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Original article

Mechanisms of *Kaempferia parviflora* extract (KPE)-induced vasorelaxation in the rat aorta

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Background: The rhizomes of *Kaempferia parviflora* (KP) have been widely used in Thai traditional medicine to treat several diseases such as hypertension. Recent studies have shown that the ethanolic extract of KP (KPE) exerts vasorelaxant effects in the rat aorta. However, the underlying mechanisms of these vascular responses remain unclear.

Objectives: Investigate the mechanisms of KPE-induced vasorelaxation in the rat aorta.

***Methods*:** Aortic rings from male Wistar rats were precontracted with methoxamine. Changes in tension were measured using an isometric force transducer and recorded on the MacLab recording system. Vasorelaxation to KPE was examined in the presence of 10 μM indomethacin, 300 μM N^G-nitro L-arginine methyl ester (L-NAME), 60 mM KCl, 5 mM tetraethylammonium chloride (TEA), 10 μM glibenclamide, 1 mM 4-aminopyridine (4-AP) or 30 μM barium chloride ($BaCl_2$). The effects of KPE on vascular responses to carbachol, sodium nitroprusside, and $CaCl_2$ were evaluated.

***Results*:** KPE (0.1-100 μg/mL) caused vasorelaxations, which were reduced with removal of the endothelium. In addition, indomethacin, L-NAME, and indomethacin plus L-NAME reduced KPE-induced vasorelaxation. Raising the extracellular KCl concentration to 60 mM, or pre-treatment with $BaCl_2$, TEA, or glibenclamide reduced relaxant responses to KPE. Contractions to $CaCl_2$ were inhibited after pre-incubation with KPE. Pre-treatment with KPE enhanced endothelium-dependent relaxations to carbachol, but not to sodium nitroprusside.

***Conclusion*:** KPE had a vasodilator effect in the rat isolated aortic rings. These effects involved endothelium-derived NO and prostanoids via a COX pathway. In addition, KPE-induced vasorelaxation was due to increasing K^+ efflux probably through K_{Ca}, K_{IR} and K_{ATP} channels. These provide pharmacological evidence for mechanism of KPE-induced vasorelaxation and support the traditional use of KPE as an antihypertensive agent.

Keywords: *Kaempferia parviflora,* vasorelaxation, endothelium, potassium channels, calcium channels, rat aorta

Kaempferia parviflora (KP) belongs to the Zingiberaceae family. The rhizomes of KP have been widely used in Thai traditional medicine as a health-promoting herb, and known as Thai ginseng. It has also been used to treat hypertension, impotence, allergy, asthma, and diarrhea [1, 2]. Recent pharmacological studies have shown that the extract of KP rhizomes (KPE) has antigastric ulcer, antimicrobacterial, and antiviral effects [3, 4]. KPE also inhibited contractions induced by acetylcholine in the rat-isolated ileum [5].

Concerning the vascular effects of KP, KPE has been shown to cause vasorelaxation in the rat-isolated aorta [5]. In human umbilical vein endothelial cells (HUVEC), KPE increases nitric oxide concentration in a dose-dependent manner after 48 hours of incubation. In addition, KPE enhances endothelial nitric oxide synthase (eNOS) mRNA and protein expression, but not inducible NOS expression in HUVEC [6]. A

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recent study has reported that intravenous injection of alcohol extract of KP increases blood flow to the testis [7]. However, the exact mechanisms underlying KPE-induced vasorelaxation remain unclear.

In this study, we investigated the role of the endothelium and endothelial-derived relaxing factors in KPE-induced responses in isolated rat aortic rings. Then, the effects of KPE on K^+ channels and extracellular Ca^{2+} influx were evaluated. Finally, the effects of KPE on endothelium-dependent and -independent vasorelaxants were evaluated.

Materials and methods

Extraction of Kaempferia parviflora

The rhizomes of *Kaempferia parviflora* were collected from Phitsanulok, Thailand in January 2007. The herbarium specimen (QSBG 15194) was kept at Queen Sirikit Botanic Garden, Chiang Mai. The plant was identified by Wittaya Pongamornkul, Queen Sirikit Botanic Garden, Chiang Mai.

The plant material was dried at 50°C. The dried material (2.6kg) was ground and macerated with 95% ethanol for three days. The alcoholic extracts were combined and evaporated until dryness under a reduced pressure. The yield of the extract was 5.8%. The extract was stored at -20°C until used [1].

Preparation of the rat aorta

Experiments were performed on male Wistar rats (250-300 g) bred and kept by the National Laboratory Animal Center, Mahidol University, Thailand. All experiments were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

Male Wistar rats were anaesthetized with Zolitil 50 mg/kg (tiletamine chloridrate and zolazepan chloridrate) into quadriceps muscle [8], and killed by cervical dislocation. Following a thoracotomy, the thoracic aorta was dissected from the rat. The aorta was cleaned of fat and connective tissue and cut into 5 mm ring segments. Each ring was transferred to 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 solution in the organ bath was exchanged every 15 minutes for one hour. The rings were mounted between two triangular stainless steel hooks that were passed through the lumen and stretched to an optimal passive tension of about one gram, and maintained at this tension for one hour. Tension was measured by isometric force transducers (MLT 0210, New South Wales, Australia), and recorded on a MacLab recording system (AD instruments, New South Wales, Australia).

Experimental protocols

Following a 1-hour equilibration period, methoxamine (10-100 μM) was used to increase tone by approximately 1 g. In the presence of 300 μM L-NAME, lower concentrations of methoxamine (10-30 μM) were required to induce equivalent levels of tone [9]. In vehicle-control experiments, dimethyl sulphoxide (DMSO) alone was added cumulatively in the same volumes as those used in the experiments with KPE. All experiments were studied in different aortic rings.

To investigate the role of the endothelium in responses to KPE, the endothelium was removed by gently rubbing the luminal surface with a cocktail stick before mounting. The preparation was considered to be endothelium-denuded if vasorelaxation to 10 μM carbachol was less than 10% of induced tone [9]. A cyclooxygenase (COX) inhibitor, indomethacin (10 μM) and an eNOS inhibitor, L-NAME (300 μM) were used to investigate the involvement of prostanoids via the COX pathway and endothelium-derived nitric oxide in KPE-induced vasorelaxation in endothelium-intact rings.

To investigate the role of K^+ channels in vasorelaxation to KPE, 60 mM KCl was used to induce tension by substituting an equimolar concentration of NaCl with KCl. In subsequent experiments, tetraethylammonium (TEA, 5 mM), a non-specific K^+ channel inhibitor, glibenclamide (10 μM), a K_{ATP} channel inhibitor, 4-aminopyridine (4-AP, 1 mM), a K_V channel inhibitor, or barium chloride ($BaCl_2$, 30 μM), a K_{IR} channel inhibitor were independently used to identify the types of K^+ channels in KPE-induced vasorelaxation.

To examine the effects of KPE on calcium influx, concentration-response curves to $CaCl_2$ (10 μM - 30 mM) were obtained in the absence and in the presence of KPE at concentrations of 10, 30, and 100 μg/mL. After aortic rings were allowed to equilibrate for 30 minutes at 1 g tension, normal Krebs solution was replaced with Ca^{2+}-free Krebs solution. The rings were washed three times at 10-minute intervals with Ca^{2+}-free Krebs solution. Then, the rings were bathed with Ca^{2+}-free, high KCl (100 mM) buffer with or

without KPE. In vehicle-control experiments, DMSO was added in the same volume as that used in the experiments with KPE. After the rings were incubated with KPE or DMSO for 30 minutes, concentration-response curves for the contractile responses to $CaCl_2$ were constructed.

To investigate the effects of KPE on endothelium-dependent and -independent vasodilators, aortic rings were incubated with KPE (1 and 10 μg/mL) for 30 minutes. Then, concentration-responses curves to endothelium-dependent vasorelaxant, carbachol (1 nM-100 μM), and an endothelium-independent vasorelaxant, sodium nitroprusside (0.1 nM-10 μM) were established.

Data and statistical analysis

The concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) and maximal responses (R_{max}) were obtained from the concentration-response curve fitted to a sigmoidal logistic equation using the GraphPad Prism package described by Tep-areenan et al. (2003) [9]. R_{max} and pEC_{50} values (negative logarithm of the EC_{50}) were compared by analysis of variance (ANOVA) with statistically significant differences between groups being determined by Bonferroni's post-hoc test. These were expressed as mean±SEM. The results were considered statistically significant when p value was less than 0.05. The number of animals in each group is represented by n.

In experiments to study the effects of indomethacin, L-NAME and K^+ channels inhibitors, data could not be fitted to any sigmoidal dose-response curves. The relaxant effects of KPE were 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, and compared by ANOVA with Bonferroni's post-hoc test.

Drugs and chemicals

All drugs and chemicals were purchased from Sigma-Aldrich Chemical Co (St**.** Louis**,** USA), but Zoletil was purchased from Virbac (Carros Cedex, France). Indomethacin was dissolved in ethanol. Glibenclamide and KPE were dissolved in DMSO. $BaCl_2$ and 4-AP were dissolved in distilled water. The remaining drugs were dissolved in the perfusion fluid. All drugs were made up on the day of the experiment.

Results

The effects of KPE on aortic rings pre-contracted with methoxamine

In the rat-isolated aorta, KPE (0.1-100 μg/mL) induced vasorelaxation in a concentration-dependent manner (**Table 1** and **Fig. 1a**). In vehicle-control experiments, DMSO caused a relaxation of 14.1±0.5% (n = 5) at the maximal concentration used (0.16 %, v/v).

The effects of endothelial denudation, indomethacin and L-NAME on vasorelaxation to KPE in rat aortic rings

Removal of the endothelium significantly inhibited vasorelaxation to KPE (see **Table 1** and **Fig. 1a**). Similarly, in endothelium-intact aortic rings, the effects of KPE were reduced by a combination of indomethacin (10 μM) and L-NAME (300 μM) (see **Table 1** and **Fig. 1a**). In addition, pre-treatment with indomethacin (10 μM) or L-NAME (300 μM) alone significantly reduced vascular responses to KPE at concentrations from 3 to 30 μg/mL (**Table 2** and **Fig 1b**).

Table 1. The concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) and maximal responses (R_{max}) to KPE after removal of the endothelium and in the presence of indomethacin, L-NAME and KCl (60 mM) in the rat aorta.

Treatments	R_{max} (%)	pEC_{50}	Number
Control	142±1	4.99±0.01	8
Denuded	116±1[a]	4.79±0.01[a]	6
Indomethacin+L-NAME	125±9[a]	4.25±0.26[a]	8
60 mM KCl	98.5±8.7[b]	4.30±0.01[c]	6
60 mM KCl+denuded	58.3±3.2[c]	4.60±0.27[b]	6

Data were shown as mean±SEM. [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$ compared with control. Statistical significance between groups was tested by ANOVA with Bonferroni's post-hoc test.

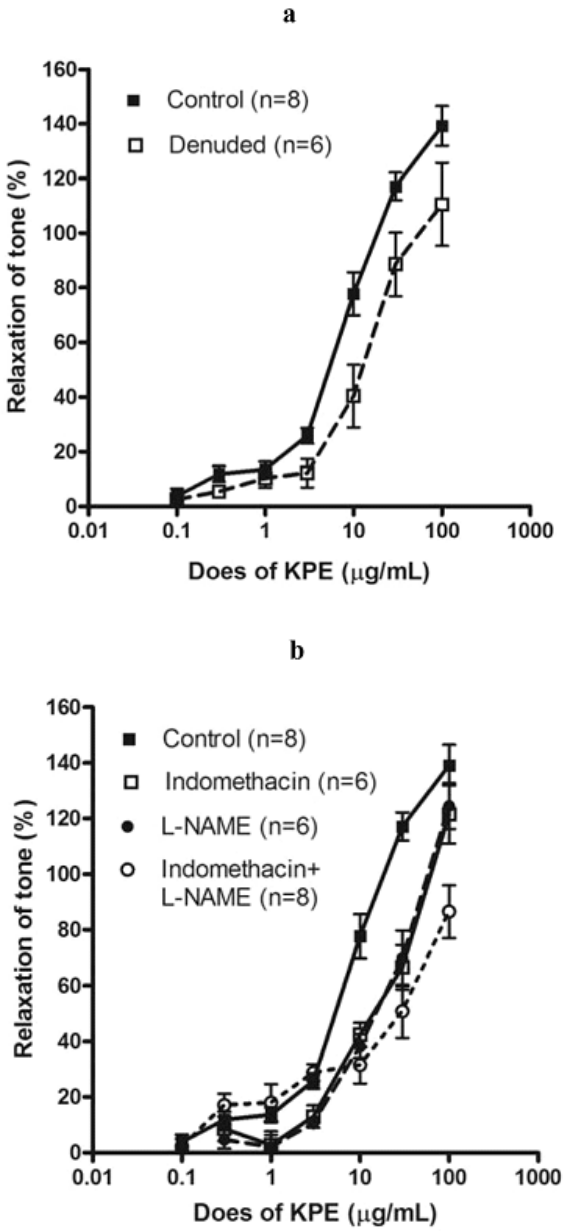

Fig. 1 The effects of endothelial denudation (**a**), and 10 μM indomethacin, 300 μM L-NAME and a combination of indomethacin and L-NAME (**b**) on KPE-induced vasorelaxation in rat aortic rings. Data are shown as mean ± SEM.

The effects of high extracellular potassium and potassium channel inhibitors on vasorelaxation to KPE in rat aortic rings

Increasing extracellular K^+ to 60 mM significantly reduced the potency of KPE-induced vasorelaxation (pEC_{50}: control = 4.99±0.01, n = 8; 60 mM KCl: 4.30 (4.16-4.45), n = 8, $p < 0.001$), and maximal response (R_{max}: control =142±1%, n = 8; 60 mM KCl = 98.5±8.7%, n = 8, $p < 0.01$) (**Fig. 2a**). In endothelium-denuded aortic rings, 60 mM KCl also significantly inhibited relaxant effects of KPE at concentrations from 3 to 30 μg/mL. There were no significant differences of KPE-induced responses between endothelium-intact and -denuded rings (**Fig. 2a**).

Pretreatment with barium chloride (30μM), glibenclamide (10 M) and TEA (5 mM) reduced relaxant responses to KPE. KPE-induced responses were not reduced by pretreatment with 4-AP (1mM) (**Table 2** and **Fig. 2b**).

Table 2. The percentage reduction of the initial tone in each ring precontracted with methoxamine in the presence of various inhibitors.

Treatments	KPE 3 μg/mL	KPE 10 μg/mL	KPE 30 μg/mL	KPE 100 μg/mL
Control (n=8)	25.8±2.8	77.7±7.9	117.0±5.0	139.0±7.0
Indomethacin (n=6)	13.0±3.9[a]	42.5±4.2[c]	66.6±7.9[c]	121.0±10.0
L-NAME (n=6)	10.5±1.9[a]	38.3±4.2[c]	69.8±10.0[b]	124.0±8.0
Tetraethylammonium (n=8)	14.5±5.0	34.8±5.3[c]	77.6±5.8[b]	128.0±7.0
Glibenclamide (n=8)	8.35±2.84[b]	26.6±8.1[c]	71.7±7.9[b]	135.7±9.6
4-aminopyridine (n=8)	21.6±3.2	54.2±3.7	97.9±7.1	151.0±8.0
Barium chloride (n=8)	9.98±1.95[a]	23.7±2.8[c]	59.9±4.4[c]	84.5±9.1[c]

Data were shown as mean±SEM. [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$ compared with control. Statistical significance between groups was tested by ANOVA with Bonferroni's post-hoc test.

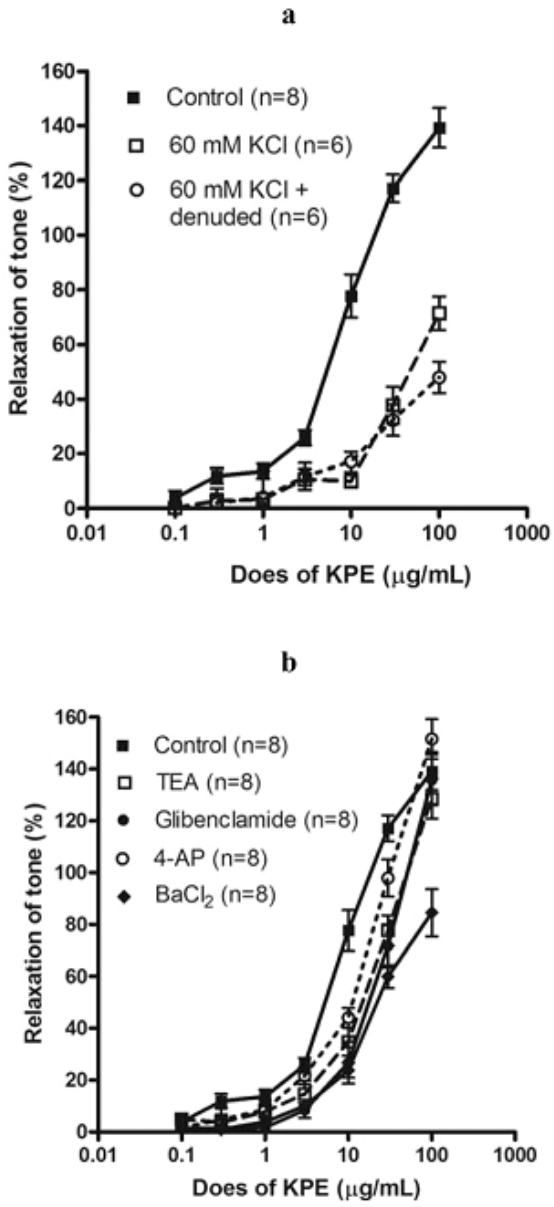

Fig. 2 The effects of 60 mM KCl in endothelium-intact and denuded aortic rings (**a**), and 10 μM glibenclamide, 5 mM TEA, 30 μM $BaCl_2$ and 1 mM 4-AP (**b**) on KPE-induced vasorelaxation in rat aortic rings. Data are shown as mean ± SEM.

The effects of KPE on $CaCl_2$-induced contraction in rat aortic rings

$CaCl_2$ (10μM-30mM) induced concentration-dependent contractions of rat aortic rings in calcium-free buffer depolarized by 100 mM KCl. Pre-incubation of the rings with KPE (10 and 30 μg/mL) significantly ($p < 0.01$) inhibited contractions induced by $CaCl_2$, such that maximal contractions were 0.94±0.01 g (control, n = 9), 0.79±0.01 g (10 μg/mL KPE, n = 9) and 0.57±0.01 g (30 μg/mL KPE, n = 6). In addition, pre-treatment with 100 μg/mL KPE abolished $CaCl_2$-induced contractions (**Fig. 3**).

The effects of KPE on endothelium-dependent and -independent vasorelaxants in rat aortic rings

Maximal relaxations to carbachol were significantly ($p < 0.001$) increased after pre-treatment with KPE at a concentration of 10 μg/mL, but not 1 μg/mL KPE (R_{max}: control = 107±1%, n = 8; 10 μg/mL KPE = 140±2%, n = 6) (**Fig. 4**). However, the potency of carbachol-induced relaxations was not affected by pre-treatment with KPE (1 and 10 μg/mL). In addition, vasorelaxations induced by sodium nitroprusside were not affected by pre-treatment with KPE (control: pEC_{50} = 7.72±0.05, with R_{max} = 135±0%, n = 8; 1 μg/mL KPE: pEC_{50} = 7.77±0.08, with R_{max} = 135±0%, n = 6; 10 μg/mL KPE: pEC_{50} = 7.95±0.07, with R_{max} = 138±1%, n = 6) (**Fig. 5**).

Discussion

In the present study using the rat aorta, we could show that KPE (0.1-100 μg/mL) caused acute concentration-dependent relaxations, which are partly endothelium-dependent. Interestingly, activation of K^+ channels and inhibition of extracellular Ca^{2+} influx also contribute to vasorelaxant effects of KPE.

High concentrations of KPE induced vasorelaxation greater than 100% of established tone. This presumably reflects relaxation of myogenic tone.

It is well known that the endothelium plays an important role in the regulation of vascular tone by synthesis and release of endothelium-derived relaxing factors (EDRFs), including NO and prostacyclin (PGI_2) [10-12]. The vascular responses of rat aortic rings to KPE are likely endothelium-dependent since removal of the endothelium partly inhibited vasorelaxation to KPE. Then, we sought to investigate EDRFs involved in KPE-induced responses. We found that either L-NAME, a NOS inhibitor, or indomethacin, a COX inhibitor reduced vasorelaxation to KPE. These findings indicate that KPE-induced vasorelaxation are mediated, in part, via NO- and COX-dependent pathways. We observed that a combination of indomethacin and L-NAME partly reduced vasorelaxation to KPE. However, there were no significant differences of the inhibitory effects among indomethacin alone, L-NAME alone, and a

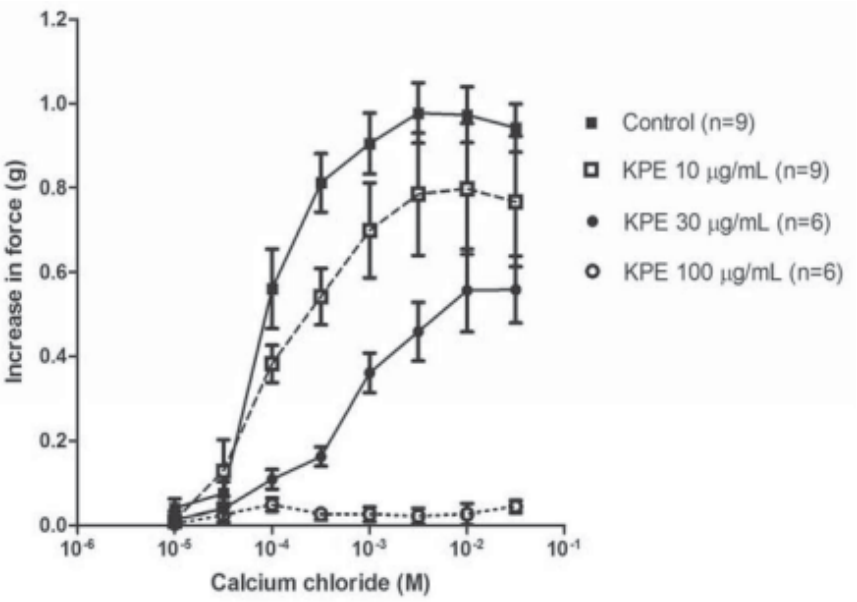

Fig. 3 The effects of KPE (10, 30 and 100 μg/mL) on $CaCl_2$-induced contraction in rat aortic rings depolarized by 100 mM KCl. Data are shown as mean ± SEM.

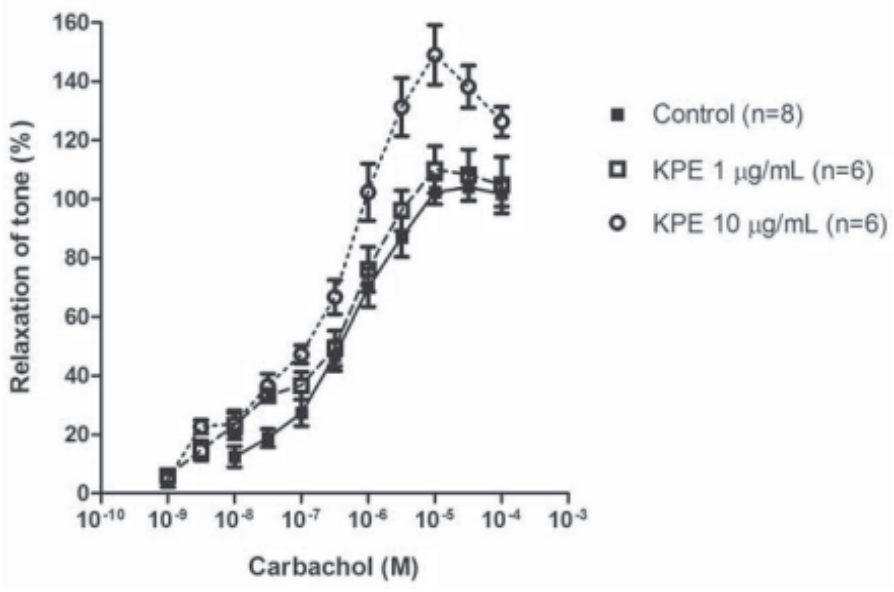

Fig. 4 The effects of pre-treatment with KPE (1 and 10 μg/mL) on carbachol-induced vasorelaxation in rat aortic rings. Data are shown as mean ± SEM.

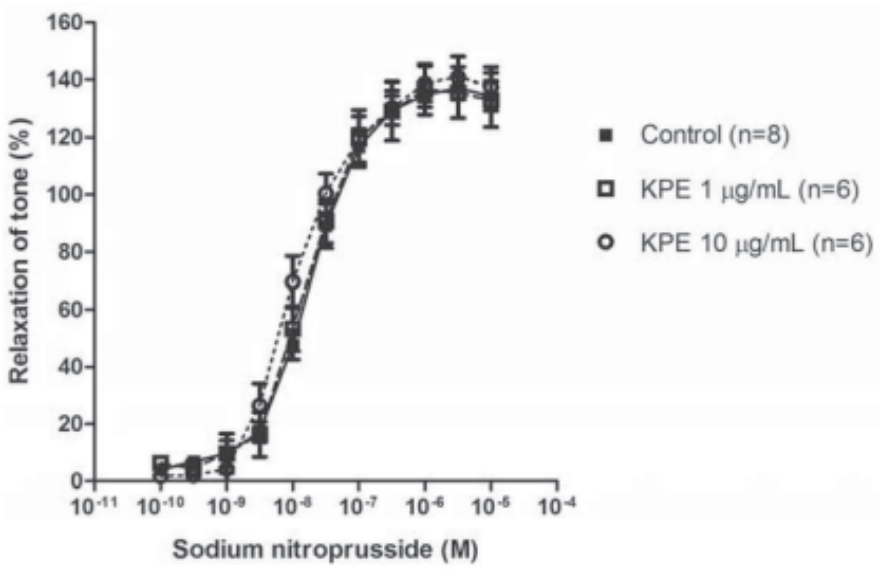

Fig. 5 The effects of pre-treatment with KPE (1 and 10 μg/mL) on vasorelaxation to sodium nitroprusside in rat aortic rings. Data are shown as mean ± SEM.

combination of indomethacin plus L-NAME on vasorelaxation induced by KPE at concentrations from 0.1-30 μg/mL, except at 100 μg/mL KPE. These indicate that endothelium-dependent vasorelaxations to KPE involve NO and vasodilator prostanoids, but not EDHF. Indeed, previous studies showed that EDHF only plays a major role in small arteries [13, 14].

The present findings clearly showed that both NO and vasodilator prostanoids contribute towards the endothelium-dependent responses to KPE. We investigated the involvement of K^+ channels in vasorelaxation to KPE. It was found that a high concentration of extracellular K^+ reduced the relaxant responses to KPE in both endothelium-intact and -denuded rings. These findings suggest that KPE causes vasorelaxation directly by increasing K^+ efflux through K^+ channels on smooth muscle cells. In addition, the contribution of K^+ channels was implicated by the ability of TEA, a non-selective K^+ channel

inhibitor, to reduce the relaxant responses to KPE. To characterize the K^+ channels contributed, we used a range of K^+ channel inhibitors. Interestingly, blockade of K_{IR} channels with barium chloride largely reduced vasorelaxation responses to KPE at concentrations from 3 to 100 μg/mL. In addition, TEA, a non-selective K^+ channels, and glibenclamide, an inhibitor of K_{ATP} channels also reduced vasorelaxation to KPE (3 to 30 g/mL). However, 4-AP, an inhibitor of K_{IR} channels did not affect KPE-induced responses. Based on these results, the relaxant responses of rat aortic rings to KPE might be mediated by increasing K^+ efflux mainly through K_{IR} channels.

As an additional mechanism of endothelium-independent relaxation, we tested the ability of KPE to inhibit calcium influx using calcium reintroduction to KCl-depolarised arteries. KPE (10, 30 and 100 μg/mL) largely inhibited contractile responses to $CaCl_2$. These indicate that in the rat aorta, KPE inhibited $CaCl_2$-induced contraction by inhibiting Ca^{2+} influx from extracellular space.

To characterize the vascular actions of KPE, we examined its effects on responses to the endothelium-dependent relaxant carbachol and the endothelium-independent agent, sodium nitroprusside. Pre-treatment of rat aortic rings with KPE increased relaxant responses to carbachol, but not responses to sodium nitroprusside. This is very interesting because KPE may augment endothelium-derived NO activity. This is independent of guanylyl cyclase, cGMP, or phophodiesterase V since SNP-induced vasorelaxation was not affected by KPE. The effects of KPE might be due to increased release of NO or bioactivity. The increased NO release could result from eNOS phosphorylation or decreased superoxide levels, resulting in increased levels of NO. It is unlikely to be a genomic effect given during the time course. It should also be noted that in a previous study using human umbilical vein endothelial cells [6], eNOS mRNA and protein expression were increased by pre-incubation with KPE after four hours.

Conclusion

In the rat aorta, KPE-induced vasorelaxation was partly mediated via cyclooxygenase- and nitric oxide-dependent pathways. In addition, the relaxant responses to KPE occurred via activation of K^+ channels, and via inhibition of extracellular Ca^{2+} influx. KPE was also shown to augment endothelial function. The present study suggests that this natural product may have therapeutic potential in the treatment of cardiovascular diseases, including hypertension.

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The authors have no conflict of interest to report.

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