N-acetyl cysteine inhibits lipopolysaccharide-induced apoptosis of human umbilical vein endothelial cells via the p38MAPK signaling pathway

TING XIONG, ZHENZHEN ZHANG, RUI ZHENG, JIALIN HUANG and LING GUO

Department of Prosthodontics, Hospital of Stomatology Affiliated to Southwest Medical University, Luzhou, Sichuan 646000, P.R. China

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Abstract. Lipopolysaccharide (LPS) can regulate the expression of apoptotic factors, including caspase-3, Bcl-2 and Bcl-2-associated X protein (Bax). Nitric oxide (NO) plays an important role in apoptosis. N-acetyl cysteine (NAC) has been shown to exhibit antioxidant effects *in vitro*. However, the effects of NAC on LPS-induced apoptosis of human umbilical vein endothelial cells (HUVECs) and the associated mechanisms are not well characterized. The present study explored the effect of NAC on LPS-induced apoptosis of HUVECs and determined the participation of the p38 mitogen-activated protein kinase (MAPK) pathway in the process of apoptosis. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. The expression of caspase-3, Bax, Bcl-2, phosphorylated (p)-p38MAPK/total (t-)p38MAPK and p-endothelial e nitric oxide synthase (eNOS)/t-eNOS proteins were determined by western blotting. The expression levels of caspase-3, Bax and Bcl-2 mRNA were determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The rate of apoptosis was determined using flow cytometry. An NO detection kit (nitric reductase method) was used to determine NO concentration. The results of CCK-8 and flow cytometric analyses showed that pretreatment of HUVECs with NAC or p38MAPK inhibitor (SB203580) attenuated LPS-induced decrease in cell viability and increase in cell apoptosis. RT-qPCR and western blotting showed that LPS promoted caspase-3 and Bax expression, but inhibited that of Bcl-2 in HUVECs; however, these effects were attenuated by pretreatment with NAC or SB203580. LPS stimulation significantly enhanced the expression of p-p38MAPK protein and reduced the expression of p-eNOS protein; however, these effects were attenuated by pretreatment with NAC or SB203580. NAC pretreatment attenuated LPS-induced inhibition of NO synthesis, which was consistent with the effects of SB203580. The results demonstrated that NAC pretreatment alleviated LPS-induced apoptosis and inhibition of NO production in HUVECs. Furthermore, these effects were proposed to be mediated via the p38MAPK signaling pathway.

Correspondence to: Professor Ling Guo, Department of Prosthodontics, Hospital of Stomatology Affiliated to Southwest Medical University, 2 Jiangyang South Road, Luzhou, Sichuan 646000, P.R. China
E-mail: 372083745@qq.com

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Introduction

Periodontal diseases are a group of chronic inflammatory diseases induced by *Porphyromonus gingivalis* (*P. gingivalis*) that affect the tooth-supporting tissues. *P. gingivalis* secrete a variety of toxic substances such as lipopolysaccharide (LPS), gingival hormone and protease (1). LPS (also referred to as endotoxin) is one of the important virulence factors that is known to mediate inflammation and apoptosis (2). Several cell types are involved in the development of periodontitis. Studies have shown that periodontitis is associated with altered expression of inflammatory mediators in gingival fibroblasts and periodontal membrane cells (3,4). However, few studies have investigated LPS-induced apoptosis of vascular endothelial cells. Furthermore, periodontitis has been shown to be associated with systemic cardiovascular disease (5-7), which suggests its close association with vascular endothelial cells.

Apoptosis refers to the self-regulated and orderly death of cells controlled by genes in order to maintain the stability of internal environment (8). The apoptosis of endothelial cells is a key event that may impair the integrity of the vessel wall and lead to the formation of atherosclerotic plaques (9). There are two typical apoptotic pathways: The mitochondrial pathway and the death receptor pathway (10,11). The death receptor pathway plays an important role in cell apoptosis (12). A series of caspases proteins have been shown to be activated by the death induced signal complex [a form of death ligand that binds to the corresponding death receptor on the cell surface (13)], which in turn induces apoptosis. Throughout this process, caspase-3 acts as an apoptotic executor (14). The mitochondrial pathway is also referred to as the endogenous apoptosis pathway (15). A recent study revealed that apoptosis can be induced by multiple pro-apoptotic stimuli affecting the

mitochondria; these induce the release of cytochrome *c* and activation of caspase enzymes, which ultimately leads to cell apoptosis (16). Moreover, migration of Bax (a pro-apoptotic member of the Bcl-2 family) from cytoplasm to mitochondria induces activation of the caspase cascade system, leading to apoptosis (15). In contrast, the anti-apoptotic member Bcl-2 binds to the mitochondria and inhibits death signals (17). Collectively, these findings indicate an integral role of caspase-3, Bcl-2-associated X (Bax) and Bcl-2 in modulating apoptosis.

Mitogen-activated protein kinases (MAPKs; including p38, JNK, ERKs) are serine/threonine protein kinases that serve a critical role in response to extracellular stimulation (18). The degree of MAPKs phosphorylation is a key determinant of cell fate: Death or survival (19,20). A review of previous studies identified that activation of p38 MAPK pathway is also related to apoptosis (21).

N-acetyl cysteine (NAC), a precursor for glutathione biosynthesis, can scavenge oxygen free radicals, control inflammatory reactions and inhibit apoptosis (22). NAC has been used as an antioxidant in neurodegenerative diseases, chronic lung diseases, cardiovascular diseases and other oxidative diseases (23-25). Apart from the antioxidant properties, NAC has been shown to induce hemodynamic improvements in a rabbit model of acute pulmonary thromboembolism; the effect was mediated via the p38MAPK pathway (26). The role of NAC in preventing endothelial apoptosis and its beneficial effect on cell survival by acting through the p38MAPK signaling pathway has evoked considerable interest in recent years. Moreover, in the vascular endothelial cells, nitic oxide (NO) is involved in modulating the vascular tone, vascular proliferation, vascular apoptosis and platelet aggregation (27). Endothelial NO synthase (eNOS) is a critical enzyme for the synthesis of vasoprotective molecule NO in the endothelial cells (27). The main features of endothelial dysfunction are loss of NO production and impaired eNOS activity (28). Therefore, it was hypothesized that NAC may also affect the function of vascular endothelial cells by regulating the production of NO derived from endothelial NO synthase.

No study, to the best of our knowledge, has systematically investigated the effect of NAC on LPS-mediated apoptosis *in vitro* by using the human umbilical vein endothelial (HUVECs) culture system. The purpose of the present study was to establish cell apoptosis model by LPS stimulation of HUVECs and to evaluate the effect of NAC in LPS-induced apoptosis and NO production in HUVECs. In addition, we investigated the underlying molecular mechanisms.

Materials and methods

Materials. HUVECs were purchased from Otwo Biotech (Shenzhen) Inc. LPS and NAC were purchased from Sigma-Aldrich (Merck KGaA). SB203580 was purchased from Selleckchem. Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc.). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from HyClone (GE Healthcare Life Sciences). Penicillin (100 U/ml) and streptomycin (100 μg/ml) were purchased from Beyotime Institute of Biotechnology. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. An NO assay kit (cat. no. A013-1) was purchased from Nanjing Jiancheng Bioengineering Institute. Antibodies against caspase-3, Bcl-2, Bax, phosphorylated (p-)-p38, total (t)-p38, p-eNOS and t-eNOS were obtained from Cell Signaling Technology, Inc. A fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit was purchased from BD Pharmingen (BD Biosciences). All other reagents were of ultrapure grade.

Cell culture. HUVECs were cultured in high-glucose DMEM containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin solution at 37˚C in a 5% CO_2 atmosphere in accordance with the manufacturer's recommendation. To select the optimal concentration of NAC for cell viability, HUVECs were incubated with high-glucose DMEM containing 10% FBS at a density of $1x10^5/cm^2$ at 37˚C in 5% CO_2 humidified air. After the cells had adhered to the wall for 24 h at 37˚C, different concentrations of NAC (0.25, 0.5, 1, 2.5 or 5 mM) were used to stimulate the cells. For the induction of apoptosis using LPS, cells were stimulated with LPS (1 μg/ml) for 24 h at 37˚C.

Six experimental groups were examined, as presented in Table I: i) Control group; ii) LPS (1 μg/ml) group; iii) NAC (1 mM) group; iv) SB203580 (10 μM) group; v) NAC (1 mM) + LPS (1 μg/ml) group; and vi) SB203580 (10 μM) + LPS (1 μg/ml) group. In the control group, cells were cultured for 24 h in DMEM containing 10% FBS and 1% penicillin-streptomycin solution. The LPS, NAC and SB203580 groups were treated for 24 h with LPS, NAC and SB203580, respectively. The NAC + LPS and SB203580 + LPS groups were treated with NAC and SB203580, respectively, for 1 h prior to LPS exposure. Cells were then incubated with LPS for 24 h. All treatments were preformed at 37˚C with 5% CO_2.

Cell viability assay. A CCK-8 assay was performed to assess cell viability. The experiments were divided into three groups: Blank, control and experimental groups (NAC and NAC + LPS groups). First, different concentrations of NAC (0.25, 0.5, 1, 2.5, 5 mM) were applied in the NAC group, respectively; after 1 h, 1 μg/ml LPS was added to the NAC + LPS group. HUVECs were seeded in a 96-well plate at a density of $1x10^5/cm^2$; the cells adhered to the plate for 24 h in 5% CO_2 at 37˚C. Subsequently, the DMEM medium was removed and CCK-8 solution (10 μl CCK-8 + 100 μl DMEM) added to each well. Then, incubation was continued for 2 h in a 5% CO_2 atmosphere at 37˚C. The absorbance at 450 nm was detected by microplate reader (Synergy H4, BioTek Instruments, Inc.). All experiments were conducted in triplicate.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). mRNA expressions of target genes (caspase-3, Bax and Bcl-2) were quantified using RT-qPCR. For RNA extraction, cells were plated in a six-well plate at a density of $1x10^5/cm^2$ in DMEM containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (Beyotime Institute of Biotechnology) for 24 h at 37˚C. Subsequently, cells were treated according to the six experimental groups. The total RNA was extracted from cultured HUVECs with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Electrophoresis was performed to examine the integrity of the RNA extracted. First, cDNA was synthesized from total RNA in each sample using the PrimeScript RT

Table I. Experimental groups employed in the present study.

Groups	Control	LPS	NAC	SB203580	NAC+LPS	SB203580 + LPS
NAC	-	-	+	-	+	-
LPS	-	+	-	-	+	+
SB203580	-	-	-	+	-	+

LPS, lipopolysaccharide; NAC, N-acetyl cysteine.

reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions incubating the samples for 1 h at 42˚C, for 5 min at 70˚C and for 1 min at 4˚C. Total RNA (500 ng) was reverse transcribed to cDNA with 5X Reaction Buffer (4 μl), Oligo DT18 primer (1 μl), dNTP Mix (2 μl), RiboLock RNase Inhibitor (1 μl), RevertAid M-MuL V RT (1 μl) and ddH_2O to supplement to a total volume of 20 μl. SYBR Green Mix (Toyobo Life Science) was used for RT-qPCR of cDNA samples following the manufacturer's manual. The amplification assay was performed using an ABI Prism 7300 sequence detection PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) The reaction conditions included 95˚C for 5 min, followed by 40 cycles of two-step PCR (95˚C for 10 sec and 60˚C for 30 sec) and a final extension at 75˚C for 10 min. The primer sequences employed for qPCR are listed in Table II. RT-qPCR for each sample was performed in triplicate. Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as an internal reference control and the relative expressions of mRNA were quantified using the $2^{-\Delta\Delta Cq}$ method (29).

Protein isolation and western blot analysis. The expression levels of target proteins (caspase-3, Bax, Bcl-2, p-p38 MAPK/t-p38MAPK and p-eNOS/t-eNOS) were determined by western blotting. The cells were harvested and the total proteins were extracted using the radioactive immunoprecipitation assay buffer (Thermo Scientific, Inc.). Total protein was boiled for 5 min and then cooled for another 5 min. A bicinchoninic acid protein concentration assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used for protein quantification and the same amount of protein (20 μg) was separated from each sample by 12% SDS-PAGE (Beyotime Institute of Biotechnology). Following electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes. These membranes were blocked with 5% non-fat dried milk in PBST (0.1% Tween 20 in PBS) for 2 h at room temperature and then incubated overnight at 4˚C with primary antibodies against caspase-3 (1:1,000; rabbit, cat. no. PAB33236; Bioswamp; Wuhan Beinglay Biological Technology Co.), Bax (1:1,000; rabbit, cat. no. PAB30861; Bioswamp; Wuhan Beinglay Biological Technology Co.), Bcl-2 (1:1,000; rabbit, cat. no. PAB33482; Bioswamp; Wuhan Beinglay Biological Technology Co.), p38MAPK (1:2,000; rabbit, cat. no. 14064-1-AP; ProteinTech Group, Inc.)/p-p38 MAPK (1:1,000; rabbit, cat. no. ab178867; Abcam), eNOS (1:1,000; rabbit, cat. no. PAB32306; Bioswamp; Wuhan Beinglay Biological Technology Co.)/p-eNOS (1:1,000; rabbit, cat. no. PAB36348-P; Bioswamp; Wuhan Beinglay Biological Technology Co.) and β-actin (1:1,000; rabbit, cat. no. PAB36265; Bioswamp; Wuhan Beinglay Biological Technology Co.), followed by washing with PBST three times (5 min each time). Subsequently, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:20,000; goat. no. PAB160011; Bioswamp; Wuhan Beinglay Biological Technology Co.). After washing with TBST for three times (5 min each time), the target bands were detected using the Clarity Western Enhanced Chemiluminescence kit (Analytik Jena AG) and the band intensities were quantified using ImageJ software. (version 1.44; National Institutes of Health). The expression levels of target proteins were determined relative to that of β-actin.

Table II. Primer Sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Control	Primer sequence (5'-3')
Caspase-3	Reverse	CTGAATGTTTCCCTGAGGTTTG
	Forward	CCAAAGATCATACATGGAAGCG
Bax	Reverse	CAGTTTGCTGGCAAAGTAGAAA
	Forward	CGAACTGGACAGTAACATGGAG
Bcl-2	Reverse	GAACTCAAAGAAGGCCACAATC
	Forward	GACTTCGCCGAGATGTCCAG
GAPDH	Reverse	AGGCGCCCAATACGACCAA
	Forward	CCACTAGGCGCTCACTGTTC

Bax, Bcl-2-associated X protein.

Cell apoptosis assay. The apoptosis rate of cells was evaluated using the Annexin V-FITC/PI apoptosis detection kit. The cells were collected and centrifuged for 5 min at 1,000 x g at 37˚C and rinsed twice with cold PBS. Subsequently, the cells at a density of $1.0x10^7$ cells/ml were resuspended in 1X Binding Buffer. Then, 100 μl of cell suspension was transferred into a 5 ml test tube and 5 μl Annexin V/FITC and 5 μl PI solution were added. The test tube was gently vortexed to mix the cells with the reagent and incubated at room temperature for 15 min in the dark. Subsequently, 400 μl of 1X Binding Buffer was added to each test tube prior to analysis using a flow cytometer (BD FACSCanto II; BD Biosciences) and FACSDiva software (version 6.1; BD Biosciences) to detect the apoptotic rate. Flow cytometry results suggested that the advanced apoptotic cells were in the upper right quadrant, and the early apoptotic cells were in the lower right quadrant. The apoptotic rate was calculated as the sum of early and advanced apoptotic cells.

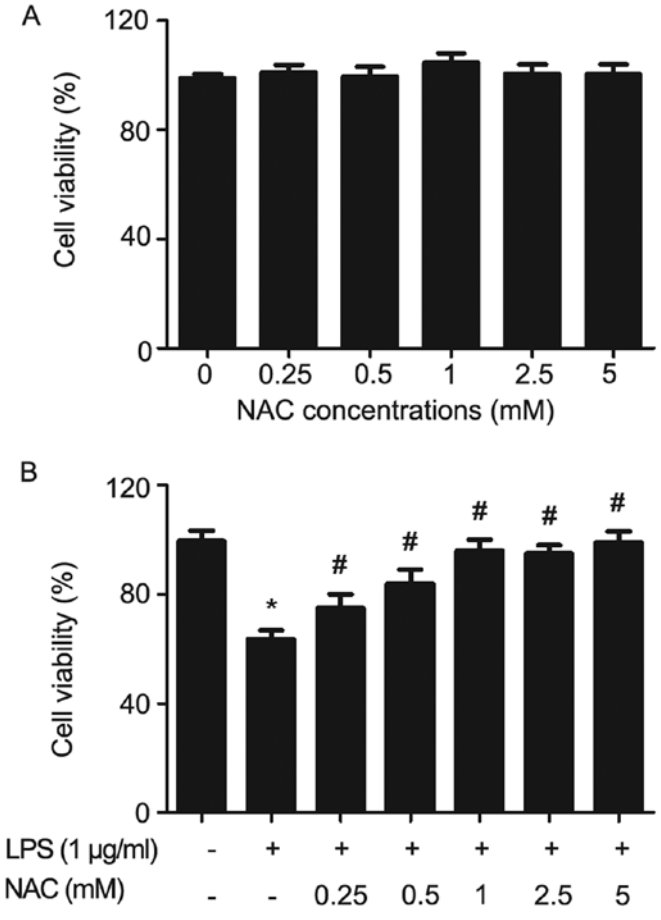

Figure 1. Effect of NAC on viability of HUVECs. (A) Effects of NAC (0.25, 0.5, 1, 2.5, 5 mM) on the viability of HUVECs at 24 h. (B) HUVECs were pretreated with NAC (0.25, 0.5, 1, 2.5, 5 mM) for 1 h, followed by stimulation with LPS (1 μg/ml) for 24 h. Mean ± standard deviation values from three independent experiments are presented. *P<0.05 vs. untreated, $^{\#}$P<0.05 vs. LPS. NAC, N-acetyl cysteine; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide.

Measurement of NO. The cell supernatants were collected and NO concentration was detected with the NO detection kit following the manufacturer's recommendation. The absorbance was measured at 540 nm with microplate reader (ELx800, General Electric Company). The concentration of NO in each sample was calculated by comparing the absorption with a standard curve line.

Statistical analysis. All data are presented as mean ± standard deviation values from three experimental replicates. One-way analysis of variance followed by a Student-Newman-Keuls post hoc test, or a Student's-test was used to analyze the experimental data using SPSS 17.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of NAC on viability of HUVECs. The results of NAC cell toxicity assay are shown in Fig. 1A. The results showed no significant changes in the viability of HUVECs after incubation with NAC alone at various concentrations (0.25, 0.5, 1, 2.5, 5 mM) for 24 h. The effects of NAC on HUVEC viability under LPS (1 μg/ml) treatment are shown in Fig. 1B. The data revealed that LPS treatment (1 μg/ml) significantly decreased the cell viability compared with the control group. Additionally, it was found that NAC pretreatment (0.25, 0.50, 1, 2.5, 5 mM) for 1 h significantly prevented the LPS-induced decline in cell viability compared with the LPS group. The cell viability remained essentially unchanged over a specific concentration range of NAC (1-5 mM). Therefore, 1 mM NAC was used in the subsequent experiments.

NAC attenuates LPS-induced increases in caspase-3 and Bax, and decreases in Bcl-2 expression in HUVECs. The relative expression levels of caspase-3, Bax and Bcl-2 are shown in Fig. 2A and C. The RT-qPCR results indicated that LPS significantly induced the expression of caspase-3 and Bax, but downregulated that of Bcl-2 compared with the control group; however, the effects of LPS were significantly attenuated by pretreatment with NAC or SB203580. In addition, pretreatment of HUVECs with NAC or SB203580 significantly inhibited the LPS-induced upregulation of caspase-3 and Bax, and the downregulation of Bcl-2 compared with LPS treatment alone (Fig. 2A). Western blotting analysis of protein expression levels were consistent with the mRNA results (Fig. 2B and C).

NAC inhibits LPS-induced apoptosis of HUVECs. It was further examined whether NAC reduces the rate of apoptosis using flow cytometry. The results showed that treatment of HUVECs with LPS alone significantly increased the rate of apoptosis compared with control group. However, pretreatment of cells with NAC for 1 h significantly decreased the apoptosis rate compared with LPS treatment alone. Similar results were observed after pretreatment with SB203580. The results suggest that NAC plays an important anti-apoptotic role (Fig. 3).

NAC inhibits LPS-induced p38MAPK phosphorylation in HUVECs. To further explore the mechanism of the anti-apoptotic effect of NAC, the protein expression levels of p-p38MAPK/t-p38MAPK were investigated (Fig. 4A and B). LPS treatment significantly increased the phosphorylation of p38MAPK, compared with the control, without altering t-p38MAPK expression in HUVECs. However, this effect of LPS was abolished by pretreatment with NAC or the p38MAPK inhibitor (SB203580). In brief, pretreatment with NAC reduced the expression of p-p38MAPK compared with cells exposed to LPS alone.

NAC alleviates LPS-induced inhibition of eNOS phosphorylation and NO production in HUVECs. The protein expression levels of p-eNOS/t-eNOS are shown in Fig. 4A and C. LPS treatment significantly inhibited the phosphorylation of eNOS without altering t-eNOS expression, compared with the control. However, NAC pretreatment significantly antagonized the decrease in LPS-induced eNOS phosphorylation; similar results were obtained after pretreatment with SB203580. The NO concentration in the cell supernatant in various groups is presented in Fig. 4D. HUVECs treated with LPS exhibited significant decreases in the synthesis of NO compared with the untreated group. Correspondingly, pretreatment of HUVECs with NAC significantly attenuated the LPS-induced reductions in NO synthesis, which was consistent with the results observed after pretreatment with SB203580.

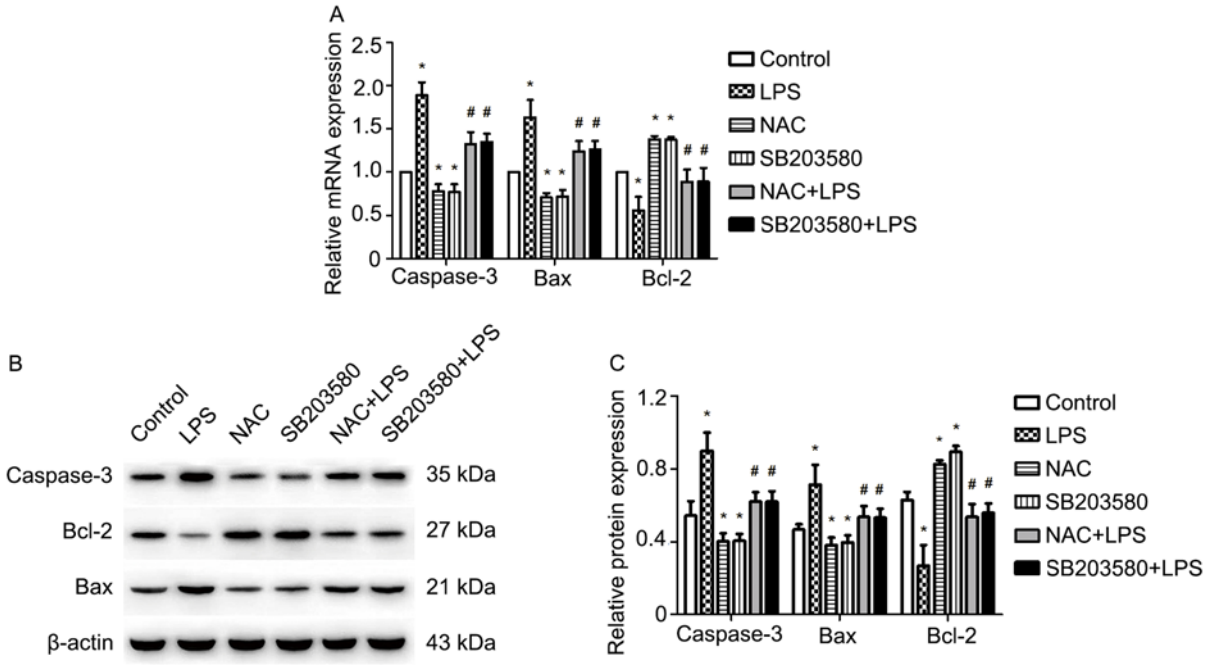

Figure 2. Effects of NAC pretreatment on the expressions of caspase-3, Bax and Bcl-2 in human umbilical vein endothelial cells stimulated with LPS. Cells were pretreated with 1 mM NAC or 10 μM SB203580 for 1 h, then co-treated with 1 μg/ml LPS for 24 h. (A) Levels of caspase-3, Bax and Bcl-2 mRNA were determined by RT-qPCR. (B) Levels of caspase-3, Bax and Bcl-2 protein were determined by western blot analysis. (C) Densitometry analysis of band intensity in western blotting was expressed as the ratio of caspase-3/β-actin, Bax/β-actin and Bcl-2/β-actin. Mean ± standard deviation values from three independent experiments are presented. *P<0.05 vs. control. #P<0.05 vs. LPS. NAC, N-acetyl cysteine; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

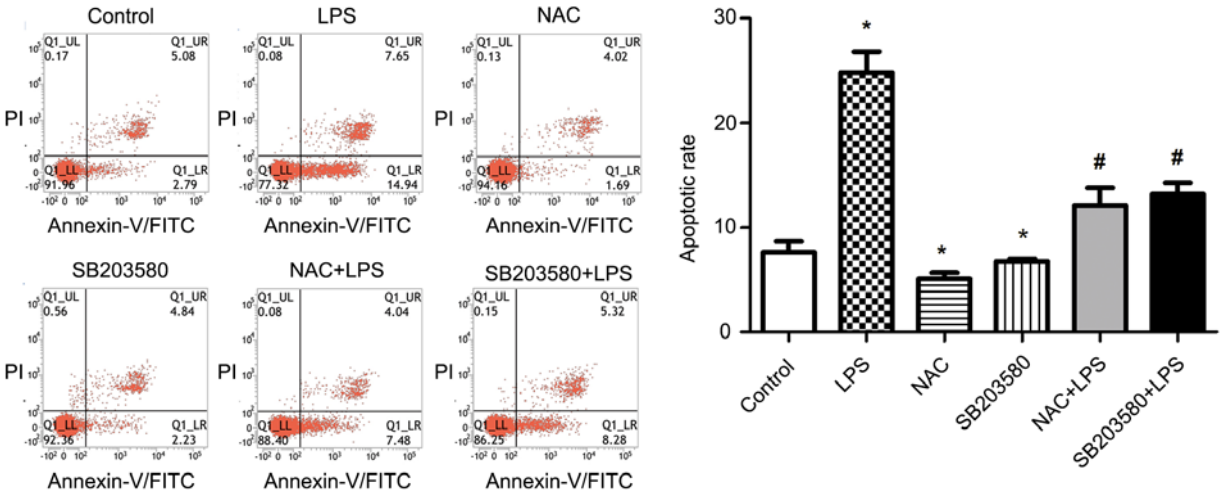

Figure 3. Effects of NAC pretreatment on the apoptosis rate of human umbilical vein endothelial cells stimulated with LPS. Cells were pretreated with 1 mM NAC or 10 μM SB203580 for 1 h, then co-treated with 1 μg/ml LPS for 24 h. The cell apoptosis rate was analyzed by flow cytometry. Mean ± standard deviation values from three independent experiments are presented. *P<0.05 vs. untreated; #P<0.05 vs. LPS. NAC, N-acetyl cysteine; LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Discussion

The current study examined the effect of NAC on LPS-induced apoptosis of HUVECs. Additionally, we investigated the potential molecular mechanisms that mediate the effects of NAC. LPS significantly promoted the synthesis of caspase-3 and Bax, while suppressing that of Bcl-2 in HUVECs. However, NAC pretreatment preempted these effects, which suggests that NAC has an anti-apoptotic effect on endothelial cells. This result is consistent with the results of flow cytometry. Moreover, it was also observed that NAC inhibited LPS-induced upregulation of p-p38MAPK signaling protein in HUVECs, an effect that was similar to that of SB203580. In addition, it was also found that NAC pretreatment significantly restored the phosphorylation of eNOS and production of NO, which suggests that the anti-apoptotic effect of NAC on HUVECs may be mediated via its effect on NO synthesis.

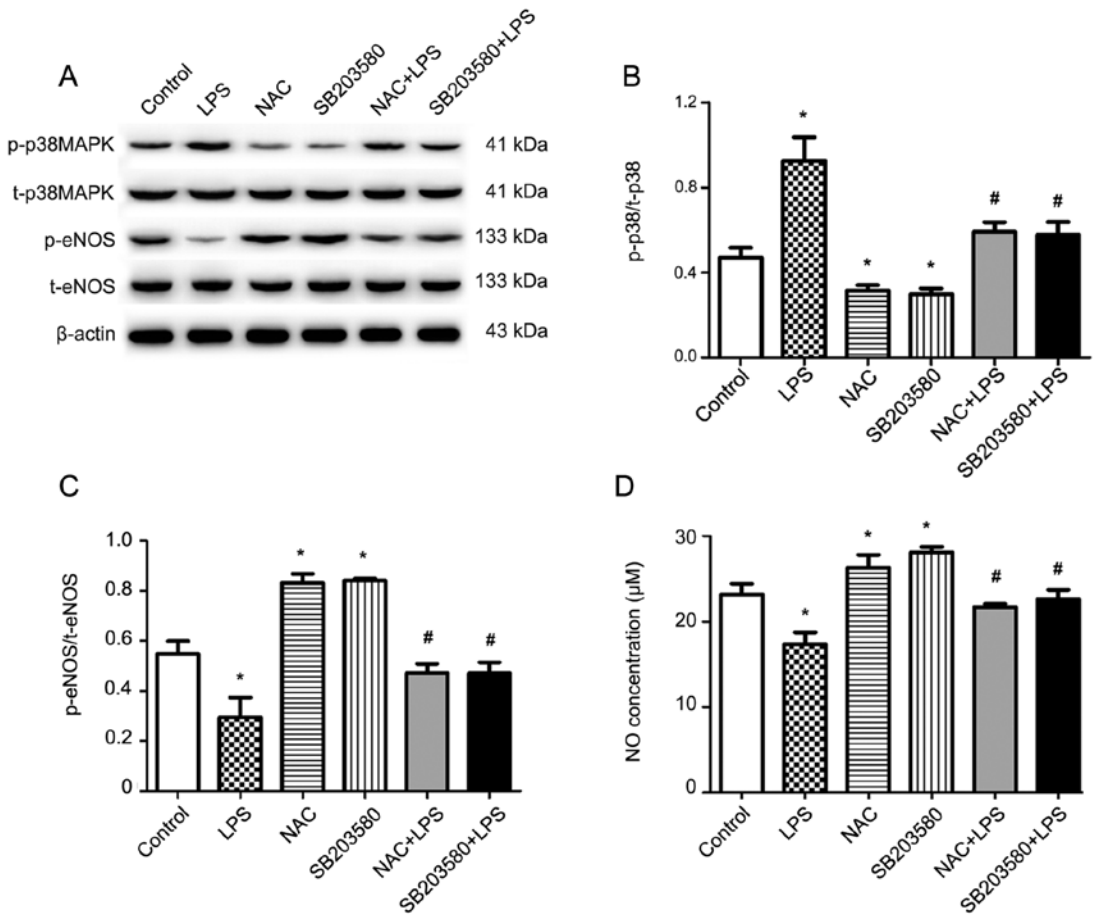

Figure 4. Effects of NAC pretreatment on the expression of p38MAPK, eNOS and NO production in HUVECs stimulated with LPS. Cells were pretreated with 1 mM NAC or 10 μM SB203580 for 1 h, then co-treated with 1 μg/ml LPS for 24 h. (A) Levels of p-p38MAPK, t-p38MAPK, p-eNOS and t-eNOS protein were determined by western blot analysis. (B) Densitometry analysis of band intensity was expressed as the ratio of p-p38/t-p38. (C) Densitometry analysis of band intensity was expressed as the ratio of p-eNOS/t-eNOS. (D) NO concentration was determined via an NO assay kit. Mean ± standard deviation values from three independent experiments are presented. *P<0.05 vs. untreated; #P<0.05 vs. LPS. NAC, N-acetyl cysteine; MAPK, mitogen-activated protein kinase; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; p-, phosphorylated; t-, total.

Periodontitis is closely related to cardiovascular diseases (30). Endothelial dysfunction and apoptosis play a vital role in the progression of atherosclerosis (31). Moreover, Bcl-2 and Caspase protein families serve an important role in the induction of apoptosis (32). Caspase-3 is considered as the main caspase-executor in apoptosis (14) and the most reliable determinant of apoptosis (33). The Bcl-2 protein family comprises of both anti-apoptotic (such as Bcl-2) and pro-apoptotic (such as Bax) members. Decreased expression of Bcl-2 and increased expression of Bax has been shown to stimulate the release of cytochrome *c* from mitochondria, which can activate caspase-9 (34). Caspase-9 catalyzes the activation of caspase-3, which eventually induces apoptosis (35,36). Previous studies have shown that overexpression of tumor necrosis factor α can increase the expression of Bax and promote cardiomyocyte apoptosis, eventually contributing to an imbalance between Bcl-2 and Bax (37). It has been shown that the imbalance between Bcl-2 and Bax determines whether the cells undergo apoptosis (38). In the present study, western blot analysis and RT-qPCR demonstrated that LPS negatively regulated the expression of Bcl-2 at the protein and the mRNA levels. In contrast, LPS appeared to have regulated the expression of caspase-3 and Bax at the protein and mRNA levels. However, these effects were suppressed by pretreatment with NAC or SB203580. Periodontal inflammation was shown to be associated with the increased concentration of important atherosclerosis biomarkers in the blood; furthermore, the concentration of these markers (such as CRP) was shown to decrease after periodontal treatment (39). The results of the present study suggested that NAC may have an anti-apoptotic effect, which might be mediated via the p38MAPK signal pathway. This conclusion is consistent with our results of flow cytometric analysis.

MAPKs (including ERK1/2, JNK and p38) play a key role in determining the cell fate, such as proliferation, differentiation, survival and apoptosis (40). Activation of p38 and JNK is considered as a pro-apoptotic signal, while activation of ERK1/2 is considered a pro-survival signal (17). The p38MAPK signaling pathway is involved in the functional response of macrophages and neutrophils, including adhesion and apoptosis (41,42), which is implicated in a variety of cardiovascular diseases (43). It has also been shown to regulate a variety of cellular biological events including cell migration, proliferation and differentiation (44-46). Phosphorylation of p38MAPK in endothelial cells can be activated by physiological stress, LPS, osmotic stress and ischemia reperfusion, which can affect the

function of endothelial cells and eventually promote cell apoptosis (47). p38MAPK not only downregulates anti-apoptotic proteins, but also upregulates pro-apoptotic proteins (48,49). The present study assessed whether the anti-apoptotic effect of NAC is partly mediated via the p38MAPK signal pathway; HUVECs were treated with LPS, NAC and SB203580 and the changes in p-p38MAPK/t-p38MAPK examined. As expected, the expression of p-p38MAPK in HUVECs was increased after treatment with LPS. However, the expression of p-p38 was downregulated by pretreatment with NAC or SB203580. The results suggested that NAC can inhibit the phosphorylation of p38MAPK signal protein. This result is consistent with a previous study in which NAC was shown to attenuate hepatocyte apoptosis by inhibiting the expression of p-p38MAPK (50); the role of p38MAPK in vascular inflammation and endothelial cell apoptosis has been reported (51,52). These results suggest that NAC can attenuate LPS-induced apoptosis by inhibiting the activation of p-p38MAPK.

NOS consists of neuronal NOS (nNOS), eNOS and inducible NOS (53). eNOS is the main subtype of NOS expressed in vascular endothelial cells and accounts for most of NO production. The critical role of NO in regulating the growth, function, apoptosis and survival of endothelial cells is well documented (54,55). Atherosclerosis stimulates endothelial cell injury, which impairs eNOS bioactivity and the synthesis of NO (56). Decreased bioavailability of NO is an important feature of vascular dysfunction (57). Decreased vascular NO bioavailability has long been considered as a common pathogenetic mechanism of endothelial dysfunction, leading to the occurrence of cardiovascular risk factors such as hypertension, atherosclerosis and diabetes (58). The regulation of NO production by activating eNOS phosphorylation is a key strategy for treating cardiovascular diseases (59). Oxidative free radical-mediated apoptosis or cellular dysfunction, which is the main cause of decreased endothelial-derived NO production and bioavailability, can lead to impaired endothelial-dependent vascular reactivity (60). NAC has an antioxidant effect, can scavenge oxygen radicals and reduce apoptosis (18). However, to the best of our knowledge, no studies have directly investigated whether the anti-apoptotic effect of NAC in endothelial cells is mediated via activation of eNOS phosphorylation and NO production. To determine whether the vasculoprotective effect of NAC is mediated via endothelium-derived NO synthesis, the phosphorylation of eNOS and was examined and NO concentration in the cell supernatant was assessed. The results showed that LPS treatment significantly reduces the expression of p-eNOS protein. Pretreatment of HUVECs with NAC or SB203580 enhanced the expression of p-eNOS protein, the result of NO concentration analysis was consistent with the changes in the expression levels of p-eNOS. A previous study showed that endothelium-derived NO plays an important physiological role in regulating vascular tension as well as endothelial cell survival and migration (61). The current study indicated that the protective effects of NAC on vascular endothelial cells may be mediated via modulation of endothelium-derived NO synthesis.

In conclusion, the findings of present study indicated that NAC attenuated LPS-induced vein endothelial cells apoptosis via the p38MAPK signaling pathway. The findings also suggested that NAC may be valuable for the prevention and treatment of periodontitis. However, other relevant mechanisms underlying the effects of NAC require further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TX and LG made substantial contributions to the design of the experiments. TX, ZZ, RZ and JH performed the experiments and collected the data. TX, ZZ and JH analyzed and interpreted the data. TX produced the manuscript. TX, ZZ, RZ, JH and LG revised the manuscript. All authors read and approved the final manuscript to be published.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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