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 of 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 treatment increased TLR2-mediated 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). 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. 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, andextracellular ATP.

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-aminoethylethyl)amine/N,N′,N′,N′-tetraacetic acid-acetoxyethyl ester; FSL-1, synthetic S-dipalmitolglyceryl-CGDHPKPSF 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-dipalmitolglyceryl-CGNDESIFSKEXF derived from Mycoplasma fermentans; Pam3CSK₄, synthetic N-palmitolyl-S-dipalmitolglyceryl-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; sTNF, small interfering RNA; ELISA, enzyme-linked immunosorbent assay; P3K, 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 classic Toll/IL-1R signaling pathway utilizing MyD88 and IRAK-1 and a control oligonucleotide were purchased from Dharmaco. 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-73122 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.

**EXPERIMENTAL PROCEDURES**

Reagents, Chemicals, and Antibodies—LTA and PGN from *Staphylococcus aureus* and LPS from *Escherichia coli* 026: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. Pam3CSK4 (16) was obtained from InvivoGen. Preparation of *Staphylococcus aureus* /H9253 was described previously (17–19). A23187, an ionophore, was purchased from Sigma. LY294002 and IFN-γ from *Salmonella enteritidis* were purchased from PEL (Cambridge). U-73122 was purchased from Calbiochem. Monoclonal antibodies to human TLR2, TLR2.1 (BD Biosciences), TLR2.3 (eBioscience), and IMG-319 (Immugenex), were purchased for a TLR2 block. The dominant negative antigen was expressed by transfection with a TLR2 siRNA, which was 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 Dharmaco. 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. The siRNAs were transfected into the cells, and luciferase activity was measured as described previously (17, 20).

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**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 monocytes THP-1 cells (2.5 × 10⁵) 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 stained with DAPI (4 μg/ml) and Alexa488-conjugated secondary antibody (Invitrogen). Images were captured by a fluorescent microscope IX71 (magnification: ×40) with DP70 image capture (Olympus) 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-HCl, 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/cm². 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 an antibody to VWF, in the cells (Fig. 1A, right) and stimulated with A23187. Each value is the mean ± S.D. (n = 3). C, HAECs transfected with MyD88 or IRAK1-specific or control siRNA were prepared. Cells were treated 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). *

**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. 1A). 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. 1B). 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 Pam3CSK4, 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

| 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)                             |
| Pam3CSK4  | 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) |

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 Pam3CSK4, 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 Pam3CSK4 (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
therefore examined the role of PLC\(\gamma\), 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 PLC\(\gamma\) inhibitor U-73122 significantly suppressed TLR2 agonist-induced VWF release (Fig. 5B). Because PLC\(\gamma\) 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 isoforms (data not shown). However, downstream of TLR/IL-1R, activation of PI3K is regulated through a MyD88-independent mechanism (30), conflicting with our results showing that Weibel-Palade body exocytosis requires MyD88 (Figs. 1D and 2A). Because enzymatic activity of PLC\(\gamma\) is also regulated by tyrosine phosphorylation (31), we tested whether this event was mediated by MyD88. Phosphorylation of PLC\(\gamma\) at the Tyr-738 residue was induced by LTA stimulation (Fig. 5C). Interestingly, this activity was efficiently suppressed by knockdown of MyD88 expression but not by knockdown of IRAK-1 expression (Fig. 5C). MyD88-dependent activation of PLC\(\gamma\) 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 activation of PLC\(\gamma\) through MyD88-dependent tyrosine phosphorylation.

We also investigated the role of PLC\(\gamma\) in TLR2-mediated NF-\(\kappa\)B signaling. U-73122 treatment clearly suppressed TLR2 agonist-induced production of the NF-\(\kappa\)B-driven chemokine IL-8 in HAECs (Fig. 5D). U-73122 treatment also suppressed LTA-driven phosphorylation and degradation of IkB\(\alpha\) in HAECs (Fig. 5E). These results suggest that the MyD88-PLC\(\gamma\) pathway also mediates inflammatory responses through NF-\(\kappa\)B 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-\(\gamma\) 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-\(\gamma\) increased TLR2 expression level in HAECs (Fig. 6A). Under this condition, the magnitude of TLR2-mediated exocytosis was significantly increased (Fig. 6B). In contrast to this, TLR2 expression slightly decreased in HAECs incubated under laminar flow (Fig. 6C), consistent with the results of a previous study (33). We found that laminar flow decreased the magnitude of TLR2-mediated exocytosis (Fig. 6D).

**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 supported 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
lipopeptide FSL-1.3 Sequentially or simultaneously, both PLCγ- and IRAK1-mediated signaling pathways activate NF-κB, by which production of various proinflammatory cytokines, and expression of adhesion molecules such as ICAM-1 are induced to promote adherence and activation of platelets and leukocytes (37). The delayed Weibel-Palade-body exocytosis with de novo protein synthesis is further activated in the cells. Therefore, endothelial TLR2 may be able to function as a primary initiator and a modulator of artery inflammation through these early-phase endothelial responses after recognition of cognate agonists.

We investigated the responsiveness of HAECS toward common bacterial constituents. For the TLR2 agonists, we prepared several compounds that have already been proposed to function as TLR2 agonists, because TLR2 forms a complicated recognition system and because human endothelial cells from different vascular beds show different degrees of responsiveness to TLR2 agonists (32, 38, 39). Unexpectedly, PGN, unlike other TLR2 agonists, could not activate either Weibel-Palade-body exocytosis or IL-8 production (Figs. 1D and 3A). The issue of recognition of PGN by TLR2 is still controversial. The existence of a pathogen-associated molecular pattern (PAMP) of an intracellular receptor for PGN (NOD2) further complicates this matter. However, Gupta’s group recently concluded that PGN is in fact recognized by TLR2 by showing that muramidase treatment of PGN abolished the TLR2-stimulating activity (8). We showed that recognition of our PGN was at least dependent on TLR2 (Fig. 3A). It has been shown that PGN directly binds TLR2 per se (40), whereas bacterial lipopeptides

3 T. Into, Y. Kanno, J. I. Dohkan, M. Nakashima, M. Inomata, K. I. Shibata, C. J. Lowenstein, and K. Katsushita, unpublished data.
TLR2 Mediates Weibel-Palade Body Exocytosis

are thought to directly interact with TLR2-associated molecules such as CD14 and LBP but not with TLR2 per se (7, 41, 42), suggesting the existence of different ligand-recognition mechanisms by TLR2. Furthermore, a novel family of PGN-binding proteins such as peptidoglycan recognition proteins has been found (43) and might enable discrimination of PGN from other TLR2 agonists. Thus, PGN may be recognized by a TLR2 recognition system different from that for LTA and lipoproteins/lipopeptides. Collectively, HAECs express functional TLR2 to respond to several TLR2 agonists, including lipopptides and LTA, but may lack a PGN-recognition system resulting in an inability to respond to PGN. Moreover, aortic endothelial cells may particularly recognize diacylglyceride-containing bacterial lipid derivatives (LTA and bacterial lipopeptides), recognition of which has recently been reported to depend on TLR6 and CD36 (11).

We also showed that the TLR4 agonist LPS did not activate Weibel-Palade body exocytosis (Fig. 1D). Although the reason for this is not clear, several lines of evidence obtained in previous studies may provide an explanation. For example, TLR4 expression has been reported to localize intracellularly in artery endothelial cells (44). This observation suggests that TLR4 in artery endothelial cells may be lacking in induction of phospholipid-dependent signaling events, including PLCγ activation, which are commonly intrinsic to the signaling receptors spanning the cell membrane. Further investigation is needed to determine the reason.

Several properties of endothelial TLR2 have been proposed to be involved in the development of atherosclerosis. First, endothelial TLR2 expression is enhanced by proinflammatory stimuli, such as TNF-α, IFN-γ, and LPS (32), and by SP-1-dependent machinery in areas of disturbed blood flow such as lesion predilection within the aortic tree and heart (33). The expression level of TLR2 is indeed increased in an atherosclerotic lesion in humans (45). Furthermore, a recent study has revealed that complete deficiency of TLR2 in atherosclerosis-prone LDLR-null mice leads to an apparent reduction in the lesion predilection within the aortic tree and heart (33). Thus, our results raise the possibility that bacterial constituent-induced Weibel-Palade body exocytosis can be physiologically or pathologically regulated in particular circumstances of the vessel wall.

In conclusion, our study focused on endothelial exocytosis induced by bacterial pathogens and showed a linkage between endothelial innate recognition of pathogens and early-phase endothelial inflammatory responses. Our results may provide a new insight into the role of endothelial TLR2 in the initiation and modulation of vascular inflammation or atherogenic responses.

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