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Original Article

Dual effects of ouabain on the regulation of proliferation and apoptosis in human umbilical vein endothelial cells: involvement of Na^+-K^+-ATPase α-subunits and NF-κB

Yan-Ping Ren[1], Ming-Juan Zhang[2], Ting Zhang[1], Ruo-Wen Huang[1]

[1]*Department of Geriatric-Cardiovascular Diseases, The First Affiliated Hospital of Medical College in Xi'an Jiaotong University, Xi'an 710061, China;* [2]*Department of Cardiology, The Second Affiliated Hospital of Medical College in Xi'an Jiaotong University, Xi'an 710004, China*

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Abstract: Purpose: To elucidate the effect of ouabain on the regulation of proliferation and apoptosis of HUVECs and involvement of different Na^+-K^+-ATPase α-subunits and NF-κB. Methods: HUVECs were isolated by collagenase perfusion, and MTT assays and cell cycle analysis were performed to study proliferation. NF-κB expression and function were examined by immunohistochemical staining and western blotting. Na^+-K^+-ATPase activity was determined by measuring released ouabain inhibitable inorganic phosphate (Pi). The expression of different α-subunits was investigated by real RT-PCR, western blotting and cell immunofluorescence. Results: 0.3 nM ouabain treatment for 0.5 h triggered the proliferation of HUVECs, peaking at 1-2 h. At 1.8 nM for 0.5 h, ouabain induced an increase of cell proliferation for a short time, and then triggered a decrease after 1 h. Cell cycle analysis show that 37% of HUVECs were in G2/M phase of the cell cycle following incubation with 1.8 nM ouabain, compared with 18% with 0.3 nM ouabain. NF-κB activity was assessed by western blot analysis of IκB expression, which was significantly reduced with 0.3 nM ouabain treatment; there was no different between 1.8 nM ouabain treatment and untreated cells. Na^+-K^+-ATPase activity in HUVECs was markedly reduced after treatment with 0.3 nM and 1.8 nM ouabain. Real RT-PCR and western blotting indicated that Na^+-K^+-ATPase α_1-subunit mRNA expression levels increased after 0.3 nM ouabain treatment and decreased after 1.8 nM ouabain treatment. However, α_2- and α_3-subunit mRNA decreased after 0.3 nM ouabain treatment and increased after 1.8 nM ouabain treatment. Conclusion: Ouabain at different concentrations caused dual effects on proliferation and apoptosis in HUVECs.

Keywords: Ouabain, endothelium, α subunit, NF-κB

Introduction

Vascular endothelial cells, of which the functional integrity is crucial for the maintenance of blood flow and antithrombotic activity, may be targets for endogenous ouabain (EO) [1]. Following an original report published in 1991 [2], EO has been isolated and detected by different laboratories. EO shares many of the properties of ouabain obtained from exogenous sources. Exogenous ouabain is a cardiac glycoside isolated from the bark and roots of the ouabain tree and the seeds of *Strophanthus gratus*. It was identified in the 1800s as one of several poisons used by African tribes to make poison arrow tips. As an endogenous regulator of Na^+-K^+-ATPase activity [3, 4], mounting evidence shows that ouabain is involved in the pathogenesis of hypertension. Chronic ouabain treatment results in hypertension [5] and triggers cardiac remodeling [6, 7], the onset of which is associated with endothelial dysfunction, which markedly affects the overall integrity and function of the cardiovascular system. Our previous study indicated that ouabain at physiological concentrations (0.3-0.9 nmol/l) stimulated the proliferation of human umbilical vein endothelial cells (HUVECs). However, at pathological concentrations (0.9-1.8 nmol/l), cell death was induced, as characterized by the swelling phenomenon and appearance of apoptotic bodies [8]. The results of mRNA profile

analysis indicated that the most up regulated genes were related to signal transduction and metabolism, including nuclear factor-κB (NF-κB) activator TANK (NM_004180). Many experiments have shown that Na^+-K^+-ATPase activity regulated by ouabain is dependent on NF-κB [9]. However, the relationship between the different Na^+-K^+-ATPase α-subunits, NF-κB and the dual effects on the endothelium by ouabain remain unclear. Our study focused on the pathway by which ouabain regulates endothelial function. We examined the effect of varying concentrations of ouabain on endothelial function according to the activity of different α-subunits and NF-κB.

Materials and methods

Cell culture and morphological analysis by light microscopy

The research was approved by the ethics committee of Xi'an Jiaotong University. All chemicals were obtained from Sigma Aldrich Co. (St. Louis, M, USA) unless otherwise specified. The collagenase perfusion method was used to isolate HUVECs [10], which were then cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. HUVECs were identified by their typical cobblestone morphology using microscopy (Olympus). After identification, HUVECs were incubated with either 0.3 nM or 1.8 nM ouabain for 2 h to study morphological differences.

MTT cell proliferation assay

HUVECs at passage three were cultured to 80% confluence and then plated at a density of 5×10^3 cells/well in 96-well multiplates. Complete growth medium was replaced with serum-free medium after the cells had reached confluency to down regulate cell proliferation and block cells in G1 phase of the cell cycle. Serum-free medium was replaced once again 24 h later, and 0.3 nmol/L or 1.8 nmol/L ouabain was added to the cells in quadruplicate; an untreated group was set up as a control. After varying incubation periods (0.5, 1, 2, 4, 8, 16, 24, and 48 h), cells were pulsed with 500 μg/ml methyl thiazolyl tetrazolium (MTT; Sigma) for 4 h, and the resulting MTT formazan was solubilized with 10% sodium dodecyl sulfate (SDS) overnight. Absorbance at 490 nm was measured with a Model 680 microplate reader. Proliferation rates (%) were calculated as (experimental absorbance/control absorbance-1) ×100%.

Cell cycle analysis

Cells were stained with propidium iodide (PI) and DNA content was analyzed using flow cytometry. Briefly, cells were fixed in 70% ethanol, incubated with PI staining solution (containing 10 μg/ml PI and 100 μg/ml RNase) for 15 min at 37°C, and analyzed by flow cytometry.

Immunohistochemical staining of NF-κB p65 subunit

Following pretreatment of glass cover slips with poly-L-lysine in 6-well multiplates, HUVECs were seeded at a density of 5×10^4 cells/well. Complete growth medium was replaced with serum-free medium once the cells had reached confluency, and plates were incubated for 6 h. Then, different doses of ouabain (0, 0.3 and 1.8 nmol/L) were added to each plate in medium containing 100 $ml{\cdot}L^{-1}$ FBS for 4 h. After washing three times with PBS, the cover slips were fixed for 30 min with cold acetone and dried naturally. NF-κB immunohistochemical staining was performed in accordance with the instructions provided by the manufacturer (Wuhan Boster Bio-engineering Co., Ltd); the primary antibody was diluted to 1:200.

NF-κB activity assay

Activation of NF-κB by ouabain was investigated by examining the degradation of its inhibitory subunit IκBα. The expression of IκB was studied by western blot analysis as described above, using 10% polyacrylamide gels and an anti-IκBα monoclonal antibody (1:200) with a goat anti-mouse secondary IgG conjugated to horseradish peroxidase (1:200) (Boster Bio-engineering Co., Ltd Wuhan, China).

Na^+-K^+-ATPase activity assay

Homogenates of HUVECs were suspended in Tris buffer (1300 mM NaCl, 200 mM KCl, 40 mM $MgCl_2$ and 150 mM histidine; pH 7.4) to a concentration of 2 mg/ml. Saponin (1%) was then added at a ratio of 1:4, and the samples were incubated at room temperature for 30 min in different concentrations of ouabain (0 nM, 0.3 nM, 1.8 nM), a specific inhibitor of the ATPase. Adenosine tri-phosphate (ATP) was then added to a final concentration of 7.5 mM,

and samples were incubated for an additional 30 min period at 37°C. At the end of incubation period, an equal volume of trichloroacetic acid (11.5%) was added to stop the reaction, and the samples were spun at 3000×g for 5 min. The amount of inorganic phosphate liberated was measured colorimetrically according to the method of Taussky and Shorr [11]. Enzymatic activity was determined by measuring the ouabain inhibitable inorganic phosphate (Pi) released.

Real RT-PCR

Total RNA was extracted from HUVECs using Sepasol-RNA and reverse-transcribed to cDNA using the Superscript III First-Strand System for RT-PCR (Life Tech, California, U.S.A.) according to the manufacturer's instructions. cDNA was amplified with a Bio-Rad iCycler (Bio-Rad Laboratories) using KOD DNA polymerase and the following primers (designed and synthesized by Beijing Sunbiotech Co.,Ltd.): α_1-subunit (forward): 5'-ACACAGCCTTCTTCGTCAGTATCG-3', α_1-subunit (reverse): 5'-ACATCCTAAGAGCAACACCCATTCC-3'; α_2-subunit (forward): 5'-GGTCTCCTTCTTCGTGCTCTCC-3', α_2-subunit (reverse): 5'-GTTCTTCACCAGGCAGTTCTTCC-3'; α_3-subunit (forward): 5'-CATCTTCCTCATCGGCATCATCG-3', α_3-subunit (reverse): 5'-GTTCTTCACCAGGCAGTTCTTCC-3'; β-actin (forward): 5'-ATCGTGCGTGACATTAAGGAGAAG-3', β-actin (reverse): 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. PCR conditions for α-subunits and β-actin were 95°C for 3 min, followed by 40 to 45 cycles of 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, and 55°C for 30 s, followed by 81 s. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and stained with 1 μg/ml ethidium bromide (Life Tech).

Preparation of cell lysates and western blotting determination of total cellular Na^+/K^+ ATPase α-subunits

HUVECs were washed once with PBS and lysed in Trion X-100 lysis buffer consisting of 50 mM Tris-HCl (PH 7.4), 1% Triton X-100, 2 mM DTT, 2 Mm sodium orthovanadate, and Complete protease inhibitor mixture (1 mM PSMF, 1 mM benzamidine-HCl, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 1 mg/ml pepstatin, 50 mM sodium fluoride). Postnuclear lysates were collected as supernatants by centrifugation (800×g for 7 min). Pellets were washed twice with HEPES buffer (20 mM HEPES-NaOH (pH 8.0), 20% glycerol, 100 Mm KCl, 1 mM EDTA, 0.5 Mm DTT, leupeptin (10 μg/ml), and 0.5 Mm PMSF) and then lysed in the HEPES buffer by sonication, followed by centrifugation (9000×g for 2 min). Western blotting was performed on whole cell lysates using procedures similar to those described by Singh et al. [12]. Protein samples (20-30 μg/lane) were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad), then blocked with 5% non-fat dehydrated milk in Tris buffered saline containing Tween 20 (TBST; 25 mM Tris (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) for 1 h at room temperature. The membranes were incubated with primary antibodies (1:150) in TBST (5% BSA) overnight at 4°C, followed by secondary antibody horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:200) (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd.) in TBST (5% non-fat dehydrated milk or 5% BSA) for 1 h at room temperature. Immunoblotting detection was performed using the ECL detection system (Bio-rad) according to the manufacturer's instructions.

Equal loading of proteins was evaluated by stripping and developing a blot for β-actin (Cell Signaling). The primary antibody used for detection of Na^+/K^+ ATPase α-subunit was obtained from Cell Signaling (1:1000). Percentage of phosphorylated α-subunit was determined in parallel samples by first developing the membrane to determine the density of phosphorylated signal, and then stripping and exposing the membrane to the Na^+/K^+ ATPase α-subunit antibody (1:1000; Cell Signaling). Densitometric analyses of α-subunits relative to β-actin were performed using ImageJ, Sigma Stat and Sigma Plot.

Cell immunofluorescence observation of Na^+/K^+ ATPase α-subunit protein expression

HUVECs were identified as described above and samples were observed under confocal laser microscopy (nIKON). The primary antibody was the same as that used for western blotting, and the secondary antibodies were FITC fluorescence antibody (goat anti-mouse, 1:100; Zhongshan Goldenbridge Biotechnology Co., Ltd.), RBITC fluorescence antibody (rabbit anti-goat, 1:100; Zhongshan Goldenbridge Biotechnology Co., Ltd.) and FITC fluorescence antibody (goat anti-mouse, 1:100; Zhongshan Goldenbridge Biotechnology Co., Ltd.).

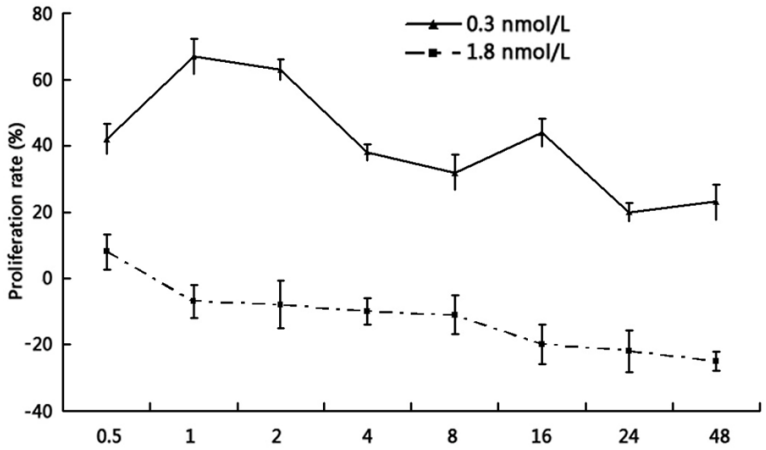

Figure 1. Ouabain affected the proliferation of HUVECs in a time-dependent manner. HUVECS were treated with 0.3 nmol/L or 1.8 nmol/L ouabain over a period of 48 h (n = 4; mean ± SEM). Ouabain at 0.3 nM triggered the proliferation of HUVECs from 0.5 h, and the most prominent increases were observed at 1-2 h. However, 1.8 nM ouabain treatment for 0.5 h induced an increase in cell proliferation for a short time, and then triggered a decrease from 1 h.

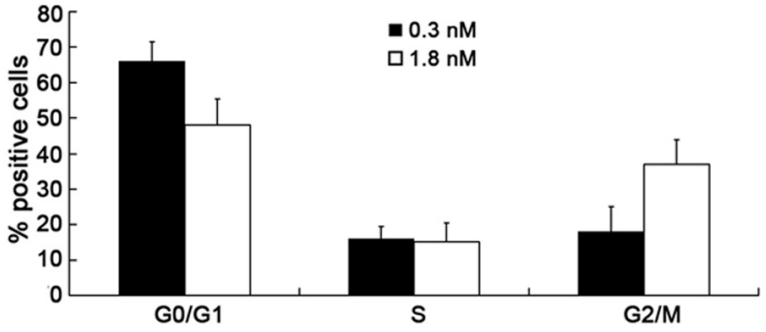

Figure 2. Effect of ouabain on cell cycle distribution. The results show that 37% of HUVECs were in G2/M phase after 1.8 nM ouabain treatment, compared with 18% of those treated with 0.3 nM ouabain (P < 0.01) after 2 h incubation. The data are expressed as a percentage of positive cells in the respective cell cycle phase. Bars correspond to the mean ± SEM. Data are representative of three samples.

Statistical analysis

Values are presented as mean ± SEM. Statistical significance was evaluated using Student's two-tail t-test and identified as P < 0.05.

Results

Ouabain has dual effects on HUVECs according to morphological evaluation, MTT cell proliferation assay and cell cycle analysis

Fluorescence microscopy showed that HUVECs had elongated spindles, and green fluorescence could be seen in the cell membrane and cytoplasm by staining with vascular-factor III. After incubation with 0.3 nM ouabain for 2 h, HUVECs at passage three grew in a cobblestone-like arrangement. However, cells treated with 1.8 nM ouabain became wider and grew slowly, and the number of dead cells increased. Compared with the control group, different doses of ouabain exhibited time-dependent changes in HUVEC proliferation as measured by MTT assay. Ouabain at 0.3 nM triggered the proliferation of HUVECs from 0.5 h, and the most prominent increases were observed at 1-2 h. This is why all further studies were conducted using 2 h treatment. However, 1.8 nM ouabain treatment for 0.5 h induced an increase in cell proliferation for a short time, and then triggered a decrease from 1 h (**Figure 1**).

To analyze the cell cycle phase in which the HUVECs were localized following stimulation with 0.3 nM and 1.8 nM ouabain, cells were cultured in RPMI-1640 containing 10% FBS and analyzed using PI staining. **Figure 2** shows that 37% of HUVECs were in G2/M phase after 1.8 nM ouabain treatment, compared with 18% of those treated with 0.3 nM ouabain (P < 0.01).

Evaluation of the involvement of NF-κB on the dual effects of ouabain in HUVECs

After incubation with 0.3 nM ouabain for 2 h, the nuclei and cytoplasm of HUVECs exhibited NF-κB p65 staining, indicating that NF-κB heterodimers had become free and trans-located to the nucleus. Hence, the nuclei and cytoplasm were all stained with NF-κB p65. However, only the cytoplasm of untreated HUVECs and those treated with 1.8 nM ouabain were

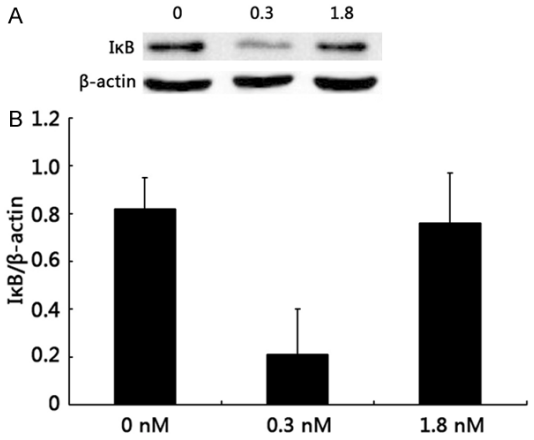

Figure 3. NF-κB activity determination by western blot analysis of IκB expression. (A) Western blot and (B) densitometric analysis of IκB expression. The expression of IκB was significantly reduced after 0.3 nM ouabain treatment (0.82 ± 0.13 versus 0.21 ± 0.19, $P < 0.01$). No effects were observed for 1.8 nM ouabain treatment or the control group (0.82 ± 0.13 versus 0.76 ± 0.21, p > 0.05). Bars not sharing a common letter are significantly different from each other ($P < 0.01$).

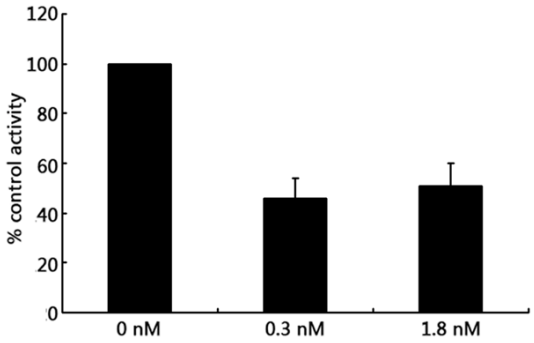

Figure 4. Effects of different concentrations of ouabain on Na^+-K^+-ATPase activity in HUVECs. The activity of Na^+-K^+-ATPase in HUVECs was significantly reduced after treatment with either 0.3 nM or 1.8 nM ouabain (46 ± 8%, 51 ± 9% versus 100%, $P < 0.01$), and there was no difference between the two groups (46 ± 8% versus 51 ± 9%, $P > 0.05$). Values shown are means ± SEM of three observations assayed in triplicate. Bars not sharing a common letter are significantly different from each other ($P < 0.01$).

stained. This phenomenon indicated that the combination of NF-κB with the IκB inhibitory proteins was confined to the cytoplasm, and NF-κB was not activated. Then western blot analysis was used to determine NF-κB activity after 2 h of incubation with two different concentrations of ouabain. IκB expression was significantly reduced after 0.3 nM ouabain treatment (0.82 ± 0.13 versus 0.21 ± 0.19, $P < 0.01$). There was no different between 1.8 nM ouabain treatment and the untreated control (0.82 ± 0.13 versus 0.76 ± 0.21, $P > 0.05$) (**Figure 3**).

Examination of the involvement of different Na^+-K^+-ATPase α-subunits on the dual effects of ouabain in HUVECs by Na^+-K^+-ATPase activity assay, RT-PCR western blot and immunofluorescence

The activity of Na^+-K^+-ATPase in HUVECs was significantly reduced after treatment with either 0.3 nM or 1.8 nM ouabain (46 ± 8%, 51 ± 9% versus 100%, $P < 0.01$), and there was no difference between the two groups (46 ± 8% versus 51 ± 9%, $P > 0.05$) (**Figure 4**). To detect the expression level of α-subunits mRNA by RT-PCR, we found that the expression level of α_1-subunit mRNA increased after 0.3 nM ouabain treatment (1.78 ± 0.61 versus 1, $P < 0.01$) and decreased after 1.8 nM ouabain treatment (0.62 ± 0.41 versus 1, $P < 0.01$). However, α_2- and α_3-subunits mRNA expression decreased after 0.3 nM ouabain treatment (0.73 ± 0.31 and 0.61 ± 0.42 versus 1, respectively; $P < 0.01$) and increased after 1.8 nM ouabain treatment (1.27 ± 0.39 and 1.87 ± 0.50 versus 1, respectively; $P < 0.01$) (**Figure 5**). Examination of α-subunit protein expression by western blot revealed that α_1-subunit protein expression levels were increased after 0.3 nM ouabain treatment (1.3 ± 0.11 versus 0.95 ± 0.08, $P < 0.01$) and decreased after 1.8 nM ouabain treatment (0.67 ± 0.23 versus 0.95 ± 0.08, $P < 0.01$). But α_2 and α_3-subunit protein decreased after 0.3 nM ouabain treatment (0.21 ± 0.12 versus 0.38 ± 0.10, and 0.25 ± 0.10 versus 0.37 ± 0.06, respectively; $P < 0.01$) and increased after 1.8 nM ouabain treatment (0.08 ± 0.19 versus 0.38 ± 0.10, and 0.57 ± 0.13 versus 0.37 ± 0.06, respectively; $P < 0.01$) (**Figure 6**). The results of further study to detect protein expression by immunofluorescence were similar to those obtained by RT-PCR and western blotting.

Discussion

EO is associated with blood pressure and cardiovascular complications both in rats infused with ouabain and in humans suffering from early and mild to long-lasting and more severe hypertension [13-16], thus causing cardiac

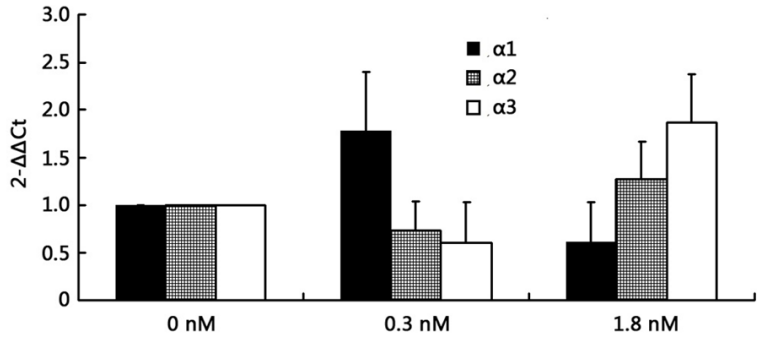

Figure 5. Effect of different concentrations of ouabain on Na^+-K^+-ATPase α-subunit mRNA expression in HUVECs. The expression level of α_1-subunit mRNA increased after 0.3 nM ouabain treatment (1.78 ± 0.61 versus 1, $P < 0.01$) and decreased after 1.8 nM ouabain treatment (0.62 ± 0.41 versus 1, $P < 0.01$). However, α_2- and α_3-subunit mRNA expression decreased after 0.3 nM ouabain treatment (0.73 ± 0.31 and 0.61 ± 0.42 versus 1, respectively; $P < 0.01$) and increased after 1.8 nM ouabain treatment (1.27 ± 0.39 and 1.87 ± 0.50 versus 1, respectively; $P < 0.01$). Values shown indicate means ± SEM of three observations assayed in triplicate. In each α-subunit group, bars not sharing a common letter are significantly different from each other ($P < 0.01$).

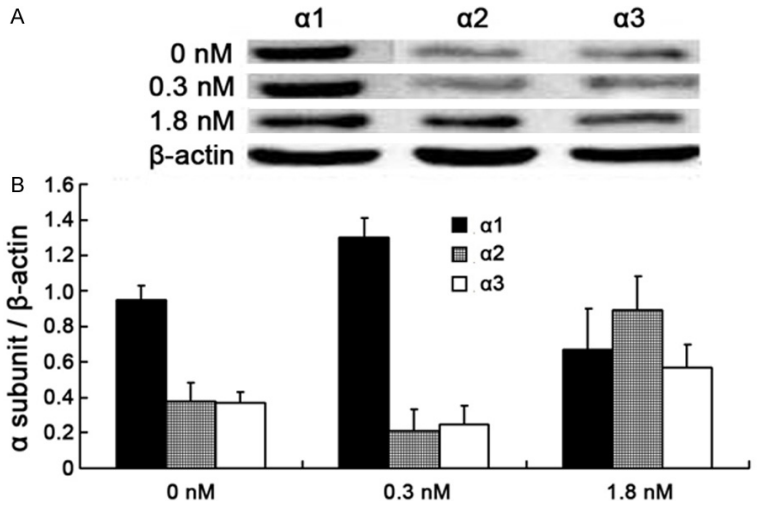

Figure 6. Analysis of α_1-, α_2- and α_3-subunits expression. (A) Western blot and (B) densitometric analysis of α_1-, α_2- and α_3-subunits expression. α_1-subunit protein expression levels were increased after 0.3 nM ouabain treatment (1.3 ± 0.11 versus 0.95 ± 0.08, $P < 0.01$) and decreased after 1.8 nM ouabain treatment (0.67 ± 0.23 versus 0.95 ± 0.08, $P < 0.01$). But α_2 and α_3-subunit protein decreased after 0.3 nM ouabain treatment (0.21 ± 0.12 versus 0.38 ± 0.10, and 0.25 ± 0.10 versus 0.37 ± 0.06, respectively; $P < 0.01$) and increased after 1.8 nM ouabain treatment (0.89 ± 0.19 versus 0.38 ± 0.10, and 0.57 ± 0.13 versus 0.37 ± 0.06, respectively; $P < 0.01$) Values shown are means ± SEM of three observations assayed in triplicate. In each α-subunit group, bars not sharing a common letter are significantly different from each other ($P < 0.01$).

damage. EO increases blood pressure in Sprague Dawley rats when infused at very low doses (15 μg/kg/day) for 4-8 weeks [17, 18].

Chronic ouabain treatment produces hypertension by acting on the central nervous system and at the vascular level. On the other hand, EO also triggers cardiac remodeling. Endothelial dysfunction is a very important factor in cardiac remodeling. The vascular endothelium is a monolayer of endothelial cells that lines in the inner surface of all blood vessels, including arteries, veins, and lymphatic vessels. The endothelial cells are continually exposed to circulating blood and must function to regulate and meet the oxygen and nutritional needs of underlying tissue. Therefore, they are not only a physical barrier between the blood and the underlying smooth muscle cells, but also an active player in the regulation of vasodilation, coagulation, inflammation, and immune response. Additionally, they are essential for cardiovascular homeostasis and maintaining vascular tone. Endothelial cells play a pivotal role in the development of atherosclerosis.

As an endogenous hormone, ouabain has different roles in endothelial cells. A previous study found that ouabain can initiate cell proliferation or apoptotic cell death in a dose-dependent manner; ouabain stimulated the proliferation of HUVEC at physiological concentrations (0.3-0.9 nmol/l) and induced cell death at pathological concentrations (0.9-1.8 nmol/l) [19]. The loss of Na^+-K^+-ATPase activity in the evolution of cellular pathology is well recognized. Thus, a graded reduction of Na^+-K^+-ATPase activity or a modulation of Na^+-K^+-ATPase function independent of pump activity [20, 21] may activate signals that promote cel-

lular proliferation and/or block programmed cell death. However, the relationship between ouabain and different Na^+-K^+-ATPase α-subunits and NF-κB in the regulation of proliferation and apoptosis of HUVECs remained unclear.

As a target for ouabain, Na^+-K^+-ATPase is a ubiquitous cell membrane enzyme involved in regulation of intracellular ion gradients. There are four isoforms of the α-subunit (α_1, α_2, α_3 and α_4) and three of the β-subunit (β1, β2 and β3) in vertebrates [22, 23]. The Na^+-K^+-ATPase α-subunit ouabain binding site has been highly conserved during the evolution of higher animals, which may indicate a fundamental role in the regulation of physiological processes [24]. Nevertheless, not all α-subunit isoforms, nor the isoforms in all species, have the same high affinity for ouabain. In rats, ouabain exhibits high affinity for the α_2/α_3 isoforms of Na^+-K^+-ATPase. We found that the mRNA and protein expression levels of α_1-subunit increased after 0.3 nM ouabain treatment and decreased after 1.8 nM ouabain treatment; in contrasts, that of the α_2- and α_3-subunits decreased after 0.3 nM ouabain treatment and increased after 1.8 nM ouabain treatment, indicating that signaling pathway of ouabain binding to α_1-subunit participates in the proliferation of HUVECs and that of the $\alpha_2/_3$-subunits participates in the death of HUVECs in dose-dependent manner.

The transcription factor NF-κB participates in the expression of a wide variety of genes that are involved in the regulation of many cellular mediators of immune and inflammatory responses [25].

I-κB exists as a complex with NF-κB heterodimer in the cytosol [26]. The NF-κB heterodimer becomes free and trans-locates to the nucleus where it activates a variety of genes responsible for inflammation as well as cancer development and progression [27]. Activation of NF-κB results from the phosphorylation of its IκB protein. This phosphorylation is mediated via IκB protein kinases (IKKs), which causes proteolytic degradation of IκB by 26S proteasomes. Subsequently, degraded IκB allows NF-κB to translocate to the nucleus, where it regulates the expression of many genes [28]. Moreover, it has been reported that ouabain is able to activate the NF-κB pathway accordingly to affect the early NF-κB signaling pathway, triggering slow calcium oscillations and preventing NF-κB-inducible protein expression by blocking Na^+-dependent amino acid transport [29-32].

The present study was designed as a follow up to our previous study to determine whether NF-κB plays a role in ouabain-mediated proliferation and apoptosis of HUVECs, and to investigate its relationship with different α-subunits. IκB expression was significantly reduced after 0.3 nM ouabain treatment. NF-κB p65 staining was observed in the nuclei and cytosol of HUVECs incubated with 0.3 nM ouabain for 2 h, but in the cytosol alone of untreated HUVECs or those treated with 1.8 nM ouabin. This phenomenon indicated that the binding of NF-κB with the IκB inhibitory proteins was confined to the cytoplasm. NF-κB was activated after ouabain binding to α_1-subunit, which induced HUVEC proliferation.

EO, an important endogenous hormone, is associated with many cardiovascular diseases such as hypertension, by its mode of action is unclear. Our results indicated that EO has dual effects on the regulation of proliferation and apoptosis in HUVECs at different concentrations. At 0.3 nM, ouabain promoted cell proliferation via activation of Na^+-K^+-ATPase α_1-subunit and the NF-κB pathway, whereas it induced cell apoptosis via activation of α_2- and α_3-subunits without participation of NF-κB at 1.8 nM. In conclusion, any mechanism altering Na^+-K^+-ATPase function and/or expression may be a candidate for hypertension pathogenesis and thus represents a potential new therapeutic target [33].

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Disclosure conflict of interest

None.

Address correspondence to: Yan-Ping Ren, Department of Geriatric-Cardiovascular Diseases, The First Affiliated Hospital of Medical College in Xi'an Jiaotong University, Xi'an 710061, China. Tel: +86 29 85324042; Fax: +86 29 85323239; E-mail: ryp0071@126.com; Ming-Juan Zhang, Department of Cardiology, The Second Affiliated Hospital of Medical College in Xi'an Jiaotong University, Xi'an 710004, China. E-mail: zhangmingjuan@mail.xjtu.edu.cn

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