

Research Article

Mechanism of kolaviron-induced relaxation of rabbit aortic smooth muscle.

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Keywords:

Kolaviron, Vascular smooth muscle, rabbit aorta, phenylephrine, high-K⁺

ABSTRACT

There is a considerable evidence linking kolaviron (KV), a biflavanoid-complex of *Garcinia kola* Heckel seed (gKola) to smooth muscle relaxation. The present study was designed to characterize the mechanism of kolaviron-induced relaxation on contractile responses in ring preparations of vascular smooth muscle (VSM) of the rabbit aorta in vitro, in standard laboratory organ bath procedure. Following Phenylephrine (PE) (10⁻⁷M), or high-K⁺ (80 mM K⁺) PSS induced contraction, KV (6, 12, 25, 50 100,200 & 400 µg/mL) was added cumulatively and relaxation responses determined in intact (+E) and endothelium-denuded (-E) aortic rings. Mechanism of KV-induced relaxation was further examined in PE or high-K⁺ precontracted +E and -E rings following 20 minutes exposures in methylene blue (MB) or ouabain (OB). To examine KV effect on extracellular Ca²⁺, tissues were exposed to a Ca²⁺-free 40 mM K⁺ depolarizing solution and Ca²⁺ as in CaCl₂ response curve constructed.

The results showed that KV causes concentration-dependent relaxation in VSM of the aorta and KV-induced relaxation was not significantly different in PE or high-K⁺ mediated responses. However, relaxation was significantly different and more potent in -E compared to +E rings in PE or high-K⁺ precontractions. KV-mediated relaxation was abolished in MB and OB incubated and precontracted rings. Ca²⁺- dependent contractions in K⁺- depolarized PSS was significantly attenuated by KV. Mechanisms of KV-induced relaxation in VSM rabbit aorta is non-specific but linked to interference in calcium exchange as well as guanylate cyclase enzyme and Na⁺-K⁺ ATPase cellular activity. Key words: Short running title: Mechanism of kolaviron-induced relaxation in VSM

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INTRODUCTION

Garcinia kola seed (gKola) also called bitter kola is known to be rich in biflavonoids complex with Kolaviron as active ingredient (Adaramoye and Medeiros, 2009, Adegboye et al., 2008, Iwu et al., 1987). The pharmacological properties and medicinal

values of gKola Heckel plant is widely reported and linked with various bioactive compounds with direct impact on organs-health. Previous studies have established the hepatoprotective, gastroprotective, hypolipidemic and hypoglycaemic effects of Kolaviron (KV) on experimental animals (Adaramoye and Adeyemi, 2006; Nwangwa, 2012; Udia et al., 2009). Recently, administration of kolaviron and sulfasalazine ameliorated Dextran-Sulphate-Sodium-induced colitis in rats by increasing antioxidant status, decreased hydrogen peroxide and lipid peroxidation levels (Falombi, 2013). Medicinal plants have been widely reported to contain varieties of health beneficiary bioactive compounds including: polyphenols, tocopherols, alkaloids, etc. (Gordana et al., 2004; Lee et al., 2007). The health benefits of flavonoids have

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been attributed to their actions as antioxidants, free radical scavengers, quencher of singlet and triplet oxygen and inhibitors of peroxidation reactions (Lichem *et al.*, 2006). Other phytochemical constituents include dimeric flavoid, xanthone, benzophenones and tocopherols (Alaba, 2007; Lee *et al.*, 2007).

There is considerable evidence linking kolaviron (KV), a biflavanoid-complex of *Garcinia kola* seed (gKola) to smooth muscle relaxation (Braide, 1989; Orié and Ekon; 1993; Adaramoye and Medeiros, 2009; Udia *et al.*, 2009). However, effects of gKola or kolaviron (KV) and the exert mechanisms of action on VSM of rabbit aorta have not been reported. The present study examines the vascular effect of kolaviron on VSM of rabbit aorta and the possible mechanism of its vasorelaxant effect.

MATERIALS AND METHODS

Extraction of Kolaviron (KV)

Garcinia Kola seeds were obtained commercially in Benin City, Nigeria and were certified by a taxonomist in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, where the herbarium sample with voucher number 13184 has been deposited. The seeds were dehulled, chopped into pieces, sundried to constant weight and pulverized using a mechanical grinder. Extraction of KV was achieved by the methods of Adaramoye and Medeiros (2009); and Iwu *et al.*, 1990). Briefly, 500g of the powder 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 x 250ml). The concentrated ethyl acetate fraction gave a yellowish solid known as kolaviron (49.79g). It was stored in a wide mouth air tight glass container to prevent absorption of moisture.

Tissue Preparation

Matured male and female rabbits were used throughout the experiment. All experimental procedures complied with the standard protocols for the use of laboratory animals (National Institute of Health USA, 2002). The study was approved by the Institutional ethical committee on the use of animals for experiments. The rabbits were sacrificed by cervical dislocation. The thoracic aorta was carefully dissected out and quickly removed, placed in Petri dish containing physiological salt solution (PSS) and freed of connective tissues, cut

into 2 mm ring segments and suspended between two L-shaped fine stainless steel holders in a 20 ml jacketed organ baths containing physiological salt solution (PSS) of the following composition (mM/L): NaCl 119.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 24.9, CaCl₂ 1.6, and glucose 11.5. The medium was bubbled continuously with 95% O₂ and 5% CO₂ gas mixture and maintained thermostatically at 37°C and pH7.4. Isometric contractions were recorded with force displacement transducers FT.03 connected to Grass model 79D polygraph (Grass instrument Co, Quincy, MA, USA) under an initial load of 1g. An equilibration period of 90 minutes was allowed before the commencement of the experimental protocols, during which ring segments were renewed with fresh prewarmed normal PSS at 20 minutes intervals (Ebeigbe and Aloamaka, 1987).

Experimental protocols

Concentration-response to phenylephrine (PE). After tissue recovery following pre-contraction with 80mMk⁺ to record the maximal contractile response. Concentration response to PE in arterial rings was examined in normal PSS by cumulative addition. Contractile responses were analysed with reference to maximal contractions induced by 80mM K⁺ in normal PSS

Vasorelaxant effect of kolaviron. Aortic rings were each precontracted with (EC₇₀) M (10⁻⁷M) phenylephrine (PE) or high-K⁺ (80 mM K⁺). When the contractions were stable, kolaviron (3.125 x 10⁻² - 4) M was added cumulatively. The resultant relaxation responses were expressed as percentage (%) of the initial tension.

Role of the endothelium: The role of vascular endothelium in the relaxation response induced by KV was studied in intact (+E) and endothelium denuded (-E) PE or high-K⁺ precontracted arterial rings. Endothelium removal was effected mechanically (Furchgott and Zawadzki, 1980) by gently rubbing the inner lining surface of the rings with a pair of forceps (Ebeigbe *et al.*, 1990). The effectiveness of de-endothelisation was confirmed by lack of relaxation response to 10⁻⁵M Acetylcholine (Ach) (Ebeigbe *et al.*, 1990; Olele *et al.*, 1998) in PE-precontracted endothelium-denuded arterial rings.

Effects of exposure to methylene blue and ouabain: The activities of soluble guanylate cyclase and Na⁺/K⁺ATPase enzymes were studied to further elucidate the possible mechanism of KV-induced relaxation in VSM of the aorta. Following 20 minutes exposure to 10⁻⁶M methylene blue or ouabain, +E and -

E rings were precontracted with PE or high-K⁺ and at stable contractions, kolaviron was cumulatively added to bath solution but no relaxation response was elicited by KV.

Effect of KV on response to restoration of calcium (Ca²⁺). In the experiment to study the effect of KV on extracellular calcium-dependent contractions arterial rings were first depleted of Ca²⁺ for 30 minutes (15 min nominal Ca²⁺-free and 15 min) period of exposure to a Ca²⁺-free 40 mM K⁺ depolarizing solution as previously described by Ebeigbe and Aloamaka, 1987: Ca²⁺ as (CaCl₂) was then added cumulatively and concentration-response curve was then constructed (Olele *et al.*, 1998, Ebeigbe and Aloamaka, 1987). In test arterial ring KV was applied 10 min before the end of Ca²⁺-free exposure and maintained throughout the duration of the protocol.

Drugs and solution

Ca²⁺-free PSS was prepared by omitting CaCl₂ from the normal PSS. In some experiments, Ca²⁺-free PSS contained 1.0mmol/L EGTA. Potassium free as well as high-K⁺ PSS were prepared by equimolar replacement of KCl by NaCl, or NaCl by KCl. Drugs used were: Acetylcholine Hydrochloride (Sigma, UK), L.phenylephrine hydrochloride (PE) (Sigma USA), histamine hydrochloride (HIST) (Sigma USA), ethyleneglycol bis(-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA)(Sigma Chemical Co; Saint Louis, MO, USA) tween-80, ouabain and methylene blue. Chemicals used for PSS are: calcium chloride, glucose, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate and sodium chloride (Sigma Chemical Co; Saint Louis, MO, USA). The solutions were prepared fresh on the day of experiments.

Data are presented as means ± SEM; Statistical analysis was performed using Student's t test and Microcal origin 8.0 statistical package.

P - Values less than 0.05 (P<0.05) were considered statistically significant; while n values denote number of animals studied. IC₅₀ (concentration producing 50% relaxation) values were derived graphically.

RESULTS

The maximal relaxation and IC₅₀ values (Table 1) for kolaviron-induced relaxation following PE or high-K⁺ contractions in intact (+E) was not significantly different, whereas in endothelium-denuded (-E) arterial rings, KV-induced relaxation was significantly (p<0.05) different.

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Table1.

Maximal contraction (E_{max}) values for 10⁻⁷M phenylephrine (PE) and high-K⁺ (80 mM K⁺) and maximal relaxation response (%), as well as IC₅₀ M (concentration producing 50% relaxant effect) values for kolaviron (KV)-induced relaxation. 0.09*, 0.27 ± 0.09*

Agent	Maximal contraction (g)	Maximal relaxation (%)	IC ₅₀ (M)
10 ⁻⁷ M PE	+E 1.73 ± 0.15 -E 1.82 ± 0.36	21.00 ± 2.26 30.07 ± 5.97	0.28 ± 0.04* 0.24 ± 0.08*
80 mM K ⁺	+E 1.83 ± 0.33 -E 1.92 ± 0.14	21.15 ± 2.01 32.40 ± 5.15	0.27 ± 0.09* 0.25 ± 0.09*

Effect of kolaviron on response to Ca²⁺ restoration.

In the contractile response to Ca²⁺ in 40mMK⁺-depolarized arterial rings as shown in Fig. 1, kolaviron addition caused a rightward shift of the Ca²⁺ concentration-dependent curve dose-dependently with a decrease in maximal tension. (N=6); means ± SEM.. Contractile responses are expressed as % of maximal contraction induced by Ca²⁺-free 40mMK⁺ depolarization.

Effect of kolaviron on response to Ca²⁺ restoration.

In the contractile response to Ca²⁺ in 40mMK⁺-depolarized arterial rings as shown in Fig.7, kolaviron addition to solution caused a rightward shift of the Ca²⁺ concentration-dependent curve dose-dependently with a decrease in maximal tension (n=6); means ± SEM; Contractile responses are expressed as % of maximal contraction induced by Ca²⁺-free 40mMK⁺ depolarization.

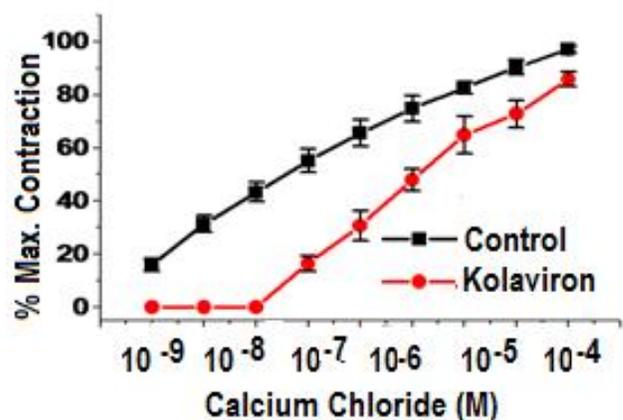


Fig. 1. Concentration response to Calcium in K⁺ depolarized rings. means ± SEM; n=e 6.

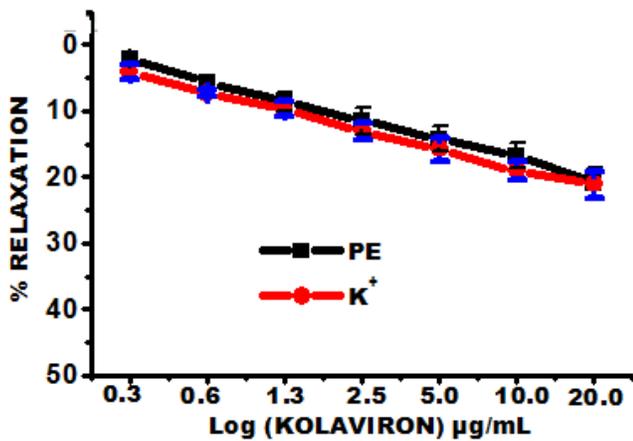


Fig. 2. Relaxation responses induced by kolaviron following pre-contraction induced by 10^{-7} M PE or high- K^+ . means \pm SEM; n=6. There was no significant difference between PE and high- K^+ curves.

In the experiment to examine the influence of vascular endothelium on KV-induced relaxation; Figures 3 shows relaxation response to cumulative addition of kolaviron in +E and -E rings following PE or 40mM K^+ -induced pre-contractions. (N=6); means \pm SEM. Values are expressed as percentage of contraction induced by PE or high- K^+ contractions. KV-induced relaxation was more in -E compared to +E rings.

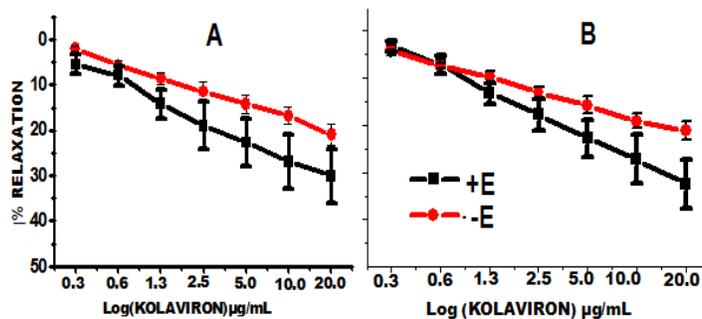


Fig. 3. Influence of endothelium on relaxation response to KV following PE (left) or high K^+ (right)-induced contraction in endothelium intact (+E) and denuded (-E) aortic rings. n = 6.

DISCUSSION AND CONCLUSION

In this present study to examine the effect and characterize the possible mechanism of kolaviron on rabbit aortic smooth muscle in vitro; we report that KV causes relaxation in vascular smooth muscle of rabbit aorta concentration-dependently in PE and high- K^+ -induced contractions. In the two modes of stimulation (PE and high- K^+) used in the study to characterise the

relaxant effect of KV, we observed that KV-induced relaxation was not significantly different in both PE and high- K^+ (80 mM k^+) contractions (figure 2) with no significant differences in maximal tension (E_{max}) of PE and high- K^+ (Table.1) suggests a similar mode of KV-mediated relaxant response in a non-specific interference with receptor-dependent and-independent Ca^{2+} -channels. Agonists-induced contractions of vascular smooth muscles can be clearly separable into intracellular- and extracellular Ca^{2+} -dependent components (Bohr, 1973 and Bolton, 1979). Previous work on smooth muscle reactivity has shown that sources of calcium in vascular smooth muscle contractions involves principally an extracellular pool, typically utilized by high- K^+ stimulation which involves membrane depolarization, eliciting calcium-influx through voltage-sensitive channels (VSCs) and an intracellular pool, typically mobilized by drugs (e.g. noradrenaline, phenylephrine, histamine, serotonin) which interact with specific receptors on the plasmalemma membrane and also stimulate Ca^{2+} - entry via receptor-operated channels (ROCs) (Kamm and Stull, 1985; Ebeigbe, 1982 Bolton, 1979; Lee et al., 2001).

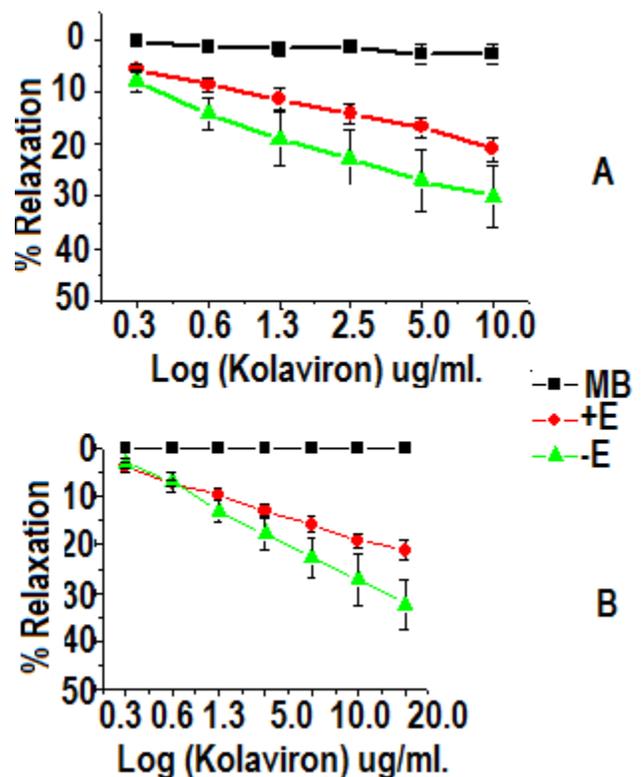


Fig. 4. Relaxation response to KV in PE (A) or high K^+ (B) pre-contracted +E or, δ E aortic rings following 20 minutes exposure to methylene blue (MB).

It is now well established that endothelial factors are of importance in the mediation and modulation of both vasoconstriction and vasodilatation (Sofola et al, 2002). However, the observation that the influence of vascular endothelium on KV-induced relaxation of PE and high- K^+ contractions occurred in both +E as well as -E arterial rings suggest that the influence of vascular endothelium on the relaxation response to KV is a non-specific interference with endothelial derived nitric oxide (EDNO)- and endothelium-derived hyperpolarising factor (EDHF). Nevertheless, we also report that KV-induced relaxation was more potent in endothelium denuded tissues compared to intact arterial rings in both PE and high- K^+ contractions (figures.3 & 4.) despite the significant enhancement in the magnitude of E_{max} of -E arterial rings; suggesting that KV-induced differential relaxation response is unconnected with altered tissue tension but decrease in responsiveness of KV to EDNO and EDHF release. The differential relaxation effect of endothelial removal on PE and high- K^+ -induced contractions has been reported by others in rat aorta where they observed that following endothelium removal, sensitivity to noradrenaline was enhanced; whereas that to K^+ was depressed: however, no explanation was provided for their observation (Ebeigbe and Aloamaka, 1987). The inhibition of KV-induced relaxation in +E and -E precontracted arterial rings in methylene blue and ouabain-incubation suggests a direct link of kolaviron relaxant action on VSM of rabbit aorta to soluble guanylate cyclase- nitric oxide synthase activation and Na^+/K^+ ATPase cellular activity. Methylene blue has been reported to inhibit both nitric oxide synthase and soluble guanylate cyclase in endothelium-dependent relaxation and the accompanying increase in cyclic GMP thus decreasing VSM relaxation due to reduction of cytosolic Ca^{2+} through activation of Ca-ATPase distributed in the membranes of internal stores (Ginimuge and Ryothi,2010; Karaki et al., 1997, Moncada et al.,1992, Ebeigbe et al., 1990, Martin et al., 1985, Holzmann, 1982) whereas ouabain, a cardiotonic steroid, inhibits Na/K -ATPase activity and/or interference with Na^+/Ca^{2+} exchanger not only in vascular tissue but in virtually all species (Lingrel, 2010). The likely increase in intracellular Na^+ caused by the inhibition of this enzyme resulted in an increase in intracellular Ca^{2+} through the Na^+/Ca^{2+} exchanger, which is also located in the plasma membrane (lingrel, 2010). Other investigators in hypertension had proposed that ouabain inhibition of Na^+/K^+ -ATPase activity in vascular tissues caused vasoconstriction and increase in blood pressure (Haddy, 1974; Lingrel

2010). In the experiment to examine kolaviron effect on extracellular calcium ion, following exposures to Ca^{2+} -free 40 mM K^+ depolarizing solution. Our result showed that calcium-induced contractile response was significantly attenuated concentration-dependently in the presence of kolaviron, which suggests extracellular calcium blockage and influx for utilization in intracellular smooth muscle contraction.

CONCLUSION

In conclusion, the present study shows that Kolaviron induces vasorelaxation in smooth muscle of rabbit aortic arterial blood vessel concentration-dependently. This inhibitory effect is non-specific as relaxation occurred in intact and endothelium-denuded aortic arterial blood vessel; which might suggest the use of gKola as a preferred ant-atherosclerotic and antihypertensive therapeutic agent. The mechanism of KV-induced relaxation in vascular smooth muscle of the aorta may be associated with calcium blockade and interference with soluble guanylate cyclase enzyme as well as Na^+-K^+ ATPase and /or Na^+-Ca^{2+} exchanger activity.

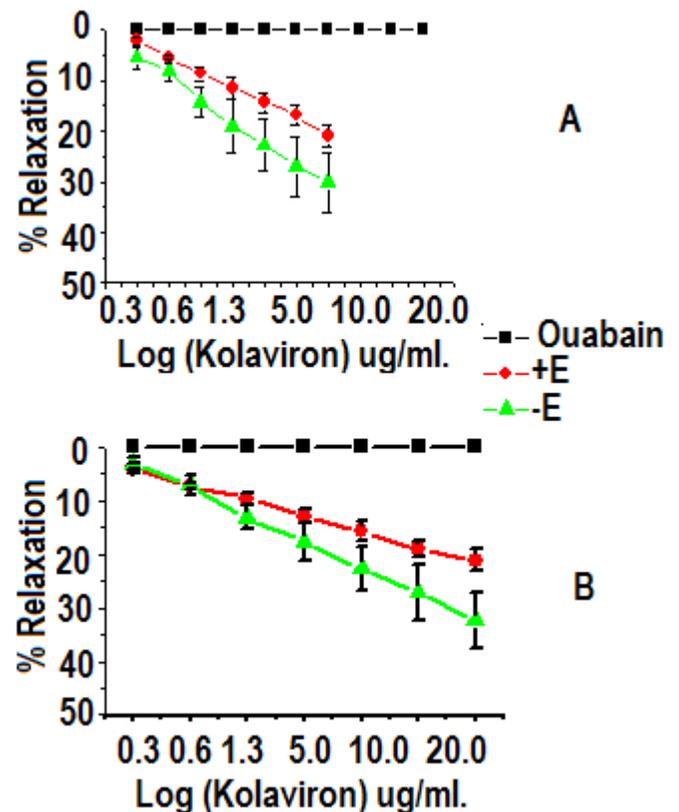


Fig. 5. Relaxation response to KV in PE (A) or high K^+ (B) precontracted +E or -E aortic rings following 20 minutes exposure to ouabain.

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