Original Paper

Diosgenin, an Activator of 1,25D₃-MARRS Receptor/ERp57, Attenuates the Effects of TNF-α by Causing ADAM10-Dependent Ectodomain Shedding of TNF Receptor 1

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Key Words

A disintegrin and metalloprotease 10 • Diosgenin • 1,25D₃-MARRS receptor/ERp57 • L-type calcium channel • Tumor necrosis factor receptor 1

Abstract

Background/Aims: We investigated how diosgenin, a steroidal sapogenin, has anti-tumor necrosis factor-α (TNF-α) effects in human aortic endothelial cells (HAECs). **Methods:** Tumor necrosis factor receptor 1 (TNFR1) was assessed by Western blot analysis. Intracellular Ca²⁺ was measured using Fluo-4 AM. Immunofluorescence staining was performed for a disintegrin and metalloprotease 10 (ADAM10). **Results:** Diosgenin (1 ~ 100 nM) induced ectodomain shedding of TNFR1 within 30 min and attenuated TNF-α-induced intercellular adhesion molecule-1 (ICAM-1) expression. Upon treatment with diosgenin, extracellular Ca²⁺ entered into the cells via L-type calcium channels, whereas diosgenin-induced ectodomain shedding of TNFR1 was almost completely inhibited by BAPTA-AM (intracellular Ca²⁺ chelator), verapamil (L-type calcium channel antagonist) and the absence of extracellular Ca²⁺. Diosgenin caused translocation of ADAM10 to the cell surface, which was mediated by extracellular Ca²⁺ influx. Depletion of ADAM10 prevented diosgenin-induced ectodomain shedding of TNFR1 and abolished the inhibitory effect of diosgenin on TNF-α-induced ICAM-1 expression. Diosgenin did not induce extracellular Ca²⁺ influx and ectodomain shedding of TNFR1 in cells depleted of 1,25D₃-membrane associated rapid response steroid-binding receptor (1,25D₃-MARRS receptor/ERp57). **Conclusion:** Diosgenin elicits L-type calcium channel-mediated extracellular Ca²⁺ influx, and thereby induces ADAM10-mediated ectodomain shedding of TNFR1. This effect of diosgenin was exerted through 1,25D₃-MARRS receptor/ERp57.

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Introduction

Diosgenin is a steroidal sapogenin abundant in plants from the genus *Dioscorea*, *Costus* and *Smilax* species [1]. It has a structural similarity with steroid hormones and can be chemically converted, for example, into progesterone, estradiol and cortisone [2]. In the past and even nowadays, it was used as a base material for making synthetic steroids [2]. In the preclinical studies, however, diosgenin itself exhibited diverse beneficial pharmacological effects [1]. In particular, it has anti-inflammatory effects and was found to have protective effect on aortic atherosclerosis in an animal model [3].

Tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine that has a pivotal role in the development of atherosclerosis [4, 5] as well as in other inflammatory diseases. *In vitro* studies have shown that diosgenin attenuates the effects of TNF- α . In vascular smooth muscle cells, preincubation with diosgenin inhibited TNF- α -induced vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1) expressions and attenuated monocyte adhesions [6]. In monocytes, diosgenin inhibited TNF- α -induced thromboplastin expression and its procoagulant activity [7]. In addition, diosgenin inhibited TNF- α -induced invasive activity of tumor cells [8]. In these studies, diosgenin had anti-TNF- α effects by inhibitions of generation of reactive oxygen species and mitogen-activated protein kinase/NF- κ B signaling pathway [6-8].

The cellular effects of TNF- α are initiated by its binding to the receptors on the cell surface. Two different receptors, tumor necrosis factor receptor 1 (TNFR1) and 2, mediate the signaling of TNF- α [9]. Recently, we found that 1,25-dihydroxyvitamin D₃ (1,25D₃) and its analogues (25-hydroxyvitamin D₃, elocalcitol) induce extracellular Ca²⁺ influx which in turn causes a disintegrin and metalloprotease 10 (ADAM10)-mediated ectodomain shedding of TNFR1 in human aortic smooth muscle cells [10, 11]. The shedding of TNFR1 ectodomain resulted in decreased responsiveness of the cells to TNF- α . Release of the ectodomain results in a decrease in the number of effective receptors on the cell surface. In addition, the released ectodomain portion of TNFR1 may bind to TNF- α in the extracellular space and neutralize its activity [12]. This nongenomic effect of vitamin D₃ was mediated by 1,25D₃-membrane associated rapid response steroid-binding receptor (1,25D₃-MARRS receptor), which is also named as endoplasmic reticulum stress protein 57 (ERp57) [11]. Diosgenin has a core structure similar to that of 1 α ,25-dihydroxylumisterol, a 6-s-cis locked derivative of 1,25D₃, and was found to activate 1,25D₃-MARRS receptor/ERp57 on neuronal cells [13]. Based on these studies, we hypothesized that diosgenin may have anti-TNF- α effects by causing ectodomain shedding of TNFR1 through Ca²⁺-dependent activation of ADAM10.

To test this possibility, we investigated whether diosgenin causes ectodomain shedding of TNFR1 in human aortic endothelial cells (HAECs) and thereby attenuates the effect of TNF- α .

Materials and Methods

Materials

Diosgenin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). It was dissolved in 99.9% ethanol heated to 37°C. Diosgenin is soluble in ethanol, but its solubility is dependent on temperature with better solubility at higher temperature [14]. To maintain its solubility during the experiments, stock solution of diosgenin as well as the culture media or Hank's balanced salt solution (HBSS) were warmed to 37 °C before it was added to the cells. Calcium chloride, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid)-acetoxymethyl ester (BAPTA-AM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF- α was from R&D Systems (Abingdon, UK). ERp57-siRNA and antibodies to human ICAM-1, TNFR1 (sc-8436; used to detect TNFR1 in cell lysate), ADAM10 (sc-16524), ERp57 and actin were from Santa Cruz Biotechnology. Rabbit polyclonal antibody to human TNFR1 (ab19139; raised against synthetic peptides corresponding to amino acids 29-43 of human TNFR1 in the N-terminus; used to detect TNFR1 in cell culture supernatant) was from Abcam, Inc.

(Cambridge, MA, USA). ADAM10-siRNA, control-siRNA (Ambion®) and Fluo-4 AM (Molecular probes®) were from Life Technologies (Paisley, UK).

Cell culture

Primary 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% fetal calf serum for 24 h. Thereafter, we replaced the medium 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 perform experiments in the presence or absence of physiological concentration of extracellular Ca^{2+} (1.10 - 1.35 mM) [15].

Transfection of siRNA

ADAM10-siRNA, ERp57-siRNA or a nonspecific, scrambled, control-siRNA was transfected to the cells using Lipofectamine® Reagent (Life Technologies). In brief, 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. Transfection of siRNAs was performed 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. The cells were further cultured for 18 h in EBM-2 endothelial growth basal medium, and then rested for 24 h in M199 Hank's medium containing 1% fetal calf serum. Thereafter, the cells were subjected to the experiments.

Western blot analysis

Treated cells were washed with phosphate buffered saline (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 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 TNFR1 in cell culture supernatant of the treated cells, equal amounts of the conditioned media were concentrated 30-fold using Amicon® Ultra 10K device (Merck Millipore Co.). Thereafter, the samples were incubated with 5x sample buffer for 30 min at 37 °C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with an antibody against N-terminus of TNFR1.

Measurement of intracellular Ca^{2+} by confocal microscopy

Fluo-4 AM was used as a Ca^{2+} indicator. HAECs were incubated with Fluo-4 AM (2 μM) for 30 min. Thereafter, the cells were washed and placed in HBSS (Ca^{2+} 1.2 mM or 0 mM). Fluo-4 becomes fluorescent when bound with Ca^{2+} . 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 diosgenin. The fluorescence intensity in each cell was measured by ZEN 2011 imaging software (Carl Zeiss, Germany). The change in intracellular Ca^{2+} concentration was represented by relative fluorescence intensity compared with the initial value.

Immunofluorescence staining

ADAM10 expression on the cell surface was examined by immunofluorescent staining. 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 goat anti-ADAM10 antibody (1:50 dilution) overnight at 4 °C, washed 3 times with PBS and then incubated with Alexa Fluor 488-conjugated anti-goat IgG secondary antibody. 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 Scheffé's multiple comparison test was used to determine statistically significant differences between groups. A p value of <0.05 was considered statistically significant.

Results

Diosgenin attenuates TNF- α -induced ICAM-1 expression

We first examined the effect of diosgenin on TNF- α -induced ICAM-1 expression. HAECs were treated with TNF- α (10 ng/ml) for 0, 4, 8 or 24 h. As shown in Fig. 1A, TNF- α stimulated ICAM-1 expression with a marked increase at 8 ~ 24 h. In contrast, pretreatment of the cells with diosgenin for 30 min significantly attenuated TNF- α -induced ICAM-1 expression in a dose-dependent manner, while ethanol (the vehicle for diosgenin) had no such effect (Fig. 1B).

Fig. 1. Diosgenin attenuates TNF- α -induced ICAM-1 expression. (A) HAECs were incubated with TNF- α (10 ng/ml) for 0, 4, 8 or 24 h (n=4). (B) HAECs were preincubated for 30 min with different concentrations of diosgenin or ethanol (0.1%, vehicle), and further incubated with TNF- α (10 ng/ml) for 8 h (n=3). Whole cell lysates were analyzed by Western blotting using anti-ICAM-1 and anti-actin antibodies. (*p<0.05 compared with control; **p<0.05 as compared with TNF- α).

Diosgenin causes ectodomain shedding of TNFR1

To test whether diosgenin causes ectodomain shedding of TNFR1, we incubated HAECs with diosgenin at the doses of 1, 10 or 100 nM for 30 min or at the dose of 100 nM for different times, and obtained whole cell lysates and the conditioned media. TNFR1 was measured by Western blot (Fig. 2). TNFR1 in cell lysate was detected at ~ 55 kDa. TNFR1 released in the culture medium was detected by an antibody recognizing N-terminus of TNFR1, and the band was located at ~ 25 kDa, indicating an N-terminal fragment of TNFR1. As shown in Fig. 2A, diosgenin dose-dependently decreased TNFR1 in the cell lysates, while it increased the fragment of TNFR1 in the culture medium. Diosgenin-induced ectodomain shedding of TNFR1 was peaked at 30 min (Fig. 2B). Thereafter, TNFR1 increased in the cell lysate gradually toward the basal level.

Extracellular Ca²⁺ influx is prerequisite for ectodomain shedding of TNFR1

We explored whether Ca²⁺ signaling is implicated in the ectodomain shedding of TNFR1 in the cells treated with diosgenin. HAECs were preincubated with or without BAPTA-AM (an intracellular Ca²⁺ chelator) or verapamil (an L-type calcium channel antagonist), and then further treated with diosgenin for 30 min. Thereafter, whole cell lysates and the conditioned media were analyzed by Western blotting using anti-TNFR1 antibody. As shown in Fig. 3A, diosgenin-induced ectodomain shedding of TNFR1 was prevented by both BAPTA-AM and verapamil.

To determine whether the extracellular Ca²⁺ influx was essential for diosgenin-induced ectodomain shedding of TNFR1, we performed the experiment in Ca²⁺-free DMEM media. As

Fig. 2. Diosgenin causes ectodomain shedding of TNFR1. (A) HAECs in M199 Hank's media (Ca^{2+} 1.26 mM) were incubated with different concentrations of diosgenin or ethanol (0.1%, vehicle) for 30 min ($n=4$). (B) HAECs in M199 Hank's media (Ca^{2+} 1.26 mM) were incubated with diosgenin (100 nM) for different times ($n=3$). Whole cell lysates and conditioned media were analyzed by Western blotting using antibodies against TNFR1 and actin ($^*p<0.05$ compared with control).

Fig. 3. Extracellular Ca^{2+} influx is prerequisite for ectodomain shedding of TNFR1. (A) HAECs in M199 Hank's media (Ca^{2+} 1.26 mM) were preincubated with or without BAPTA-AM (10 μM), DMSO (0.1%, vehicle) for 30 min or verapamil (1 μM) for 2 min, and then further treated with diosgenin (100 nM) for 30 min ($n=4$). (B) HAECs were incubated with or without diosgenin (100 nM) for 30 min in DMEM media containing 1.2 mM Ca^{2+} or no Ca^{2+} . Whole cell lysates and conditioned media were analyzed by Western blotting using antibodies against TNFR1 and actin ($n=4$). ($^*p<0.05$ compared with control).

shown in Fig. 3B, diosgenin did not induce ectodomain shedding of TNFR1 in the absence of extracellular Ca^{2+} .

Diosgenin induces extracellular Ca^{2+} influx via L-type calcium channels

Next, we investigated the effect of diosgenin on intracellular Ca^{2+} level in HAECs, using Fluo-4 AM, a calcium fluorescent indicator (Fig. 4). In the presence of extracellular Ca^{2+} (1.2 mM), diosgenin markedly increased intracellular Ca^{2+} concentration within a minute. This effect of diosgenin was abolished by both BAPTA-AM and verapamil.

In the absence of extracellular Ca^{2+} , diosgenin-induced intracellular Ca^{2+} elevation was negligible, but became remarkable after addition of Ca^{2+} to the extracellular media, which indicates that diosgenin induced the increase in intracellular Ca^{2+} through extracellular Ca^{2+} influx.

ADAM10 is the enzyme responsible for ectodomain cleavage of TNFR1

Because Ca^{2+} influx is known to activate ADAM10 [16], we investigated the role of ADAM10 in diosgenin-induced ectodomain shedding of TNFR1. By immunofluorescent

Fig. 4. Diosgenin induces extracellular Ca^{2+} influx via L-type calcium channels. HAECs were loaded with Fluo-4 AM (2 μM). While fluorescent images of the cells were obtained every 10 sec, the cells were treated with ethanol (0.1%, vehicle) or diosgenin (100 nM), as indicated by arrows. The experiments were performed on the cells in 1.2 mM Ca^{2+} -containing HBSS or Ca^{2+} -free HBSS, as indicated. BAPTA-AM (10 μM) was preincubated for 30 min before Fluo-4 AM loading. Verapamil (1 μM) was preincubated for 2 min before diosgenin treatment. In case of Ca^{2+} -free experiment, CaCl_2 was added after monitoring the response to diosgenin to measure the effect of presence of extracellular Ca^{2+} . (A) Representative images before and after diosgenin treatment. (B) The fluorescence intensity in each cell representing intracellular Ca^{2+} concentration was measured for 6 min. Each point represents mean $\pm$ SE of the relative fluorescence intensity compared with the initial value. ($n = 174 \sim 211$ cells in each group from three independent experiments).

Fig. 5. Temporal changes of ADAM10 expression on the cell surface following treatment with diosgenin. HAECs in M199 Hank's media (Ca^{2+} 1.26 mM) were treated with diosgenin (100 nM) for different times. After then, 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.

staining and confocal microscopy, the cell surface expression of ADAM10 was visualized in nonpermeabilized HAECs. As shown in Fig. 5, ADAM10 was weakly detected in untreated cells. On treatment with diosgenin, however, there was a marked increase in ADAM10

Fig. 6. ADAM10 is the enzyme responsible for diosgenin-induced ectodomain cleavage of TNFR1. (A) Diosgenin induces Ca²⁺-dependent ADAM10 translocation to the cell surface. HAECs in DMEM media containing 1.2 mM Ca²⁺ or no Ca²⁺ were preincubated with or without BAPTA-AM (10 μ M) for 30 min or verapamil (1 μ M) for 2 min, and then further treated with diosgenin (100 nM) or ethanol (0.1%, vehicle as a control) for 10 min. After then, 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. (B) Depletion of ADAM10 abolishes diosgenin-induced ectodomain shedding of TNFR1. HAECs were transfected with control-siRNA or ADAM10-siRNA, and then incubated with diosgenin (100 nM) for 30 min in M199 Hank's media (Ca²⁺ 1.26 mM). Whole cell lysates and conditioned media were analyzed by Western blotting using anti-TNFR1, anti-ADAM10 and anti-actin antibodies (n=4). (C) Depletion of ADAM10 abolishes the inhibitory effect of diosgenin against TNF- α . HAECs were transfected with control-siRNA or ADAM10-siRNA, and then incubated with or without diosgenin (100 nM) for 30 min in M199 Hank's media (Ca²⁺ 1.26 mM) and further treated with TNF- α (10 ng/ml) for 8 h. Whole cell lysates were analyzed by Western blotting using anti-ICAM-1, anti-ADAM10 and anti-actin antibodies (n=3). (*p<0.05 compared with control).

immunoreactivity on the cell surface at 10 ~ 30 min. This effect of diosgenin subsided at 60 min and thereafter.

In the absence of extracellular Ca²⁺, however, diosgenin failed to increase ADAM10 expression at 10 min (Fig. 6A). Preincubation of the cells with BAPTA-AM or verapamil before diosgenin treatment also abolished the increase in ADAM10 cell surface expression (Fig. 6A).

Though treatment of the cells with diosgenin markedly increased ADAM10 at the cell surface in 10 min, it had no such effect on the amount of ADAM10 in the whole cell lysates (Fig. 6B). 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.

To determine whether ADAM10 is involved in diosgenin-induced ectodomain shedding of TNFR1, we transfected HAECs with control-siRNA or ADAM10-siRNA, and treated them

Fig. 7. Depletion of ERp57 prevents diosgenin-induced extracellular Ca^{2+} influx. HAECs were transfected with control-siRNA or ERp57-siRNA and loaded with Fluo-4 AM ($2 \mu\text{M}$). While fluorescent images of the cells were obtained every 10 sec, the cells were treated with diosgenin (100 nM) as indicated by arrows. (A) Representative images before and after diosgenin treatment. (B) The fluorescence intensity in each cell representing intracellular Ca^{2+} concentration was measured for 6 min. Each point represents mean $\pm$ SE of the relative fluorescence intensity compared with the initial value. ($n = 153$ cells in control-siRNA group; 131 cells in ERp57-siRNA group from three independent experiments) (C) Whole cell lysates were analyzed by Western blotting using anti-ERp57 and anti-actin antibodies ($n=3$).

Fig. 8. Depletion of ERp57 abolishes diosgenin-induced ectodomain shedding of TNFR1 and the inhibitory effect of diosgenin against TNF- α . (A) HAECs were transfected with control-siRNA or ERp57-siRNA, and then incubated with diosgenin (100 nM) for 30 min in M199 Hank's media (Ca^{2+} 1.26 mM). Whole cell lysates and conditioned media were analyzed by Western blotting using anti-TNFR1, anti-ERp57 and anti-actin antibodies ($n=4$). (B) HAECs were transfected with control-siRNA or ERp57-siRNA, and then incubated with or without diosgenin (100 nM) for 30 min in M199 Hank's media (Ca^{2+} 1.26 mM) and further treated with TNF- α (10 ng/ml) for 8 h. Whole cell lysates were analyzed by Western blotting using anti-ICAM-1, anti-ERp57 and anti-actin antibodies ($n=3$). ($p < 0.05$ compared with control).

with diosgenin. Depletion of ADAM10 prevented diosgenin-induced ectodomain shedding of TNFR1 (Fig. 6B).

We next investigated whether depletion of ADAM10 reverses the inhibitory effect of diosgenin on TNF- α -induced ICAM-1 expression. As shown in Fig. 6C, diosgenin did not inhibit TNF- α -induced ICAM-1 expression when ADAM10 was depleted by siRNA transfection.

1,25D₃-MARRS receptor/ERp57 mediates the effects of diosgenin

Because diosgenin was reported to activate 1,25D₃-MARRS receptor/ERp57, we evaluated the involvement of 1,25D₃-MARRS receptor/ERp57 in the diosgenin-induced ectodomain shedding of TNFR1. We first tested the effect of 1,25D₃-MARRS receptor/ERp57

depletion on diosgenin-induced extracellular Ca^{2+} influx. As shown in Fig. 7, diosgenin did not induce extracellular Ca^{2+} influx in the cells depleted of 1,25D₃-MARRS receptor/ERp57.

Next, we examined the effect of 1,25D₃-MARRS receptor/ERp57 depletion on diosgenin-induced ectodomain shedding of TNFR1. As shown in Fig. 8A, diosgenin did not induce ectodomain shedding of TNFR1 in the cells depleted of 1,25D₃-MARRS receptor/ERp57.

Finally, we examined the effect of 1,25D₃-MARRS receptor/ERp57 depletion on the inhibitory effect of diosgenin on TNF- α -induced ICAM-1 expression. As shown in Fig. 8B, diosgenin did not have an inhibitory effect on TNF- α -induced ICAM-1 expression when 1,25D₃-MARRS receptor/ERp57 was depleted by siRNA transfection.

Discussion

In the present study, we explored the mechanism underlying anti-TNF- α effects of diosgenin in HAECs. We found that diosgenin attenuates the cellular effect of TNF- α by inducing ADAM10-mediated ectodomain shedding of TNFR1.

Diosgenin has been shown to suppress the effects of TNF- α in different cell types [6-8], but its regulatory effect on TNF receptors has not yet been addressed. Our results indicate that diosgenin induces ectodomain shedding of TNFR1 as it caused a rapid reduction of TNFR1 in the cell lysate with accompanying increase of N-terminal fragment of TNFR1 in cell culture supernatant. Ectodomain shedding of TNFR1 accounted for anti-TNF- α effect of diosgenin because the inhibitory effect of diosgenin was absent when ectodomain cleavage of TNFR1 was prevented by depletion of ADAM10 or ERp57.

Ectodomain shedding of TNFR1 was preceded by a rapid increase in intracellular Ca^{2+} . In the presence of physiological concentration of Ca^{2+} , diosgenin markedly increased intracellular Ca^{2+} , but had no such effect in the absence of extracellular Ca^{2+} , suggesting that diosgenin increases intracellular Ca^{2+} primarily through the influx across the cell membrane. Our findings with HAECs are consistent with the previous studies. In human cortical neuron cells [17], erythrocytes [18] and mesenteric endothelial cells [19], diosgenin (10 μM , 15 μM or 1 mM, respectively) was shown to increase intracellular Ca^{2+} concentration. In these studies [17, 18], diosgenin induced little or no change in intracellular Ca^{2+} in Ca^{2+} -free solution as in the present study. However, it was not clear how diosgenin induces extracellular Ca^{2+} influx. In the present study, diosgenin-induced extracellular Ca^{2+} influx was completely abolished by the L-type calcium channel antagonist verapamil, and it suggests that diosgenin activates mainly L-type calcium channels. This effect of diosgenin in HAECs was significant at the concentration of 100 nM which is much lower than those tested in the previous studies of other cell types [17-19].

ADAMs are the metalloproteinases that regulate the expression of transmembrane proteins including cell surface receptors by cleaving the extracellular portion of them. Increase in cytosolic Ca^{2+} either by extracellular Ca^{2+} influx induced by vitamin D₃ or Bay K8644 or by release of intracellular stored Ca^{2+} induced by epigallocatechin-3-gallate, thapsigargin or cyclopiazonic acid was shown to activate ADAM10 in our previous studies [10, 11, 20, 21]. The findings in the present study are consistent with these studies. Diosgenin caused translocation of ADAM10 to the cell surface where TNFR1 is located. Diosgenin-induced ADAM10 translocation and ectodomain shedding of TNFR1 were dependent on its ability to induce extracellular Ca^{2+} influx as they were prevented by BAPTA-AM, verapamil and the absence of extracellular Ca^{2+} . On the other hand, depletion of ADAM10 by siRNA transfection prevented diosgenin-induced TNFR1 ectodomain shedding and also abolished the inhibitory effect of diosgenin on TNF- α -induced ICAM-1 production. The findings together indicate that extracellular Ca^{2+} influx induced by diosgenin leads to ADAM10-mediated ectodomain cleavage of TNFR1.

ERp57 is a member of protein disulfide isomerase found in the endoplasmic reticulum where its main function is to modulate folding of newly synthesized glycoproteins [22]. Besides endoplasmic reticulum, ERp57 is located in the cell surface, cytosol, mitochondria

and nucleus, and has diverse regulatory functions [22]. $1,25D_3$ binds to its receptor on the cell surface and exerts rapid nongenomic effects such as Ca^{2+} uptake in mammalian intestine. The receptor was named as a $1,25D_3$ -MARRS receptor which was afterwards found to be identical with ERp57 [23]. Diosgenin has a structure similar with steroid hormones and can be used to synthesize vitamin D analogs [24]. Interestingly, a docking simulation suggested that diosgenin may bind $1,25D_3$ -MARRS receptor/ERp57 more potently than $1,25D_3$ [13]. In cultured neuronal cells, diosgenin was shown to compete for $1,25D_3$ -MARRS receptor/ERp57 with $1,25D_3$ and activated the receptor pathway leading to axonal growth [13]. Consistent with it, $1,25D_3$ -MARRS receptor/ERp57 was essential for the cellular effects of diosgenin in HAECS, as depletion of ERp57 abolished diosgenin-induced extracellular Ca^{2+} influx and ectodomain shedding of TNFR1 and its inhibitory effect on TNF- α -induced ICAM-1 expression.

Diosgenin-induced ectodomain shedding of TNFR1 occurred rapidly and reached a peak in 30 min. Thereafter, this effect of diosgenin disappeared with accompanying decrease in ADAM10 on the cell surface, while the loss of TNFR1 in the cell lysate was restored gradually. The latter may be because the cleaved TNFR1 was replaced by newly synthesized TNFR1. The loss of diosgenin effect in terms of TNFR1 cleavage beyond 30 min may be related with its transient effect on extracellular Ca^{2+} influx that affects cell membrane translocation of ADAM10. On treatment with diosgenin, intracellular Ca^{2+} concentration increased rapidly with a peak within a minute, and then declined with time. After 5 min, intracellular Ca^{2+} concentration was still elevated as compared with control, but not greatly. On the other hand, ADAM10 translocated to the cell surface could be subject to proteolysis by other cell surface metalloproteases including ADAM9 and ADAM15 [25] or internalized by endocytosis [26].

By causing extracellular Ca^{2+} influx, diosgenin activated ADAM10, which may be linked to its diverse cellular effects. For example, diosgenin has anti-inflammatory effects in animal studies [27-29]. TNF- α , a proinflammatory cytokine, plays a pivotal role in the inflammatory processes [30], while diosgenin may have anti-inflammatory effects by reducing the responsiveness of the cells to TNF- α via ADAM10-dependent ectodomain shedding of TNFR1. Diosgenin was also shown to improve Alzheimer's disease by activating ERp57 in a mouse model [13], in which ADAM10 may have a role as it protects against Alzheimer's disease through cleavage of amyloid precursor protein [16, 31].

In the present study, diosgenin was effective at the concentration of 10 or 100 nM. To have an implication in the clinical setting, these concentrations should be attainable at the tissue level after administration of diosgenin. In the toxicity studies, oral administration of diosgenin was well tolerated [32, 33] and no evident toxicities were observed in mice given daily oral steroidal saponins (including 28 % diosgenin) extracted from rhizomes of *Dioscorea zingiberensis* at a dose of 127.5 mg/kg for 30 days [32]. In a pharmacokinetic study of a rat model, the peak plasma level of diosgenin was 23 ng/ml (55 nM) after oral administration of a dose of 10 mg/kg [34]. Formation of liquid crystals using glyceryl monooleate or β -cyclodextrin inclusion complex improved the bioavailability of diosgenin, and increased the peak plasma level to 239 ng/ml (550 nM) at the same dose [34]. These data support that the concentrations of diosgenin tested in the present study are physiologically relevant levels.

Clinically, L-type calcium channel antagonists are commonly used to control hypertension or supraventricular tachycardia. Our data also suggest that anti-inflammatory effects of diosgenin may diminish by taking L-type calcium channel antagonists, and this interaction needs to be considered in human studies for diosgenin.

Conclusion

Diosgenin elicits L-type calcium channel-mediated extracellular Ca^{2+} influx through $1,25D_3$ -MARRS receptor/ERp57. Extracellular Ca^{2+} influx in turn triggers ADAM10-mediated ectodomain shedding of TNFR1 and thereby reduces the responsiveness of the cells to TNF- α .

By inducing ectodomain shedding of TNFR1, diosgenin may downregulate the inflammatory response of vascular endothelial cells.

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Disclosure Statement

The authors declare no potential Disclosure Statement.

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