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Effects of octacosanol on HMG-CoA reductase and cyclooxygenase-2 activities in the HT-29 human colorectal cancer cell line

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ABSTRACT: Octacosanol (OCT) is a major component of policosanol which has been reported to possess anti-inflammatory and lipid-lowering effects. Therefore, it was our interest to evaluate the effects of OCT on HMG-CoA reductase (HMGR) and cyclooxygenase-2 (COX-2) activities in a human colorectal cancer cell line (HT-29). Our results demonstrate that 100 μM OCT decreased viability of HT-29 cells as analyzed by sulforhodamine B colorimetric assay with more pronounced effects seen in cells treated with atorvastatin (AST) or celecoxib (CLX), the inhibitors to HMGR and COX-2, respectively. Additionally, the activity of HMGR was found to be inhibited in cells treated with OCT, while COX-2 activity was unaffected; these effects were also more pronounced in AST- and CLX-treated cells. In cells treated with OCT, a significant decrease in HMGR protein expression was observed, but there was no alteration in COX-2 protein expression as determined by Western blot. Taken together, our results show that OCT inhibited HT-29 cell growth and that this effect might be attributed to the reduction of HMGR protein expression and activity.

KEYWORDS: octacosanol, HMG-CoA reductase, cyclooxygenase, colorectal cancer

INTRODUCTION

Octacosanol (OCT) is a straight-chain aliphatic 28-carbon primary fatty alcohol, the main component of policosanol [1] found in plants, especially sugar cane and rice. OCT has been reported to possess many physiological functions including lipid-lowering and anti-inflammatory effects [2].

Nuclear factor-kappaB (NF-κB) regulates the expression of inflammatory genes including cyclooxygenase-2 (COX-2). The anti-inflammatory effect of OCT has been suggested to involve downregulation of inflammatory factor expression and blockade of the MAPK/NF-κB/AP-1 signaling pathway [3]. OCT isolated from plants has been shown to inhibit nuclear translocation of NF-κB as well as its DNA binding activity, resulting in downregulation of gene expression downstream of this signaling [4]. Additionally, policosanol has been reported to significantly inhibit COX-1 activity in a dose-dependent manner as well as reduce COX-2 activity by up to 39%, suggesting some inhibitory action on this target [3].

The lipid-lowering effects of policosanol have been documented in several studies, comprising data from both animal models and human trails [4–7]. Apart from polycosanol, reduction of cholesterol level has been reported in mice by feeding the diet with extracts from *Antheraea pernyi* plant containing many bioactive substances such as hormone and protein mediating immune function [8]. HMG-CoA reductase (HMGR) plays a role in the cellular synthesis of cholesterol. At the molecular level, policosanol treatment reduces cellular HMGR activity through activation of AMP-kinase [9].

The effects of non-steroidal anti-inflammatory drugs (NSAIDs) in prevention of human cancers have been emphasized in several studies. Inhibition of COX enzymes (both COX-1 and COX-2) resulting in the altered metabolism of arachidonic acid is a basic conceivable mechanism for the anti-tumor effects of NSAIDs [10, 11]. COX-2 is inducible and is frequently overexpressed in a variety of tumors. COX-2 inhibitors such as celecoxib (CLX) have been shown to interfere with tumorigenesis and induce apoptosis in colon cancer; therefore, COX-2 is an interesting target for therapeutic and chemoprotective strategies [12].

Statins, potent cholesterol-lowering drugs, exert their effect via inhibition of HMGR. Statins such as atorvastatin (AST) have been shown to exhibit anti-tumor effects and attenuate metastasis in several studies including both *in vitro* and *in vivo* experiments as well as human studies [13–16]. HMGR involves in the production of cholesterol and non-steroidal isoprenoid derivatives, which are necessary for cell growth and differentiation [17]. *In vitro* and *in vivo* studies have demonstrated that simvastatin suppresses cancer cell growth by inducing apoptosis and inhibiting cell cycle progression [18, 19].

Since policosanol has been suggested to inhibit COX and HMGR activities and OCT is a major component in policosanol, we were interested in evaluating

the effects of OCT on COX-2 and HMGR activities and thereby its anti-cancer potential, using the HT-29 colon cancer cell line.

MATERIALS AND METHODS

Chemicals

OCT (1-Octacosanol, > 99%, CAS#03379) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The HMGR assay kit (CS1090) was purchased from Sigma-Aldrich. The COX activity assay kit (ab 204699) was purchased from Abcam (Cambridge, UK). COX-2 and HMGR antibodies were purchased from Santa Cruz Biotechnology, Inc., USA.

Cell culture

Human colon carcinoma (HT-29) cells were obtained from American Type Culture Collection (ATCC) (Rockville, CT, USA). The cells were maintained in McCoy's 5A Medium supplemented with 100 U/ml penicillin, 100 unit/ml streptomycin sulfate and 10% fetal calf serum at 37 °C in a 5% carbon dioxide atmosphere incubator. OCT was diluted in ethanol at 37 °C and shaken in an ultrasonic bath. The stock solution was stored below −20 °C; the final amount of ethanol added to the cells was not greater than 0.1% (v/v). This research project was approved by the institutional biosafety committee of Thammasat University (TU-IBC) (allowance no. 057-2561).

Cell viability assay

Cells were seeded onto 96-well culture plates. After an overnight culture, the cells were treated with OCT, AST or CLX for 24 h. Cell viability was investigated by Sulforhodamine B (SRB) assay. Briefly, the cells were washed using phosphate buffered saline (PBS) and then fixed with 10% (w/v) trichloroacetic acid. Next, the cells were stained with 0.4% SRB in 1% acetic acid for 30 min. To remove any excess dye, the cells were then washed repeatedly with 1% (v/v) acetic acid. The protein-bound dye was then dissolved in 10 mM Tris base solution pH 10.5 and incubated for 20 min. The OD was determined at 540 nm using a microplate reader. Cells treated with vehicle (0.1% ethanol) were used as a control.

COX and HMGR activity assay

After the 24 h treatment with OCT, AST or CLX, the cells were washed with cold PBS and then lysed in cell lysis buffer. The lysate was loaded into 96 well plates for further assay. The Pierce™ BCA Protein Assay Kit (Thermo-Scientific) was used to measure protein content; the assay was performed according to the manufacturer's protocol. Cells treated with vehicle (0.1% ethanol) were used as a control.

The enzymatic activity of HMGR was investigated according to the manufacturer's instructions (HMGR assay kit, CS1090, Sigma-Aldrich, St. Louis, MO, USA). Results were expressed as (mol/min)/μg protein. Determination of COX activity was performed according to the manufacturer's protocol (COX Activity Assay Kit Fluorimetric, Abcam ab204699, Cambridge, UK). The total amount of COX activity was expressed as (pmol/min)/μg protein.

Western blot analysis

After an overnight culture in 6 well plates, the cells were treated with OCT, AST or CLX for 24 h. Cells treated with vehicle (0.1% ethanol) were used as a control. The cells were then washed with cold PBS and lysed in RIPA cell lysis buffer with protease inhibitor cocktail. Total protein content was estimated by Pierce™ BCA Protein Assay Kit (Thermo-Scientific) following the manufacturer's protocol. Protein (15 μg) from whole-cell lysates was used for SDS-PAGE. After electrophoresis, samples were transferred to a PVDF membrane (Millipore, Boston, MA, USA) and then blocked using 5% skim milk for 2 h. After blocking, the membrane was rinsed 3 times with TBS-T buffer and then incubated with antibodies (anti-β-actin (sc-47778 HRP), anti-COX-2 (sc-376861 HRP) and anti-HMGR (sc271595 HRP) (Santa Cruz Biotechnology, Inc. USA) in a dilution of 1:1000 at 4 °C overnight. Subsequently, the membrane was washed 3 times and then developed using Western Blotting Substrate (ECL Prime Western Blotting reagent, Amersham, Buckinghamshire, UK). β-actin was used as a control to ensure equal protein loading.

Statistical analysis

Data are presented as mean ± SD. An analysis of variance (ANOVA) was used to determine significant differences between each experimental group. The level of significance was set at $p < 0.05$.

RESULTS

Effect of octacosanol on inhibition of HT-29 cell growth

To investigate the inhibitory effects of OCT on HT-29 cell growth, the cells were treated with OCT, AST or CLX in various concentrations for 24 h. Treatment of HT-29 cells with 10 or 50 μM OCT for 24 h did not affect cell viability, while treatment with 100 μM OCT did have an affect. Exposure to AST or CLX results in cell growth inhibition in a dose-dependent manner. At the highest concentration of OCT tested (100 μM), cell growth was reduced to 78% of control (approximately 20% inhibition). Meanwhile, the highest tested concentration of AST and CLX (50 μM in both cases) reduced cell growth to 47% (about 50% inhibition) and 38% (about 60% inhibition) of control, respectively (Fig. 1).

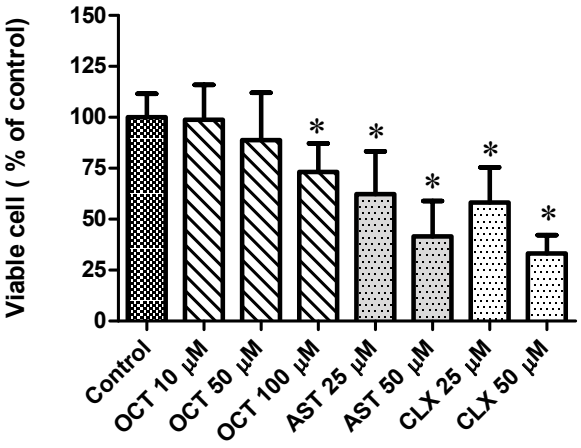

Fig. 1 Inhibitory effect of octacosanol on HT-29 cell growth. HT-29 cells were pretreated with various concentrations of octacosanol (OCT), atorvastatin (ATS) or celecoxib (CLX) for 24 h. Cell viability was analyzed by sulforhodamine B assay. Each bar represents the mean ± SD from 3 experiments. * $p <$ 0.05 compared with control.

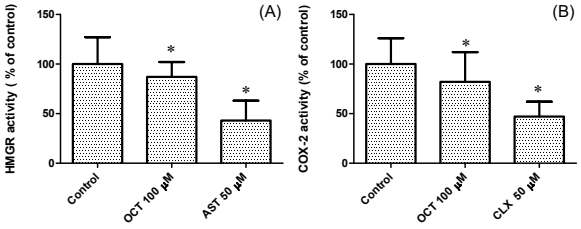

Fig. 2 Effect of OCT on HMGR and COX-2 activities. HT-29 cells were cultured in 35 mm dishes and then incubated with 100 μM octacosanol (OCT), 50 μM atorvastatin (AST) or 50 μM celecoxib (CLX) for 24 h, and the activities of (COX-2) were determined using commercial kits. Each bar represents the mean ± SD from 3 experiments. * $p < 0.05$ compared with the control.

Effect of octacosanol on HMGR and COX-2 activities

OCT has been suggested to inhibit HMGR and COX-2 activity [20, 21]. Inhibition of HMGR and COX-2 activities is thought to inhibit cancer cell growth and cell cycle progression; therefore, inhibition of these enzymes may be involved in the cell growth inhibition of OCT. Cells were incubated with OCT, AST or CLX for 24 h. Treatment with OCT or AST significantly reduced HMGR activity when compared with control (Fig. 2A). Regarding COX-2, treatment with CLX significantly reduced enzyme activity when compared with control, whereas OCT treatment demonstrated a trend towards reduced COX-2 activity ($p = 0.076$) (Fig. 2B). Additionally, AST and CLX demonstrated more potent abilities than OCT in terms of HMGR and COX-2 inhibition, respectively.

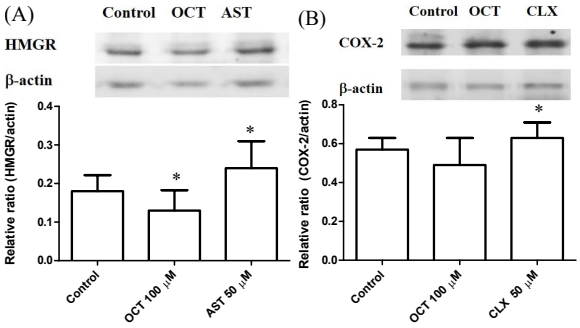

Fig. 3 Effect of octacosanol on HMGR and COX-2 protein expression. HT-29 cells were incubated with 100 μM octacosanol (OCT), 50 μM atorvastatin (AST) or 50 μM celecoxib (CLX) for 24 h, and then expression of HMGR (A) and COX-2 (B) proteins were determined by Western blot analysis. Each bar represents the mean ± SD from 3 experiments. * $p < 0.05$ compared with the control. Representative images from one experiment are shown.

Effect of octacosanol on HMGR and COX-2 protein expression

To examine how OCT inhibits the activity of HMGR and COX-2, the protein expressions of HMGR and COX-2 were investigated. Treatment with OCT resulted in significant decreases in HMGR expression as evidenced by Western blot analysis of HMGR protein in the treated HT-29 cells (Fig. 3A). Additionally, induced HMGR protein expression was observed in HT-29 cells treated with AST (Fig. 3A), while induced COX-2 protein expression was found in the CLX-treated cells (Fig. 3B). Treatment with OCT, however, did not demonstrate any significant effects on COX-2 protein expression (Fig. 3B).

DISCUSSION

Octacosanol (OCT), a long-chain fatty alcohol isolated from natural products, has multiple pharmacological functions including anti-inflammatory [19] and lipid-lowering effects [20]. Atorvastatin belongs to the statin family and exerts lipid-lowering effects by inhibiting the activity of HMGR, the rate limiting enzyme in cholesterol biosynthesis. Recently, a study has demonstrated that statins exert cancer-preventing effects through regulation of cellular events including cell proliferation, differentiation, inflammation and program cell death [21]. Studies using hepatoma cells and a mice model have shown that policosanol inhibited cholesterol synthesis by down regulation of HMGR [7, 9]. Moreover, Octacosa-10,19-dien-1-ol, an unsaturated analogue of 1-octacosanol, has shown inhibitory effects on HMGR upregulation, inducing phosphorylation of AMPK and downregulation of HMGR mRNA using a similar mechanism with policosanol [21].

Induction of prostaglandin synthesis by COX-2

stimulates cancer cell proliferation, promotes angiogenesis, inhibits programmed cell death and increases metastatic potential [22]. COX-2 protein expression is under the regulation of NF-κB. Furthermore, MAPKs play an essential role in NF-κB activation. CLX, a selective COX-2 inhibitor, has exhibited anti-cancer activity in several cancer models [22]; these effects may be mediated via inhibition of COX-2 and, at least partially, regulation of NF-κB activity [23]. In our study, we observed no significant differences in COX-2 activity or protein expression following OCT treatment when compared with control. Previous studies have reported anti-inflammatory effects of OCT via molecular mechanisms involving blockade of NF-κB signaling pathway [20, 21]. Additionally, policosanol, the main component of which is OCT, has been reported to inhibit COX activity *in vitro* [3]. In the current study, OCT treatment concentrations were limited to 100 μM due to the compound's solubility; therefore, the tested concentrations may have been insufficient to provide an inhibitory effect on COX-2.

Interestingly, in the cells treated with AST or CLX, inhibition of HMGR and COX-2 activities triggered an increase in the production level of these proteins. This finding is consistent with the previous studies by Gobel et al and Ramer et al which proposed that the increase in protein levels is possibly for compensating the loss of its activity [24, 25].

CONCLUSION

Our data suggested that OCT possesses anti-cancer activity. This effect may be attributed to many factors including the ability of OCT to inhibit HMGR activity. However, the exact mechanisms underlying the effects of OCT on HMGR activity require further investigation.

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