Tetramethylpyrazine inhibits angiotensin II-induced nuclear factor-κB activation and bone morphogenetic protein-2 downregulation in rat vascular smooth muscle cells

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Abstract: Tetramethylpyrazine (TMP), an effective component of traditional Chinese medicine Chuanxiong, is commonly used to resolve embolism. Its possible therapeutic effect against atherosclerosis has received considerable attention recently. Angiotensin II (Ang II) is highly implicated in the proliferation of vascular smooth muscle cells (VSMCs), resulting in atherosclerosis. The mechanisms of TMP in the proliferation of VSMCs induced by Ang II remain to be defined. The present study was aimed to study the effect of TMP on Ang II-induced VSMC proliferation through detection of nuclear factor-κB (NF-κB) activity and bone morphogenetic protein-2 (BMP-2) expression. Primary cultured rat aortic smooth muscle cells were divided into the control group, Ang II group, Ang II + TMP group and TMP group. Cells in each group were harvested at different time points (15, 30 and 60 min for detection of NF-κB activity; 6, 12 and 24 h for measurement of BMP-2 expression). NF-κB activation was identified as nuclear staining by immunohistochemistry. BMP-2 expression was observed through Western blot, immunohistochemistry and in situ hybridization. The results showed that: (1) Ang II stimulated the activation of NF-κB. Translocation of NF-κB p65 subunit from cytoplasm to nucleus appeared as early as 15 min, peaked at 30 min (P<0.01) and declined after 1 h. (2) TMP inhibited Ang II-induced NF-κB activation (P<0.01). (3) Ang II increased BMP-2 expression at 6 h but declined it significantly at 12 and 24 h (P<0.01). (4) BMP-2 expression was also kept at high level at 6 h in Ang II + TMP group but maintained at the normal level at 12 and 24 h. (5) There was no significant difference in NF-κB activation and BMP-2 expression between the control group and TMP group. These results indicate that TMP inhibits Ang II-induced VSMC proliferation through repression of NF-κB activation and BMP-2 reduction, and BMP-2 expression is independent of the NF-κB pathway. In conclusion, TMP has therapeutic potential for the treatment of atherosclerosis.

Key words: bone morphogenetic protein-2; nuclear factor-κB; angiotensin II; atherosclerosis; tetramethylpyrazine

川芎嗪抑制血管紧张素II诱导的平滑肌细胞NF-κB激活和骨形成蛋白-2表达降低

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摘要：本文旨在观察血管紧张素II（angiotensin II, Ang II）对血管平滑肌细胞核转录因子-κB（nuclear factor-κB, NF-κB）的活性及骨形成蛋白-2（bone morphogenetic protein-2, BMP-2）表达的影响，以探讨Ang II参与动脉粥样硬化化的机制，并探讨川芎嗪是否能抑制Ang II的促血管粥样硬化作用。采用Western blot、免疫组化和原位杂交等方法分别检测Ang II刺激和川芎嗪干预后NF-κB活性、BMP-2蛋白和mRNA表达的变化。结果显示：（1）Ang II刺激激活 NF-κB。Ang II刺激15 min后 NF-κB p65 核移入，30 min达高峰（P<0.01），1 h后减退。川芎嗪抑制 Ang II诱导的 NF-κB 激活，与Ang II组比较，川芎嗪+ Ang II组 NF-κB活性显著降低（P<0.01）。（2）Ang II刺激6 h后 BMP-2表达增强（P<0.05），12 h后减弱（P<0.01），24 h时更弱（P<0.01）。川芎嗪+ Ang II组中，川芎嗪干预6 h后 BMP-2表达亦增强，12与24 h时保持正常水平。（3）川芎嗪对正常细胞的 NF-κB 活
性和BMP-2表达无影响。以上结果表明，Ang II刺激后激活NF-κB并最终使生长抑制因子BMP-2表达下降，这可能是其参与动脉粥样硬化发生机制之一。BMP-2一过性增高可能不依赖NF-κB通路的激活。川芎嗪可抑制Ang II诱导的NF-κB激活与BMP-2表达降低，提示它在动脉粥样硬化形成中起重要作用。

关键词：骨形成蛋白-2；核转录因子-κB；血管紧张素II；动脉粥样硬化；川芎嗪

In the development of atherosclerosis (AS), vascular smooth muscle cells (VSMCs) initially migrate into intima after intimal injury, then transform from contractile phenotype to synthetic and proliferative phenotype. At this time they can scavenge lipid, deposit collagen, and produce inflammatory cytokines, which attract monocytes. Finally, the abnormal VSMCs lead to form the lipid core and fibrous cap. Inhibition of the proliferation of VSMCs is thus important for the therapy of AS. A number of growth factors and growth inhibitors have been detected within vascular proliferative lesions. Among them, platelet-derived growth factor, basic fibroblast growth factor and insulin-like growth factor-I are strongly mitogenic for VSMCs, and may play a critical role in the in vivo proliferation of VSMCs[1]. On the other hand, natriuretic peptides, nitric oxide (NO) and interferon-γ have growth-suppressive actions on VSMCs[2,3]. Bone morphogenetic protein-2 (BMP-2), which belongs to transforming growth factor β (TGF-β) superfamily, is a newly found growth inhibitor. In addition to bone induction, BMP-2 has been observed to be involved in the development of AS recently[4]. Further research demonstrated that BMP-2 had a potent inhibitory action on the growth of VSMCs from rat and human[5,6].

Angiotensin II (Ang II) is the main effector peptide of rennin-angiotensin system, not only as a regulator in blood pressure and water-electrolyte balance, but also as a cytokine involved in cardiovascular diseases[7]. It takes part in AS development via inducing inflammation, producing reactive oxygen species, etc[8-10]. Ang II can also stimulate abnormal proliferation of VSMCs and extracellular matrix synthesis possibly through inducing cellular growth factor production and nuclear factor-κB (NF-κB) activation. But the effect of Ang II on growth inhibitors such as BMP-2 and the related cell signaling pathway is not yet clear.

Tetramethylpyrazine (TMP) is one of the effective ingredients of Chuanxiong, a traditional Chinese medicine. It is mainly used in clinic to treat embolism diseases. The effect is correlated not only to its action of anti-congestion but also to inhibition of VSMC growth. It has been demonstrated by many in vivo and in vitro studies. The inhibition effect on VSMC proliferation indicates its role in anti-AS therapy[11]. But the mechanism is not yet known.

For the reasons mentioned above, the effect of TMP on Ang II-induced proliferation of VSMCs was observed by detection of NF-κB activation and BMP-2 expression in the present study.

1 MATERIALS AND METHODS

1.1 Materials
Sprague-Dawley (SD) rats were provided by Tongji Experimental Animal Center. SMC growth medium was consisted of M199 and 10% fetal calf serum (Gibco Technologies Inc., America). Ang II (Sigma Technologies Inc., America) was dissolved in 1×PBS and stored at -20 ºC until use. TMP was purchased from the Fourth Medicine Production Factory in Beijing. The rabbit anti-human BMP-2 NF-κB, α and β-actin polyclonal antibodies were from Beijing Zhongshan Biological Company. BMP-2 hybridization probe was from Wuhan Borston Company. Streptavidin peroxidase (SP) kits were purchased from Beijing Zhongshan Biological Company.

1.2 Primary culture of rat VSMCs
Rat aortic VSMCs were prepared from 4-6 week-old SD rat by the explanted method[12], and were cultured in M199 supplemented with 10% FBS, 0.03% glutamine at 37 ºC in 95% air/5% CO₂ atmosphere. The cells from passages 2-4 were used in experiments. Purity of isolation was identified by the typical “hill and valley” morphology revealed by phase-contrast microscopy and uniform phalloidin staining for smooth muscle α-actin.

1.3 Experimental protocols
VSMCs were seeded onto 1 cm × 2 cm glass plates at a density of 1×10⁴ cells/cm² and maintained in M199 containing 10% FBS for 24 h for attachment. Then VSMCs grown under standard conditions were serum-starved for 24 h to achieve synchronous growth arrest. Cells were divided into 4 groups: control group (serum-starved for 24 h in the presence of solvent control), Ang II group (treated by 1×10⁻⁶ mol/L Ang II[13]), Ang II + TMP group (adding 1×10⁻⁶ mol/ L Ang II and 200 µg/mL TMP to cells at the same time) and TMP group (treated by 200 µg/mL TMP).
Cells were harvested at different time points (15, 30, 60 min for NF-κB activity; 6, 12, 24 h for detection of BMP-2 expression). The optimal dose of TMP (200 µg/mL) was selected by previous study, at which VSMC proliferation was significantly inhibited[14,15].

1.4 **Immunohistochemical staining**

Harvested cells were fixed in ice acetone for 15 min, and stored at -20 ºC until use. Fixed cells were incubated with 10% non-immune rabbit serum for 20 min and then treated with one of the primary polyclonal antibodies overnight at 4 ºC and incubated with the secondary biotinylated goat anti-rabbit antibody for 30 min after washing in PBS (3×5 min). After further washing in PBS, SP conjugate was applied for 30 min. Then cells were washed again with PBS and counter-stained by 3,3’-diaminobenzidine (DAB) for 5-10 min. In negative controls of immunohistochemical staining, the same method was used except omitting incubation with the primary antibody.

1.5 **In situ hybridization**

Harvested cells were fixed in 4% paraformaldehyde (prepared in DEPC-treated water) for 30 min, and stored at -20 ºC. Sense and anti-sense digoxigenin-labeled cDNA probes for BMP-2 were transcribed from the T7 promoter of pGEM3z. After hybridization, results were visualized with DAB under microscope.

1.6 **BMP-2 protein expression by Western blot**

The cells in dishes were washed three times in PBS, lysed in 250 µL lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium vandate, 0.1% SDS, 0.5% deoxycholic acid, 100 µg/mL PMSF, 0.2 µg/mL leupeptin, 1% NP-40). Samples were separated on 12% SDS-PAGE gel, and transferred onto PVDF filters by a wet transferring system[16]. The membrane was blocked with Blotto-Tween (5% nonfat milk and 0.05% Tween 20 in PBS) and incubated with the primary antibodies against BMP-2 and β-actin for 2 h at room temperature. After that, horseradish peroxidase-conjugated secondary IgG was added for 1 h at room temperature. The blots were developed according to the enhanced chemiluminescence protocol (Zhongshan Biotechnology, Beijing).

1.7 **Calculation and statistics**

Each experiment was repeated at least three times. BMP-2-positive cells were shown as brown granular substances in cytoplasm either by immunohistochemistry or in situ hybridization. Mean absorbance values of NF-κB p65 subunit in nuclei and BMP-2 in cytoplasm were evaluated by using HPIAS1000 pathological image analysis system. Cells in four randomly selected high-power fields were calculated by the image software. All data were expressed as means±SEM. For comparison, SPSS 11.0 analysis system was used. Data were submitted to one-way ANOVA followed by Dunnet’s post hoc test. Differences were considered statistically significant at P<0.05.

2 RESULTS

2.1 **Identification of VSMCs**

VSMCs migrated out from tissue pieces of tunica after 3 to 4 d of culture (Fig.1A), and then became confluent in 7 d. By immunocytochemical examination on the 10th day in vitro, almost all the cells were smooth muscle α-actin-positive (Fig.1B), identified as VSMCs. The α-actin-positive cells (purity>96%) were used for experiments.

2.2 **Detection of NF-κB activity by immunohistochemistry**

NF-κB p65 subunit was stained as brown granular in the cytoplasm of the control cells. When NF-κB was activated, the brown substances appeared in the nuclei. Ang II increased NF-κB nuclear staining as early as 15 min, peaked at 30 min (P<0.01) and declined after 60 min. In Ang II + TMP group, TMP significantly blocked Ang II-induced NF-κB nuclear translocation at all three time points (P<0.01) (Table 1 and Fig.2). TMP alone had no influence on the activation of NF-κB.

2.3 **Detection of BMP-2 expression by Western blot, immunohistochemistry and in situ hybridization**

Western blot analysis (Fig.3) showed that the normal VSMCs expressed BMP-2 abundantly. Treatment of quie-
Fig. 1. Identification of vascular smooth muscle cells. A: Vascular smooth muscle cells migrated out from tissue pieces 4 d after primary culture. Scale bar, 50 µm. B: Immunocytochemical staining of α-actin-positive smooth muscle cells. Scale bar, 50 µm.

Fig. 2. Effects of Ang II on NF-κB activation in vascular smooth muscle cells (immunohistochemical staining). The arrows indicated nuclear NF-κB staining. As early as 15 min after Ang II stimulation, nuclear NF-κB staining was observed, peaked at 30 min and cytoplasmic staining reappeared 60 min after Ang II stimulation. Scale bar, 50 µm.

Fig. 3. Detection of BMP-2 expression at different time points by Western blot. A: Western blot of BMP-2 expression. B: Integrated optical density of Western blot. *P<0.05 vs control, ▲ P<0.05 vs Ang II, ＃ P<0.01 vs 6 h, ￥P<0.01 vs 12 h.

Fig. 4. Mean optical density of immunohistochemical staining of BMP-2 expression. *P<0.05, **P<0.01 vs control; ￥P<0.01 vs 6 h; ￥P<0.01 vs 12 h, ▲P<0.05 vs Ang II.

Fig. 5. mRNA expression changes of BMP-2 at different time points by in situ hybridization. A: The results of in situ hybridization visualized by DAB. Scale bar, 50 µm. B: Mean optical density of BMP-2. *P<0.01 vs control, ￥P<0.01 vs 6 h, ￥P<0.01 vs 12 h, ▲P<0.05 vs Ang II.
scent VSMCs with Ang II increased BMP-2 expression at 6 h but decreased it later. In Ang II + TMP group, TMP did not prevent the augment of BMP-2 expression at 6 h but inhibited its reduction induced by Ang II at 12 and 24 h. As a complementary support to the Western blot results, immunochemical analysis also exhibited the similar changes (Fig.4). The results of in situ hybridization indicated that the changes of mRNA expression were consistent with changes of protein expression (Fig.5). There was no significant difference in protein or mRNA level of BMP-2 between the control and TMP groups.

3 DISCUSSION

Although Ang II is considered as an important risk factor of AS, the exact acting mechanism remains poorly understood. The abnormal proliferation of VSMCs is an important character of AS. In the present study it is suggested that Ang II might participate in the pathogenesis of AS through breaking the balance between growth factor and growth inhibitor.

Firstly, Ang II was observed inducing NF-κB activation. The activated NF-κB in the process of AS is a prominent growth factor inducer. It also participates in cardiovascular pathophysiology through regulation of several genes, including cytokines, adhesion proteins, NO synthase and angiotensinogen, as well as other products involved in AS, inflammation and immune response[17]. Ang II-induced increase of NF-κB binding activity in cultured VSMCs was also reported by Ruiz-Ortega et al.[19] and Hernandez-Presa et al.[19], respectively.

Secondly, Ang II distinctly decreased the expression of BMP-2, a growth inhibitor. BMP-2 is a major member of morphogenetic protein, which was initially isolated from bone matrix and can induce ectopic bone formation such as skeletal muscle. Scientists show great interest in BMPs using gene knockout technology. Further study showed that BMP-2 was involved in the pathogenesis of arterial calcification and AS[20]. It has been demonstrated that BMP-2 inhibits VSMC proliferation not only under basal conditions but also when VSMCs are stimulated by serum, TGF-β or LDL[8,20]. Vascular bone formation is absent in the intact arteries, whereas it occurs in certain areas of atherosclerotic lesions with upregulation of BMP-2. Thus changes in BMP-2 expression possibly play a critical role in plaque stability or lesion regression.

The regulating mechanism of BMP-2 is not yet clear. It has been reported that NF-κB positively regulates the expression of BMP-2[21,22]. Our results indicated that BMP-2 could be regulated independent of NF-κB activation. Ang II + TMP also increased BMP-2 expression at 6 h without NF-κB activation. Additionally, TMP alone had no effect on BMP-2 expression. These results suggest that some unknown cell signaling pathways which possibly protect the body from Ang II-induced harmful stimulation are activated at the same time and up-regulate BMP-2 expression. The long-term effect of Ang II on vascular smooth muscle is achieved through decreasing the expression of BMP-2 coinciding with its role in VSMC proliferation.

The present study also for the first time provided evidence that TMP might inhibit Ang II-induced proliferation in rat VSMCs through suppression of NF-κB activation and BMP-2 reduction. The growth inhibitory effect of TMP on Ang II-stimulated VSMCs is in consistent with the results obtained in the previous studies[23]. Moreover, our research showed that TMP could protect VSMCs from injury by Ang II.

Taken together, we find that Ang II has dual effects on the expression of BMP-2 in VSMCs. Ang II can induce BMP-2 expression except for inflammatory cytokines shortly after stimulation, suggesting there is an balanced regulatory system when the body initially responds to injury. With continuous stimulation of Ang II, the balance is destroyed and diseases happen. The perfect molecular mechanism, which needs to be studied further, would give a better understanding on the mechanism of Ang II-induced AS. TMP can strongly inhibit Ang II-stimulated NF-κB activation and downregulation of BMP-2 expression. The results obtained can explain the inhibitory effect of TMP on VSMC proliferation and suggest therapeutic potential for the therapy of AS.

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