Kaempferia parviflora ethanolic extract promoted nitric oxide production in human umbilical vein endothelial cells

Suvara K. Wattanapitayakul [a,*], Maneewan Suwatronnakorn [b], Linda Chularojmontri [c], Angkana Herunsalee [d], Somchit Niumsakul [d], Suphan Charuchongkolwongse [d], Nuchattra Chansuvanich [d]

[a] *Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Wattana, Bangkok 10110, Thailand*
[b] *Graduate Program in Biomedical Science, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand*
[c] *Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Pratumthani 12121, Thailand*
[d] *Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health, Nonthaburi 11000, Thailand*

Abstract

The rhizomes of *Kaempferia parviflora* (KP) (Zingiberaceae) have been used in Thai traditional medicine for health promotion and for the treatment of digestive disorders and gastric ulcer. This study investigated effect of KP on endothelial function. Studies in human umbilical vein endothelial cells (HUVEC) showed that KP dose-dependently increased nitrite concentrations in culture media after 48 h incubation. eNOS mRNA and protein expression were also enhanced. The induction of eNOS mRNA was detected at 4 h and plateau at 48 h while iNOS expression was not observed. These data demonstrate that KP has a great potential for a supplemental use in vascular endothelial health promotion.

Keywords: *Kaempferia parviflora*; Nitric oxide; HUVEC

1. Plant

Kaempferia parviflora Wall. Ex Baker (KP) (Zingiberaceae) was botanically identified and a voucher specimen was deposited at Medical Plant Research Institute, Thailand (N. Chansuvanich 641; DMSC 1575). Fresh rhizomes of the plant were harvested in October 2001, from a plantation in Loei province, Thailand. The rhizomes were cut into thin slices, oven dried, and ground into powder. The ethanolic extract was obtained using 95% ethanol as a solvent and reflux in Soxhlet apparatus until exhausted. The KP extract was then dried under vacuum (yield 17.5%).

2. Uses in traditional medicine

Rhizomes of KP have long been used in Thai traditional medicine. Its ethnopharmacological uses include nerve stimulation, antiflatulence, digestive disorders, gastric ulcer, diuresis, and tonic (Yenjai et al., 2004; Rujjanawate et al., 2005). Daily drink of alcoholic macerated KP rhizome is believed to improve erectile function. While it is well established that endothelial dysfunction is one of the major causes of erectile dysfunction there is no scientific evidence to support the use. This prompted us to investigate the effect of KP on endothelial function.

3. Previously isolated classes of constituents

Chalcones and flavonoids, derivatives of methoxyflavones, were isolated from the rhizomes of KP (Yenjai et al., 2004).

4. Materials and methods

KP ethanolic extract was dissolved in DMSO (10–50 mg/ml) as stock solutions. All other chemicals and reagents used in the study were obtained in high quality grade from Sigma Aldrich (St. Louis, MO) or otherwise indicated. Oligonucleotide primers were synthesized by Gibco Invitrogen. Anti-eNOS antibody was purchased from Santa Cruz Biotechnology Inc.

* Corresponding author. Tel.: +662 260 2233x4803; fax: +662 260 0125.
E-mail address: suvara@swu.ac.th (S.K. Wattanapitayakul).

4.1. Cell culture and treatment protocols

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh human umbilical cord veins. Cells were characterized by the expression of von Willebrand factor using standard immunohistochemistry technique (Wagner et al., 1982). Briefly, the vein was flushed with lactate ringer solution and then the endothelial cells were isolated by 0.1% collagenase digestion at 37 °C for 15 min. Cells were collected by centrifugation and cultured in M199 medium, supplemented with 20% fetal bovine serum (FBS) with antibiotic and antimycotic agents, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The typical endothelial cell cobblestone morphology was observed. For further endothelial cell characterization, cells were grown on glass slides in 6-well plate. Following 4% paraformaldehyde fixation, slides were incubated in blocking buffer (1% BSA in PBS) for 1 h. Anti-von Willebrand factor antibody (Chemicon) was added and incubated for 2 h. The slides were washed for 3 times and then FITC-conjugated goat anti-rabbit IgG (Chemicon) was added to the slides and incubated for 30 min. The immunofluorescence of the cells was evaluated under fluorescent microscope. Immunoreactivity was observed in approximatedly 90–95% of cell population using phase contrast view for comparison. For further experiments, low serum medium (1% FBS) was replaced to the culture 24 h prior to any treatment. KP extract was used at the concentrations that did not cause significant cell death (>90% survival) as evaluated by crystal violet assay (Wattanapitayakul et al., 2005). KP extract was incubated in the culture medium at the concentrations and times indicated in the figures.

4.2. Nitric oxide production measurement

Nitrate (NO_3^-) and nitrite (NO_2^-) are primary and non-volatile breakdown products of NO. This study measure nitrite in the culture medium based on a diazotization reaction originally described by Griess in 1879 with modifications (Granger et al., 1996). Briefly, 50 μl of each sample was added to 96-well microplate in triplicate. Then, 50 μl of the sulfanilamide solution (1% in 5% phosphoric acid) was dispensed to all samples and standards. Following 5–10 min incubation at room temperature (protected from light), another 50 μl of the 0.1% *N*-1-napthylethylenediamine dihydrochloride solution was added to all wells. The reaction mixtures were incubated for 10 min at room temperature. A magenta color was developed and the absorbance was measured within 30 min in a plate reader with a 550 nm filter (Bio-Rad model 550).

4.3. RT-PCR

Total RNA was isolated from the cells using RNeasy mini kit (Qiagen) according to the manufacturer's handbook and quantified by spectrophotometry. RT-PCR was performed using one-step RT-PCR kit (Qiagen). GAPDH gene amplifications were used as an internal standard. The following sequences were used as primers for eNOS, 5′-GAAGAGGAAGGAGTCCAGTAACACAGAC-3′(sense) and 5′-GGACTTGCTGCTTTGCAGGTTTTC-3′ (antisense) (GenBank: NM000603) (Binion et al., 1998); iNOS, 5′-CGCCAAGAACGTGTTCACCA-3′(sense) and 5′-AGCAGGCACACGCAATGATG-3′ (antisense) (GenBank: AF051164) (Reuter et al., 2001); GAPDH, 5′-CCCTTCATCGACCTCAACTACATGGT-3′ (sense) and 5′-GAGGGCCATCCACAGTCTTCTG -3′ (antisense) (GenBank: BC020308) (Esposito et al., 2002). The reverse transcription step was performed at 50 °C for 30 min. The PCR amplification was run for 30 cycles as follows; denaturation at 94 °C for 90 s, annealing at 58 °C for 90 s, and extension at 72 °C for 90 s. Final extension step was carried out at 72 °C for 10 min. The PCR products were separated by 1% agarose gel and stained with ethidium bromide. The relative expression levels were determined by densitometry.

4.4. eNOS Western blot analysis

Cells were harvested by washing twice with PBS and solubilized in the lysis buffer (100 mM Tris, 1 mM EDTA, 0.2% Triton-X100), supplemented with 1% protease inhibitor cocktail (Sigma). After determination of protein content using Bio-Rad protein assay reagent, protein samples were separated by 6% SDS-PAGE, transferred to PVDF membrane sheets (Schleicher & Schuell, Dassel, Germany), and blocked with 5% non-fat dry milk in PBS-Tween 0.1%. The membrane was then incubated with anti-eNOS antibodies (Santa Cruz Biotechnology Inc., CA, USA) at 4 °C overnight. After washing with wash buffer (0.1% Tween 20 in PBS), the membrane was incubated with peroxidase-conjugated anti-mouse IgG (Zymed, CA, USA) for 1 h at room temperature. The membrane was then washed several times with wash buffer. The immunoreactivity on the protein bands was detected by Opti-4CN kit (Bio-Rad) and quantified by a densitometer.

4.5. Statistical analysis

Each data point was performed in duplicate or triplicate and in at least three separate experiments. Statistical analysis between two groups was performed with unpaired Student *t*-test using SigmaStat Software (Jandel Scientific Inc., San Rafael, CA). Probability values of $p < 0.05$ is considered to be significant.

5. Results

5.1. KP increased NO production

Shown in Fig. 1 is the effect of KP extract on endothelial nitric oxide production evaluated by Griess reaction. KP extract was co-incubated with HUVEC for 48 h and total nitrite concentrations were determined in the cell culture media. Acetylcholine (ACH) and L-NAME were used as positive and negative controls of endothelial-dependent NO production, respectively. ACH (10–1000 μM) dose-dependently increased nitrite concentrations in HUVEC culture media while L-NAME (100 μM) significantly decreased NO production both in the presence and absence of ACH stimulation (Fig. 1). Although only one con-

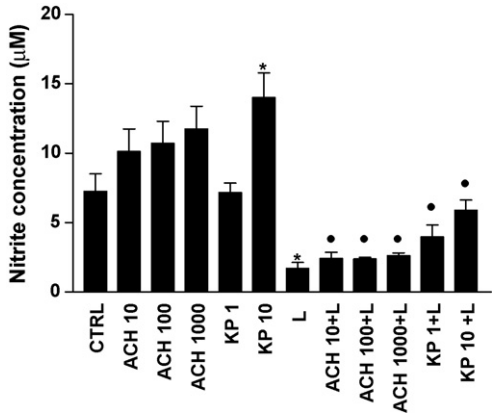

Fig. 1. Nitrite concentrations in HUVEC culture media. Nitrite contents were measured by Griess reaction in vehicle-treated cells (CTRL); acethylcholine-treated cells at concentrations 10 μM (ACH 10), 100 μM (ACH 100), and 1000 μM (ACH 1000); *Kaempferia parviflora* (KP) extract-treated cells at concentrations 1 μg/ml (KP 1) and 10 μg/ml (KP 10). Additional series of experiments, cells were co-incubated with 100 μM L-NAME (L). Data are mean ± S.E.M., $n = 6$; (*) $p < 0.05$ vs. CTRL; (●) $p < 0.05$ vs. corresponding treatments without L-NAME.

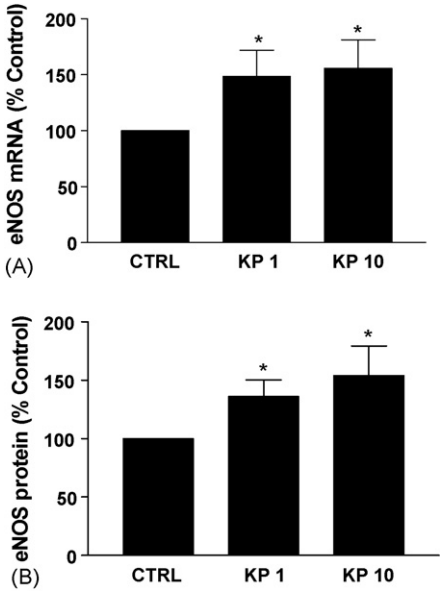

Fig. 2. Effects of *Kaempferia parviflora* (KP) extract on eNOS mRNA and protein expression. HUVEC were treated with vehicle (CTRL) or KP at the concentrations of 1 μg/ml (KP1) or 10 μg/ml (KP10). Cells were collected and relative eNOS mRNA expression was determine by RT-PRC method using GAPDH as internal standard. (A) KP extract (1 and 10 μg/ml) treatment for 48 h significantly increased eNOS mRNA and protein expression (*$p < 0.05$ vs. CTRL). (B) Following 48 h KP extract treatment, eNOS protein expression was determined by Western blot analysis as described in Section 4 (*$p < 0.05$ vs. CTRL).

centration of KP extract (10 μg/ml) significantly increased NO production this effect was inhibited by L-NAME.

5.2. KP enhanced eNOS mRNA and protein expression

The relative eNOS mRNA and protein expression was semiquantified by RT-PCR and western blot analysis, respectively. When assessed at 48 h, KP extract (1 and 10 μg/ml) significantly increased eNOS mRNA by 148 ± 23.5% and 155.76 ± 25.44%, respectively (Fig. 2A). Time-course study showed dose- and time-dependent increases in mRNA expression (data not shown). It appears that the activation of eNOS gene expression was as early as 4 h and plateau at 48 h. Similarly, eNOS protein expression was enhanced by KP extract (1 and 10 μg/ml) (136.07 ± 14.17% and 153.95 ± 25.49%, respectively) (Fig. 2B). The iNOS mRNA expression was not observed in controls and all KP treated cells (data not shown).

6. Discussion

It is well recognized that endothelial nitric oxide (NO) plays a significant role in maintaining normal vascular function and preventing cardiovascular disease (Landmesser and Drexler, 2005). The present study suggests that KP may improve endothelial function by the activation of NO production in association with eNOS mRNA and protein expression but not iNOS expression.

NO is a signaling molecule synthesized by three isoforms of NO synthases (NOS), i.e., nNOS (NOSI or NOS1), iNOS (NOSII or NOS2), and eNOS (NOSIII or NOS3). NO production from each NOS isoform is tightly controlled at the transcription, translation, and posttranslational levels (Kone et al., 2003). NO produced by eNOS is described as "low output" pathway whereas iNOS generates NO in a "high output" manner which causes cell or organ dysfunction and apoptosis (Alderton et al., 2001). Alterations of NOS isoform expression (particularly iNOS) and oversupply of NO is evident in some pathologic conditions (Burgner et al., 1999; Mungrue et al., 2002; Liu et al., 2005). Here we demonstrate that KP selectively enhanced eNOS expression. Thus, KP may potentially be used as a supplement for vascular endothelial health promotion.

The major phytoconstituents of KP are methoxyflavone derivatives. Some of them showed antiplasmodial and antifungal activities (Yenjai et al., 2004). However, it has not been tested whether these compounds may have additional pharmacological effects similar to methoxyflavones found in other plant species. For example, studies have shown that wogonin (5,7-dihydroxy-8-methoxyflavone) and chrysin (5-hydroxy-7-methoxyflavone) exerted anti-inflammatory effect by inhibiting iNOS, COX2, and proinflammatory cytokine production (Kim et al., 2001; Cho et al., 2004; Chun et al., 2005). The flavonoid isokaempferide (5,7,4′-trihydroxy-3-methoxyflavone) induces relaxation of guinea-pig isolated trachea (Leal et al., 2006). Thus, further studies of bioactive compounds from KP are warranted.

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References

Alderton, W.K., Cooper, C.E., Knowles, R.G., 2001. Nitric oxide synthases: structure, function and inhibition. Biochemical Journal 357, 593–615.

Binion, D.G., Fu, S., Ramanujam, K.S., Chai, Y.C., Dweik, R.A., Drazba, J.A., Wade, J.G., Ziats, N.P., Erzurum, S.C., Wilson, K.T., 1998. iNOS expression in human intestinal microvascular endothelial cells inhibits leukocyte adhesion. American Journal of Physiology 275, G592–G603.

Burgner, D., Rockett, K., Kwiatkowski, D., 1999. Nitric oxide and infectious diseases. Archives of Disease in Childhood 81, 185–188.

Cho, H., Yun, C.W., Park, W.K., Kong, J.Y., Kim, K.S., Park, Y., Lee, S., Kim, B.K., 2004. Modulation of the activity of pro-inflammatory enzymes, COX-2 and iNOS, by chrysin derivatives. Pharmacological Research 49, 37–43.

Chun, W., Lee, H.J., Kong, P.J., Lee, G.H., Cheong, I.Y., Park, H., Kim, S.S., 2005. Synthetic wogonin derivatives suppress lipopolysaccharide-induced nitric oxide production and hydrogen peroxide-induced cytotoxicity. Archives of Pharmacal Research 28, 216–219.

Esposito, G., Ligresti, A., Izzo, A.A., Bisogno, T., Ruvo, M., Di Rosa, M., Di Marzo, V., Iuvone, T., 2002. The endocannabinoid system protects rat glioma cells against HIV-1 Tat protein-induced cytotoxicity. Mechanism and regulation. Journal of Biological Chemistry 277, 50348–50354.

Granger, D.L., Taintor, R.R., Boockvar, K.S., Hibbs Jr., J.B., 1996. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. Methods in Enzymology 268, 142–151.

Kim, H., Kim, Y.S., Kim, S.Y., Suk, K., 2001. The plant flavonoid wogonin suppresses death of activated C6 rat glial cells by inhibiting nitric oxide production. Neuroscience Letters 309, 67–71.

Kone, B.C., Kuncewicz, T., Zhang, W., Yu, Z.Y., 2003. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. American Journal of Physiology Renal Physiology 285, F178–F190.

Landmesser, U., Drexler, H., 2005. The clinical significance of endothelial dysfunction. Current Opinion in Cardiology 20, 547–551.

Leal, L.K., Costa, M.F., Pitombeira, M., Barroso, V.M., Silveira, E.R., Canuto, K.M., Viana, G.S., 2006. Mechanisms underlying the relaxation induced by isokaempferide from Amburana cearensis in the guinea-pig isolated trachea. Life Science 79, 98–104.

Liu, Y.H., Carretero, O.A., Cingolani, O.H., Liao, T.D., Sun, Y., Xu, J., Li, L.Y., Pagano, P.J., Yang, J.J., Yang, X.P., 2005. Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction. American Journal of Physiology Heart Circulation Physiology 289, H2616–H2623.

Mungrue, I.N., Husain, M., Stewart, D.J., 2002. The role of NOS in heart failure: lessons from murine genetic models. Heart Failure Review 7, 407–422.

Reuter, U., Bolay, H., Jansen-Olesen, I., Chiarugi, A., Sanchez del Rio, M., Letourneau, R., Theoharides, T.C., Waeber, C., Moskowitz, M.A., 2001. Delayed inflammation in rat meninges: implications for migraine pathophysiology. Brain 124, 2490–2502.

Rujjanawate, C., Kanjanapothi, D., Amornlerdpison, D., Pojanagaroon, S., 2005. Anti-gastric ulcer effect of *Kaempferia parviflora*. Journal of Ethnopharmacology 102, 120–122.

Wagner, D.D., Olmsted, J.B., Marder, V.J., 1982. Immunolocalization of von Willebrand protein in Weibel–Palade bodies of human endothelial cells. Journal of Cell Biology 95, 355–360.

Wattanapitayakul, S.K., Chularojmontri, L., Herunsalee, A., Charuchongkolwongse, S., Niumsakul, S., Bauer, J.A., 2005. Screening of antioxidants from medicinal plants for cardioprotective effect against doxorubicin toxicity. Basic and Clinical Pharmacology and Toxicology 96, 80–87.

Yenjai, C., Prasanphen, K., Daodee, S., Wongpanich, V., Kittakoop, P., 2004. Bioactive flavonoids from *Kaempferia parviflora*. Fitoterapia 75, 89–92.