

Tetramethylpyrazine protects CoCl₂-induced apoptosis in human umbilical vein endothelial cells by regulating the PHD2/HIF-1 α -VEGF pathway

CHENG YANG^{1,2*}, YUE XU^{1*}, HUANJIAO ZHOU¹, LU YANG¹, SHANSHAN YU¹,
YI GAO¹, YONGSHENG HUANG¹, LIN LU¹ and XIAOLING LIANG¹

¹State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University;

²Department of Ophthalmology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510000, P.R. China

Received January 13, 2015; Accepted November 17, 2015

DOI: 10.3892/mmr.2015.4679

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 CoCl₂-induced apoptosis. Following pre-incubation with CoCl₂ (150 μ M/ml) for 4 h, the HUVECs were treated with TMP at different concentrations (50, 100 and 200 μ M/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-1 α 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)-1 α and VEGF by TMP. In addition, TMP protected CoCl₂-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 CoCl₂-induced HUVECs was, at least in part, via the regulation of the PHD2/HIF-1 α signaling pathway.

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)-1 α 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-1 α regulates the production of VEGF (4-7). Silencing HIF-1 α 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-1 α 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-1 α

Correspondence to: Professor Xiaoling Liang or Professor Lin Lu, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 Xianlie Road, Guangzhou, Guangdong 510000, P.R. China
E-mail: liangxlsums@qq.com
E-mail: lulin888@126.com

*Contributed equally

Key words: tetramethylpyrazine, human umbilical vein endothelial cells, apoptosis, prolyl hydroxylase, hypoxia inducible factor-1 α

in an oxygen-dependent manner. In normoxia or hyperoxia, PHD2 hydroxylates the proline residues of HIF-1 α , 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 O₂ and, therefore, HIF-1 α 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-1 α 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-1 α 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-1 α signaling pathway.

The present study confirmed the antiapoptotic effect of TMP in human umbilical vein endothelial cells (HUVECs) following pre-incubation with CoCl₂ and revealed that its antiapoptotic effect was, at least in part, via the regulation of the PHD2/HIF-1 α 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. CoCl₂ 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-1 α (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% CO₂ 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 CoCl₂ (150 μ M/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 μ M/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 (1x10⁴ cells/well) were cultured in 96-well plates at 37°C for 24 h, and were subsequently divided into four groups: i) control; ii) CoCl₂ (only pre-incubated with 150 μ M/ml CoCl₂ for 4 h); iii) CoCl₂ + TMP (pre-incubated with 150 μ M/ml CoCl₂ for 4 h and subsequently treated with 200 μ M/ml TMP for 8 h); iv) CoCl₂ + TMP + siRNA (PHD2 gene silencing with siRNA, pre-incubation with 150 μ M/ml CoCl₂ for 4 h and subsequently treated with 200 μ M/ml TMP for 8 h). Following treatments, the medium was removed and 10 μ l MTT solution (5 mg/ml) was added and the plates were incubated at 37°C for 4 h in a humidified 5% CO₂ 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 μ M 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 GAPDH and the $2^{-\Delta\Delta C_q}$ 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 AAGTGAACATTTTCGGTGAC-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 EDTA Na_2 , 1 mM DTT, 1 μ g/ml leupeptine, 1 mM Benzamidine and 2 μ g/ml aprotinin. The protein concentration was determined using Bradford reagent, as previously described (26). The proteins (30 μ g) 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 \pm 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-1 α and VEGF in CoCl $_2$ -treated HUVECs. The mRNA expression levels of PHD2, HIF-1 α 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-1 α and VEGF compared with the control group ($^*P < 0.05$; Fig. 1B and C). Treatment with TMP (50, 100 and 200 μ M/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-1 α ($P > 0.05$; 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-1 α 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-1 α and VEGF ($^*P < 0.05$; Fig. 1D, F and G) compared with the control group. Treatment with TMP (50, 100 and 200 μ M/ml) significantly increased the protein expression of PHD2 ($^*P < 0.05$; Fig. 1D and E) and decreased the protein expression levels of HIF-1 α 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-1 α , resulting in the inhibition of VEGF.

PHD2-siRNA lentiviral vector inhibits the effect of TMP on the expression levels of PHD2, HIF-1 α and VEGF in CoCl $_2$ -treated HUVECs. To further assess the role of TMP in the regulation of the PHD2/HIF-1 α 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-1 α and, therefore, HIF-1 α 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-1 α protein ($^*P < 0.05$; Fig. 2F and G), and not HIF-1 α mRNA (Fig. 2D) was observed between the NC and PHD2 siRNA group.

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

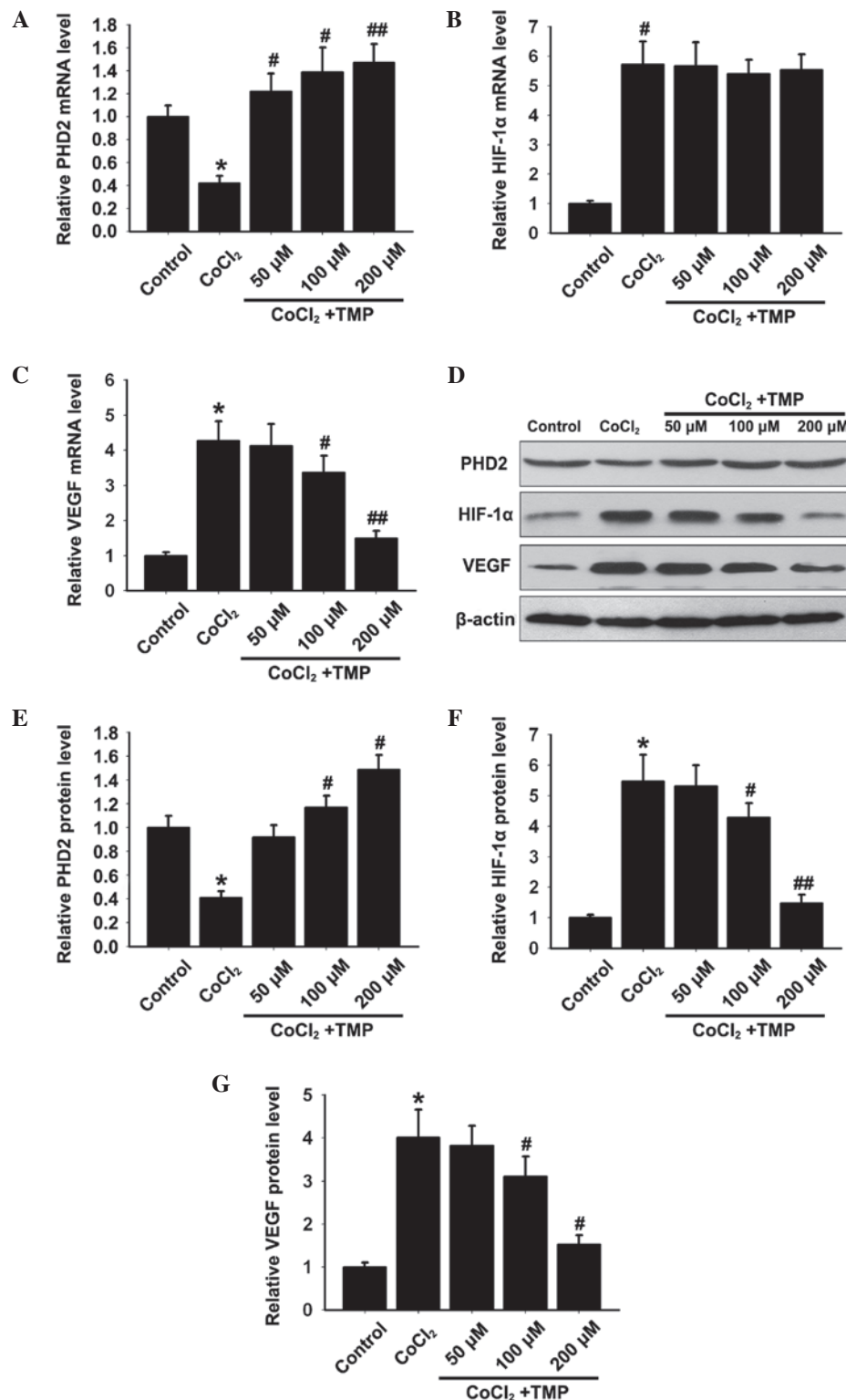


Figure 1. Effect of TMP on the expression levels of PHD2, HIF-1 α and VEGF in CoCl₂-treated HUVECs. Following pre-incubation with CoCl₂ (150 μ M/ml) for 4 h (h), the HUVECs were treated with TMP at different concentrations (50, 100 and 200 μ M/ml) for 8 h. The mRNA expression levels of (A) PHD2, (B) HIF-1 α 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-1 α 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-1 α 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 \pm standard deviation (* P <0.05, vs. control group; # P <0.05 and ## P <0.01, vs. CoCl₂ 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-1 α in the NC and TMP groups increased significantly

following CoCl₂ treatment, which remained unaffected by the PHD2-siRNA lentiviral vector transfection (P >0.05; Fig. 2D). These results further indicated that TMP initiated the

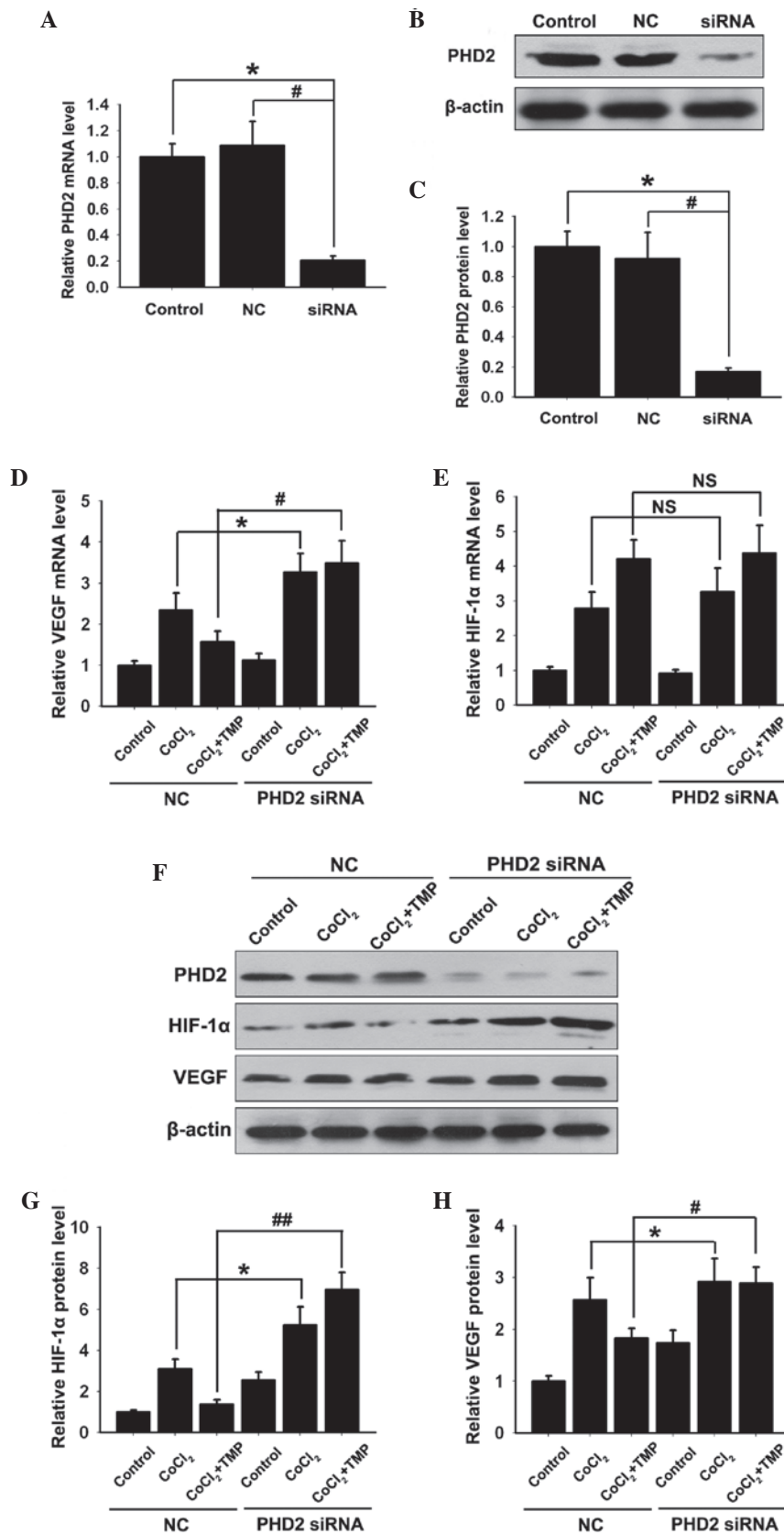


Figure 2. Effect of TMP on the expression levels of HIF-1 α and VEGF in PHD2-siRNA transfected HUVECs. The HUVECs were transfected with PHD2-siRNA lentiviral vector and were subsequently pre-incubated with CoCl_2 (150 $\mu\text{M}/\text{ml}$) for 4 h, followed by TMP (200 $\mu\text{M}/\text{ml}$) for 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-1 α in each group were measured by RT-qPCR. (F) The protein expression levels of PHD2, HIF-1 α and VEGF in each group were measured by western blotting. Quantification graphs of the intensity of (G) HIF-1 α 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 \pm standard deviation (* 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.

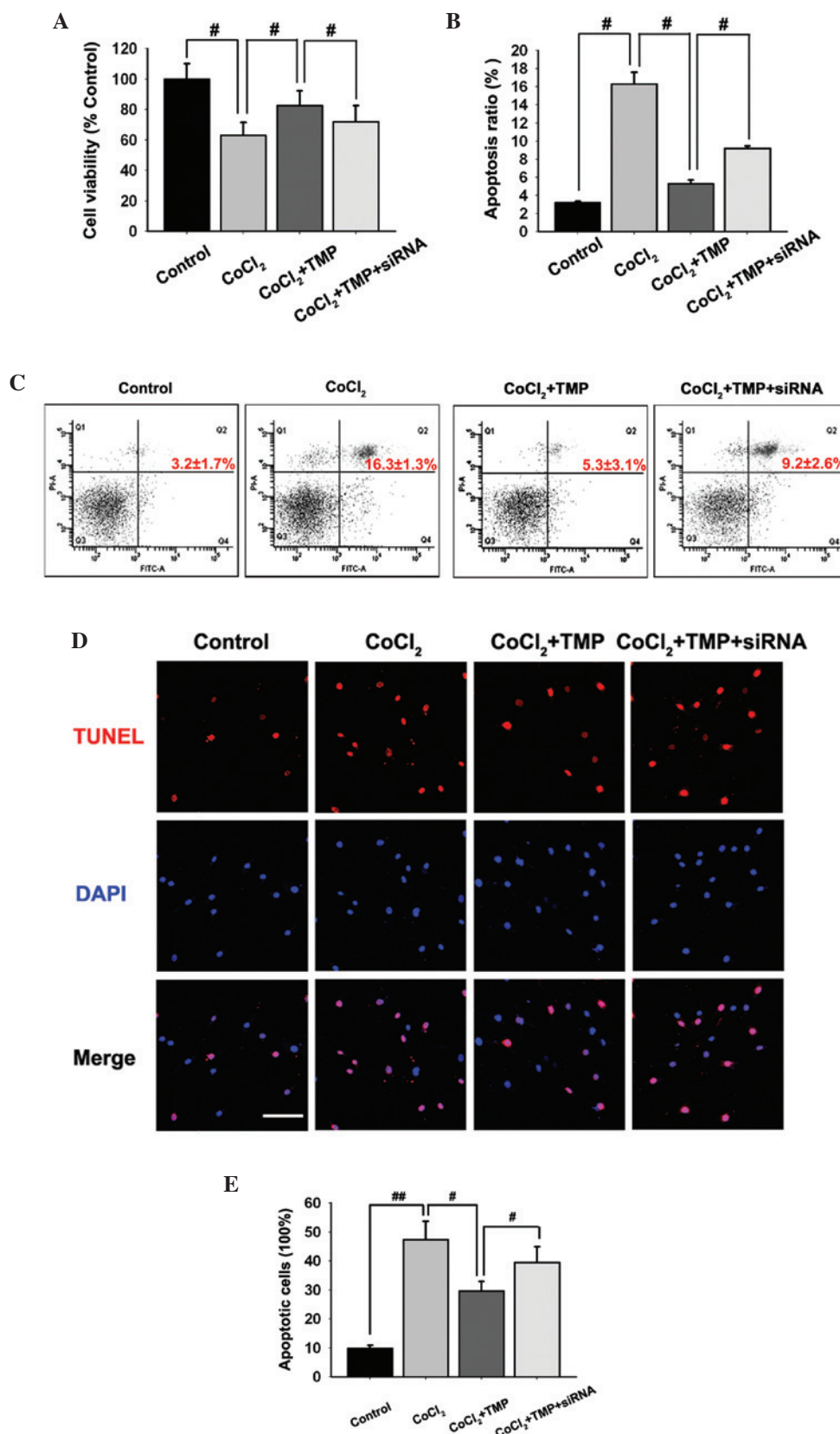


Figure 3. Effect of TMP on the apoptosis of CoCl₂-induced HUVECs. The HUVECs were transfected with PHD2-siRNA lentiviral vector and were subsequently pre-incubated with CoCl₂ (150 μ M/ml) for 4 h, followed by TMP (200 μ M/ml) for 8 h. (A) The effect of TMP on CoCl₂-induced loss of cell viability in HUVECs was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide assay. (B and C) The apoptotic cells in CoCl₂-induced HUVECs were detected by flow cytometry following staining with PI. (D and E) The apoptotic cells in CoCl₂-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 μ m) 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 \pm 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.

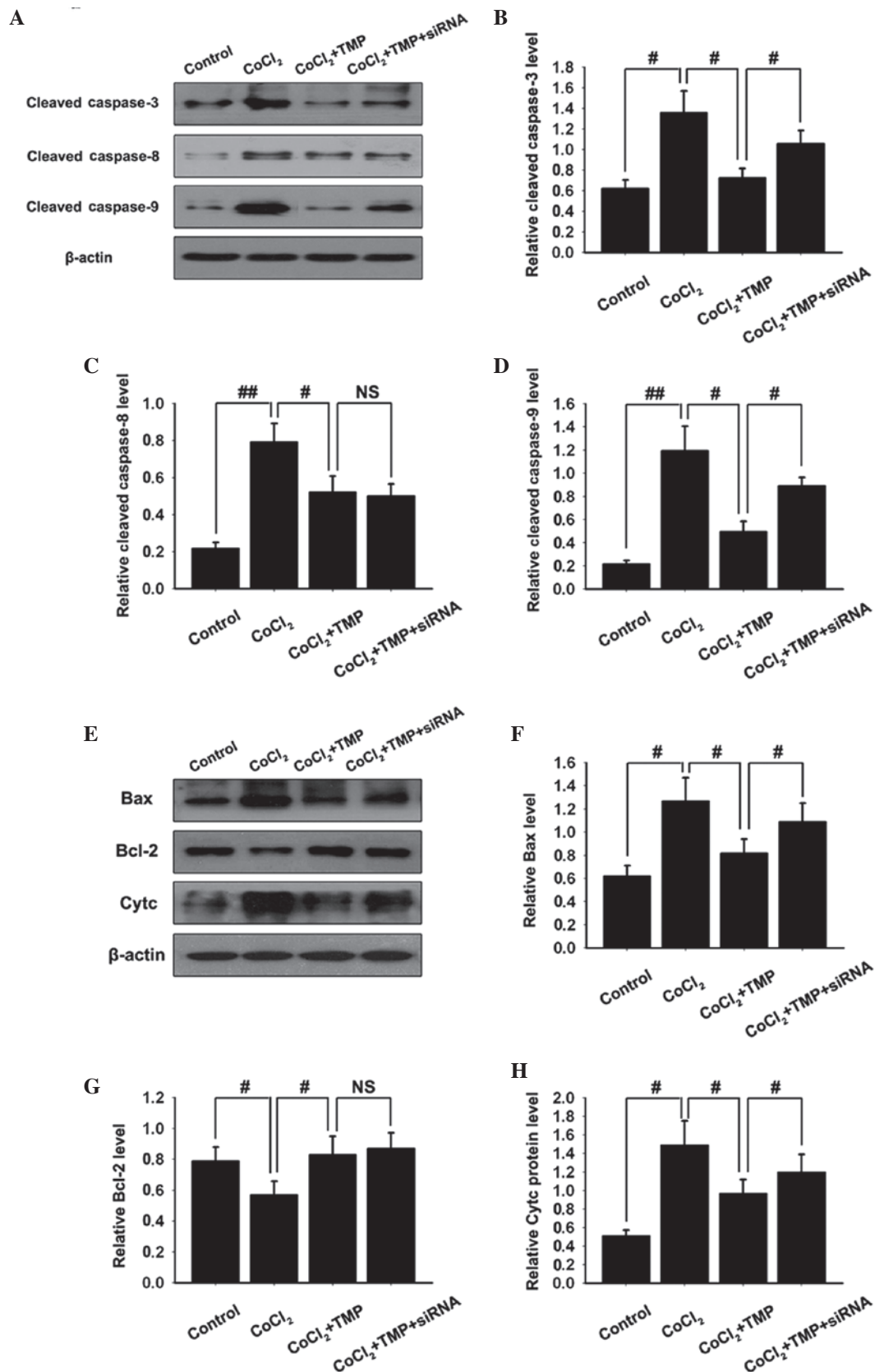


Figure 4. Effect of TMP on the expression levels of apoptotic associated proteins in CoCl₂-induced HUVECs. The HUVECs were transfected with PHD2-siRNA lentiviral vector and were subsequently pre-incubated with CoCl₂ (150 μ M/ml) for 4 h, followed by TMP (200 μ M/ml) for 8 h. (A) The protein expression levels of activated caspase family members (caspase-3, caspase-8 and caspase-9) in the CoCl₂-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 CoCl₂-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 \pm 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-1 α and the inhibition of VEGF by upregulating the expression of PHD2 in CoCl₂-treated HUVECs.

TMP protects cell viability in CoCl₂-treated HUVECs. An MTT assay was used to investigate the effect of TMP on the viability of HUVECs. Pre-incubation with CoCl₂ inhibited the cell viability of HUVECs ($^{\#}P<0.05$; Fig. 3A). Treatment with TMP (200 μ M/ml) for 8 h following pre-incubation with CoCl₂ 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 CoCl₂. 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 CoCl₂ induced apoptosis in HUVECs (Fig. 3B and C). Following treatment with TMP (200 μ M/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 CoCl₂-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 CoCl₂-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-1 α signaling pathway.

TMP inhibits the activation of the caspase family in CoCl₂-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 CoCl₂ 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 ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\text{NS}}P>0.05$; 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 CoCl₂-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 CoCl₂-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 CoCl₂ resulted in an almost 3-fold increase in the levels of cytochrome *c* compared with the control group. Treatment with TMP inhibited this CoCl₂-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 CoCl₂-induced HUVECs, at least in part, via the regulation of the PHD2/HIF-1 α 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- κ B (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-1 α (31-33). The HIF-1 α 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-1 α 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-1 α , resulting in decreased expression of VEGF in CoCl₂-treated HUVECs. However, the mRNA expression of HIF-1 α was unaffected following treatment with TMP. It was hypothesized that TMP abolished the HIF-1 α accumulation associated with its role in upregulating

the expression of PHD2, which could degrade the protein expression of HIF-1 α , however, not the mRNA expression in CoCl₂-treated HUVECs. Furthermore, selective knockdown of PHD2 with siRNAs abolished the production of HIF-1 α and VEGF reduced by TMP. These results indicated that TMP initiated the degradation of HIF-1 α and the inhibition of VEGF by upregulating the expression of PHD2 in CoCl₂-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-1 α is associated with severe hypoxia-induced apoptosis of endothelial cells (9-11). HIF-1 α suppresses apoptosis by activating multiple antiapoptotic genes, including VEGF (36) and Bcl-xL (37), which leads to the protection against hypoxic injury. Notably, HIF-1 α 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-1 α genes being knocked out (38). In the present study, following pre-incubation with CoCl₂, 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 CoCl₂-induced apoptosis of HUVECs, and the antiapoptotic effect of TMP on CoCl₂-induced HUVECs was, at least in part, via the regulation of the PHD2/HIF-1 α signaling pathway.

Acknowledgements

This work was supported by the Science and Technology Planning Project of Guangdong Province (no. 2013B021800056).

References

- Nyengaard JR, Ido Y, Kilo C and Williamson JR: Interactions between hyperglycemia and hypoxia: Implications for diabetic retinopathy. *Diabetes* 53: 2931-2938, 2004.
- Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY and Kim RY; MARINA Study Group: Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* 355: 1419-1431, 2006.
- Arjamaa O and Nikinmaa M: Oxygen-dependent diseases in the retina: Role of hypoxia-inducible factors. *Exp Eye Res* 83: 473-483, 2006.
- Forooghian F, Razavi R and Timms L: Hypoxia-inducible factor expression in human RPE cells. *Br J Ophthalmol* 91: 1406-1410, 2007.
- Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 16: 4604-4613, 1996.
- Liu LZ, Jing Y, Jiang LL, Jiang XE, Jiang Y, Rojanasakul Y and Jiang BH: Acacetin inhibits VEGF expression, tumor angiogenesis and growth through AKT/HIF-1 α pathway. *Biochem Biophys Res Commun* 413: 299-305, 2011.
- Lin M, Chen Y, Jin J, Hu Y, Zhou KK, Zhu M, Le YZ, Ge J, Johnson RS and Ma JX: Ischaemia-induced retinal neovascularisation and diabetic retinopathy in mice with conditional knockout of hypoxia-inducible factor-1 in retinal Müller cells. *Diabetologia* 54: 1554-1566, 2011.
- Forooghian F and Das B: Anti-angiogenic effects of ribonucleic acid interference targeting vascular endothelial growth factor and hypoxia-inducible factor-1 α . *Am J Ophthalmol* 144: 761-768, 2007.
- Yanyan C, Guoxian Q, Yang G and Leting W: Mechanism of hypoxia-induced factor 1 α expression in endothelial cells of the human umbilical vein and its induction of apoptosis. *Mol Biol Rep* 35: 285-290, 2008.
- Zhu X, Zhou W, Cui Y, Zhu L, Li J, Feng X, Shao B, Qi H, Zheng J, Wang H and Chen H: Pilocarpine protects cobalt chloride-induced apoptosis of RGC-5 cells: Involvement of muscarinic receptors and HIF-1 α pathway. *Cell Mol Neurobiol* 30: 427-435, 2010.
- Jin Y, An X, Ye Z, Cully B, Wu J and Li J: RGS5, a hypoxia-inducible apoptotic stimulator in endothelial cells. *J Biol Chem* 284: 23436-23443, 2009.
- Toffoli S and Michiels C: Intermittent hypoxia is a key regulator of cancer cell and endothelial cell interplay in tumours. *FEBS J* 275: 2991-3002, 2008.
- Mazure NM, Brahimi-Horn MC, Berta MA, Benizri E, Bilton RL, Dayan F, Ginouvès A, Berra E and Pouyssegur J: HIF-1: Master and commander of the hypoxic world. A pharmacological approach to its regulation by siRNAs. *Biochem Pharmacol* 68: 971-980, 2004.
- Li SY, Jia YH, Sun WG, Tang Y, An GS, Ni JH and Jia HT: Stabilization of mitochondrial function by tetramethylpyrazine protects against kainate-induced oxidative lesions in the rat hippocampus. *Free Radic Biol Med* 48: 597-608, 2010.
- Li WM, Liu HT, Li XY, Wu JY, Xu G, Teng YZ, Ding ST and Yu C: The effect of tetramethylpyrazine on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells. *Basic Clin Pharmacol Toxicol* 106: 45-52, 2010.
- Liao SL, Kao TK, Chen WY, Lin YS, Chen SY, Raung SL, Wu CW, Lu HC and Chen CJ: Tetramethylpyrazine reduces ischemic brain injury in rats. *Neurosci Lett* 372: 40-45, 2004.
- Zou Y, Jiang W and Chiou GC: Effect of tetramethylpyrazine on rat experimental choroidal neovascularization in vivo and endothelial cell cultures in vitro. *Curr Eye Res* 32: 71-75, 2007.
- Liang X, Zhou H, Ding Y, Li J, Yang C, Luo Y, Li S, Sun G, Liao X and Min W: TMP prevents retinal neovascularization and imparts neuroprotection in an oxygen-induced retinopathy model. *Invest Ophthalmol Vis Sci* 53: 2157-2169, 2012.
- Li M, Zhao MQ, Kumar Durairajan SS, Xie LX, Zhang HX, Kum WF, Goto S and Liao FL: Protective effect of tetramethylpyrazine and salvianolic acid B on apoptosis of rat cerebral microvascular endothelial cell under high shear stress. *Clin Hemorheol Microcirc* 38: 177-187, 2008.
- Yang J, Yang S and Yuan YJ: Integrated investigation of lipidome and related signaling pathways uncovers molecular mechanisms of tetramethylpyrazine and butylidenephthalide protecting endothelial cells under oxidative stress. *Mol Biosyst* 8: 1789-1797, 2012.
- Ou YL, Dong X, Liu XY, Cheng XC, Cheng YN, Yu LG and Guo XL: Mechanism of tetramethylpyrazine analogue CXC195 inhibition of hydrogen peroxide-induced apoptosis in human endothelial cells. *Biol Pharm Bull* 33: 432-438, 2010.
- Kang Y, Hu M, Zhu Y, Gao X and Wang MW: Antioxidative effect of the herbal remedy Qin Huo Yi Hao and its active component tetramethylpyrazine on high glucose-treated endothelial cells. *Life Sci* 84: 428-436, 2009.
- Chen L, Lu Y, Wu JM, Xu B, Zhang LJ, Gao M, Zheng SZ, Wang AY, Zhang CB, Zhang WW and Lei N: Ligustrazine inhibits B16F10 melanoma metastasis and suppresses angiogenesis induced by vascular endothelial growth factor. *Biochem Biophys Res Commun* 386: 374-379, 2009.
- de Martin R, Vanhove B, Cheng Q, Hofer E, Csizmadia V, Winkler H and Bach FH: Cytokine-inducible expression in endothelial cells of an I kappaB α -like gene is regulated by NF kappa B. *EMBO J* 12: 2773-2779, 1993.
- Dai H, Yu Z, Fan X, Liu N, Yan M, Chen Z, Lo EH, Hajjar KA and Wang X: Dysfunction of annexin A2 contributes to hyperglycaemia-induced loss of human endothelial cell surface fibrinolytic activity. *Thromb Haemost* 109: 1070-1078, 2013.

26. Hedenbjörk-Lager A, Björndal L, Gustafsson A, Sorsa T, Tjäderhane L, Åkerman S and Ericson D: Caries correlates strongly to salivary levels of matrix metalloproteinase-8. *Caries Res* 49: 1-8, 2015.
27. Gottlieb E, Armour SM, Harris MH and Thompson CB: Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. *Cell Death Differ* 10: 709-717, 2003.
28. Zhang Z, Wei T, Hou J, Li G, Yu S and Xin W: Iron-induced oxidative damage and apoptosis in cerebellar granule cells: Attenuation by tetramethylpyrazine and ferulic acid. *Eur J Pharmacol* 467: 41-47, 2003.
29. Dang SC, Zhang JX, Qu JG, Wang XQ and Fan X: Ligustrazine alleviates gastric mucosal injury in a rat model of acute necrotizing pancreatitis. *Hepatobiliary Pancreat Dis Int* 6: 213-218, 2007.
30. Guo SK, Chen KJ, Qian ZH, Weng WL and Qian MY: Tetramethylpyrazine in the treatment of cardiovascular and cerebrovascular diseases. *Planta Med* 47: 89, 1983.
31. Brahimi-Horn MC and Pouyssegur J: HIF at a glance. *J Cell Sci* 122: 1055-1057, 2009.
32. Semenza GL: HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* 13: 167-171, 2001.
33. Semenza GL: Hypoxia-inducible factor 1: Oxygen homeostasis and disease pathophysiology. *Trends Mol Med* 7: 345-350, 2001.
34. Lee C, Cheng W, Chang M, Su Y, Chen C and Hsieh F: Hypoxia-induced apoptosis in endothelial cells and embryonic stem cells. *Apoptosis* 10: 887-894, 2005.
35. Matsushita H, Morishita R, Nata T, Aoki M, Nakagami H, Taniyama Y, Yamamoto K, Higaki J, Yasufumi K and Ogihara T: Hypoxia-induced endothelial apoptosis through nuclear factor-kappaB (NF-kappaB)-mediated bcl-2 suppression: In vivo evidence of the importance of NF-kappaB in endothelial cell regulation. *Circ Res* 86: 974-981, 2000.
36. Wang D, Weng Q, Zhang L, He Q and Yang B: VEGF and Bcl-2 interact via MAPKs signaling pathway in the response to hypoxia in neuroblastoma. *Cell Mol Neurobiol* 29: 391-401, 2009.
37. Chen N, Chen X, Huang R, Zeng H, Gong J, Meng W, Lu Y, Zhao F, Wang L and Zhou Q: BCL-xL is a target gene regulated by hypoxia-inducible factor-1{alpha}. *J Biol Chem* 284: 10004-10012, 2009.
38. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, *et al*: Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394: 485-490, 1998.