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Carvacrol induces mitochondria-mediated apoptosis in HL-60 promyelocytic and Jurkat T lymphoma cells

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ABSTRACT

The aim of the present study was to investigate the effect of carvacrol, a phenolic monoterpenoid on the induction of apoptosis in HL-60 (Human acute promyelocytic leukemia cells) and Jurkat (human T lymphocyte cells) cells. Carvacrol showed a potent cytotoxic effect on both cells with dose-dependent increase in the level of free radical formation as measured by an oxidation sensitive fluorescent dye, 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) levels. The reduction in the level of antioxidants such as catalase (CAT) and superoxide dismutase (SOD) ($P < 0.05$) was observed in carvacrol-treated cells. The major cytotoxic effect appears to be intervened by the induction of apoptotic cell death as assessed by annexin-V labeling assay using flow cytometry. Western blot analysis showed that Bax expression was increased, whereas Bcl-2 expression was significantly decreased in carvacrol exposed HL-60 cells and Jurkat cells. Further studies revealed that the dissipation of mitochondrial membrane potential of intact cells was accompanied by the activation of caspase-3. Our results found that the potential mechanism of cellular apoptosis induced by carvacrol is mediated by caspase-3 and is associated with the collapse of mitochondrial membrane potential, generation of free radicals, and depletion of the intracellular anti-oxidant pool.

1. Introduction

Phytochemicals from medicinal plants are normally non-toxic and have been reported for its potential against various diseases and disorders including cancer (Lai and Roy, 2004). It is now apparent that many natural products preferentially inhibit the growth of tumor cells by targeting one or more signaling cascades leading to induction of apoptosis (Khan et al., 2007). Since the imbalance between cell proliferation and apoptosis are correlated with cancer progression, identifying chemopreventive agents is of great importance. Numerous evidences reported that apoptosis induction is considered to be one of the promising therapeutic strategies against cancer (Pan and Ho, 2008). In such a way polyphenols have been reported for their promising effects of induction of apoptosis in cancer cells by altering phase I and phase II drug-metabolizing enzymes; depleting antioxidants; inhibition of protein kinases; blocking of receptor mediated functions; alteration of cell cycle checkpoint controls, inhibition of signaling pathways, suppression of cell proliferation and apoptosis induction; inhibition of angiogenesis, invasion, and metastasis; and epigenetic changes in promoter methylation and chromatin remodeling (Agarwal et al., 2000; Rajput and Mandal, 2012). Many plants and plant polyphenolic compounds such as epigallocatechin-3-gallate, quercetin, resveratrol, catechins, have been proved for their chemopreventive action by pursuing one of the above stated mechanisms (Gokbulut et al., 2013; Vijaya Padma et al., 2007). There have also been a number of reports suggesting that dietary phenolics exhibit prooxidant and cytotoxic properties under certain conditions. The antioxidant/prooxidant activity of phytophenolics can depend on such factors as metal-reducing potential, chelating behavior, pH, and solubility characteristics (Summers and Felton, 1994). Khan et al., demonstrated that several anticancer polyphenolic compounds from fruits and vegetables induce tumor growth arrest largely through the generation of ROS (Khan et al., 2012). In addition, resveratrol, quercetin and curcumin also reported to suppress cancer cell proliferation by inducing oxidative stress (Su et al., 2006; Zamin et al., 2009).

A monoterpenoid phenolic phytochemical, carvacrol abundant

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in the oil of oregano, thyme, pepperwort etc (De Vincenzi et al., 2004) has been known to possess diverse medicinal values like analgesic, anti-inflammatory, anti-angiogenic, anti-oxidant, anti-elastase, insecticidal, AChE inhibitor and anti-tumor activity (Baser, 2008; Sokmen et al., 2004). The effect of carvacrol against cancer has been studied on various cell lines such as non-small cell lung cancer cells (Koparal and Zeytinoglu, 2003), hepatocellular carcinoma cells (Yin et al., 2012), human metastatic breast cancer cells (Arunasree, 2010) and chronic myeloid leukemia cells. (Horvathova et al., 2007) However, the exact mechanism of apoptosis induction by carvacrol against leukemia remains unknown. Hence we aimed to investigate the molecular mechanism of carvacrol against leukemia using HL-60 cells (human acute promyelocytic leukemia cell line) and Jurkat cells (immortalized human T cell lymphocyte cell line). This study further elucidates the therapeutic benefit of carvacrol by identifying its apoptotic potential in leukemic cells.

2. Materials and methods

2.1. Chemicals

Carvacrol was purchased from Sigma-Aldrich (St. Louis, MO). The stock solution was prepared in dimethyl sulphoxide (DMSO) and later dilutions were made with fresh culture medium. The concentration of DMSO in the final culture medium was maintained as $<1\%$.

2.2. Cell culture conditions

The HL-60 and Jurkat cells were obtained from National Centre for Cell Sciences, Pune, India and cultured at 37 °C under a humidified, 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Isolation and treatment of lymphocytes

Peripheral blood samples (5 ml) were obtained from four healthy non-smoking, male volunteers not exceeding the age of 35 years. Lymphocytes were isolated from blood samples by centrifugation using Ficoll-based density gradient, washed twice with serum-free RPMI-1640 medium and treated independently. To assess cytotoxicity of carvacrol, lymphocytes ($\sim 1\times 10^4$ cells/well) were resuspended in 1 ml fresh serum-free RPMI-1640 to which 10 μl aliquots of the chosen concentrations of carvacrol (0–200 μM) was added. After 24 h incubation at 37 °C in the dark, cells were separated by centrifugation and immediately were analyzed using a cytotoxicity assay with trypan blue.

2.4. Assessment of carvacrol-induced cytotoxicity

Cytotoxicity of carvacrol in HL-60 and Jurkat cells was assessed by the MTT assay. HL-60/ Jurkat cells ($\sim 1\times 10^4$ cells/well) were cultured in 96-well plates at 37 °C for 24 h and then exposed with different concentrations of carvacrol for another 24 h. Then the medium was removed, MTT solution was added (5 mg/ml) and the plates were incubated at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. Then 100 μl of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals formed, and the absorbance was read at 570 nm using a microplate ELISA reader.

2.5. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were measured using an oxidation sensitive fluorescent dye, 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA). Briefly, HL-60/Jurkat cells were treated with carvacrol (0–100 μM) for 24 h, H2DCFDA (20 μM) was added to the cells and further incubated for 30 min at 37 °C. Then the reaction was stopped with phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS). The cells were pelleted by centrifugation (800 g, 10 min), washed, and resuspended in PBS. The resultant fluorescence intensity was assessed by FACS analysis. A greater shift in fluorescence intensity implies higher amount of DCF and greater ROS generation.

2.6. Determination of antioxidants

For the estimation of antioxidants (superoxide dismutase, SOD; catalase, CAT), 24 h cultures of HL-60/Jurkat cells were treated with different concentrations of carvacrol for 24 h at 37 °C. At the end of incubation, the cells were centrifuged and the supernatant was removed. The cell pellet was resuspended in PBS and sonicated.

Total SOD activity was assayed according to the method of Durak (Durak et al., 1993) based on the inhibition of the formation of nitro blue tetrazolium formazan. One unit was taken as the amount of enzyme that produced 50% inhibition of nitro blue tetrazolium reduction per milligram of protein.

CAT was assayed colorimetrically at 620 nm and expressed as moles of H_2O_2 consumed per min per mg of protein, as described by Sinha (Sinha, 1972). This method is based on the determination of H_2O_2 decomposed and remained after stopping the enzyme reaction on substrate (mixture of potassium dichromate and glacial acetic acid). One unit of enzyme activity is the enzyme quantity that decomposes one micromole of H_2O_2 for one min at pH 7. The results are expressed in units per milligram of protein.

2.7. Measurement of annexin-V binding

Phosphatidylserine redistribution in the membrane was measured by the binding of annexin-V fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (Miltenyl Biotec, Germany). The cells ($\sim 1\times 10^3$ cells/well) were treated with carvacrol (0–100 μM) for 24 h at 37 °C. Then the medium was removed and replaced with fresh medium, incubated for 24 h and subjected for the analysis. The cells incubated in RPMI-1640 medium only were used as the control. In all cytofluorometric determinations, cell debris and cell clumps were excluded from the analysis by suitable gating. After the treatment protocol, the cells were washed and resuspended in 100 μl of HEPES buffer (10 mM HEPES (pH 7.4), 140 mM NaCl and 5 mM CaCl2) containing annexin-V-FITC (10 μl). Samples were mixed gently and incubated at room temperature in dark for 15 min. Immediately before analysis by flow cytometry (FACSVantage SE, BD Biosciences, San Jose, CA USA), 5 μl of propidium iodide (PI) (100 μg/ml) was added to each sample. For each experiment, 10,000 events were collected and analyzed.

2.8. Protein extraction and Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Briefly, the protein lysates (50 μg) were resolved electrophoretically on 15% denaturing SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking in 5% milk,