Endothelium-independent vasodilation induced by kolaviron, a biflavonoid complex from *Garcinia kola* seeds, in rat superior mesenteric arteries

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Abstract

Previous studies have established the hepatoprotective, gastroprotective, hypolipidemic and hypoglycemic effects of kolaviron (KV), a biflavonoid complex from *Garcinia kola* seeds. In this study, we investigated the mechanisms involved in the vasorelaxant effects of KV in isolated superior mesenteric arteries from normotensive rats. KV (1, 10, 30, 100, 300, 500 and 1,000 $\mu\text{g/ml}$) concentration-dependently inhibited the contractions induced by phenylephrine (PHE) (10 μM) and KCl (80 mM) in both endothelium-intact ($E_{\text{max}} = 58.3 \pm 1.7\%$ and $51.4 \pm 1.3\%$, respectively) and -denuded rings ($E_{\text{max}} = 59.3 \pm 5.5\%$ and $64.3 \pm 2.4\%$, respectively). Furthermore, KV reduced CaCl_2 -induced contraction in Ca^{2+} -free medium containing KCl 60 mM, thus acting as a Ca^{2+} -antagonist. In addition, KV inhibited the transient contraction by PHE in Ca^{2+} -free medium containing EGTA, suggesting a possible action on the release of intracellular Ca^{2+} via the inositol-1,4,5-triphosphate (IP_3) pathway. KV is not a specific α -adrenoceptor blocker, since it also caused a concentration-dependent inhibition of contractile responses to KCl, suggesting that KV also blocks the L-type Ca^{2+} -channel. As a Ca^{2+} antagonist, KV (100 $\mu\text{g/ml}$) potentiates the relaxant effects of nifedipine in denuded rings ($E_{\text{max}} = 97.6 \pm 1.2\%$; control = $75.1 \pm 3.0\%$, $P < 0.05$). Also, the vasorelaxation induced by KV was significantly inhibited after pre-treatment of the denuded rings with 4-aminopyridine (4-AP) 1 mM, a selective blocker of voltage-dependent K^+ (K_v) channels and, tetraethylammonium (TEA) 1 mM or charybdotoxin (ChTX) 0.1 μM , non-selective blockers of large and intermediate conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels. In contrast, neither glibenclamide (10 μM), BaCl_2 (1 mM) nor apamin (0.1 μM), blockers of K_{ATP} , K_{IR} and SK_{Ca} channels, respectively affected the KV-induced vasorelaxation. In conclusion, our results provide functional evidence that the vasorelaxant effects by KV involve extracellular Ca^{2+} influx blockade, inhibition of intracellular Ca^{2+} release and the opening of K^+ channels sensitive to 4-AP and ChTX with a resultant membrane hyperpolarization/ repolarization.

Key words: Ca^{2+} influx, endothelium-independent, *Garcinia kola*, mesenteric artery, vasodilation

Introduction

Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in alleviating oxidative stress-induced pathologies (Lans *et al.*, 2007; Lee *et al.*, 2007). Several studies revealed that medicinal plants contain diverse classes of bioactive compounds such as polyphenols, tocopherols, alkaloids, *etc.* (Gordana *et al.*, 2004; Lee *et al.*, 2007). Among them, flavonoids are particularly attractive as they are known to exhibit various pharmacological properties such as vasoprotection, anti-carcinogenic, anti-microbial, anti-inflammatory and anti-proliferative effects (Gordana *et al.*, 2004). The health benefits of flavonoids have been linked to their actions as antioxidants, free radical scavengers, quencher of singlet and triplet oxygen and inhibitors of peroxidation reactions (Li-chem *et al.*, 2006). *Garcinia kola* Heckel (Family; *Guttiferae*) is a herb grown in Nigeria with a characteristic astringent, bitter and resinous taste. This seed, also called “bitter kola” is eaten raw by the people with the belief that it promotes longevity. Extracts of the plant are used in traditional African medicine for the treatment of laryngitis, cough and liver diseases (Iwu and Igboko, 1982). Chemical investigations of the seed revealed the presence of *Garcinia* biflavanone (GB), xanthones, triterpenes and benzophenones (Cotterhill *et al.*, 1978). The biflavanones are the most dominant in most *Garcinia* species (Waterman and Hussain, 1983). Kolaviron (KV), the predominant constituent in *Garcinia kola* is a biflavonoid complex that has been reported to prevent hepatotoxicity mediated by several toxins (Iwu *et al.*, 1987; Adaramoye and Adeyemi, 2006a). Likewise, KV is known to exhibit hypoglycemic effects in normal, alloxan and streptozotocin-diabetic animals (Iwu *et al.*, 1990; Adaramoye and Adeyemi, 2006b). Also, KV has been reported to elicit strong antioxidant activity in both *in vivo* and *in vitro* experimental models (Adaramoye *et al.*, 2005a). In a preliminary study, we demonstrated that KV elicited hypocholesterolemic effects and reduced the relative weight of the heart in cholesterol fed animals (Adaramoye *et al.*, 2005b), thus providing a background to explore the possible cardiovascular effects of this biflavonoid complex in rats. Accordingly, this study was designed to investigate the mechanisms involved in the vasorelaxant effects induced by KV in isolated rat superior mesenteric arteries.

Methods

Animals

Male Wistar rats (250–300 g) were used for all experiments. The animals were kept in well-ventilated cages at controlled temperature ($21 \pm 1^\circ\text{C}$) and under controlled light cycles (12 h light/12 h dark). They were maintained on normal laboratory chow (PURINA-Brazil) and tap water *ad libitum*. The study was approved by the Committee of Ethics for Animal Research (Comitê De Ética Em Pesquisa Animal) CEPA N° 0805/07, Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba, João Pessoa, Brazil. The protocols conform to the 1985 guidelines for laboratory animal care of the National Institute of Health (NIH).

Drugs

The drugs used were: L-phenylephrine hydrochloride, acetylcholine hydrochloride, Tween-80, nifedipine, barium chloride, tetraethylammonium, glibenclamide, charybdotoxin, apamin, 4-aminopyridine, caffeine, ethyleneglycol bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma Chemical Co., Saint Louis, MO, USA). Chemicals used for preparing Tyrode's solutions are; calcium chloride, glucose, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate and sodium chloride (E. Merck, Darmstadt, Germany). The stock solutions were dissolved in distilled water, except nifedipine and glibenclamide that were dissolved in ethanol. The solutions were prepared fresh on the day of experiments.

Extraction of kolaviron (KV)

Garcinia kola seeds were obtained commercially in Ibadan, Nigeria and certified at the herbarium in the Department of Botany, University of Ibadan, Nigeria, where a voucher specimen already exists (UI-00138/01). Three kilogram of peeled seeds was sliced, pulverized with an electric blender and air-dried in the laboratory (25–28°C). Extraction of KV was achieved by the methods of Cotterhill *et al.* (1978) and Iwu *et al.* (1990). Briefly, powdered seeds were extracted with light petroleum ether (bp 40–60°C) in a soxhlet extractor. The defatted, dried marc was repacked and then extracted with methanol. The extract was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate (6 $\times$ 250 ml). The concentrated ethyl acetate fraction gave a yellow solid known as kolaviron (KV) (Fig. 1) with a percentage yield of 6%. KV was dissolved in 2–3 drops of tween-80 and diluted to desire concentrations with distilled water to give a water-soluble fraction. The drug solutions were prepared fresh on the day of experiments.

Preparation of isolated rat superior mesenteric artery rings

The superior mesenteric arteries from the second order branches were quickly removed and cleaned of adherent connective tissues and fat. Mesenteric rings (2–4 mm length) were obtained and suspended by cotton threads in organ bath containing 10 ml Tyrode's solution, maintained at 37°C and gassed with a 95% O₂ + 5% CO₂ mixture (pH 7.4). The composition of the Tyrode's solution (in mM): NaCl 158.3; KCl 4.0; CaCl₂·2H₂O 2.0; MgCl₂·6H₂O 1.05; NaHCO₃ 10.0; NaH₂PO₄·H₂O 0.42 and Glucose 5.6 (Tanaka *et al.*, 1999). Rings were stabilized with a resting tension of 0.75 g, for at least 60 min, with constant changing of Tyrode's solution (every 15 min) to prevent the accumulation of metabolites that could otherwise lead to misinterpretation of results (Altura and Altura, 1970). The isometric tension was recorded by a force-displacement transducer (Miobath-4, WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Transbridge-4; WPI, Sarasota, FL, USA) and a computer equipped with an analog-to-digital converter board (AD16JR; WPI, Sarasota, FL, USA). In some experiments, the endothelium layer of the rings was removed by gently rubbing the external surface with a finger moistened with Tyrode's solution. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (10 μ M) in the presence of contractile tone induced by phenylephrine (PHE, 10 μ M). Rings were considered to be endothelium-denuded when acetylcholine-induced relaxant effects were less than 10% and, endothelium-intact when the

	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Fig. 1. Structure of kolaviron.

relaxant effects were more than 90%.

Effect of KV on sustained contractions induced by phenylephrine or KCl in isolated preparations from rat superior mesenteric arteries

After equilibration, steady tension was evoked by PHE (10 μ M) for endothelium-intact and -denuded rings to induce contraction of similar magnitude and KV was added cumulatively (1, 10, 30, 100, 300, 500 and 1,000 μ g/ml). The ability of KV to attenuate the 80 mM KCl-induced sustained contraction in the rings was also examined. For comparison, the vasorelaxant effect of nifedipine (10^{-14} – 10^{-5} M), a blocker of voltage-dependent Ca^{2+} channels (Priviero *et al.*, 2006), was also evaluated against the contractions induced by PHE or KCl, in both, endothelium-intact and -denuded rings. Furthermore, the ability of KV (10, 30 and 100 μ g/ml) to ameliorate the vasorelaxant effects of nifedipine (10^{-14} – 10^{-5} M) in endothelium denuded rings pre-contracted with PHE was studied. The relaxations were measured by comparing the developed tension before and after addition of KV or nifedipine.

Investigation of the role of K⁺ channels in the KV-induced vasorelaxant response in isolated rat superior mesenteric arteries

In another set of experiments, the rings without endothelium were pre-contracted with PHE (10 μ M) for 30 min after being pre-incubated with one of the following inhibitors: glibenclamide (10 μ M), selective blocker of K_{ATP} channels; BaCl₂ (1 mM), selective blocker of K_{IR} channels; tetraethylammonium (TEA) (1 mM) or charybdotoxin (ChTX) 0.1 μ M, non-selective blockers of BK_{Ca} channels, 4-aminopyridine (4-AP) (1 mM), a selective blocker of K_v channels, ouabain (100 μ M), a selective inhibitor of Na⁺- K⁺ ATPase and apamin (0.1 μ M), a specific blocker of SK_{Ca} channels. The concentration used for each blocker/inhibitor of K⁺ channels is sufficient to antagonize selectively those channels in arterial smooth muscle (Nelson and Quayle, 1995; Lagaud *et al.*, 1999; Cortes *et al.*, 2001; Oliveira *et al.*, 2006). After stabilization of the tonic contraction induced by PHE, increasing cumulative concentrations of KV (1, 10, 30, 100, 300, 500 and 1,000 μ g/ml) were added to the organ bath. The concentrations were chosen on the basis of previous studies in our laboratories (Santos *et al.*, 2006; Santos *et al.*, 2007).

Effect of KV on sustained contractions induced by CaCl₂, and Ca²⁺ release from intracellular stores sensitive to phenylephrine and caffeine

To further investigate the mechanism of vasorelaxation induced by KV, concentration-response curves to CaCl₂ were constructed using endothelium-denuded rings (Lagaud *et al.*, 1999). Briefly, the rings were pre-contracted with 60 mM KCl to confirm tissue viability. The Tyrode's solution was replaced with depolarizing Tyrode's solution (KCl 60 mM) nominally without Ca²⁺ (15 min). Thereafter, concentration-response curves to CaCl₂ (1 μ M–10 mM) were constructed in the absence or presence of KV. To determine whether KV could interfere with Ca²⁺ release from intracellular stores, the denuded rings were pre-contracted with KCl, washed and exposed to Ca²⁺-free Tyrode's solution containing EGTA (1 mM). The rings were then stimulated with phenylephrine (10 μ M) or caffeine (20 mM) (Hugdins and Weiss, 1968). The contractions of both agonists were obtained in the absence (control) or after incubation with KV.

Statistical analysis

Two pharmacological parameters were analyzed in this study; $E_{\max}$ (Maximal effect generated by agonist) and pD_2 (-log EC₅₀). Values are expressed as means $\pm$ standard error of the mean (S.E.M.). Statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's *t*-test using GraphPad Prism TM 4.0 version software, San Diego, CA, USA. Post hoc comparisons were performed after ANOVA using Dunnett's test as indicated in the text. The level of significance considered in all the tests was 0.05 ($P < 0.05$).

Results

Effect of KV on sustained contractions induced by phenylephrine or KCl

KV inhibited the sustained contractions induced by phenylephrine (PHE, 10 μ M) in isolated rat mesenteric arterial rings in a concentration dependent manner. The pD_2 and $E_{\max}$ values of KV for contractions induced by PHE were 2.13 ± 0.06 and $58.3 \pm 1.7\%$ for endothelium-intact and,

Fig. 2. Relaxation responses induced in isolated rat mesenteric arteries; (A) by kolaviron (KV) in endothelium-intact and -denuded rings pre-contracted with phenylephrine (PHE) (10 μM), (B) denuded rings pre-constricted with either KCl (80 mM), or pre-incubated with KCl (20 mM) before constricted with PHE, (C) intact rings pre-constricted with either KCl (80 mM), or pre-incubated with KCl (20 mM) before constricted with PHE, (D) by nifedipine in endothelium intact and denuded rings pre-contracted with KCl or PHE (E).

Table 1. Effect of TEA (1 mM), glibenclamide (10 μ M), BaCl₂ (1 mM), charybdotoxin (0.1 μ M), KCl (20 mM), 4-AP (1 mM), ouabain (100 μ M) and apamin (0.1 μ M) on $E_{\max}$ (%) and pD_2 values of kolaviron (KV)-induced relaxant responses in endothelium-denuded mesenteric rings pre-contracted with phenylephrine (10 μ M)

Groups	$E_{\max}$ (%)	pD_2
Control	59.3 $\pm$ 5.5	2.48 $\pm$ 0.05
KCl (20 mM)	39.0 $\pm$ 1.9*	1.95 $\pm$ 0.06*
TEA (1 mM)	35.4 $\pm$ 1.7*	1.84 $\pm$ 0.02*
Glibenclamide (10 μ M)	53.8 $\pm$ 9.2	2.37 $\pm$ 0.05
BaCl ₂ (1 mM)	52.9 $\pm$ 3.5	2.26 $\pm$ 0.06
Charybdotoxin (0.1 μ M)	39.0 $\pm$ 1.7*	2.01 $\pm$ 0.05*
4-AP (1 mM)	43.5 $\pm$ 1.4*	2.29 $\pm$ 0.03
Charybdotoxin + 4-AP	35.4 $\pm$ 1.8*	1.86 $\pm$ 0.04*
Ouabain (100 μ M)	56.1 $\pm$ 1.4	2.20 $\pm$ 0.04
Apamin (0.1 μ M)	55.7 $\pm$ 1.7	2.25 $\pm$ 0.03

Values are the Mean $\pm$ S.E.M., n=6–8 experiments. *Significantly different from control ($P<0.05$). ANOVA followed by Dunnett's Multiple Comparison Test.

2.48 $\pm$ 0.05 and 59.3 $\pm$ 5.5%, for denuded rings, respectively (Fig. 2A). The pD_2 and $E_{\max}$ values of KV for PHE-induced contractions did not differ significantly in endothelium-intact from denuded rings. Similarly, no significant difference ($P>0.05$) was found in the pD_2 and $E_{\max}$ values of KV for KCl-induced contractions in endothelium-intact (2.09 $\pm$ 0.04 and 57.4 $\pm$ 2.3%) when compared with denuded (2.14 $\pm$ 0.05 and 64.3 $\pm$ 2.4%) rings, respectively (Figs. 2B and 2C). The effect of the solvent (vehicle) used to prepare KV was insignificant with $E_{\max}$ value of 6.3 $\pm$ 1.1% (Fig. 2A). Nifedipine significantly relaxed KCl-induced tonic contraction in intact and denuded rings (pD_2 and $E_{\max}$ values = 9.23 $\pm$ 0.08 and 92.0 $\pm$ 2.1%, 9.58 $\pm$ 0.07 and 95.6 $\pm$ 1.5%, respectively), but its relaxation of PHE-induced contraction was lesser (pD_2 and $E_{\max}$ values = 8.89 $\pm$ 0.10 and 77.3 $\pm$ 0.9%, 8.74 $\pm$ 0.11 and 75.1 $\pm$ 3.0%, for intact and denuded rings respectively) (Figs. 2D and 2E).

Effect of KV (10, 30 and 100 μ g/ml) on nifedipine-induced vasorelaxant response in denuded rings

As shown in Fig. 3A and Table 2, nifedipine (10⁻¹⁴–10⁻⁵ M) concentration-dependently relaxed PHE-induced contraction of endothelium-denuded rings ($E_{\max}$ and pD_2 values = 75.1 $\pm$ 3.0% and 8.74 $\pm$ 0.11, respectively). Furthermore, the relaxation induced by nifedipine remains unaltered when denuded rings were pre-incubated with KV at 10 μ g/ml ($E_{\max}$ and pD_2 values = 79.3 $\pm$ 1.0% and 8.51 $\pm$ 0.04, respectively) and, KV at 30 μ g/ml ($E_{\max}$ and pD_2 values = 80.2 $\pm$ 2.0% and 8.54 $\pm$ 0.09, respectively). However, KV at 100 μ g/ml shifted the concentration-response curve to nifedipine to the left with significant increases ($P<0.05$) in $E_{\max}$ and pD_2 values (97.6 $\pm$ 1.2% and 9.49 $\pm$ 0.02, respectively) (Fig. 3B and Table 2).

Effect of K⁺ channel blockers on the vasorelaxant response induced by KV

In denuded rings pre-contracted with PHE in the presence of KCl 20 mM, the concentration-response curve for KV was significantly shifted to the right with decreased $E_{\max}$ and pD_2 values

Fig. 3. Effects of kolaviron (KV) (10 and 30 $\mu\text{g/ml}$) (A) and, 100 $\mu\text{g/ml}$ (B) pre-incubated for 30 min on nifedipine-induced relaxant responses in denuded mesenteric rings. Steady tension was evoked by phenylephrine (10 μM) and nifedipine (10^{-14} – 10^{-5} M) was added cumulatively. Control, represents rings that were relaxed by nifedipine in the absence of KV. *, Significantly different from control ($P < 0.05$), ANOVA followed by Dunnett's multiple comparison test.

= $39.0 \pm 1.9\%$ and 1.95 ± 0.06 , respectively when compared to the control ($59.3 \pm 5.5\%$ and 2.48 ± 0.05 , respectively) (Fig. 2B and Table 1), indicating the involvement of K^+ channels in the vasorelaxant effects of KV. Furthermore, in denuded rings pre-incubated with TEA (1 mM), ChTX (0.1 μM), 4-AP (1 mM) and ChTX + 4-AP before contracted with PHE, produced a rightward displacement of the concentration-response curves for KV with decreased E_{max} and pD_2 values = $35.4 \pm 1.7\%$ and 1.84 ± 0.02 , $39.0 \pm 1.7\%$ and 2.01 ± 0.05 , $43.5 \pm 1.4\%$ and 2.29 ± 0.03 , $35.4 \pm 1.8\%$ and 1.86 ± 0.04 , respectively (Fig. 4 and Table 1). On the other hand, glibenclamide

Table 2. Effect of pre-incubation of KV (10, 30 and 100 $\mu\text{g/ml}$) on the E_{max} and pD_2 values of nifedipine-induced relaxant responses in denuded rat mesenteric rings pre-contracted with phenylephrine (10 μM)

Groups	E_{max} (%)	pD_2
Control	75.1 ± 3.0	8.74 ± 0.11
After KV (10 $\mu\text{g/ml}$)	79.3 ± 1.0	8.51 ± 0.04
After KV (30 $\mu\text{g/ml}$)	80.2 ± 2.0	8.54 ± 0.09
After KV (100 $\mu\text{g/ml}$)	$97.6 \pm 1.2^*$	$9.49 \pm 0.02^*$

Values are the Mean $\pm$ S.E.M., n= 6 experiments. * Significantly different from control ($P<0.05$). ANOVA followed by Dunnett's Multiple Comparison Test.

(10 μM), BaCl_2 (1 mM), apamin (0.1 μM) and ouabain (100 μM) did not alter the vasorelaxant effects of KV in the denuded rings (Fig. 4).

Effect of KV on CaCl_2 -induced contractile response and Ca^{2+} release from intracellular stores in endothelium denuded rings

In Fig. 5, pre-incubation of endothelium denuded rings with KV attenuated CaCl_2 -induced contraction in Ca^{2+} -free medium containing KCl 60 mM. CaCl_2 -induced a concentration-dependent contraction of mesenteric rings ($E_{\text{max}} = 100\%$; $pD_2 = 3.45 \pm 0.05$, control, Table 3). Pre-incubation of the rings with KV at 1, 10, 30, 100, 300, 500 and 1,000 $\mu\text{g/ml}$ altered the concentration-response curves of CaCl_2 , but with significant effects ($P<0.05$) at any concentrations of KV (Fig. 5). The effects of KV on the E_{max} and pD_2 values for CaCl_2 -induced contractions are given in Table 3. Figure 6 shows that KV significantly ($P<0.05$) antagonized the transient contractions induced by PHE in endothelium denuded rings in Ca^{2+} -free media containing EGTA (1 mM). In contrast, KV produced no significant effect ($P>0.05$) on the transient contractions induced by caffeine (20 mM) under similar conditions.

Discussion

The present study provides the first direct evidence that KV induces vasorelaxation in isolated rat blood vessels. Our findings indicate that KV elicits a concentration-dependent relaxation of rat superior mesenteric arteries that is mediated by the blockade of Ca^{2+} influx from extracellular medium as well as inhibition of Ca^{2+} release from intracellular stores sensitive to phenylephrine. In addition, the vasorelaxant effect of KV is linked to the opening of K^+ channels sensitive to 4-aminopyridine and charybdotoxin.

The pD_2 and E_{max} values of KV for inhibiting the PHE or KCl induced contractions of rat mesenteric arteries with or without functional endothelium are similar, which suggest that the vasorelaxant effects of KV are independent of endothelium. Furthermore, the pD_2 and E_{max} values of nifedipine on intact or denuded rings pre-contracted with PHE or KCl were higher than with KV, indicating that nifedipine may be a better vasorelaxant agent. We investigated the possible link between KV-induced vasorelaxation and the inhibition of Ca^{2+} influx from the extracellular medium. We observed that CaCl_2 -induced contractions in Ca^{2+} -free media

Fig. 4. Relaxation responses induced by kolaviron (KV) on denuded rat mesenteric arterial rings pre-contracted with phenylephrine in the presence of tetraethylammonium (TEA) (1 mM) (A), charybdotoxin (ChTX) (0.1 μ M) (B), ChTX + 4-aminopyridine (4-AP) (1 mM) (C), glibenclamide (10 μ M) and BaCl_2 (1 mM) (D), 4-AP (1 mM) (E) and, ouabain (100 μ M) and apamin (0.1 μ M) (F). The rings were pre-incubated with the inhibitors for 30 min. Steady tension was evoked by phenylephrine (10 μ M) and KV (1, 10, 30, 100, 300, 500 and 1,000 μ g/ml) was added cumulatively. *, Significantly different from control ($P < 0.05$), ANOVA followed by Dunnett's multiple comparison test.

containing KCl 60 mM were significantly inhibited in a concentration-dependent manner by KV. The rightward shift in the concentration-response curves for CaCl_2 with a decrease in the E_{max} values, indicates that the biflavonoid behaves as a calcium antagonist. These results support the notion that KV can block Ca^{2+} influx through Ca^{2+} channels present in the vascular smooth

Fig. 5. Effect of kolaviron (KV) on CaCl_2 -induced contractile response in endothelium-denuded mesenteric arterial rings. Concentration-response curves for CaCl_2 were determined in Ca^{2+} -free solution containing KCl (60 mM). The curves were determined in the absence (control) and after incubation with KV (1, 10, 30, 100, 300, 500 and 1,000 $\mu\text{g/ml}$). *, Significantly different from control ($P < 0.05$), ANOVA followed by Dunnett's multiple comparison test.

Table 3. Effect of KV on E_{max} and pD_2 for CaCl_2 -induced contraction in endothelium denuded mesenteric rings in Ca^{2+} -free medium

KV ($\mu\text{g/ml}$)	pD_2	E_{max} (%)
Control	3.45 ± 0.05	100
1	3.31 ± 0.03	98.1 ± 2.9
10	3.56 ± 0.04	88.1 ± 4.2
30	3.24 ± 0.04	83.8 ± 3.2
100	$2.80 \pm 0.06^*$	$74.2 \pm 2.4^*$
300	$2.63 \pm 0.09^*$	$61.3 \pm 3.2^*$
500	$2.33 \pm 0.07^*$	$57.7 \pm 1.9^*$
1000	$2.01 \pm 0.11^*$	$47.8 \pm 1.6^*$

Values are the Mean $\pm$ S.E.M., $n=42$ for control; $n=6$ for others. *Significantly different from control ($P < 0.05$). ANOVA followed by Dunnett's Multiple Comparison Test.

muscle cells (VSMCs). However, this behaviour does not rule out the possibility that KV may reduce the sensitivity of the contractile filaments to Ca^{2+} . Contractions of VSMCs induced by KCl rely almost exclusively on Ca^{2+} influx through activation of voltage-sensitive channels

* Significantly different from control ($p < 0.05$)

(ANOVA followed by Dunnett's multiple comparison test)

PHE = Phenylephrine (10 μ M); CAF = Caffeine (20 mM)

Fig. 6. Effect of KV (1, 10, 30, 100, 300, 500 and 1,000 μ g/ml) on phenylephrine (10 μ M) and caffeine (20 mM) induced transient contractions in endothelium-denuded mesenteric arterial rings in Ca^{2+} -free medium containing EGTA (1 mM). *, Significantly different from control ($P < 0.05$), ANOVA followed by Dunnett's multiple comparison test.

(Hirata *et al.*, 1998), whereas contractions induced by PHE are mediated by an increase in Ca^{2+} influx through both receptor-operated channels (Lee *et al.*, 2001a) and voltage-sensitive channels (Eckert *et al.*, 2000; Lee *et al.*, 2001b). Since KV relaxed mesenteric rings precontracted with PHE and KCl (80 mM) with statistically similar E_{max} and pD_2 values, it could be suggested that the biflavonoid blocks Ca^{2+} influx through interference with both voltage- and receptor-operated channels. This led us to investigate whether KV could exert its vasorelaxant effects by interfering with the release of intracellular calcium, $[\text{Ca}^{2+}]_i$ via the phosphoinositide-dependent or -independent pathway following receptors activation. In Ca^{2+} -free media containing EGTA, the biflavonoid significantly decreased the transient contractions induced by PHE, which stimulates inositol-1,4,5-triphosphate (IP_3)-dependent Ca^{2+} release from intracellular stores (Leijten and van Breemen, 1984), whereas, the caffeine-induced contractions, which releases $[\text{Ca}^{2+}]_i$ by an (IP_3)-independent mechanism (Standen *et al.*, 1989) were unaltered. Thus, it seems likely that the vascular effects of KV involve a reduction in Ca^{2+} release from

intracellular stores sensitive to PHE. This result does not rule out the possibility that KV may interfere with other elements in the sarco-endoplasmic reticulum, especially the Ca^{2+} -ATPases. This observation needs further evaluation.

In order to analyze the contribution of different types of K^+ channels to the KV-induced endothelium-independent relaxation in the mesenteric rings, we used agents that are known to possess a K^+ channel-blocking activity. Glibenclamide is known to be one of the most selective blockers of K_{ATP} channels, but it can also block voltage-dependent K^+ (K_v) channels in concentrations higher than $10 \mu\text{M}$ (Schaffer *et al.*, 1999; Schuldt *et al.*, 2005). In the present study, glibenclamide did not produce any significant effect on concentration-response curves of KV, suggesting that K_{ATP} channels are not involved in the vasorelaxation induced by the biflavonoid. Similarly, neither apamin, BaCl_2 nor ouabain, blockers of SK_{ca} , K_{IR} channels and Na^+ - K^+ ATPase, respectively (Cortes *et al.*, 2001; Tirapelli *et al.*, 2004; Oliveira *et al.*, 2006) affected the concentration-response curves of KV, hence these channels and the ATPase are not involved in the vasorelaxant effects of KV. TEA and ChTX, at these concentrations are non-selective blockers of large and intermediate conductance Ca^{2+} -activated K^+ channels (BK_{ca}). Both TEA and ChTX significantly inhibited the concentration-response curves of KV, indicating that the opening of these channels is required for the vasorelaxation induced by the biflavonoid. Similarly, 4-AP, a selective blocker of voltage-dependent K^+ -channels significantly affected KV-induced relaxation, thus the activation of K_v channels also plays a role in KV-induced relaxation.

Clinically, Ca^{2+} antagonists and K^+ channels openers are used for the treatment of hypertension due to their ability to induce vascular smooth muscle relaxation. It is therefore possible to suggest, considering its vascular effects, that KV is a potential agent that could exert antihypertensive action *in vivo*. It should be noted that KV ($100 \mu\text{g}/\text{ml}$) potentiates the relaxant effect of nifedipine in denuded rings pre-contracted with PHE. This observation calls for a caution in the intake of *Garcinia kola* seeds, which contain KV, by hypertensive patients on Ca^{2+} channel blockers in order to avoid hypotension.

In summary, the vasorelaxation-induced by KV occurs through an endothelium-independent mechanism that involves the blockade of extracellular Ca^{2+} influx by interfering with both voltage- and receptor-operated channels. In addition, it inhibited Ca^{2+} release from intracellular stores via the IP_3 pathway and, opened BK_{ca} and K_v channels with a resultant membrane hyperpolarization/ repolarization. However, further studies are needed to evaluate the involvement of other K^+ channel-independent pathways in the vasorelaxation induced by KV.

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