Original Paper

1,25-Dihydroxyvitamin D₃ Attenuates the Effects of Lipopolysaccharide by Causing ADAM10-Dependent Ectodomain Shedding of Toll-Like Receptor 4

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Key Words

A disintegrin and metalloprotease 10 • 1,25-Dihydroxyvitamin D₃ • Ectodomain shedding • Lipopolysaccharide • L-type calcium channel • Toll-like receptor 4

Abstract

Background/Aims: We investigated how 1,25-dihydroxyvitamin D₃ (1,25D₃) inhibits the effects of lipopolysaccharide (LPS) in human aortic endothelial cells. **Methods:** Cellular signaling was explored by determination of protein abundance with Western blot, measurement of cytosolic Ca²⁺ concentration and immunofluorescence staining for a disintegrin and metalloprotease 10 (ADAM10). **Results:** LPS stimulated the expression of intercellular adhesion molecule 1 (ICAM-1) through toll-like receptor 4 (TLR4) and subsequent activation of p38 mitogen-activated protein kinase (p38 MAPK). Pretreatment with 1,25D₃ attenuated LPS-induced p38 MAPK activation and ICAM-1 expression by causing ectodomain shedding of TLR4. This effect of 1,25D₃ depended on its ability to induce a rapid extracellular Ca²⁺ influx through L-type calcium channels because the ectodomain shedding was prevented by the absence of extracellular Ca²⁺ or the presence of verapamil. TLR4 ectodomain shedding was also induced by Bay K8644 (L-type calcium channel agonist). Both 1,25D₃ and Bay K8644 caused extracellular Ca²⁺ influx-dependent ADAM10 translocation to the cell surface. Depletion of ADAM10 by siRNA transfection prevented 1,25D₃- and Bay K8644-induced ectodomain shedding of TLR4, and abolished the inhibitory effect of 1,25D₃ on LPS-induced ICAM-1 expression. **Conclusion:** 1,25D₃ causes ectodomain shedding of TLR4 and thereby decreases the responsiveness of cells to LPS. ADAM10, activated by extracellular Ca²⁺ influx, was implicated in the ectodomain cleavage of TLR4.

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Introduction

Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria [1]. Gram-negative bacteria are common causes of sepsis in which LPS in the bloodstream stimulates vascular endothelial cells as well as monocytes/macrophages to produce a variety of inflammatory cytokines, causing systemic inflammatory responses, and if severe, multiorgan failure and even death [2].

1,25-Dihydroxyvitamin D₃ (1,25D₃) is the hormone that regulates calcium and phosphate homeostasis [3]. Besides the mineral metabolism, it has anti-inflammatory effects [4, 5]. Studies of cultured cells and animal models have shown that 1,25D₃ attenuates the effects of LPS. In human umbilical vein endothelial cells, preincubation with 1,25D₃ for 30 min inhibited LPS-induced mRNA expressions of interleukin-6 and receptor for advanced glycation end products [6]. In an animal model of sepsis-induced acute kidney injury, 1,25D₃ pretreatment suppressed LPS-induced tubular epithelial cell apoptosis [7]. In another rat experiment, 1,25D₃ pretreatment suppressed LPS-induced elevations of interleukin-12 and interferon-γ in the serum and reduced mortality [8]. In these studies, 1,25D₃ was shown to prevent LPS effects by inhibiting p38 mitogen-activated protein kinase (p38 MAPK) activation [6], by alleviating oxidative stress [7], or by regulation of nuclear factor-κB and Ca²⁺/nuclear factor of activated T-cells signaling pathway [8].

The cellular effects of LPS are initiated by its binding to toll-like receptor 4 (TLR4) on the cell surface [9]. In the bloodstream, LPS-binding protein recognizes LPS and transfers it to cluster of differentiation 14 (CD14). Formation of LPS-CD14 complex facilitates the transfer of LPS to TLR4/myeloid differentiation-2 complex, which activates TLR4 [10]. TLR4 is type I transmembrane protein with a cytoplasmic Toll/interleukin-1 receptor domain that recruits adaptor molecules inducing cascades of signal transduction [9]. In general, the expressions of the cell surface receptors including type I transmembrane protein are regulated by proteolytic cleavage of ectodomain as well as by its synthesis [11].

A disintegrin and metalloproteases (ADAMs) are the enzymes that cleave the extracellular domain of various molecules on the cell surface [11]. Recently, we found that 1,25D₃ and its derivatives activate ADAM10 by inducing extracellular Ca²⁺ influx in human aortic smooth muscle cells [12, 13]. Activation of ADAM10 caused ectodomain shedding of tumor necrosis factor receptor 1, leading to decreased response of the cells to tumor necrosis factor-α. Interestingly, extracellular Ca²⁺ influx was also reported to attenuate the effect of LPS in macrophage. In this study [14], estradiol triggered extracellular Ca²⁺ influx and inhibited LPS-induced p38 MAPK phosphorylation and tumor necrosis factor-α production. The inhibitory effects of estradiol were dependent on extracellular Ca²⁺ influx, as BAPTA-AM, an intracellular Ca²⁺ chelator, prevented these effects. Based on these studies, we hypothesized that extracellular Ca²⁺ influx-activated ADAM10 may cause the ectodomain shedding of TLR4 and thereby diminish the effects of LPS.

In the present study, therefore, we evaluated the effect of 1,25D₃ on LPS-induced intercellular adhesion molecule 1 (ICAM-1) expression in cultured human aortic endothelial cells (HAECs), and investigated whether ectodomain cleavage of TLR4 is implicated in the effect of 1,25D₃.

Materials and Methods

Materials

1,25D₃ was purchased from Tocris Bioscience (Bristol, UK). (±)-Bay K8644 (Bay K8644), SB203580 and dimethyl sulfoxide (DMSO) were from EMD Chemicals (Darmstadt, Germany). LPS (from *Escherichia coli* O111:B4), calcium chloride, verapamil and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were from Sigma Chemical Co. (St. Louis, MO, USA). 1,25D₃, Bay K8644, verapamil and SB203580 were dissolved in DMSO. LPS was dissolved in phosphate buffered saline (PBS). Antibodies to human ICAM-1, ADAM10, TLR4 (H-80: a rabbit polyclonal antibody against amino acids 242 - 321 of human TLR4; 76B357.1: a mouse

monoclonal antibody against amino acids 100 - 200 of human TLR4), p38 MAPK and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-p38 MAPK (Thr180/Tyr182) was from Cell Signaling Technology (Denver, MA, USA). ADAM10-siRNA, control-siRNA (Ambion®) and Fluo-4 AM (Molecular probes®) were from Life Technologies (Paisley, UK).

Cell culture and treatments

HAECs were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA) and cultured in EBM-2 endothelial growth basal medium (Lonza Walkersville, Inc.). After 3-5 passages, the cells were used for the experiments. Before each experiment, the cells were rested in M199 Hank's medium (Life Technologies) containing 1% FBS for 16 h. To perform the experiments at the presence or absence of physiological concentration of extracellular Ca^{2+} (1.10 - 1.35 mM) [15], the medium was replaced with serum-free M199 Hank's medium (Ca^{2+} 1.26 mM) or DMEM media (Life Technologies; Ca^{2+} 0 or 1.2 mM). To examine LPS signaling pathway, 1,25D₃, Bay K8644, verapamil or SB203580 was preincubated for the indicated times and then stimulated with LPS in the presence of these agents.

Transfection of siRNA

TLR4-siRNA, ADAM10-siRNA or a nonspecific, scrambled, control-siRNA was transfected to the cells using Lipofectamine® Reagent (Life Technologies). Briefly, 1×10^5 cells were seeded in each 6-well plate and cultured for 24 h. To prepare siRNA-lipofectamine complexes, siRNAs (100 pmol) were incubated for 15 min with lipofectamine reagent diluted in Opti-MEM® medium (Life Technologies) at room temperature. siRNAs were transfected by adding the siRNA-lipofectamine complexes to the cells in serum-free culture medium and incubating the cells for 6 h at 37 °C in a CO₂ incubator. After siRNA transfection, the cells were cultured for 18 h in EBM-2 endothelial growth basal medium. Thereafter, the cells were rested for 16 h in M199 Hank's medium containing 1% FBS and then subjected to the experiments.

Western blot analysis

To obtain whole cell lysate, the cells were washed with PBS and incubated on ice for 10 min with cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, protease and phosphatase inhibitors). The lysed cells were collected using a plastic cell scraper, transferred to a microcentrifuge tube and centrifuged at 4 °C ($10,000 \times g$) for 20 min, and the supernatant (whole cell lysate) was obtained. The cell lysates containing equal amount of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Merck Millipore Co., Darmstadt, Germany). Then, the membrane was incubated with the primary antibody, followed by washing and further incubation with horseradish peroxidase conjugated secondary antibody. Thereafter, the protein bands were visualized using the enhanced chemiluminescence agent (Luminata™ Forte Western HRP Substrate; Merck Millipore Co.).

To detect TLR4 in culture media of the treated cells, equal amounts of culture supernatant were concentrated 30-fold using Amicon® Ultra 10K device (Merck Millipore Co.). Thereafter, the samples were incubated with 5× sample buffer for 30 min at 37 °C and subjected to immunoblot with anti-TLR4 antibody (76B357.1).

Measurement of cytosolic Ca^{2+} concentration

Fluo-4 AM is permeable to the cell membrane due to the acetoxymethyl (AM) ester. Within the cells, the AM ester is cleaved by esterase in the cytoplasm, and Fluo-4 is entrapped within the cells and becomes fluorescent when bound with Ca^{2+} . To load the Ca^{2+} indicator into the cells, HAECs were incubated with Fluo-4 AM (2 μM) for 30 min. Thereafter, the cells were washed and placed in Hank's balanced salt solution (HBSS; Ca^{2+} 1.2 mM or 0 mM). While fluorescence images (excitation 494 nm, emission 506 nm) were obtained every 10 sec using Zeiss LSM710 laser-scanning confocal microscope (Carl Zeiss, Germany), the cells were treated with 1,25D₃ or Bay K8644. The fluorescence intensity in each cell was measured by ZEN 2011 imaging software (Carl Zeiss, Germany).

Immunofluorescence staining

Cell surface expressions of TLR4 and ADAM10 were examined by immunofluorescent staining as follows. Treated cells were fixed with 4% paraformaldehyde for 10 min (without permeabilization), and

incubated with 1% bovine serum albumin in PBS for 60 min to block nonspecific binding. Thereafter, the cells were incubated with a mouse antibody against the amino acids in the extracellular domain of human TLR4 (76B357.1, 1:100 dilution) or a goat anti-ADAM10 antibody (sc-16524, 1:50 dilution) overnight at 4 °C, washed 3 times with PBS and then incubated with Alexa Fluor 488-conjugated anti-mouse IgG or anti-goat IgG secondary antibody, as appropriately. The fluorescence images were obtained with Zeiss LSM710 laser-scanning confocal microscope.

Statistical analysis

Data are presented as means $\pm$ SE (standard error), with n representing the number of different experiments. An analysis of variance with Scheffe's multiple-comparison test was used to determine statistically significant differences between groups. A p value of <0.05 was considered statistically significant.

Results

1,25D₃ inhibits LPS-induced ICAM-1 expression

First, we tested the effect of 1,25D₃ on LPS-induced ICAM-1 expression. HAECs were treated with LPS (100 ng/ml) for 0, 4, 8 and 24 h. As shown in Fig. 1A, LPS stimulated ICAM-1 expression with a peak at 8 h. In contrast, pretreatment of the cells with 1,25D₃ for 30 min significantly attenuated LPS-induced ICAM-1 expression in a dose-dependent manner, while DMSO (vehicle for 1,25D₃) had no effect (Fig. 1B).

LPS induces ICAM-1 expression through TLR4-p38 MAPK axis

We tested whether TLR4 and p38 MAPK are involved in LPS-induced ICAM-1 expression. Both depletion of TLR4 by siRNA transfection (Fig. 2A) and inhibition of p38 MAPK by SB203580 (Fig. 2B) suppressed LPS-induced ICAM-1 expression, whereas LPS activated p38 MAPK as evidenced by increased phosphorylation of it. On the other hand, siRNA depletion of TLR4 prevented LPS-induced p38 MAPK activation, indicating that p38 MAPK is downstream of TLR4 in LPS signal transduction (Fig. 2C).

1,25D₃ inhibits LPS-induced p38 MAPK activation: involvement of L-type calcium channel

Next, we tested the effect of 1,25D₃ on LPS-induced p38 MAPK activation. Pretreatment of the cells with 1,25D₃ at the doses 1, 10 or 100 nM for 30 min significantly attenuated LPS-induced p38 MAPK activation, in a dose-dependent manner (Fig. 3A).

Fig. 1. 1,25D₃ inhibits LPS-induced ICAM-1 expression. (A) HAECs were incubated with LPS (100 ng/ml) for 0, 4, 8 and 24 h. (B) HAECs were preincubated for 30 min with different concentrations of 1,25D₃ or DMSO (vehicle), and further incubated with LPS (100 ng/ml) for 8 h. Whole cell lysates were immunoblotted with antibody to ICAM-1 or actin. (n=3, *p < 0.05 compared with control; **p < 0.05 as compared with LPS).

Fig. 2. LPS induces ICAM-1 expression through TLR4-p38 MAPK axis. (A) HAECs were transfected with control-siRNA or TLR4-siRNA, and then incubated with or without LPS (100 ng/ml) for 8 h (n=3). (B) HAECs were preincubated for 30 min with different concentrations of SB203580, and further incubated with LPS (100 ng/ml) for 8 h (n=3). (C) HAECs were transfected with control-siRNA or TLR4-siRNA, and then incubated with or without LPS (100 ng/ml) for 15 min (n=5). Whole cell lysates were immunoblotted with antibody to ICAM-1, TLR4, actin, p-p38 MAPK or p38 MAPK. (*p < 0.05 compared with control-siRNA only, **p < 0.05 as compared with control-siRNA plus LPS, #p < 0.05 as compared with control, ##p < 0.05 as compared with LPS).

Fig. 3. 1,25D₃ inhibits LPS-induced p38 MAPK activation: involvement of L-type calcium channel. (A) HAECs were preincubated for 30 min with different concentrations of 1,25D₃, and further incubated with LPS (100 ng/ml) for 15 min. (B) HAECs were preincubated with or without verapamil (1 μM) for 2 min, followed by incubation with or without 1,25D₃ (100 nM) or Bay K8644 (1 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 15 min. Whole cell lysates were immunoblotted with antibody to p-p38 MAPK or p38 MAPK. (n=3, *p < 0.05 compared with control; **p < 0.05 as compared with LPS; #p < 0.05 as compared with 1,25D₃ + LPS).

Because 1,25D₃ is known to activate L-type calcium channels [16], we tested the effect of verapamil, an L-type calcium channel inhibitor, on 1,25D₃ inhibition of LPS-induced p38 MAPK activation and the effect of Bay K8644, an L-type calcium channel activator, on LPS-induced p38 MAPK activation. The cells were preincubated with or without verapamil for

Fig. 4. 1,25D₃ stimulates extracellular Ca²⁺ influx through L-type calcium channels. HAECs were loaded with Fluo-4 AM (2 μM) and preincubated with or without verapamil (1 μM) for 2 min. While fluorescent images of the cells were obtained every 10 sec, the cells were treated with DMSO (vehicle), 1,25D₃ (100 nM) or Bay K8644 (1 μM), as indicated by arrows. The experiments were performed on the cells in 1.2 mM Ca²⁺-containing HBSS or Ca²⁺-free HBSS. The fluorescence intensity in each cell representing cytosolic Ca²⁺ concentration was measured for 6 min. (mean ± SE of n = 106 ~134 cells in each group from three independent experiments).

2 min and then further incubated with 1,25D₃, or incubated with Bay K8644 for 30 min. Thereafter, the cells were stimulated with LPS for 15 min. As shown in Fig. 3B, verapamil attenuated the inhibitory effect of 1,25D₃ on LPS-induced p38 MAPK activation. Conversely, Bay K8644 inhibited LPS-induced p38 MAPK activation.

1,25D₃ stimulates extracellular Ca²⁺ influx through L-type calcium channels

Because verapamil inhibited the effect of 1,25D₃ while Bay K8644 mimicked 1,25D₃, L-type calcium channel-mediated extracellular Ca²⁺ influx may have a critical role in the action of 1,25D₃. In the following experiments, we checked whether 1,25D₃ induces extracellular Ca²⁺ influx through L-type calcium channels in HAECs, using Fluo-4 AM, a calcium fluorescent indicator. In the presence of physiological extracellular Ca²⁺ (1.2 mM), 1,25D₃ induced a significant increase in the cytosolic Ca²⁺ concentration, while DMSO (vehicle) did not (Fig. 4). Cytosolic Ca²⁺ concentration began to rise rapidly and reached a maximum within a minute after 1,25D₃ treatment, and then declined with time. In the absence of extracellular Ca²⁺, however, 1,25D₃-induced increase in cytosolic Ca²⁺ was minimal, which indicates that cytosolic Ca²⁺ was elevated mainly by extracellular Ca²⁺ influx. To determine whether the extracellular Ca²⁺ influx occurs through L-type calcium channels, we tested the effects of verapamil and Bay K8644 on the extracellular Ca²⁺ influx. As shown in Fig. 4, pretreatment with verapamil greatly suppressed 1,25D₃-induced Ca²⁺ influx. In contrast, Bay K8644 increased cytosolic Ca²⁺, while this increase was suppressed by pretreatment with verapamil.

Extracellular Ca²⁺ influx causes ectodomain shedding of TLR4

To test whether 1,25D₃ causes ectodomain shedding of TLR4, we incubated HAECs with 1,25D₃ (100 nM) for 0, 2.5, 5, 10, or 30 min and obtained the conditioned media and whole cell lysates. TLR4 in the cell lysate was measured by Western blot using a polyclonal antibody against TLR4. As shown in Fig. 5A, 1,25D₃ decreased TLR4 in the cell lysates in a time-dependent manner. To determine whether this reduction was caused by ectodomain shedding of the receptor, the conditioned medium was subjected to Western blot using a monoclonal antibody against the epitope in the N-terminus of TLR4. In the culture medium, the antibody detected a ~48 kDa N-terminal fragment of TLR4. 1,25D₃ increased the TLR4 fragment in the culture medium in a time-dependent manner.

Fig. 5. Extracellular Ca²⁺ influx causes ectodomain shedding of TLR4. (A) HAECs in M199 Hank's media (Ca²⁺ 1.26 mM) were incubated with 1,25D₃ (100 nM) for different times (n=3). (B) HAECs in M199 Hank's media (Ca²⁺ 1.26 mM) were preincubated with or without verapamil (1 μM) for 2 min, followed by incubation with different concentrations of 1,25D₃ or Bay K8644 (1 μM) for 30 min (n=3). (C) HAECs were incubated with 1,25D₃ (100 nM) for 30 min in 1.2 mM Ca²⁺-containing DMEM media or Ca²⁺-free DMEM media (n=4). Cell lysates and culture media were analyzed by Western blotting using a polyclonal anti-TLR4 antibody (cell lysate) or a monoclonal antibody directed against the N-terminal region of human TLR4 (culture media). *p < 0.05 compared with control; **p < 0.05 as compared with 1,25D₃ (100 nM).

Next, we examined ectodomain shedding of TLR4 at different doses of 1,25D₃ and the involvement of L-type calcium channel in this effect. HAECs were preincubated with or without verapamil (1 μM) for 2 min and then further incubated with different doses of 1,25D₃ (0.1, 1, 10 or 100 nM) or Bay K8644 (1 μM) for 30 min. TLR4 was measured in the conditioned media and whole cell lysates. 1,25D₃ caused ectodomain shedding of TLR4 in a dose-dependent manner. 1,25D₃-induced ectodomain shedding of TLR4 was abrogated by verapamil. Like 1,25D₃, Bay K8644 decreased cellular TLR4 with accompanying increase in TLR4 fragment in the culture supernatant (Fig. 5B).

To determine whether extracellular Ca²⁺ is required for 1,25D₃-induced ectodomain shedding of TLR4, we tested the effect of 1,25D₃ on the cells in Ca²⁺-free media. As shown in Fig. 5C, the absence of extracellular Ca²⁺ markedly reduced 1,25D₃-induced ectodomain shedding of TLR4.

We next examined the effect of 1,25D₃ and Bay K8644 on cell surface expression of TLR4 by immunofluorescent staining. Treated cells were fixed without permeabilization of the cell membrane, and immunostained using a mouse antibody against the amino acids in the extracellular domain of human TLR4 (76B357.1). In our previous study [17], the specificity of this antibody was validated as the expression of TLR4 on the cell surface was markedly reduced in the cells depleted of TLR4 by siRNA transfection. As shown in Fig. 6, both 1,25D₃ and Bay K8644 reduced TLR4 on the cell surface while this effect was prevented by verapamil and the absence of extracellular Ca²⁺. The findings are consistent with the data of Western blotting.

Fig. 6. 1,25D₃ induces a marked reduction in TLR4 at the cell surface. The experiments were performed on the cells in 1.2 mM Ca^{2+} -containing DMEM media or Ca^{2+} -free DMEM media, as indicated. HAECs were preincubated with or without verapamil (1 μM) for 2 min and then incubated for 30 min with DMSO (control), 1,25D₃ (100 nM) or Bay K8644 (1 μM). Thereafter, the cells were fixed with 4% paraformaldehyde and not permeabilized. Fixed cells were then immunostained for TLR4 using a monoclonal antibody directed against the N-terminal region of human TLR4, and visualized by confocal microscopy. Cell nuclei were stained with DAPI. Representative images from three independent experiments with similar results are shown.

Fig. 7. 1,25D₃ increases ADAM10 cell surface expression by inducing extracellular Ca^{2+} influx. The experiments were performed on the cells in 1.2 mM Ca^{2+} -containing DMEM media or Ca^{2+} -free DMEM media, as indicated. HAECs were preincubated with or without verapamil (1 μM) for 2 min and then incubated for 10 min with DMSO (control), 1,25D₃ (100 nM) or Bay K8644 (1 μM). Thereafter, the cells were fixed with 4% paraformaldehyde, and not permeabilized. Fixed cells were then immunostained for ADAM10, and visualized with confocal microscopy. Cell nuclei were stained with DAPI. Representative images from three independent experiments with similar results are shown.

Fig. 8. ADAM10 is implicated in 1,25D₃-induced ectodomain shedding of TLR4. (A,B) Depletion of ADAM10 abolishes 1,25D₃- or Bay K8644-induced ectodomain shedding of TLR4. HAECs were transfected with control-siRNA or ADAM10-siRNA, and then incubated with or without 1,25D₃ (100 nM) (A) or Bay K8644 (1 μM) (B) for 30 min. Cell lysates and culture media were analyzed by Western blotting using a polyclonal anti-TLR4 antibody (cell lysate) or a monoclonal antibody directed against the N-terminal region of human TLR4 (culture media). (n=3, *p < 0.05 compared with control). (C) Depletion of ADAM10 abolishes the inhibitory effect of 1,25D₃ on LPS-induced ICAM-1 expression. HAECs transfected with control-siRNA or ADAM10-siRNA were preincubated with or without 1,25D₃ (100 nM) for 30 min and further incubated with LPS (100 ng/ml) for 8 h. Whole cell lysates were immunoblotted with antibody to ICAM-1, ADAM10 or actin. (n=3, *p < 0.05 compared with control).

Ectodomain shedding of TLR4 is mediated by ADAM10

Because calcium influx induced by 1,25D₃ was shown to activate ADAM10 in our previous study [12, 13], we investigated the role of ADAM10 in 1,25D₃-induced ectodomain shedding of TLR4. We first assessed cell surface expression of ADAM10 by immunofluorescent staining in nonpermeabilized HAECs (Fig. 7). ADAM10 was weakly detected in HAECs. On treatment with 1,25D₃ for 10 min, there was a marked increase in ADAM10 immunoreactivity at the cell surface. In the absence of extracellular Ca²⁺, however, 1,25D₃ failed to increase ADAM10

expression. Preincubation of the cells with verapamil for 2 min before 1,25D₃ treatment also abolished the increase in ADAM10 cell surface expression. Like 1,25D₃, Bay K8644 increased ADAM10 cell surface expression, while this effect was prevented by preincubation with verapamil.

Though treatment of the cells with 1,25D₃ or Bay K8644 markedly increased ADAM10 at the cell surface in 10 min, it had no such effect on ADAM10 in the whole cell lysates (Fig. 8). It suggests that the rapid increase in cell surface expression of ADAM10 occurs by translocation of ADAM10 to the cell surface from the intracellular pool.

Next, we examined the effect of specific depletion of ADAM10 on 1,25D₃-induced ectodomain shedding of TLR4. To deplete ADAM10, we transfected the cells with control-siRNA or ADAM10-siRNA, and treated them with 1,25D₃ or Bay K8644. Depletion of ADAM10 markedly decreased 1,25D₃- and Bay K8644-induced ectodomain shedding of TLR4 (Fig. 8A & 8B).

We further investigated whether depletion of ADAM10 reverses the inhibitory effect of 1,25D₃ on LPS-induced ICAM-1 expression. As shown in Fig. 8C, 1,25D₃ had little effect on LPS-induced ICAM-1 expression when ADAM10 was depleted.

Discussion

In HAECs, pretreatment of the cells with 1,25D₃ inhibited LPS-induced ICAM-1 expression. As the mechanism for it, our data demonstrated that 1,25D₃ attenuates the responsiveness of the cells to LPS by causing ectodomain shedding of TLR4. 1,25D₃ rapidly induced extracellular Ca²⁺ influx through L-type calcium channels, and the elevation of cytosolic Ca²⁺ led to the translocation of ADAM10 to the cell surface where TLR4 is located. ADAM10 was implicated in the ectodomain shedding of TLR4.

Enzymatic cleavage of cell surface receptor is important for the regulation of biologic response of cells [18]. The new finding in the present study was that TLR4, the receptor for LPS, undergoes ectodomain shedding upon stimulation with 1,25D₃. TLR4 belongs to type I transmembrane protein [9]. Cell membrane receptors like type I transmembrane protein are commonly subjected to proteolytic cleavage, resulting in shedding of the ectodomain portion of it. The released ectodomain fragments of the receptors are detected as soluble receptors in the supernatant of cultured cells and body fluid. Soluble receptors can also be generated by release of full-length receptor as a component of exosome-like vesicles or by alternative splicing of mRNA transcripts [19]. Like other cell surface receptors, TLR4 was found as soluble receptor in the supernatant of cultured peripheral blood mononuclear cells [20], plasma [20, 21], amniotic fluid [22], saliva [23] and pleural fluid [24], but it is obscure how soluble TLR4 is formed. The reported sizes of TLR4 fragments were different, suggesting that different enzymes may be involved in this process. So far, neutrophil elastase and prostatic (a serine protease) have been identified to cleave the extracellular region of TLR4 [25, 26]. The present study clearly demonstrates that 1,25D₃ causes ectodomain shedding of TLR4. 1,25D₃ decreased cellular TLR4 with accompanying increase in TLR4 immunoreactivity in the culture supernatant. TLR4 in the culture supernatant detected by the monoclonal antibody recognizing the N-terminus of TLR4 was ~ 48 kDa in size, which is smaller than the full-length TLR4 (95 kDa) and indicates a fragment of TLR4 containing N-terminal portion produced by cleavage of the ectodomain region. 1,25D₃-induced ectodomain shedding of TLR4 was also supported by the reduction in cell surface expression of TLR4 following exposure to 1,25D₃ in the immunofluorescent staining.

As in other studies [27, 28], LPS stimulated the expression of ICAM-1 in HAECs. This effect of LPS was mediated by TLR4 and subsequent p38 MAPK activation. Besides inducing ectodomain shedding of TLR4, 1,25D₃ suppressed LPS-induced p38 MAPK activation and ICAM-1 expression, the downstream steps of TLR4. Thus, the ectodomain shedding of TLR4 was accompanied by a decrease in the receptor response.

1,25D₃-induced extracellular Ca²⁺ influx through L-type calcium channels has been demonstrated in osteoblasts [29] and vascular smooth muscle cells [12, 13]. 1,25D₃-membrane associated rapid response steroid-binding receptor (endoplasmic reticulum stress protein 57) mediated this effect of 1,25D₃ [13]. Likewise, 1,25D₃ induced L-type calcium channel-mediated extracellular Ca²⁺ influx in HAECs. 1,25D₃-induced ectodomain shedding of TLR4 was dependent on the extracellular Ca²⁺ influx as it was greatly suppressed by verapamil and the absence of extracellular Ca²⁺. In addition, L-type calcium channel agonist Bay K8644 mimicked 1,25D₃ by causing ectodomain shedding of TLR4.

Of 12 proteolytically active ADAMs identified, ADAM10 is known to be activated by elevated cytosolic Ca²⁺ [30, 31]. The present study indicated that the extracellular Ca²⁺ influx-dependent ectodomain cleavage of TLR4 is mediated by ADAM10. To cleave the ectodomain of TLR4, the responsible sheddase has to be located at the cell surface. In untreated cells, ADAM10 was weakly detected at the cell surface. On treatment with 1,25D₃, ADAM10 was markedly increased at the cell surface by translocation from the intracellular pool. ADAM10 translocation was dependent on extracellular Ca²⁺ influx because 1,25D₃ failed to translocate ADAM10 in the absence of extracellular Ca²⁺ or after preincubation with verapamil. Furthermore, the L-type calcium channel agonist Bay K8644 also increased ADAM10 translocation to the cell surface while verapamil inhibited this effect. On the other hand, specific depletion of ADAM10 by transfection of ADAM10-siRNA prevented both 1,25D₃- and Bay K8644-induced ectodomain shedding of TLR4 and abolished the suppressive effect of 1,25D₃ on LPS-induced ICAM-1 expression.

Ectodomain shedding of a receptor commonly result in a decreased response of the cells to the ligand as the number of binding receptor is decreased on the cell surface. In addition, the released ectodomain of the receptor may be able to bind the ligand and thereby acts as a decoy receptor [18]. On the contrary, the cleavage of ectodomain may cause constitutive activation of the receptor by removal of an inhibitory domain [32]. As noted above, ADAM10-mediated ectodomain shedding of TLR4 resulted in a significant decrease in the response of cells to LPS in terms of p38 phosphorylation and ICAM-1 expression. In HepG2 cells [26], the ectodomain cleavage of TLR4 mediated by the serine protease prostasin was also shown to decrease the receptor activity. In contrast, the ectodomain cleavage of TLR4 mediated by neutrophil elastase in macrophage was accompanied by an increased production of inflammatory cytokines [25]. However, it is not clear whether the stimulatory effect on cytokine production was caused by ectodomain shedding of TLR4 or by other unknown mechanisms including secondary mediators produced by neutrophil elastase. In another study [32], the deletion of ectodomain at specific sites was shown to cause constitutive activation of the receptor. TLR4 consists of an extracellular domain of 624 amino acids (residues 1-624), a transmembrane domain of 33 amino acids (residues 625-658) and a cytoplasmic domain of 178 amino acids (residues 659-838) [9]. The transmembrane and cytoplasmic domains of TLR4 were shown to have an intrinsic dimerization propensity leading to activation of the receptor, while the ectodomain inhibited the spontaneous receptor dimerization [32]. The inhibitory part of the ectodomain was the N-terminal amino acid residues between 542 and 563 of TLR4 because deletion of N-terminal amino acid residues 563 or 569 caused constitutive activation of the receptor, while deletion of amino acid residues 542 or its distal did not. The fragment of TLR4 deleted of N-terminal amino acid residues 542 was about ~ 35 kDa. Considering the whole size is 95 kDa, the deleted ectodomain is estimated to be ~ 60 kDa in size. Accordingly, deletions of amino acid residues 563 or 569 causing constitutive activation is expected to produce the ectodomain fragment larger than this size. In the present study, N-terminal fragment of TLR4 produced by 1,25D₃ treatment was detected at the size of ~ 48 kDa, suggesting that the cleavage occurs distal to the inhibition domain of the receptor.

In summary, 1,25D₃ causes ectodomain shedding of TLR4 in HAECs and thereby decreases the responsiveness of the cells to LPS. ADAM10, activated by extracellular Ca²⁺ influx, was implicated in the ectodomain cleavage of TLR4.

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Disclosure Statement

The authors declare no potential conflict of interest.

References

- Heine H, Rietschel ET, Ulmer AJ: The biology of endotoxin. *Mol Biotechnol* 2001;19:279-296.
- Shukla P, Rao GM, Pandey G, Sharma S, Mittapelly N, Shegokar R, Mishra PR: Therapeutic interventions in sepsis: current and anticipated pharmacological agents. *Br J Pharmacol* 2014;171:5011-5031.
- DeLuca HF: Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr* 2004;80:1689S-1696S.
- Vojinovic J: Vitamin D receptor agonists' anti-inflammatory properties. *Ann N Y Acad Sci* 2014;1317:47-56.
- Hu F, Yan L, Lu S, Ma W, Wang Y, Wei Y, Yan X, Zhao X, Chen Z, Wang Z, Cheng B: Effects of 1,25-dihydroxyvitamin D₃ on experimental autoimmune myocarditis in mice. *Cell Physiol Biochem* 2016;38:2219-2229.
- Talmor Y, Bernheim J, Klein O, Green J, Rashid G: Calcitriol blunts pro-atherosclerotic parameters through NFκB and p38 in vitro. *Eur J Clin Invest* 2008;38:548-554.
- Xu S, Chen YH, Tan ZX, Xie DD, Zhang C, Xia MZ, Wang H, Zhao H, Xu DX, Yu DX: Vitamin D₃ pretreatment alleviates renal oxidative stress in lipopolysaccharide-induced acute kidney injury. *J Steroid Biochem Mol Biol* 2015;152:133-141.
- Qi XP, Li P, Li G, Sun Z, Li JS: 1,25-dihydroxyvitamin D₃ regulates LPS-induced cytokine production and reduces mortality in rats. *World J Gastroenterol* 2008;14:3897-3902.
- Vaure C, Liu Y: A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Front Immunol* 2014;5:316.
- Park BS, Lee JO: Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med* 2013;45:e66.
- van der Vorst EP, Keijbeek AA, de Winther MP, Donners MM: A disintegrin and metalloproteases: molecular scissors in angiogenesis, inflammation and atherosclerosis. *Atherosclerosis* 2012;224:302-308.
- Yang WS, Kim HW, Lee JM, Han NJ, Lee MJ, Park SK: 1,25-dihydroxyvitamin D₃ causes ADAM10-dependent ectodomain shedding of tumor necrosis factor receptor 1 in vascular smooth muscle cells. *Mol Pharmacol* 2015;87:533-542.
- Yang WS, Yu H, Kim JJ, Lee MJ, Park SK: Vitamin D-induced ectodomain shedding of TNF receptor 1 as a nongenomic action: D₃ vs D₂ derivatives. *J Steroid Biochem Mol Biol* 2016;155:18-25.
- Liu L, Zhao Y, Xie K, Sun X, Gao Y, Wang Z: Estrogen-induced nongenomic calcium signaling inhibits lipopolysaccharide-stimulated tumor necrosis factor α production in macrophages. *PLoS One* 2013;8:e83072.
- Peacock M: Calcium metabolism in health and disease. *Clin J Am Soc Nephrol* 2010;5:S23-S30.
- De Boland AR, Boland RL: Non-genomic signal transduction pathway of vitamin D in muscle. *Cell Signal* 1994;6:717-724.
- Yang WS, Han NJ, Kim JJ, Lee MJ, Park SK: TNF-α activates high-mobility group box 1 - toll-like receptor 4 signaling pathway in human aortic endothelial cells. *Cell Physiol Biochem* 2016;38:2139-2151.
- Hayashida K, Bartlett AH, Chen Y, Park PW: Molecular and cellular mechanisms of ectodomain shedding. *Anat Rec* 2010;293:925-937.
- Levine SJ: Mechanisms of soluble cytokine receptor generation. *J Immunol* 2004;173:5343-5348.
- Ten Oever J, Kox M, van de Veerdonk FL, Mothapo KM, Slavcovic A, Jansen TL, Tweehuysen L, Giamarellos-Bourboulis EJ, Schneeberger PM, Wever PC, Stoffels M, Simon A, van der Meer JW, Johnson MD, Kullberg BJ, Pickkers P, Pachot A, Joosten LA, Netea MG: The discriminative capacity of soluble Toll-like receptor (sTLR)2 and sTLR4 in inflammatory diseases. *BMC Immunol* 2014;15:55.

- 21 Yakut M, Örmeci N, Erdal H, Keskin O, Karayel Z, Tutkak H, Soykan I: The association between precancerous gastric lesions and serum pepsinogens, serum gastrin, vascular endothelial growth factor, serum interleukin-1 Beta, serum toll-like receptor-4 levels and Helicobacter pylori Cag A status. *Clin Res Hepatol Gastroenterol* 2013;37:302-311.
- 22 Kacerovsky M, Andrys C, Hornychova H, Pliskova L, Lancz K, Musilova I, Drahosova M, Bolehovska R, Tambor V, Jacobsson B: Amniotic fluid soluble Toll-like receptor 4 in pregnancies complicated by preterm prelabor rupture of the membranes. *J Matern Fetal Neonatal Med* 2012;25:1148-1155.
- 23 Zunt SL, Burton LV, Goldblatt LI, Dobbins EE, Srinivasan M: Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumour necrosis factor- α secretion by macrophage-like cells. *Clin Exp Immunol* 2009;156:285-293.
- 24 Yang HB, Xie KQ, Deng JM, Qin SM: Expression of soluble Toll-like receptors in pleural effusions. *Chin Med J* 2010;123:2225-2230.
- 25 Benabid R, Wartelle J, Malleret L, Guyot N, Gangloff S, Lebargy F, Belaouaj A: Neutrophil elastase modulates cytokine expression: contribution to host defense against *Pseudomonas aeruginosa*-induced pneumonia. *J Biol Chem* 2012;287:34883-34894.
- 26 Uchimura K, Hayata M, Mizumoto T, Miyasato Y, Kakizoe Y, Morinaga J, Onoue T, Yamazoe R, Ueda M, Adachi M, Miyoshi T, Shiraishi N, Ogawa W, Fukuda K, Kondo T, Matsumura T, Araki E, Tomita K, Kitamura K: The serine protease prostaticin regulates hepatic insulin sensitivity by modulating TLR4 signalling. *Nat Commun* 2014;5:3428.
- 27 Takeuchi S, Kawashima S, Rikitake Y, Ueyama T, Inoue N, Hirata K, Yokoyama M: Cerivastatin suppresses lipopolysaccharide-induced ICAM-1 expression through inhibition of Rho GTPase in BAEC. *Biochem Biophys Res Commun* 2000;269:97-102.
- 28 Park HS, Chun JN, Jung HY, Choi C, Bae YS: Role of NADPH oxidase 4 in lipopolysaccharide-induced proinflammatory responses by human aortic endothelial cells. *Cardiovasc Res* 2006;72:447-455.
- 29 Liu R, Li W, Karin NJ, Bergh JJ, Adler-Storthz K, Farach-Carson MC: Ribozyme ablation demonstrates that the cardiac subtype of the voltage-sensitive calcium channel is the molecular transducer of 1,25-dihydroxyvitamin D₃-stimulated calcium influx in osteoblastic cells. *J Biol Chem* 2000;275:8711-8718.
- 30 Nagano O, Murakami D, Hartmann D, De Strooper B, Saftig P, Iwatsubo T, Nakajima M, Shinohara M, Saya H: Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca²⁺ influx and PKC activation. *J Cell Biol* 2004;165:893-902.
- 31 Yang WS, Moon SY, Lee MJ, Park SK: Epigallocatechin-3-gallate attenuates the effects of TNF- α in vascular endothelial cells by causing ectodomain shedding of TNF receptor 1. *Cell Physiol Biochem* 2016;38:1963-1974.
- 32 Panter G, Jerala R: The ectodomain of the toll-like receptor 4 prevents constitutive receptor activation. *J Biol Chem* 2011;286:23334-23344.