Uric acid enhances PKC-dependent eNOS phosphorylation and mediates cellular ER stress: A mechanism for uric acid-induced endothelial dysfunction

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Abstract. The mechanism by which hyperuricemia induced-endothelial dysfunction contributes to cardiovascular diseases (CVDs) is not yet fully understood. In the present study, we used uric acid (UA) to trigger endothelial dysfunction in cultured endothelial cells, and investigated the effects of induced reactive oxygen species (ROS) generation, endoplasmic reticulum (ER) stress induction, and the protein kinase C (PKC)-dependent endothelial nitric oxide synthase (eNOS) phosphorylation. However, PKC-dependent eNOS phosphorylation at Thr495 was greatly enhanced, and consequently interaction between eNOS and calmodulin (CaM) was reduced. Cellular ROS depletion, ER stress inhibition and PKC activity restoration inhibited the effect of UA on eNOS activity, NO release and apoptosis in HUVECs. Thus, we concluded that UA induced HUVEC apoptosis and endothelial dysfunction by triggering oxidative and ER stress through PKC/eNOS-mediated eNOS activity and NO production.

Introduction

Uric acid (UA) is the end product of purine metabolism in humans and higher primates, and humans tend to have higher blood UA levels than other mammals (1). Pathological accumulation of UA in the blood (hyperuricemia) is associated with gout, lithiuria, and UA nephropathy. Hyperuricemia has recently been reported to be a risk factor for cardiovascular diseases (CVDs), chronic kidney diseases (CKDs), hypertension and kidney disease (1-6), but the mechanisms are not yet fully understood.

The relatively high physiological level of UA in humans has been suggested to play an antioxidative defense role (7,8), but previous research has reported that higher levels of UA trigger oxidative stress in cells, and contribute to endothelial dysfunction (9). High concentrations of UA enhance reactive oxygen species (ROS) generation in neutrophils (10), and UA induced oxidative stress and inhibited growth of pancreatic β-cells (11). In addition, in endothelial cells, hyperuricemia has been reported to trigger cellular oxidative stress and endothelial dysfunction (12).

Cellular stresses trigger endoplasmic reticulum (ER) stress, which activates a complex signaling network, known as the unfolded protein response (UPR), to reduce ER stress and restore homeostasis (13), but sustained and unresolvable ER stress promotes apoptosis, elevates CCAAT-enhancer-binding protein homologous protein (CHOP) and activates transcription factor 6 (ATF-6) expression, inducing endothelial dysfunction (14-16). ER stress also activates protein kinase C (PKC) and AKT signaling pathways (17-19). UA was reported to induce ER stress in glomerular mesangial cells and hepatocytes (20,21).

Endothelial dysfunction is characterized by decreased nitric oxide (NO) levels and cellular activity, and contributes to the pathogenesis of CVD. Hyperuricemia has been reported to induce endothelial dysfunction in several studies (6,9,22,23). Allopurinol therapy, which reduces UA levels, has been noted to improve endothelial function (24,25). In endothelial cells, a high concentration of UA increased ROS levels, and decreased eNOS activity and NO production (22,26). One possible

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mechanism involves the phosphorylation of eNOS (27, 28). In renal proximal tubule cells, UA has been reported to inhibit proliferation through the PKC signaling pathway (29). UA does not alter eNOS expression in human umbilical vein endothelial cells (HUVECs), but abolished the interaction of eNOS with calmodulin (CaM) and NO release (22).

Therefore, in the present study, we investigated the effect of UA on eNOS phosphorylation, eNOS activity, and NO production in primary culture HUVECs. We detected ER stress in HUVECs incubated with UA. We noted that UA triggers oxidative and ER stress through PKC/eNOS-mediated eNOS activity and NO production, causing HUVEC apoptosis.

Materials and methods

Reagents. UA (>99% pure, without endotoxin), polyethylene glycol-superoxide dismutase (PEG-SOD), 4-phenylbutyric acid (4-PBA), and polymyxin B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3, acetoxyethyl (AM) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Antibodies against p-eNOS-Thr1177 (ab138430), p-eNOS-Ser1177 (ab195944), eNOS (ab66127), caspase-12 (ab62484), CHOP (ab1419), and ATF-6 (ab19099) were obtained from Abcam (Cambridge, UK). Primary monoclonal antibody against human CaM (05-173) was from Millipore Corp. (Billerica, MA, USA). Primary polyclonal antibodies against PKC (ab2056) and p-PKC (#9375) and HRP-linked secondary antibodies against rabbit IgG (#7074), mouse IgG (#7076) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). The Annexin V-FITC Apoptosis Detection Kit was from BD Pharmingen (San Diego, CA, USA), and the Nitric Oxide Synthase Assay kit was from Beyotime Biotech (Jiangsu, China). All chemicals were of analytical grade.

HUVEC culture. Primary HUVECs were purchased from PromoCell (Heidelberg, Germany) and cultured at 37°C in 5% CO2 in Endothelial Cell Growth Medium (Cat. no. C-22010; PromoCell), which contains 2% fetal bovine serum, 5 ng/ml epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml Long R3 IGF-1, and 0.5 ng/ml epidermal growth factor (VEGF). Cells at passage 3-7 were used for experiments.

UA solution. The UA solution was prepared as follows: 0.4 g of NaOH was dissolved in 10 ml ultrapure water to obtain 1 mM NaOH solution. Subsequently, 150 mg UA was added, fully dissolved in a warm water bath under agitation. The final UA concentration was 15 mg/ml. The solution was filtered using a 0.22-µm filter under a sterile hood for eventual removal of microorganisms. The solution was stored at room temperature. Prior to the experiment, the stock solution was used to obtain culture media at 6, 9 and 12 mg/dl.

UA stimulation and pharmacological inhibition. UA was added to HUVEC cultures 24 h after passage, at final concentrations of 6, 9 or 12 mg/dl. Where indicated, 30 min prior to the addition of UA, the following inhibitors were added at the indicated final concentrations: 100 U/ml of the PEG-SOD antioxidant, 10 mM of the ER stress inhibitor 4-PBA, or 20 µg/ml of the PKC inhibitor polymyxin B. Untreated HUVECs were used as the control.

Analysis of apoptosis using flow cytometry. Trypsin-dispersed HUVECs (5x10^4/sample) were resuspended in 200 µl Annexin V-FITC and 10 µl propidium iodide and subsequently incubated at room temperature (20-25°C) in the dark for 20 min. Staining was then analyzed using a Becton-Dickinson flow cytometer (Becton-Dickinson, San Diego, CA, USA).

Measurement of NO release. Cell culture supernatant NO content was measured using a Total Nitric Oxide Assay kit (nitrate/nitrite assay kit; Beyotime Biotech) according to the manufacturer’s instructions. Total NO production was estimated according to the accumulation of nitrite and nitrate in the cell culture supernatant using Griess reagent (Beyotime Biotech). Nitrate was converted enzymatically into nitrite by nitrate reductase. Nitrite is a stable metabolite of NO, and thus is an indicator of released NO in the media, as previously described (30).

Measurement of eNOS activity. eNOS activity was measured using a Nitric Oxide Synthase Assay kit (Beyotime Biotech) according to the manufacturer’s instructions. As previously described (31), HUVECs were seeded in a 96-well plate. Cells were resuspended in 100 µl NOS detection buffer and 100 µl reaction buffer containing NOS substrates (5µl 0.1 mM NADPH and L-arginine, respectively) and a cell-permeable fluorescent substance (5 µl 3-amino 14-aminomethyl-2',7'-difluorescein diacetate) and incubated at 37°C in the dark for 40 min. Fluorescence intensity (FI) was measured at Ex 495 nm/Em 515 nm on a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The relative activity (RA) of eNOS was calculated as:

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RA = \frac{FI (sample) - FI (negative)}{FI (control) - FI (negative)}
\]

Measurement of ROS. The intracellular ROS levels were measured using CM-H2DCFDA (Sigma, Carlsbad, CA, USA). HUVECs were seeded on 35-mm confocal culture dishes at 4x10^4 cells/ml in 2 ml media per dish 24 h before UA stimulation. After stimulation, culture media was replaced with dichloro-dihydro-fluorescein diacetate (DCFH-DA), diluted in serum-free media to a final concentration of 10 µM, and cells were incubated at 37°C in the dark. After 20 min, the cells were washed with serum-free media three times and FI was measured using a Nikon A1 confocal microscope (purchased from Nikon Instruments, Inc., Tokyo, Japan) at Ex 488 nm/Em 515 nm.

Measurement of Ca^{2+} concentration. The intracellular level of Ca^{2+} was measured using the Ca^{2+}-specific fluorescent probe Fluo-3, AM, as previously described (26). HUVECs were cultured in the presence or absence of 12 mg/dl of UA for 6, 12 or 24 h, then incubated with 50 µM Fluo-3, AM at 37°C in the dark. After 1 h, cells were washed three times in Hanks' solution to remove unloaded probes. The FI of Fluo-3, AM was measured at Ex 488 nm/Em 525 nm using a Nikon A1 confocal microscope (Nikon Instruments, Inc.).

Co-immunoprecipitation assay. HUVECs were harvested with cell lysis buffer containing protease inhibitors (#5871; Cell Signaling Technology, Inc., Danvers, MA, USA) on ice for
30 min. The supernatant protein lysates were collected after 30 min of centrifugation at 1,200 x g. Lysate (500 µg) was incubated overnight with 3 µg eNOS-directed antibody or preimmune IgG (Cell Sciences, Canton, MA, USA) at 4˚C. Labeled lysates were incubated with 25 µl of protein A agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C. After 3 h, protein A-agarose beads were washed three times with lysis buffer and resuspended in 15 µl of 2X SDS loading buffer, boiled for 5 min, and analyzed by SDS-PAGE and western blot analysis.

**Western blot analysis.** HUVECs were lysed in RIPA buffer with phosphatase inhibitor cocktail (100X) (#5870; Cell Signaling Technology, Inc., Danvers, MA, USA) and stored at -70°C after determining the protein concentration using the BCA reagent (Beyotime Biotech). As described previously (32), proteins were separated on SDS-PAGE with a 5% stacking gel and a 12% resolving gel. After separation, proteins were electrophoretically transferred onto PVDF membranes (Millipore Corp.), and incubated in blocking buffer (Beyotime Biotech). Blots were probed with primary antibodies (against p-eNOS-Thr495, p-eNOS-Ser1177, eNOS, caspase-12, CHOP, ATF-6, CaM, PKC, p-PKC and β-actin) overnight at 4˚C, then corresponding HRP-labeled secondary antibodies (against rabbit IgG or mouse IgG), and developed using enhanced chemiluminescence reagents (Millipore Corp.). Band intensity was analyzed using the Touch Gel Dock system (UVP LLC, Upland, CA, USA). β-actin was used as the internal reference; the relative expression of protein was represented as the ratio of the target protein to β-actin (target protein/β-actin).

**Statistical analysis.** All data in the present study are presented as the means ± standard deviation (SD). Statistical analysis was performed using SPSS. Group characteristics were compared
Results

UA enhances apoptosis and reduces NO production. As shown in Fig. 1A, in the present study we examined the effect of UA on vascular endothelial cellular function and NO production by treating in vitro cultured HUVECs with various concentrations of UA for different durations of time. We found that the rate of apoptosis was increased in HUVECs incubated with UA in a time- and concentration-dependent manner. Significantly increased HUVEC apoptosis was detected within 48 h in HUVECs incubated with 6 mg/dl of UA, within 24 h in HUVECs incubated with 9 mg/dl of UA, and within 12 h in HUVECs incubated with 12 mg/dl of UA (all $P<0.05$) (Fig. 1A).

Incubation of HUVECs with UA reduced NO production in a time- and concentration-dependent manner (Fig. 1B) and reduced eNOS activity (Fig. 1E and F), whereas even 12 mg/dl UA barely affected the protein expression of eNOS (Fig. 1C and D).

UA increases intracellular ROS generation. Oxidative stress is known to play an important role in UA-induced endothelial dysfunction (12,26). We measured intracellular ROS levels in order to estimate oxidative stress using a specific probe, CM-H2DCFDA, in primary HUVEC cultures. Intracellular ROS levels were significantly increased 3 h after incubation with 12 mg/dl of UA, and remained significantly elevated for 24 h ($P<0.05$) (Fig. 2A and C). Pretreatment of HUVECs with the cell-permeable ROS scavenger PEG-SOD (26) for 30 min significantly ameliorated the UA-induced increase in intracellular ROS accumulation (Fig. 2B and D).

UA-induced oxidative stress triggers ER stress in HUVECs. UA has previously been reported to regulate cell function through the ER stress-signaling network in glomerular mesangial cells and hepatocytes, and oxidative stress is one of the key drivers of ER stress (20,21). To investigate the role of ER stress in UA-induced endothelial dysfunction, we measured the cellular content of the ER stress biomarkers ATF-6 and CHOP, and the ER stress-induced apoptotic marker caspase-12, by western blot analysis. UA (12 mg/dl) increased the levels of ER stress markers in HUVECs in a time-dependent manner (Fig. 3A). The cellular content of ATF-6 and CHOP was significantly increased after 6 h of incubation with UA, rose again at 12 h, and remained elevated for 24 h; for CHOP, expression peaked at 12 h and for ATF-6 at 24 h. Cellular levels of caspase-12 were significantly increased at 12 h and peaked at 24 h. HUVECs were pre-treated with the antioxidant PEG-SOD or ER stress inhibitor 4-PBA in order to explore the association between UA-induced oxidative stress and ER stress. Both PEG-SOD and 4-PBA effectively inhibited UA-induced ATF-6, CHOP, and caspase-12 upregulation (Fig. 3B). UA-induced oxidative stress was observed after 3 h, and ER stress was observed after 6 h. These results suggest that UA stimulation induces oxidative stress, which triggers ER stress in HUVECs.

Figure 2. Effect of uric acid (UA) on intracellular reactive oxygen species (ROS) generation in human umbilical vein endothelial cells (HUVECs). (A and C) Intracellular ROS levels were detected using the specific probe CM-H2DCFDA. (B and D) HUVECs were pre-treated with or without 100 U/ml polyethylene glycol-superoxide dismutase (PEG-SOD) for 30 min before incubation with UA. *$P<0.05$ vs. control; $^\#P<0.05$ vs. 3 h and UA; $^\&P<0.05$ vs. 6 h; n=3.
ROS scavenging and ER stress inhibition attenuated UA-induced apoptosis and decrease of NO production. To further confirm the roles of oxidative stress and ER stress in UA-induced apoptosis and endothelial dysfunction, we assessed the rate of apoptosis and NO production in HUVECs pre-treated with PEG-SOD and 4-PBA. While HUVECs incubated with 12 mg/dl UA for 48 h exhibited increased apoptosis and reduced NO production (P<0.05), in cells pre-incubated with PEG-SOD or 4-PBA this effect was not observed (Fig. 4), suggesting that UA induces endothelial dysfunction by triggering oxidative stress and ER stress.

UA decreases eNOS activity by increasing Thr495 phosphorylation. Ca2+, CaM, eNOS, and eNOS phosphorylation state are known to act as key regulators of eNOS activity (22,33) and were thus examined in HUVECs in the present study. HUVECs incubated with 12 mg/dl UA for 6, 12 or 24 h contained higher levels of eNOS and CaM. Although the intracellular Ca2+ concentration, the rate of eNOS phosphorylation at Ser1177, and CaM expression were almost unaltered (Fig. 5A-C), eNOS phosphorylation at Thr495 was increased in a time-dependent manner between 6 and 24 h (P<0.05) (Fig. 5A). Binding of eNOS and CaM was assessed by co-immunoprecipitation, and we noted that UA also reduced eNOS and CaM binding in a time-dependent manner (P<0.05) (Fig. 5A). These findings indicated that UA inhibits eNOS activity through phosphorylation of eNOS at Thr495 and prohibits its interaction with CaM.

UA decreases eNOS activity through the ER stress/PKC pathway in HUVECs. PKC activates eNOS enzymatic activity by phosphorylating eNOS at Thr495 (28). To further investigate the mechanism by which UA modulates eNOS activity, PKC expression and phosphorylation, HUVECs were incubated with the antioxidant PEG-SOD, the ER stress inhibitor 4-PBA, and the PKC inhibitor polymyxin B prior to incubation with UA. The PKC expression level was almost unaltered by incubation with UA, whereas the level of p-PKC was elevated after 6 h and peaked at 12 h (P<0.05) (Fig. 6A). Moreover, PEG-SOD, 4-PBA, or polymyxin B pretreatment inhibited UA-induced PKC phosphorylation (Fig. 6B-D). These results suggest that UA triggers ER stress and PKC phosphorylation by inducing ROS generation. Active PKC increases phosphorylation of eNOS at Thr495, and reduces the interaction between eNOS and CaM, thus inhibiting eNOS activity and NO production.

Discussion

In the present study, we sought to determine the mechanisms by which UA induces endothelial dysfunction, and also to investigate the effects of UA on eNOS phosphorylation, eNOS activity, and NO production in primary culture HUVECs. UA increased the rate of apoptosis, and decreased eNOS activity.
Figure 5. Uric acid (UA) decreases endothelial nitric oxide synthase (eNOS) activity by phosphorylation at Thr\(^{495}\). Human umbilical vein endothelial cells (HUVECs) were incubated with 12 mg/dl of UA for 6, 12 and 24 h. (A) Cellular content of p-eNOS-Ser\(^{1177}\), p-eNOS-Thr\(^{495}\) and eNOS was assessed by western blot analysis. (B) Intracellular (Ca\(^{2+}\)) was assessed using the specific probe Fluo-3, AM by laser confocal microscope. (C) Cellular content of calmodulin (CaM) was assessed by western blot analysis. (D) Co-immunoprecipitation of eNOS and CaM from lysates of UA-treated HUVECs using an eNOS-directed antibody, and detected using a CaM-directed antibody. *P<0.05 vs. control; †P<0.05 vs. 6 h; ‡P<0.05 vs. 12 h; n=3. NC, HUVECs not stimulated with UA.

Figure 4. Effect of polyethylene glycol-superoxide dismutase (PEG-SOD) and 4-phenylbutyric acid (4-PBA) on cell apoptosis and nitric oxide (NO) release in human umbilical vein endothelial cells (HUVECs). NC indicates HUVECs which were incubated without UA stimulation, just with different pharmacological pretreatment. (A and B) HUVECs were incubated with 12 mg/dl of uric acid (UA) with or without a 30-min pre-incubation with PEG-SOD or 4-PBA. HUVEC apoptosis was measured with an Annexin V-FITC kit. (C and D), and endothelial nitric oxide (NO) synthase (eNOS) activity and NO release were measured using a NOS activity assay kit and Griess reagent, respectively. *P<0.05 vs. control; †P<0.05 vs. UA; n=3. NC, HUVECs not stimulated with UA.
and NO production in a dose- and time-dependent manner. A high concentration of UA induced intracellular ROS accumulation, likely triggering ER stress, and increasing eNOS Thr495 phosphorylation via a PKC-dependent pathway, and reduced eNOS CaM binding and activity. Moreover, we found that pharmacological suppression of oxidative stress and ER stress by PEG-SOD and 4-PBA improved UA-induced apoptosis, eNOS inhibition, and NO production.

Hyperuricemia is a risk factor for the pathogenesis of CVD and CKD (34,35). However, the molecular mechanisms responsible for these observations have not been clearly defined, limiting the development of interventions targeting this process (22). UA has been shown to be involved in oxidative stress and ER stress via PEG-SOD and 4-PBA improved UA-induced apoptosis, eNOS inhibition, and NO production.

![Figure 6](https://example.com/figure6.png)

Figure 6. Effect of pharmacological inhibition on the protein kinase C (PKC)/endothelial nitric oxide synthase (eNOS) pathway in uric acid (UA)-stimulated human umbilical vein endothelial cells (HUVECs). NC indicates HUVECs which were incubated without UA stimulation, just with different pharmacological pretreatment. (A) HUVECs were incubated with 12 mg/dl of UA for the indicated time periods. Cellular content of PKC and p-PKC was assessed by western blot analysis. (B) HUVECs were incubated with 12 mg/dl of UA with or without a 30-min pre-incubation with polyethylene glycol-superoxide dismutase (PEG-SOD) or 4-phenylbutyric acid (4-PBA). Cellular content of PKC and p-PKC was assessed by western blot analysis. (C) HUVECs were incubated with 12 mg/dl of UA with or without a 30-min pre-incubation with PEG-SOD, 4-PBA, or polymyxin B. Cellular content of p-eNOS-Ser1177, p-eNOS-Thr495, and eNOS was assessed by western blot analysis. (D) eNOS activity was detected in HUVECs incubated with 12 mg/dl UA with or without a 30-min pre-incubation with PEG-SOD, 4-PBA, or polymyxin B. *P<0.05 vs. control; †P<0.05 vs. 6 h and UA; n=3. NC, HUVECs not stimulated with UA.

to reduce NO production in vascular endothelial cells in a dose-dependent manner, possibly through the interaction of eNOS and CaM, although UA did not alter the cellular content of Ca²⁺ or CaM in endothelial cells (22,26). Similarly, in the present study, a high concentration of UA did not markedly alter the cellular content of Ca²⁺, CaM, or eNOS, but inhibited eNOS binding to CaM.

We also found that UA enhanced eNOS phosphorylation at Thr495, but not at Ser1177. Phosphorylation of eNOS at Thr495 is PKC-dependent, and inhibition of PKC activity reduced UA-induced eNOS phosphorylation at Thr495. Taken together, these findings suggest that in endothelial cells UA activates PKC to phosphorylate eNOS at Thr495. Phosphorylated eNOS cannot bind to CaM (27), and thus remains enzymatically inactive.

Previous research has suggested that UA inhibits eNOS phosphorylation at Ser1177 and NO production in acetylcholine-stimulated HUVECs (23). Additionally, insulin-stimulated eNOS phosphorylation and NO production in vascular endothelial cells was inhibited by UA (41). The discrepancies between our results and these previous results probably lie in the different kinases and downstream signaling pathways which UA affects; acetylcholine and insulin activate the PI3K/Akt pathway to phosphorylate eNOS at Ser1177, while PKC is
activated by UA and PKC inactivates eNOS enzymatic activity by phosphorylation at Thr\(^{95}\).

In a murine model of hyperuricemia, serum UA levels were negatively correlated with serum NO and superoxide dismutase levels. In pancreatic β-cells, UA has previously been reported to induce oxidative stress and growth inhibition by activating the AMP-activated protein kinase (AMPK) signaling pathway (11).

In this study, we noted that in primary cultured HUVECs, UA reduced NO production and induced apoptosis in a dose- and time-dependent manner. UA also induced ROS generation, suggesting a damaging, rather than anti-oxidative, effect, as previously reported elsewhere (6,9,11). We also noted that the antioxidant PEG-SOD significantly inhibited the effect of UA on apoptosis and NO release, suggesting that oxidative stress is key to UA-stimulated endothelial dysfunction.

UA has previously been reported to trigger the ER stress response in rat glomerular mesangial cells in vitro (20). In the present study, we noted that UA upregulated the ER stress markers CHOP and ATF-6, and the ER stress-induced apoptosis marker caspase-12. Elevation of free radicals is one of the factors that trigger ER stress (42). We found that ER stress markers ATF-6 and CHOP began to significantly increase after 6 h of UA stimulation, and caspase-12 levels began to significantly increase after 12 h. However, even 3 h of UA stimulation triggered ROS generation, and inhibition of ROS generation with PEG-SOD attenuated UA-induced ER stress and apoptosis. These findings suggest that UA-stimulated oxidative stress precedes ER stress. ER stress modulates cellular function through the PKC signaling pathway, and knockdown of PKC attenuates ER stress-induced cellular damage (17-19). In this study, we found that in cultured HUVECs, UA triggered PKC phosphorylation in a time-dependent manner. Antioxidant and ER stress inhibitors inhibited UA-induced PKC activation and phosphorylation of eNOS at Thr\(^{95}\), thus preventing UA-mediated depression of NO production.

In conclusion, the present study confirmed previous reports that high levels of UA trigger endothelial dysfunction, inducing apoptosis and reducing eNOS activity and NO production. We were able to implicate further molecular mechanisms in this process, and hypothesize that UA activates the PKC pathway to trigger oxidative stress and ER stress signaling network to induce endothelial dysfunction in HUVECs. The implications of these mechanisms in vivo remain to be seen, and whether the concentrations of UA employed in this study accurately represent the stresses endothelial cells experience in vivo also remains to be determined.

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