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Effects of *Kaempferia parviflora* Wall. Ex Baker on endothelial dysfunction in streptozotocin-induced diabetic rats

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ABSTRACT

Aim of the study: The aim of the present study was to investigate an ethanolic extract of *Kaempferia parviflora* (KPE) reduces oxidative stress and preserves endothelial function in aortae from diabetic rats.
Materials and methods: Diabetes was induced in Sprague–Dawley rats by streptozotocin (STZ) treatment (55 mg/kg i.v.). Vascular reactivity and superoxide generation were assessed in aortic rings using standard organ bath techniques and lucigenin-enhanced chemiluminescence, respectively.
Results: Eight weeks after STZ treatment blood glucose was elevated compared to citrate treated control rats and there was an increased aortic generation of superoxide anion. In aortic rings acetylcholine-induced relaxation was impaired whereas endothelium-independent relaxation to sodium nitroprusside was unaffected. When aortic rings were acutely exposed to KPE (1, 10 and 100 μg/ml) there was a significant reduction in the detection of superoxide anion and enhanced relaxation to acetylcholine. Two separate groups of rats (control and diabetic) were orally administered daily with KPE (100 mg/kg body weight) for 4 weeks. KPE treatment reduced superoxide generation and increased the nitrite levels in diabetic aortae, and enhanced acetylcholine-induced relaxation. In the presence of N^G-nitro-L-arginine (L-NNA), the relaxation to acetylcholine in aortic rings of diabetic rats was only partially inhibited, but was totally abolished in aortic rings from the KPE-treated diabetic rats. Indomethacin did not affect relaxation to acetylcholine in aortic rings of any group.
Conclusions: These results suggest that KPE, acutely *in vitro* or after 4 weeks administration *in vivo*, reduces oxidant stress, increases NO bioavailability and preserves endothelium-dependent relaxation in aortae from diabetic rats.

1. Introduction

Diabetes mellitus profoundly increases the risk of cardiovascular disease (Deedwania, 2004; Crawford et al., 2009). It is becoming increasingly clear that oxidative stress is an important contributor to the onset and development of micro- and macrovascular complications of the disease (Channon and Guzik, 2002; Crawford et al., 2009). An accumulating body of evidence indicates that endothelium-dependent relaxation is impaired in diabetic patients (McVeigh et al., 1992; Johnstone et al., 1993; Morris et al., 1995) and in animal models of diabetes (McVeigh et al., 1992; Johnstone et al., 1993; Malakul et al., 2008; Woodman et al., 2008). A number of cellular mechanisms account for the impaired endothelium-dependent vasodilation in diabetic vasculature including a loss in the bioavailability of nitric oxide (NO), the most important endogenous vasodilator agent (Malakul et al., 2008; Woodman et al., 2008). The bioavailability of NO can be affected by cellular metabolism including increases in the generation of reactive oxygen species (ROS) (Beckman et al., 1990; Pacher and Szabo, 2006). In particular, the endothelial dysfunction induced by hyperglycaemia and diabetes have been demonstrated to be abrogated by antioxidants (Yakubu et al., 2004; Woodman and Malakul, 2009).

The phytomedicine has been extensively used to prevent and heal of various cardiovascular problems for over a millennium in oriental countries. *Kaempferia parviflora* belongs to the Zingiberaceae family, recommended in Thai folk medicine for the treatment of allergy, asthma, impotence, gout, diarrhea, peptic ulcer, diabetes, antioxidant and vasorelaxant (Pengcharoen, 2002; Pojanagaroon and Rujjanawate, 2005; Vichitphan et al., 2007; Wattanapitayakul et al., 2008). Moreover, the ethanolic extract of this plant promoted

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nitric oxide (NO) production in human umbilical vein endothelial cells (Wattanapitayakul et al., 2007). These beneficial effects of *Kaempferia parviflora* may partly contribute to vascular protection in addition to its antioxidant activity. We hypothesized that *Kaempferia parviflora* may cause reducing vascular oxidant stress, increasing NO bioavailability and improving vascular dysfunction in diabetes. However, to our best knowledge, there are no reports on effect of *Kaempferia parviflora* on STZ-diabetic model wherein free radical generation is one of main causes of endothelial dysfunction. Therefore the aim of the present study was to investigate whether either acute *in vitro* or chronic (4 weeks) *in vivo* treatment with *Kaempferia parviflora* preserves endothelial function in aortae from diabetic rats and whether that can be correlated with a reduction in vascular oxidant stress.

2. Materials and methods

2.1. Plant material and extraction

Kaempferia parviflora Wall. Ex Baker rhizomes were collected from Phitsanulok, Thailand in January 2007. The herbarium specimen (QSBG.15194) was kept at Queen Sirikit Botanic Garden, Chiangmai. The plant was identified by Wittaya Pongamornkul, Queen Sirikit Botanic Garden, Chiangmai. The rhizomes were washed thoroughly in tap water, shade-dried and powdered. The rhizome powder was extracted with 95% ethanol, evaporated in vacuo at 55 °C and lyophilized to obtain a dry extract (7.89% yield) which from now on is referred as KPE. The KPE was kept at −20 °C and was suspended in 15% Tween-20 to the required concentrations on the day of experiments.

2.2. HPLC analysis

HPLC analysis of KPE was conducted on a Shimadzu HPLC system (Shimadzu, Japan) equipped with a SPD-M20A photodiode array detector (PDA), an LC-10ATVP pump and a Rheodyne injector with a 20 μL loop. A Luna RP-18 column (150 mm × 4.6 mm, 5 μm particle size) was used together with a Phenomenex RP-18 guard column. The mobile phase consisted of methanol and water (70:30, v/v). The flow rate was adjusted to 1.0 ml/min and the detector was set at 210 nm. The standard flavones obtained from Department of Chemistry, Faculty of Sciences, Chulalongkorn University (Sawasdee et al., 2009) were used for peak identification of the HPLC fingerprint.

2.3. Antioxidant activity of KPE

The free radical scavenging activity of KPE (0.1–100 μg/ml) was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method, as previously described (Hsu, 2006). Ascorbic acid was used as a reference standard. The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve.

2.4. Animals

Male Sprague–Dawley rats weighing 180–200 g were housed at a constant temperature of 22 ± 2 °C, with a 12 h light/dark cycle, and fed on standard pellet chow and water *ad libitum*. All procedures used in this study were complied with the Animal Ethics Committee of Naresuan University and the National Research Council, Thailand guidelines for the care and use of laboratory animals.

2.5. Drugs and chemicals used

All drugs and chemicals were purchased from Sigma Chemical (St. Louis, USA) except the nitric oxide assay kit which was purchased from Calbiochem (San Diego, California, USA).

2.6. Induction of diabetes

Diabetes mellitus was induced in rats by injecting 55 mg/kg streptozotocin (STZ), dissolved in 0.1 M citrate buffer (pH 4.6) into the tail vein. Non-diabetic control rats were injected with the buffer alone. Diabetes was confirmed by the presence of hyperglycemia, polyphagia, polydipsis, polyuria and weight loss. About 72 h after STZ treatment, blood samples were taken from caudal vein, and glucose levels were measured with a glucometer (Roche, Mannheim, Germany). Rats with glucose levels higher than 15 mM were considered diabetic. Normal and hyperglycemic rats were randomly allocated and similarly grouped into four groups (eight in each): control, KPE-treated control, diabetic, and KPE-treated diabetic. After 4 weeks of induced diabetes, KPE was orally administered (using gavage needle) daily at a dosage of 100 mg/kg body weight dissolved in 15% Tween-20 throughout the experimental period for 4 weeks.

2.7. Preparation of aortic rings

At the end of the study, the rats were killed by exposure to 80% CO_2/20% O_2 for 5 min and the chests were opened to isolate the descending thoracic aorta. Superficial connective tissue and fat surrounding the aorta was then removed. The thoracic aorta was cut in 3–4 mm long ring segments and placed in ice-cold Krebs–HEPES buffer [composition (mM): NaCl 99.0, KCl 4.7, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 1.2, D-glucose 11.0, $NaHCO_3$ 25.0, $CaCl_2{\cdot}2H_2O$ 2.5, Na-HEPES 20.0] for O_2^- assay experiments, or placed in Krebs–bicarbonate solution [composition (mM): NaCl 118.0, NaH_2CO_3 25.0, glucose 11.0, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2 and $MgSO_4$ 1.18] to measure relaxations.

2.8. Superoxide anion generation in aorta

Superoxide anion generation was measured in isolated aortic rings by lucigenin-enhanced chemiluminescence method, as previously described (Chan et al., 2003). Aortic rings were preincubated for 45 min at 37 °C in Krebs–HEPES buffer containing diethylthiocarbamic acid (DETCA, 1 mM) to inactivate superoxide dismutase and β-nicotinamide adenine dinucleotide phosphate (NADPH, 0.1 mM) as a substrate for NADPH oxidase and either vehicle (15% Tween-20), KPE (0.1–100 μg/ml) or diphenylene iodonium (DPI; 5×10^{-6} M) as an inhibitor of NADPH oxidase. Aliquots (0.3 ml) of Krebs–HEPES buffer containing lucigenin (5 μM) and vehicle or DPI (5×10^{-6} M)were placed into separate wells of a 96-well Optiplate which was loaded into a TopCount single photon counter to measure background photon emission over 20 min. After background counting was completed, a single ring segment of aorta was transferred to each appropriate well and photon emission, as a measure of superoxide anion production, was re-counted for 20 min. Tissues were then dried for 48 h at 65 °C so that superoxide anion production could be calculated as average relative light units per mg of vessel dry weight.

2.9. Nitric oxide assay (measured as NO_2^-)

The nitric oxide assay kit was purchased from Calbiochem (San Diego, California, USA) and utilized for determination of nitrite levels produced by aorta according to the manufacturer's instructions. Arteries from all groups were incubated in 0.5 ml Krebs–Henseleit

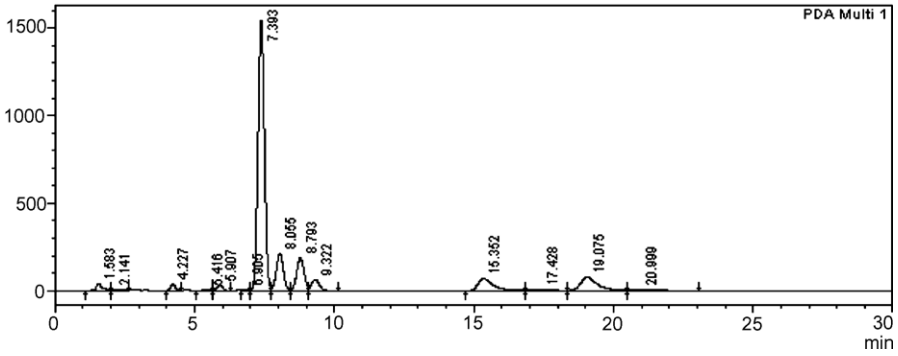

Fig. 1. HPLC chromatogram of *Kaempferia parviflora* extract (1 mg/ml) detected at 210 nm.

solution for 30 min at 37 °C and the solution was then replaced by 0.5 ml Krebs–Henseleit containing 10 μM ACh (37 °C). After reaching equilibration for 5 min, 100 μl of the perfusate was mixed with 100 μl of Griess reagent. Spectrophotometric measurements were made using a microplate reader set at 540 nm.

2.10. Relaxation responses of aorta

Aortic rings were mounted in standard 10 ml organ baths filled with Krebs–bicarbonate solution. The bath medium was maintained at 37 °C with a pH of 7.4, and continuously bubbled with 95% O_2 and 5% CO_2. Aortic rings were allowed to equilibrate for 90 min at a resting tension of 1 g, with the bath medium changed every 20 min. All preparations were then maximally contracted with isotonic, high potassium physiological saline solution (KPSS, 123 mM) in which all of the NaCl is replaced with KCl (123 mM) to achieve the maximum tension. Endothelial integrity was assessed by the ability of acetylcholine (ACh, 10 μM) to induce more than 60% of relaxation of rings precontracted submaximally with phenylephrine (PE, 10–100 nM). Cumulative concentration–response curves to the endothelium dependent relaxant ACh (1 nM–10 μM) and the endothelium-independent relaxant sodium nitroprusside (SNP, 0.1 nM–10 μM) were determined in aortic rings contracted with PE (50–60% of maximum response). To determine the participation of endothelial vasodilator factors in response to ACh, rings were incubated 20 min before the experiment with N^G-nitro-L-arginine (L-NNA, 10 μM, a non-selective nitric oxide synthase inhibitor) or indomethacin (10 μM, a non-selective cyclo-oxygenase inhibitor).

In separate *in vitro* experiments, the acute effect of KPE on ACh-induced endothelium-dependent and SNP-induced endothelium-independent relaxation of aorta from 8-week diabetic and non-diabetic rat was determined. Cumulative concentration–response curves to ACh (1 nM–10 μM) and SNP (0.1 nM–10 μM) were determined in the absence or presence of KPE (1–100 μg/ml), ascorbic acid (1 μM) or vehicle.

2.11. Statistical analysis

Results are expressed as the mean ± SEM. Concentration–response curves from rat isolated thoracic aortic rings were computer fitted to a sigmoidal curve using non-linear regression (Prism version 4.0; GraphPad Software Inc., San Diego, CA, USA) to enable the calculation of the agonist sensitivity (pEC_{50}). The maximum relaxation (R_{max}) to agonists was measured as a percentage of the contraction with U46619 alone. The calculated values of pEC_{50} and R_{max} were compared among treatments using the one-way analysis of variance (ANOVA) with post hoc multiple comparison using Newman–Keuls or Dunnett's test (Prism version 4.0; GraphPad Software Inc., San Diego, CA, USA). In all cases, statistical differences were accepted when $P < 0.05$.

3. Results

3.1. HPLC analysis

The HPLC fingerprint of KPE was obtained (Fig. 1). The major peak at 7.39 min was identified as 5,7-dimethoxyflavone by comparison with the reference standard. The result was in agreement with the report from (Sawasdee et al., 2009). Other peaks at 8.06, 8.79, 9.32, 15.35 and 19.08 min also showed typical spectrum of flavones via PDA. This indicated that flavones were presence as major components in the extract.

3.2. Antioxidant activity (DPPH assay) of KPE

The IC_{50} values (the concentration required to inhibit radical formation by 50%) of KPE are 161.9 ± 5.9 μg/ml. In comparison, the positive control, ascorbic acid had an IC_{50} values of 50 ± 5.6 μg/ml.

3.3. Rat body weights and blood glucose levels

There was no difference in the starting weights in the animals from the four groups. At the end of the study, STZ rats gained significantly less weight than control rats (control: 600 ± 11 g, diabetic: 280 ± 9 g, $P < 0.01$). Treatment with KPE did not alter the body weight gain of control or diabetic rats (KPE-treated control rats: 590 ± 7 g, KPE-treated diabetic rats: 312 ± 7 g). All rats exhibited increased blood glucose at 4 weeks of induced diabetes (control: 5.4 ± 0.4 mM, diabetic: 38 ± 0.8 mM, $P < 0.01$). Chronic KPE treatment did not affect the final blood glucose levels in either control or diabetic rats (KPE-treated control rats: 5.2 ± 0.9 mM, KPE-treated diabetic rats: 36 ± 1.8 mM).

3.4. Superoxide anion generation

The level of superoxide generated by thoracic aortic rings from diabetic rats was approximately double that of control rats. The ability of KPE (0.1–100 μg/ml) *in vitro* to acutely reduce superoxide production was assessed in aortae from control and diabetic rats. KPE caused a concentration-dependent decrease in superoxide from aortae isolated from both control and diabetic rats (Fig. 2).

Chronic KPE treatment of the rats did not affect superoxide production by aortae from control rats but significantly reduced levels generated by diabetic rat aortae to levels observed from control rats. In all groups DPI, applied *in vitro*, significantly reduced superoxide generation to low levels, suggesting that NADPH oxidase was a likely major source of the detected superoxide (Fig. 3).

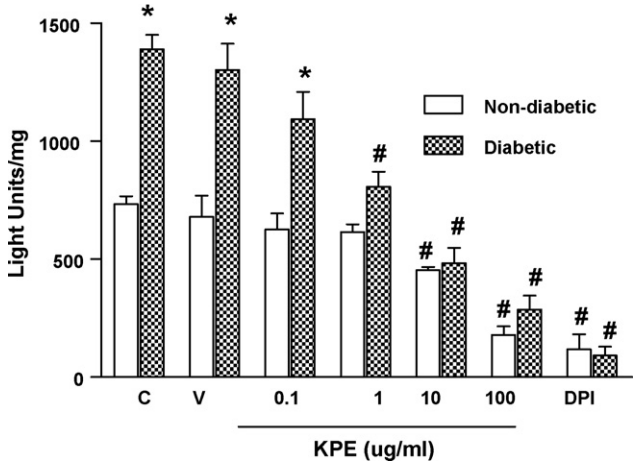

Fig. 2. Levels of superoxide generated by aortic rings from diabetic (open bars, $n = 5$) and non-diabetic rats (cross hatched bars, $n = 5$), using lucigenin-enhanced chemiluminescence method, in the absence (C) and the presence of the ethanolic extract of *Kaempferia parviflora* rhizomes (KPE, 0.1–100 μg/ml), diphenylene iodonium (DPI, 5 μM) or vehicle (V, 15% Tween-20). $^{*}P < 0.01$ compared with the non-diabetic group within the same treatment (Dunnett's test); $^{\#}P < 0.01$ compared with vehicle treatment within the same group (Dunnett's test).

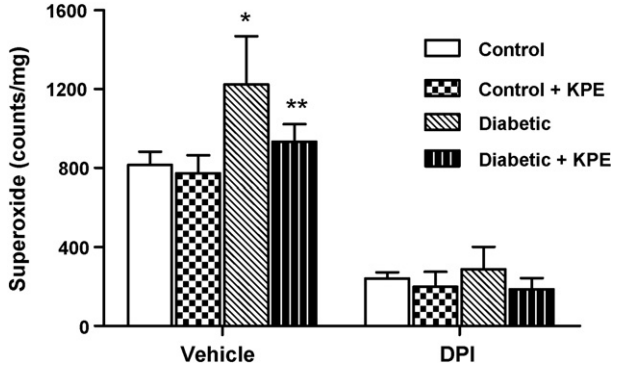

Fig. 3. Measurement of superoxide generation by segments of rat aorta using lucigenin-enhanced chemiluminescence. The generation of superoxide detected in diabetic rats was twice that from control rats ($n = 8$). Treatment of the rats with KPE (100 mg/kg, daily for 4 weeks) significantly reduced generation of superoxide by rat aortic rings from diabetic rats but had no effect in control rats. The NADPH oxidase inhibitor diphenyleneiodonium (DPI, 10 μM) reduced superoxide generation in tissue from all groups. $^{*}P < 0.01$ compared to control (Newman–Keuls test). $^{**}P < 0.01$ compared to diabetic (Newman–Keuls test).

Table 1
Comparison of sensitivity (pEC_{50}) and maximum relaxation (R_{max}) to ACh and SNP in the absence (vehicle) or presence of KPE (1, 10 and 100 μg/ml) or ascorbic acid (1 μM) in aortic rings from non-diabetic and diabetic rats.

	ACh		SNP	
	pEC_{50}	R_{max}	pEC_{50}	R_{max}
Non-diabetic				
+ Vehicle	7.03 ± 0.16	87 ± 7	7.63 ± 0.07	98 ± 2
+ KPE 1 μg/ml	7.01 ± 0.01	91 ± 5	7.65 ± 0.11	100 ± 1
+ KPE 10 μg/ml	7.11 ± 0.05	96 ± 4	7.96 ± 0.06	100 ± 2
+ KPE 100 μg/ml	6.98 ± 0.07	100 ± 1	7.42 ± 0.12	101 ± 1
+ Ascorbic acid	7.23 ± 0.10	89 ± 2	7.44 ± 0.11	103 ± 2
Diabetic				
+ Vehicle	6.84 ± 0.13	61 ± 8^{+}	7.73 ± 0.15	100 ± 1
+ KPE 1 μg/ml	7.04 ± 0.24	83 ± 4*	7.68 ± 0.12	99 ± 1
+ KPE 10 μg/ml	6.82 ± 0.29	88 ± 1*	7.87 ± 0.11	101 ± 1
+ KPE 100 μg/ml	7.03 ± 0.18	93 ± 1*	7.34 ± 0.13	102 ± 2
+ Ascorbic acid	7.07 ± 0.08	91 ± 2*	7.97 ± 0.06	101 ± 1

All data are shown as the mean ± SEM for 8 rats.
$^{+}$ $P < 0.01$ compared with the non-diabetic group within the same treatment (Dunnett's test).
* $P < 0.01$ compared with vehicle treatment within the same group (Dunnett's test).

3.5. Determination of nitrite (NO_2^-) level

The nitrite levels due to ACh stimulation of aortae from non-diabetic rats were significantly higher than those from diabetic rats (control: 0.10 ± 0.03, diabetic: 0.06 ± 0.01 μM/mg vessel dry weight; $P < 0.05$). Chronic KPE treatment increased the nitrite levels in aortae from diabetic rats to a level similar to non-diabetic groups (KPE-treated diabetic: 0.11 ± 0.02, control: 0.10 ± 0.03 and KPE-treated control: 0.12 ± 0.02 μM/mg vessel dry weight).

3.6. Vascular relaxation

To determine whether the antioxidant activity of KPE could acutely influence endothelium-dependent relaxation, responses to ACh were examined in aortic rings from control and diabetic rats in the absence and presence of KPE (1, 10 and 100 μg/ml). The maximum relaxation to ACh was significantly reduced in the diabetic rats in comparison to the control rats. The presence of KPE did not affect endothelium-dependent relaxation in control rats but significantly increased the maximum relaxation to ACh in diabetic rats. Moreover, the effect of KPE on relaxation to ACh in diabetic rats was dose dependent. To compare antioxidant efficacy with a known free radical scavenger, ascorbic acid (1 μM) was also tested and it produced a similar effect in both groups (Table 1 and Fig. 4). The presence of KPE and ascorbic acid did not affect the

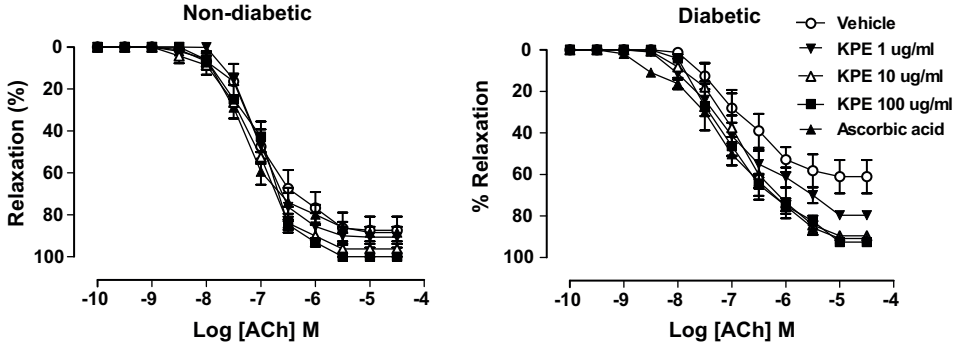

Fig. 4. Concentration–response curves to acetylcholine (ACh) in aortic segments from non-diabetic (left panel) and diabetic rats (right panel). Responses to acetylcholine were determined in the absence or presence of KPE (1, 10 and 100 μg/ml) or ascorbic acid (1 μM). In each group of experiments the aortic rings were precontracted to a similar level using PE (50 ± 1% of KPSS). All data are shown as the mean ± SEM. The pEC_{50} and R_{max} values determined from the data presented in these graphs are given in Table 1.

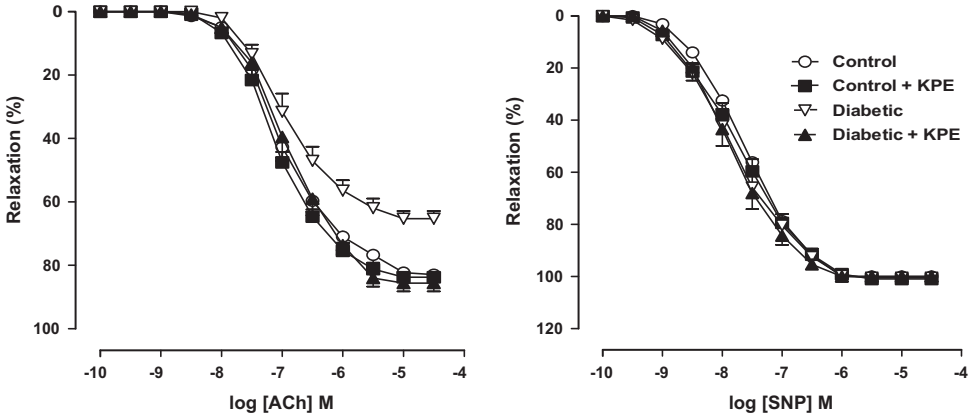

Fig. 5. Concentration–response curves to the endothelium-dependent dilator acetylcholine (ACh; left panel) and the endothelium-independent dilator sodium nitroprusside (SNP; right panel) in aortic rings from control and diabetic rats that had been orally treated with KPE (100 mg/kg) or its vehicle (15% Tween-20) daily for 4 weeks. In each group of experiments the aortic rings were precontracted to a similar level using PE (50 ± 1% of KPSS). The pEC_{50} and R_{max} values determined from the data presented in these graphs are given in Table 2.

responses to SNP in aortic rings from both control and diabetic rats (Table 1).

In addition the chronic effect of KPE on endothelial function was assessed by oral administration of KPE (100 mg/kg/day) for 4 weeks (Table 2 and Fig. 5). Treatment with KPE in control rats did not affect responses to ACh. However, in aortic rings from diabetic rats treated with KPE, the maximum relaxation to ACh was significantly increased in comparison to the response in aortae from untreated diabetic rats, such that the endothelium-dependent relaxation was not different to that observed in control rats. KPE treatment did not affect response to SNP in control and diabetic rats (Table 2 and Fig. 5). In the presence of L-NNA, the relaxation to ACh in aortic rings of non-diabetic rats was totally abolished, but was only

Table 2
A comparison of the sensitivity (pEC_{50}) and maximum response to Ach and SNP in aortic rings from control and diabetic rats with or without prior 4 weeks treatment with oral KPE (100 mg/kg daily).

Group	Ach		SNP	
	pEC_{50}	R_{max}	pEC_{50}	R_{max}
Control	6.93 ± 0.10	83 ± 1	7.6 ± 0.09	100 ± 1
Control + KPE	7.09 ± 0.06	84 ± 1	7.70 ± 0.10	101 ± 1
Diabetic	6.90 ± 0.11	65 ± 2*	7.81 ± 0.08	101 ± 1
Diabetic + KPE	6.90 ± 0.10	88 ± 3#	7.87 ± 0.11	100 ± 2

* $P < 0.001$ compared to control, Newman–Keuls test.
\# $P < 0.001$ compared to diabetic, Newman–Keuls test.

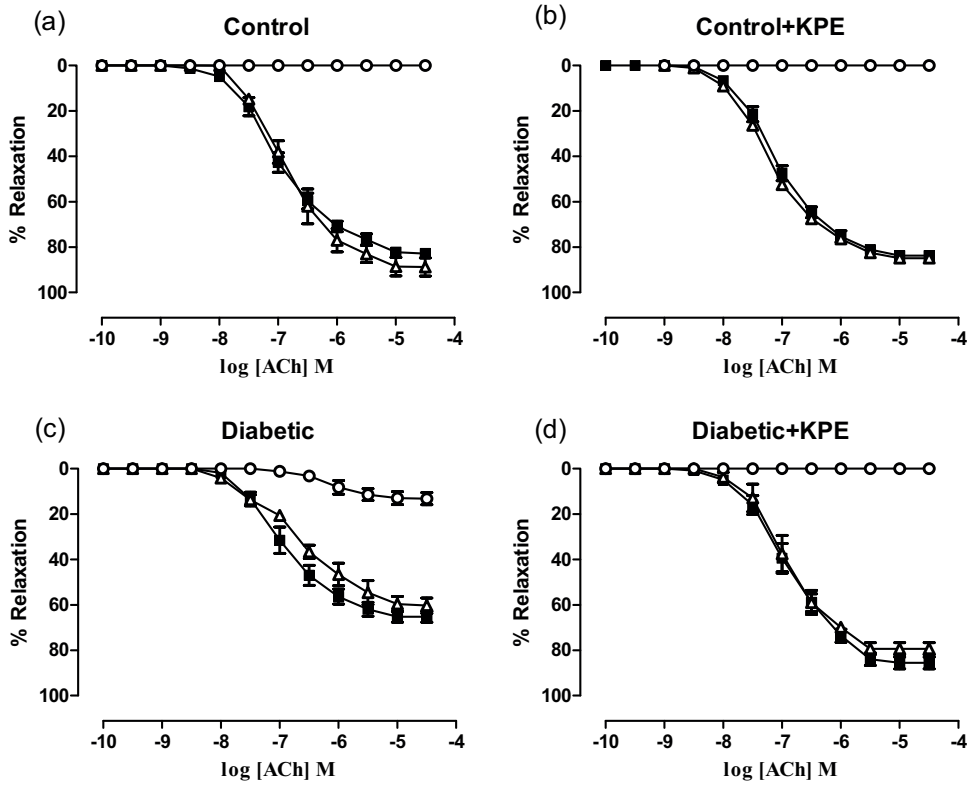

Fig. 6. Concentration–response curves for acetylcholine in phenylephrine-contrated aortic rings, alone (■) or in the presence of indomethacin (△) or NG-nitro-L-arginine (L-NNA; ○) in (a) control rats, (b) KPE-treated control rats, (c) diabetic rats and (d) KPE-treated diabetic rats. All data are shown as the mean ± SEM.

partially inhibited in aortic rings from the diabetic groups. In KPE-treated diabetic groups, ACh-induced relaxation was completely inhibited by L-NNA. The results indicate that the important role of endothelium-derived NO in the vascular effect of KPE in diabetes. Indomethacin did not affect relaxation to ACh in aortic rings of any group, indicating that cyclo-oxygenase products, including prostacyclin, are not involved in endothelium-dependent relaxation under any of the conditions tested (Fig. 6.).

4. Discussion

This study has demonstrated that KPE acutely reduces the levels of oxidative stress and improves endothelium-dependent relaxation in aortae from diabetic rats. Furthermore, oral administration of KPE to diabetic rats over a 4-week periods reduced vascular oxidative stress, and increased the nitrite levels and improved endothelial function, independently of any effect on blood glucose levels.

Type I diabetes including that produced by streptozotocin causes an elevation of plasma glucose to a similar level as shown here, and increases oxidative stress and depletion of antioxidant systems in both blood and tissues (Malakul et al., 2008; Woodman et al., 2008; Wang et al., 2009). Hyperglycemia causes an increase in the levels of oxygen radicals through several pathways such as glucose auto-oxidation, increased shunting the polyol pathway and advanced glycation end products (Tesfamariam and Cohen, 1992). NO is an important vascular target for reactive oxygen species. Superoxide neutralizes NO and the peroxynitrite formed is a source of hydroxyl radicals that can cause endothelial damage (Beckman et al., 1990; Pacher and Szabo, 2006). Oxidative stress therefore diminishes vessel endothelium-dependent relaxation, which is apparent in some experimental preparations even after acute exposure to hyperglycemia (Tesfamariam and Cohen, 1992; Taylor and Poston, 1994). Defective endothelium-dependent relaxation has been observed in chronic diabetic animals (McVeigh et al., 1992; Johnstone et al., 1993; Malakul et al., 2008; Woodman et al., 2008) and also in type 1 and type 2 diabetic patients (McVeigh et al., 1992; Johnstone et al., 1993; Morris et al., 1995).

KPE has been demonstrated to have free radical scavenging and antioxidant properties that protect tissues against oxidative damage (Vichitphan et al., 2007). In our study KPE possessed radical scavenging activity against the DPPH stable free radical, but its scavenging activity was lower than that of ascorbic acid, the standard antioxidant, of 3 times. However, the presence of KPE applied *in vitro* lowered superoxide anion levels in aortic rings from control and diabetic rats. The ability of DPI to markedly attenuate the superoxide signal indicated that NADPH oxidase was likely to be a major source of oxidant stress in aorta (Gao and Mann, 2009). In addition our study showed that the endothelium-dependent relaxant response was reduced in aortae from STZ-induced diabetic rats, similar to some other studies (McVeigh et al., 1992; Malakul et al., 2008; Woodman et al., 2008). Acute application of KPE restores endothelium-dependent relaxation of aortic rings from diabetic rats without affecting vessels from control rats. We also demonstrated that KPE had no effects on endothelium-independent relaxation in response to SNP, which causes smooth muscle relaxation directly, suggesting that the protective actions of KPE may not involve vascular muscle but target endothelium. Given that superoxide anions are involved in the reduced endothelium-dependent relaxation of diabetic arteries to ACh, the free radical scavenging action may explain the effects of KPE on ACh-induced relaxation in diabetic rat aortic rings in the present study.

We also examined whether an impaired endothelium-dependent relaxation in diabetic aortae might be improved by chronic KPE treatment. According to our preliminary study, we have observed the reduction in endothelium-dependent relaxation in aortae at 4 weeks of diabetes. Therefore, in this study, KPE was orally administrated for 4 weeks starting 4 weeks after the STZ injection. Chronic administration of KPE for 4 weeks restored ACh-induced relaxation in diabetic rat aortic rings to levels similar to those observed in euglycemic rat aortic. There are several proposed mechanisms by which KPE restores endothelial function, including increasing the sensitivity of ACh receptors or augmenting the sensitivity of vascular cells to NO. However, KPE did not alter the sensitivity (pEC_{50}) to either ACh or SNP, indicating that the endothelial sensitivity to ACh or the smooth muscle sensitivity to NO was not changed by KPE treatment. We then explored the possibility that KPE increased the bioavailability of NO by reducing superoxide-mediated metabolism of NO. Our study demonstrated that the presence of L-NNA partially inhibited the relaxation to ACh in aortic rings from the diabetic groups, similar to some other studies (Malakul et al., 2008). However, ACh-induced relaxation was completely inhibited by L-NNA in KPE-treated diabetic groups, indicating that KPE-enhanced ACh-induced relaxation in diabetic rat aortic rings was dependent on endothelium-derived NO. Previous studies demonstrated that KPE increased nitrite concentrations, and eNOS mRNA and protein expression in human umbilical vein endothelial cells (Wattanapitayakul et al., 2007). In this study, we found that a significant increase of nitrite levels in KPE-treated diabetic rats in comparison to untreated ones. Therefore, the present data indicate that the protective effect of KPE on endothelial dysfunction in diabetes depended on improved the bioavailability of endothelium-derived NO. Furthermore treatment of the rats with KPE significantly reduced superoxide levels generated by diabetic rat aortae to levels observed from control rats. Decreasing superoxide production may contributed to reduced vascular NO bioavailability and endothelial dysfunction in diabetic rats treated KPE.

In conclusion, this is the first study to report that KPE prevents endothelial dysfunction, assessed by relaxant responses to ACh, in rats with STZ-induced diabetes. The protective actions of KPE involve reducing vascular superoxide production and increasing NO bioavailability in diabetes. The beneficial effect of KPE found in this study indicates that it has potential as a therapeutic agent for use in the prevention of the vascular complications of diabetes.

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