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ORIGINAL ARTICLE

Mechanism of vasorelaxation induced by 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in the rats aortic ring assay

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Abstract

Previous studies have demonstrated that 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) content in *Orthosiphon stamineus* fractions correlate with its vasorelaxation activity. Even with the availability of previous studies, there is still very little information on the vasorelaxation effect of TMF, and few scientific studies have been carried out. Therefore, the present study was designed to investigate the vasorelaxation activity and mechanism of action of the TMF. The vasorelaxation activity and the underlying mechanisms of TMF were evaluated on thoracic aortic rings isolated from Sprague Dawley rats. TMF caused the relaxation of aortic rings with endothelium pre-contracted with phenylephrine. However, the vasorelaxant effect of TMF was significantly decreased in PE-primed endothelium-denuded and potassium chloride-primed endothelium-intact aortic rings. In the presence of Nω-nitro-L-arginine methyl ester, methylene blue, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, indomethacin, tetraethylammonium, 4-aminopyridine, barium chloride, atropine and propranolol, the relaxation stimulated by TMF was significantly reduced. TMF was also found to reduce Ca^{2+} release from sarcoplasmic reticulum (via IP_3R) and block calcium channels (VOCC). The present study demonstrates the vasorelaxant effect of TMF involves NO/sGC/cGMP and prostacyclin pathways, calcium and potassium channels and muscarinic and beta-adrenergic receptors.

Keywords 3′-hydroxy-5,6,7,4′-tetramethoxyflavone · NO/sGC/cGMP pathway · Calcium and potassium channels · Muscarinic and beta-adrenergic receptors

Introduction

Orthosiphon stamineus is a medicinal plant that has been widely used in Southeast Asia as a diuretic and antihypertensive agent. In clinical studies, it has been tested as an additional regimen for antihypertensive treatment (Trimarco et al. 2012). Our previous results showed that the active chloroform fraction of *O. stamineus* (consisting three important compounds, namely eupatorin, sinensetin and 3′-hydroxy-5,6,7,4′-tetramethoxy flavone) possesses excellent vasodilatory effects on the in vitro isolated rat aorta model (Yam et al. 2016a). The most noteworthy previous publications showed that eupatorin and sinensetin were not the only two active vasorelaxants present in the chloroform fraction (Yam et al. 2016b, 2018). For instance, the chloroform fraction at 8.75 μg/mL (containing 0.262 and 0.408 μg/mL of eupatorin and sinensetin, respectively) will cause a 69.19% reduction in phenylephrine-induced vasocontraction in aortic ring, but eupatorin and sinensetin will not cause any vasodilation at 0.262 and 0.408 μg/mL. This alludes to the fact that there may be other compounds present in the chloroform extract which contributed a larger portion to the vasodilatory effect. Furthermore, statistical analysis from the previous study revealed that the amount of 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in the fractions correlated with the vasodilatory effect of the fractions. Considering the chemical structure of 3′-hydroxy-5,6,7,4′-tetramethoxyflavone fulfilled the criteria of C_4=O and C_2=C_3

Highlight of the manuscript:
- TMF might be one of the antihypertensive compounds from *Orthosiphon stamineus* (Jawa tea).
- TMF exhibits vasorelaxant effect on aortic ring assay.
- The vasorelaxant effect of TMF involves multi-pathways.
- TMF induces vasorelaxation via K_v, K_{ir} and K_{Ca} VOCC channels and IP_3 receptor.
- The vasorelaxant effect of TMF involves NO/sGC/cGMP pathway

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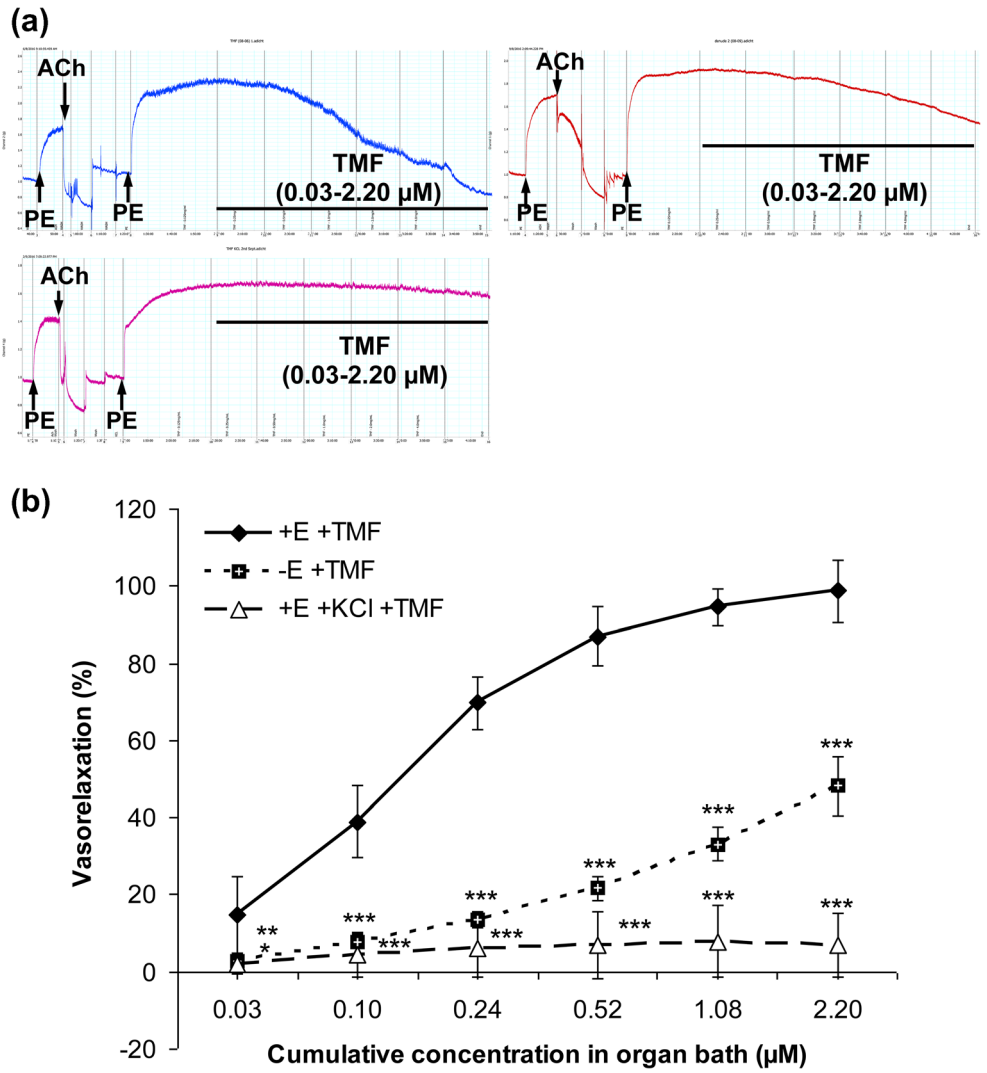

Fig. 1 Original isometric force recordings (**a**) and vasorelaxant effect (**b**) of TMF in endothelium-intact and denuded rat aortic rings pre-constricted by PE and endothelium-intact aortic ring pre-constricted by KCl ($n = 8$). Significance at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, as compared to the endothelium-intact aortic ring group

suggested by Xu et al. (2007), we hypothesised that 3′-hydroxy-5,6,7,4′-tetramethoxyflavone may be one of the other active ingredients of *O. stamineus* contributing to its vasorelaxant activity. Therefore, we designed the present study to investigate the vasodilatory activity and mechanism of action of 3′-hydroxy-5,6,7,4′-tetramethoxyflavone.

Methods

Chemicals and drugs

Acetylcholine chloride (ACh), phenylephrine hydrochloride (PE) and nifedipine were purchased from Acros Organics (Belgium). Nω-nitro-L-arginine methyl ester (L-NAME), indomethacin, tetraethylammonium chloride (TEA), barium chloride ($BaCl_2$), glibenclamide, 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), 2-aminoethoxy diphenyl borate (2-APB), atropine and propranolol hydrochloride were purchased from Sigma Aldrich (USA). Potassium bromide (KBr), 95% ethanol and 4-aminopyridine (4-AP) were purchased from Merck (Germany). Ethylene glycol tetraacetic acid (EGTA) was purchased from Calbiochem (Germany). Methylene blue was purchased from Promedipharm Sdn. Bhd. (Malaysia). 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) was purchased from INDOFINE (USA).

Methylene blue, ACh, PE, L-NAME, TEA, propranolol, atropine, 4-AP, KCl and $BaCl_2$ were prepared in distilled water. Indomethacin, nifedipine, ODQ, 2-ABP and glibenclamide were dissolved with 1% Tween 80. TMF was prepared at a concentration of 8 mg/ml in 1% Tween 80 as the stock solution. The stock solution was diluted to working concentration with distilled water.

Animals

Adult male Sprague-Dawley (SD) rats (200–240 g) were used in this experiment. The animals were kept under a 12/12 h light/

Table 1 pD2 and maximum response for TMF-induced vasorelaxation in different conditions or pre-treated with various antagonists in aortic ring

	pD2	E_{MAX}
Endothelium intact	6.48 ± 0.14	98.78 ± 8.13
Endothelium denude	5.28 ± 0.29	48.24 ± 7.68***
KCl-induced contraction	< 5.66	6.95 ± 8.2***
Indomethacin	7.09 ± 0.19	104.09 ± 9.45
L-NAME	5.44 ± 0.33	48.87 ± 9.09***
ODQ	6.42 ± 0.12	93.27 ± 12.84
Methylene blue	5.74 ± 0.45	55.49 ± 8.59***
TEA	6.61 ± 0.1	103.49 ± 5.41
4-AP	< 5.66	45.81 ± 5.8***
Glibenclamide	6.91 ± 0.14	100.51 ± 10.86
$BaCl_2$	6.45 ± 0.21	86.06 ± 10.74*
Atropine	6.08 ± 0.14	67.66 ± 8.23***
Propranolol	6.57 ± 0.16	78.76 ± 10.78**

Results are expressed as mean ± S.D ($n = 8$); *, ** and *** indicate significant level at $P < 0.05$, 0.01 and 0.001, respective compare to without pre-treatment of antagonist

Significant level at $^{*}P < 0.05$; $^{**}P$ 0.01; $^{***}P$ 0.001, compared to without pre-treatment of antagonist

dark cycle and allowed free access to food and water. All the procedure related to animal adheres to and is in compliance with guidelines set by Universiti Sains Malaysia Animal Ethics Committee (2016/(103)(772)).

Preparation of aortic rings

Healthy male Sprague-Dawley rats were used in this experiment. The rat was restrained in a restrainer and supplied with 8 psi of CO_2 for 2 min to euthanise the animal. The isolated aorta was immersed in Krebs-Henseleit (Krebs') solution in a petri dish to remove excess blood, and nearby tissue and fats were carefully excised. The isolated aorta was then cut into 3–5-mm-long segments. The clean aortic ring was incubated in an organ bath containing 10 mL Krebs' solution, which was continuously aerated with carbogen (95% O_2 and 5% CO_2) at 37 °C. The suspended aortic ring was allowed to equilibrate for 30 min under a tension of 1 g. The Krebs' solution was replaced every 10 min, and the tension was readjusted to 1 g if necessary. The condition of the aortic ring's endothelium was tested by pre-contracting the ring with phenylephrine (PE, 1 μM), followed by relaxation with the addition of acetylcholine (ACh, 1 μM). Aortic rings that produced more than 60% of relaxation in response to ACh were considered to be endothelium-intact. In some preparations, the endothelium was mechanically removed, and the removal was confirmed by the absence or lower than 10% of relaxation by ACh (1 μM) after being pre-contracted with PE (1 μM). Then, the aortic ring was rinsed with Krebs' solution at least three times to allow the tension to return to baseline before it was pre-contracted with PE (Yam et al. 2016a, b; Tan et al. 2017). When the contraction achieved a plateau, a concentration-response curve was produced by the cumulative addition of TMF (100 μL of 0.125–4.000 mg/mL or equivalent to 0.3–2.20 μM, pipetted into the organ bath) at 20-min intervals for each concentration. The tension was measured with a PowerLab system (AD Instrument, Australia) equipped with a force-displacement transducer (GRASS Force-Displacement Transducer FT03 C) and LabChart 5 (AD Instrument, Australia).

Vasorelaxation, a measure of inhibition of contraction in aortic rings pre-contracted with PE or KCl (80 mM), was measured in percentage and calculated as follows:

$$\% \text{ of vasorelaxation} = \left(\frac{C_o - C_i}{C_0}\right) \times 100$$

where C_i = the contraction of aortic rings with treatment; C_0 = plateau contraction of aortic rings after pre-contraction with PE.

Determination of the effect of TMF on endothelium-dependent PE-induced contraction

To determine the contribution of an endothelium-dependent pathway, via PGI_2 and NO/sGC/cGMP, on the vasorelaxant effect of TMF, endothelium-intact aortic rings were incubated with indomethacin (COX inhibitor) (10 μM), L-NAME (NO synthase inhibitor) (10 μM), ODQ (sGC inhibitor) (10 μM) or methylene blue (cGMP-lowering agent) (10 μM) for 20 min prior to pre-contraction with PE (Jin et al. 2011; Tan et al. 2017). Comparisons were made between the cumulative concentration-response of TMF on aortic rings with and without pre-incubation with the above-mentioned inhibitors.

Determination of the effect of TMF on PE-induced contraction in the presence of propranolol and atropine

The effects of TMF on muscarinic and β_2-adrenergic receptors were assessed with endothelium-intact aortic rings. The experiments were designed to trigger the effect of TMF as either a vasodilator or an agonist when bound to these two receptors. The aortic rings were pre-incubated with atropine (antagonist of muscarinic receptor) (1 μM) or propranolol (antagonist of β_2-adrenergic) (1 μM) for 20 min prior to pre-contraction with PE (Loh et al. 2017). Comparisons were made between the cumulative concentration-response of sinensetin on aortic rings with and without pre-incubation with the above-mentioned inhibitors and with the control.

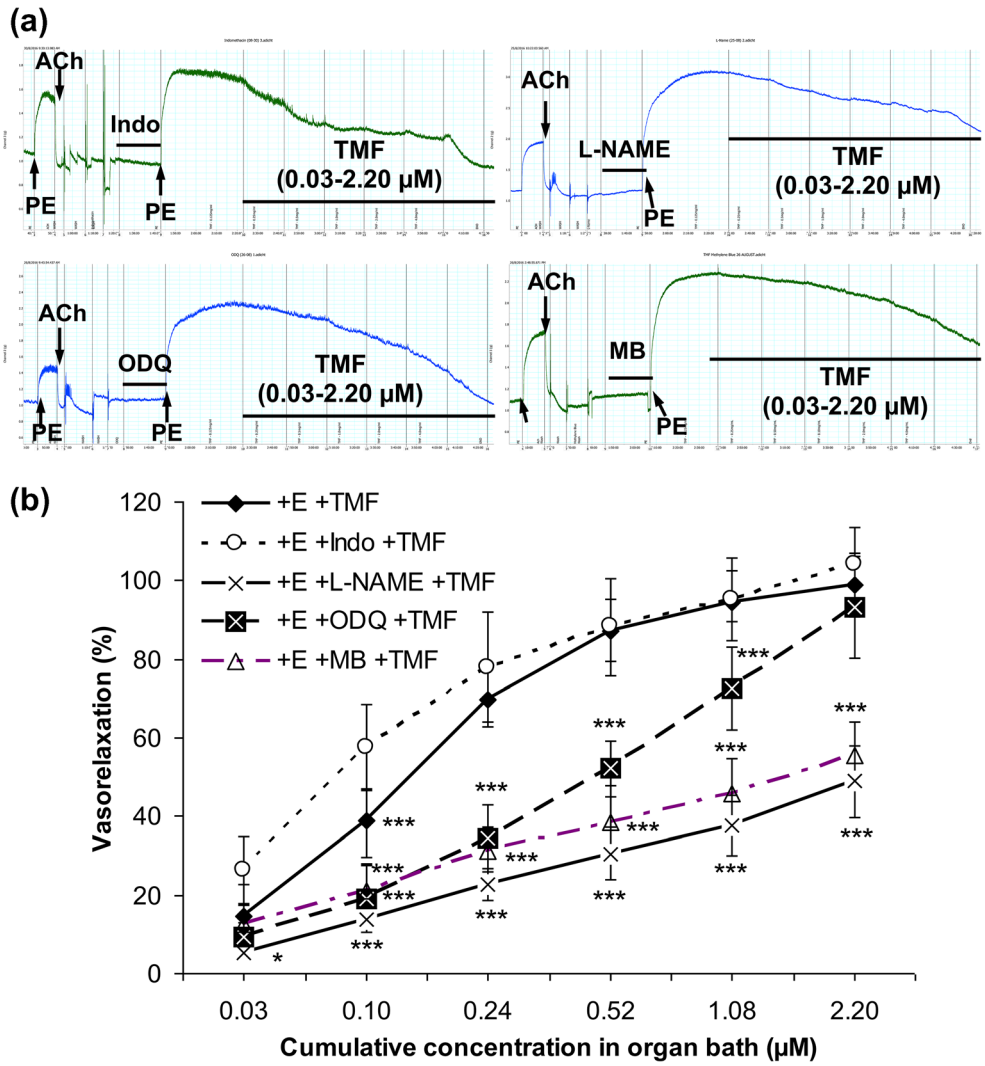

Fig. 2 Original isometric force recordings showing the influence of indomethacin, L-NAME, methylene blue and ODQ (**a**) on the vasorelaxant effect of TMF in endothelium-intact aortic rings ($n = 8$) (**b**). Significance at $^{*}P < 0.05$ and $^{***}P < 0.001$, as compared to the endothelium-intact aortic ring group

Determination of the effect of TMF on PE-induced contraction in the presence of K^+ channel blockers

To determine the involvement of K^+ channels in the vasorelaxant effect of TMF, TEA (K_{Ca} blocker) (1 mM), 4-AP (K_V blocker) (1 mM), $BaCl_2$ (K_{ir} blocker) (10 μM) or glibenclamide (non-selective K_{ATP}) (10 μM) were applied to the endothelium-intact aortic rings for 20 min prior to pre-contraction with PE (Koon et al. 2014). Comparisons were made between the cumulative concentration-response of sinensetin on the aorta rings with and without pre-incubation with the above-mentioned inhibitors.

Determination of the effect of TMF on extracellular Ca^{2+}-induced vasoconstriction

To investigate the effect of TMF on L-type calcium channel, three sets of experiments were carried out, namely the control, nifedipine and TMF groups. For the control, the endothelium-intact aortic rings were allowed to stabilise in normal Krebs' solution for 30 min. The solution was then replaced with Ca^{2+}-free Krebs' solution containing EGTA (0.2 mM) for 30 min (the solution in the organ bath was washed and replaced twice, 15 min each) in order to remove the Ca^{2+} from the tissues. The aortic rings were then rinsed in a Ca^{2+}-free, K^+-rich (50 mM) Krebs' solution for 30 min (the solution in the organ bath was washed and replaced twice, 15 min each). Then, Ca^{2+} (0.01–10.0 mM) was added cumulatively into the organ bath at 3-min intervals. For the nifedipine group, the effects of the cumulative additions of Ca^{2+} (0.01–10.0 mM) into the organ bath were recorded for 3 min for each concentration prior to incubation with nifedipine (0.1, 0.3, and 1 μM) for 20 min (Yam et al. 2016a, b; Tan et al. 2017). The experiment for the TMF group was carried out in a way similar to that of the nifedipine group, but with the aortic ring pre-incubated with TMF (0.03, 0.14 and 0.56 μM) instead of nifedipine before contraction with Ca^{2+}.

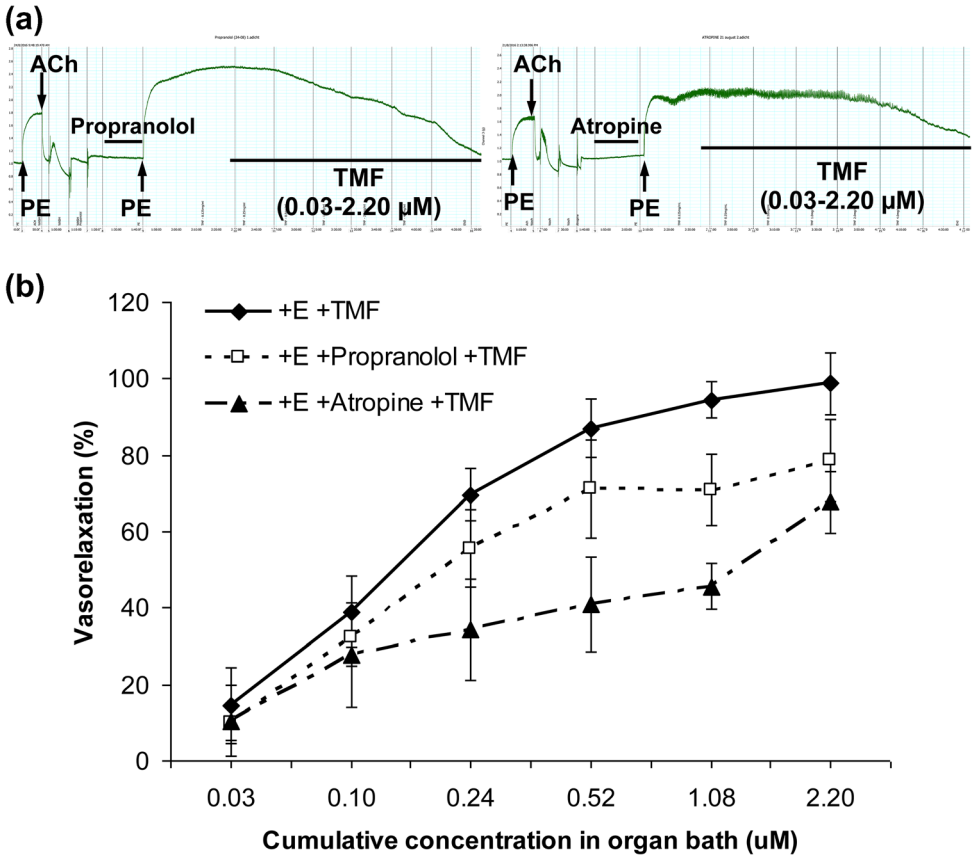

Fig. 3 Original isometric force recordings showing the influence of atropine and propranolol (**a**) on the vasorelaxant effect of TMF in endothelium-intact aortic rings ($n = 8$) (**b**). Significance at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{**}P < 0.001$, as compared to the endothelium-intact aortic ring group

Determination of vasorelaxant effect of TMF in presence of inhibition of on intracellular Ca^{2+} release

The experiment was conducted to determine the relaxation effect of TMF on the inhibition of intracellular Ca^{2+} release. The endothelium-denuded aortic rings were allowed to stabilise in a Ca^{2+}-free Krebs' solution for 20 min. The Krebs' solution was then replaced with EGTA (0.2 mM) and Ca^{2+}-free Krebs' solution for 10 min. TMF (0.03, 0.14, and 0.56 μM) or 2-APB (100 μM) was used to pre-incubate the aortic rings for 20 min before PE (1 μM) was added. The group without incubation with TMF was considered to be the control (Senejoux et al. 2013).

Statistical analysis

The values were expressed as mean ± S.D. Statistical analysis was performed by using one-way ANOVA, and Dunnett's test was conducted post hoc using the SPSS version 20 software. All tests were two-tailed and the significance was set at $P < 0.05$. pD2 value was calculated using the formula $pD2 = -\log(EC_{50})$, where EC_{50} was the concentration that produces the half-maximal response. The data were then tabulated using Microsoft Excel 2013.

Result

Vasorelaxant effect of TMF on PE/KCl-induced contraction in aortic rings

Figure 1 shows the vasorelaxation effect of TMF from the lowest dose to the highest dose (0.03–2.2 μM). The concentration-dependent vasorelaxant effect of PE-constricted aortic rings had a pD_2 value of 6.48 ± 0.14 and E_{max} value of 98.78 ± 8.13% (Table 1). To investigate the involvement of endothelium in TMF-induced vasorelaxation, the experiment was repeated with the endothelium-denuded aortic rings. As shown in Fig. 1, the vasorelaxant effect was markedly inhibited by endothelial denudation ($pD2 = 5.28 \pm 0.29$ and $E_{max} = 48.24 \pm 7.68$) ($P < 0.001$). Besides using PE to pre-contract the aortic rings, the vasorelaxation of TMF was also studied by using KCl-constricted aortic rings ($pD_2 < 5.66$ and $E_{max} = 6.95 \pm 8.2\%$) ($P < 0.001$) (Table 1).

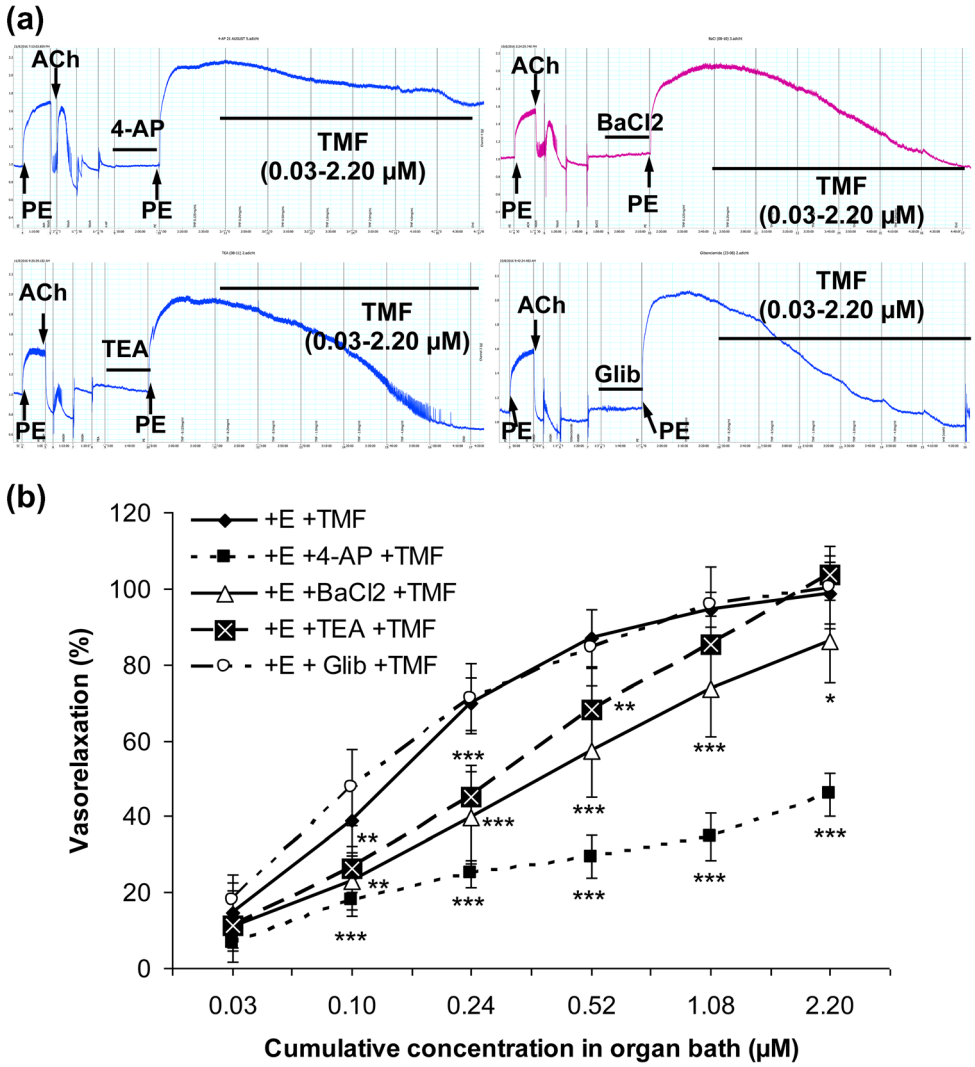

Fig. 4 Original isometric force recordings showing influences of TEA, 4-AP, glibenclamide and $BaCl_2$ (**a**) on the vasorelaxant effect of TMF in endothelium-intact aortic rings ($n = 8$) (**b**). Significance at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, as compared to the endothelium-intact aortic ring group

Role of EDRF in the vasorelaxant effects of TMF

The vasorelaxant effect of TMF was strongly attenuated by L-NAME ($pD_2 = 5.44 \pm 0.33$ and $E_{max} = 48.87 \pm 9.09\%$) ($P < 0.001$), methylene blue ($pD_2 = 5.74 \pm 0.45$ and $E_{max} = 55.49 \pm 8.59\%$) ($P < 0.001$) and ODQ ($pD_2 = 6.42 \pm 0.12$ and $E_{max} = 93.27 \pm 12.84\%$) (Fig. 2). However, the vasorelaxation of TMF was not statistically different when compared to the indomethacin-treated group ($pD_2 = 7.09 \pm 0.19$ and $E_{max} = 104.09 \pm 9.45\%$) and control group ($pD_2 = 6.48 \pm 0.14$ and $E_{max} = 98.78 \pm 8.13$) (Table 1). By comparing the vasorelaxation mediated by EDRFs, the blocking effects of their respective antagonists could be arranged in the order of L-NAME > methylene blue > ODQ > indomethacin as shown in Fig. 2.

Role of muscarinic and β-adrenergic receptors on TMF-induced vasorelaxation

Incubation with propranolol affected the vasorelaxant effect of TMF with an E_{max} value of $78.76 \pm 10.78\%$ and pD_2 value of 6.57 ± 0.16. However, Fig. 3 shows that the vasorelaxant effect of TMF was also affected by atropine with an E_{max} value of $67.66 \pm 8.23\%$ and pD_2 value of 6.08 ± 0.14.

Role of K^+ channels in TMF-induced vasorelaxant effects

There were no changes in TMF-induced vasorelaxation following pre-treatment with glibenclamide ($pD_2 = 6.91 \pm 0.14$ and $E_{max} = 100.51 \pm 10.86\%$). A similar blocking effect was obtained in the presence of K_{Ca} or K_{ir} blockers (Fig. 4) with pD_2 values of 6.61 ± 0.10 and 6.45 ± 0.21, respectively. This result suggested that the higher the concentration of TMF, the higher the vasorelaxant activity. Besides that, the 4-AP also significantly affected the vasorelaxant effect of TMF with $pD_2 < 5.66$ and $E_{max} = 45.81 \pm 5.80$ (Fig. 4 and Table 1).

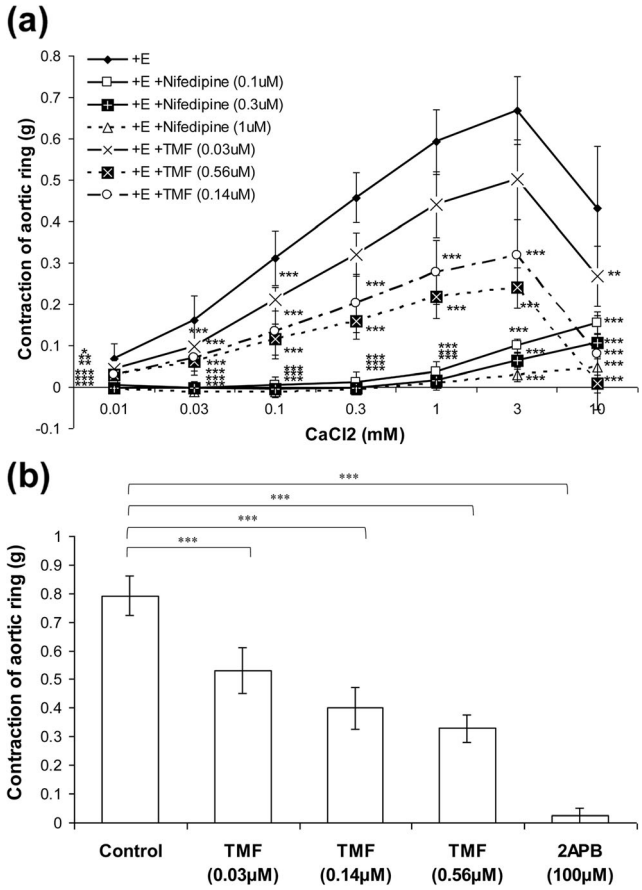

Fig. 5 Effect of TMF on Ca^{2+}-induced vasoconstriction in isolated aortic ring (**a**) and vasorelaxant effect of TMF on PE pre-contracted endothelium-intact aortic rings in Ca^{2+}-free Kreb's solution (**b**) ($n = 8$). Significance at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{**}P < 0.001$, respectively, as compared to the group without incubation with antagonists (control)

Role of Ca^{2+} channels in TMF-induced vasorelaxation

Addition of calcium (0.01–3.00 mM) caused a concentration-dependent contraction of the intact aortic rings. However, aortic rings started to relax at a high concentration of calcium (10 mM). As shown in Fig. 5a, incubation of nifedipine at concentration of 0.1, 0.3 and 1.0 μM was able to inhibit the contraction caused by the influx of Ca^{2+}. A similar effect was observed in the TMF-treated group.

The intracellular calcium release study result is shown in Fig. 5b. In short, 2-APB at 100 μM was able to suppress the aortic ring contraction evoked by PE. Also, TMF at concentrations of 0.03, 0.14, and 0.56 μM significantly suppressed the contraction induced by PE at $P < 0.001$.

Discussion

In this study, the vasorelaxant effects of TMF were examined by using aortic ring assay. It is one of the more convenient ways to study the pharmacological effect of active compounds. Also, vascular tone can be detected using this aortic ring assay. Vascular tone is determined by both the vascular endothelium and vascular smooth muscle cells (VSCMs) within blood vessel wall. Vasoactive compounds stimulate it by acting on the channels, receptors or enzymes in the blood vessel (Loh et al. 2016).

Apart from that, the mechanism that can cause vasorelaxation can be separated into two categories, namely endothelium-dependent (direct vasorelaxation) and endothelium-independent (indirect vasorelaxation) (Yam et al. 2016a). In order to study the complete mechanism of the vasorelaxant effect of TMF, both endothelium-dependent and endothelium-independent experiments should be carried out. As shown in Fig. 2, TMF was able to induce vasorelaxation in PE-evoked vasoconstriction in endothelium-intact aortic rings. However, TMF hardly caused vasorelaxation in KCl-evoked vasoconstriction in endothelium-intact aortic rings. Also, approximately half of the vasorelaxant effect of TMF was inhibited in aortic rings with endothelium removed (denuded). This indicates that part of the vasorelaxation factors were contributed by endothelium-dependent pathways.

Endothelium is of particular importance since it regulates vascular smooth muscle tone by releasing the EDRFs, namely nitric oxide (NO) and prostacyclin (PGI_2) (Ignarro et al. 1981; Senejoux et al. 2013; Loh et al. 2016). NO plays a major role in endothelium-dependent vasorelaxation. It is produced from the breakdown of L-arginine which is catalysed by endothelial NO synthase (eNOS). The diffusion of NO from endothelium into VSMC will activate the sGC to produce cGMP. The increase of cGMP will cause a reduction of Ca^{2+} in VSMC, resulting in vasorelaxation (Gruetter et al. 1979; Jin et al. 2011). The results show that the vasorelaxant effect of TMF was markedly inhibited by pre-treatment with L-NAME or methylene blue in the intact aortic rings. This means that TMF possesses the ability to activate NOS, while also being able to lower cGMP production in order to induce vasorelaxation (Jin et al. 2011). The activation of sGC can be inhibited using antagonist such as ODQ. However, the ODQ effect was negated when a high concentration of TMF was added. Besides that, PGI_2 is a cyclooxygenase (COX)-derived product, which also participates in the vasorelaxant effect. Indomethacin (non-selective COX antagonist) was used to study the contribution of the release of COX-derived product. As shown in Fig. 2, the contribution of the release of COX products is ruled out since there is not much relaxation present in the result.

G-protein-coupled receptors (GPCR) are located on the intracellular surface of the cell membrane. The most frequently studied GPCRs are muscarinic and β_2-adrenergic receptors. Activation of β_2-adrenergic receptors will stimulate vasorelaxation by activating a cascade of signalling pathways. The activity of adenylyl cyclase (AC) will be activated to catalyse the breakdown of adenosine triphosphate (ATP) to form cyclic

3′,5′-adenosine monophosphate (cAMP), hence resulting in vasorelaxation. Furthermore, vasorelaxation can take place by stimulating the phospholipase C signalling pathway cascade via muscarinic receptors (Ishii and Kurachi 2006). Based on the results obtained, the reduction of vasorelaxant effect of TMF by propranolol (β_2-adrenergic receptor antagonist) was less than atropine (muscarinic receptors antagonist). This suggests that TMF vasorelaxation is dependent on muscarinic receptors more than the β_2-adrenergic receptor, but the vasorelaxant effect of TMF reduced by propranolol cannot be ruled out since there is a 21.24% reduction.

As shown in Fig. 4, around 54% of the vasorelaxant effect of TMF was suppressed by incubation with 4-AP, as well as by BaCl and TEA. The majority of the hyperpolarising current induced by TMF was attributed via K_v channel, followed by K_{ir} and K_{Ca} channels, hence causing vasodilatory effects. This result suggests involvement of K^+ channels, which cause vasorelaxant activity due to membrane hyperpolarisation subsequent to K^+ channel opening. Hence, TMF induces vasorelaxant effect in a manner similar to 4-AP, BaCl and TEA. However, the concentration of TMF did affect the overall vasorelaxant effect. High concentration of TMF could negate the effect of several antagonists, such as BaCl and TEA. This means that the higher the concentration of TMF, the better the vasorelaxant effect in aortic rings. Meanwhile, the presence of glibenclamide did not affect the vasorelaxant activity of TMF. This finding suggests that TMF induces vasorelaxation via Kv, Kir and K_{Ca}.

In addition to K^+ channels, Ca^{2+} channels also play a vital role in controlling vascular tone in blood vessels. However, the function of Ca^{2+} channels is the opposite of K^+ channels. Ca^{2+} in VSMCs is important for muscle contraction. There are two common ways for the entry of calcium into the cytosol in VSMC via voltage-operated calcium channel (VOCC) by an extracellular influx of Ca^{2+} and releasing Ca^{2+} from intracellular sarcoplasmic reticulum stores (Nelson et al. 1988; McFadzean and Gibson 2002). Iwamuro et al. (1998) claimed that pre-treatment with 1 μM of nifedipine was able to fully inhibit the L-type VOCC. The contraction induced by addition of Ca^{2+} was clearly suppressed by nifedipine in Ca^{2+}-free Krebs' solution. Although the effect of TMF was not as strong as nifedipine, it still played a role in suppressing the contraction of aortic rings. The result proved that the higher the concentration of TMF, the better the suppression of contraction caused by the VOCC in aortic rings. In addition, an α-adrenergic receptor agonist (PE, 1 μM) causes the aortic ring contraction by Ca^{2+} influx through Ca^{2+} channel and activating the release of Ca^{2+} from sarcoplasmic reticulum stores via membrane-bound IP_3 receptors (Yildiz et al. 2013). An antagonist (2-APB, 100 μM) was used to inhibit the contraction of aortic rings evoked by PE. Based on the result shown in Fig. 5b, incubation of aortic rings with TMF (0.03, 0.14 and 0.56 μM) significantly inhibits the contraction of PE in Ca^{2+}-free Krebs' solution. This implies that TMF has the ability to inhibit the Ca^{2+} release from sarcoplasmic reticulum stores. Hence, the contraction of aortic rings could be minimised.

Other than sinensetin and eupatorin, 3′-hydroxy-5,6,7,4′-tetramethoxyflavone is another important phytochemical found in *Orthosiphon stamineus*. In clinical studies, *O. stamineus* has been tested as an additional regimen for antihypertensive treatment (Trimarco et al. 2012). Our previous results showed that the active chloroform fraction of *O. stamineus* and its isolated compounds, sinensetin and eupatorin, possesses excellent vasodilatory effects on in vitro isolated rat aorta model (Yam et al. 2016a, b). The most noteworthy previous publications showed that the vasodilation effect of *O. stamineus* chloroform fraction, sinensetin and eupatorin was dependent on multi-pathways. *O. stamineus* chloroform fraction mostly involves K_{ir}, K_{ca} and K_v and calcium channels, muscarinic receptor and NO pathway. The vasodilation effects of sinensetin and eupatorin also utilise multi-pathways but mainly depends on NO/sGC/cGMP pathway (pD2 values of NO, sGC and cGMP for sinensetin and eupatorin were 6.42, 6.18 and 5.52 and < 4.6, 5.84 and 6.02, respectively) and calcium receptor, which is not similar to the general trend of *O. stamineus* (Yam et al. 2016a, b, 2018). This eludes to the fact that sinensetin and eupatorin may not be the only vasodilative phytochemicals present in *O. stamineus*. This is further supported by looking at the fact that *O. stamineus* chloroform fraction at 8.75 μg/mL (containing 0.262 and 0.408 μg/mL of sinensetin and eupatorin, respectively) will cause 69.19% of vasodilation in PE-induced aortic ring vasocontraction model which is much higher than the vasodilation effect of sinensetin and eupatorin at 0.262 and 0.408 μg/mL, respectively. This indicated that the vasorelaxation effect of *O. stamineus* is also mediated by TMF, and the effect is not solely endothelium-dependent, but may also be partially affected by endothelium-independent relaxant factors. Hence, it can be concluded that TMF exerts its effects via multiple mechanisms on rat aortic rings. Even though the vasorelaxant action of the TMF was employed through multiple mechanisms, the pD2 results (value < 6.0) indicated that it is mainly endothelium-dependent, while also using the NO/cGMP pathway, voltage-dependent K^+ channel and calcium channels.

Compliance with ethical standards

All the procedure described herein were approved by animal ethics committee USM (no. of animal ethics approval: USM/Animal Ethics Approval/2016/(103)(772).

Conflict of interest The authors declare that they have no conflict of interest.

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