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Effect of Tetramethylpyrazine on Blood Pressure in Hypertensive Rats and Calcium Mobilization in Vascular Smooth Muscle Cells

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Background: Tetramethylpyrazine (TMP) is a plant alkaloid isolated from the traditional Chinese herb Chuang Xiong (川芎). It has been proved to have antihypertensive effect on anestheized animals. This study was undertaken to evaluate its effect on ambulatory hypertensive rats and its mechanism of antihypertension.

Materials and Methods: Deoxycorticosterone acetate (DOCA)- induced hypertensive rats comprising 4 groups (each n = 8) were used for study. TMP dissolved in normal saline was given intraperitoneally at different dosages (1,5,10 mg/kg) and blood pressure was recorded by a noninvasive tail-cuff monitor. Aortic smooth muscle cells (A7r5) were employed to evaluate calcium influx inhibition by using Fura-2 calcium-sensitive dye.

Results: The hypotensive effect of TMP on DOCA-salt sensitive hypertensive rats was dose-dependent; the maximal blood pressure decrease was 28.2 ± 5.0 mm Hg at the dosage of 10 mg/kg. By using calcium influx stimulating agents phenylephrine (an α adrenergic agonist) and arginine vasopressin to induce calcium influx, TMP showed a dose-dependent calcium influx inhibition.

Conclusion: TMP isolated from traditional herb has an effective antihypertensive action and this antihypertension is probably due to intracellular calcium influx inhibition.

Key Words: Tetramethylpyrazine; DOCA salt- sensitive hypertensive rats; Calcium influx.

Introduction

Chuang Xiong (川芎), is the dried rhizome of *Ligusticum chuanxiong Hort.* (Umbelliferae). The rhizome of *L. chuanxiong* contains phthalides, alkaloids and acids. The phthalides include ligustilide, chuanxiongol, butylphthalide, butylidene phthalide, senkyunolide, neocnidilide, 3-butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide, ligustilidiol, and a dimeric phthlide named diligustilide.[1-6] Alkaloids and other nitrogen-containing substances obtained from the rhizome are tetramethylpyrazine (TMP) or ligustrazine, L-isoleucy-L-valine anhydride, perlolyrine, L-valyl-L-valine anhydride, uridine, trimethylamine hydrochloride, choline chloride and 1-acetyl-β-carboline.[2,7]

TMP inhibits ADP- or collagen-induced platelet aggregation in rabbits, healthy people and patients with coronary heart disease. It also inhibits platelet

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aggregation induced by TXA_2-like substances.[8] TMP prevents arterial thrombus formation, probably by inhibiting platelet aggregation.[9] Although it has no significant effect on specific thrombosis, TMP decrease the size and weight of thrombi.[8]

The alkaloids of the herb and TMP decreased vascular resistance in anesthetized dogs and increased blood flow in the brain, femoral artery and lower limbs. The alkaloids or phenolic fraction of the herb and TMP also inhibited the constrictor effect of KCl and epinephrine on isolated thoracic aorta strips of rabbits.

Intramuscular or intravenous administration of the concentrated decoction, aqueous extract, ethanolic extract and the alkaloids of the herb produced significant and prolonged hypotensive effects on anesthetized dogs, cats and rabbits. Oral administration of the aqueous extract also reduced blood pressure in renal hypertensive dogs and rats.[8] This previous study in unconscions animals showed that TMP was hypotensive and had a direct vascular effect. It not only blocked the entry of extracellular calcium through calcium channels but also inhibited the release of intracellular stored calcium in the vascular smooth muscle cells.[10] It is a true calcium antagonist.[10] Although such a study may suggest that TMP could reduce blood pressure, it is quite unclear whether TMP could work as an antihypertensive agent. Moreover, the effects of TMP in conscious animals are also unclear. The purpose of this study was to evaluate whether TMP is also effective for blood pressure lowering in ambulatory hypertensive animals.

Materials and Methods

Preparation of Animals

Male Wistar rats 12 to 24 weeks old (about 250-400 g) were used in the present study. They were obtained from the animal center of the National Cheng-Kung University Medical College. Rats were housed in a temperature-controlled room (25 ± 1 °C) and kept on a 12 hr- 12 hr light-dark cycle (light on at 0600 h). Food (Purina Rat Chow) and water were available *ad labitum* throughout the experiment. Pentobarbital of 30 mg/kg was administered intraperitoneally for anesthesia, then unilateral ligation of renal artery was performed.[11] Deoxycorticosterone acetate (DOCA) was administered intra-peritoneally weekly for 10 weeks.[11] The blood pressure and body weight of rats were recorded at every 10 minutes.

Measurement of Systemic Blood Pressure

As described previously,[12] systemic blood pressure was measured by a noninvasive tail-cuff monitor (UR-5000, Ueda Company, Japan) in conscious DOCA-salt sensitive hypertensive rats. The systolic blood pressure and heart rate could be recorded simultaneously. When the blood pressure was stable (varied less than 5 mmHg between 20-minute intervals), different dosages of TMP (1,5,10 mg/kg, dissolved in normal saline) were administered intraperitoneally into DOCA salt- sensitive hypertensive rats. The other group of DOCA salt -sensitive hypertensive rats (n = 8) receiving the same volume of normal saline was used as control. The systolic blood pressure was measured repeatedly after the injection of TMP.

Rat Aortic Smooth Muscle Cells (A7r5)

Cells from the A7r5 aortic smooth muscle cell line,[13] were obtained from the Food Industry Research Institute (Hsin-Chu, Taiwan). The cells were cultured as described previously.[13]

Measurement of Cytosolic Ca^{2+} with Fura-2 in A7r5

Measurements of Ca^{2+} in aortic smooth muscle cells were performed at room temperature using the calcium-sensitive dye fura 2-acetoxymethyl ester (Fura 2-AM, Molecular Probes, Eugene, Oregon, USA), as previously described.[14,15] After collagenase treatment, the cells were kept on ice for 15 min before incubation with 4 μM Pluronic acid in PBS for 60 min in the dark at room temperature. Then the solution was centrifuged for 2-3 min, and the Fura solution was removed. The pellet of cells was put on ice, and after 10-15 min, 300 μl of physiological salt solution (PSS) were added slowly back to the cells over 2-3

min. Harvested cells were suspended in Ca^{2+}-containing PSS for 30 min up to 4 hr before Fura-2 determinations. The cells were maintained on ice until immediately before an experiment.

For measurements of Ca^{2+}, 10 μl of cell suspension were placed on a glass coverslip and centered in the optical field of a 40x oil-immersion fluorescence objective of an inverted microscope (Olympus IX 70). The cells were excited alternatively with light of 340- and 380-nm wavelengths from a dual-excitation wavelength Delta-Scan equipped with dual monochronometers and a chopper [Photon Technology International (PTI)]. Fluorescence was detected by a photometer after passing signals through a barrier emission filter (510 nm). Fluorescence signal intensity was acquired, stored, and processed by an IBM-compatible Pentium computer and Felix software (PTI). The Ca^{2+} was calculated based on the ratio at 340/380 nm, according to the formula $Ca^{2+} = [(R-R_{min})/R_{max}-R] \times (Sf/Sb) \times K_d]$, described by Grynkiewicz et al., using external calibration.[14]

The separate effects of Ca^{2+} influx stimulating agents (vasopressin, phenylephrine were evaluated in Ca^{2+}-containing PSS.

Materials and Solutions

Drugs used in this study were: TMP (Aldrich, Milwaukee, USA) (Fig. 1), HEPES, L-phenylephrine hydrochloride (both from Sigma, USA); fetal bovine serum, FBS (Hyclone, Utah, USA); Fura-2/AM (Molecular Probes Inc., Eugene, Oregon, USA); arginine vasopressin (Parke-Davis Co., USA). The standard PSS contained (in mM): 140 NaCl, 5.9 KCl, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 1.4 $MgCl_2$, 1.8 $CaCl_2$, 11.5 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH). For Ca^{2+}-free solutions, $CaCl_2$ was replaced by 1.8 mM $MgCl_2$ (total 3.2 mM) and 0.5 mM EDTA was added. The ionic composition of the Krebs solution was as following (mM): NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 20, D-glucose 10 and EDTA-2Na 0.026. The solution was aerated with O_2 containing 5% CO_2, and the pH of the solution was maintained at 7.4.

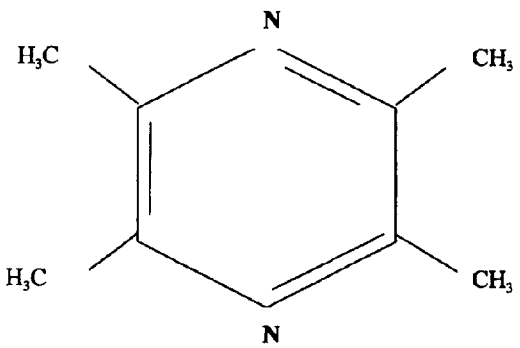

Fig. 1. The chemical structure of tetramethylpyrazine (TMP).

Statisics

All values were presented as mean ± standard error of mean. For all experimental animal groups or experiments, n = 8 unless specified. ANOVA and Dunnetts post-hoc test was used to evaluate between groups. A p values less than 0.05 was regarded as significant.

Results

Effect of TMP on blood pressure of DOCA salt-sensitive hypertensive rats

Using intraperitoneal administration of TMP at different dosages of 1,5,10 mg/kg to DOCA salt- sensitive hypertensive rats, remarkable blood pressure lowering was observed. This blood pressure lowering showed dose-dependent manner (Fig. 2). The maximal blood pressure reduction was 28.2 ± 5.0 mmHg at the dosage of 10 mg/kg.

Effect of TMP on intracellular Ca^{2+} concentrations in A7r5 cells

Vascular smooth muscle cells play an important role in control of vasorelaxation. Intracellular Ca^{2+} plays a vital role in this aspect. By using vasopressin or phenylephrine (adrenergic α_1 agonist) stimulation, the intracellular Ca^{2+} concentration in A7r5 cells increased from 214.4 ± 51.4 nM to 1271.7 ± 69.4 nM (vasopressin) and 820.9 ± 83.1 nM (phenylephrine), respectively. However, after pretreatment with TMP, the intracellular Ca^{2+} increase showed a significant

dose-dependent reduction. (Fig. 3), revealing that TMP could inhibit Ca^{2+} influx in A7r5. This Ca^{2+} influx inhibitory effect became saturated when TMP was administered at the dosage of 10^{-5}M.

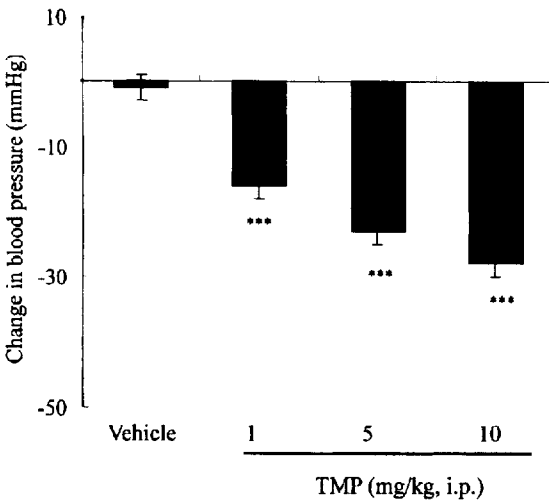

Fig. 2. Changes of blood pressure after intraperitoneal injection of TMP in DOCA salt - sensitive hypertensive rats (n=8) ***$p < 0.001$.

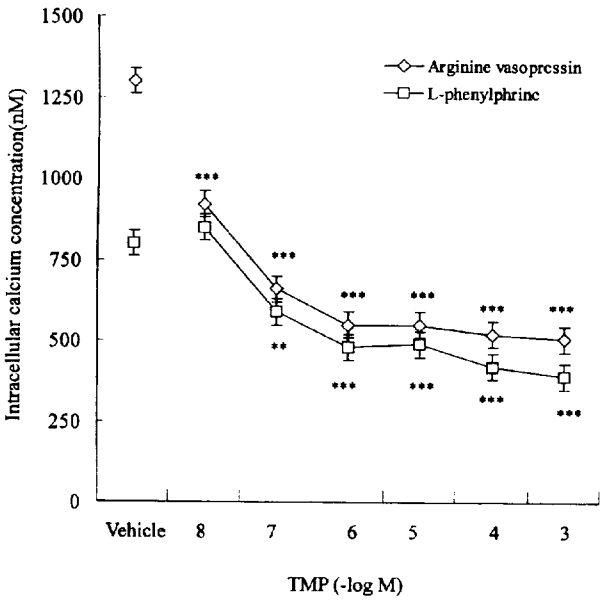

Fig. 3. Using Fura-2/AM in A7r5 cells to observe the effect of TMP on intracellular Ca^{2+} concentration under the effect of Ca^{2+} influx stimulators such as vasopressin and phenylephrine. The number of experiments = 8. **$p < 0.01$, ***$p < 0.001$.

Discussion

"In Chinese medicine,"*Chuan Xiong* has a pungent taste and a warm property, acting on the liver, gallbladder and pericardium channels.[16] It has functions such as promoting blood circulation and the flow of Qi, is used to treat menstrual disorder, dysmenorrhea, abdominal pain with mass formation, pricking pain in the chest, swelling and pain due to traumatic injury, for eliminating wound and relieving pain, using in headache and rheumatic arthralgia.[16] It is also used to treat angina pectoris of atherosclerotic coronary heart disease and cerebrovascular diseases.[16]

TMP is rapidly absorbed from the gastrointestinal tract and uniformly distributed into the cortex and cerebellum. The peak effect is observed within 1 to 3 hrs after oral administration. The biological half-life, $t_{1/2}$, is 29 min.[17] The drug is eliminated primarily by metabolism after absorption. Metabolites are excreted both in the urine and bile.

This herb is relatively nontoxic. The LD_{50} in mice is 65.9 ± 31.3 mg/kg for intraperitoneal administration and 66.4 ± 3.2 mg/kg for intramuscular administration. TMP has an LD_{50} in mice of 239 mg/kg (intraperitoneal administration).[17] Female patients taking this herb occasionally show early menstruation, and it is therefore not recommended for use by females suffering from dysmenorrhea or other hemorrhagic diseases.[17]

Previous studies in dog mesenteric arterial rings[18,19] and rat aortic smooth muscle[18,20] also have demonstrated that TMP may suppress vessel contraction. These findings were consistent with our study on aortic smooth muscle. However, most previous studies about the effects of TMP were evaluated in isolated vessels. Although Pang et al. have investigated the effects of TMP in unconscious normal rats in vivo, the antihypertensive effects in hypertensive conscious animals have not been investigated. In this study, TMP was clearly demonstrated to reduce blood pressure in our conscious animals. In addition, a similar effect of TMP was found, in our other study (unpublished data), wherein TMP also had significant anti-hypertensive effect on spontaneously hyperten-

sive rats. These findings suggest that TMP is an effective antihypertensive agent both in conscious and unconscious state. Therefore, our results suggest that TMP may be a potential drug for the control of blood pressure.

In the in vitro study, pretreatment of TMP prevented the increase of intracellular calcium due to the administration of α-agonist. This effect is similar to that noted in a previous study, wherein TMP was demonstrated to inhibit L-type calcium channel by the patch clamp method and decrease intracellular calcium by the Fure-2 method.[10] This observation may suggest that TMP could regulate the mobilization of calcium ions. However, without direct measurement of the calcium currents, it is still unclear whether this effect may arise from the inhibition of calcium influx or release of calcium from sarcoplasmic reticulum.

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川芎嗪抑制細胞外鈣離子流入以降低 DOCA 高血壓大鼠之血壓

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背景: Tetramethylpyrazine (TMP) 是從傳統中藥"川芎"所提煉出來的物質，在動物實驗中已經證實有降血壓的效果。本實驗將評估 TMP 對高血壓老鼠的降壓效果及其降壓機制。

方法: 本實驗使用 Deoxycorticosterone acetate (DOCA) 引發高血壓的老鼠共 8 隻。將 TMP 溶於生理食鹽水，再稀釋成不同濃度(1, 5, 10 毫克/公斤)注入老鼠腹腔內，利用血壓監視器測量血壓。同時利用主動脈平滑肌細胞(A7r5)來評估 Fura-2-鈣離子敏感染料來測量鈣離子流的抑制情形。

結果: TMP 在 DOCA 引發高血壓老鼠的降壓效果是與劑量相關，最大降壓效果在使用劑量 10 毫克/公斤下，血壓下降 28.2 ± 5.0 毫米汞柱，在使用鈣離子刺激劑 phenylephrine 及 arginine vasopressin 可使鈣離子流增加，而 TMP 則可以抑制鈣離子流，並且呈現與劑量成正比關係。

結論: 從傳統中藥提煉出來的 TMP 具有降血壓效果，而這種降壓效果可能與抑制細胞內鈣離子流有關。

關鍵詞: 川芎嗪；DOCA 高血壓鼠；鈣離子內流。

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Comment

Effect of Tetramethylpyrazine on Blood Pressure in Hypertensive Rats and Calcium Mobilization in Vascular Smooth Muscle Cells

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The study by Tsai and colleagues,[1] "Effect of Tetramethylpyrazine on Blood Pressure in Hypertensive Rats and Calcium Mobilization in Vascular Smooth Muscle Cells" in the January 2001 issue of the Journal (pp 3-8) addresses one important question concerning the cellular mechanisms for the antihypertensive effect of tetramethylpyrazine (TMP) – effect on calcium mobilization in vascular smooth muscle. TMP, an active component of a Chinese herbal medicine Chuang Xiong (川芎), can be synthesized chemically and is available for therapeutic[2] and other commercial uses. As indicated in the section of Materials and Methods, the chemical used in the present study was obtained from Aldrich Chemical (Milwaukee, USA), rather than isolated from the herb.

In the article by Tsai and colleagues,[1] the hypotensive effect of TMP (1-10 mg/kg, given intraperitoneally) on deoxycorticosterone acetate (DOCA)-induced hypertensive rats was evaluated. It was found that TMP induced a half maximum hypotensive effect at a dose of 1 mg/kg. To explore the cellular mechanisms responsible for the hypotensive action of TMP, effect of TMP on intracellular Ca^{2+} concentration of aortic smooth muscle cells (A7r5) were tested by using Fura-2 calcium-sensitive dye in the presence of "calcium influx stimulating agents" phenylephrine and arginine vasopressin. It was discovered that indeed TMP inhibited intracellular Ca^{2+} with an IC_{50} around 10~100 nM.

To my knowledge only one published paper[3] addressed specially to the effect of TMP on intracellular Ca^{2+} of vascular smooth muscle cells. In intact experimental animals, TMP has been shown to increase the coronary blood flow,[4,5] the heart rate and the maximum left ventricular dP/dt. The latter effects were attributed to a reflex activation of the sympathetic nerveous system.[5] In isolated canine ventricular tissues, TMP (0.3-3 mM) has been shown to induce a sustained increase in the contractile force of ventricular muscle but decreased force in Purkinje fibers after a brief initial increase.[6,7] In partially depolarized human atrial muscle fibers with slow response action potentials, TMP reduced force of contraction in standard physiological salt solution but increased force in the presence of 0.1 μM epinephrine.[8] The decrease in contractile force in the absence of epinephrine was attributed to an inhibition of Ca^{2+} reuptake by the sarcoplasmic reticulum and an enhanced release of Ca^{2+} with the consequent depletion of the intracellular Ca stores (similar to the actions of theophylline).[8] An activation of the adenylyl cyclase with the consequence elevation of the cyclic AMP level was assumed to be the underlying mechanism responsible for the positive inotropic action of TMP in the presence of epinephrine. This assumption was supported by the biochemical evidence of a significant reduction in cAMP-phosphodiesterase activity with a subsequent elevation of cAMP level (but not cGMP level) in the presence of TMP plus epinephrine in human and dog atria.[7] Similarly, a correlation between vasorelaxation and inhibition of cAMP- phosphodiesterase activity was found in dog coronary arteries.[7] These observations are in agreement with the

findings of Wu et al.[9] in rat aorta in which TMP (0.05-2 mM) relaxed in a concentration-dependant manner the aortic rings precontracted by phenylephrine (10 μM). However, to establish the cellular mechanisms responsible for the actions of TMP in cardiovascular system, a measurement of changes in intracellular Ca^{2+} in cardiac or smooth muscle cells treated with TMP is essential. Results of the present experiments show that indeed in aortic smooth muscle cells pretreated with TMP, the changes in intracellular Ca^{2+} induced by phenylephrine or vasopressin was markedly inhibited. However, it was not clear how TMP exerted its effect to inhibit changes in intracellular Ca^{2+}. As indicated by Pang et al.,[9] TMP could act via a blockade of Ca^{2+} influx across sarcolemmal calcium channel or through an inhibition of the release of Ca^{2+} from intracellular stores. Thus, strictly speaking, it is not justified from the present data to conclude in the article that "TMP showed a calcium influx inhibition".

There are several questions remain unanswered in the present article: (1) The maximum decrease in blood pressure induced by TMP was only 28 ± 5 mmHg even at the highest dose tested (10 mg/kg) in the conscious DODC-salt sensitive hypertensive rats. It is not clear whether this relatively mild hypotensive effect is specific for this model of hypertensive rats. Also it is a pity that a comparative study on the effect of TMP in control healthy Wistar rats was not mentioned; (2) In the intracellular Ca^{2+} measurement in aortic smooth muscle cells, 0.1 μM of TMP induced a half maximum inhibitory effect but a wide range of higher concentrations (1-1000 μM) induced similar inhibitory effect (around 60 % inhibition) (Figure 3). This could be due to the concentrations of stimulating agents (phenylephrine or vasopressin, concentration not specified) used. Another possibility is that the efficacy of TMP was relatively low as compared to other vasodilators or inotropic agents. Nevertheless, because of its multiple actions on intracellular Ca^{2+} regulation and inducible NO synthase expression,[10] TMP may exert beneficial effects in certain diseased states such as circulatory failure.

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