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Vasorelaxant Effects of 5,7,4′-Trimethoxyflavone from *Kaepmferia parviflora* in the Rat Aorta

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Abstract: The aim of the present study was to investigate mechanisms underlying vasorelaxation induced by 5,7,4′-trimethoxyflavone (TMF), a major compound isolated from KPE, in the isolated rat aorta. TMF (1-100 μM) caused concentration-dependent vasorelaxations which were reduced by removal of the endothelium and addition of 300 μM N^G-nitro L-arginine methyl ester, or 10 μM 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one. However, the effects of TMF were not inhibited by pretreatment with 10 μM indomethacin, 100 μM aminoguanidine, 100 μM 7-nitroindazole. In addition, vasorelaxnt responses to TMF were inhibited by a high concentration of KCl (60 mM), 5 mM tetraethylammonium and 30 μM barium chloride, but not 10 μM glibenclamide and 1 mM 4-aminopyridine. Interestingly, incubation with TMF (10 and 100 μM) for 30 min significantly inhibited contractions to $CaCl_2$ in a Ca^{2+}-free, high K^+ buffer. The present findings have shown for the first time that TMF-induced vasorelaxations are partly mediated via endothelium-derived NO, at least in part, through cGMP-dependent pathway. Moreover, activation of K^+ efflux and inhibition of extracellular Ca^{2+} influx are involved in the vasorelaxant effects of TMF. From these findings, TMF acts as a vasodilator and may play an important role in the vasorelaxant effects of KPE.

Key words: Endothelium, nitric oxide, K^+ channels, Ca^{2+} influx, vasorelaxation

INTRODUCTION

Kaempferia parviflora Wall. ex Baker (KP) (Zingiberaceae), locally known in Thailand as kra-chai-dum, is a perennial herb and formerly known as *Boesenbergia pandurata* black rhizome. The alcoholic macerated rhizome of KP has been widely recognized in Thailand as a tonic drink for rectifying male impotence. As regards its previous studies, ethanolic extract of KP rhizomes (KPE) has been reported to exert a variety of biological activities, such as anti-gastric ulcer (Rujjanawate *et al*., 2005) activity, promotion of Nitric Oxide (NO) production (Wattanapitayakul *et al*., 2007), antispasmodic effects (Wattanapitayakul *et al*., 2008) and anti-allergic activity (Tewtrakul and Subhadhirasakul, 2007).

Phytochemical investigations on KP have revealed the presence of methoxyflavones as the major components which are involved in various pharmacological effects of KP. Indeed Yenjai *et al.* (2004) has shown that 5,7,4′-trimethoxyflavone exerts anti-plasmodial, anti-fungal and anti-microbial activities. 5-hydroxy-3,7,3′,4′-tetramethoxyflavone was found to possess the potent anti-allergic (Tewtrakul *et al*., 2008) and anti-inflammatory effects (Tewtrakul *et al*., 2009). Recently, our group (Sawasdee *et al*., 2009) has reported anticholinesterase activities of 7-methoxyflavones isolated from KP.

Regarding to the vascular effects of KP, Wattanapitayakul *et al.* (2008) have reported that KPE inhibits contraction to phenylephrine in the rat aorta. In human umbilical vein endothelial cells (HUVEC), KPE increases nitric oxide production (Wattanapitayakul *et al*., 2007). Moreover, chronic treatment of KP in male rats enhances blood flow to the testes (Chaturapanich *et al*., 2008). A recent study in the isolated rat aorta has demonstrated that KPE directly causes vasorelaxation via endothelium-derived NO. In addition, the vasorelaxant effects of KPE involve increasing K^+ efflux and inhibiting Ca^{2+} efflux from the extracellular space (Tep-areenan *et al*., 2010). However, it is not known which constituents of KPE cause these effects. In the present study, we examine whether 5,7,4′-trimethoxyflavone (TMF), a major compound isolated from KP, could be the active component that causes the vasorelaxant effect of KPE. Then, mechanisms underlying vasorelaxation to TMF were investigated in the isolated rat aorta.

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MATERIALS AND METHODS

Extraction of 5,7,4′-trimethoxyflavone: *Kaempferia parviflora* rhizomes, locally grown in Loei province, were purchased from Thai drug shop, Chao-Krom-Per, Bangkok, Thailand, in 2005. A herbarium number 013201 (BCU) has been deposited at Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

The extraction and isolation of TMF was described in our previous study (Sawasdee *et al*., 2009). The chemical structure of TMF (Fig. 1) was identified by comparison of its physical and spectroscopic data with previous literature values (Yenjai *et al*., 2004; Sutthanut *et al*., 2007; Sawasdee *et al*., 2009).

Tissue preparation: In 2008, Experiments were performed using aorta obtained from male Wistar rats (300 - 350 g) purchased from National Laboratory Animal Center, Mahidol University, Thailand. Rats were housed in standard environmental condition (25°C) under 12 h light/dark cycles and fed with standard laboratory rat chow and tap water *ad libitum*. All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

The rats were anaesthetized with zolitil (50 mg kg^{-1} i.m.) into quadriceps muscle and killed by cervical dislocation. The thoracic aorta was carefully dissected from the rat, cleaned of fat and connective tissue and cut into 4-5 mm long rings. Each ring was suspended horizontally in a jacketed organ bath filled with 20 mL of modified Krebs-Henseleit solution (composition, mM; NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10) that was maintained at 37°C and bubbled continuously with 95% O_2 and 5% CO_2 mixture. The rings were mounted between two triangular stainless steel hooks that were passed through the lumen, stretched to an optimal passive tension of about 1 g and then allowed to equilibrate for 60 min. During this period, the buffer in the organ bath was exchanged every 15 min for 1 h. Tension was recorded isometrically by a force transducer (MLT0210) connected to a MacLab recording system (AD instruments, New South Wales, Australia).

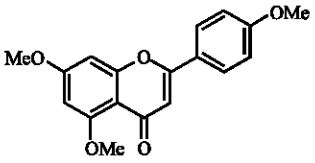

Fig. 1: Chemical structure of 5,7,4′-trimethoxyflavone (Sawasdee *et al*., 2009)

Experimental protocol: After equilibration, aortic rings were contracted with methoxamine to increase the vascular tone. Once a stable contraction was achieved, TMF (1-100 μM) was added cumulatively. To characterize the mechanisms involved in TMF-induced vasorelaxation, aortic rings were incubated with various inhibitors added to the organ bath for 30 min before methoxamine was added to increase tone. In vehicle-control experiments, dimethyl sulphoxide (DMSO) alone was added cumulatively in the same volumes as those used in the experiments with TMF.

To investigate the involvement of the endothelium in vasorelaxation to TMF, the endothelium was removed by rubbing the luminal surface with a cocktail stick. The preparation was considered to be endothelium-denuded if vasorelaxation to 10 μM carbachol was less than 10% of the induced tone. To evaluate the participation of vasodilator prostanoids via the cyclooxygenase (COX) pathway in the relaxant effects of TMF, aortic rings were treated with indomethacin (10 μM), a COX inhibitor. The contribution of nitric oxide in TMF-induced response were assessed using N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), an inhibitor of endothelial nitric oxide synthase (eNOS), aminoguanidine (100 μM), a selective inhibitor of inducible NOS (iNOS) and 7-nitroingazole (100 μM), an inhibitor of neuronal NOS (nNOS) added to the bath. In addition, a guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 10 μM) was used to investigate the involvement of the GC/cGMP-dependent pathway in TMF-induced vasorelaxation.

To investigate the role of K^+ channels, vasorelaxation to TMF was performed in aortic rings pre-contracted with a high K^+ (60 mM) Krebs solution, which was prepared by replacing an equimolar concentration of NaCl with KCl (Tep-areenan *et al*., 2003). To identify the types of K^+ channels involved in TMF-induced vasorelaxation, concentration-response curves to TMF were constructed after incubation with tetraethylammonium (5 mM), a non-specific K^+ channel inhibitor, 4-aminopyridine (1 mM), a K_V channel inhibitor, glibenclamide (10 μM), a K_{ATP} inhibitor, or barium chloride (30 μM), a K_{IR} channel inhibitor.

To examine the vascular effect of TMF on Ca^{2+} influx from the extracellular space, concentration-response curve to $CaCl_2$ (10 μM-30 mM) were performed in the presence and absence of TMF (10 and 100 μM) for 30 min. Aortic rings were first allowed to equilibrate at 1 g tension in a Ca^{2+}-free Krebs solution and then the rings were bathed with Ca^{2+}-free, high KCl (100 mM) Krebs solution. In vehicle-control experiments, DMSO was added in the same volume as that used in the experiments with TMF.

Data and statistical analysis: The relaxant effect of KPE was presented as the percentage reduction of the initial tone in each ring precontracted with methoxamine. Mean response at each concentration was expressed as mean±SEM. Statistical evaluation of data was the Student's unpaired t-test or analysis of variance (ANOVA) with statistically significant differences between groups being determined by Bonferroni's post-hoc test. The number of animals in each group is represented by n.

In some experiments, the concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) and maximal relaxations was obtained from the concentration-response curve. The curve-fitting by linear regression analysis was carried out using the graphical package GraphPad Prism.

Chemicals: All compounds were purchased from Sigma Chemical Company (St. Louis, MO, USA), except zoletil was purchased from Virbac (Carros Cedex, France). Indomethacin was dissolved in ethanol. TMF and glibenclamide were dissolved in DMSO. $BaCl_2$ and 4-AP were dissolved in distilled water. The remaining drugs were dissolved in the Krebs solution. All drugs were made up on the day of the experiment.

RESULTS

Effects of endothelial denudation, indomethacin, NOS inhibitors and ODQ on vasorelaxation to TMF in rat aortic rings: In the rat isolated aorta, TMF (1-100 μM) produced vasorelaxation in a concentration-dependent manner. Removal of the endothelium significantly reduced vasorelaxation to TMF at concentrations from 10 to 100 μM, but not lower concentrations (Fig. 2). Vasorelaxant responses to high concentrations of TMF (10-100 μM) were significantly reduced by L-NAME (300 μM) (Fig. 2). However, vasorelaxations to TMF (1-100 μM) were not affected by pretreatment with 10 μM indomethacin (Fig. 2).

As shown in Fig 3, pretreatment with a guanylyl cyclase inhibitor, ODQ (10 μM) significantly inhibited vasorelaxations induced by TMF at concentrations from 10 to 100 μM. However, aminoguanidine (100 μM), or 7-nitroindazole (100 μM) did not affect vasorelaxant responses to TMF (Fig. 3).

Effects of high extracellular potassium and potassium channel inhibitors on vasorelaxation to TMF in rat aortic rings: When aortic rings were precontracted with 60 mM KCl, vasorelaxant responses to TMF (10-100 μM) were significantly inhibited. Then, types of K^+ channels

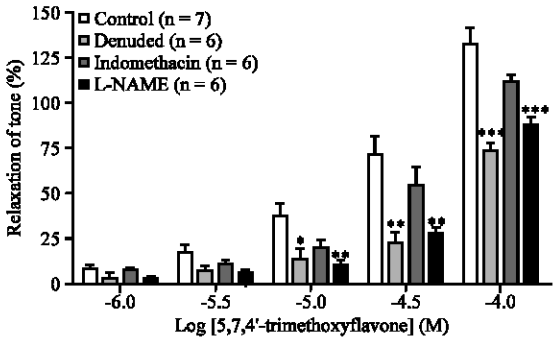

Fig. 2: Effects of removal of the endothelium, indomethacin (10 μM) and N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) on vasorelaxation to 5,7,4′-trimethoxyflavone in aortic rings precontracted with methoxamine. Data were shown as mean±SEM and analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. *p<0.05, **p<0.01 and ***p<0.001 versus control

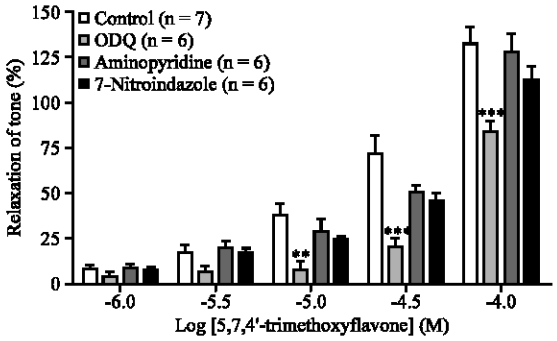

Fig. 3: Effectsof1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 10 μM), aminoguanidine (100 μM) and 7-nitroindazole (100 μM) on vasorelaxation to 5,7,4'-trimethoxyflavone in aortic rings precontracted with methoxamine. Data were shown as mean±SEM and analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. **p<0.01 and ***p<0.001 versus control

involved in TMF-induced vasorelaxation were investigated. Pretreatment with tetraethylammonium (5 mM) significantly reduced vasorelaxations induced by TMF at concentrations of 30 and 100 μM (Fig. 4). Similarly, vasorelaxant responses to high concentrations of TMF (30 and 100 μM) were significantly reduced by barium chloride (30 μM). However, addition of 4-aminopyridine (1 mM) or glibenclamide (10 μM) did not affect the relaxant effects of TMF.

Effects of TMF on extracellular Ca^{2+} influx in rat aortic rings: $CaCl_2$ (10 μM-30 mM) induced concentration-

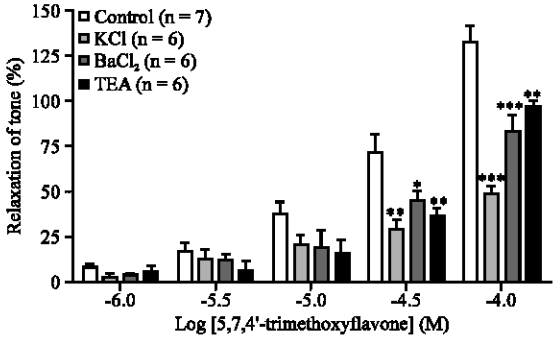

Fig. 4: Effects of 60 mM KCl, barium chloride ($BaCl_2$, 10 μM) and tetraethylammonium (TEA, 5 mM) on vasorelaxation to 5,7,4'-trimethoxyflavone in aortic rings precontracted with methoxamine. Data were shown as mean±SEM and analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* test. *p<0.05, **p<0.01 and ***p<0.001 versus control

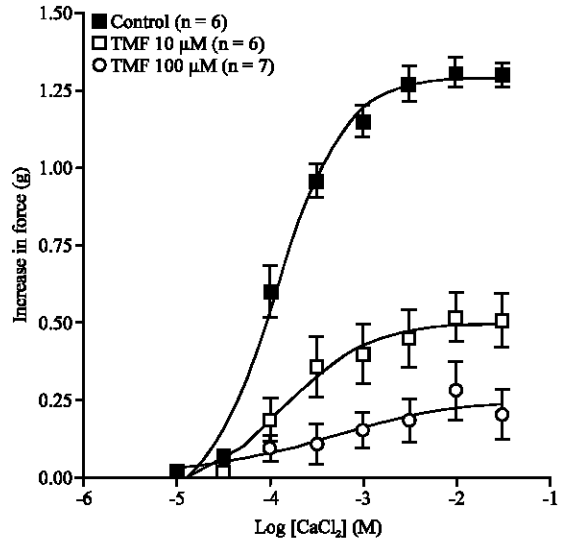

Fig. 5: Effects of 5,7,4'-trimethoxyflavone (TMF) on $CaCl_2$-induced contraction in aortic rings depolarized by 100 mM KCl. Data were shown as mean±SEM and analyzed by one-way ANOVA followed by Bonferroni's post-hoc test

dependent contractions of rat aortic rings in calcium-free buffer depolarized by 100 mM KCl. The maximal contraction elicited by $CaCl_2$ was 1.28±0.08 g and this was attenuated to 0.49±0.12 g in the presence of 10 μM TMF. However, the potency of $CaCl_2$-induced contraction was not affected by pretreatment with 10 μM TMF (pEC_{50}: control = 3.93±0.06, n = 6; 10 μM TMF: pEC_{50} = 3.82±0.31, n = 6; Fig. 5).

Similarly, 100 μM TMF significantly ($p<0.01$) inhibited maximal contractions to $CaCl_2$, but not the potency of $CaCl_2$-induced contraction (control: pEC_{50} = 3.93±0.06, with R_{max} = 1.28±0.08 g, n = 6; 100 μM TMF pEC_{50} = 3.33±0.89, with R_{max} = 0.25±0.11 g, n = 7; Fig. 5).

DISCUSSION

This study reports for the first time on the mechanisms underlying vasorelaxation induced by TMF in the rat aorta. The results of the present study demonstrate that vasorelaxant effects of TMF are mediated via NO-cGMP dependent pathway and by opening K_{Ca} and K_V channels. Inhibition of extracellular Ca^{2+} influx is also largely involved in TMF-induced vasorelaxation.

The present study demonstrated that TMF, a major methoxyflavone extracted from KP, caused vasorelaxation in the isolated rat aorta. These findings are in agreement with several earlier studies with plant extract showing that flavonoids exert a vasorelaxant property (Herrera *et al*., 1996; Ajay *et al*., 2003; Anwarul *et al*., 2006; Morello *et al*., 2006; Tep-areenan *et al*., 2010). In addition, present findings showed that the magnitude of vasorelaxation induced by TMF was very similar to that of KPE, as reported in our previous study (Tep-areenan *et al*., 2010). Thus, these results indicate that TMF seems to be the major compound involved in KPE-induced vasorelaxation.

The endothelium, lining the luminal surface of blood vessels, plays an essential function in modulating tone of vascular smooth muscle cells by releasing endothelium-derived relaxing factors, such as nitric oxide and prostacyclin (Mitchell *et al*., 2009; Vanhoutte *et al*., 2009). To determine the significance of the endothelium in vascular responses to TMF, its effects were examined in endothelium-denuded rings and in the rings that were incubated with COX and NOS inhibitors. In the present study, removal of the endothelium reduced relaxant responses to TMF, thus suggesting that the effects of TMF may be mediated via endothelium-dependent pathway. However, pretreatment with indomethacin, an inhibitor of a COX pathway, had no effect on the vasorelaxant effect of TMF. These results suggest that vasodilator prostanoids via a COX pathway do not play a part in TMF-induced responses.

NO is synthesized through the conversion of L-arginine to L-cirtulline by the enzyme, NOS. In human, there are three different isoforms of NOS, which are classified as eNOS, nNOS and iNOS (Förstermann *et al*., 1994; Mitchell *et al*., 2009). In the present study, L-NAME, an eNOS inhibitor, aminoguanidine, an iNOS inhibitor and 7-nitroindazole, a nNOS inhibitor, were used to investigate the types of NOS involved in the effects of TMF. In endothelium-intact rings, the vascular responses

to TMF were reduced by L-NAME, However, aminoguamidine and 7-nitroindazole did not affect TMF-induced responses. In addition, vascular responses to TMF in the presence of L-NAME were similar to those in denuded rings. These results suggest that endothelium-dependent vasorelaxation induced by TMF are largely mediated by endothelium-derived NO. These finding are in agreement with a previous study by Wattanapitayakul *et al*. (2007) showing that KP increases nitric oxide production in HUVEC.

Endothelium-derived NO causes relaxation of vascular smooth muscle cells mainly via activation of soluble guanylyl cyclase, leading to increase cGMP levels and lower intracellular Ca^{2+} levels and the sensitivity of to contractile proteins (Gewaltig and Kojda, 2002; Raghavan and Dikshit, 2004). Since we found that TMF-induced vasorelaxation was reduced by pre-treatment with L-NAME, we then examined whether TMF stimulates soluble guanylyl cyclase using ODQ, a guanylyl cyclase inhibitor. Pretreatment with ODQ reduced vasorelaxation to TMF as similar effect as L-NAME did. These findings suggest that TMF-induced vasorelaxation is mediated by endothelium-derived NO, at least in part, through the cGMP-dependent pathway.

Our findings demonstrated that vasorelaxant effects of TMF was present in endothelium-denuded rings and in the presence of NOS or COX inhibitors, suggesting that TMF-induced vasorelaxation is likely due to a direct effect on vascular smooth muscle cells. Activation of K^+ channels causes relaxation of vascular smooth muscle cells by increasing K^+ efflux, leading to membrane potential hyperpolarization (Jackson, 2000; Ko *et al*., 2008).We then investigate the role of K^+ channels in vasorelaxation induced by TMF. We found that relaxant responses to TMF were reduced when aortic rings were contracted with a high concentration of K^+ (60 mM), suggesting activation of K^+ channels. In addition, vasorelaxation to TMF was reduced by tetraethylammonium, a non-specific K^+ channel inhibitor and barium chloride, a K_{IR} channel inhibitor. However, glibenclamide, a K_{ATP} channel inhibitor and 4-aminopyridine, a K_V channel inhibitor, had no effects on TMF-induced responses. Taken together, these findings suggest that DMF-induced vasorelaxation is mediated by increasing K^+ efflux, at least in part, via K_{ca} and K_{IR} channels, but not via K_{ATP} and K_V channels.

An increase in extracellular Ca^{2+} influx plays an important role in contraction of vascular smooth muscle cells. In the present study, the effects of TMF on Ca^{2+} influx from the extracellular space were investigated in rat aortic rings bathed with Ca^{2+}-free, high KCl (100 mM) Krebs solution. The present results showed that TMF inhibited contractile responses of aortic rings to the re-introduction of calcium was reduced by 10 μM TMF and largely inhibited by 100 μM DMF. These results suggest that DMF likely induces vasorelaxation through direct inhibition of extracellular Ca^{2+} influx.

In conclusion, the present study has clearly shown for the first time that TMF induces endothelium-dependent vasorelaxation in the rat aorta. The mechanism of this effect involves the NO-cGMP pathway. In addition, the relaxant responses to TMF are largely due to increasing K^+ efflux as well as inhibition of Ca^{2+} channels. These findings provide a rational for the protective effects of TMF on the cardiovascular system and support its use as an antihypertensive agent.

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