Synergistic effects of lovastatin and celecoxib on caveolin-1 and its down-stream signaling molecules: Implications for colon cancer prevention

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Abstract. Progression of colon cancer is associated with the up-regulation of cyclooxygenase-2 (COX-2) and hydroxy-methyl glutaryl CoA reductase (HMG-R). Clinical and preclinical evidence shows that a combination of COX-2 and HMG-R inhibitors provide additive/synergistic chemopreventive effects against colorectal cancer. However, the mechanism by which statins and NSAIDs inhibit cancer growth is not yet fully understood. We aimed to identify critical molecules and signal pathways modulated by a combination of lovastatin and celecoxib in the human HCT-116 colon cancer cell line. HCT-116 cells were exposed to 50 μM celecoxib, 25 μM lovastatin or a combination of both to assess their effect in modulating caveolin-1 expression and its down-stream signaling pathways. Our results suggest that a combination of lovastatin and/or celecoxib suppressed caveolin-1 expression and membrane localization profoundly when compared to either agent alone. Lovastatin and/or celecoxib also inhibited caveolin-1-dependent cell survival signals mediated through Akt activation as well as its down-stream effectors such as phosphorylated ERK and STAT3 in HCT-116 cells. Treatment with lovastatin or celecoxib decreased the levels of cyclin D1, CDK2, pRb and E2F1, while the combination treatment showed more pronounced suppression. In addition, lovastatin and celecoxib also decreased the amount of cholesterol rich cytoplasmic lipid bodies (storehouses of esterified arachidonates) by 80%, while the combination showed a complete inhibition. Overall, our data suggest that a combination of COX-2 and HMG-R inhibitors synergistically inhibits caveolin-1 and its associated signaling pathways.

Introduction

Colon cancer is one of the leading causes of cancer-related death in the Western world, including the United States. According to the American Cancer Society, ~112,340 new cases of colon cancer will be diagnosed with 52,180 deaths expected during 2008 (1). Since colorectal cancers develop slowly over a long period, at least 10 years in most people (2), early intervention strategies such as a chemopreventive approach is an ideal choice to prevent colon cancer mortality.

Several studies from our laboratory and others have shown that combination of chemopreventive agents exerts greater efficacy than a single agent alone (3,4). The combinational approaches are particularly significant when agents are combined at low-dose levels possessing diverse modes of action. Targeting different pathways of tumor cell survival may provide better efficacy and possibly without any side effects. In an earlier study, we have shown that a low-dose combination of celecoxib and lovastatin synergistically suppressed growth and induced apoptosis in HT-29 human cancer cells (5). Furthermore, studies using preclinical animal models carried out in our laboratory suggested that a combination of COX-1/2 inhibitor with HMG-R inhibitor provides synergistic colon tumor inhibitory effects (Proc Am Assoc Cancer Res, Abs. 2362, April, 2006). In studies with APCMin mice, a complete inhibition of colon tumor formation was observed using a combination of atorvastatin and celecoxib when compared to either agent alone (6). In another study, a low-dose of atorvastatin in combination with celecoxib and/or aspirin acted synergistically in suppressing azoxymethane-induced colon adenocarcinoma incidence and multiplicity in rats (7). Therefore, it is important to unravel the molecules and/or signaling pathways that play a role in exerting the combinational synergistic efficacy. This is particularly important given the concerns with life threatening side effects as observed in patients using high doses of the COX-2 selective inhibitor Rofecoxib.
The identification of a particular cellular target modulated by a combination of statin and COX-2 inhibitors provides unique advantages for designing future chemopreventive strategies. We utilized a global gene array to identify several key targets that are modulated by a combination treatment of lovastatin and celecoxib in rat AOM-induced colon tumors. Based on the results from this experiment, we observed in rats fed a combination of 200 ppm lovastatin and 300 ppm celecoxib that the expression of caveolin-1 was more effectively suppressed than either agent administered alone (Proc Am Assoc Cancer Res, Abs. 2362, April, 2006). Caveolin-1 and cholesterol plays a crucial role in the formation of membrane microdomains such as lipid rafts and caveolae. Caveolin-1 contains a scaffolding domain to which a number of signaling molecules such as G-protein-coupled receptors, heterotrimeric G proteins, receptor tyrosine kinases, components of the Ras-mitogen-activated protein (MAP) kinase pathway, Src-like kinases, protein kinase C (PKC), nitric oxide synthase (NOS), H-Ras and eNOS are bound and concentrated (8-11). Previously, we have shown that caveolin-1 is overexpressed in rat colon tumors and is significantly associated with proliferative rates of human colon cancer cell lines (12). Caveolin-1 plays pivotal roles in intracellular signal transduction, angiogenesis and tumor invasion (13,14). Therefore, we studied the effect of lovastatin and/or celecoxib on caveolin-1, Akt, Erk, Stat3, cyclin D1 and other cell cycle regulators. Excessive lipid body formation in cells associated with inflammation reactions have been reported (15,16). Lipid bodies are spherical structures containing a mixture of triglycerides, eicosanoids and cholesterol esters encased in a thin phospholipids membrane (17). Since both cholesterol and caveolin-1 are essential for lipid body formation, we studied the effect of lovastatin, celecoxib and their combination on lipid body formation in human colon cancer cell lines.

In this study, we demonstrate for the first time that lovastatin and celecoxib both disrupt membrane caveolae and decrease the expression of Cav-1 associated signaling molecules. In particular, both drugs decreased phosphorylated AKT and its down-stream effector proteins. There was a significant suppression of lipid body formation by both lovastatin and celecoxib.

Materials and methods

Cell cultures. Human colon carcinoma cell line, HCT-116, was obtained from American Type Culture Collection. The cells were maintained in McCoys (Gibco-BRL, Grand Island, NY) supplemented with 10% FBS and 1% streptomycin and penicillin antibiotics at 37°C in a 5% CO₂ incubator. Cultures at 70-80% confluence were used for all experiments.

Cell viability assay. Cell viability was measured by 3-(4,5)-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, HCT-116 cells were grown in the presence or absence of various concentrations of celecoxib and lovastatin or a combination of both. After 24 h, MTT reagent was added to the cell culture plate and incubated for 4 h at 37°C. Subsequently, the culture plates were incubated overnight with 10% sodium dodecyl sulphate (SDS) solution. The absorbance at 540 nm was measured by a microtiter plate reader (BMG, Germany).

Immunofluorescence. HCT-116 cells were grown on six-well tissue culture plates. The cells were treated with 25 μM lovastatin and/or 50 μM celecoxib when they were 80% confluent. After 24 h incubation, the cultures were fixed in formalin. Immunofluorescence analysis was performed by tissue incubation in PBS containing 0.2% saponin, 1% BSA, followed by overnight incubation with Cav-1 (1:500; Santa Cruz Biotechnology Inc, USA) primary antibody at 4°C. The goat anti-rabbit tagged to fluorescein (FITC) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used for the secondary antibody. Following incubation with secondary antibody, nuclei were stained with 10 μg/ml DAPI for 5 min. After each incubation step, the tissue was washed in PBS three times. Finally, the tissue was covered with aqueous mounting medium (Biomed, CA) and examined under an Olympus IX71 fluorescent microscope.

Western blot analysis. Cells exposed to 25 μM lovastatin and/or 50 μM celecoxib were lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT and a protease inhibitor cocktail) and the protein content was quantitated using the Bio-Rad protein assay kit (Hercules, CA). Separation of proteins (50 μg) was resolved on a SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with a solution containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Tween-20 and 5% dry milk and incubated for 1 h with primary antibody (1:1000), anti-actin (1:1000). After washing the blots with TBST they were then incubated with anti-mouse and rabbit HRP-conjugated secondary antibody followed by washing again with TBST. Washed blots were incubated with Super Signal West Pico Chemiluminescence Substrate (Pierce, Rockford, IL) for 5 min and exposed to Kodak X-ray film.

Lipid body staining. HCT-116 cells were fixed in 4% formaldehyde and immersed in 1.0% thio-carbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO₄ for 3 min, rinsed in distilled water and then dried and mounted. Cell morphology was observed and lipid bodies were enumerated by microscopy.

Results

Growth inhibition of HCT-116 cells by lovastatin and celecoxib. To compare the growth inhibitory effect of HCT-116 cells in response to lovastatin and celecoxib, we used the MTT assay. In the MTT assay, thiazolyl blue (MTT) is reduced by mitochondrial dehydrogenases. The uptake of the vital dye MTT is a measure of the mitochondrial dehydrogenase activity within the cell and provides an indication of the proliferation status of the cells. The HCT-116 cell line was exposed to lovastatin (0-100 μM) and celecoxib (0-150 μM) for 24 h. The IC₅₀ value for HCT-116 cell growth inhibition was 25 μM for lovastatin and 50 μM for celecoxib. Hence we used 25 μM lovastatin and 50 μM celecoxib for all the experiments.
Lovastatin and celecoxib decrease caveolin-1. The effect of lovastatin and celecoxib or a combination of both agents on caveolin-1 expression and localization is summarized in Fig. 1A-E. Cells exposed to celecoxib and its combination with lovastatin for 24 h showed a significant decrease in immunofluorescence of caveolin-1 protein (Fig. 1A-D). These results were further confirmed by Western blot analysis (Fig. 1E upper panel, 24 h treatment). While cells exposed to lovastatin for 24 h do not show any decrease in total immunofluorescence, it did lead to the disruption of membrane bound caveolin-1 into the cytoplasm (Fig. 1B). Notably, Western blot analysis of HCT-116 cells treated with lovastatin for 48 h showed a significant decrease of caveolin-1 protein (Fig. 1E, lower panel). Taken together, our results clearly suggest that the combination of lovastatin and celecoxib suppresses caveolin-1 expression more effectively than either agent alone (Fig. 1A-E).

Akt signaling is down-regulated by celecoxib and lovastatin. To understand whether molecules that are directly influenced by caveolin-1 are modulated by celecoxib and lovastatin, we investigated the effect of these agents on the AKT survival pathway. We examined the effect of lovastatin, celecoxib and their combination on EGF-induced AKT phosphorylation and its down-stream effector molecules. Our results show that, while total AKT levels are unaffected by either individual or combination treatment, both lovastatin and celecoxib decreased phosphorylation of AKT while the combination treatment showed total inhibition of AKT phosphorylation (Fig. 2). Similar to AKT phosphorylation, treatment of HCT-116 with lovastatin and/or celecoxib also decreased the levels of phosphorylated Erk1/2 (Fig. 2). Also, treatment with lovastatin and/or celecoxib at concentrations that block ERK-phosphorylation in HCT-116 cells inhibited STAT3 activation, indicating that the AKT-ERK-STAT3 pathway is involved in the proliferative effects of HCT-116 colon cancer cells (Fig. 2).

Effect of lovastatin and celecoxib on cell cycle and apoptosis proteins. Lovastatin and celecoxib showed a modest decrease in cyclin D1, while the combination treatment showed a robust
Lovastatin and celecoxib decrease lipid body formation. Lovastatin and/or celecoxib decreased the size as well as the number of lipid bodies, which correlated well with the inhibitory effects of the compounds. As shown in Fig. 4, while there was a considerable amount of lipid bodies in control cells, there was a significant decrease in lipid body formation with lovastatin and/or celecoxib treatment. Lovastatin and celecoxib showed a 90% decrease in the amount of accumulated lipid bodies, while the combination treatment showed a complete inhibition.

Discussion

Previous studies from our laboratory have shown that combination of statins and/or celecoxib can reduce colon tumors in rodent models (6). However, the molecular mechanism by which synergistic inhibition occurs is not clearly known. In the present study, we show that lovastatin and celecoxib modulate caveolin-1 expression and thereby alter the signaling molecules associated with caveolin-1. The phosphorylation of AKT, a protein that is associated with the scaffolding domain of caveolin-1 promotes the activation of ERK and subsequently STAT3. We also show that lovastatin and/or celecoxib decreases cyclin D1, pRb and E2F1.

Caveolin-1 and its associated signaling molecules have been linked with cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis and chemoresistance in several cancer types. There is evidence suggesting that caveolin-1 plays an important role in the promotion of colon tumorigenesis. In support of this, caveolin-1 is overexpressed in colon cancer cells (18,12) and has been shown to promote cancer in an animal model of prostate cancer (19). In addition, caveolin-1 promotes cancer growth through inhibition of apoptosis through induction of cyclin D1 (20). In the current study, we observed that 24 h treatment of HCT-116 colon cancer cells with either celecoxib or the combination with lovastatin decreased caveolin-1 expression. Although overnight treatment with lovastatin did not decrease caveolin-1 expression, immunofluorescence studies showed that lovastatin induced membrane disassociation of caveolin-1 into the cytoplasm. In contrast, cells treated for 48 h with lovastatin completely decreased caveolin-1 expression. The reason for the above observations could be that lovastatin alters the membrane stability by blocking cholesterol biosynthesis, thereby releasing caveolin-1 into the cytoplasm which is later degraded. However, the mechanism by which celecoxib decreases caveolin-1 is not yet understood.

The interaction between caveolin-1 and various signaling molecules that are bound to its scaffolding domain plays an important role in caveolin-1-mediated signaling involving growth and survival. Several signaling molecules such as Akt, G-protein-coupled receptors, heterotrimeric G proteins, receptor tyrosine kinases, components of the Ras-mitogen-activated protein (MAP) kinase pathway, Src-like kinases, protein kinase C (PKC), nitric oxide synthase (NOS), H-Ras and eNOS are bound and concentrated (8-11) and are known to bind to the scaffolding domain of caveolin-1. Akt (protein kinase B), a serine/threonine kinase, has emerged as a critical enzyme in several signal transduction pathways involved in cell proliferation, apoptosis, angiogenesis and diabetes. Increased Akt activity has been shown to be responsible for caveolin-1-mediated cell survival. Caveolin-1 has been shown to increase the half-life of phosphorylated PDK1 and Akt after treatment with lovastatin, celecoxib and their combination and analyzed for cyclin D1, CDK2, pRb, E2F1 GADD45, Bcl-2 and BAX was studied by Western blotting.

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metastatic cancer cells express high amounts of activated Akt (22). Activation of Akt occurs through phosphorylation at two regulatory residues, a threonine residue on the kinase domain and a serine residue on the hydrophobic motif (22). The principal role of Akt is to facilitate growth factor-mediated cell survival and block apoptotic cell death. Akt achieves this by phosphorylating a variety of substrates, including Erk, Bad, and STAT3 transcription factors (23-25). It therefore seems rational to hypothesize that lovastatin and celecoxib, by decreasing caveolin-1 expression, may also decrease the levels of the activated Akt protein. In agreement with this hypothesis, we found that both lovastatin and/or celecoxib decreased the phosphorylation of Akt.

The Erk signaling cascade is critical for transducing signals that are essential for differentiation, plasticity and survival (26,27). The Erk signaling cascade is activated by a wide variety of receptors involved in growth and differentiation such as receptor tyrosine kinases (RTKs). Activated Erk can regulate targets in the cytosol and also translocate to the nucleus where it phosphorylates a variety of transcription factors regulating expression of genes such as STAT3 (28). In this context, we studied the phosphorylation of ERK 1/2 after lovastatin and celecoxib treatment. Our results show that both lovastatin and celecoxib treatment led to a decrease in the phosphorylation of ERK1/2, which points to an inhibition of their kinase activity. The results from this study also show that both lovastatin and celecoxib decreased Bcl-2, while increasing BAX expression. Since Erk activation has been shown to be important for survival and is known to induce Bcl-2, the decrease in the phosphorylation of ERK observed in the
current study may be involved in the apoptotic induction by lovastatin and celecoxib.

Induction of cyclin D1 by growth factors and oncogenes is PI3'-kinase-dependent, which suggests that the PI3'-kinase/Akt pathway may play an important role in the control of cell cycle progression (29,30). Further, the ability of Akt to directly phosphorylate key mediators of the apoptotic response provides strong circumstantial evidence for the importance of the PI3'-kinase-Akt pathway in the aberrant behavior of cancer cells. The progression of cells into S phase requires the activity of cyclin D1 (31). The ability of Akt to promote cell cycle progression is correlated with its ability to increase expression of cyclin D1. We show that celecoxib and/or lovastatin alters the levels of cyclin D1, and subsequently pRb and E2F1. Without active nuclear E2F-1 function, crucial S phase activities such as DNA repair are impaired, leading to eventual cell death. For instance, the toxicity of celecoxib has been shown to be in part through the down-stream inhibition of pRb and E2F-1 activity within the nucleus (32).

One important aspect of cholesterol regulation is intracellular cholesterol storage in lipid storage organelles, called lipid droplets or lipid bodies, which contain a pool of proteins with a wide range of biochemical activities. So far, only a few of these proteins have been identified and little is known about their structural properties and functions. High concentrations of lipid bodies develop in cells associated with inflammation reactions (15,16). Various cytokines and pro-inflammatory stimuli trigger the synthesis of COX-2, leading to increased formation of eicosanoids. There is increasing evidence that lipid bodies are sites of intracellular localization of COX-2 (33,34) as well as repositories of esterified arachidonates (35). Thus, lipid bodies serve as novel putative sites for eicosanoid biosynthesis in cells involved in inflammation (36-39). Caveolin-1 plays an important role in the modulation of lipolysis and lipid droplet formation (38). In support of this, caveolin-1 has been shown to be redirected from the plasma membrane caveolae to intracellular lipid droplets and mice lacking caveolin-1 have lean bodies with adipocyte abnormalities (38-40). Lipid bodies are sites at which key eicosanoid-forming enzymes are localized. The arachidonate-releasing enzyme cPLA₂ and its activating MAP kinases also localizes to lipid bodies. PI3K has been shown to localize to lipid bodies of myeloid-derived cells, which would support a role for PI3K in generating phosphoinositide signaling molecules within lipid bodies. Therefore, it is likely that regulated signal transduction responses occur within lipid body domains. In this context, our findings that lovastatin and/or celecoxib inhibit lipid body formation are important for understanding the regulation of signaling molecules associated with lipid bodies (Fig. 5). Since lipid bodies contain cholesterol...
and COX-2 as well as caveolin-1, statins will be useful in disrupting the membrane integrity of the lipid bodies while COX-2 inhibitors can be used to block the activity of the COX-2 enzyme.

The increased expression of HMG-coA reductase and COX-2 are important pathological components in colon cancer. In this regard, our current findings provide further insights into the possible use of lovastatin and celecoxib in a low-dose combination for colon cancer chemoprevention and treatment.

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References