Tetramethylpyrazine protects $CoCl_2$-induced apoptosis in human umbilical vein endothelial cells by regulating the PHD2/HIF/1α-VEGF pathway

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Abstract. Tetramethylpyrazine (TMP), one of the active ingredients isolated from a Chinese herbal prescription, possesses protective effects against apoptosis in endothelial cells. However, the underlying mechanism of its protective effects in endothelial cells remains to be elucidated. Using human umbilical vein endothelial cells (HUVECs), the present study assessed the protective effects of TMP on $CoCl_2$-induced apoptosis. Following pre-incubation with $CoCl_2$ (150 μM/ml) for 4 h, the HUVECs were treated with TMP at different concentrations (50, 100 and 200 μM/ml) for 8 h. TMP upregulated the expression of prolyl hydroxylase (PHD)2, reduced the protein and mRNA expression levels of vascular endothelial growth factor (VEGF), and reduced the expression of HIF-1α only at the protein level, not at the mRNA level in HUVECs, in a concentration-dependent manner. Furthermore, silencing of the PHD2 gene with small interfering (si)RNAs abolished the reduction in the expression of hypoxia-inducible factor (HIF)-1α and VEGF by TMP. In addition, TMP protected $CoCl_2$-induced HUVEC injury via an apoptosis pathway, as characterized by the increased ratio of cell viability and the reduced percentage of apoptotic and terminal deoxynucleotidyl transferase dUTP nick end labeling-positive HUVECs, activation of caspase-3, -8 and -9, B-cell lymphoma (Bcl)-2/Bcl-2-activated X protein expression, as well as the release of cytochrome *c*. The protective properties of TMP were partially attributed to the mRNA and protein expression levels of PHD, since silencing of the PHD2 gene with siRNAs abolished these effects. The present study demonstrated that the antiapoptotic effect of TMP in $CoCl_2$-induced HUVECs was, at least in part, via the regulation of the PHD2/HIF-1α signaling pathway.

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Introduction

Retinal neovascularization is one of the leading causes of visual impairment in numerous diseases, including proliferative diabetic retinopathy (PDR), neovascular age-related macular degeneration (NVAMD), central retinal vein occlusion and retinopathy of prematurity (1). Vascular endothelial growth factor (VEGF) is an important stimulator of new vessel growth in the processes of these diseases, and the development of anti-VEGF treatment has provided substantial benefits in patients with these diseases (2). However, the side effects, including loss of peripheral vision and reduction of night vision, greatly limit its value and more importantly, there remains a lack of methods for a final cure. Hypoxia is an important pathophysiological signal and can cascade down in a series of angiogenic processes in retinal neovascularization diseases. Therefore, exploring the biological alterations of endothelial cells under hypoxic conditions may be helpful to provide an improved understanding of the mechanism of retinal neovascularization diseases and provide potential molecular therapies.

Hypoxia inducible factor (HIF)-1α is a key oxygen sensor and is important in the response to retinal hypoxia, and the regulation of angiogenesis in PDR and NVAMD (3). Through the hypoxia response element binding site, HIF-1α regulates the production of VEGF (4-7). Silencing HIF-1α by RNA interference during hypoxia reduces the expression levels of VEGF and other clinically important angiogenic factors, leading to the inhibition of angiogenesis (8). Notably, HIF-1α activates diverse genes involved in both cell growth and cell apoptosis under hypoxic conditions (9-11).

Prolyl hydroxylase (PHD)2 also serves as an oxygen sensor, which regulates the stability or degradation of HIF-1α

in an oxygen-dependent manner. In normoxia or hyperoxia, PHD2 hydroxylates the proline residues of HIF-1α, which are captured by the Von Hippel Lindau protein (pVHL) ubiquitin E3 ligase complex and are degraded by the proteasome. By contrast, in hypoxia, PHD2 fails to initiate this reaction due to a shortage of O_2 and, therefore, HIF-1α is stabilized (12,13).

Tetramethylpyrazine (TMP), one of the most important active ingredients of Chuan Xiong and is applied widely in the treatment of neurovascular disorders, including ischemic stroke and pulmonary hypertension secondary to chronic obstructive pulmonary diseases in China (14-16). Previous studies have suggested the potential antiangiogenic properties of TMP in choroidal and retinal neovascularization *in vivo* (17,18). Our previous study demonstrated that TMP improved neurovascular recovery by preventing neovascularization, and protecting retinal astroglia cells and neurons from ischemia-induced cell death, partially due to the downregulation of the expression levels of HIF-1α and VEGF (18). In addition, several previous studies revealed that TMP can protect endothelial cells from apoptosis under hyperoxia and oxidative stress *in vitro* (15,19-23). However, the molecular and cellular mechanisms of TMP in the protection of endothelial cells remain to be elucidated. As a result of the protection of TMP in apoptotic endothelial cells, and the downregulation of HIF-1α and VEGF following TMP treatment, it is reasonable to hypothesize that the antiapoptotic effect of TMP in endothelial cells occurs via the regulation of the PHD2/HIF-1α signaling pathway.

The present study confirmed the antiapoptotic effect of TMP in human umbilical vein endothelial cells (HUVECs) following pre-incubation with $CoCl_2$ and revealed that its antiapoptotic effect was, at least in part, via the regulation of the of PHD2/HIF-1α signaling pathway. These results may provide useful insight into the pathology of endothelial cell apoptosis and may serve as a potential novel therapeutic target in retinal neovascularization diseases.

Materials and methods

Reagents. $CoCl_2$ and TMP were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Rabbit monoclonal PHD2 (cat. no. 4835; 1:500), HIF-1α (cat. no. 3716; 1:500) and VEGF (cat. no. 2839; 1:500) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA), rabbit monoclonal cleaved caspase-3 (AB3623; 1:500), rabbit polyclonal cleaved caspase-8 (ASP387; 1:500) and rabbit polyclonal cleaved caspase-9 (ASP315; 1:500) were purchased from EMD Millipore (Billerica, MA, USA), and the mouse monoclonal β-actin (sc-69879; 1:500) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using the *in situ* Cell Death Detection kit (EMD Millipore).

Cell culture and drug treatment. Normal human umbilical cords were obtained from The Third Affiliated Hospital of Sun Yat-sen University, Zhujiang Hospital of Southern Medical University, and Foshan Nanhai Maternity and Child Health Hospital. HUVECs were isolated from the vein of normal human umbilical cord, as described previously (24). The cells were cultured in DMEM-F12, supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin (Thermo Fisher Scientific, Inc.), 30 lg/ml endothelial cell growth supplement (Gibco; Thermo Fisher Scientific, Inc.) and 5 units/ml heparin (Thermo Fisher Scientific, Inc.) at 37˚C, with 5% CO_2 and 95% air. The cells used in the present study were between passage 3 and 4. Once the cells had grown to 90% confluence, they were pre-incubated with $CoCl_2$ (150 μM/ml) for 4 h. Following incubation, the cells were washed twice with phosphate-buffered saline (pH 7.4) and were subsequently exposed to TMP at different concentrations (50, 100 and 200 μM/ml) for 8 h. TMP was dissolved in dimethyl sulfoxide (<0.1%), which caused no deleterious effect on the viability of HUVECs in preliminary studies.

The present study was performed in accordance with the Declaration of Helsinki. The institutional review board of Sun Yat-sen University approved the protocol for collection human umbilical cords. Written informed consent was obtained from all patients following an explanation of the purpose and procedures of the investigation.

Cell viability assay. Cell viability was assessed using an MTT assay (25). HUVECs ($1x10^4$ cells/well) were cultured in 96-well plates at 37˚C for 24 h, and were subsequently divided into four groups: i) control; ii) $CoCl_2$ (only pre-incubated with 150 μM/ml $CoCl_2$ for 4 h); iii) $CoCl_2$ + TMP (pre-incubated with 150 μm/ml $CoCl_2$ for 4 h and subsequently treated with 200 μM/ml TMP for 8 h); iv) $CoCl_2$ + TMP + siRNA (PHD2 gene silencing with siRNA, pre-incubation with 150 μM/ml $CoCl_2$ for 4 h and subsequently treated with 200 μM/ml TMP for 8 h). Following treatments, the medium was removed and 10 μl MTT solution (5 mg/ml) was added and the plates were incubated at 37˚C for 4 h in a humidified 5% CO_2 atmosphere. Next, the cells were assessed in a microtiter plate reader (LabSystems; Thermo Fisher Scientific, Inc.) and scanned to visualize the color development. The cell survival rates were expressed as the percentages of the value of normal cells.

Analysis of cell apoptosis by flow cytometry. A FACScan Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used for quantifying apoptotic cells by determining the DNA content of the cells. The apoptotic rate of HUVECs was detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Santa Cruz Biotechnology, Inc.). Following the different treatments, the cells were harvested, washed and double-stained with annexin V-FITC and propidium iodide in a dark at room temperature for 20 min. Following staining, the samples were analyzed at an excitation wavelength of 488 nm and an emission wavelength of 530 nm using the EL340 Microtiter Plate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Analysis of cell apoptosis by TUNEL. The apoptosis of HUVECs was assessed using a TUNEL assay kit, according

to the manufacturer's protocol. Following the different treatments, the cells were seeded into 24-well plates and incubated overnight. The cells were subsequently fixed and incubated with 100 μM enzyme solution for 30 min at 37˚C. Following incubation, the cells were washed three times with phosphate-buffered saline and incubated with labeled solution for 1 h and 37˚C. At the same time, the cell nucleus was labeled with DAPI (1:1,000). The ratio of cell apoptosis was calculated by comparing the number of positively labeled cells with the total number of cells.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total cellular RNA was isolated using TRIzol reagent, according to manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). The purity of the RNA was confirmed by the ratio of optical densities at 260 and 280 nm. The primers for target genes were obtained from the NCBI GeneBank database (http://www.ncbi.nlm.nih.gov/genbank/). The cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The fluorescence signal was detected during the extension step in each cycle. The data were normalized against GADPH and the $2^{-\Delta\Delta Cq}$ method was used to calculate target gene expression. The result was represented as the relative value compared with the control.

The primers of the target genes were as follows: Bcl-2 (Genbank Accession no. NM_000657), forward: 5'-AGG AAGTGAACATTTCGGTGAC-3' and reverse: 5'-GCTCAG TTCCAGGACCAGGC-3'; Bax (Genbank Accession no. NM_138763), forward: 5'-TGCTTCAGGGTTTCATCCAG-3' and reverse: 5'-GGCGGCAATCATCCTCTG-3'.

Western blotting. Following the appropriate treatment, the cell lysates were prepared in non-denaturing buffer comprising 10 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTANa_2, 1 mM DTT, 1 μg/ml leupeptine, 1 mM Benzamidine and 2 μg/ml aprotinin. The protein concentration was determined using Bradford reagent, as previously described (26). The proteins (30 μg) were subsequently separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (EMD Millipore), and were transferred onto polyvinylidene difluoride membrane (EMD Millipore). The 5% non-fat milk powder dissolved in Tris-buffered saline containing Tween-20 (TBST) buffer was used to block the membrane for 1-2 h at 37˚C to reduce non-specific binding. Specific primary antibodies against each target protein were used and the membrane was incubated with these antibodies at 4˚C overnight. In order to remove any unbound primary antibody, the membrane was washed three times with TBST for 15 min, and the membranes were subsequently incubated with secondary antibodies, which were conjugated to horseradish peroxidase (EMD Millipore) for 4 h. Enhanced chemiluminescence (EMD Millipore) was used for protein visualization, according to the manufacturer's protocol. β-actin was used as the endogenous reference protein.

Statistical analysis. All results are representative of three independent experiments and the data are expressed as the mean ± standard deviation. The data were analyzed statistically using one-way analysis of variance, followed by the Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Effect of TMP on the expression levels of PHD2, HIF-1α and VEGF in $CoCl_2$-treated HUVECs. The mRNA expression levels of PHD2, HIF-1α and VEGF in HUVECs were determined by RT-qPCR. The $CoCl_2$ group showed significantly decreased mRNA expression of PHD2 ($^{*}P<0.05$; Fig. 1A) and increased mRNA expression levels of HIF-1α and VEGF compared with the control group ($^{*}P<0.05$; Fig. 1B and C). Treatment with TMP (50, 100 and 200 μM/ml) for 8 h, increased the mRNA expression of PHD2 ($^{\#}P<0.05$, $^{\#\#}P<0.01$; Fig. 1A) and reduced the expression of VEGF ($^{\#}P<0.05$, $^{\#\#}P<0.01$; Fig. 1C) in a dose-dependent manner. Notably, the mRNA expression of HIF-1α ($P>0.05$; Fig. 1B) remained unaffected compared with the $CoCl_2$ group.

On the basis of the above mRNA expression results, the effect of TMP on the protein expression levels of PHD2, HIF-1α and VEGF in HUVECs was investigated by western blotting. The $CoCl_2$ group showed significantly reduced protein expression of PHD2 ($^{*}P<0.05$; Fig. 1D and E), and increased the protein expression levels of HIF-1α and VEGF ($^{*}P<0.05$; Fig. 1D, F and G) compared with the control group. Treatment with TMP (50, 100 and s200 μM/ml) significantly increased the protein expression of PHD2 ($^{\#}P<0.05$; Fig. 1D and E) and decreased the protein expression levels of HIF-1α and VEGF ($^{\#}P<0.05$, $^{\#\#}P<0.01$; Fig. 1D, F and G) in a dose-dependent manner. These results indicated that TMP upregulated the expression of PHD2 and initiated the degradation of HIF-1α, resulting in the inhibition of VEGF.

PHD2-siRNA lentiviral vector inhibits the effect of TMP on the expression levels of PHD2, HIF-1α and VEGF in $CoCl_2$-treated HUVECs. To further assess the role of TMP in the regulation of the PHD2/HIF-1α signaling pathway, PHD2-siRNA lentiviral vector or the negative control (NC) vector was transfected into the HUVECs. Compared with the control group, no significant difference was detected in the mRNA expression of PHD2 ($P>0.05$; Fig. 2A) or the protein expression ($P>0.05$; Fig. 2B-C) in the NC group. However, the mRNA ($P<0.01$; Fig. 2A) and protein ($P<0.01$; Fig. 2B and C) expression levels of PHD2 decreased significantly in the PHD2-siRNA group ($P<0.01$).

Previous studies showed that during hypoxia, PHD2 fails to initiate the degradation of HIF-1α and, therefore, HIF-1α is stabilized and upregulated (12-13), resulting in the expression of VEGF. In the present study, compared with the NC group, the upregulation of the mRNA and protein expression levels of VEGF was observed following transfection with the PHD2-siRNA lentiviral vector in $CoCl_2$-treated HUVECs ($^{*}P<0.05$; Fig. 2E, F and H). However, only the upregulation of the HIF-1α protein ($^{*}P<0.05$; Fig. 2F and G), and not HIF-1α mRNA (Fig. 2D) was observed between the NC and PHD2 siRNA group.

Notably, compared with the NC group, the TMP induced degradation of HIF-1α ($^{\#\#}P<0.01$; Fig. 2F and G) and the

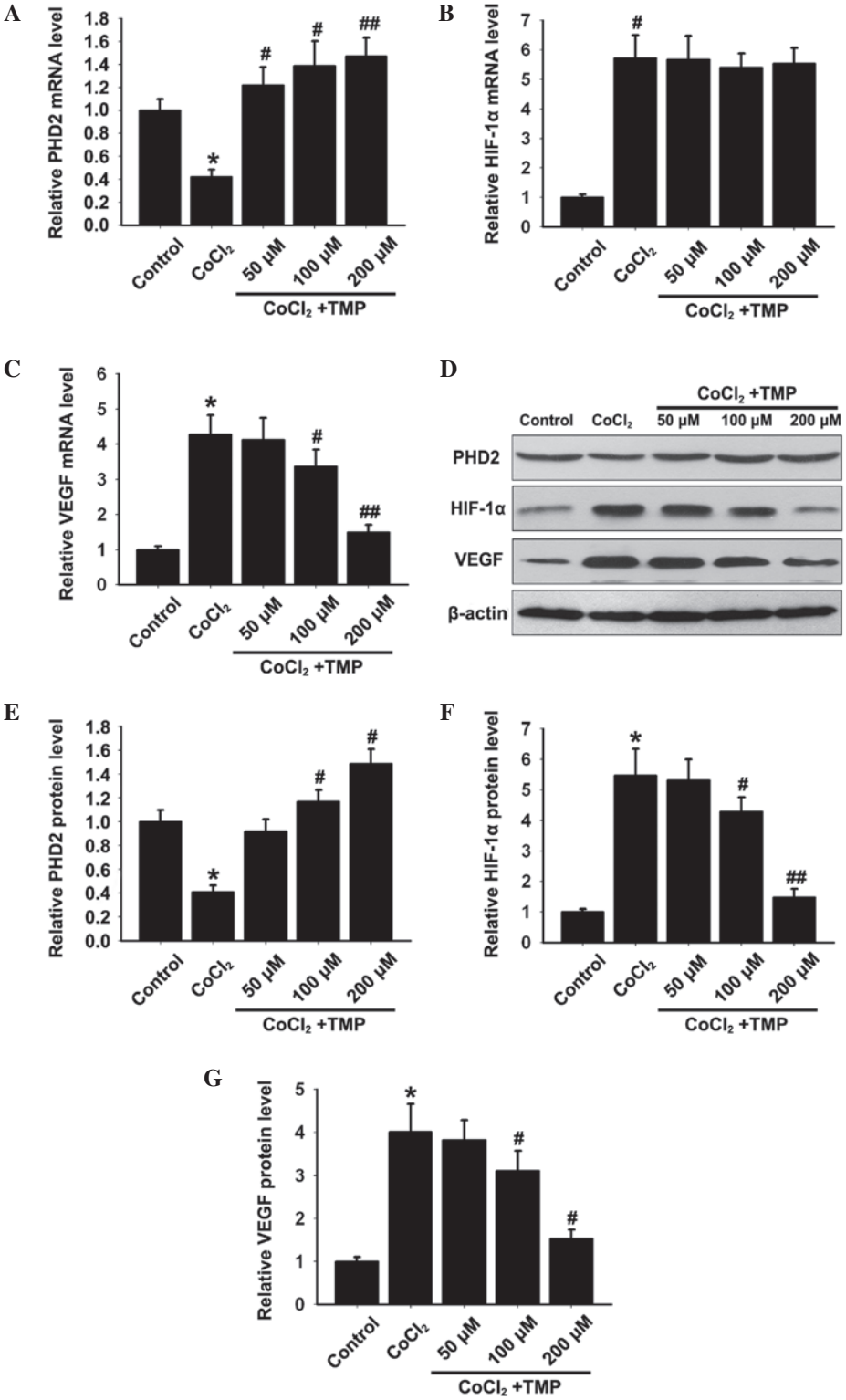

Figure 1. Effect of TMP on the expression levels of PHD2, HIF-1α and VEGF in $CoCl_2$-treated HUVECs. Following pre-incubation with $CoCl_2$ (150 μM/ml) for 4 h (h), the HUVECs were treated with TMP at different concentrations (50, 100 and 200 μM/ml) for 8 h. The mRNA expression levels of (A) PHD2, (B) HIF-1α and (C) VEGF in each group were measured by RT-qPCR. β-actin was used as internal standard. (D) The protein expression levels of PHD2, HIF-1α and VEGF in each group were determined by western blotting. β-actin was used as internal standard. Quantification graphs of the expression levels of (E) PHD2, (F) HIF-1α and (G) VEGF against β-actin in each group. The results are representative of three independent experiments and the data are expressed as the mean ± standard deviation (*P<0.05, vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. $CoCl_2$ group). TMP, tetramethylpyrazine; HUVEC, human umbilical vein endothelial cells; PHD, prolyl hydroxylase; HIF, hypoxia inducible factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor.

inhibition of VEGF ($^{\#}$P<0.01; Fig. 2F and H) was abolished in the PHD2-siRNA group. However, the mRNA expression of HIF-1α in the NC and TMP groups increased significantly following $CoCl_2$ treatment, which remained unaffected by the PHD2-siRNA lentiviral vector transfection (P>0.05; Fig. 2D). These results further indicated that TMP initiated the

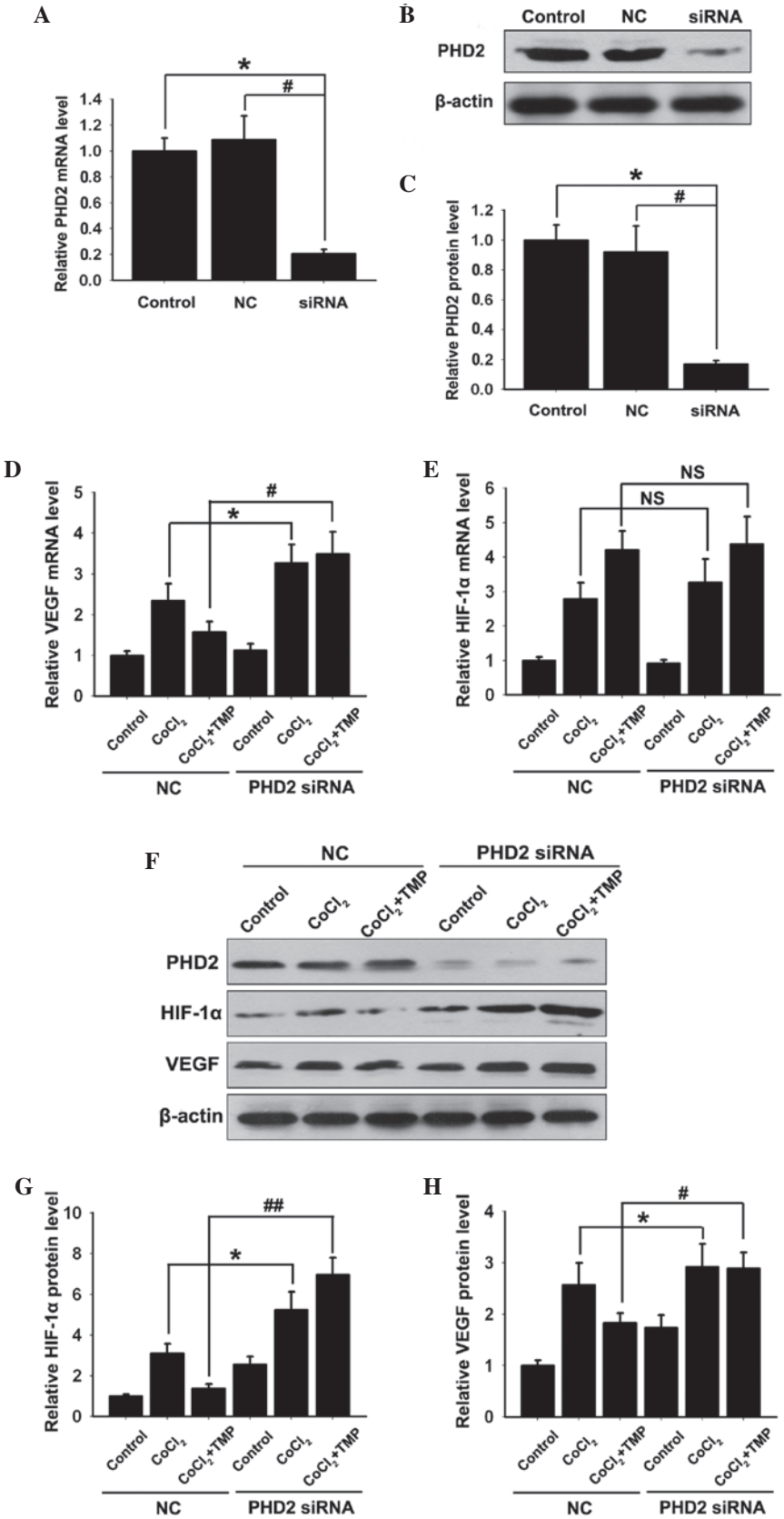

Figure 2. Effect of TMP on the expression levels of HIF-1α and VEGF in PHD2-siRNA transfected HUVECs. The HUVECs were transfected with PHD2-siRNA lentiviral vector and were subsequently pre-incubated with $CoCl_2$ (150 μM/ml) for 4 h, followed by TMP (200 μM/ml) for 8 h. (A) The mRNA expression of PHD2 was measured by RT-qPCR. (B) The protein expression of PHD2 was determined by western blotting. β-actin was used as an internal standard. (C) Quantification graph of PHD2 against β-actin. The mRNA expression levels of (D) VEGF and (E) HIF-1α in each group were measured by RT-qPCR. (F) The protein expression levels of PHD2, HIF-1α and VEGF in each group were measured by western blotting. Quantification graphs of the intensity of (G) HIF-1α and (H) VEGF bands against β-actin in each group. The results are representative of three independent experiments. The data are expressed as the mean ± standard deviation (*P<0.05, $^{\#}$P<0.05, $^{\#\#}$P<0.01, ^{NS}P >0.05). NS, non-significant; NC, negative control; TMP, tetramethylpyrazine; HUVEC, human umbilical vein endothelial cells; PHD, prolyl hydroxylase; HIF, hypoxia inducible factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA.

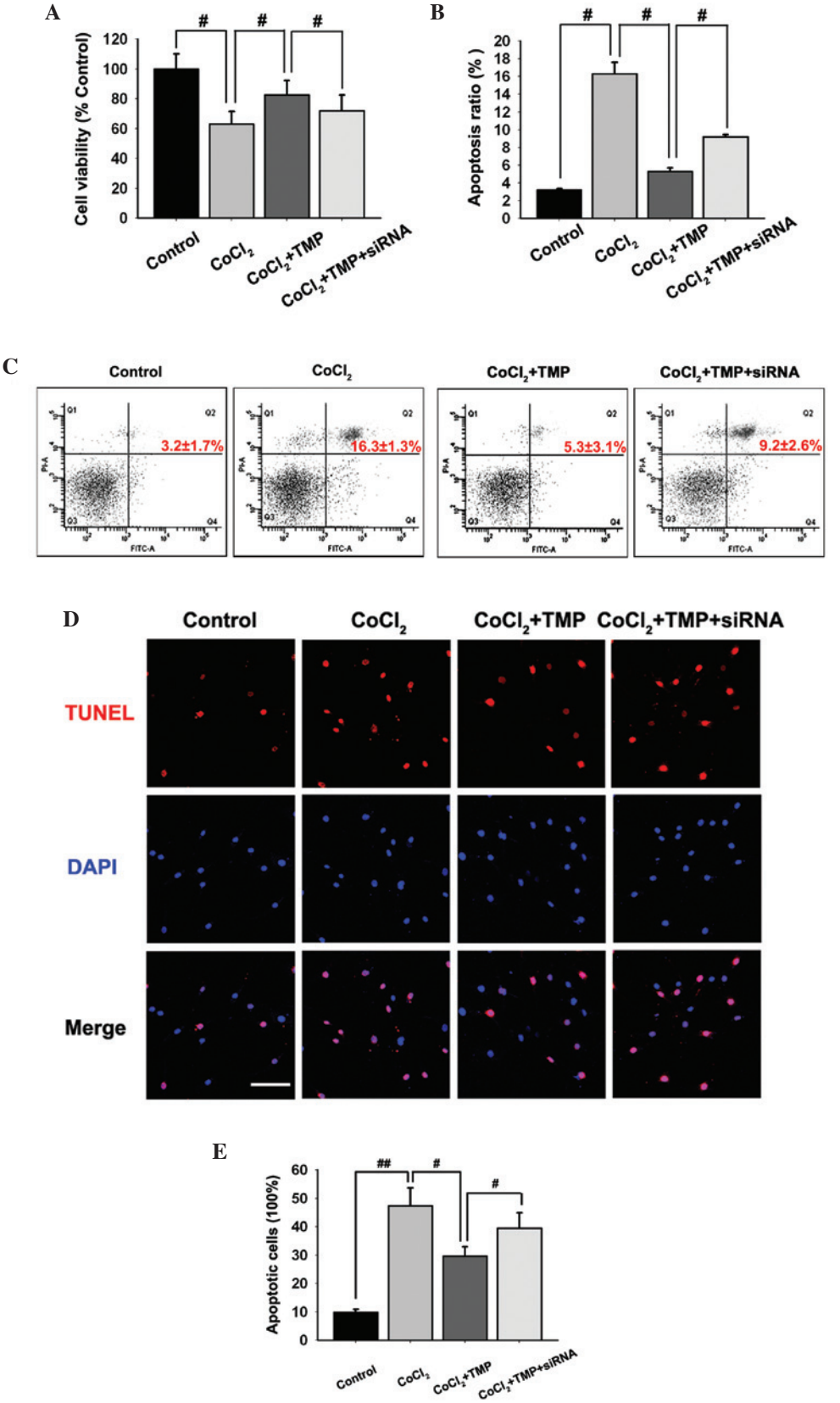

Figure 3. Effect of TMP on the apoptosis of $CoCl_2$-induced HUVECs. The HUVECs were transfected with PHD2-siRNA lentiviral vector and were subsequently pre-incubated with $CoCl_2$ (150 μM/ml) for 4 h, followed by TMP (200 μM/ml) for 8 h. (A) The effect of TMP on $CoCl_2$-induced loss of cell viability in HUVECs was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide assay. (B and C) The apoptotic cells in $CoCl_2$-induced HUVECs were detected by flow cytometry following staining with PI. (D and E) The apoptotic cells in $CoCl_2$-induced HUVECs were observed by TUNEL. The HUVECs in different groups were stained with TUNEL (red) and DAPI (blue). Images were captured (scale bar, 100 μm) and the ratio of TUNEL-positive cells against the number of cell nuclei in each group was calculated. The data are expressed as the mean ± standard deviation of three independent experiments ($^{\#}P<0.05$ and $^{\#\#}P<0.01$). TMP, tetramethylpyrazine; HUVEC, human umbilical vein endothelial cells; PHD, prolyl hydroxylase; HIF, hypoxia inducible factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

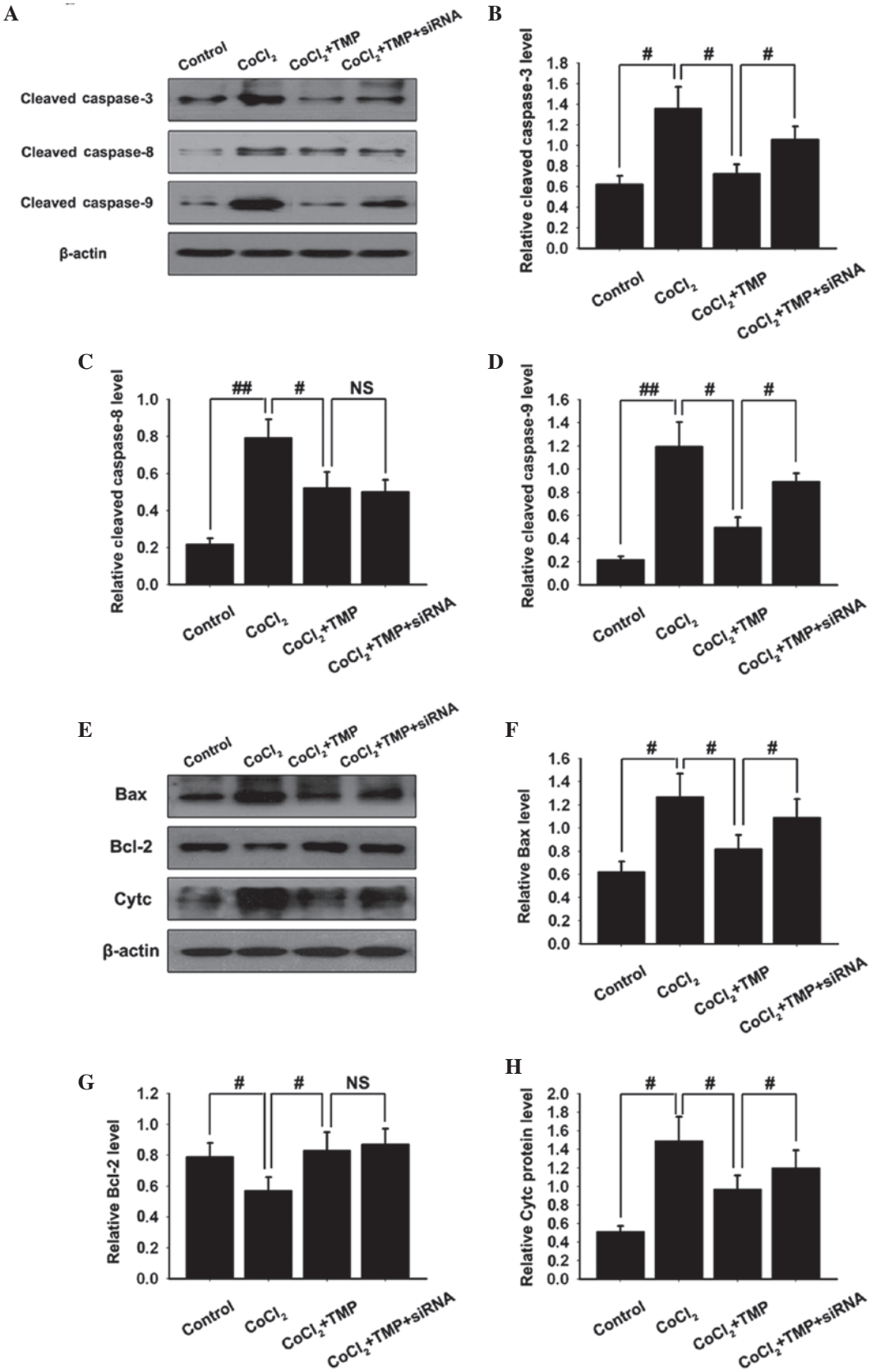

Figure 4. Effect of TMP on the expression levels of apoptotic associated proteins in $CoCl_2$-induced HUVECs. The HUVECs were transfected with PHD2-siRNA lentiviral vector and were subsequently pre-incubated with $CoCl_2$ (150 μM/ml) for 4 h, followed by TMP (200 μM/ml) for 8 h. (A) The protein expression levels of activated caspase family members (caspase-3, caspase-8 and caspase-9) in the $CoCl_2$-induced HUVECs were measured by western blotting. β-actin was used as internal standard. Quantification graphs of the band intensities of activated (B) caspase-3, (C) caspase-8 and (D) caspase-9 against β-actin in each group. (E) The protein expression levels of Bax, Bcl-2 and Cytc in $CoCl_2$-induced HUVECs were measured by western blotting. β-actin was used as internal standard. Quantification graphs of the intensity of staining of (F) Bax, (G) Bcl-2 and (H) Cytc against β-actin in each group. The results are representative of three independent experiments. The data are expressed as the mean ± standard deviation ($^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{NS}P>0.05$). Cytc: cytochrome *c*; NS, non-significant; NC, negative control; TMP, tetramethylpyrazine; HUVEC, human umbilical vein endothelial cells; PHD, prolyl hydroxylase; Bcl, B-cell lymphoma. Bax, Bcl-2-associated X protein; siRNA, small interfering RNA.

degradation of HIF-1α and the inhibition of VEGF by upregulating the expression of PHD2 in $CoCl_2$-treated HUVECs.

TMP protects cell viability in $CoCl_2$-treated HUVECs. An MTT assay was used to investigate the effect of TMP on the viability of HUVECs. Pre-incubation with $CoCl_2$ inhibited the cell viability of HUVECs ($^{\#}P<0.05$; Fig. 3A). Treatment with TMP (200 μM/ml) for 8 h following pre-incubation with $CoCl_2$ protected cell viability in HUVECs ($^{\#}P<0.05$; Fig. 3A). However, following transfection with the PHD2-siRNA lentiviral vector, the protection of TMP was partly abolished ($^{\#}P<0.05$; Fig. 3A).

Effect of TMP on the apoptosis of HUVECs induced by $CoCl_2$. The present study determined whether TMP protected from the apoptosis of HUVECs by annexin V-FITC and PI staining. Flow cytometric analysis results indicated that $CoCl_2$ induced apoptosis in HUVECs (Fig. 3B and C). Following treatment with TMP (200 μM/ml), the apoptosis in HUVECs was markedly inhibited ($^{\#}P<0.05$; Fig. 3B and C). However, the PHD2-siRNA lentiviral vector transfection partly abolished the protection of TMP in $CoCl_2$-induced HUVECs apoptosis ($^{\#}P<0.05$; Fig. 3B and C).

To further investigate the apoptotic ratio of TMP-treated HUVECs, TUNEL staining was performed. It was revealed that TMP reduced the apoptosis of HUVECs following $CoCl_2$-treatment (Fig. 3D). Additionally, the ratio of TUNEL-positive HUVECs reduced by TMP was partly upregulated following transfection with the PHD2-siRNA lentiviral vector ($^{\#}P<0.05$, $^{\#\#}P<0.05$; Fig. 3E). The results suggested that TMP protected HUVECs from apoptosis, at least in part, via the regulation of the PHD2/HIF-1α signaling pathway.

TMP inhibits the activation of the caspase family in $CoCl_2$-treated HUVECs. To further investigate downstream apoptotic signaling in TMP-treated HUVECs, the activation of caspase-3, -8 and-9, hallmark apoptotic execution enzymes, were assessed by western blotting. Pre-incubation with $CoCl_2$ markedly stimulated the activation of all caspases in HUVECs. Treatment with TMP clearly inhibited the activation of all caspases. Following transfection with PHD2-siRNA lentiviral vector, the inhibition of TMP on the activation of caspase-3 and -9, however not caspase-8, was partly abolished ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{NS}P>0.05$; Fig. 4A-D). The results suggested that TMP protected HUVECs from apoptosis, at least in part, via the regulation of the caspase family.

TMP treatment modifies the expression levels of Bcl-2 and Bax, and cytochrome c release in $CoCl_2$-treated HUVECs. The Bcl-2 family proteins have either proapoptotic or antiapoptotic activities and modulate the mitochondrial apoptosis signaling pathway. The balance between antiapoptotic and proapoptotic proteins is critical in determining the susceptibility of cells to death signals. Western blotting analysis demonstrated the upregulation of the proapoptotic Bax protein and the downregulation of antiapoptotic Bcl-2 in $CoCl_2$-treated HUVECs. TMP treatment resulted in the increased protein expression of Bcl-2 and the decreased protein expression of Bax. Following transfection with the PHD2-siRNA lentiviral vector, the inhibition of TMP on the activation of Bax, however, not Bcl-2 was partly abolished ($^{\#}P<0.05$; Fig. 4E-G). The results suggested that TMP protected HUVECs from apoptosis by regulating the expression of the Bcl-2 family.

The disruption of the mitochondrial membrane function is known to result in the release of the mitochondrial enzyme, cytochrome *c* (27). As detected by western blotting, stimulation of HUVECs with $CoCl_2$ resulted in an almost 3-fold increase in the levels of cytochrome *c* compared with the control group. Treatment with TMP inhibited this $CoCl_2$-induced cytochrome *c* release. Following transfection with the PHD2-siRNA lentiviral vector, the inhibition of TMP on the release of cytochrome *c* was partly abolished ($^{\#}P<0.05$; Fig. 4E and H). These results suggested that TMP protected HUVECs from apoptosis by inhibiting the release of cytochrome *c*.

Discussion

Endothelial cells, the specialized cell type in vessels, are involved in the physiological and pathological processes of retinal neovascularization. It was reported that endothelial cells are highly sensitive to the harmful effects of hypoxia. As a traditional Chinese herb, TMP and its protective effect against apoptosis in endothelial cells has been previously investigated *in vitro* and *in vivo* (15,19,20,22,28,29). For the first time, to the best of our knowledge, the present study showed that TMP effectively ameliorated the apoptosis of $CoCl_2$-induced HUVECs, at least in part, via the regulation of the PHD2/HIF-1α signaling pathway.

TMP, the active ingredient of a traditional Chinese herb from *Ligustium wallichii* Franch, has long been widely used in the treatment of patients with cerebral and cardiac ischemic diseases in China (30). Previous studies have demonstrated that TMP protected endothelial cells against oxidative stress through scavenging the reactive oxygen species, downregulating the phosphorylation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase, and inhibiting nuclear factor-κB (15,23). Although these previous studies revealed that TMP regulated certain signaling pathways during antioxidative stress, the further upstream signaling pathways regarding the protection effect of TMP in endothelial cells remain to be elucidated. To further confirm this, it is pivotal for us to understand the most effective protective role of TMP in treating retinal neovascularization.

Considerable evidence has implicated that hypoxia, known to be associated with angiogenesis, has a key role in the development of retinal neovascularization. It has been well accepted that the cellular effects of hypoxia are mostly mediated by HIF-1α (31-33). The HIF-1α protein has been determined to coexist with hypoxia and is associated with the development of retinal neovascularization. PHD2, one of the PHD family members (PHD1, 2, and 3), regulates the stability of HIF-1α in an oxygen-dependent manner. In the present study, it was demonstrated that TMP increased the expression of PHD2 and almost completely abolished the accumulation of HIF-1α, resulting in decreased expression of VEGF in $CoCl_2$-treated HUVECs. However, the mRNA expression of HIF-1α was unaffected following treatment with TMP. It was hypothesized that TMP abolished the HIF-1α accumulation associated with its role in upregulating

the expression of PHD2, which could degrade the protein expression of HIF-1α, however, not the mRNA expression in $CoCl_2$-treated HUVECs. Furthermore, selective knockdown of PHD2 with siRNAs abolished the production of HIF-1α and VEGF reduced by TMP. These results indicated that TMP initiated the degradation of HIF-1α and the inhibition of VEGF by upregulating the expression of PHD2 in $CoCl_2$-treated HUVECs.

Previous studies also showed that hypoxia not only led to the activation of several transcription factors, but also initiated the complex apoptotic cascade in endothelial cells (34,35). HIF-1α is associated with severe hypoxia-induced apoptosis of endothelial cells (9-11). HIF-1α suppresses apoptosis by activating multiple antiapoptotic genes, including VEGF (36) and Bcl-xL (37), which leads to the protection against hypoxic injury. Notably, HIF-1α has also been shown to be a factor mediating hypoxia-induced apoptosis. Hypoxia increases apoptosis of embryonic stem cells, however, certain apoptotic effects disappear following the HIF-1α genes being knocked out (38). In the present study, following pre-incubation with $CoCl_2$, HUVECs treated with TMP showed significantly decreased cell viability and apoptosis ratio. Treatment with TMP also clearly inhibited the activation of the proapoptotic caspase family (caspase-3, -8 and-9) and proapoptotic Bcl-2 family (Bax) and the release of the cytochrome *c*. Additionally, the expression of the antiapoptotic Bcl-2 family (Bax) was increased. However, selective knockdown of PHD2 with siRNAs almost inverted cell viability, apoptosis ratio and most of the hallmark apoptotic execution enzymes, with the exception of caspase-8 and Bcl-2 following treatment with TMP.

In conclusion, the present study demonstrated that TMP had effective protection against $CoCl_2$-induced apoptosis of HUVECs, and the antiapoptotic effect of TMP on $CoCl_2$-induced HUVECs was, at least in part, via the regulation of the PHD2/HIF-1α signaling pathway.

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