

The IL-24 gene protects human umbilical vein endothelial cells against H₂O₂-induced injury and may be useful as a treatment for cardiovascular disease

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Abstract. The aim of the present study was to investigate the protective effects of interleukin-24 (IL-24) on hydrogen peroxide (H₂O₂)-induced vascular endothelial injury and to examine the association between IL-24 and cardiovascular disease. Human umbilical vein endothelial cells (HUVECs) were exposed to increasing concentrations of H₂O₂ in the presence or absence of IL-24, which was introduced via Lipofectamine[®] 2000-mediated transfection. The successful uptake of the IL-24 plasmid was confirmed by RT-PCR at 24 h post-transfection. The effects of H₂O₂ and IL-24 on the proliferation and migration of the HUVECs was determined using cell migration assays. Cell viability was determined using a Cell Counting Kit-8 (CCK-8). Apoptosis and the measurement of the intracellular reactive oxygen species (ROS) levels were determined by flow cytometry, and the levels of caspase-3, which is associated with apoptosis, were determined by western blot analysis. Real-time PCR and western blot analysis were also used to measure the levels of multiple cardiovascular disease-associated factors. *In vivo* experiments were also performed using a rat model of hypertension which was constructed by angiotensin II infusion using an osmotic pump. The mRNA and protein levels of IL-24 were measured in both the control and hypertensive rats; the effects of treatment with enalapril and nifedipine on the IL-24 levels were also examined. Our results revealed that IL-24 protected against the H₂O₂-mediated abnormal increase in HUVEC proliferation. IL-24 also antagonized H₂O₂ by reducing the content of ROS in the cells, thus decreasing cellular oxidative damage, improving the cellular survival rate, reducing apoptosis and decreasing the expression of cardiovascular disease-related factors. The results from our *in vivo* animal experiments revealed that

IL-24 expression was lower in the hypertensive rats compared to the healthy controls. Additionally, the IL-24 levels increased following anti-hypertensive therapy. The findings of our study indicate that IL-24 protects against H₂O₂-mediated endothelial cell damage and may thus provide a novel therapeutic strategy for treatment of cardiovascular disease.

Introduction

Hypertension is an important risk factor for the development of cardiovascular disease. In addition to causing damage to the heart, hypertension can also cause stress and damage to several other organs and tissues, including the brain, liver, kidneys and vascular tissue (1,2). Previous studies have described the effects of high blood pressure on heart and brain tissue damage, showing that such effects can lead to myocardial hypertrophy and stroke (3). There are significant data showing that treatment with anti-hypertensive drugs leads to changes in gene expression in the kidneys and bladder tissue (4,5). Additionally, a recent study demonstrated that treatment with such drugs may also result in changes in gene expression directly in the heart, brain and liver tissues (6). The authors reported on differentially expressed genes following treatment with anti-hypertensive drugs. While 33 of these genes were previously linked to cardiovascular disease, this analysis also led to the identification of 16 novel genes with no known link to cardiovascular disease; some of the 16 newly identified genes included interleukin (IL)-24, cathepsin Z (Ctsz), and secretory carrier membrane protein (Scamp2). Genes that are differentially expressed following treatment with anti-hypertensive drugs may be potential biomarkers and novel targets for the prevention of cardiovascular disease. Moreover, such genes may provide valuable information for the development of new anti-hypertensive therapy (6).

Initially identified in 1995 during a melanoma cell treatment regimen, IL-24 [also known as melanoma differentiation associated gene-7 (MDA-7)] possesses tumor suppressor characteristics (7). IL-24 cDNA encodes a protein of 206 amino acids with molecular weight of 23.8 kDa. N-terminal signal peptide sequences have indicated that IL-24 is a secreted protein (8). The transfer of recombinant IL-24 into mononuclear cells in human peripheral blood has been shown to lead to the release of IL-6, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF), lending to its characterization as

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a bona fide cytokine (8). Adenovirus-mediated IL-24 expression has been shown to inhibit the growth of vascular endothelial cells in tumor tissues, suggesting that IL-24 may inhibit angiogenesis (9). In human melanoma, the loss of IL-24 has been shown to be closely associated with tumor invasion and metastasis, suggesting that IL-24 may be a tumor suppressor in this setting as well (10).

Since IL-24 inhibits cancer cell proliferation through multiple biological pathways, it may also exert anti-proliferative effects in occlusive vascular disease. It has been demonstrated that both atherosclerosis and cancer may originate from local tissue injury, which can promote inflammation and genomic instability (11). Whereas some researchers have worked to elucidate the role of IL-24 in cancer (12-14,49), its role in cardiovascular disease remains unclear.

A number of cardiovascular diseases, including ischemia/reperfusion injury and myocardial infarction, have been shown to be closely associated with cellular apoptosis (15). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), can lead to the development of cardiovascular disease by promoting the apoptosis of endothelial cells (16,17). Protecting endothelial cells from apoptosis has a positive clinical significance in the prevention and treatment of cardiovascular disease. Some have proposed repairing endothelial cell injury as part of hypertension treatment and have suggested that the degree of injury may be indicative of disease severity (18,47). As such, the detection of endothelial cell dysfunction is clinically important.

To date, to the best of our knowledge, there are no studies available describing the protective effects of IL-24 on vascular endothelial cells following injury. Previous studies have suggested that the adenovirus-mediated introduction of tumor suppressor genes, such as retinoblastoma (RB), p53, p21 and phosphatase and tensin homolog (PTEN) inhibits vascular smooth muscle cell (VSMC) proliferation and neointima formation following vascular injury *in vivo* (19-22). However, the potential effects of IL-24 on oxidative damage and the abnormal proliferation of vascular endothelial cells have not yet been reported.

In the present study, we examined whether IL-24 exerts protective effects on vascular endothelial injury induced by oxidative stress, and whether it is a therapeutic target of anti-hypertensive drugs in the cardiovascular system. Performing cellular experiments, we found that IL-24 protected against H_2O_2 -induced endothelial cell damage, and that the expression of IL-24 could be altered by an increase in blood pressure and anti-hypertensive drug therapy in a rat model of hypertension. Our results revealed that the IL-24 gene is closely related to cardiovascular disease and may thus provide a novel therapeutic target for the treatment of cardiovascular disease.

Materials and methods

Materials and reagents. Human umbilical vein endothelial cells (HUVECs) were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, China. Normal male Sprague Dawley (SD) rats (n=36, 6-7 weeks old; weight, 225 ± 25 g) were purchased from the Academy of Military Medical Sciences (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and trypsin were from Gibco

Life Technologies (Grand Island, NY, USA); fetal bovine serum (FBS) was obtained from (HyClone (Logan, UT, USA); the hypertension construction pump (ALZET mini-osmotic pump) was from Durect Corp., (Cupertino, CA, USA); angiotensin II was purchased from Sigma-Aldrich (St. Louis, MO, USA); the IL-24 recombinant shuttle plasmid, pDC316-h IL-24, and the empty plasmid, pDC316, were obtained from Benyuan Zhengyang Gene Technology Co. Ltd. (Beijing China); the plasmid extraction and purification kit was purchased from Bioer Technology Co., Ltd. (Hangzhou, China); the liposome transfection reagent, Lipofectamine® 2000, was from Invitrogen (Carlsbad, CA, USA); the Cell Counting Kit-8 (CCK-8) was obtained from the Beyotime Institute of Biotechnology (Shanghai China); the ROS test kit was from Beijing Applygen Technology Ltd. (Beijing, China); the Annexin V/PI staining kit was obtained from Nanjing KGI Biotechnology Development Co., Ltd. (Nanjing, China). The anti-cleaved caspase-3 antibody [(Asp175) Western Blot Detection Kit] was from Cell Signaling Technology, Inc., (Danvers, MA, USA); anti-IL-24 [EPR13281] antibody (ab182567) was from Abcam (Cambridge, UK); anti-endothelin-1/ET-1 (N-8) (sc-21625), anti-angiotensin II type 1 receptor-associated protein (AGTRAP/ATRAP) (F-6) (sc-271367), anti-angiotensin (H-12) (sc-374511) and anti-platelet-derived growth factor (PDGF-A) (H-77) (sc-7958) antibodies were all from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

Cell culture. The HUVECs were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 . All experiments were performed using cells cultured for 3-4 days at approximately 80-90% confluency.

Cell experimental groups. The cells were divided into the following groups: in group A, control cells cultured in DMEM with 10% FBS. In group B, cells were exposed to H_2O_2 (0.10 or 0.30 mmol/l). In group C, the cells were exposed to H_2O_2 (same concentrations of H_2O_2 described above) and also treated with IL-24. The cells in this group were transfected with the recombinant plasmid, pDC316-h IL-24 (100 ng/ml) using Lipofectamine 2000 to promote IL-24 expression. In group D, the cells were treated with H_2O_2 , but transfected with the empty plasmid (served as a control). The other 2 control groups were group E and F. In group E, the cells were transfected only with the recombinant plasmid, pDC316-h IL-24. In group F, the cells were transfected only with the empty plasmid. The cells were examined by fluorescence microscopy for transfection efficiency at 24 h post-transfection. The cells incubated for 48 h to determine the protective effects of IL-24 against H_2O_2 .

Animal grouping and intervention. The normal SD rats were divided into a control group (n=12) and a hypertension group (n=24). An osmotic micropump was used to generate the model of hypertension, as previously described (23,24). The osmotic pump with the pre-placement of angiotensin II was implanted subcutaneously. The infusion of angiotensin II was carried out by subcutaneous implantation [500 ng/(kg·min), 7 days]. Blood pressure was recorded daily using a BP-6 animal non-invasive blood pressure measuring instrument (Chengdu Taimeng Technology Co., Ltd., Chengdu, China). The tail artery blood

Table I. Sequences of primers used for RT-PCR.

Gene	Primer sequences (5'→3')
IL-24	F: GCCAAGCTTATGAATTTTCAACAGAGG R: GCCGTCGACCTAGAGCTTGTAGAATTT
GAPDH	F: TGAACGGGAAGCTCACTGG R: TCCACCACCCTGTTGCTGGA

IL-24, interleukin-24; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

pressure of the rats was measured using the tail-cuff method. The rats are arranged in the heating device, which was heated to 37°C for 3 min, then the tail was connected with the sensor, while the rats were in a quiescent state; food and water was made available in the device, which helped to keep the rats calm. Blood pressure was measured without anesthesia with the rats in a quiescent state. The blood pressure of each rat was continuously measured at least 10 times and the average values were calculated.

After successfully establishing the model of hypertension, 12 rats were sacrificed by cervical dislocation 2 weeks after they experienced a spike in blood pressure. The heart and kidney tissues were harvested from these animals, and real-time PCR was performed to detect the expression of IL-24. The remaining 12 hypertensive rats were administered enalapril (35 mg/kg/day; Shanghai Modern Pharmaceutical Ltd., Shanghai, China) and nifedipine (30 mg/kg/day; Beijing Bayer Healthcare Co., Ltd., Beijing, China) orally each day for 3 weeks. This leads to a steady decline in blood pressure; 1 week after the blood pressure had decreased and stabilized, the rats were sacrificed and IL-24 gene expression was examined.

Ethics statement. All surgical and animal care procedures were approved by the Institutional Ethics Committee. All research was carried out in strict accordance with the provided guidelines. We abided by all relevant provisions set forth by the Medical Ethics Committee of Shanxi Medical University (Shanxi, China).

Determination of IL-24 transfection efficiency by RT-PCR. Total RNA was extracted from the cells using TRIzol reagent. RNA concentration and purity were determined by UV spectrophotometry prior to reverse transcription and cDNA synthesis. The reaction conditions were as follows: 95°C denaturation for 5 min, followed by 95°C denaturation 30 sec, 58°C annealing 30 sec, 72°C extension 60 sec, cycle 32 times, 72°C terminal extension 7 min, 4°C hold. PCR products were analyzed by 1.0% agarose gel electrophoresis and sequenced by imaging analysis using the IS-10,000 multifunction gel image analysis system (Shanghai Tianneng Technology Co., Ltd., Shanghai, China). The primers used for RT-PCR are listed in Table I. The amplified fragment length of IL-24 was 624 bp and that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was 307 bp.

Cell migration assay. The study by Burdon and Gill indicated that H₂O₂ is an 'information contact' between cells, and that

H₂O₂ is one of the essential materials for cell maintenance (25). H₂O₂ at low concentration may play the role of a mitogen and can stimulate cell proliferation and migration. We wished to determine whether H₂O₂ can stimulate cell proliferation and migration. We performed a scratch test to determine the effects of H₂O₂ (0.1 mmol/l) with or without the presence of IL-24 on HUVEC migration. Briefly, a small scratch was made using a 10- μ l pipette tip in the culture dish at time 0, and the distance the cells had migrated was observed at 24 h.

Cytotoxicity assays. Cell viability was measured by CCK-8 assay, according to the manufacturer's instructions (Beyotime Institute of Biotechnology). The cells were seeded in a 96-well plate and transfected with the empty plasmid or IL-24 plasmid the following day using Lipofectamine 2000. At 24 h post-transfection, the cells were switched to serum-free medium for 24 h. They were then exposed to H₂O₂ (0.30 mmol/l) for 12 h. CCK-8 solution (10 μ l) was then added to each well 1 h before the absorbance was measured at 450 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Cell viability was determined as follows: cell viability (%) = treatment group OD/control group OD x100.

Apoptosis assay. Annexin V/PI staining was performed to detect apoptosis. The treated cells were collected, washed and then stained with Annexin V/PI for 20 min in the dark at room temperature. The percentage of apoptotic cells was analyzed by flow cytometry (BD FACSCalibur flow cytometer, FACS101; BIO-RAD Corporation, Hercules, CA, USA).

Determination of intracellular ROS production. Intracellular ROS production was measured by flow cytometry and fluorescence microscopy. Following treatment, the cells were washed and incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 10 μ mol/l; Sigma-Aldrich) for 20 min at 37°C in the dark. The fluorescence corresponding to the intracellular ROS levels was monitored using a light microscope (Olympus BX51; Olympus, Tokyo, Japan) and analyzed by flow cytometry (BD FACSCalibur flow cytometer, FACS101; BIO-RAD Corporation).

Real-time PCR. We used real-time PCR to detect the expression of the hypertension-related factors, angiotensinogen, endothelin-1, ATRAP and PDGF in the HUVECs, as well as IL-24 gene expression in the heart and kidney tissue of the rats in our model of hypertension and following anti-hypertensive therapy. In the cell experiments, total RNA was isolated from the treated HUVECs using RNeasy spin columns obtained from Qiagen (Hilden, Germany). Real-time PCR was performed in triplicate on a Stratagene Mx3005P Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA) using 100 ng RNA, 12.5 μ l 2X QuantiFast SYBR-Green PCR Master Mix (Qiagen) and 1 μ mol each of forward and reverse primers (Table II) in a total final volume of 25 μ l. For the tissues, the tissue samples were incubated at 94°C for 10 min. Subsequently, denaturation was performed for 45 cycles at 94°C for 15 sec followed by annealing and extension at 60°C for 60 sec. The amplification products were normalized to β -actin. The expression of the target gene, normalized to β -actin, was calculated using the 2^{- $\Delta\Delta$ Ct} method, as previously described (26).

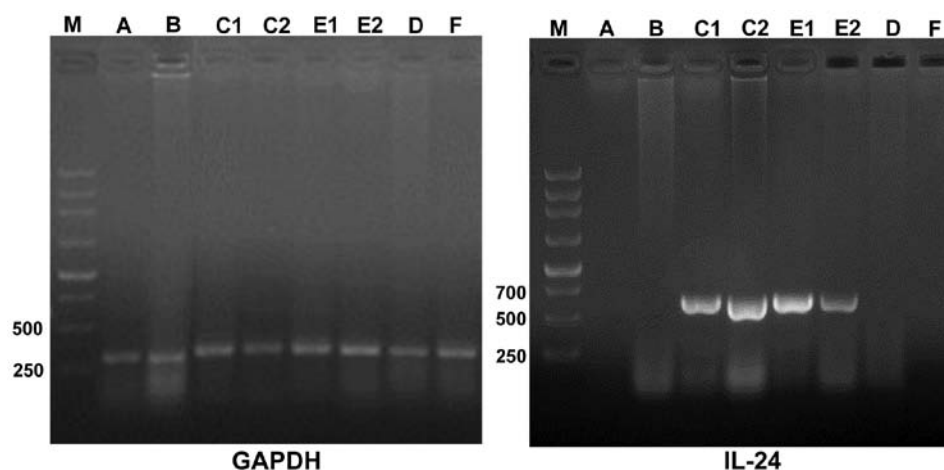


Figure 1. IL-24 gene transcription in endothelial cells M, DNA marker; A, control group; B, H_2O_2 group; C, H_2O_2 + IL-24 group (C1 and C2 are double test groups); D, H_2O_2 + empty plasmid group; E, IL-24 group (E1 and E2 are double test groups); F, empty plasmid group. Each sample shows the expression of the housekeeping gene, GAPDH, 307 bp, indicating that cDNA extraction was complete. Transfection of IL-24, amplified 620 bp bands, indicating that IL-24 was successfully transfected into the cells and was expressed in vascular endothelial cells. IL-24, interleukin-24; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table II. Sequences of primers used for real-time PCR.

Gene	Primer sequences (5'→3')
Angiotensinogen	F: CTTCCAGCACTGGAGTGACA R: AGAGGCATAGTGAGGCTGGA
Endothelin-1	F: AATGCCATGCTTCCCATTAG R: CCCTGAGGACAACAGGGATA
ATRAP	F: GGGTTTCCTTGGGTCTTCTC R: CAAGAGACGGAGGTCAGGAG
PDGF	F: GGTGGTCACAGGTGCTTTTT R: TCCAAGACATTCTGCTTCC
β -actin	F: GTCAGGTCATCACTATCGGCAAT R: AGAGGTCTTTACGGATGTCAACGT
IL-24	F: AATGCCATGCTTCCCATTAG R: CCCTGAGGACAACAGGGATA

ATRAP, angiotensin II type 1 receptor-associated protein; PDGF, platelet-derived growth factor; IL-24, interleukin-24; F, forward; R, reverse.

Western blot analysis. We used western blot analysis to detect the expression of cleaved caspase-3, angiotensinogen, endothelin-1, ATRAP and PDGF in the HUVECs, and that of IL-24 in the heart and kidney tissue of the rats. In the cell experiments, protein was extracted from the treated HUVECs and the concentration was determined. Equal amounts of total protein were analyzed on SDS-PAGE gels. The samples were transferred onto a membrane, which was incubated with first primary and then secondary antibodies. The protein bands were visualized by enhanced chemiluminescence (ECL) and autoradiography. Protein bands were analyzed using Quantity One image analysis software. In the animal experiments, specific intervention and treatment times were the same as those described for the determination IL-24 mRNA expression by RT-PCR. Protein was harvested from the rat

kidney and heart tissues and was analyzed as described for the cell experiments.

Statistical analysis. We used SPSS 19 software for statistical analysis. Data are presented as the means $\pm$ standard deviation. Data were analyzed by one way analysis of variance (ANOVA) and Student's t-test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Detection of IL-24 gene transcription in endothelial cells. RT-PCR confirmed the successful transfection and overexpression of IL-24 in endothelial cells in both the H_2O_2 + IL-24 (group C) and IL-24 (group E) groups (Fig. 1).

IL-24 attenuates the promoting effects of H_2O_2 on endothelial cell proliferation. Low concentrations of H_2O_2 (0.1-0.15 mmol/l) can stimulate abnormal cell proliferation and migration, which was determined in our pre-experiments. Compared with the control group, following 24 h of exposure to H_2O_2 , cell proliferation and migration were markedly increased in the H_2O_2 group compared to the controls (controls, 0.2575 ± 0.05123 ; H_2O_2 group, 0.3875 ± 0.01250 mm; $P = 0.1026 > 0.05$). We found that the overexpression of IL-24 significantly inhibited the H_2O_2 -induced proliferation of the HUVECs (0.0800 ± 0.05715 mm; H_2O_2 group compared with H_2O_2 + IL-24 group; $P = 0.0042 < 0.05$) (Fig. 2).

Cell viability determined by CCK-8 assay. IL-24 significantly decreased the apoptosis induced by oxidative stress, thus improving the survival rate of the cells (Fig. 3). Cell survival in the H_2O_2 oxidative stress injury group was lowest with a viability of $51 \pm 5.57\%$ of the control group ($P < 0.001$). Cell injury due to oxidative stress was significantly attenuated by IL-24, leading to an increase in cell viability to $81.66 \pm 4.81\%$ of the control group. The differences in cell viability between the H_2O_2 and H_2O_2 + IL-24 groups were statistically significant ($P = 0.0019 < 0.05$).

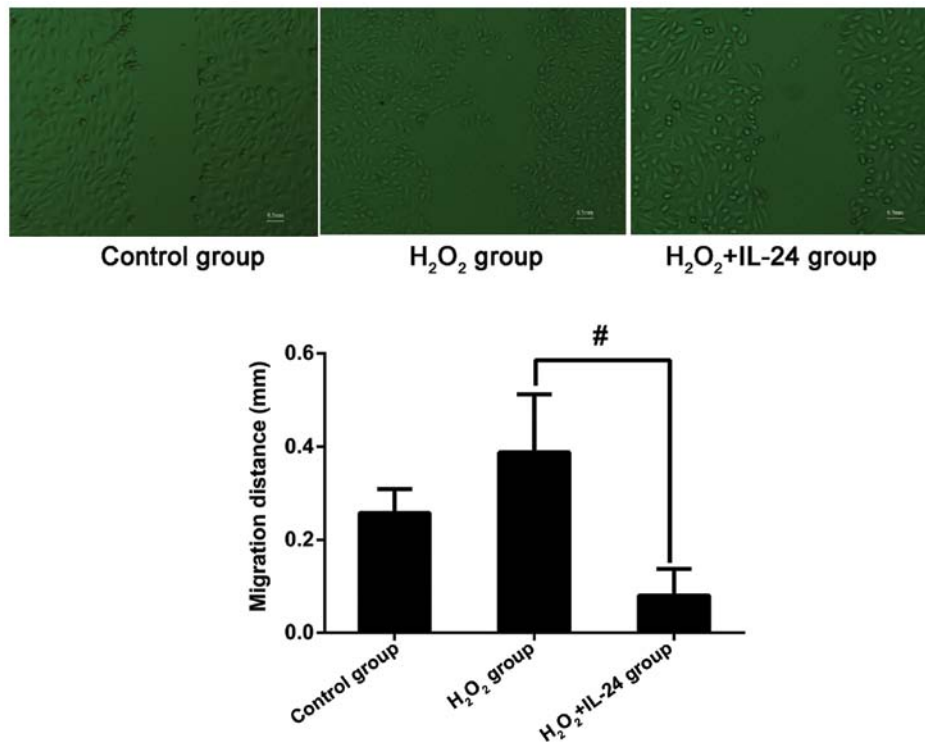


Figure 2. Low concentration of H₂O₂ stimulates abnormal cell proliferation and migration. After 24 h, compared with the control group, cell proliferation and migration were markedly increased in the H₂O₂ group (0.2575±0.05123 and 0.3875±0.01250 mm, respectively) (P=0.1026>0.05). IL-24 significantly inhibited cell proliferation (0.0800±0.05715 mm); H₂O₂ group compared with the H₂O₂ + IL-24 group, [#]P=0.0042<0.05. Magnification, x40. Bar, 0.5 mm. IL-24, interleukin-24.

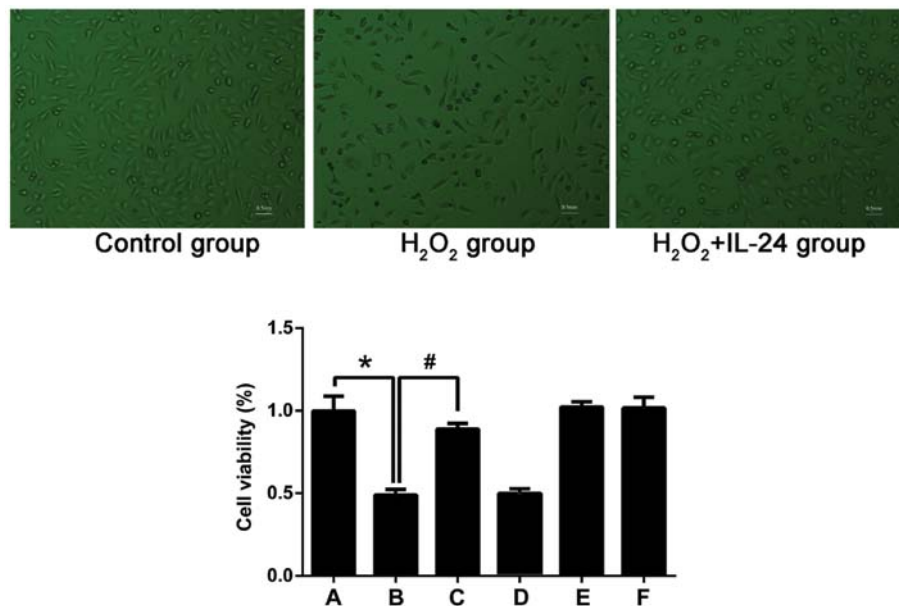


Figure 3. Cell viability. A, control group; B, H₂O₂ group; C, H₂O₂ + IL-24 group; D, H₂O₂ + empty plasmid group; E, IL-24 group; F, empty plasmid group. In the H₂O₂ oxidative stress injury group (H₂O₂, 0.3 mmol/l), the cell survival rate was lowest; cell activity for this group was 51±5.57% of the control group (^{*}P<0.001). Cell injury due to oxidative stress was significantly reduced by IL-24; the cell survival rate was significantly increased to 81.66±4.81% of the control group. There was a significant difference between the H₂O₂ injury group and the H₂O₂ + IL-24 group ([#]P=0.0019<0.05). Magnification, x40. Bar, 0.5 mm. IL-24, interleukin-24.

Cell apoptosis measured by flow cytometry. The amount of apoptosis induced by H₂O₂ was significantly higher than the basal level of apoptosis observed in the control group (51.38±1.39 vs. 13.01±0.50%; P<0.001; Fig. 4). IL-24 was able to protect the cells against H₂O₂-induced apoptosis (51.38±1.39% in the

H₂O₂ group compared to 25.93±1.902% in the H₂O₂ + IL-24 group; P<0.001).

Cleaved caspase-3 expression determined by western blot analysis. H₂O₂ can significantly induced the activation and

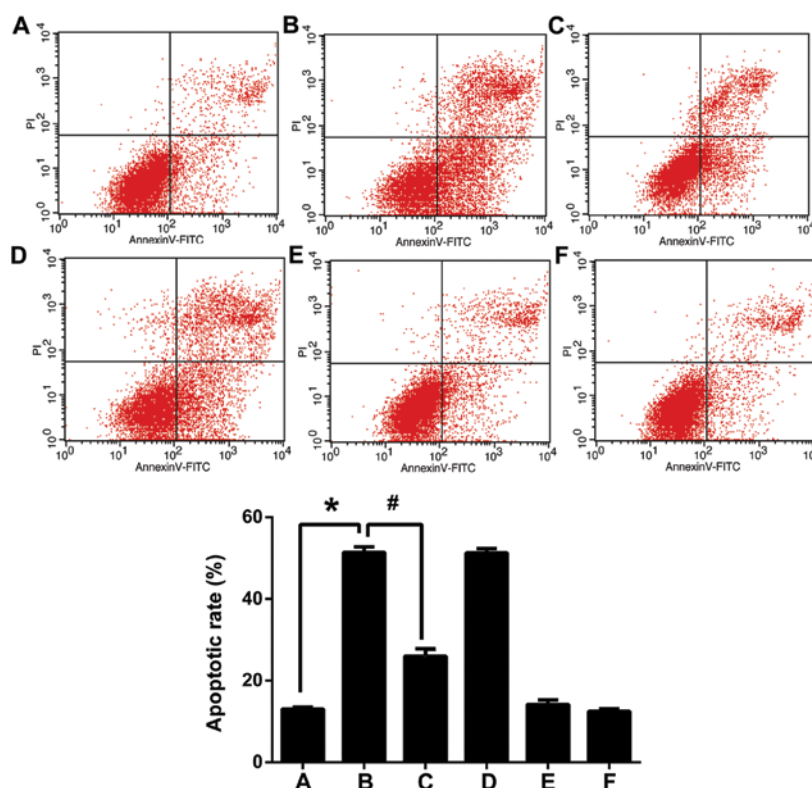


Figure 4. Cell apoptosis. A, control group; B, H₂O₂ group; C, H₂O₂ + IL-24 group; D, H₂O₂ + empty plasmid group; E, IL-24 group; F, empty plasmid group. Cell apoptosis rate in the H₂O₂ group was significantly higher than that of the control group (51.38 ± 1.39 and $13.01 \pm 0.50\%$, respectively) (* $P < 0.001$). There was a statistically significant difference between the H₂O₂ injury group and the H₂O₂ + IL-24 protective group (51.38 ± 1.39 and $25.93 \pm 1.902\%$, respectively) (# $P < 0.001$). IL-24, interleukin-24.

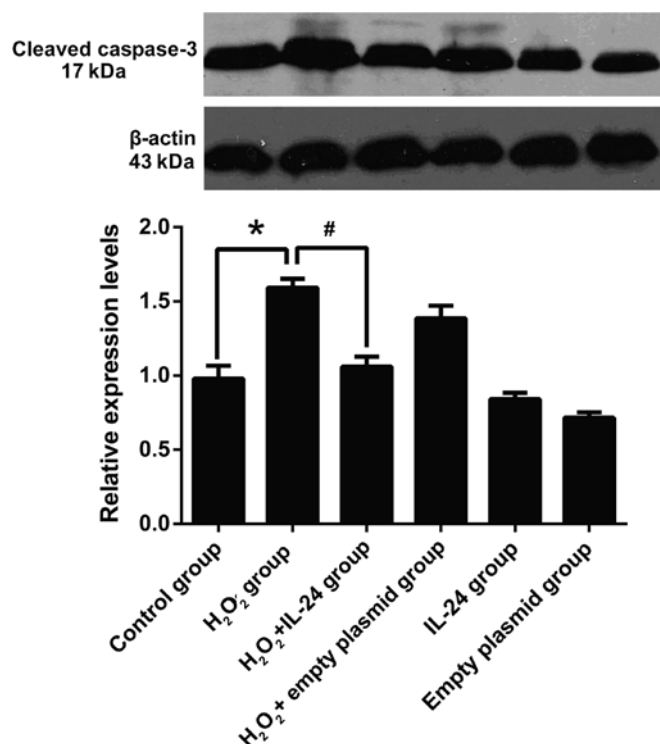


Figure 5. Effect of H₂O₂ and IL-24 on the cleaved expression of caspase-3. H₂O₂ significantly induced the activation and expression of caspase-3, while IL-24 attenuated these effects. There were significant differences between the control group and the H₂O₂ group (0.98 ± 0.086 and 1.59 ± 0.061 , respectively) (* $P < 0.001$). The difference between the H₂O₂ group and H₂O₂ + IL-24 group was statistically significant. Mean values were 1.59 ± 0.061 and 1.06 ± 0.067 , respectively (# $P < 0.001$). IL-24, interleukin-24.

expression of caspase-3, while this effect was attenuated in H₂O₂ + IL-24 group. There were significant differences between the control group and the H₂O₂ group (control group, 0.98 ± 0.086 ; H₂O₂ group, 1.59 ± 0.061 ; $P < 0.001$). The difference between the H₂O₂ group and the H₂O₂ + IL-24 group was statistically significant. The mean values were 1.59 ± 0.061 , 1.06 ± 0.067 , respectively ($P < 0.001$; Fig. 5).

Flow cytometry to measure ROS production in HUVECs. The quantitative detection of ROS production in each treatment group was performed (Fig. 6). The ROS content in the H₂O₂ group was significantly higher than in any other group. The difference between the control group and the H₂O₂ + IL-24 group was statistically significant. The mean values of the ROS content in the H₂O₂, control and H₂O₂ + IL-24 groups were 59.43 ± 4.39 , 7.97 ± 1.78 and 27.92 ± 3.35 , respectively ($P < 0.001$). Taken together, these results indicate that IL-24 decreases cellular ROS levels induced by H₂O₂, thus reducing damage induced by oxidative stress.

ROS activity in HUVECs detected by fluorescence microscopy. The overexpression of IL-24 attenuated the H₂O₂-induced increase in the intracellular ROS levels. The ROS levels were qualitatively observed by fluorescence microscopy (Fig. 6).

mRNA expression of cardiovascular disease-related factors. Real-time PCR was performed to assess the levels of angiotensinogen, endothelin-1, ATRAP and PDGF. The exposure

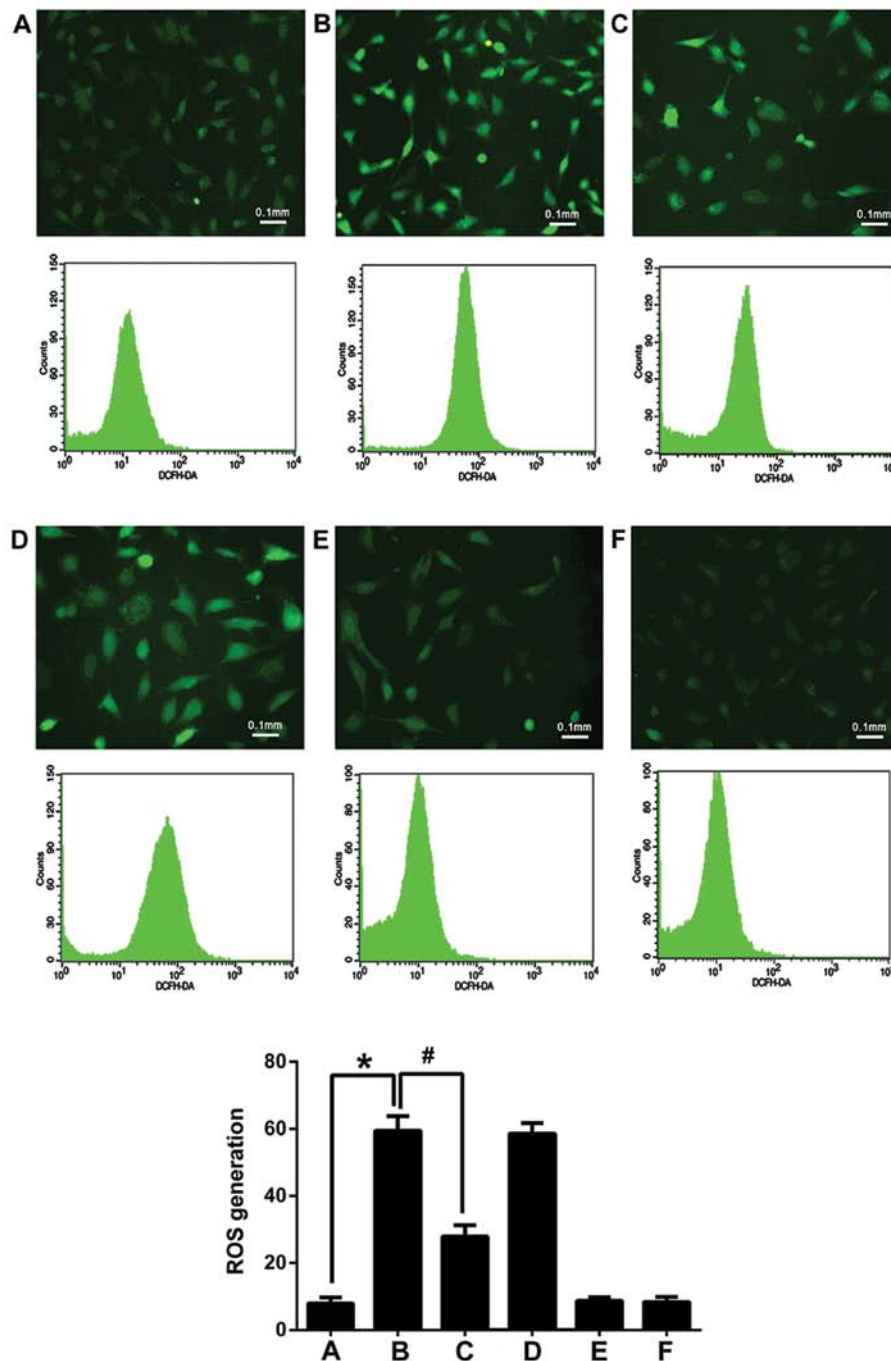


Figure 6. ROS generation. A, control group; B, H_2O_2 group; C, H_2O_2 + IL-24 group; D, H_2O_2 + empty plasmid group; E, IL-24 group; F, empty plasmid group. The ROS content in the H_2O_2 group was significantly higher than the other groups; the difference between the control group and the H_2O_2 + IL-24 group was statistically significant. Mean values were 59.43 ± 4.39 , 7.97 ± 1.78 and 27.92 ± 3.35 , respectively ($^{*}P < 0.001$). Fluorescence images show that H_2O_2 damage causes an increase in intracellular ROS generation, while IL-24 attenuates this effect. Magnification, $\times 100$. Bar, 0.1 mm. IL-24, interleukin-24.

of the HUVECs to H_2O_2 significantly increased the mRNA expression of each of these cardiovascular disease-related factors ($P < 0.001$). Importantly, IL-24 attenuated the increase in the levels of these factors ($P < 0.001$; Fig. 7A).

Protein expression of cardiovascular disease-related factors. Western blot analysis was performed to measure the protein expression of angiotensinogen, endothelin-1, ATRAP and PDGF. Consistent with the results observed at the mRNA level, oxidative stress induced by H_2O_2 -induced damage increased expression of these cardiovascular disease-associ-

ated factors at the protein level as well. Compared with the control group, each protein was normalized to the levels of β -actin, and the relative expression of angiotensinogen, endothelin-1, ATRAP and PDGF was $2.10 \pm 0.097/1.48 \pm 0.112$, $1.66 \pm 0.063/0.78 \pm 0.065$, $1.28 \pm 0.068/0.80 \pm 0.023$ and $1.33 \pm 0.031/0.63 \pm 0.020$, respectively ($P < 0.001$). Compared with the H_2O_2 group, the relative expression of angiotensinogen, endothelin-1, ATRAP and PDGF in the H_2O_2 + IL-24 group was $1.28 \pm 0.064/2.10 \pm 0.097$, $0.76 \pm 0.046/1.66 \pm 0.063$, $0.82 \pm 0.027/1.28 \pm 0.068$ and $0.88 \pm 0.058/1.33 \pm 0.031$, respectively ($P < 0.001$). Taken together, our results revealed that

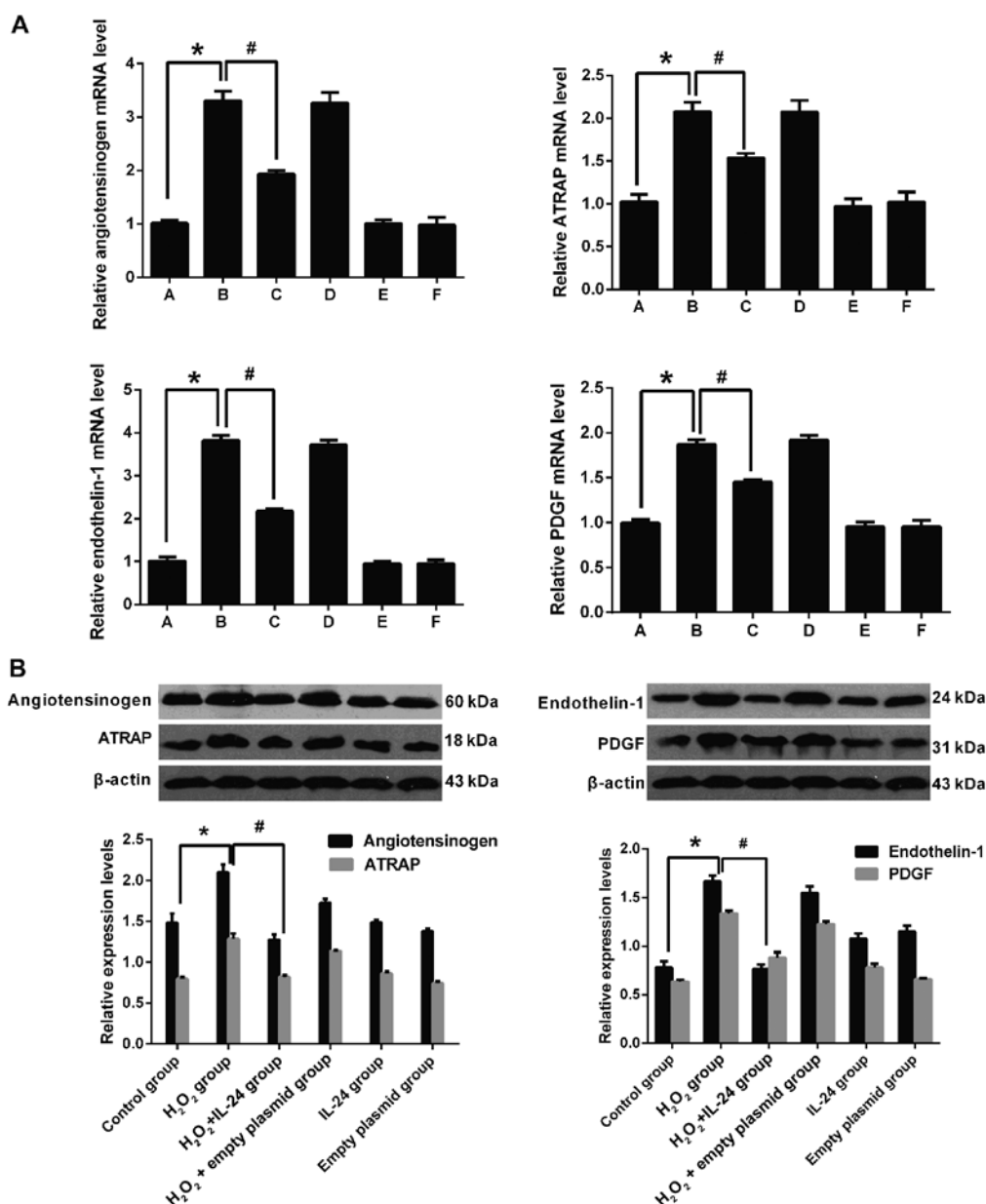


Figure 7. (A) H_2O_2 significantly stimulated the mRNA expression of cardiovascular disease-related factors in HUVECs ($^*P<0.001$). IL-24 attenuated this affect ($^*P<0.001$). (B) Oxidative stress induced by H_2O_2 damage induced the upregulation of vascular disease-associated protein expression in HUVECs compared with the control group. Compared with the control group, the relative expression of angiotensinogen, endothelin-1, ATRAP and PDGF in the H_2O_2 group was $2.10\pm0.097/1.48\pm0.112$, $1.66\pm0.063/0.78\pm0.065$, $1.28\pm0.068/0.80\pm0.023$ and $1.33\pm0.031/0.63\pm0.020$, respectively ($^*P<0.001$). Differences in protein expression in the H_2O_2 + IL-24 group compared with the H_2O_2 group were as follows: angiotensinogen ($1.28\pm0.064/2.10\pm0.097$), endothelin-1 ($0.76\pm0.046/1.66\pm0.063$), ATRAP ($0.82\pm0.027/1.28\pm0.068$) and PDGF ($0.88\pm0.058/1.33\pm0.031$) ($^*P<0.001$); all proteins normalized to β -actin. HUVECs, human umbilical vein endothelial cells; ATRAP, angiotensin II type 1 receptor-associated protein; PDGF, platelet-derived growth factor; IL-24, interleukin-24.

IL-24 significantly decreased the expression of cardiovascular disease-related proteins (Fig. 7B).

Successful establishment of the rat model of hypertension. The blood pressure of the normal rats was generally 90-115/67-90 mmHg. Blood pressure was measured at the second day following the infusion of angiotensin II, and blood pressure began to rise thereafter. As shown by continuous monitoring for 7 days, blood pressure was significantly elevated ($P<0.05$), which shows that the model of hypertension was successfully established. Blood pressure in the control group was 91.72 ± 4.89 mmHg, whereas blood pressure in the hypertension group was 147.35 ± 9.58 mmHg (Table III).

Table III. Comparison of systolic blood pressure (SBP, mmHg).

Group	N	Pre-angiotensin II administration	Post-angiotensin II administration
Control group	12	90.87 ± 4.76	91.23 ± 4.85^a
Hypertensive group	24	91.72 ± 4.89^b	$147.35\pm9.58^{a,b}$

After the establishment of the model of hypertension, the differences in SBP between the control group and the hypertensive group were statistically significant ($^*P<0.05$). Compared to pre-angiotensin II and post-angiotensin II administration, the difference in SBP in the hypertensive group was also statistically significant ($^bP<0.05$).

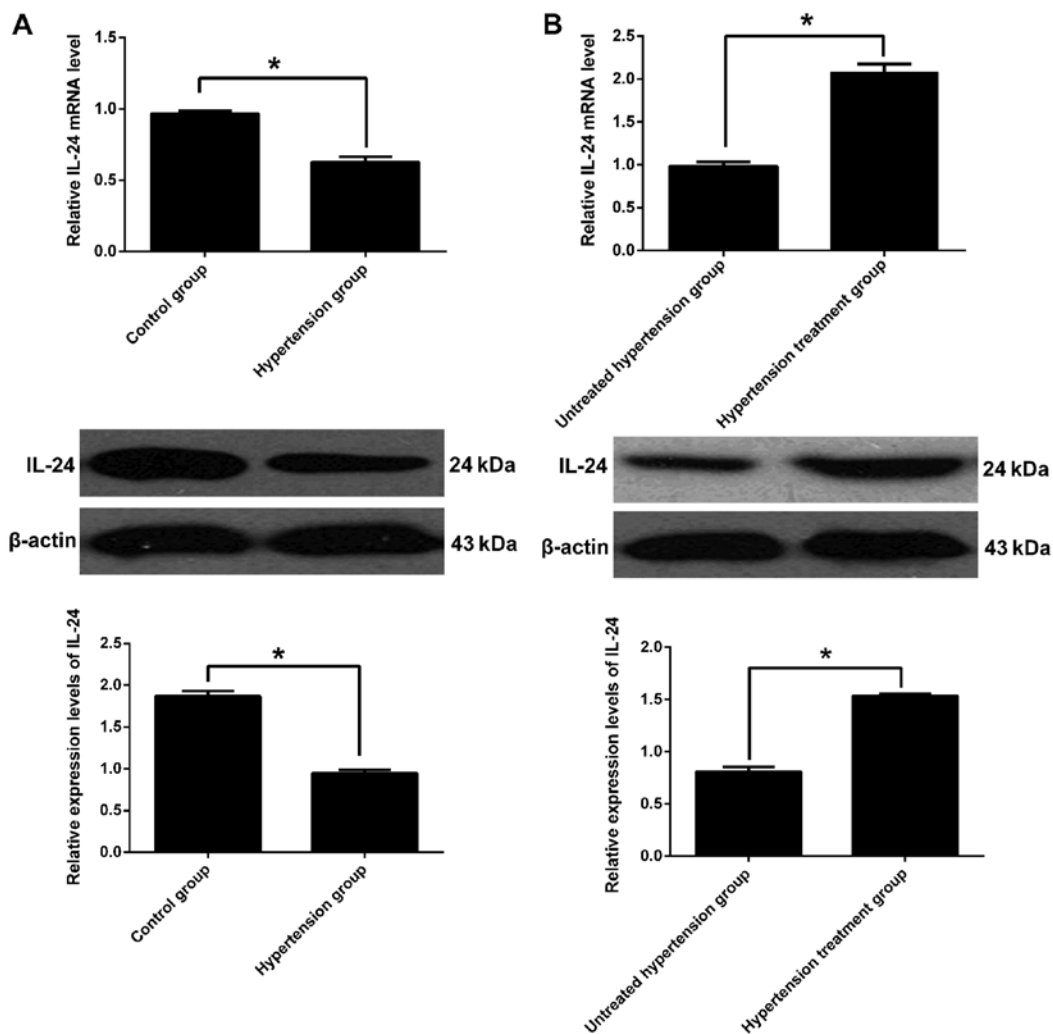


Figure 8. (A, top panel) IL-24 gene expression was decreased in heart and kidney tissue of hypertensive rats ($P<0.001$). (B, bottom panel) IL-24 protein expression was also decreased ($0.95\pm0.041/1.87\pm0.062$; $P<0.001$). (B, top panel) Anti-hypertensive therapy increased the expression of IL-24 ($*P<0.001$). (B, bottom panel) IL-24 protein expression was also upregulated ($1.54\pm0.014/0.81\pm0.042$; $*P<0.001$). IL-24, interleukin-24.

IL-24 mRNA levels in heart and kidney tissue. The transcript levels of IL-24 in the heart and kidney tissue from both the control and hypertensive rats were measured by real-time PCR. The effects of anti-hypertensive therapy were also examined. Compared to the rats in the control group, the IL-24 transcript levels were significantly decreased in the heart and kidney tissue from the hypertensive rats (Fig. 8A, top panel). Following anti-hypertensive therapy, the expression of IL-24 was significantly increased compared to the untreated group ($P<0.001$; Fig. 8B, top panel).

IL-24 protein levels in heart and kidney tissue. IL-24 protein expression was significantly lower in the hypertensive rats compared to the control rats. The relative expression of IL-24 in the hypertension group, normalized to β -actin, was $0.95\pm0.041/1.87\pm0.062$ ($P<0.001$; Fig. 8A, bottom panel). Anti-hypertensive therapy increased IL-24 protein expression; the relative expression in this setting was $1.54\pm0.014/0.81\pm0.042$ ($P<0.001$; Fig. 8B, bottom panel). Taken together, our data indicate that IL-24 expression is closely associated with hypertension.

Discussion

ROS play an important role in the pathophysiology of cardiovascular diseases, such as hyperlipidemia, hypertension, ischemic heart disease and chronic heart failure (27). Exogenous H_2O_2 activates the NAD(P)H oxidase-induced cell production of endogenous H_2O_2 (27). Intracellular ROS, such as superoxide oxygen ion (O_2^-), H_2O_2 , and hydroxyl radical ($OH^\cdot$), are associated with the pathogenesis of a number of diseases, such as cardiovascular diseases, including hypertension, heart failure and atherosclerosis, as well as diabetes (28). ROS can cause damage to blood vessel walls; in turn, vascular wall damage can induce changes in the expression of genes associated with cardiovascular diseases (29,30). These genes can stimulate VSMC migration and proliferation, and vascular intima hyperplasia, which lead to the development of cardiovascular disease (31-34).

Methods to reduce and prevent these pathophysiological processes include drug therapy and gene therapy. Compared to drug treatment, gene therapy has the advantage of promoting long-term efficacy with no systemic toxicity (19). Studies have demonstrated that several growth regulatory genes,

such as nitric oxide (NO) synthase, Rb and p53 may play a role in preventing neointima formation both *in vitro* and *in vivo* (35,36). However, the mechanisms through which these pathophysiological processes can be prevented are still unclear. Theoretically speaking, interfering with multiple biological processes, such as migration, proliferation and apoptosis, should provide the best efficacy. Along these lines, IL-24 has emerged as a promising candidate.

Toxins, such as cigarette smoke or a high-fat diet, can induce molecular pathways that are common to both cardiovascular disease and cancer; such pathways result in oxidative stress and cell damage. Atherosclerosis may arise from the damage or infection of a single arterial smooth muscle cell; in a similar manner, a tumor may arise from an acquired mutation within a single cell. Regulators of cell proliferation, commonly implicated in cancer, are also involved in the progression of atherosclerosis, vascular stenosis, and vascular restenosis after angioplasty. Likewise, cell adhesion molecules are closely related to both the formation of plaques and thrombus and to tumor invasion and metastasis (11,37,38).

Considering the common pathogenesis of cancer and cardiovascular disease (38), some researchers have suggested that IL-24 may play a potential therapeutic role in the treatment of cardiovascular diseases, such as atherosclerosis, arterial disease following organ transplants, hypertension and post-operative valve restenosis (39,40). Chen *et al* found that IL-24 inhibited the growth of PAC1 cells, a rat pulmonary artery smooth muscle cell line, through an intracellular pathway, while having no effect on normal primary human coronary artery cells or rat aortic smooth muscle cells (41). Ramesh *et al* found that IL-24 inhibited the formation of two cell capillary structure of cultured HUVECs and human vein endothelial cells in a dose-dependent manner (42). Their study also demonstrated that the anti-angiogenic effects of IL-24 were more prominent than the effects of endostatin. Transwell migration assays revealed that IL-24 significantly inhibited vascular endothelial growth factor (VEGF)-induced HUVEC migration (42). Vascular calcification is an important marker of cardiovascular disease. In a previous study, the rat model of β -glycerophosphate (β -GP)-induced VSMC calcification model was confirmed by Von Kossa staining and the detection of the calcium content; the authors showed that IL-24 significantly inhibited the calcification of VSMCs in this model (43). IL-24 inhibited calcification, osteogenic cell marker expression and apoptosis induced by β -GP. It also blocked the activation of the Wnt/ β -catenin signaling pathway, thereby inhibiting vascular calcification. This research suggested that IL-24 may be a potential therapeutic agent in the calcification of VSMCs. Besides this, another study demonstrated that IL-24 suppressed the growth of normal VSMCs by inhibiting H_2O_2 -induced ROS production through the regulation of mitochondrial ROS production and the expression of antioxidant enzymes (44). In a recent study, the researchers found that IL-24 polymorphisms were associated with cardiometabolic parameters and cardiovascular risk factors (45). These results indicate that IL-24 plays an important role in cardiovascular disease.

In a previous study, IL-24 was demonstrated to inhibit tumor cell growth and blood vessel formation; it also induces tumor cell apoptosis and stimulates the expression of several cytokine genes (46).

In recent years, much attention has been paid to obtaining a better understanding of the association between endothelial cell dysfunction and hypertension. Some groups have suggested that endothelial cell injury is a result of hypertension (47). In contrast, others have proposed that endothelial cell dysfunction is an important risk factor for hypertension, and not merely the result of hypertension (48).

Burdon and Gill demonstrated that H_2O_2 , along with a superoxide anion, play a 'contact information' role, so as to maintain a normal life state of cells (25). ROS, such as H_2O_2 , promote the proliferation of both cancer cells and normal cells in the body, including VSMCs and vascular endothelial cells. IL-24 has been shown to significantly inhibit cell proliferation in some studies (49-51). In this study, we demonstrated that IL-24 inhibited the abnormal proliferation of vascular endothelial cells induced by H_2O_2 , a regulator of cell proliferation involved in the progression of atherosclerotic plaques, vascular stenosis, vascular restenosis following angioplasty and cancer. As such, IL-24 may play a role in the treatment of occlusive vascular disease through this mechanism.

H_2O_2 is the intermediate product of oxidative metabolism in the body, and it can easily permeate the cell membrane. If the intracellular levels of H_2O_2 accumulate to toxic levels, it can have dire consequences for vascular endothelial cells and myocardial cells. H_2O_2 at 0.3 mmol/l can stimulate intracellular ROS production, causing oxidative stress damage and resulting in cellular injury, apoptosis and death (52-54). In this study, we demonstrated that IL-24 significantly attenuated H_2O_2 -induced cell apoptosis. IL-24 intervention significantly reduced the protein expression of cleaved caspase-3. Caspase-3 is one of the most key executioners in the process of apoptosis, and the activation of caspase-3 is often used as an important indicator of apoptosis. Importantly, IL-24 may reduce apoptosis by reducing the content of intracellular ROS. Taken together, our data indicate that IL-24 is an effective protective agent and therapeutic target for vascular endothelial cells and for the vascular system in general.

Several genes, including angiotensinogen, endothelin-1, ATRAP and PDGF are closely related to the pathogenesis of cardiovascular disease (31-34). We found that exposure of the HUVECs to H_2O_2 increased the expression of these genes, and may thus contribute to the development of hypertension. Importantly, we demonstrated that the ectopic overexpression of IL-24 significantly reduced the expression levels of these genes, indicating that IL-24 may reduce the incidence of hypertension by affecting the expression of related genes.

In this study, we also examined the effects of H_2O_2 and IL-24 in an *in vivo* rat model of hypertension. We found that IL-24 expression was significantly reduced in the tissues of hypertensive rats compared to the healthy controls. Additionally, we found that treatment with anti-hypertensive drugs increased the IL-24 levels. These data suggest that IL-24 may be clinically useful.

In conclusion, our data demonstrate that IL-24 inhibits the the abnormal proliferation of vascular endothelial cells induced by low concentrations of H_2O_2 . It also inhibits apoptosis via the inhibition of ROS production in vascular endothelial cells. IL-24 can also downregulate the expression of several cardiovascular disease-related genes. Considering the common molecular mechanisms underlying the pathogenesis of cancer

and cardiovascular disease, and given that IL-24 inhibits ROS production, IL-24 may provide a basic therapeutic strategy for the treatment of vascular disease and cancer caused by ROS overproduction.

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