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Inhibition of cyclooxygenase-1 lowers proliferation and induces macroautophagy in colon cancer cells

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ABSTRACT

Evolving evidence supports that cyclooxygenase-1 (COX-1) takes part in colon carcinogenesis. The effects of COX-1 inhibition on colon cancer cells, however, remains obscured. In this study, we demonstrate that COX-1 inhibitor sc-560 inhibited colon cancer cell proliferation with concomitant G_0/G_1-phase cell cycle arrest. The anti-proliferative effect was associated with down-regulation of c-Fos, cyclin E_2 and E_2F-1 and up-regulation of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. In addition, sc-560 induced macroautophagy, an emerging mechanism of tumor suppression, as evidenced by the formation of $LC3^+$ autophagic vacuoles, enhanced LC3 processing, and the accumulation of acidic vesicular organelles and autolysosomes. In this connection, 3-methyladenine, a Class III phosphoinositide 3-kinase inhibitor, significantly abolished the formation of $LC3^+$ autophagic vacuoles and the processing of LC3 induced by sc-560. To conclude, this study reveals the unreported relationship between COX-1 and proliferation/macroautophagy of colon cancer cells.

Introduction

Recent findings supports that cyclooxygenase-1 (COX-1) plays an active role in colon carcinogenesis. For instances, experimental mouse study using genetic ablation of the *COX-1* plus *Apc* genes have demonstrated that COX-1 is involved in intestinal polyp formation [1]. In addition, inhibition of COX-1 by the selective blocker mofezolac suppresses the development of azoxymethane-induced colonic aberrant crypt foci [2] and colon cancer formation [1] in F344 rats, and intestinal polyp development in *APC1309* mice with a truncated *Apc* gene [3]. Up-regulation of COX-1 also protects intestinal stem cells from the DNA-damaging effect of azoxymethane and may play a key role in the early phase of intestinal tumorigenesis [4]. Moreover, clinical studies have found that inhibiting both COX-1 and COX-2 with conventional non-steroidal anti-inflammatory drugs, such as indomethacin or sulindac, reduce the number of intestinal polyps in familial adenomatous polyposis patients more effectively than by COX-2 selective inhibitors such as celecoxib and nimesulide [5–8].

Macroautophagy is a catabolic process by which the cell degrades its organelles through the lysosomal machinery [9]. Overactivation of macroautophagy can lead to cell death and may serve as an alternative to apoptosis to eliminate transformed cells [9,10]. Moreover, tumorigenesis is often associated with a down-regulation in macroautophagy while genes that are involved in the execution of macroautophagy are found to be tumor suppressors [9,10]. In colon cancer, mutations of *UVRAG*, a macroautophagy-related gene, frequently occur in a subset of patients with high microsatellite instability [11]. Morphologically, macroautophagy is characterized by the formation of $LC3^+$ autophagic vacuoles [12] and the accumulation of acidic vesicular organelles [13] and autolysosomes [14]. In relation to its regulation, induction of macroautophagy is associated with the suppression of mammalian target of rapamycin (mTOR) pathway [15]. The relationship between macroautophagy and COX-mediated signaling, however, remains unexplored. In this study, we evaluated the effects of COX-1 inhibition on colon cancer cell proliferation and macroautophagy by treating HT-29 colon cancer cells with the COX-1-specific inhibitor sc-560.

Materials and methods

Reagents. Antibodies for the detection of c-Fos, cyclin D_1, cyclin E_2, cyclin-dependent kinase-2 (CDK-2), LC3, Beclin-1, phosphorylated and total mammalian target of rapamycin (mTOR), and β-actin were

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purchased from Cell Signaling Technology (Beverley, MA, USA). Other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acridine orange and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Invitrogen, Carlsbad, CA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

Cell culture and proliferation assay. The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cell proliferation was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as previously described [16]. Necrotic cell death was determined by measuring lactate dehydrogenase released from cells into the culture medium using a cytotoxicity detection kit (Roche Diagnostics Corp., Indianapolis, IN, USA).

Colony-formation assay. HT-29 cells were then seeded in six-well plates at the density of 1×10^4 cells per well. After attachment, cells were treated with or without sc-560 for 24 h and then allowed to grow for another 6 days, after which the cells were fixed with absolute methanol and stained in hematoxylin for 30 min and the cultures were photographed under white-transillumination using ChemiDoc XRS system (Bio-Rad). Colonies were then counted using Quantity One software (Bio-Rad).

Apoptosis assay. Apoptosis was measured using the Cell Death Detection ELISA plus system (Roche Diagnostics Corp.), a spectrophotometric enzyme-immunoassay for the *in vitro* determination of cytoplasmic histone-associated DNA-fragments of mono- and oligonucleosomes after induced cell death.

Cell cycle analysis. HT-29 cells were fixed with ice-cold 70% ethanol in phosphate buffered saline followed by incubation with 50 μg/ml propidium iodide, 3.8 mmol/L sodium citrate, and 0.5 μg/ml RNase A at 4 °C for 3 h and analyzed by flow cytometry (Beckman Coulter, Fullerton, CA, USA). The resultant DNA histograms were generated using ModFit software (Verity Software House, Topsham, ME, USA).

Western blot analysis. Cells were harvested in radioimmunoprecipitation buffer containing proteinase and phosphatase inhibitors as previously described [16]. Equal amounts of protein (50 μg/lane) were resolved by SDS–PAGE, and transferred to Hybond C nitrocellulose membranes (Amersham Corporation). The membranes were probed with primary antibodies overnight at 4 °C and incubated for 1 h with secondary peroxidase-conjugated antibodies. Chemiluminescent signals were then developed with Lumiglo reagent (Cell Signaling Technology) and detected by the ChemiDoc XRS gel documentation system (Bio-rad).

Immunofluorescence for LC3⁺ autophagic vacuoles. Cells grown on 96-well plates were fixed with 4% (v/v) paraformaldehyde for 30 min and then made permeable with methanol at −20 °C for 10 min. The cells were then covered with 10% (v/v) goat serum for 30 min at room temperature to block nonspecific adsorption of antibodies to the cells. After this procedure, the cells were incubated with primary antibody against LC3 at 4 °C overnight. Cells were then probed with Alexa Fluor 488 goat anti-rabbit secondary antibodies and incubated at room temperature for another 2 h. Fluorescent signals were detected using a fluorescence microscope (Nikon TS100-F). Macroautophagy was quantified by counting the number of $LC3^+$ dots or vacuoles per cells (a minimum of 100 cells per preparation).

Acridine orange staining for acidic vesicular organelles. Acridine orange was added at a final concentration of 1 μg/ml for a period of 15 min. Pictures were obtained with a fluorescence microscope (Nikon TS100-F) equipped with a 450–490-nm band-pass blue

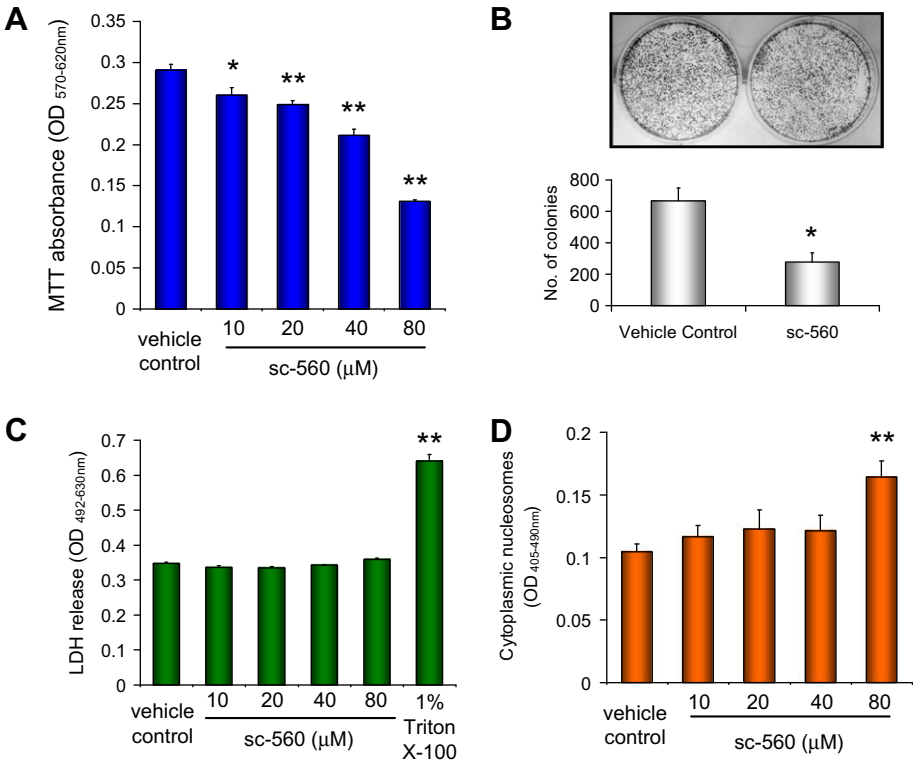

Fig. 1. Inhibition of HT-29 cell proliferation by COX-1-specific inhibitor sc-560. (A) Incubation of sc-560 for 24 h dose-dependently lowered cell proliferation as determined by MTT assay. (B) Twenty-four hours treatment with sc-560 (80 μmol L^{-1}) significantly reduced the number of cell colonies. (C) sc-560 did not affect necrotic cell death as determined by lactate dehydrogenase release assay. Triton X-100 (1%, v/v) was used as a positive control. (D) sc-560 only modestly increased apoptosis at high concentration. $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from respective control group.

excitation filters, a 505-nm dichroic mirror, a 520-nm long pass-barrier filter, and a digital camera (Nikon DS-5Mc).

Monodansylcadaverine (MDC) staining for autolysosomes. HT-29 cells were stained with a 0.05 mM final concentration of MDC in PBS for 30 min at 37 °C. Cells were then washed with PBS two times to remove excess MDC and fixed with 4% (v/v) paraformaldehyde for 30 min. Pictures were obtained using a fluorescence microscope (Nikon TS100-F) equipped with a 330–380 band-pass ultraviolet excitation filter, a 400-nm dichroic mirror, and a 420-nm long pass-barrier filter.

Statistical analysis. Results were expressed as means ± SEM. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Turkey's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

COX-1 inhibitor sc-560 inhibited colon cancer cell proliferation

As shown in Fig. 1A, sc-560 significantly reduced HT-29 cell MTT tetrazolium salt formation. At the dose of 80 μmol L^{-1}, 24-h treatment of sc-560 inhibited HT-29 cell proliferation by about 50%, when compared with vehicle control (0.4% v/v dimethyl sulfoxide). The anti-mitogenic effect of sc-560 could be detected at the concentration as lowest as 10 μmol L^{-1}. To further confirm the anti-mitogenic action of sc-560, colony-formation assay was performed. Results showed that 24-h treatment of sc-560 significantly reduced the colony-forming ability of HT-29 (Fig. 1B). Necrotic cell death in all treatment groups was confirmed to be unaffected by sc-560 treatment using lactate dehydrogenase release assay (Fig. 1C). Furthermore, quantitation of cytoplasmic nucleosomes indicated that sc-560 at 80 μmol L^{-1} only modestly increased apoptosis (Fig. 1D). At low doses, ranging from 10 to 40 μmol L^{-1}, sc-560 did not significantly induce apoptosis.

COX-1 inhibitor sc-560 induced G_0/G_1-phase cell cycle arrest and down-regulated proteins related to G_1–S transition

Results from flow cytometry-based cell cycle analysis showed that sc-560 at the concentrations of 40 and 80 μmol L^{-1} induced a substantial accumulation of HT-29 cells at the G_0/G_1-phase. A reciprocal reduction of proportion of cells in S-phase was also observed in sc-560-treated cells (Fig. 2A) which showed an indistinct G_2/M peak. In addition, sc-560 at the various concentrations substantially decreased the expression of c-Fos, cyclin E_2 and E_2F-1. Moreover, sc-560 enhanced the expression of cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. The expression of c-Myc, c-Jun, cyclin D_1, and CDK-2, -4, -6 were not affected by sc-560 treatment (Fig. 2B).

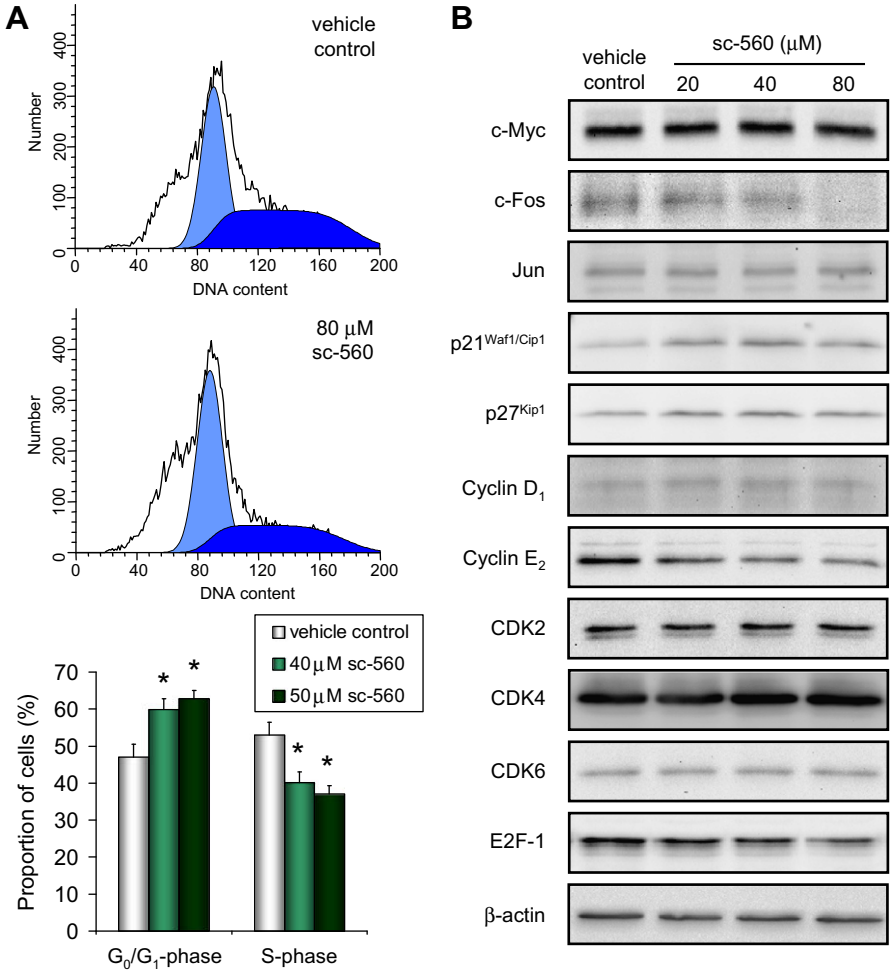

Fig. 2. Inhibition of HT-29 cell cycle progression by COX-1-specific inhibitor sc-560. (A) DNA histogram shows the accumulation of G_0/G_1-phase cells induced by sc-560 in HT-29. Cells were treated without or with 40 or 80 μmol L^{-1} sc-560 for 24 h, and their DNA contents were determined by flow cytometry analysis. (B) sc-560 at various concentrations down-regulated the protein expression of c-Fos, cyclin E_2, and E_2F-1 and up-regulated $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. The expression of other cell cycle regulators was not affected. $^{*}P < 0.05$, significantly different from the control group. The results are the representative of two independent experiments.

COX-1 inhibitor sc-560 induced the formation of autophagic vacuoles, enhanced LC3 processing and increased the accumulation of acidic vesicular organelles and autolysomes

To determine the effect of COX-1 inhibition on macroautophagy, we analyzed the formation of $LC3^+$ autophagic vacuoles and the accumulation of acidic vesicular organelles and autolysosomes. Results showed that sc-560 significantly increased the formation of $LC3^+$ autophagic vacuoles in HT-29 (Fig. 3A) as determined by immunofluorescence staining. In this regard, the number of $LC3^+$ dots or vacuoles increased from 0.75/cell to 4.38/cell after 24-h treatment with 80 μmol L^{-1} sc-560 in HT-29 cells. As the amount of LC3 protein, particularly LC3-II, has been shown previously to correlate with the extent of macroautophagy [17], the effect of sc-560 on LC3 protein expression in HT-29 cells was studied. Results showed that sc-560 at the concentrations of 40 and 80 μmol L^{-1} significantly induced LC3-II protein expression (Fig. 3B). In contrast, the expression of Beclin-1, another protein involved in macroautophagy, was not significantly affected. Moreover, treating HT-29 cells with comparable pharmacologically active concentrations of the COX-2-specific inhibitor sc-236 did not alter LC3 processing (data not shown), suggesting that the macroautophagy-inducing activity of sc-560 was not mediated through interference with COX-2 activity. For detection of the

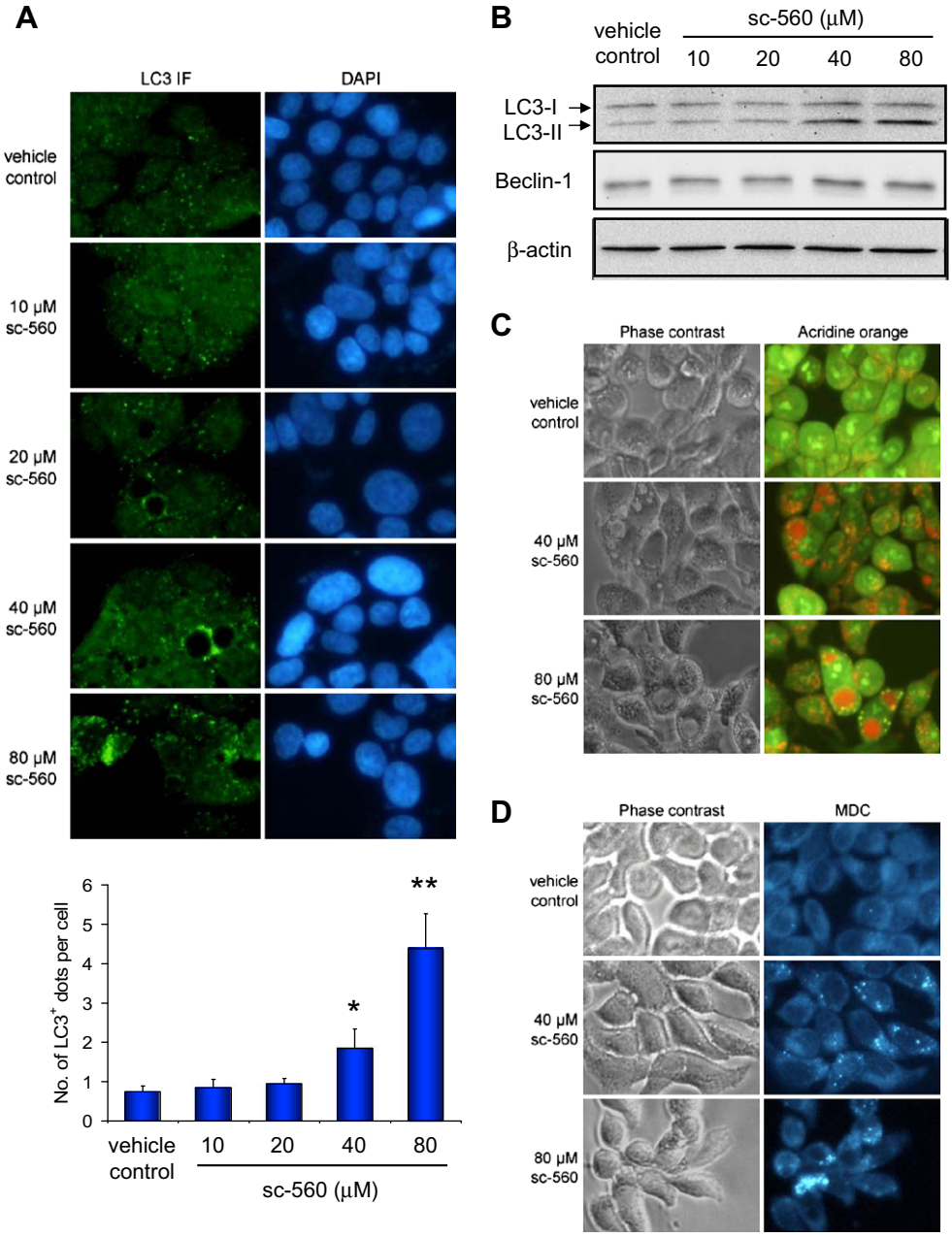

Fig. 3. Determination of sc-560-induced autophagy in HT-29 cells. (A) Treating the cells with sc-560 for 24 h prominently enhanced the formation of autophagic vacuoles as determined by immunofluorescent staining for LC3. Counting $LC3^+$ dots or vacuoles showed a dose-dependent increase in autophagic vacuoles in sc-560-treated cells. (B) sc-560 dose-dependently increased the protein expression of LC3-II. The expression of Beclin-1, however, was not altered. (C) The accumulation of acidic vesicular organelles, which emitted bright red fluorescence, was visualized by acridine orange staining. (D) Effects of sc-560 on the formation of autolysosomes were determined by monodansylcadaverine staining in paraformaldehyde-fixed cells. $^*P < 0.05$; $^{**}P < 0.01$, significantly different from respective control group. The results are the representative of two independent experiments.

acidic cellular compartment and autolysosomes, we used the acridine orange and monodansylcadaverine staining, respectively. Acridine orange emitted bright red fluorescence in acidic vesicles but fluoresced green in cytoplasm and nucleus [13]. Vital staining of HT-29 cells with acridine orange revealed the appearance of acidic vesicular organelles after sc-560 treatment (Fig. 3C). Conversely, the majority of control cells exhibited only minimal red fluorescence. For monodansylcadaverine staining, sc-560-treated HT-29 cells showed a substantial increase in fluorescent dots or vacuoles when compared with the control cells (Fig. 3D).

3-Methyladenine blocked sc-560-induced macroautophagy and processing of LC3

Class III phosphoinositide 3-kinase (PI3K), which produces phosphatidylinositol-3-phosphate, has been implicated in the initiation and propagation of macroautophagy [18]. We therefore studied the involvement of this enzyme in sc-560-induced macroautophagy. In this connection, 3-methyladenine, a Class III PI3K inhibitor, significantly reduced LC3-II protein expression and the formation of $LC3^+$ autophagic vacuoles induced by sc-560 as determined by Western blot (Fig. 4A) and immunofluorescence (Fig. 4B). The data presented so far indicated that COX-1 inhibition induced G_0/G_1 cell cycle arrest and macroautophagy. A common link of these two processes is that they can be modulated by mTOR signaling. We therefore examined the effect of sc-560 on the activity of mTOR by determining the phosphorylation at Ser2448 and Ser2481. Contrary to our expectations, our results showed that sc-560 did not alter the phosphorylation or expression of mTOR (Fig. 4C).

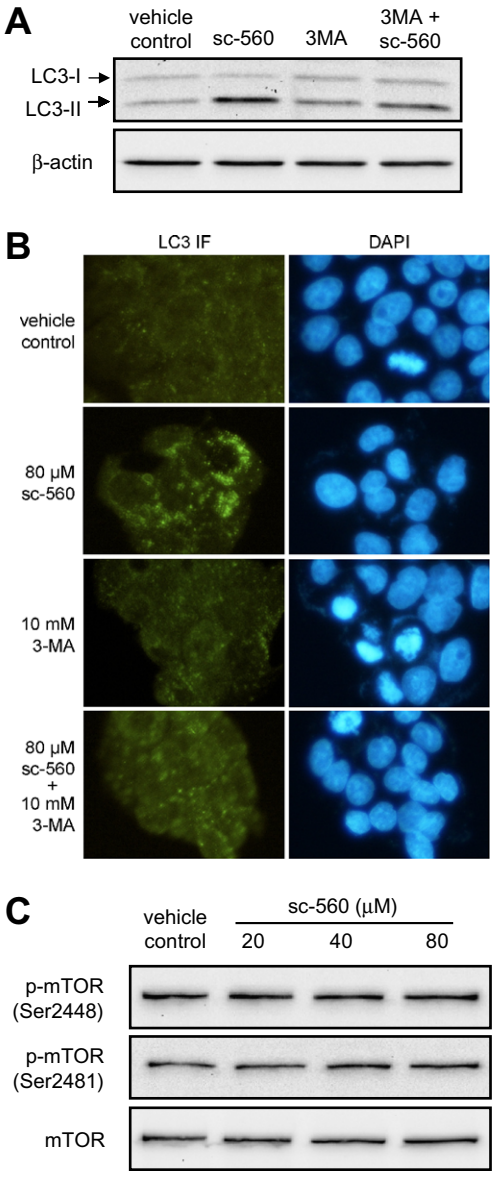

Fig. 4. Mechanism of sc-560-induced autophagy in HT-29 cells. (A) 3-Methyladenine (10 mmol/L) blocked the conversion of LC3-I to LC3-II induced by sc-560. (B) Immunofluorescent staining for LC3 demonstrated the prevention of sc-560-induced autophagic vacuole formation by 3-methyladenine (10 mmol/L). (C) The total expression and phosphorylation of mTOR were not affected by sc-560 treatment. The results are the representative of two independent experiments.

Discussion

Numerous studies have suggested that COX-1 plays an important role in colon carcinogenesis [1–4]. The molecular and cellular mechanism underlying the oncogenic action of COX-1 in colon cancer was unclear. Here we show that inhibition of COX-1 lowers the proliferation and induces G_0/G_1 arrest in colon cancer cells. The expression of a number of proto-oncogenes related to G_1–S transition is down-regulated. The anti-mitogenic effect of COX-1 inhibition is associated with the induction of macroautophagy as evidenced by the increased formation of $LC3^+$ autophagic vacuoles, accumulation of acidic vesicular organelles and autolysosomes, as well as up-regulation of LC3-II protein. The effects of COX-1 inhibition on macroautophagy induction are marked and coincide with the anti-mitogenic activity. In our view the most significant finding of the present study is that treatment of cells with COX-1 inhibitor did not induce overt apoptosis, a response commonly associated with COX-2 inhibition [19–21], but rather induced morphological and biochemical alterations characteristic of macroautophagy, a cellular process that recently becomes a subject of intense investigation because of its role in various pathological states including cancer. In fact, there are recent suggestions that defective macroautophagy contributes to carcinogenesis. In this regard, our experimental findings are consistent with the role of macroautophagy as a tumor-suppressing mechanism in colon cancer and shed new light on the unreported relationship between COX-1 and macroautophagy.

In the present study, macroautophagy induced by COX-1 inhibition is associated with the inhibition of G_1–S transition in colon cancer cells. This finding is in line with previous studies reporting that cysmethynil and temsirolimus induce both macroautophagy and G_0/G_1-phase cell cycle arrest in prostate cancer cells [22] and mantle cell lymphoma cells [23], respectively. Moreover, pharmacological inhibition or targeted disruption of the p27 E3 ligase SCF^{Skp2} result in macroautophagy and accumulation of myeloma cells in G_0/G_1 phase [24]. These data along with our experimental findings indicate that induction of macroautophagy is associated with inhibition of cell proliferation and G_0/G_1 cell cycle arrest. The accumulation of cells in G_0/G_1-phase that we detected appears in part to be mediated through down-regulation of c-Fos, cyclin E_2, and E_2F-1, all of which are essential for successful G_1–S transition. In addition, the expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ are up-regulated by COX-1 inhibitor which further reduces the activities of CDKs.

Mechanistically, we demonstrated that COX-1 inhibition by sc-560 activates macroautophagy in colon cancer cells and such action depends on Class III PI3K. In this regard, Class III PI3K is known to be required for the induction of macroautophagy during nutrient deprivation in both yeast and mammalian cells [18]. Our results also indicate that inhibition of Class III PI3K by 3-methyladenine

suppresses the formation of $LC3^+$ autophagic vacuoles and the conversion of LC3-II from LC3-I induced by COX-1 inhibitor. These findings fit well with the reported role of Class III PI3K in macroautophagy. Aside from Class III PI3K, we also explored the relationship between COX-1 inhibition and mTOR signaling. Inhibition of mTOR signaling has been shown to activate macroautophagy. Our results show that COX-1 inhibition has no effect on mTOR phosphorylation, suggesting that the macroautophagy-promoting effect of sc-560 is mTOR-independent.

In summary, we report that COX-1 inhibition lowers the proliferation of colon cancer cells, a phenomenon associated with G_0/G_1-phase cell cycle arrest and induction of macroautophagy. Moreover, Class III PI3K is involved in macroautophagy associated with COX-1 inhibition. The current study clarifies the functional role of COX-1 as a molecular target in the treatment and prevention of colorectal cancer and improves our understanding about the linkage between COX-1 and macroautophagy in relation to the pathogenesis of colorectal cancer. Information derived from this study may also lead us to further refine our current clinical guidelines regarding the use of COX inhibitors in the prophylaxis and treatment of colorectal cancer as non-selective COX inhibitors may have some benefits over COX-2 selective inhibitors.

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