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Tetramethylpyrazine protects against high glucose-induced vascular smooth muscle cell injury through inhibiting the phosphorylation of JNK, p38MAPK, and ERK

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Abstract

Objectives: High glucose-induced alterations in vascular smooth muscle cell behavior have not been fully characterized. We explored the protective mechanism of tetramethylpyrazine (TMP) on rat smooth muscle cell injury induced by high glucose via the mitogen-activated protein kinase (MAPK) signaling pathway.

Methods: Vascular smooth muscle cells (VSMCs) isolated from rat thoracic aortas were divided into control, high glucose (HG), and pre-hatching TMP groups. The effect of different glucose concentrations on cell viability and on the migration activity of VSMC cells was examined using MTT analysis and the wound scratch assay, respectively. Superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured using enzyme-linked immunoassays. The levels of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38MAPK, and MAPK phosphorylation were assessed by western blotting.

Results: Cell proliferation was remarkably increased by increased glucose concentrations. Compared with the HG group, the migratory ability of VSMC cells was reduced in the presence of TMP. TMP also decreased the MDA content in the supernatant, but significantly increased the SOD activity. Western blotting showed that TMP inhibited the phosphorylation of JNK, p38MAPK, and ERK.

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Conclusions: TMP appears to protect against HG-induced VSMC injury through inhibiting reactive oxygen species overproduction, and p38MAPK/JNK/ERK phosphorylation.

Keywords
Tetramethylpyrazine, vascular smooth muscle cells, high glucose, p38MAPK signaling, reactive oxygen species, traditional Chinese medicine

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Introduction

The incidence of type 2 diabetes mellitus (T2DM) is increasing gradually worldwide, and its related complications place a large economic burden on public health.[1] T2DM is closely associated with a range of cardiovascular diseases, including peripheral vascular disease, cerebrovascular disease, coronary artery disease,[2] and sclerosing mesenteritis.[3] High glucose (HG) levels have been shown to damage vascular endothelial cells and vascular smooth muscle cells (VSMCs), and this injury to VSMCs correlates with disease acceleration in T2DM patients.[4] Moreover, emerging clinical evidence suggests that VSMCs may be functionally impaired in diabetes, and that this contributes to increased peripheral vascular disease.[5,6]

High blood glucose levels facilitate the formation of reactive oxygen species (ROS), resulting in the uncoupling of mitochondrial oxidative phosphorylation and generating further ROS. Furthermore, ROS causes vascular damage, which triggers an inflammatory reaction and the subsequent release of chemoattractants, thus increasing the risk of vascular disease in patients with T2DM.[7] Therefore, an understanding of the mechanism underlying HG-induced VSMC injury and the identification of an effective drug to regulate the subsequent oxidative stress response is a key challenge.

The Chinese herb *Rhizoma Chuanxiong* has long been used in traditional Chinese herbal medicine for the prevention and treatment of ischemic neural disorders and cardiovascular diseases including ischemic stroke and pulmonary hypertension. Tetramethylpyrazine (TMP) is one of the pharmacologically active components isolated from the rhizomes of *Rhizoma Chuanxiong*.[8] Its cardioprotective effects are believed to be related to its vasodilating, anti-inflammatory, anticoagulant, free radical-scavenging, and microcirculatory properties.[9–12] However, despite the extensive use of TMP in the prevention and treatment of cardiovascular diseases, its mechanisms and protective effects on VSMCs in diabetes are not completely understood.

Therefore, in the present study, we aimed to explore whether TMP can attenuate HG-induced oxidative stress and proliferation in VSMCs, and we investigated the underlying molecular mechanisms.

Materials and methods

Drugs

TMP was purchased from Sigma-Aldrich (St Louis, MO, USA) and used to make a stock solution by dissolving in dimethyl sulfoxide at a concentration of 1.0 mM and storing at –20°C.

Vascular smooth muscle cell culture and treatments

All animals used in this study were provided and cared for by the Yantai University Animal Center, and protocols were approved by the Animal Care and Use Committee of Yantai University. VSMCs were isolated from the aorta of male Sprague–Dawley rats (6–8 weeks old, 200–250 g body weight) by enzymatic digestion as described previously.[12] The VSMC primary culture was validated by assessing for the expression of α-smooth muscle cell actin using western blotting. Cells were cultured in DMEM medium (GIBCO® Cell Culture, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France) and penicillin–streptomycin (100 IU/mL-0.1 mg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Experiments were performed using VSMCs between passage 3 and 5 at 70% confluency.

VSMCs were divided into the following groups: control group (CN), DMEM medium with 5.5 mM D-glucose; high glucose group (HG), DMEM medium with 25 mM D-glucose; and the pre-hatching TMP group (TMP 15 μM), DMEM medium with 25 mM D-glucose and 15 μM TMP.

MTT assay

VSMCs were seeded and cultured in 96-well plates at a density of 5×10^3 cells per well. After adhering, the cells were incubated in medium with different concentrations of glucose (5.5 mM, 11 mM, and 25 mM) for 24 hours. VSMCs pretreated with HG were administrated TMP for 24 hours. Cell proliferation was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[13]

Cell migration analysis

A wound scratch assay was used to measure the migration of VSMCs. Cells were plated into 6-well culture dishes at a density of 2.0×10^5 cells/well. After adhering, cells were grown to about 80% confluency, then a sterile plastic 10 μL micropipette tip was used to create a 1 mm scratch as a homogeneous wound. Cells were washed twice with phosphate-buffered saline after wounding, and were further incubated for 24 hours. The wound widths were then measured under microscope and images of the wound were then taken using the IX51 inverted microscope (Olympus Optical Co., Tokyo, Japan) at magnification ×100; the wound width was used to calculate cell migration as a wound healing percentage as follows: (0 hours wound width – 24 hours wound width)/0 hours wound width × 100%.

Determination of oxidative stress biomarkers

VSMCs seeded in 6-well plates at 3×10^5 cells per well were pretreated with or without TMP for 24 hours followed by stimulation with 25 mM glucose for 30 minutes. Cells were then collected and lysed using cell lysis buffer (pH 7.0) containing 1 mM EDTA, 0.5 mg/mL leupeptin, 10 mg/mL aprotinin, 0.7 mg/mL pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation at 10,000 ×*g* for 5 minutes, the supernatant was collected to determine malonyldialdehyde (MDA) and superoxide dismutase (SOD) levels using a competitive enzyme-linked immunoassay kit (ELISA; Cell Biolabs Inc., San Diego, CA, USA).[14]

Western blotting

Total protein was extracted using an extraction kit (Beyotime, China) according to the manufacturer's protocols, and the protein concentration was determined by a BCA

protein assay kit (Roche, Basel, Switzerland). Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (40 μg per lane), then transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat dried milk in Tris-buffered saline with Tween-20 for 1 hour, the membranes were incubated with the appropriate primary antibody. Antibodies against β-actin (1:1000), c-Jun N-terminal kinase (JNK; 1:1000), p38 mitogen-activated protein kinase (MAPK; 1:1000), extracellular signal-regulated kinase (ERK; 1:1000), phospho-JNK (1:500), phospho-p38MAPK (1:500), and phospho-ERK (1:500) were purchased from Cell Signaling Technology® (Danvers, MA, USA). They were then incubated with horseradish peroxidase-conjugated secondary antibody (Bioss, Beijing, China) and visualized using the ECL plus detection kit (Pierce Protein Research Products, Rockford, IL, USA). Protein expression was quantified by densitometry using a ChemiDoc XRSþ image analyzer (Bio-Rad, Hercules, CA, USA) with β-actin as an internal loading control.[15]

Statistical analysis

All data were described as means ± standard deviation (SD). Statistical analysis was performed with SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The data were analyzed by one-way analysis of variance, and differences between the two groups were analyzed using the Least Significant Difference method. Values of $P<0.05$ were considered statistically significant.

Results

High glucose promoted VSMC proliferation

Compared with the normal glucose control group with a relatively low concentration of glucose (CN, 5.5 mM), the effect of middle glucose (MG, 11 mM) on cell proliferation was not significant. However, HG (25 mM) significantly promoted cell proliferation compared with the control group ($p<0.05$). Cell viability was increased by 8% and 39% in cells treated with 11 mM and 25 mM D-glucose, respectively. These findings indicate that HG treatment promotes the proliferation of VSMCs in a dose-dependent manner (Figure 1).

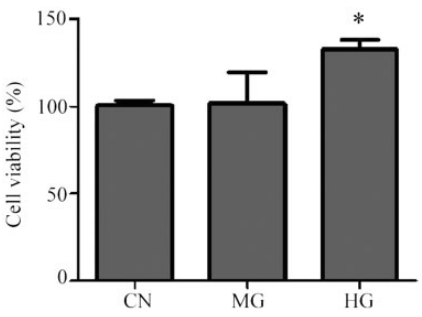

Figure 1. Effects of HG on viability in VSMCs. Cells were treated with D-glucose for 24 hours. Cell viability was detected using the MTT assay. CN: treated with DMEM and 5.5 mM D-glucose; MG: middle glucose, 11 mM D-glucose; HG: high glucose, 25 mM D-glucose.
Values are expressed as means ± SD from triplicate experiments. *$p < 0.05$ vs. CN group.

TMP diminished VSMC proliferation after HG stimulation

VSMCs were pretreated with 15 μM TMP followed by incubation with DMEM HG medium containing 25 mM D-glucose for 24 hours. Compared with the CN group, proliferation of the HG group was significantly inhibited ($p<0.01$). Moreover, compared with the HG group, TMP significantly attenuated the glucose-induced cell proliferation ($p<0.05$) (Figure 2).

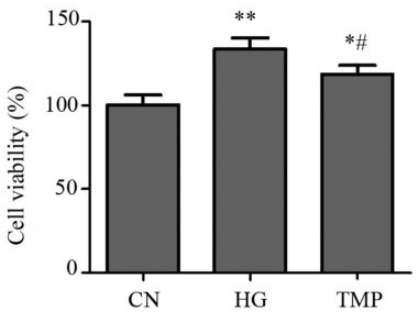

Figure 2. Effects of TMP on viability in VSMCs stimulated with HG were detected by the MTT assay. VSMCs cells were pretreated with 15 μM TMP, then incubated with DMEM containing 25 mM D-glucose for 24 hours.
CN: control group, HG: high glucose group, TMP: pre-hatching tetramethylpyrazine high glucose group.
Values are expressed as means ±SD of triplicate experiments. $^{*}p < 0.05$ vs. CN group; $^{**}p < 0.01$ vs. CN group; $^{\#}p < 0.05$ vs. HG group.

Effect of TMP on the migratory ability of VSMCs

HG significantly stimulated VSMC migration compared with the control group ($p<0.05$). TMP treatment abolished the increased migration ability of VSMCs to some extent, with markedly reduced cell migration distances observed in the TMP treatment group compared with the HG group (Figure 3).

TMP influenced the levels of MDA and SOD in VSMCs after HG stimulation

The content of MDA in the supernatant of VSMCs after HG stimulation was significantly increased compared with the control group, while the activity of SOD was significantly decreased ($p<0.05$). After treatment with TMP, the activity of MDA was markedly decreased, and the activity of SOD was significantly increased ($p<0.05$) (Table 1). These results indicated that TMP significantly inhibited the oxidative stress induced by HG in VSMCs.

TMP inhibited the phosphorylation of ERK, JNK, and p38MAPK in VSMCs after HG stimulation

The levels of p-ERK, p-JNK, and p-p38MAPK in VSMCs were significantly increased after HG treatment ($p<0.05$ vs control group). However, TMP significantly suppressed the phosphorylation of p-JNK, p-p38MAPK, and p-ERK compared with the HG group ($p<0.05$) (Figure 4). These results suggested that the protective effect mechanism of TMP on VSMC injury might be associated with MAPK signaling pathway inhibition.

Discussion

In this study, we showed that TMP could attenuate cell proliferation, inhibit cell migration, and weaken oxidative stress in VSMCs triggered by a HG stimulus. The major findings of this study were that TMP significantly decreased MDA levels, and increased SOD activity in VSMCs after HG stimulation. Additionally, the levels of p-ERK, p-JNK, and p-p38MAPK were increased in VSMCs after HG treatment ($p<0.05$ vs control group), but this was inhibited by TMP.

Approximately 422 million people worldwide have diabetes mellitus, with the majority diagnosed with T2DM.[16] Deaths caused by diabetes-associated complications account for approximately 6% of worldwide mortality.[17] The vascular diseases coronary artery disease, and cerebrovascular and peripheral vascular diseases are the pivotal causes of mortality and morbidity in T2DM patients. Because the proliferation and migration of VSMCs are some of the most critical events to occur during progressive intima thickening and arterial wall sclerosis development, VSMC injuries play an important role in the pathogenesis of vascular lesions.[18] The disorganized production of advanced glycation end

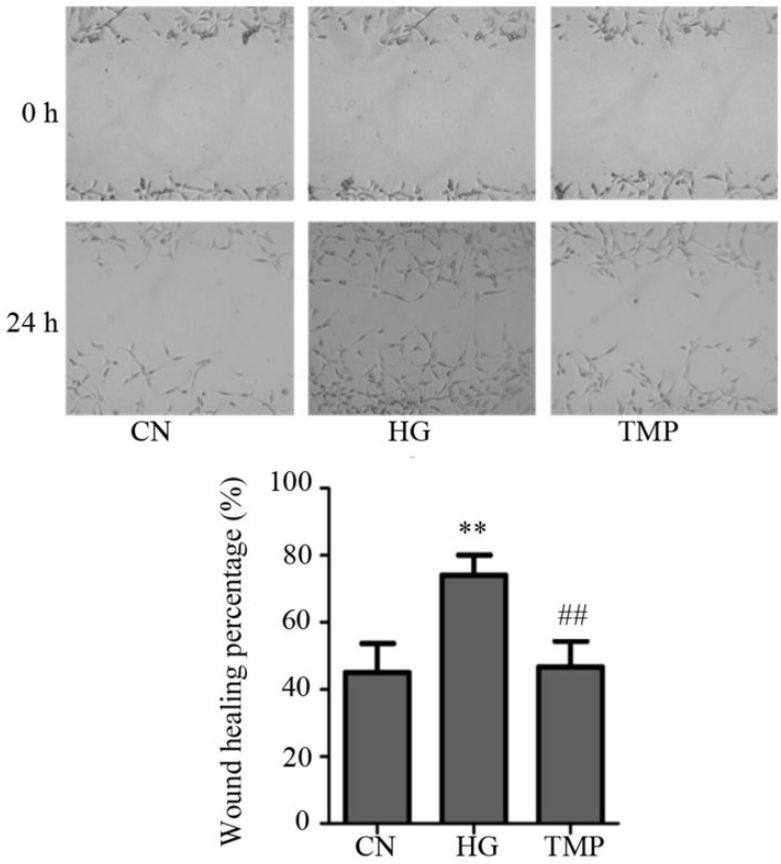

Figure 3. Effects of TMP on VSMC migration induced by HG were analyzed by the wound healing assay. Wound widths were measured under a microscope using an ocular grid (magnification, ×100). Bars indicate means ±SD (n= 3).
CN: control group, HG: high glucose group, TMP: pre-hatching tetramethylpyrazine high glucose group.
$^{**}p < 0.01$ vs. CN group. $^{\#\#}p < 0.01$ vs. HG group.

Table 1. Effects of TMP on the antioxidant activity of VSMCs

Group	MDA (nmol/L)	SOD (U/mL)
CN	0.701±0.096	27.4±0.222
HG	1.215±0.138*	22.6±0.501*
TMP	0.837±0.112$^{\#}$	26.0±0.427$^{\#}$

Values are expressed as means ± S.D. of triplicate experiments. $^{*}p < 0.05$ vs. CN. $^{**}p < 0.01$ vs. CN. $^{\#}p < 0.05$ vs. HG.
CN: control group, HG: high glucose group, TMP: pre-hatching tetramethylpyrazine high glucose group.

products, elevated production of ROS, and the abnormal stimulation of hemodynamic regulatory systems can cause the aberrant activation of multiple signaling cascades affecting VSMCs.[19] Therefore, the prevention of VSMC injuries is an effective therapy to improve diabetes-associated vascular dysfunction. However, no suitable drugs are currently available. The quality of life may be improved for patients with diabetes, and macrovascular and microvascular diseases by prescribing individualized exercise

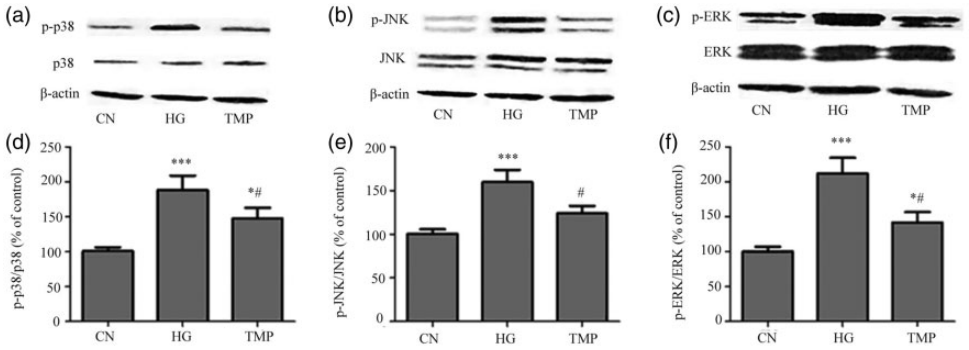

Figure 4. Detection of MAPK-related proteins in VSMCs after TMP treatment. (a–c). Expression of p-p38/p38, p-JNK/JNK, and p-ERK/ERK detected by western blotting; (d, e, f). Semi-quantitative analysis of protein expression by densitometric analyses of p-p38/p38, p-JNK/JNK, and p-ERK/ERK bands. Bars indicate means ± SD. of triplicate experiments.
CN: control group, HG: high glucose group, TMP: pre-hatching tetramethylpyrazine high glucose group.
$^{*}p < 0.05$ vs. CN group; $^{***}p < 0.001$ vs. CN group; $^{\#}p < 0.05$ vs. HG.

programs, while exercise training may also benefit diabetes patients by decreasing hyperglycemia, improving insulin resistance, and adjusting dyslipidemia and hypertension. These could all lower the vascular disease risk profile.[20]

Many reports have shown that TMP acts a protective mechanism on multiple organs through inhibiting platelet aggregation and blood vessel dilation, improving microcirculation, increasing coronary and cerebral blood flow, and vascular recanalization, and scavenging ROS.[21] The antioxidant activity of TMP receives continued public interest, and TMP has shown potential in treating stroke both *in vivo* and *in vitro*; however, there is still a need for further preclinical resarch.[22] In the present study, HG increased MDA levels while significantly decreasing SOD activity, suggesting that the level of oxidative stress in rats receiving HG is much higher than under control conditions. The antioxidant activity of TMP was verified by its ability to decrease the enhanced MDA levels and increase reduced SOD activity after HG stimulation. These results are in agreement with previous reports in other disease models.[23,24] However, the exact mechanisms associated with TMP antioxidant activity have not been fully elucidated.

VSMC growth and migration are correlated with the activation of ERK1/2, p38MAPK, and JNK, as well as the focal adhesion kinase family, proline-rich tyrosine kinase 2, protein tyrosine kinase, and c-Src.[25–27] HG stimulation caused the up-regulation of p-ERK, p-JNK, and p-p38MAPK levels in VSMCs in the present study, which likely reflects increased intracellular ROS and the subsequent activation of downstream kinases.[28–30] Other reports found that TMP could accelerate the proliferation and migration of cerebral endothelial cells, and that its actions were strengthened when combined with soluble Fas ligand. TMP was also shown to reverse oxygen–glucose deprivation-induced Cx32 expression and apoptosis in cultured hippocampal neurons by regulating the ERK1/2 and p38MAPK pathways.[31] Taken together, our findings showed that inhibition of the p38MAPK pathway by TMP was involved in blocking HG-induced VSMC injury through

attenuating cell proliferation, inhibiting cell migration, and reducing oxidative damage.

Conclusions

In summary, our present study indicated that TMP inhibits HG-induced oxidative injury and VSMC proliferation by inhibiting ROS overproduction and p38MAPK/JNK/ERK phosphorylation. Our findings therefore provide new insights into the protective properties of TMP on VSMCs, which may constitute a novel mechanism for the clinical application of TMP in the treatment of diabetic vascular complications.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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