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Original Article

A key mediator, PTX3, of IKK/IκB/NF-κB exacerbates human umbilical vein endothelial cell injury and dysfunction

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Abstract: Objective: This study was performed to investigate PTX3-mediated iNOS expression and IKK/IκB/NF-κB activation in PA-induced atherosclerotic HUVECs injury model. Methods: The cell viability was detected by the CCK8 assay. The cell apoptosis was assessed by annexin V-PI double-labeling staining. Expression of genes and proteins were analyzed by real-time PCR and western blotting respectively. Cells were transfected with siRNAs as a gene silencing methods. Results: PA induced cell apoptosis in human umbilical vein endothelial cells in a time and dose-dependent manner. PA also induced upregulation expression of PTX3. TPCA-1, an inhibitor of IKK-2, could suppress the expression of PTX3 and phospho-IκB-α in PA-induced endothelial dysfunction cell model. We also found that transfection of cells with PTX3 siRNA reduced the expression of iNOS and NO, and protected PA-induced cell apoptosis in HUVECs. Conclusions: PTX3 could exacerbate endothelial dysfunction, at least partially, through IKK/IκB/NF-κB activation and overexpression of iNOS and NO, and advance the development of atherosclerosis.

Keywords: PTX3, NF-κB, iNOS, atherosclerosis, HUVECs

Introduction

Atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation [1, 2]. Hypercholesterolemia is the main contributing factor to the development of atherosclerosis. It can accumulate in blood vessels and induce endothelial dysfunction that leads to atherosclerosis. Free fatty acids trigger endothelial apoptosis and inhibit cell cycle progression [3]. Palmitic acid (PA) is the main saturated free fatty acid in the bloodstream. The exposure of endothelial cells to PA leads to cell necrosis [4] and the release of inflammatory cytokines IL-6 [5]. Moreover, PA-induced increase in the generation of reactive oxygen species, the activation of NADPH oxidase, the up-regulation of inducible nitric oxide synthase (iNOS) and down-regulation endothelial nitric oxide synthase (eNOS) [3]. In in vivo studies, the exacerbation of the progression of endothelial dysfunction was reported in C57BL/6 mice after long-term exposure to high-calorie and high-cholesterol diets [6]. Similarly, type 2 diabetic mice induced by a high-fat diet combined with a single injection of low-dose streptozotocin exposure to exacerbate coronary endothelial dysfunction and increase mitochondrial reactive oxygen species (ROS) concentration [7].

Nuclear factor-κB (NF-κB), an oxidative stress sensitive transcription factor, controls the expression of a wide variety of genes active, such as IL-1, IL-8, TNF-α and iNOS. These observations suggest that NF-κB is a suitable target to prevent or reduce an inflammatory response [1]. In endothelial cells, there are several transcriptional factor-binding sites in the cytokine promoter, including NF-κB [8]. Activated NF-κB may bind to the cytokine promoter, which is critically involved in cytokine gene regulation by various stimuli, such as low density lipoprotein [1], particulate matter [2] and palmitic acid [3]. Pharmacological research dispalys that losartan protects against sLDL cholesterol-inducing endothelial cell injury by inhibiting NF-κB activation [9], and flavonoids suppress angiotensin

II-induced fractalkine production by inhibiting the ROS/NF-κB pathway in human umbilical vein endothelial cells (HUVECs) [8].

The pentraxin3 (PTX3) are useful biomarkers for CVD, particularly ischemic heart disease and heart failure are deeply involved in the pathogenesis of CVD linked to inflammation and innate immunity. Circulating elevated PTX3 levels can provide prognostic information for a variety of clinical settings and facilitate the diagnosis of CVD [10]. Moreover, PTX-3 demonstrates to be more specifically associated with advanced atherosclerosis [11]. PTX-3 is highly expressed in advanced atherosclerosis tissues, including macrophages [12] and surviving endothelial cells [13]. Clinical research shows that plasma PTX3 levels were detected in patients with unstable angina pectoris [14], and in the coronary artery at sites distal from the plaque lesion, PTX3 levels were significantly elevated compared with proximal sites, suggesting that it originated from the atherosclerotic plaque itself and may reflect active atherosclerosis [14]. In addition, PTX3 may represent an early marker of myocardial lesion [15] and peripheral artery disease [16]; higher PTX3 levels (> 10.73 ng/mL) were associated with increased 3-month mortality in patients with acute myocardial infarction [17]. Taken together, these data suggest that PTX3 may be involved in the pathogenesis of atherosclerosis. However, there are many evidences to suggest a potential role for PTX3 in atherosclerosis. Moreover, the relationship between PTX3 and IKK/IκB/NF-kB pathways in HUVECs is unknown.

In this study, HUVECs were chosen to investigate PA-induced damage to endothelial cells as the atherosclerotic endothelial cell injury model. A series of evaluations, including cell viability, NO production, and apoptosis response, in HUVECs after exposure to AP were investigated. Moreover, several proteins involved in early atherosclerosis and in the activation of NF-kB were measured to determine whether AP-induced injury and dysfunction of HUVECs through PTX3 via IKK/IκB/NF-kB pathways.

Materials and methods

Cell culture

The human umbilical vein endothelial cells (HUVECs) was obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China), and maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO_2, 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with various concentrations of palmitic acid (Sigma, USA).

Cell viability detection by CCK8

HUVECs (5.0×10^3/well) were plated and treated in 96-well plates (three wells per group) with various concentrations of palmitic acid (0 μg/ml, 20 μg/ml, 40 μg/ml or 80 μg/ml) for 24 h, 48 h or 72 h respectively. 10 μL of CCK8 (Beyotime, China) was added to the cells, and the OD value of the cells was measured at 450 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells. HUVECs (5.0×10^5/well, 1 ml) were plated and treated in 6-well plates (three wells per group) with palmitic acid (0 μg/ml, 20 μg/ml, 40 μg/ml or 80 μg/ml) for 48 h. After treatment, cells were washed twice with PBS (pH=7.4), and re-suspended in staining buffer containing 10 μl PI and 5 μl annexin V-FITC. Double-labeling was performed at room temperature for 15 min in the dark before the flow cytometric analysis. Cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson). Quantitative assessment of apoptotic cells was also assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert™ DNA Fragmentation Assay Kit. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodiumcitrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with flow cytometer.

Quantitative real-time PCR

HUVECs (5.0×10^5/well) were plated and treated in 6-well plates (three wells per group) after 24 h with palmitic acid (80 μg/ml) for 48 h. The HUVECs RNA extraction was performed according to the TRIzol manufacturer's protocol

(Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Promega, Switzerland) with oligo dT (15) primers (Fermentas) as described by the manufacturer. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 9700). The cycling conditions were 2-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 55°C for 60 s. PCR with the following primers: PTX3, Forward 5'-TTGCGATTCTGTTTTGTGCT-3' and Reverse 5'-GTGGGGTCCTCAGTGGG-3'; β-actin, Forward 5'-ACAGGGGAGGTGATAGCATT-3' and Reverse 5'-GACCAAAAGCCTTCATACATCTC-3'. β-actin as an internal control was used to norm alize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the $2^{-\Delta\Delta ct}$ method.

Western blotting

The HUVECs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to PVDF Transfer Membrane (Millipore). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, PTX3 (Enzo Life Sciences, Switzerland), phospho-IκB-α, IκB-α and iNOS (Abcam, UK), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800 CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1 hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (Bioworld Technology, USA).

RNA interference

The small interfering (si) RNA for human PTX3 or scramble siRNA was obtained from Dharmacon (Lafayette, USA). The small in terfering with the following primers: PTX3-1, Forward 5'-CACUCUGAGUGGGACAAGCUCUUCA-3' and Reverse 5'-UGAAGAGCUUGUCCCACUCAGAGUG-3'; PTX3-2, Forward 5'-GCUAUCAGUCC-ACUGUGCUUGUGGU-3' and Reverse 5'ACCACAAGCACAGUGGACUGAUAGC-3', PTX33, Forward 5'-CCUCAGCUAUCAGUCCACUGUGCUU-3' and Reverse 5'-AAGCACAGUGGACUGAUAGCUGAGG-3'; scramble, Forward 5'-CAC GAGUGGGUAACACUCGUCUUCA-3' and Reverse 5'-UGAAGACGAGUGUUACCCACUCGUG-3'. The siRNA oligonucleotides (at a final concentration of 100 nM) were transfected into human umbilical vein endothelial cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Determination of PTX-3 expression by ELISA assay

HUVECs (1.0×10^4/well) were plated and treated in 96-well plates (three wells per group) with for 48 h with DMSO or kinase inhibitors (30 μM) from kinase inhibitor library, followed centrifugation to obtain the supernatant. PTX-3 levels were measured by the Sandwich ELISA kit at 450 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Nitric oxide quantification

HUVECs (5.0×10^5/well) were plated and treated in 96-well plates (three wells per group), and were stimulated with palmitic acid (80 μg/ml) and si-PTX3-1 in the presence or absence. Forty-eight hours later centrifugate to obtain the supernatant, and the level of nitric oxide was measured by nitrite production using the Griess reagent (Invitrogen, USA) at 540 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Results

PA-induced cell apoptosis in HUVECs

To evaluate the potential cell apoptosis of PA in HUVECs, we analyzed the effect of PA on cell survival in HUVECs. The CCK8 assay was used to measure cell viability. The viabilities of HUVECs treated with PA were significantly lower than those of untreatment group. Treatment of HUVECs with PA induced cell death in a time

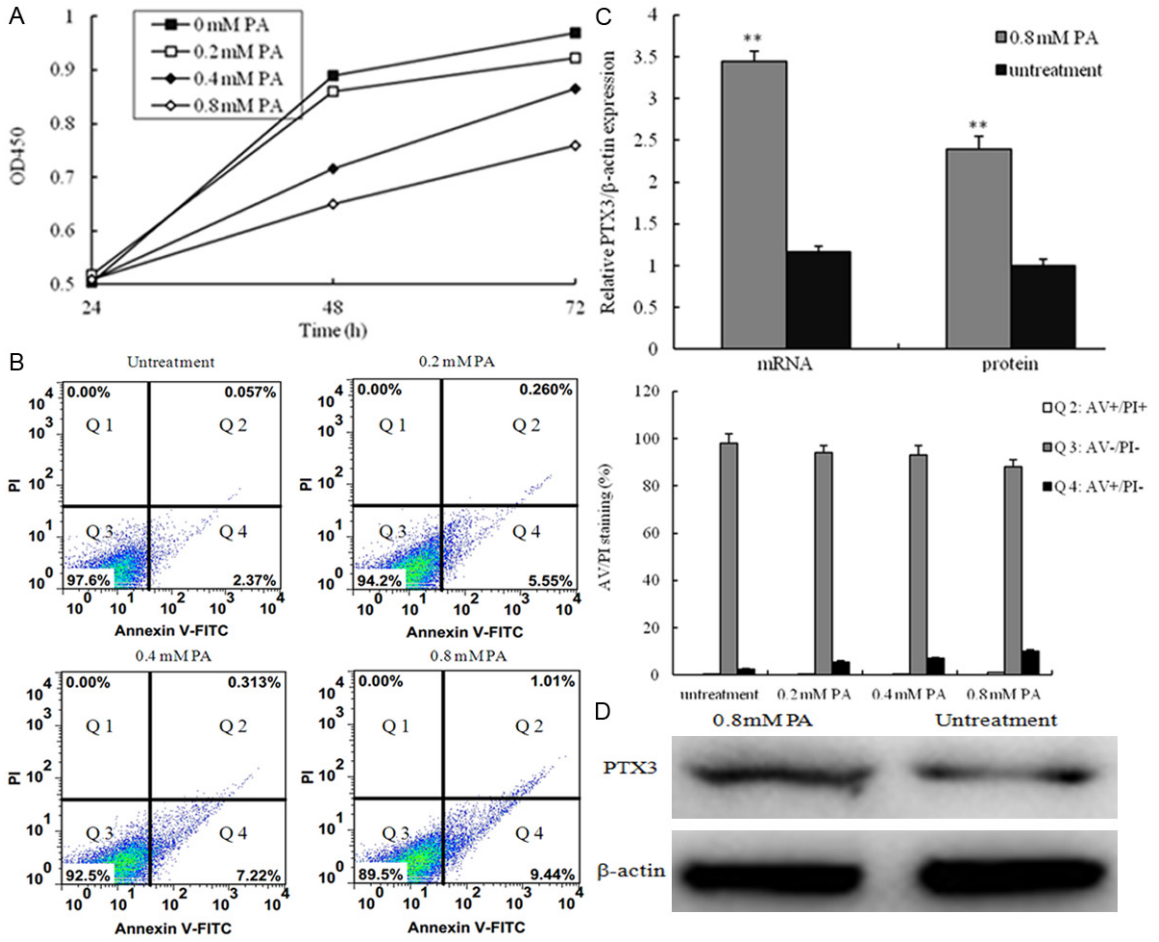

Figure 1. Palmitic acid-induced the apoptosis of human umbilical vein endothelial cells (HUVECs). HUVECs were incubated with various concentrations of Palmitic acid (PA) for 24 h, 48 h or 72 h, and the cell viability was examined by CCK8 assay (A). Cells were treated with vehicle, 0.2 mM PA, 0.4 mM PA or 0.8 mM PA for 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (B). mRNA and protein expression of pentraxin3 (PTX3) in HUVECs. Cells were treated with PA (80 mM) for 48 h. mRNA (C) and protein (C and D) expression were measured by Quantitative real-time PCR and western blotting respectively. Values are expressed as mean ± SEM, n=3 in each group. $^{*}P < 0.05$, versus untreatment group.

and dose-dependent manner by using CCK8 assay (**Figure 1A**). We next investigated whether PA induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. Consistent with the CCK8 assay, the results showed that the proportion of the early phase of apoptosis cells had gained as compared to untreatment group (**Figure 1B**). Moreover, the percentage of the early phase of apoptosis cells in a dose-dependent manner.

mRNA and protein expression of PTX3 in HUVECs

Pentraxin protein family is highly associated with CVD, and PTX-3 is highly expressed in advanced atherosclerosis tissues. The current study suggested that PTX3 was associated with PA-induced atherosclerosis. The mRNA and protein expression of PTX3 was significantly higher in HUVECs with PA (0.8 mM) than those of untreatment group (**Figure 1C** and **1D**). Therefore, our data suggest that up-regulation the expression of PTX3 was involved in PA-induced cell death.

Inhibition the function of IKK with TPCA-1 regulation PTX3 expression

PTX3 is abundantly produced by various cells in atherosclerotic lesions, including monocytes, macrophages, endothelial cells, vascular smooth muscle cells, fibroblasts. These findings suggest that PTX3 levels reflect local inflamma-

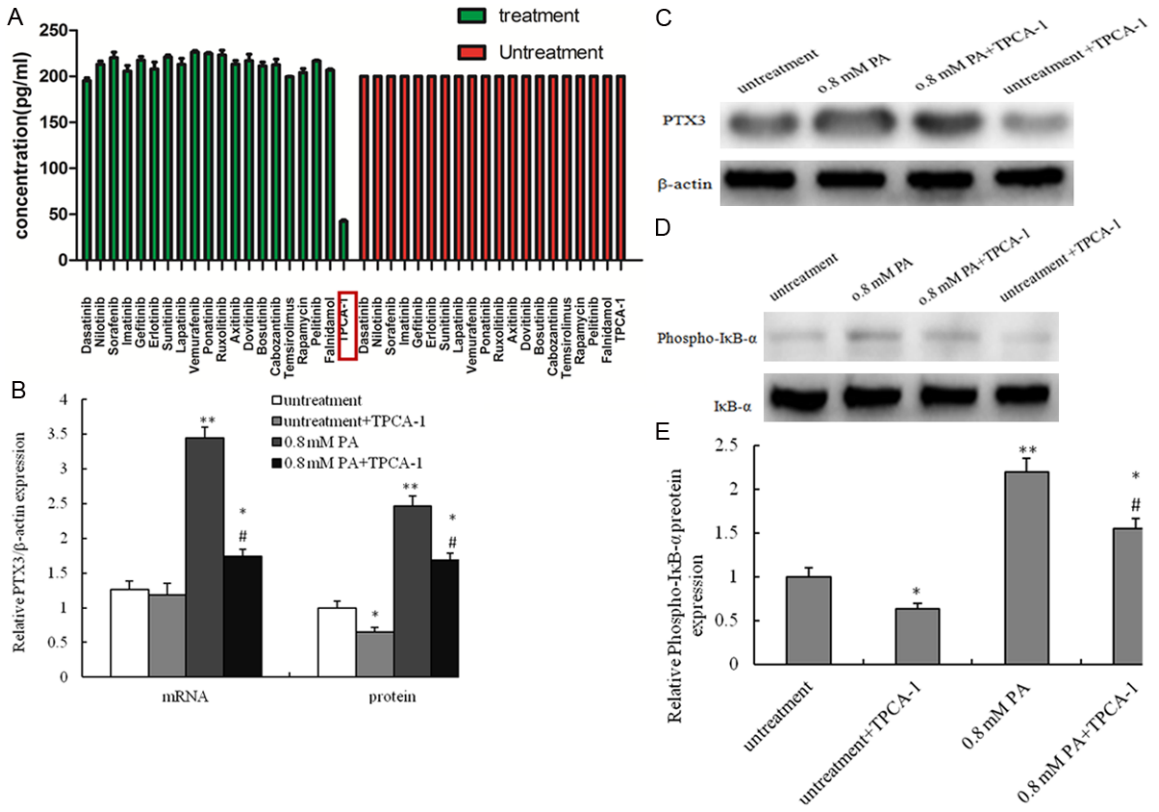

Figure 2. Screening an inhibitor of I kappa B kinase-2 (IKK-2) library and validation functions of TPCA-1. HUVECs were adopted with PA (80 mM) for 48 h, made as the atherosclerotic endothelial cell injury model. Cells were treated with vehicle or inhibitor of IKK-2 (30 μM) for 48 h, followed centrifugation to obtain the supernatant. PTX-3 levels were measured by the ELISA assay (A). HUVECs were treated with untreatment, TPCA-1 only, 0.8 mM PA only and 0.8 mM PA plus TPCA-1 for 48 h, the mRNA (B) and protein (B and C) expression were measured by Quantitative real-time PCR and western blotting respectively, and the protein expression of phospho-IκB-α and IκB-α were measured by western blotting (D and E). Values are expressed as mean ± SEM, n=3 in each group. $^{*}P < 0.05$, versus untreatment group.

tion at atherosclerotic lesions more accurately than does C-reactive protein [18]. We set out to screen an inhibitor of I kappa B kinase-2 (IKK-2) library representing the full complement of 20 human inhibitor for IKK-2 the inhibition of which might impair PTX3 expression in HUVECs, and analyzed PTX3 level in HUVECs after treatment with kinase inhibitors (including TPCA-1) at 30 μM for 48 hours. The results showed that the expression of PTX3 (40 pg/ml) was remarkably lower in HUVECs with TPCA-1 than those of untreatment group (200 pg/ml) (**Figure 2A**). Consistent with the ELISA assay results, inhibition the function of IKK with TPCA-1 induced strong and specific suppression of mRNA and protein expression of PTX3 in the PA combination with TPCA-1-treated group as compared to PA single treatment group (**Figure 2B** and **2C**). Moreover, the protein expression of phospho-IκB-α was significantly higher in HUVECs with PA (0.8 mM) than those of untreatment group (**Figure 2D**), and was statistically inhibited in the PA combination with TPCA-1-treated group as compared to PA single treatment group (**Figure 2D**). Therefore, our data suggest that up-regulation the expression of phospho-IκB-α was involved in PA-induced HUVECs death.

Identification of PTX3 in the regulation of HUVECs dysfunction

In this work, knock-out of endogenous PTX3 with small-interfering RNA (siRNA), the expression of PTX3 was down-regulated (**Figure 3A**). Inhibition the function of PTX3 with si-PTX3 induced suppression of protein expression of iNOS in the PA treatment group (**Figure 3B**). Consistent with the western blotting results, inhibition the function of PTX3 with si-PTX3 protected against PA-induced endothelial-derived

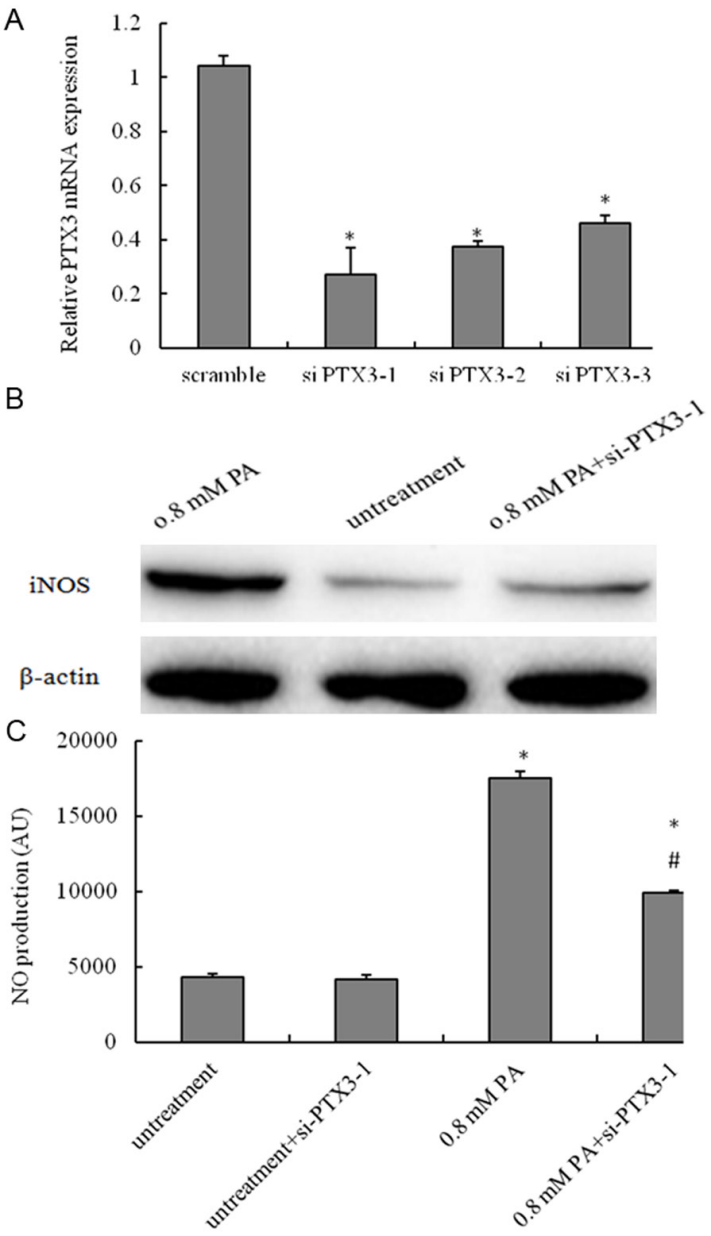

Figure 3. The small interfering RNA for suppressing the function of PTX3 (si-PTX3). Three different small interfering RNA were transfected into HUVECs suppressing the mRNA expression of PTX3 (A). HUVECs were treated with untreatment, 0.8 mM PA only and 0.8 mM PA plus si-PTX3 for 48 h, the protein expression was measured by western blotting (B), and the NO concentration was detected by ELISA assay (C). Values are expressed as mean ± SEM, n=3 in each group. $^{*}P < 0.05$, versus untreatment group.

NO dysfunction, concentrations of NO was decreased in PA+ si-PTX3 group (**Figure 3C**). To evaluate the potential protective mechanisms of inhibition the function of PTX3 in HUVECs, the CCK8 assay was used to measure cell viability. The viabilities of HUVECs inhibited with PA were protected by si-PTX3 (**Figure 4A**). Consistent with the CCK8 assay, the Annexin V-PI double-labeling results showed that inhibition the function of PTX3 with si-PTX3 could decrease the proportion of the early phase of apoptosis cells inducing by PA treatment (**Figure 4B**).

Discussion

In this study, HUVECs were considered to approximately represent the endothelial monolayer in blood vessels. The exposure of PA to HUVECs has been demonstrated to cause a series of endothelial cell injuries [3-5], and trends to make as the atherosclerotic endothelial cell injury model. First, we found that PA could induce HUVECs apoptosis in a dose dependent manner. Endothelial cell apoptosis was thought to be involved in atherosclerosis [19]. Thus, apoptosis was measured in the present study to better confirm and to analyze the endothelial cell injury by PA. We found that the proportion of the early phase of apoptotic cells was increased.

The IKKα kinase, a subunit of the NF-κB-activating IKK complex, has emerged as an important regulator of inflammatory gene expression [20]. In our work, TPCA-1, an inhibitor of IKK2, was exploited for inhibiting the function of IKK. TPCA-1 has been shown to possess potent anticancer [21] and anti-viral action [22]. Interestingly, IKK-2 activity is increased in the subgroup of aspirin sensitive nasal epithelial. IL-8 and GRO-α responses were repressed by IKK-2 inhibitor TPCA-1 in vitro [23]. The results showed that the expression of PTX3 and phospho-IκB-α was inhibited with TPCA-1 treatment. Therefore, our data suggest that up-regulation the expression of PTX3 and phospho-IκB-α was involved in PA-induced HUVECs death, and TPCA-1 inhibited PA-induced phospho-IκB-α activation in HUVECs. TPCA-1 might represent a potential therapeutic drug for treatment and prevention atherosclerosis.

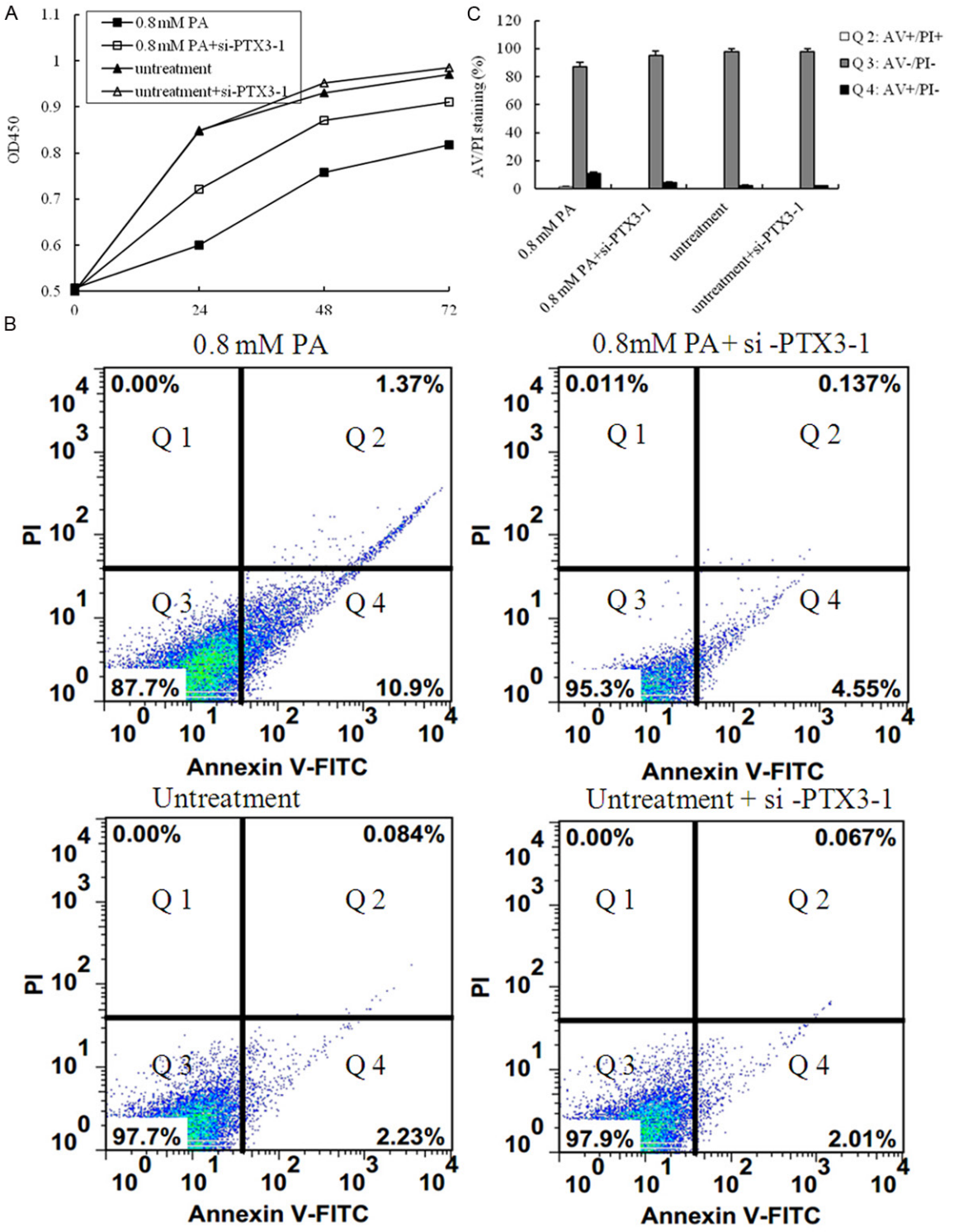

Figure 4. PTX3-induced the apoptosis of human umbilical vein endothelial cells (HUVECs). HUVECs were treated with untreatment, si-PTX3 only, 0.8 mM PA only and 0.8 mM PA plus si-PTX3 for 48 h, and the cell viability was examined by CCK8 assay (A). The apoptotic cells were detected by flow cytometric analysis of annexin V/PI double staining (B). (C) The percntage of apoptotic cells was also analyzed by annexin V/PI double staining (n=3).

The presence of PTX3 protein was demonstrated in the advanced atherosclerotic plaques and myocardial tissues of patients with acute myocardial infarction [12]. Previous reports

points out a potential protective effect of PTX-3 in the atherosclerotic, which PTX-3 deficiency is associated with increased atherosclerosis in apolipoprotein-E-deficient mice and increased macrophage accumulation in the atherosclerotic lesions [11]. On the contrary, our data have demonstrated that the knock-out of PTX3 could effectively inhibit the expression of iNOS and NO, suggesting that iNOS played a key role in PTX3-mediated endothelial cell injury. The ability to generate NO has served as a marker for healthy endothelia. Endothelial-derived NO is produced by eNOS and regulates vascular tone. Studies have shown that nanomolar concentrations of NO have anti-inflammatory. In contrast, iNOS led to inflammatory responses via NF-κB, which is the key transcription factor for major proinflammatory cytokines and adhesion molecules [24, 25]. Functional study show that tetramethylpyrazine (TMP), a compound derived from chuanxiong, suppressed TNF-α-induced expression of iNOS by inhibiting IκB kinase (IKK) phosphorylation, IκB degradation and nuclear factor κB (NF-κB) nuclear translocation, which were required for NO gene transcription [25]. The roles of the IKKs in NF-κB activation have been investigated in mice lacking IKKβ and IKKα [24]. Murine embryos genetically null for IKKβ succumbed to severe liver apoptosis in uterus due to a virtually complete block in NF-κB activation [26]. In contrast to the IKKβ, IKKα null embryos appeared to be phenotypically normal for both cytokine induced IκBα degradation, NF-κB nuclear translocation and NF-κB DNA binding activity [24]. These observations suggest that IKK/NF-κB pathway is a suitable target to prevent or reduce an inflammatory response and cardiovascular disease (CVD).

This result further confirmed the important role of PTX3 in the PA-induced injury of HUVECs. In summary, we found that PTX3 could be activated by IKK/IκB/NF-κB pathway, then inhibit cell proliferation and cause apoptosis through upregulation the expression of iNOS and NO.

Disclosure of conflict of interest

None.

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