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Kolaviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-κB and AP-1

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ABSTRACT

Aims: Kolaviron, a bioflavonoid isolated from the seeds of *Garcinia kola* has been reported to possess anti-inflammatory, antioxidant, antigenotoxic and hepatoprotective activities in model systems via multiple biochemical mechanisms. The present study investigated the possible molecular mechanisms underlying the hepatoprotective effects of kolaviron.
Main methods: Biomarkers of hepatic oxidative injury, histological and immunohistochemical techniques were used. In addition, the protein expression levels of cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) were evaluated by western blotting while DNA-binding activities of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) were determined by electrophoretic mobility shift assay.
Key findings: Kolaviron administered orally at doses of 100 and 200 mg/kg for 7 days significantly lowered the activities of serum transaminases and γ-glutamyl tranferase induced by single intraperitoneal administration of dimethyl nitrosamine (DMN) (20 mg/kg) and preserved the integrity of the hepatocytes. Also, kolaviron at both doses reduced the DMN induced elevated hepatic levels of malondialdehyde and reversed DMN mediated decrease in hepatic glutathione. The hepatoprotective effect of kolaviron was compared to that of curcumin, an established hepatoprotective agent. Kolaviron inhibited the DMN induced expression of COX-2 and iNOS. Immunohistochemical staining of rat liver verified the inhibitory effect of kolaviron on DMN-induced hepatic COX-2 expression. Furthermore, kolaviron abrogated DMN induced binding activity of NF-κB as well as AP-1.
Significance: The ability of kolaviron to inhibit COX-2 and iNOS expression through down regulation of NF-κB and AP-1 DNA binding activities could be a mechanism for the hepatoprotective properties of kolaviron.

Introduction

Dimethyl nitrosamine (DMN) is a representative chemical of a family of N-nitroso compounds and has been found in processed meats and industrial products. It is a potent hepatotoxin, carcinogen and mutagen (George et al., 2001). DMN exerts carcinogenic effects and induces hepatic necrosis through metabolic activation by CYP2E1 (Guengerich et al., 1991) in experimental animals. The formation of reactive oxygen species (ROS) like H_2O_2, superoxide anion (O_2^-) and hydroxyl radicals ($OH^{\cdot}$) has been demonstrated during the metabolism of nitrosamines resulting in oxidative stress, which may be one of the key factors in the induction of pathological conditions such as hepatocellular necrosis, carcinogenicity, neoplastic changes, and tumor formation (Nakae et al., 1997; Pradeep et al., 2007; Wills et al., 2006). Teufelhofer et al. (2005) also demonstrated that metabolism of the nitrosamine by CYP2E1 in mouse liver stimulated Kupffer cells leading to generation of superoxide and other ROS capable of damaging liver cells.

Cyclooxygenase-2 is an enzyme involved in inflammatory processes and a rate limiting enzyme in prostaglandin biosynthesis from arachidonic acid. Inappropriate up-regulation of COX-2 has been frequently observed in various premalignant and malignant tissues (Mohan and Epstein, 2003). COX-2 has been further implicated in tumorigenesis by increased susceptibility of COX-2 over-expressing transgenic mice (Muller-Decker et al., 2002) and relative resistance of COX-2 knockout animals to spontaneous or experimentally-induced carcinogenesis (Tiano et al., 2002).

Like other early-response gene products, COX-2 can be induced rapidly and transiently by proinflammatory mediators, endotoxins as well as carcinogens (Kim et al., 2005). Studies have demonstrated carcinogen-induced expression of COX-2 by activating nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (Chun et al., 2003, 2004; Kim et al., 2005). Therefore targeted inhibition of COX-2 and modulation of abnormal up-regulation of NF-κB and AP-1 have now been recognized as the molecular basis of chemoprevention by structurally diverse dietary phytochemicals. A number of dietary phytochemicals have been shown to inhibit COX-2

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and transcription factors (NF-κB and AP-1) controlling this gene in both in vivo and in vitro models of carcinogenesis (Chun et al., 2004; Surh, 2003). Inducible nitric oxide synthase (iNOS) is another inducible enzyme that causes the overproduction of nitric oxide during inflammation and tumor development (Chung et al., 2007). Nitric oxide has been implicated in initiation, promotional stage of neoplasmic transformation (Mordan et al., 1993) and in tumor progression by regulating angiogenesis (Chin et al., 1997). Therefore, suppression of the induction and activity of COX-2 and/or iNOS has been considered a new paradigm in cancer chemoprevention in several organs (Chung et al., 2007).

Garcinia kola Heckel (Guttiferae) is a largely cultivated tree and highly valued in west and central Africa for its edible nuts. The seed, commonly known, as 'bitter kola' is eaten by many and it is culturally acceptable in Nigeria. Extractives of the plant have been employed in the African herbal medicine for the treatment of ailments such as laryngitis, liver diseases, cough and hoarseness of voice (Iwu, 1982; Farombi, 2003).

Kolaviron is a fraction of the defatted ethanol extract, containing *Garcinia* biflavonoids GB1, GB2 and kolaflavanone. Several studies have demonstrated the protective effects of kolaviron against hepatotoxicity and oxidative stress induced by 2-acetylaminofluorene (Farombi et al., 2000b), carbontetrachloride (Farombi, 2000), aflatoxin B_1 (Farombi et al., 2005), potassium bromate-induced nephrotoxicity (Farombi et al., 2002) and dibutylphthalate-induced testicular oxidative stress (Farombi et al., 2007) in animal model. Furthermore, kolaviron has been shown to exhibit potent antioxidant and metal chelating activities in vitro (Farombi and Nwaokeafor, 2005) as well as inhibition of carcinogen-induced genotoxicity in human liver-derived HepG2 cells (Nwankwo et al., 2000). However the molecular mechanisms underlying the hepatoprotective and other chemopreventive/chemoprotective effects of kolaviron remain largely unknown. In the present study, we report that kolaviron inhibits DMN-induced liver injury in rats by suppressing COX-2 and iNOS expression via NF-κB and AP-1.

Materials and methods

DMN was purchased from Sigma Chemical Co. (St Louis, MO, USA). Rabbit polyclonal COX-2 antibodies were products of Cayman Chemical Co. (Ann Arbor, MI, USA). Anti-rabbit, anti-goat and anti-mouse horseradish peroxidase-conjugated secondary antibodies were products of Zymed Laboratories (San Francisco, CA, USA). ARE oligonucleotide was obtained from Bionics (Seoul, Korea). Enhanced chemiluminescence (ECL) detection kit and [γ-^{32}P] ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents used were in the purest form available commercially.

Extraction of kolaviron

Kolaviron was isolated according to published procedure (Iwu, 1985). Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60 °C) in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded a golden yellow solid termed kolaviron which has been shown to consist of *Garcinia* biflavonoid GB-1 (3″,4′,4‴,5,5″,7,7″-heptahydroxy-3,8″ biflavanone), GB-2 (3″,4′,4‴,5,5″,5‴,7,7″-octa-hydroxy-3,8″-biflavanone), and kolaflavanone (3″,4′,4‴,5,5″,5‴,7,7″octahydroxy-4‴-methoxy-3,8″-biflavanone). Kolaviron was identified by direct comparison of the ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and electron ionization (EI)-mass spectral results with previously published data (Iwu, 1985).

Animal treatment

Male albino rats Wistar strain were purchased from Charles River (Tokyo, Japan). They were housed in a temperature-controlled (25 °C) room with alternating 12-h light/12-h dark cycles. All animals were acclimatized for 7 days before experiment, fed standard pellet chow and given fresh water ad libitum. The experimental protocols were approved by the Animal Care and Use Committee of the Seoul National University.

Thirty (30) male rats were divided into five groups. Control rats received the vehicle only. Rats in group 3 and 4 were administered 100 and 200 mg/kg kolaviron respectively for 7 consecutive days (Farombi et al., 2005, 2004). Rats in groups 2, 3, 4 and 5 received a single intraperitoneal dose of DMN (20 mg/kg) 48 h before sacrifice. The protective effect of kolaviron on DMN-induced hepatotoxicity was compared to that of curcumin (200 mg/kg) which has been confirmed to have hepatoprotective effects (Park et al., 2000). At the end of each experiment, rats were sacrificed by cervical dislocation. Blood was collected by heart puncture for serum isolation. Liver was excised, rinsed in physiological saline and stored at −80 °C until use for western blot analysis and nuclear extract preparation.

Measurement of serum enzyme activities

Serum was prepared from the whole blood by centrifugation at 3000 *g* for 10 min at 4 °C. Serum aspartate amino transferase (AST), alanine amino transferase (ALT) and γ-glutamyl transferase (γ-GT) activities were determined using commercially available kits.

Determination of lipid peroxidation

Lipid peroxidation was assessed in terms of malondialdehyde (MDA) formation in the rat liver 10,000 *g* supernatant fraction. The measurement of thiobarbituric acid reacting substances (TBARS) was

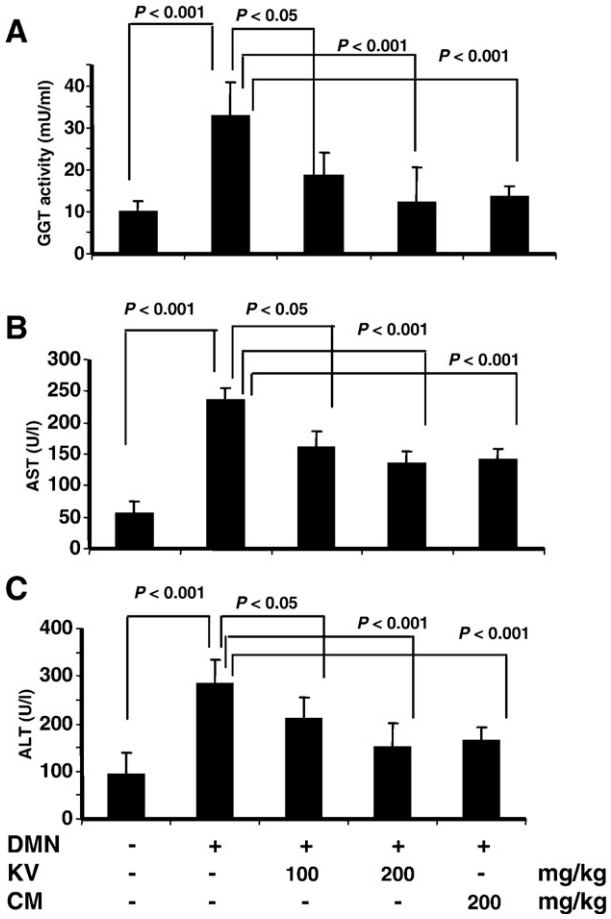

Fig. 1. Effect of kolaviron and curcumin on DMN-induced increases in the activities of γ-glutamyl transferase (A), serum aspartate amino transferase (B) and serum alanine amino transferase (C). Values are means±SD of 6 rats.