylinositol 3-kinases (PI3Ks) (29), TLR2-mediated exocytosis was suppressed by the chemical inhibitor of PI3K LY294002 (data not shown). However, downstream of TLR/IL-1R, activation of PI3K is regulated through a MyD88-independent machinery (30), conflicting with our results showing that Weibel-Palade body exocytosis requires MyD88 (Figs. 1*D* and 2*A*). Because enzymatic activity of PLCγ is also regulated by tyrosine phosphorylation (31), we tested whether this event was mediated by MyD88. Phosphorylation of PLCγ1 at the Tyr-738 residue was induced by LTA stimulation (Fig. 5*C*). Interestingly, this activity was efficiently suppressed by knockdown of MyD88 expression but not by knockdown of IRAK-1 expression (Fig. 5*C*). MyD88-dependent activation of PLCγ was also observed in TLR2-overexpressed 293 cells used as non-endothelial cells (data not shown). These results suggest that TLR2-mediated rapid Weibel-Palade body exocytosis is regulated by

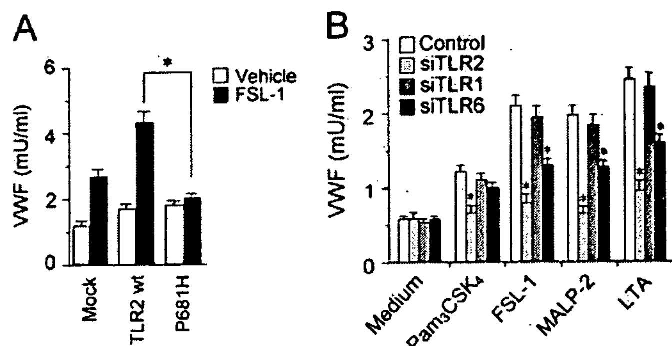

FIGURE 4. **Involvement of TLR2 in Weibel-Palade body exocytosis.** *A*, HUVECs transfected with WT or P681H mutant of TLR2 or with a control plasmid were stimulated with 1 μg/ml FSL-1 for 60 min, and then the amounts of VWF released into the media were measured. Each value is the mean ± S.D. ($n = 3$). *B*, HAECs transfected with TLR1-, TLR2-, or TLR6-specific or control siRNA were stimulated with Pam_3CSK_4 (10 μg/ml), FSL-1 (1 μg/ml), MALP-2 (1 μg/ml), and LTA (10 μg/ml) for 60 min, and then the amounts of VWF released into the media were measured. Each value is the mean ± S.D. ($n = 3$). *, $p < 0.05$.

activation of PLCγ through MyD88-dependent tyrosine phosphorylation.

We also investigated the role of PLCγ in TLR2-mediated NF-κB signaling. U-73122 treatment clearly suppressed TLR2 agonist-induced production of the NF-κB-driven chemokine IL-8 in HAECs (Fig. 5*D*). U-73122 treatment also suppressed LTA-induced phosphorylation and degradation of IκBα in HAECs (Fig. 5*E*). These results suggest that the MyD88-PLCγ pathway also mediates inflammatory responses through NF-κB activation in endothelial cells.

Regulation of TLR2-mediated Weibel-Palade Body Exocytosis—The results shown in Fig. 4 (*A* and *B*) raised the possibility that alteration of endothelial TLR2 expression affects the magnitude of Weibel-Palade body exocytosis. We examined TLR2-mediated exocytosis in the presence of vascular modulators, IFN-γ or laminar flow, which are known to affect TLR2 expression in endothelial cells of human origin. Consistent with the results of a previous study (32), treatment with IFN-γ increased TLR2 expression level in HAECs (Fig. 6*A*). Under this condition, the magnitude of TLR2-mediated exocytosis was significantly increased (Fig. 6*B*). In contrast to this, TLR2 expression slightly decreased in HAECs incubated under laminar flow (Fig. 6*C*), consistent with the results of a previous study (33). We found that laminar flow decreased the magnitude of TLR2-mediated exocytosis (Fig. 6*D*).

DISCUSSION

The major finding of this study is that aortic endothelial cells respond to several bacterial constituents that stimulate TLR2, leading to induction of Weibel-Palade body exocytosis through a MyD88-dependent mechanism without *de novo* protein synthesis. During this process, release of VWF and externalization of P-selectin were induced, by which rolling and adhesion of platelets and leukocytes and thrombus formation in the local vessel walls may be promoted (34, 35). The pathological role of this phenomenon *in vivo* may be support by the observations in mouse experiments, *i.e.* slight increases of local leukocyte-endothelial interaction after LTA administration (36) and soluble P-selectin level in serum after administration of the synthetic