Kolaviron, Biflavonoid Complex from the Seed of Garcinia kola Attenuated Angiotensin II- and Lypopolysaccharide-induced Vascular Smooth Muscle Cell Proliferation and Nitric Oxide Production

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INTRODUCTION

Kolaviron (KV), a biflavonoid from Garcinia kola seed extract is reportedly known as the most active phytochemical present in Garcinia kola seed.[9,10] KV has been extensively reported for its various pharmacological and medicinal properties including radio-protective, protection against reproductive toxicant, hypoglycaemic, hypolipidemic, and gastro protective.[12,13] The chemopreventive potentials and medicinal properties of Garcinia kola and Kolaviron have also been documented elsewhere.[11-13]

Cardiovascular disease condition involves various processes that lead to the release of mitogenic agents which can act under favourable conditions to generate free radicals as well as activate and propagate inflammatory processes manifesting in various cardiovascular diseases such as hypertension, stroke, diabetes etc.[9,12] Despite research advances, care/management of these conditions is still difficult to achieve. Majority of population suffering from cardiovascular diseases live in developing countries where access to modern medications are limited. Often, this population resort to use of herbal products to manage their health conditions. However, the mechanism/mode of action of these plants derived remedies is lacking. GK is one of the plant derived remedies that is used for various disease conditions.[11] In the present study, we have evaluated the effect of Kolaviron, a biflavonoid fraction from GK on mitogen-induced proliferation of VSMCs. Ag II and LPS are known mitogens, pro-inflammatory, pro-oxidants, proliferative and they possible act through activations of cascade of signalling pathways initially separately, converging later to activate common pathways that may regulate cellular functions possibly via common pathways that may regulate cellular functions possibly via

**Abbreviations Used:** VSMCs: Vascular Smooth Muscle Cells, Ag II: Angiotensin II, KV: Kolaviron, LPS: lypopolysaccharide, NO: Nitric Oxide, DMEM: Dulbecco’s modified Eagle’s medium, MTT: 3-(4,5-dimethylthiazolyl)-2, 5-diphenyl tetrazolium bromide, DMSO: Dimethylsulfoxide, GB1: Garcinia kola biflavonoid-1, GB2: Garcinia kola biflavonoid-2, ROS: Reactive oxygen species, ET-1: Endothelin-1, NF-kB: Nuclear factor-kappa beta, COX-2: Cyclooxygenase-2

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**SUMMARY**

- Angiotensin-induced cell proliferation
- Kolaviron mitigates angiotensin-induced cell proliferation
- Kolaviron ameliorates nitric oxide production
- Kolaviron offers antioxidant activity.

**Keywords:** Angiotensin II, cardiovascular diseases, cell proliferation, kolaviron, lypopolysaccharide, nitric oxide, vascular smooth muscle cells
transcription factors.[9,11-14] As cardiovascular pathologies involve these myriad of pathways, Ang II and LPS activated pathways will be a good model for testing the possible actions of KV in cardiovascular dysfunction in vitro.

MATERIALS AND METHODS

Chemicals and reagents

Reagents used in this study were Dulbecco’s modified Eagle’s medium (DMEM), MTT, Anti-biotoic and anti-mycoptic consisting of 100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulphate, 2.5 mg/mL amphotericin B and Trypsin-EDTA. They were purchased from Sigma-Aldrich, St Louis, MO. Matrigel (BD Biosciences, Franklin Lakes, NJ). All other chemicals and reagents were of pure analytical grade.

Extraction of *Garcinia kola* and isolation of Kolaviron

Kolaviron was extracted from the seeds of *Garcinia Kola* according to the method of Iwu with slight modification.[15] The seeds were sliced, air-dried and powdered. The powdered seeds were defatted by extraction using n-hexane in a Soxhlet extractor apparatus for 24 hours. The defatted dried marc was repacked and extracted with methanol. Kolaviron was fractionated from concentrated methanolic extract using chloroform to give a golden brown solid which consists of *Garcinia* biflavanones – GB1, GB2 and kolaflavanone.

METHODS

Vascular smooth muscle cell culture

VSMC was a gift from Dr. Ranganna of the RCMI Core Lab at TSU, Houston. The cells were cultured and maintained as previously described.[15] Briefly, VSMC were culture in a culture flask T75 and maintained at 37°C in a humidified 5% CO₂ incubator in a 20% FBS conditioned DMEM plus anti-biotic consisting of 100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulphate, and 2.5 mg/mL amphotericin B until confluent. Confluent cells were trypsinized and plated in a 96-well conditioned DMEM plus anti-biotic consisting of 100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulphate, and 2.5 mg/mL amphotericin B and Trypsin-EDTA. They were purchased from Sigma-Aldrich, St Louis, MO. Matrigel (BD Biosciences, Franklin Lakes, NJ). All other chemicals and reagents were of pure analytical grade.

Determination of nitric oxide (NO)

NO level in the media was determined using the Griess assay as describe previously.[15] Briefly, assay samples were mixed with an equal volume of the Griess reagent [0.1% N (1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 3% H₃PO₄] and incubated to yield a chromophore. Using a Bio-Tek Instruments plate reader (model ELX 800, BioTek Instruments, Winooski, Vermont, USA). The proliferation assay was conducted in triplicates, experiments repeated at least 4 times and expressed as mean of % change in VSMC growth.

Statistical analysis

Data are presented as mean ± S.E.M of n = 4-6. Differences between groups were assessed using one-way ANOVA followed by Turkey comparison tests. A value of P < 0.05 was considered significant.

RESULTS

Effects of KV VSMC proliferation

Figure 1 shows effects of treatment of VSMC with KV (25, 50, 100 µg/ml) for 24 hours. VSMC growth was reduced by 14, 6, and 15.4% following treatments with 25, 50, 100 µg/ml KV respectively. This reduction in cell growth was significantly (P < 0.05) different from the control [Figure 1, n = 4].
Effects of KV on LPS-induced VSMC proliferation

Figure 4 shows effects of LPS-induced NO production. 4 Hours incubation of VSMC with LPS 100 µg/mL resulted in significant increase in NO. NO levels in the media were significantly (P < 0.05) increased from 33.0 ± 0.3 nM/mL in the control to 36.4 ± 0.4 nM/mL in LPS treatment. LPS-induced increases in NO production was significantly attenuated by KV reducing the NO levels from 36.4 ± 0.4 nM/mL (LPS) to 32.4 ± 0.2, 31.2 ± 1.0, 31.4 ± 0.3 nM/mL for KV 25, 50, 100 µg/mL respectively [Figure 4, n = 4]. The KV-induced attenuation of LPS-induced increases in NO levels brought the NO levels to a level comparable to that observed in the control.

DISCUSSION

In the present study, we found that: (1) Treatment of VSMC with KV resulted in reduced VSMC growth, (2) KV attenuated Ag II-induced VSMC proliferation in a concentration and time-dependent manner, (3) 24 hours LPS treatment increased VSMC proliferation and NO production and were attenuated by KV treatment. Thus, these results demonstrated that KV possesses anti-mitogenic agents’-induced proliferation of smooth muscle cells as well as NO production via LPS mediated activation of inflammatory processes. Our findings suggest that KV possibly mediate its effects by regulating molecular signalling pathways that regulates diverse cellular functions.

Despite advances in knowledge and therapeutic drug development, cardiovascular diseases still remain a huge burden to individual and society. The processes that contribute to the initiation and maintenance of cardiovascular diseases become a target for therapeutic intervention. Cardiovascular pathologies are characterised by vascular cell proliferation, inflammation, and/or increased oxidative stress. Inflammation contributes critically to all stages of atherogenesis and cardiovascular remodelling. Metabolic disorders such as dyslipidemia promote activation of circulating monocytes, endothelial cells and adhesion of these cell types leading to accumulation of macrophages. Activation of macrophages can lead to the production of proinflammatory cytokines, NO, mitogens, and reactive oxygen species. Cellular oxidative stress as well as other vasoactive agents can activate neighbouring cells including endothelial cells and further promotes monocyte recruitment. In addition, these processes can lead to production of mitogenic agents (Ag II, ET-1 etc.) and further propagating atherogenesis. Such an uncontrolled amplification mechanism represents combined proliferative, inflammatory and oxidative stress aspects of atherosclerosis and cardiovascular disease pathologies. Numerous studies have been designed to investigate the involvement of inflammation, proliferation, and oxidative stress in cardiovascular diseases with the aim to developing agents that can prevent the development of atherosclerosis and its complications have resulted in unsatisfactory results. This probably could be due to the multi-factorial nature of the pathogenesis of cardiovascular diseases; hence, single remedy focused on alleviating one of these factors will result in unsatisfactory outcomes. Therefore, the target should be the development or identification of possible therapeutic agents that possess a wide range of actions against this plethora of factors and possibly mitigating against activation of common mechanistic pathways. In the present study, we have evaluated the anti-proliferative and anti-inflammatory effects of KV in cultured VSMC. We found that KV attenuated VSMC growth and sequential cell proliferation induced by Ag II and LPS as well as LPS-induced increased NO production which could mediate inflammatory processes.

It is generally accepted that Ang II and LPS as well as other mitogens such as ET-1 could activate cellular processes involved in the production of growth factors, cytokines, chemokines, and adhesion molecules, which...
Arterial wall production of Ang II is important in the normal regulation of arterial tone as well as its involvement in the pathogenesis of atherosclerosis. Ang II regulates many processes implicated in vascular pathophysiology, including cell growth/apoptosis of vascular cells, migration of vascular smooth muscle cells, inflammatory responses, and extracellular matrix (ECM) remodelling. As a result of its important role in the regulation and pathogenesis of cardiovascular diseases, drugs that block Ang II actions, such as ACE inhibitors or Ang II receptor antagonists, are currently employed in the treatment of hypertension, heart failure, atherosclerosis, and other cardiovascular diseases. Despite the widespread use of Ang II agents in clinical practice, its’ mechanism(s) of action is not completely defined by which of the multiple pathways Ang II exerts its effects in the vasculature. Similarly, LPS exert its action through series of interference with vascular signalling pathways involving activation of different kinases culminating in the systemic dysfunctions observed. However, we have shown in the present studies that KV possesses both anti-proliferative and anti-inflammatory properties in addition to its well known anti-oxidant effects as Ang II and LPS are pro-oxidants. The role of oxidative stress in the pathogenesis of vascular diseases is well recognized. Ang II and LPS stimulates the production of reactive oxygen species (ROS) via induction of vascular NADH oxidase mediated by gp91phox of NADPH oxidase and other subunits are mainly responsible for stimulated vascular oxidative stress and smooth muscle cells growth in vivo. Furthermore, LPS is known to modulate pathological conditions through activation of ROS, cellular proliferation and inflammatory processes mediated by excessive production of NO. In the present study, we have shown that KV treatment prevents mitogen induced VSMC proliferation and attenuated LPS-induced generation of NO and by extension, reduction cellular stress. These actions of KV indicate that it can be useful in conditions that involve cellular proliferation, enhanced oxidative stress, and proinflammatory processes. According to our present findings, LPS-induced nitric oxide production was quenched by KV, clearly demonstrating the anti-oxidant and anti-inflammatory properties of KV. These plethoric actions of KV observed could not be possibly linked to actions on a single pathway mediated via activation of proliferative, inflammatory, oxidative processes etc. The actions of KV observed can be attributed to inhibition of a converging single pathway that all of these signalling processes recruit to mediate these actions – a
transcription factor probably. Nuclear factor-kB (NF-kB) consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival. Evidences suggesting the potential role of NF-kB as a mediator of proliferative and inflammatory processes are rippled. Increases in NF-kB activity in rat’s vessels has been reported following systemic infusion of Ang II and LPS administration.[20–25] Ang II and LPS activates NF-kB in several cell types, including vascular smooth muscle, endothelial, renal, macrophages, and mononuclear cells in mediating oxidative, inflammatory, and proliferative processes.[19–24] Although, Ang II acts through binding to two main specific receptors, AT1 and AT2, both receptors share a common molecular pathway, the activation of NF-kB.[23] LPS has been suggested to activate NF-kB in regulating proinflammatory cytokines, NO, COX-2 etc., via series of kinase activation in pathophysiology of systemic shock.[22] There are possibilities that Ang II and LPS mediated vascular dysfunctions involves activation of proliferative and inflammatory responses via redox mechanisms and NF-kB pathways. Given the large number of signals that activate NF-kB, the list of target genes controlled by NF-kB; targeting NF-kB will be a viable opportunity for prevention and treatment of cardiovascular pathology. From our present results, KV-induced attenuation of LPS and Ang II-induced proliferation and NO production possibly involves inhibition of NF-kB activation. Consistent with this possibility, the observed ability of KV treatment to significantly reduce activation and expression of NF-kB in a cancer cell line (unpublished observation). Thus, the actions of KV observed in this study may well be mediated via inhibition of NF-kB activation but further studies are warranted to understand the molecular mechanism involve in KV-induced anti-proliferative and -inflammatory with possible role of NF-kB.

CONCLUSION

Taken together, these results showed that KV inhibited cell proliferation and prevented the generation reactive oxygen species (ROS) and nitric oxide production mediated by LPS activation of inducible nitric oxide synthase (iNOS). In conclusion, KV possesses possible anti-oxidant, anti-proliferative and anti-inflammatory properties which would be useful in alleviating cardiovascular disease conditions and further studies are warranted to investigate the mechanism involved in the KV actions.

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Conflicts of interest

There are no conflicts of interest.

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