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Original Article

Vitamin K2-induced inhibition of colorectal cancer cell proliferation and its underlying mechanisms

Bai-Chun Liu, Ying Song, Feng Li, Li-Juan Wei, Jun-An Li

Department of Gastroenterology, Second Affiliated Hospital of Jilin University, Changchun, Jilin, China

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Abstract: Epidermal growth factor receptor (EGFR) signalling promotes growth of colorectal cancer (CRC) cells and also is known to induce the production of a tumorigenic cytokine parathyroid hormone related peptide (PTHrP) in some malignancies. Sphingosine-1-phosphate (S1P), through its G-protein-coupled receptors S1PR, has been shown to transactivate multiple membrane-bound receptors, notably EGFR. S1P promotes tumorigenic and inflammatory responses that favour cell transformation and malignancy progression. We hypothesize that blocking EGFR signalling and PTHrP production in CRC could mitigate malignant growth. We found that vitamin K2 (menaquinone-4, MK4) reduced the proliferation of KRAS mutant LoVo cells and KRAS wild type Caco 2 cells induced by EGF and S1P. In both cell lines, MK4 attenuated EGF-induced proliferation, survival, phosphorylation of EGFR and production of PTHrP. The EGF-induced PTHrP secretion was responsible for EGF's proliferation-promoting effect that has been demonstrated by the suppression of EGF-induced cell growth by PTH receptor antagonist, PTH (7-34). S1P, via its receptor S1PR1, increased proliferation and transactivation in CRC cell lines. Transactivation of EGFR by S1PR1 was dependent on the activation of matrix metalloproteases (MMP), particularly MMP-2 and MMP-9 (albeit partially). S1P-induced EGFR transactivation was blocked by S1P receptor 1 (S1PR1) inhibitor which led to inhibition of PTHrP secretion. MK4 suppressed transactivation of EGFR by S1P, leading to inhibition of S1P-induced PTHrP secretion and cell proliferation. Taken together, our data show that MK4 attenuated the pro-malignant effects of EGF and S1P on CRC lines by attenuating their ability to activate EGFR signalling and the downstream production of PTHrP.

Keywords: Epidermal growth factor receptor (EGFR), sphingosine-1-phosphate (S1P), menaquinone-4 (MK4), KRAS, matrix metalloproteases (MMP)

Introduction

Colorectal cancer (CRC) is the most frequent form of malignancy of the large bowel and one of the most prevalent cancers in developed countries [1]. Colorectal carcinogenesisis is a multi-step process which includes proliferation of aberrant intestinal epithelial cells, apoptosis, differentiation and migration [2]. Migration and invasion are critical for the ability of the colorectal cancer cells to metastasize to other organs. Several growth factors determine metastatic fate of CRC cells, one of which is parathyroid hormone related peptide (PTHrP) [3, 4]. Over-expression of PTHrP correlates with the severity of colorectal carcinoma reflected by the level of cell differentiation, and the depth of invasion and metastasis (lymphatic and hepatic) [5]. In human colon adenocarcinoma cell line Caco-2, PTHrP treatment had a mitogenic effect mediated by ERK1/2, p38 MAPK and PI3-kinase [6]. In a metastatic human colorectal carcinoma LoVo cell line, the PTHrP expression correlates with the activity of Rho GTPase, Rac1 [5, 7]. Elevated Rac1 and overexpression of Tiam-1, a Rac1-specific GEF have also been reported [8]. In LoVo cells, PTHrP increased Rac1 activation by upregulating integrin α6β4 [5]. Together, the published data indicate that decreasing the PTHrP signalling in colon cancer may have therapeutic effect.

Expression of ErbB family member of epidermal growth factor receptor (EGFR) has been reported to be associated with poor outcomes in patients with stage IV of CRC [9]. EGFR overexpression has also been associated with tumor-node-metastasis T3 [10]. Anti-EGFR antibody such as cetuximab that binds the extracellular domain of EGFR is an effective therapy in the

treatment of CRC [11]. However, the clinical advantage of this therapy is limited by drug refractoriness due to the emergence of point mutation in KRAS gene [12]. EGF has been reported to stimulate the synthesis and secretion of PTHrP in a variety of malignant and normal cells [13].

Bioactive sphingolipids including ceramide and its metabolite sphingosine promote apoptosis of colorectal cancer cells [14]. Epithelial cells of gastrointestinal (GI) tract are exposed to various sphingolipid metabolites that are generated by the breakdown of sphingolipids from dietary sources. Enzymes in the brush border of epithelial cells convert sphingolipids to sphingosine which enters cells and gets converted to sphingosine-1-phosphate (S1P) by sphingosine kinases [15]. In contrast to the protective effect of sphingosine during cancer development, S1P inhibits cancer cell apoptosis, promotes angiogenesis and enhances inflammatory signalling, which culminate in the progression of colorectal cancers [16]. S1P signals through S1P receptor 1 (S1PR1), a G-protein-coupled receptor (GPCR), and there are several reports showing transactivation of receptor tyrosine kinases (RTKs), of which EGFR is a member, by GPCR [17].

We hypothesized that S1P could transactivate EGFR signalling and, in the process, stimulate cell growth and PTHrP production in Caco 2 (KRAS wild type) and LoVo (KRAS mutant) cells. We first studied whether the effect of EGF on CRC cell growth is mediated by PTHrP production. Since vitamin K2 (menaquinone, MK4) has been reported to inhibit the growth of CRC cells by apoptosis and autophagy [18], and vitamin K-related quinones could regulate EGFR function [19, 20], we tested whether MK4 could attenuate the proliferative and PTHrP-inducing effect of EGF in CRC cell lines. We next studied the effect of S1P on transactivation of EGFR and resultant CRC growth and PTHrP production, as well as the effect of MK4 on regulating of this process. Overarching goal of our study was to assess the cell growth inhibitory role of MK4 and the underlying mechanisms.

Materials and methods

Materials

Fetal bovine serum (FBS) and NuSerum were obtained from Atlanta Biologicals (Norcross, GA, USA) and BD Biosciences (San Diego, CA, USA), respectively. Tissue culture supplies were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). Antibodies for Western blot analysis and immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA), and Bethyl Laboratories (Montgomery, TX, USA). Human PTHrP ELISA kit was purchased from Cusabio Life Science (Hubei Province, P.R. China). Human total EGFR ELISA kit was from Abcam (USA). MK4, S1P were from Sigma-Aldrich (USA). PTH 7-34 peptide (PTH receptor antagonist) was purchased from Bachem AG (Bubendorf, Switzerland). Bromodeoxyuridine (BrdU) incorporation assay kit (colorimetric) was from Roche Diagnostics (Shanghai, China). W146 (inhibitor of S1PR1) and GM 6001 (broad spectrum matrix metalloproteinase (MMP) inhibitor) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). MMP-2/9 inhibitor II (MMP-2/9 In) was purchased from Merck Millipore (Germany).

Cell culture

LoVo cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown at 37°C in a humidified 95% air/5% CO_2 atmosphere in Ham's F12 medium supplemented with 10% FBS and L-glutamine. Caco 2 cells were maintained in α-MEM with 20% FBS and 1% penicillin and streptomycin.

MTT cytotoxicity assay

Cells (1×10^4 cells/well) were seeded in 96-well plates in growth medium. Plates were incubated at 37°C in 5% CO_2 for 24 h. Subsequently, medium was discarded and cells were cultured in DMEM containing 0.5% FCS (serum deprived condition). Cultures were incubated for 4 h and then various treatments were given as described in the Results section. At the end of treatments, 5 mg/ml MTT dye in phosphate buffer saline (PBS) was added (10 µl/well) and incubated for 4 h. DMSO (200 µl/well) was added to dissolve formazan crystals formed due to mitochondrial enzyme activity. After 15-20 min of incubation, optical density at 540 nm was recorded [21].

Cell proliferation assay

Cells were cultured for different durations in various growth media as described above. The

cells were pulsed with BrdU for 4 h before termination. Cell proliferation was measured at 450 nm with reference wavelength at 690 nm using BrdU ELISA kit, according to the manufacturer's instructions.

Cell cycle analysis

CRC cell lines were given various treatments and at the end of the incubation adherent cells were trypsinized and combined with any floating cells present and then washed with cold PBS. Cells were fixed in 70% ethanol, incubated with RNase A and stained with 50 μg/ml propidium iodide (PI) for 30 min before acquiring the flow cytometry reading (FACScan, BD Biosciences, USA).

Western blotting

CRC cell lines were grown to 60-70% confluence in complete growth medium. The cells then were homogenized with triton lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 mg/mL of aprotinin, and 1 mg/mL of aminoethylbenzenesulfonyl fluoride), and protein quantification was performed using BCA protein assay kit (Sigma, USA). Aliquots of 30 μg of protein in the form of cell lysate were resolved by SDS-PAGE under reducing conditions and then transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Watford, UK). The membranes were incubated with primary antibody against anti-rabbit S1P receptor 1 (also known as EDG1) at 1:1000 dilution overnight at 4°C in blocking buffer, as previously described [22]. Anti-rabbit secondary IgG was used at 1:2000 dilution, and Western blot signals were detected using the ECL-Plus (Millipore, USA) and developed on Chemidoc MP Western blotting imaging system (Bio-Rad, USA).

Receptor enzyme-linked immunosorbent assay (ELISA)

CRC cells were seeded in 6-well plates and, upon reaching 80% confluence, treated with various agents in serum-free medium containing 0.5% BSA. To determine the maximum phosphorylation of EGFR with EGF and S1P, cells were exposed to these agents at various time points (see supplementary figures). After the end of incubation, cells were washed with ice-cold PBS and lysed in pre-chilled cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% w/v NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% v/v glycerol, 1 × protease inhibitor cocktail). Protein concentration was determined by BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amount of cell lysates were used for determination of phosphor-EGF receptor (panTyr) using a Sandwich ELISA kit and total EGFR using ELISA kits according to manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). Values for receptor phosphorylation were determined by measuring absorbance at 450 nm using ELISA plate reader (μQuant, BioTek, USA) and normalized with total EGFR protein [23].

Measurement of PTHrP in the conditioned medium

Cells were seeded in 24-well plates (1×10^5 cells/well) in 0.5 ml growth medium. After 24 h, the growth medium was replaced with 0.5 ml of serum-deprived medium (0.5% FBS) and cultured for 4 h. This medium was then replaced with serum-deprived medium plus various treatments as described in the Results section for additional 24 h. After this the conditioned medium was removed for determination of PTHrP release. To avoid PTHrP degradation, the PTHrP assays were set up immediately after the conditioned medium was removed from cultures following manufacturer's protocol.

Statistics

The data are shown as means ± SE of the indicated number of experiments. Data were analyzed by 1) one-way ANOVA followed by post hoc Tukey multiple comparison test of significance, or 2) Student's *t*-test when appropriate using GraphPad prism 5. A P value of < 0.05 was considered statistically significant.

Results

EGF-induced cell growth is dependent on increased PTHrP production

LoVo and Caco 2 cells at 50-60% confluence were treated with EGF, and PTHrP was measured in the conditioned medium as a function of time. **Table 1** shows that EGF robustly increased PTHrP secretion from both CRC cell lines, and the increase was much greater in

Table 1. PTHrP secretion by CRC cells in response to EGF exposure

	24 h	48 h
Caco 2	1.7 ± 0.6*	2.1 ± 0.8*
LoVo	2.6 ± 0.8*,a	2.9 ± 0.3*,a

*$P < 0.001$ vs. respective control cells, and a$P < 0.001$ vs. Caco 2 cells.

LoVo cells compared to Caco 2 cells. With time, there was no significant increase in PTHrP secretion in either cell type. We thus studied the effect of EGF and PTHrP antagonist (PTH 7-34) on the proliferation of LoVo (**Figure 1A**) and Caco 2 cells (**Figure 1B**) at 48 h. Incubation of the cells with EGF increased their proliferation, as assessed by BrdU incorporation. Presence of EGFR inhibitor AG1478 completely attenuated the EGF-induced increase in proliferation. Co-treating the cells with EGF and PTH receptor (PTHR) antagonist, PTH (7-34), completely abrogated the proliferation-stimulating effect of EGF on these two cell types. These data suggested that EGF stimulates growth of colon cancer cells via the induction of PTHrP production in an autocrine mechanism.

MK4 attenuated EGF-induced proliferation, EGFR phosphorylation and EGF-induced PTHrP secretion in CRC cell lines

EGF induced proliferation in both cell lines but its effect was stronger in LoVo cells than in Caco 2 cells (**Figure 2A**). Increasing concentrations of MK4 (2.5- and 5 μM, RDA range) suppressed the proliferation of LoVo and Caco-2 cells in response to EGF treatment in a concentration-dependent manner, as assessed by BrdU incorporation assay (**Figure 2A**). The time course of EGFR phosphorylation by EGF showed that the maximum phosphorylation was reached at 15 min and lasted for 60 min. Thereafter, it declined in both cell lines (Supplementary Figure 1). We selected 30 min point to study the effect of MK4 on the EGF-induced EGFR phosphorylation in both cell lines. EGF-induced EGFR phosphorylation was stronger in LoVo cells compared to Caco 2 cells (**Figure 2B**). MK4 alone at 5 μM concentration had no effect on EGFR phosphorylation but it suppressed EGF-induced EGFR phosphorylation in both cell lines (**Figure 2B**). This suppression by MK4 in LoVo cells was also significant but, unlike Caco 2 cells, was not complete (**Figure 2B**).

We next studied the effect of MK4 on PTHrP secretion by the two cell lines. Basal levels of PTHrP were higher in LoVo cells compared with Caco-2 cells (**Figure 2C**). EGF robustly induced PTHrP secretion in both CRC cell lines, as assessed by ELISA measurements in conditioned media. Induction of PTHrP secretion by EGF was significantly higher in LoVo cells than in Caco 2 cells. Presence of MK4 completely blocked the EGF-induced PTHrP secretion in Caco 2 cells, whereas it was partially yet significantly reduced in LoVo cells (**Figure 2C**).

MK4 inhibited pro-survival effect of EGF in CRC lines

We next studied whether the EGF-induced proliferation that was blocked by MK4 (as shown in **Figure 2A**) is associated with apoptosis. To this aim, apoptosis was determined by annexin V/PI staining of cells followed by flow cytometric analysis. CRC cell lines were treated with serum deficient medium (0.5% FBS), which served as a stimulus for apoptosis (control). **Figure 3A** showed the representative dot plots of flow cytometric analysis, and the data quantification is presented in **Figure 3B**. EGF was very effective in protecting both types of cells from apoptosis. MK4 reversed the EGF-induced protection of cells, as significantly higher numbers of apoptotic cells were observed in the EGF+MK4 treatment compared to treatment with EGF alone (**Figure 3A**, **3B**).

Analysis of the cell cycle phase distribution by flow cytometry revealed that EGF significantly reduced the percentage of cells in G1 phase, suggesting that EGF protected CRC cell lines from serum deprivation-induced G1 arrest and consequent apoptosis (**Figure 3C**, **3D**). Co-treatment with MK4 and EGF significantly increased the percentage of cells in G1 phase compared to the EGF treatment alone, suggesting that MK4 significantly antagonized the apoptosis protection/pro-survival action of EGF by increasing G1 arrest in the cells (**Figure 3C**, **3D**).

S1P stimulates growth of CRC cell lines by transactivating EGFR

S1P significantly increased the proliferation of CRC cells, as assessed by BrdU incorporation assay (**Figure 4A**). Maximal increase in the BrdU incorporation was obtained at 10 μM concentration of S1P. We next studied how MK4

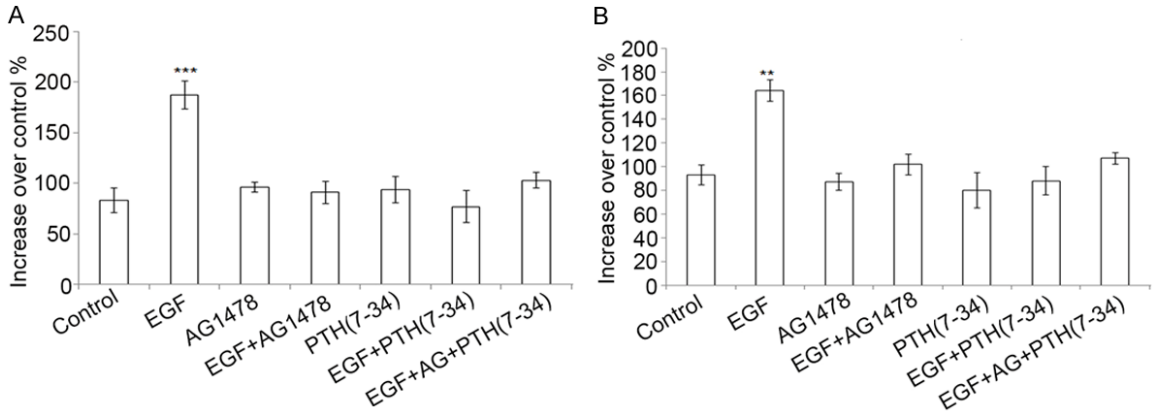

Figure 1. Attenuation of EGF-induced CRC cells proliferation by PTH receptor 1 antagonist. A. LoVo; B. Caco 2 cells were treated with various compounds as indicated. BrdU incorporation was performed to assess cell proliferation. Data are presented as mean ± SE of three independent experiments; $^{**}P < 0.01$ and $^{***}P < 0.001$. Control-cells receiving vehicle, EGF-40 ng/ml, AG1478-1 μM, PTH (7-34)-1 μM.

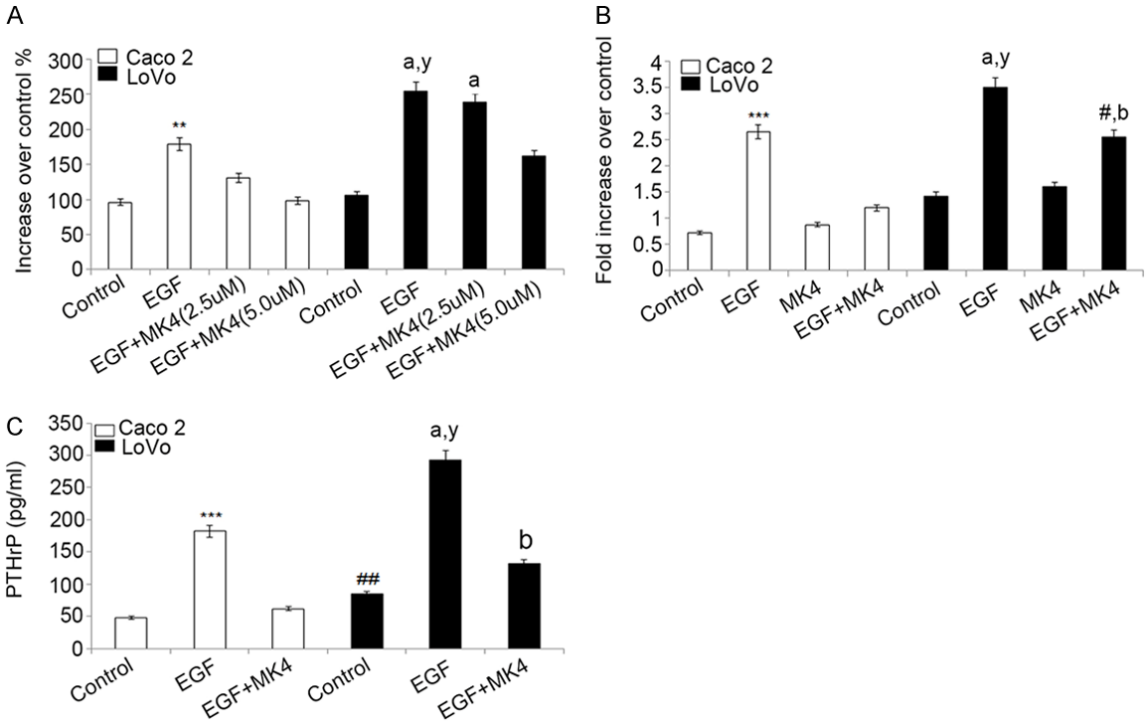

Figure 2. Inhibition of EGF-induced proliferation, EGFR phosphorylation and PTHrP secretion by MK4 in CRC cell lines. A. Effect of MK4 on EGF-induced proliferation of Caco 2 and LoVo cells as assessed by BrdU incorporation. Data are presented as mean ± SE of three independent experiments; $^{**}P < 0.01$ vs. both controls, $^{a}P < 0.001$ vs. both controls, $^{y}P < 0.01$ vs. EGF treated Caco 2 cells. B. Effect of MK4 on EGF-induced phosphorylation of EGFR in Caco 2 and LoVo cells as assessed by PathScan®Phospho-EGF Receptor (panTyr) Sandwich ELISA. Data are presented as mean ± SE of three independent experiments; $^{***}P < 0.01$ vs. Caco 2 control, $^{a}P < 0.001$ vs. both controls, $^{y}P < 0.01$ vs. EGF treated Caco 2 cells, $^{\#}P < 0.01$ vs. LoVo control, $^{b}P < 0.01$ vs. EGF treated LoVo cells. C. Effect of MK4 on EGF-induced PTHrP secretion by Caco 2 and LoVo cells as assessed in conditioned media after 24 h treatment by ELISA. Data are presented as mean ± SE of two independent experiments and each treatment was run in triplicate; $^{***}P < 0.01$ and $\#P < 0.05$ vs. Caco 2 control, $^{a}P < 0.001$ vs. both controls, $^{y}P < 0.01$ vs. EGF treated Caco 2 cells, $^{b}P < 0.01$ vs. EGF treated LoVo cells. Control-vehicle, EGF-40 ng/ml, MK4-5 μM.

influences the S1P-induced cell proliferation. As shown in **Figure 4A**, MK4 completely suppressed the proliferation induced by S1P in both cell lines. Regarding the time course anal-

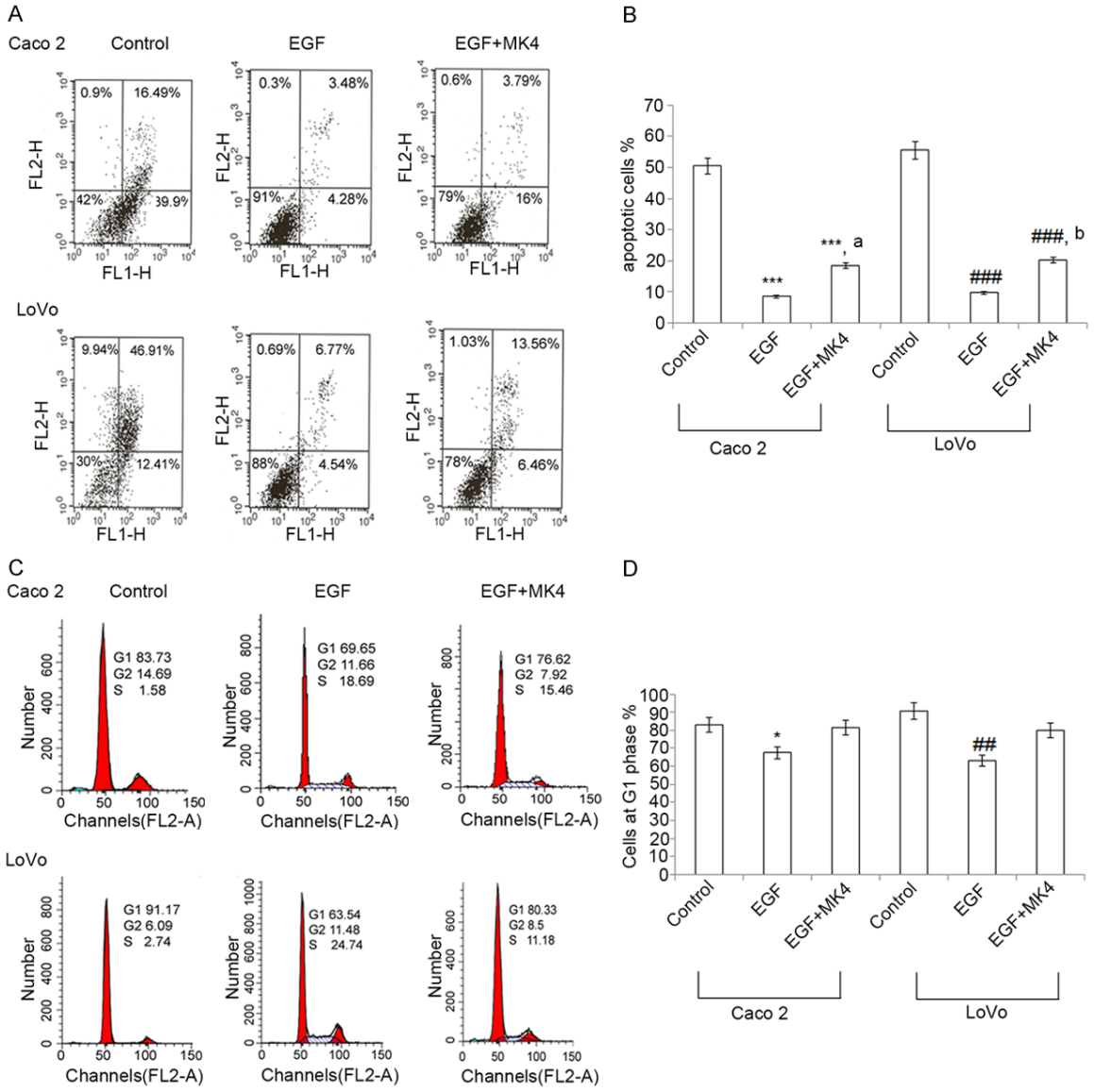

Figure 3. Inhibition of EGF-induced survival and induction of cell cycle arrest in CRC cell lines by MK4. A. The level of apoptosis in Caco 2 and LoVo cells after 36 h treatments as determined by flow cytometry using Annexin-V (FL1-H channel) and PI (FL2-H channel) in a Becton Dickinson FACS Calibur. B. Quantification of apoptotic cells by the treatments showing that EGF treatment prevented CRC lines from serum deprivation-induced apoptosis and MK4 significantly reversed the anti-apoptotic effect of EGF. $^{***}P < 0.001$ vs. Caco 2 control and EGF treated Caco 2, $^{a}P < 0.05$ vs. EGF treated Caco 2, $^{###}P < 0.001$ vs. LoVo control and EGF treated LoVo, $^{b}P < 0.01$ vs. EGF treated LoVo. C and D. Assessment of cell cycle distribution by flow cytometry and quantification of cells at G1 phase. Co-treatment of EGF and MK4 resulted in significantly increased G1 arrest over EGF treated cells in both cell types. $^{*}P < 0.05$ vs. all other treatments given to Caco 2 cells and $^{##}P < 0.01$ vs. all other treatments given to LoVo cells. Data presented are mean ± SE of three independent experiments. EGF-40 ng/ml, MK4-5 μM.

ysis of EGFR phosphorylation by S1P showed that the maximum phosphorylation was attained at 45 min, lasted for 120 min and declined thereafter (Supplementary Figure 2). We selected 60 min point to study the effect of S1P on the EGF-induced EGFR phosphorylation in both CRC cell lines. When treated with 10 μM S1P, the cells showed significant increase in EGFR phosphorylation (**Figure 4B**). EGFR phosphorylation by S1P was greater in LoVo cells than Caco-2 cells (**Figure 4B**). Up-regulation of expression of S1P receptor 1 has been previously reported to be associated with increased invasiveness and metastasis of CRC [24]. We

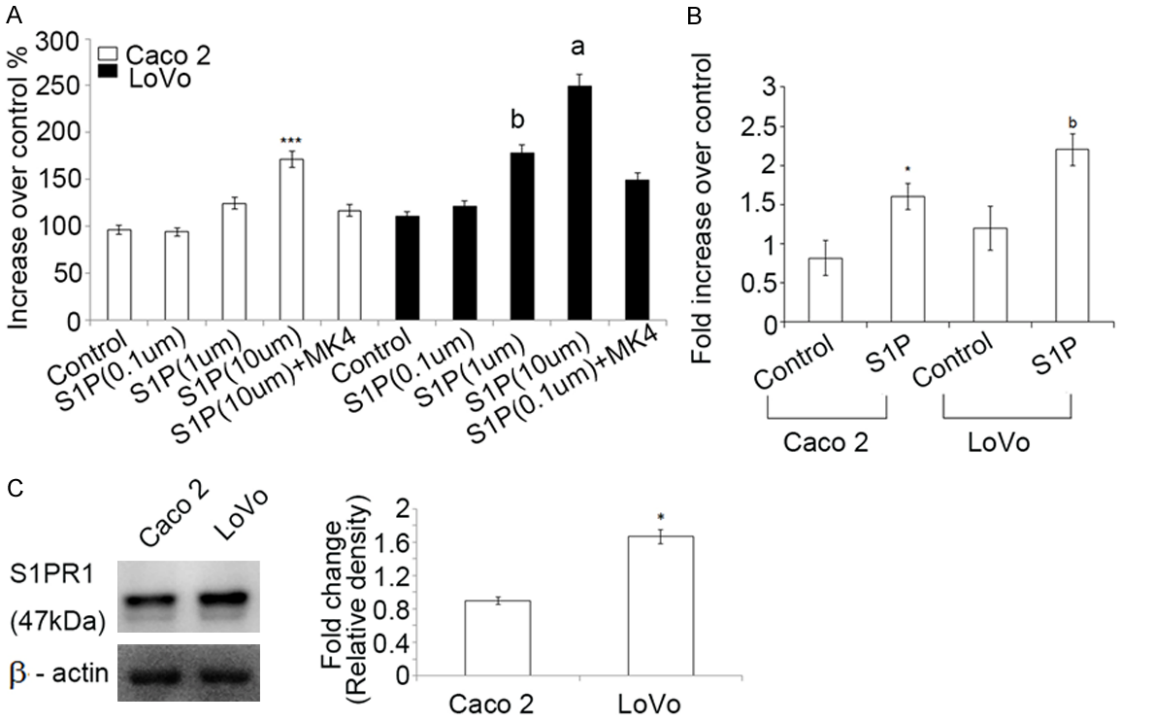

Figure 4. Proliferation stimulating effect of S1P on CRC cells and attenuating effect of MK4. A. Both CRC lines showed maximum proliferative response to S1P at 10 μM (BrdU assay). MK4 (5 μM) completely mitigated S1P-induced proliferation in both cells. Data are presented as mean ± SE of three independent experiments; $^{***}P <$ 0.001 vs. both controls, $^{b}P < 0.01$ and $^{a}P < 0.001$ vs. both control. B. S1P treatment resulted in increased EGFR phosphorylation in CRC lines. $^{*}P < 0.05$ vs. Caco 2 control and $^{b}P < 0.05$ vs. control LoVo and S1P treated Caco 2 cells. C. S1PR1 expression in CRC cells showed that LoVo cells expressed significantly higher S1P1R compared with Caco 2 cells as assessed by Western blotting. Representative Western blot (upper panel) and densitometric quantification of S1P1R band after normalization with β-actin band. $^{*}P < 0.05$. In all panels, data are presented as mean ± SE of three independent experiments.

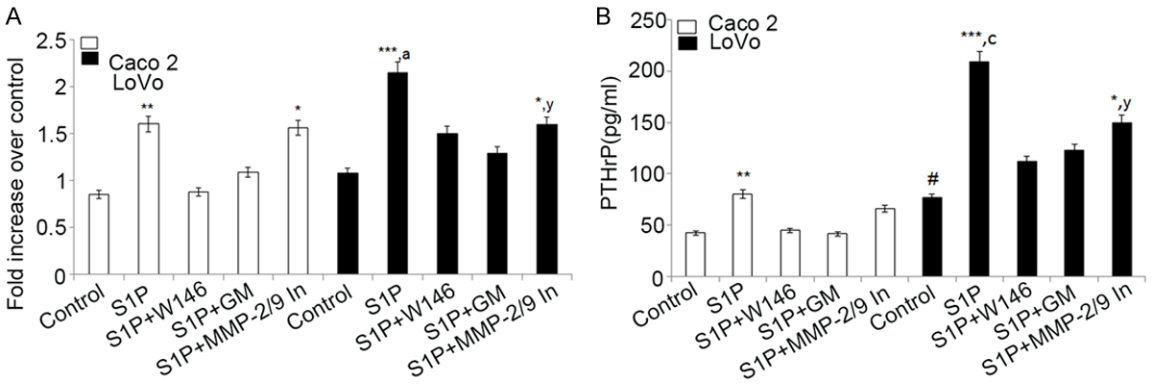

Figure 5. Transactivation of EGFR and stimulation of PTHrP secretion by S1PR1 in CRC cells, and attenuating effect of MK4. A. Phospho-EGFR level was elevated by S1P in both Caco 2 and LoVo cells. Stimulation of phospho-EGFR by S1P was greater in LoVo compared to Caco 2 cells. Data are presented as mean ± SE of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. controls of both Caco 2 and LoVo cells, $^{a}P < 0.001$ vs. S1P treated Caco 2 cells, $^{y}P < 0.05$ vs. S1P treated LoVo cells. B. PTHrP secretion was stimulated by S1P in both Caco 2 and LoVo cells. Stimulation of PTHrP by S1P was greater in LoVo compared to Caco 2 cells. Data are presented as mean ± SE of two independent experiments and each treatment was run in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. controls of both Caco 2 and LoVo cells, $^{\#}P < 0.05$ vs. control Caco 2 cells, $^{c}P < 0.001$ vs. S1P treated Caco 2 cells, $^{y}P < 0.05$ vs. S1P treated LoVo cells. EGF-40 ng/ml, S1P-10 μM; W146-1 μM; GM60001-10 μM, MMP-2/9 inhibitor-1 μM.

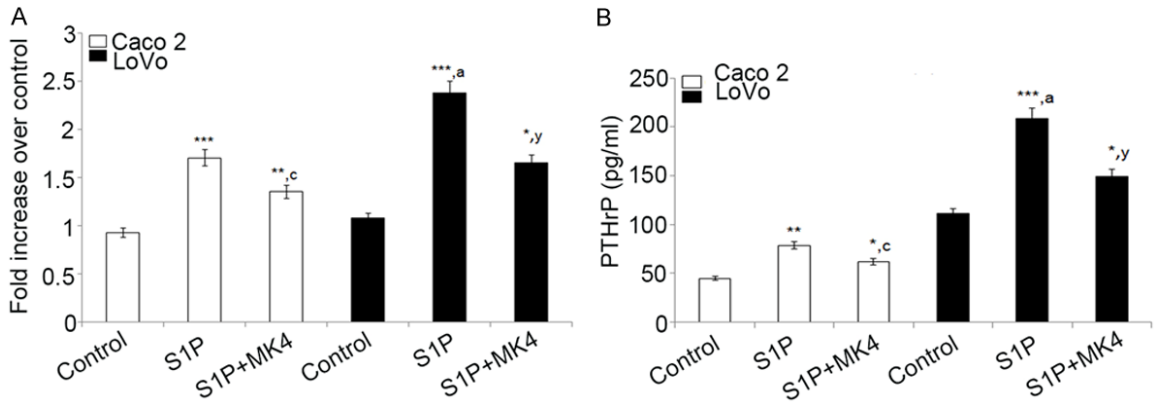

Figure 6. Attenuation of S1P-induced EGFR phosphorylation and PTHrP secretion in CRC cell lines by MK4. (A) S1P induced EGFR phosphorylation in both cell types. The effect was more significant in LoVo cells. MK4 significantly attenuated EGF-induced phosphorylation in both cell lines. (B) S1P-induced PTHrP secretion showed attenuation in Caco 2 and LoVo cells in the presence of MK4. Data are presented as mean ± SE of three independent experiments in (A) and of two independent experiments in (B). Each treatment was run in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. controls of both Caco 2 and LoVo cells, $^{c}P < 0.05$ vs. control Caco 2 cells, $^{a}P < 0.01$ vs. S1P treated Caco 2 cells, $^{y}P < 0.01$ vs. S1P treated LoVo cells.

found that S1PR1 expression was significantly higher in LoVo cells compared to Caco 2 cells (**Figure 4C**).

Presence of a potent and selective inhibitor of S1PR1, W146, blocked the S1P-induced EGFR phosphorylation in the CRC cell lines (**Figure 5A**). Presence of GM6001, a broad spectrum inhibitor of MMPs, abolished the S1P-induced EGFR phosphorylation completely in Caco 2 cells and partially yet significantly reduced it in LoVo cells. Among the MMPs, MMP-2 and MMP-9 are associated with the invasion in CRC [25]. We observed that presence of MMP-2/9 inhibitor (MMP-2/9 In) partially yet significantly blocked the S1P-induced EGFR phosphorylation in LoVo cells but failed to do so in Caco 2 cells (**Figure 5A**).

Next, we studied the effect of S1P on PTHrP secretion and observed that both LoVo and Caco 2 cells produced significantly increased amounts of PTHrP in the conditioned medium upon treatment with S1P (**Figure 5B**). In Caco 2 cells, W146 and GM6001 completely abrogated the S1P-induced PTHrP secretion. MMP-2/9 inhibitor also showed an inhibitory trend, although it did not reach statistical significance. In LoVo cells, the S1P-induced PTHrP secretion was completely blocked by W146, whereas GM6001 and MMP-2/9 inhibitor significantly (although not completely) reduced the stimulatory effect of S1P on PTHrP secretion (**Figure 5B**). These data suggest that S1PR1 transactivates EGFR via MMPs and stimulates the production of PTHrP.

MK4 inhibits S1P-induced EGFR transactivation and PTHrP secretion by CRC cell lines

In Caco 2 cells, MK4 modestly yet significantly inhibited the EGFR phosphorylation induced by S1P (**Figure 6A**). In LoVo cells, MK4 significantly inhibited the S1P-induced EGFR phosphorylation, although the effect was not complete. PTHrP secretion induced by S1P in these two CRC cell lines was significantly suppressed by MK4, although the inhibitory effect was partial (**Figure 6B**).

Discussion

In KRAS wild type (Caco 2) and KRAS mutant (LoVo) cell lines, we observed that EGF stimulated cell proliferation and survival. EGF stimulated PTHrP secretion from these cells which, in an autocrine and paracrine mode, promoted growth and survivability of the CRC cell lines. S1P, via its receptor S1P1R, stimulated cell proliferation and also triggered transactivation of EGFR. MK4 suppressed the proliferation of CRC cells by acting at two levels: by suppressing the EGFR signalling, and by inhibiting the S1PR1-mediated transactivation of EGFR.

CRCs that are at advanced stages and have become refractory to chemotherapy can be

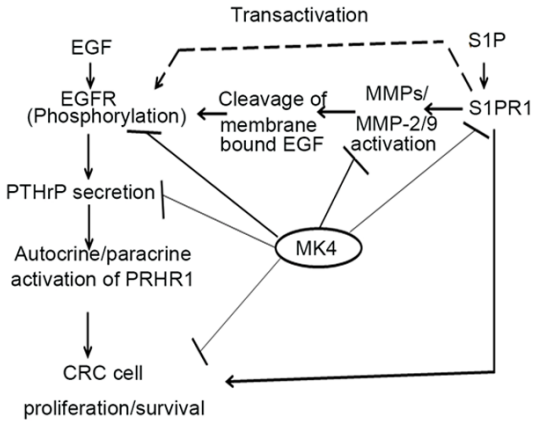

Figure 7. Schematic diagram showing the mechanism by which MK4 inhibits EGF-induced growth of CRC cells. EGF stimulates PTHrP secretion which stimulates growth and survival of CRC cells, probably via autocrine/paracrine mode. S1P also stimulates proliferation of CRC by stimulating S1P1R. S1P1R could also transactivate EGFR by activating MMP-2/9 which then cleaves membrane-bound EGF to release EGF and activate EGFR by an autocrine/paracrine manner. MK4 inhibits EGFR phosphorylation resulting in the suppression of PTHrP secretion and limiting the proliferation of CRC cells. MK4 inhibits S1P1R-induced transactivation of EGFR which also prevents S1P-induced proliferation of CRC.

treated by targeted anti-EGFR drugs cetuximab or panitumumab [26]. Blocking of EGFR signalling is a major strategy to treat these types of cancer. Anti-EGFR treatment is most pronounced in the KRAS wild type tumours, while KRAS mutant tumours are not responsive [27, 28]. Therefore, an agent that could antagonize the EGFR signalling in CRC and most particularly in the KRAS mutants will have a potential for CRC chemotherapy.

EGFR signalling has been shown to induce the production of PTHrP [29]. Expression of PTHrP correlates with the severity of CRC [30]. Expression of PTH/PTHrP receptor have been shown in human colon tumours [31]. PTHrP prevents Caco 2 cells from apoptosis under oxidative stress conditions and could contribute to colon cancer progression [29]. Our data showed that EGF stimulated PTHrP secretion in CRC cells. MK4 blocked EGFR phosphorylation, which then resulted in the inhibition of PTHrP secretion in these cells. The increased PTHrP secretion-mediated EGF-induced proliferation of the CRC cell lines was demonstrated by the abolition of EGF's effect on proliferation by treatments with PTH (7-34), an antagonist of PTH/PTHrP receptor. Furthermore, EGF is one of the growth factors that is known to protect colon cancer cells from apoptotic stimuli [32], and our data demonstrated that MK4 increased apoptosis in EGF-treated CRC cells via G1 arrest. Taken together, these data suggest that MK4 inhibits cell growth and EGF-induced survivability by directly blocking the EGFR activation and subsequent suppression of PTHrP secretion.

In addition to EGF and TGFα, the ligands of EGFR, the receptor could also be activated through transactivation by GPCRs, even though the agonists of the GPCRs do not directly interact with EGFR. One example is transactivation of EGFR in breast and prostate cancer cells by calcium-sensing receptor, a GPCR [33, 34]. In cancer cells, transactivation of EGFR by GPCRs could mediate critical downstream signals culminating in increased malignant cell growth. Inhibition of metalloproteases, such as those that inhibit disintegrins and matrix metalloproteinases, could block EGFR transactivation induced by GPCRs [35]. In colitis-associated cancers, via persistent activation of transcription factor STAT3 achieved by NF-κB, S1P results in the regulation of IL-6 production that in turn leads to upregulation of S1P1R. This represents a "malicious" loop resulting in the growth of this tumour [36]. Our data showed that S1P induces the proliferation of CRC cells, and this process could be blocked by MK4. S1P1R was expressed in both cells but significantly stronger in LoVo cells, thus suggesting that KRAS mutation up-regulates this receptor. However, at this stage more definitive evidence to claim that KRAS mutation contributed to up-regulation of S1P1R is required. S1P significantly induced EGFR phosphorylation in both cell lines, but the induction was significantly higher in LoVo cells than in Caco 2 cells. This correlated with elevated S1P1R levels in the former. S1P-induced EGFR phosphorylation was blocked by S1P1R antagonist W146 and pan-MMP inhibitor GM6001 in both cell lines, which suggested the transactivation of EGFR by S1P1R. MMP-2 and MMP-9 are the members of gelatinase sub-family of MMPs, and their high levels in tumours correlates with a very poor outcomes in terms of metastases [25]. MMP-2 and MMP-9 are expressed in Caco 2 [37] and LoVo cells [38]. Interestingly, inhibitor of MMP-2/9 abolished the S1P-induced

EGFR phosphorylation in LoVo but not Caco 2 cells suggesting that in the later cell type the EGFR transactivation by S1P1R was MMP-dependent but not specifically depended on MMP-2/9. Future studies will determine which MMP types mediate the transactivation of EGFR by S1P1R in Caco 2 cells. On the other hand, partial yet significant abrogation of EGFR activation by MMP-2/9 inhibitor in LoVo cells suggested that these two isozymes do mediate the S1P1R-induced transactivation of EGFR although other MMP isozymes could be involved as well.

S1P was found to increase PTHrP secretion in both cell lines but more in LoVo cells. In Caco 2 cells, this stimulation was blocked by W146 and GM6001 but not MMP-2/9 inhibitor. This suggests that EGFR downstream signalling upon transactivation by S1P1R was dependent on MMPs in general but not MMP-2/9. This data were consistent with EGFR phosphorylation data. Together, these data suggested that S1P-induced PTHrP production was a result to EGFR transactivation by S1P1R. On the other hand, in LoVo cells, S1P-induced PTHrP production was completely blocked by W146 but only partially (albeit significantly) by GM6001 and MMP-2/9 inhibitor. This suggests that additional factors downstream of S1P1R, others than MMP activation, stimulated the PTHrP production. EGFR transactivation by GPCRs has been shown to be induced by intracellular Ca^{2+} [39, 40], protein kinase C activation [41] and generation of reactive oxygen species [42, 43]. In LoVo cells, any of these mechanisms could mediate EGFR transactivation by S1P1R.

Additionally, MK4 significantly inhibited S1P-induced EGFR phosphorylation and PTHrP production. However, the stimulatory effects were not completely suppressed, which suggested that the anti-proliferative mechanism of the compound may not be completely dependent on the abrogation of EGFR pathway and EGFR transactivation by S1P1R. A schematic diagram showing various signalling points that are inhibited by MK4 as studied here is shown in **Figure 7**.

Based on our findings, we conclude that MK4 inhibits growth of CRC lines by suppressing EGFR signalling and inhibiting EGFR transactivation by S1P1R. Future studies are required to determine the anti-cancer effect of MK4 in appropriate animal models of colorectal cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ying Song, Department of Gastroenterology, Second Affiliated Hospital of Jilin University, 218 Ziqiang Road, Changchun 130041, Jilin, China. E-mail: tltwoya@sina.com

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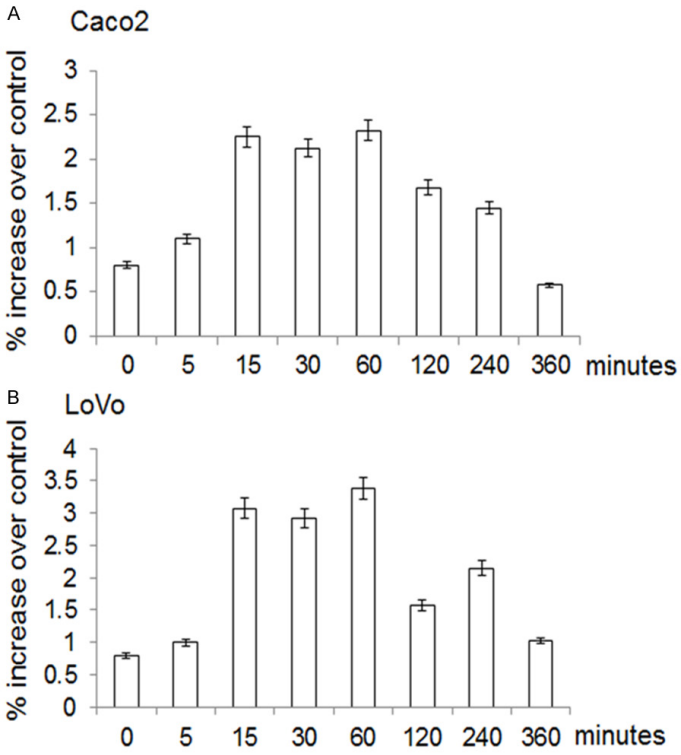
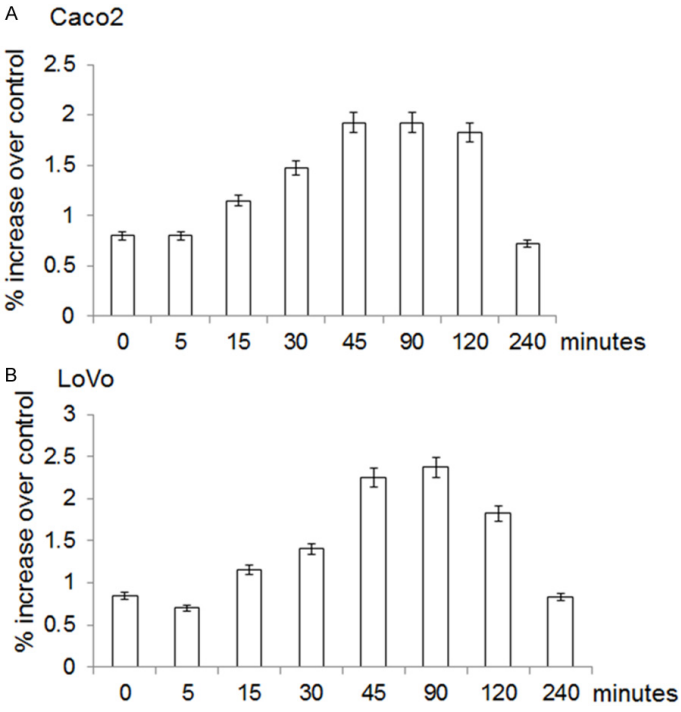


Supplementary Figure 1. 50-60% confluent cells were treated with EGF (40 ng/ml) as described in **Figure 2B**, at indicated time points. Two independent experiments were performed in triplicate for each time point. Data are mean ± SE.



Supplementary Figure 2. 50-60% confluent cells were treated with S1P (10 μM) as described in **Figure 4B**, at indicated time points. Two independent experiments were performed in triplicate for each time point. Data are mean ± SE.