

Selenium Regulates Cyclooxygenase-2 and Extracellular Signal-Regulated Kinase Signaling Pathways by Activating AMP-Activated Protein Kinase in Colon Cancer Cells

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Abstract

Epidemiologic and experimental evidences indicate that selenium, an essential trace element, can reduce the risk of a variety of cancers. Protection against certain types of cancers, particularly colorectal cancers, is closely associated with pathways involving cyclooxygenase-2 (COX-2). We found that AMP-activated protein kinase (AMPK), which functions as a cellular energy sensor, mediates critical anticancer effects of selenium via a COX-2/prostaglandin E₂ signaling pathway. Selenium activated AMPK in tumor xenografts as well as in colon cancer cell lines, and this activation seemed to be essential to the decrease in COX-2 expressions. Transduction with dominant-negative AMPK into colon cancer cells or application of *cox-2*^{-/-}-negative cells supported the evidence that AMPK is an upstream signal of COX-2 and inhibits cell proliferation. In HT-29 colon cancer cells, carcinogenic agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activated extracellular signal-regulated kinase (ERK) that led to COX-2 expression and selenium blocked the TPA-induced ERK and COX-2 activation via AMPK. We also showed the role of a reactive oxygen species as an AMPK activation signal in selenium-treated cells. We propose that AMPK is a novel and critical regulatory component in selenium-induced cancer cell death, further implying AMPK as a prime target of tumorigenesis. (Cancer Res 2006; 66(20): 10057-63)

Introduction

Selenium, an essential trace element, has been shown to inhibit tumorigenesis in a variety of experimental models (1, 2). However, its precise mechanism of anticarcinogenic activity has not been resolved. Accumulated evidences suggest that selenium and arachidonic metabolisms are linked to colonic tumorigenesis (3). Colorectal cancer displays elevated cyclooxygenase-2 (COX-2) expressions, and decreases in COX-2 and prostaglandin E₂ (PGE₂) by selenium supplementation are associated with the inhibition of proliferation in colon cancer cells (4). AMP-activated protein kinase (AMPK) is a sensor of cellular energy status, and

recently published data indicate that it also plays a crucial role in many diseases (5–7). As a metabolic sensing signal, AMPK is involved in cancer cell apoptosis. Recently, it has emerged as an important anticarcinogenic molecule due to its relationship with tumor suppressor genes, *LKB* and *TSC2* (7). We have tested the ability of selenium in inhibiting colon cancer cell proliferation through the modulation of AMPK and COX-2/PGE₂ signaling. We observed that the AMPK activation by selenate is crucial for inhibiting COX-2 and PGE₂ modulation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

Materials and Methods

Cell culture and reagents. The HT-29 human colon cancer cell line, MCF-7 human breast cancer cell line, and HeLa human cervical cancer cell line were purchased from American Type Culture Collection (Gaithersburg, MD). Cells were cultured in RPMI 1640 containing 10% fetal bovine serum under normoxic conditions. Sodium selenate, sodium selenite, 5-fluorouracil (FU), etoposide, Hoechst 33342, 2',7'-dichlorofluorescein (DCFH), propidium iodide (PI), *N*-acetylcysteine (NAC), PD98059, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) were obtained from Sigma (St. Louis, MO). TPA was obtained from Alexis Biochemicals (San Diego, CA). Celecoxib (Celebrex) was supplied from Pharmacia (Seoul, Korea). Prostaglandin (PGE₂) enzyme assay kit was purchased from Amersham Biosciences (Buckinghamshire, United Kingdom). The anti-phosphorylated specific antibodies that recognize phosphorylated acetyl-CoA carboxylase (ACC)-Ser⁷⁹, AMPK, and extracellular signal-regulated kinase (ERK) were from Cell Signaling Technology (Danvers, MA). Antibodies for COX-2, poly(ADP-ribose) polymerase (PARP), caspase-3, p53, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

COX-2-positive and COX-2-negative cells. COX-2-positive and COX-2-negative mouse fibroblast cells were gifts from Dr. Zigang Dong (University of Minnesota, Minneapolis, MN). Genotypes of these cells were confirmed using PCR primers of 5'-ATCCTAGCACTGCATCTGC-3', 5'-CACCATA-GAATCCAGTCCGC-3', and 5'-CTTGGGTGGAGAGGCTATTC-3'.

Protein extract and Western blotting. Cells were rinsed twice with ice-cold PBS and scraped with radioimmunoprecipitation assay buffer [25 mmol/L Tris (pH 7.4), 150 mmol/L KCl, 5 mmol/L EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] or NP40 lysis buffer [10 mmol/L HEPES (pH 7.4), 142 mmol/L KCl, 1 mmol/L EGTA, 1 mmol/L DTT, 0.2% NP40] and subjected to the Western blot analysis.

RNA isolation and reverse transcription-PCR. Total RNA was extracted from cells with Trizol reagent (Life Technologies, Glasgow, United Kingdom) according to the manufacturer's instructions. The cDNA fragment was amplified by PCR using the following specific primers: vascular endothelial growth factor (VEGF), 5'-AGGAGGGCAGAATCAT-CACG-3' (sense) and 5'-CAAGGCCACAGGGATTCT-3' (antisense); p53, 5'-CCTTCCCAGAAAACCTACCA-3' (sense) and 5'-TCATAGGGCAC-CACCACACT-3' (antisense); p21, 5'-GCGACTGTGATGCGCTAAT-3' (sense)

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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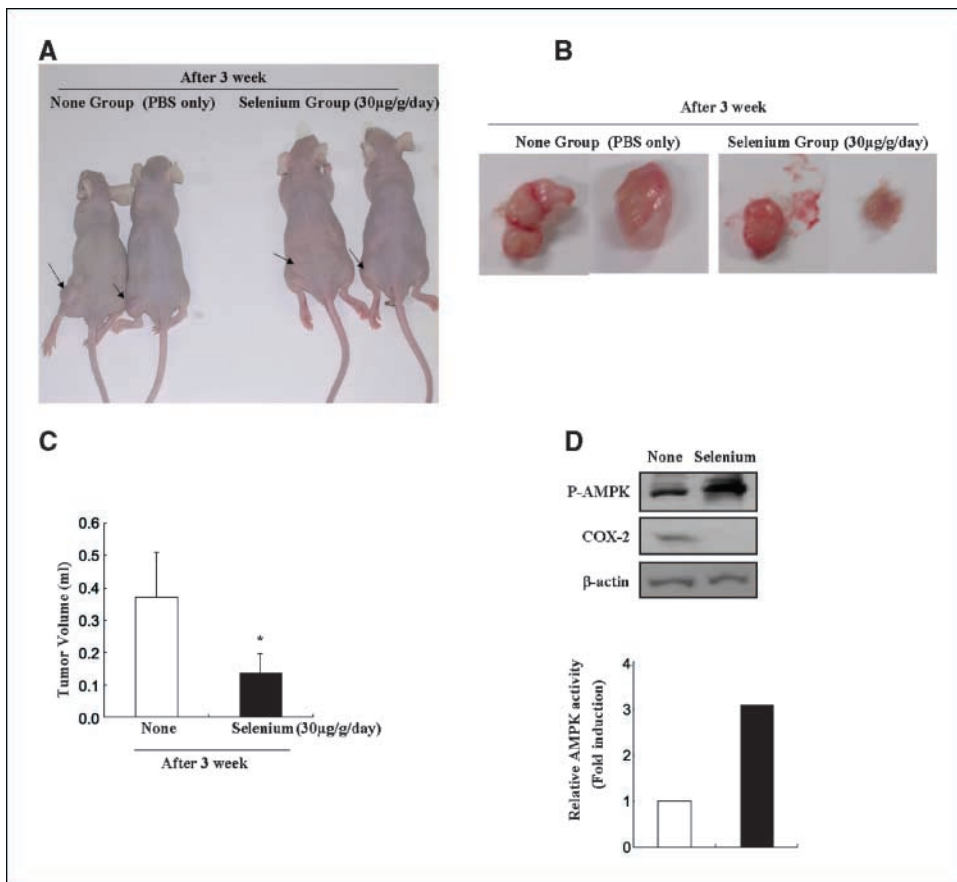


Figure 1. Selenate abrogates the growth of colon tumor xenografts in nude mice. A to C, HT-29 cells (2×10^6) were injected s.c. to 5-week male nude mice of five per group. After 1 week, mice were treated i.p. with selenate (30 µg/g body weight) every day. The control group received vehicle only (PBS). Tumor volumes were calculated as described in Materials and methods. *, $P < 0.05$, compared with none. D, under the same conditions, homogenized cell lysates were prepared from the control and selenate-treated tumor tissue from two different sets of nude mice and AMPK phosphorylation and COX-2 expression were measured by Western blot analysis.

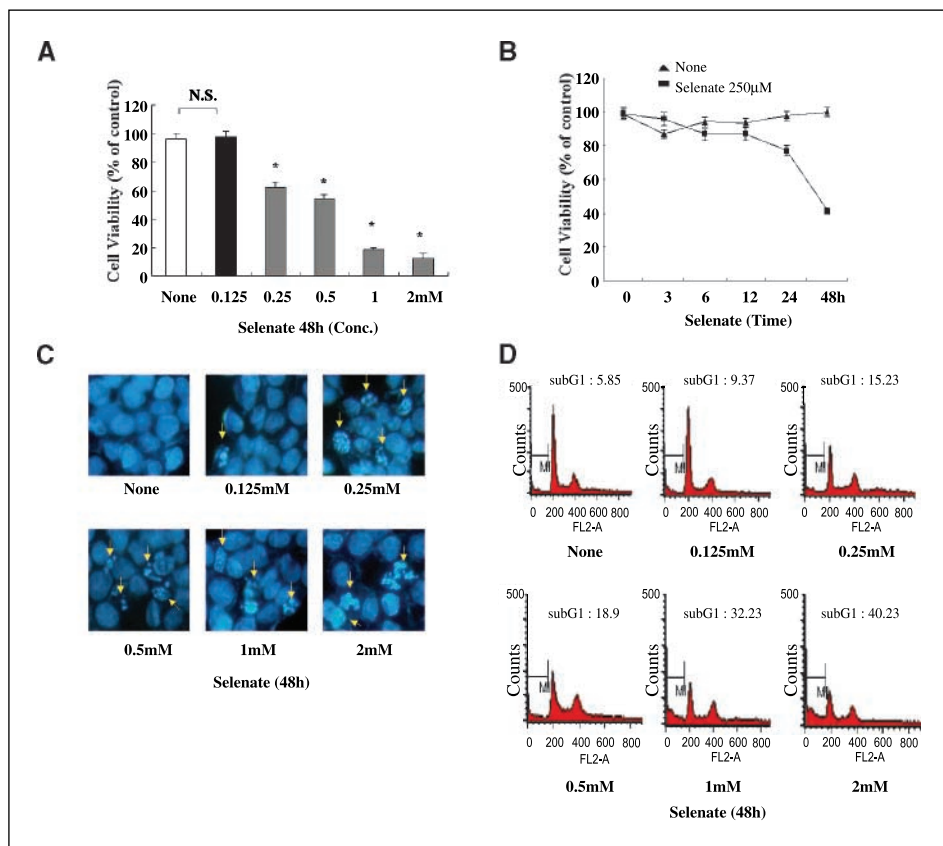


Figure 2. Selenate increases apoptosis in HT-29 colon cancer cells. A and B, cells were treated at either different concentrations or different times with selenate and then cell death patterns of selenate-treated cells were measured by MTT. Columns and points, mean of triplicate measurements of two experiments; bars, SD. *, $P < 0.05$, compared with control cells. Under the same conditions, apoptotic bodies (C) are shown with cells treated with Hoechst 33342, and fluorescence-activated cell sorting (FACS) histograms of PI-stained cancer cells are presented (D). Results are representatives of three independent experiments. N.S., not significant.

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and 5'-GGCGTTTGGAGTGGTAGAAA-3' (antisense); and β -actin, 5'-GTGGGGGCGCCAGGCACCA-3' (sense) and 5'-CTCCTTAATGTCACG-CACCATTTTC-3' (antisense).

Prostaglandin ELISA assay. The supernatants of control and treated cell cultures were added to appropriate wells and PGE₂ conjugates were pipetted into wells excluding blank wells. Finally, monoclonal antibody against PGE₂ was added to all wells, except wells for blank and nonspecific binding, followed by incubation for 18 hours at 4°C. The plates were washed four times and color reaction was developed by the addition of tetramethylbenzidine substrate. After 30 minutes of incubation at room temperature, the reaction was quenched by addition of 1 mol/L sulfuric acid. Absorbance was measured at 450 nm on an ELx800 reader (BioTek Instruments, Inc., Winooski, VT).

Chromatin staining with Hoechst 33342. Apoptosis was observed by chromatin staining with Hoechst 33342 as described previously. After incubation, the supernatants were discarded and cells were fixed with 3.5% formaldehyde in PBS for 30 minutes at room temperature, washed four times with PBS, and exposed to Hoechst 33342 at 10 μ mol/L for 30 minutes at room temperature. Cell preparations were examined under UV illumination with a fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

Fluorescence-activated cell sorting analysis. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS, after 70% ethanol fixed and resuspended in PBS containing 10 μ g/mL PI. After sorting out the viable cells, fluorescence intensity was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) using excitation and emission wavelengths of 488 and 525 nm, respectively.

Adenovirus-mediated gene transfer. AMPK wild-type (WT) α 1 subunit, a dominant-negative (DN) form, was generated by PCR as described previously (8). Recombinant adenovirus was prepared and purified as described previously (9). Infections with Ad- α 1WT or Ad- α 1DN were conducted at 100 plaque-forming units per cell in PBS for 30 minutes at 37°C, and then fresh serum-free medium was added for the indicated times.

Cell proliferation by a MTT assay. Cells seeded on 96-well microplates at 4,000 per well were incubated with the test compounds for indicated times. Respective medium was removed and then incubated with 100 μ L MTT solution (2 mg/mL MTT in PBS) for 4 hours. Absorbance was determined using an autoreader.

Reactive oxygen species measurement. Cells were seeded on 12-well microplate on cover glasses, after stimulated for indicated times, respectively, cells were incubated with 10 μ mol/L of DCFH diacetate (DCFH-DA; Sigma) for 30 minutes, and washed with PBS, and fluorescence was measured with a fluorescence microscope.

Tumor models in vivo. HT-29 colon cancer cells (2×10^6) were inoculated s.c. in 5-week male *nu/nu* mice at both flanks. Five mice were used per group. After 1 week, selenate was dissolved in PBS and given i.p. (30 μ g/g body weight). The control group received vehicle only (PBS). Tumor dimensions were measured with calipers and the volume was calculated using formula: volume = $l \times w \times d \times \pi / 6$, with l being the maximal length, w being the width, and d being the depth of tumor. Five-week-old BALB/c-*nu* mice were obtained from SLC (Tokyo, Japan). All animal experiments were approved by the Ethics Committee for Animal Experimentation of the Hannam University (Daejeon, South Korea).

Statistical analysis. Tumor volumes were compared using the Mann-Whitney Wilcoxon tests with a significance level at $P < 0.05$. Above all analyses were done using Statistical Package for Social Science (SPSS, Chicago, IL).

Results

Selenate activated AMPK and attenuated the growth of cancer cells in nude mice. We examined the effects of selenate on the growth of cancer cells in *in vivo*. Immunodeficient nude mice were inoculated with HT-29 colon cancer cells s.c. After the growth of tumors, mice were undergone i.p. treatment of selenate (30 μ g/g body weight; Fig. 1A-C). The tumor volume of selenate-treated group was significantly reduced in comparison with that of the vehicle-

treated control group; we also found the fact that selenate induced AMPK activation and suppressed COX-2 expression (Fig. 1D). There were no significant differences in body weight of animals between the control and selenate-treated groups (data not shown).

Induction of apoptosis by selenium in HT-29 colon cancer cells. We then investigated the effects of selenate on HT-29 colon cancer cell proliferation. As shown in Fig. 2A and B, treatment of HT-29 colon cancer cells with selenate markedly reduced cell viability in a dose- and time-dependent manners. In these cells, selenium treatment resulted in chromatin condensation and nuclear fragmentation dose dependently (Fig. 2C). Under the same conditions, selenate-treated total cell populations were converged in a sub-G₁ region (Fig. 2D).

AMPK activation is critical for selenate-induced apoptosis in HT-29 colon cancer cells. The activation of AMPK was shown previously to trigger apoptosis via inhibition of proliferating protein expression (10). We investigated the association of the apoptosis signal pathway and the AMPK activation in selenate-induced HT-29 cancer cell death. We assessed the expression levels or phosphorylation status of AMPK, ACC, PARP, caspase-3, and p53. As shown in Fig. 3A or B, selenate increased both phosphorylation of AMPK and ACC and also the levels of PARP, caspase-3, and p53 concentration time dependently. We also found out the fact that

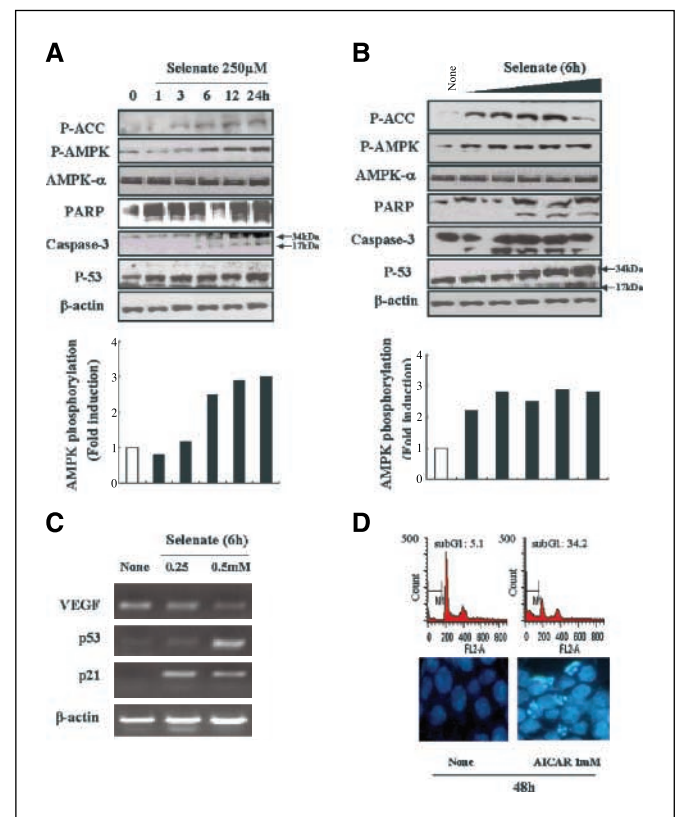


Figure 3. AMPK activation by selenium accompanied by the alteration of various apoptotic protein or gene expression. **A** and **B**, protein expression levels of phosphorylated ACC (*P-ACC*) or AMPK (*P-AMPK*), 33PARP, or caspase-3 cleavage and p53 of HT-29 cells treated with selenate. **C**, mRNA levels of *VEGF*, *p53*, *p21*, and β -actin genes were examined by reverse transcription-PCR using specific primers. **D**, HT-29 cells were treated with 1 mmol/L AICAR (an AMPK activator) for 24 hours, and then cells were incubated to 10 μ mol/L Hoechst 33342 dye or 10 μ mol/L PI dye for 30 minutes, and changes in fluorescence were measured by either fluorescence microscopic images or FACS analysis.

selenate reduced mRNA levels of survival genes, such as *VEGF*, but increased those of proapoptotic genes, such as *p53* and *p21* (Fig. 3C). To determine whether AMPK activation is necessary for cancer cell apoptosis, the effects of the AMPK activator AICAR on HT-29 colon cancer cell growth were examined. As shown in Fig. 3D, the direct AICAR treatment elevated sub-G₁ population as well as DNA fragmentation in HT-29 colon cancer cells.

Role of AMPK activated by selenate or AICAR in attenuation of COX-2 expression or PGE₂ release. Inhibition of PGE₂ released by selenate was observed dose dependently (Fig. 4A). HT-29 cells treated with TPA exhibited increased COX-2 expression and ERK phosphorylation (Fig. 4B, left). The pharmacologic ERK inhibitor PD98059 reduced COX-2 expression as well as ERK activation (Fig. 4B, right). The results indicated that ERK signal is necessary for TPA-induced COX-2 expressions. Stimulation of AMPK by AICAR (Fig. 4B, bottom) or selenate (Fig. 4C and D) abrogated TPA-stimulated ERK phosphorylation and COX-2 expressions. Celecoxib was used as a positive control of COX-2 inhibition (Fig. 4B, bottom; Fig. 4C and D).

AMPK-COX-2 pathway in selenium-induced apoptosis. To examine the association of AMPK with the inhibitory effect of selenium on COX-2 and PGE₂ regulation, COX-2 dominant-positive or COX-2 DN fibroblast cells or adenoviral-mediated DN AMPK (ad-dn AMPK) cells were treated with selenium or AICAR before TPA treatment. AMPK activation was not dependent on the presence of *cox-2* gene (Fig. 5A). In addition, transfection with ad-dn AMPK completely abolished the selenium-induced COX-2 expression and ERK phosphorylation (Fig. 5B). Attenuation of cell survival or inhibition of PGE₂ release by selenium was disappeared in ad-dn AMPK-infected cells (Fig. 5C and D). These results strongly suggest that AMPK activity is likely to be necessary for the selenate-mediated inhibition of ERK phosphorylation and COX-2 expression in TPA-treated colon cancer cells.

Selenate inhibited COX-2 expression via reactive oxygen species in TPA-treated cancer cells. We found that selenium increased intracellular reactive oxygen species (ROS) in HT-29 cells. As shown in Fig. 6A, selenate markedly increased DCF fluorescence indicating the ROS generation. To show a direct role

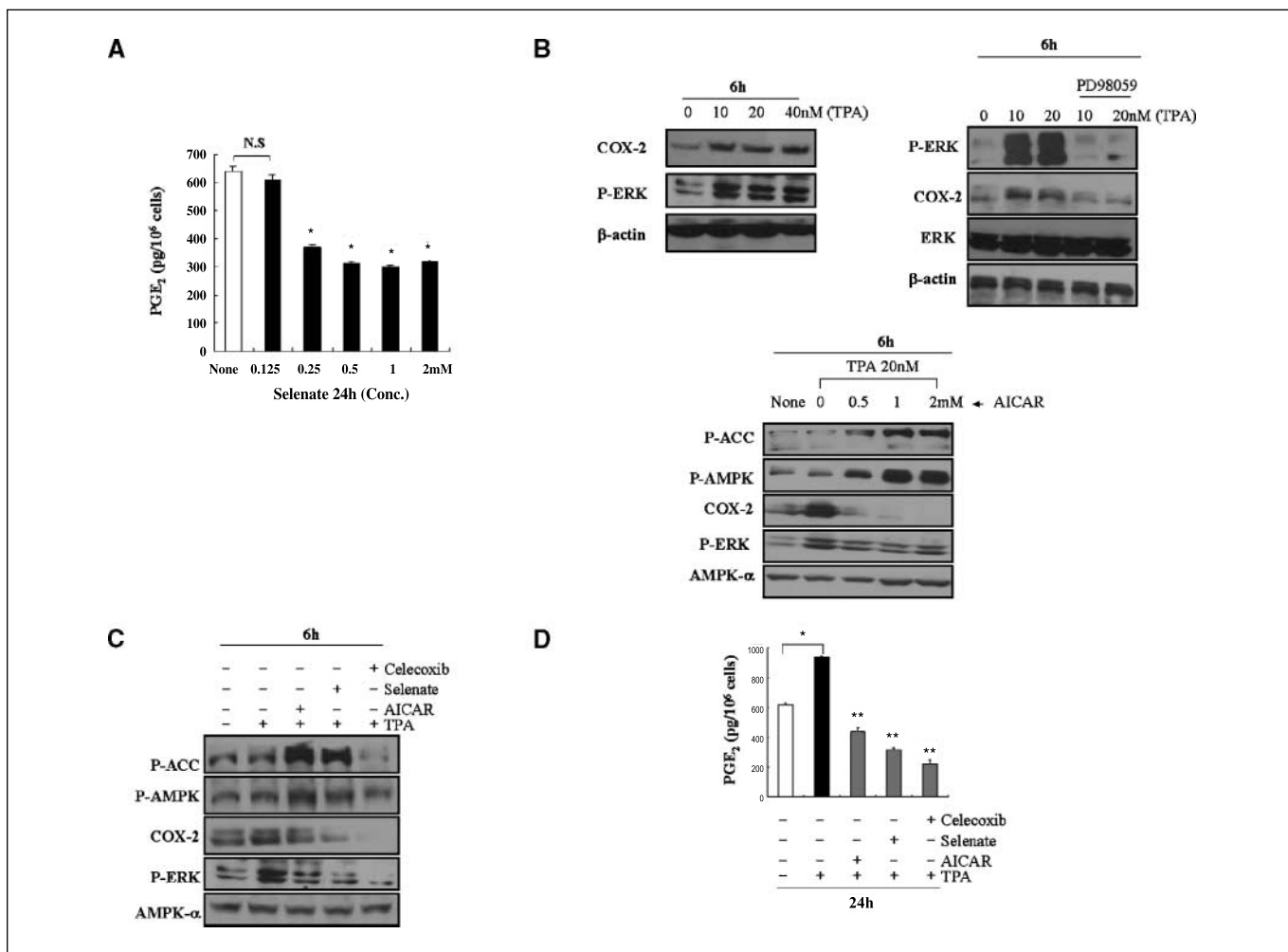
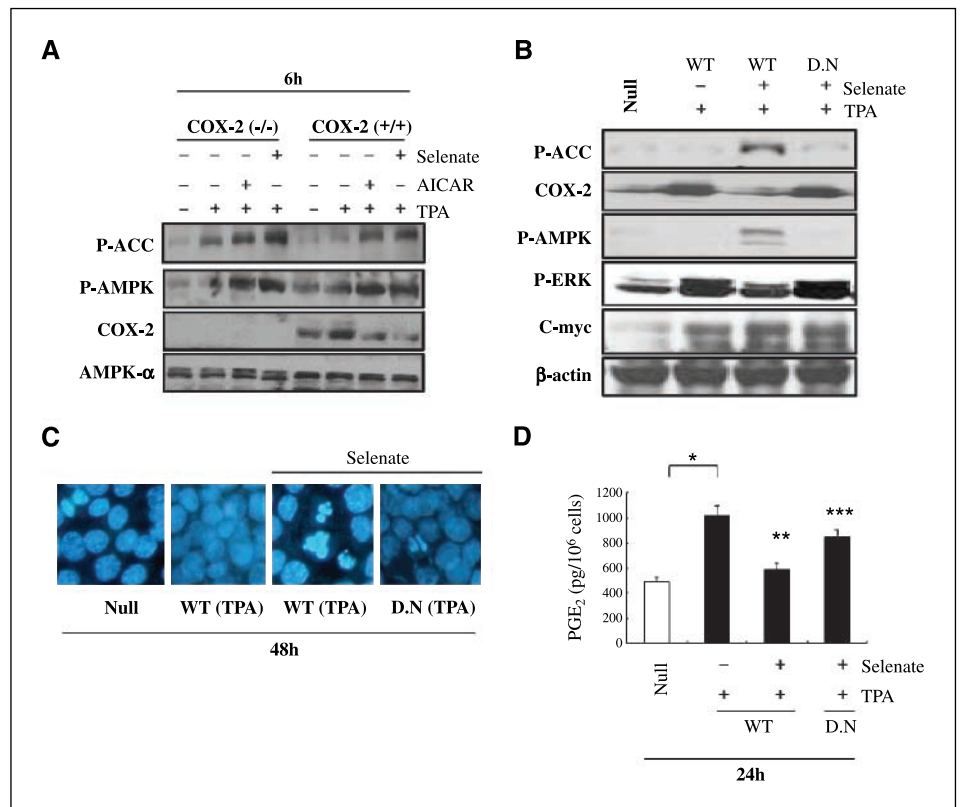


Figure 4. AMPK activation by selenate and AICAR dramatically abrogates the elevated levels of COX-2 expression and PGE₂ release in TPA-treated HT-29 cells. A, HT-29 cells were exposed to different concentrations of selenate for 24 hours, and the amount of PGE₂ was assayed with ELISA. B, cells were treated with 10, 20, and 40 nmol/L TPA for 6 hours (left), and cells were pretreated with 25 μmol/L PD98059 for 30 minutes and then exposed to 20 or 40 nmol/L TPA for 6 hours, and Western blot analysis of the designated proteins were carried out (right). B, bottom and C, effects of various combinations of celecoxib, selenate, and AICAR on phosphorylated ERK (P-ERK), COX-2, phosphorylated AMPK, and phosphorylated ACC expression. D, PGE₂ levels by either selenate or AICAR were examined in TPA-pretreated HT-29 cells. Data are representative of three separate experiments done in triplicates. *, P < 0.05, compared with control cells; **, P < 0.05, compared with TPA-treated cells.

Figure 5. Role of AMPK on COX-2 expression using both COX-2 knockout fibroblast (*cox-2^{-/-}*) or ad-dn AMPK cells. **A**, phosphorylation of ACC, AMPK, and ERK and expression of COX-2 by either selenate or AICAR in TPA-pretreated COX-2 WT or knockout mouse fibroblast cells. **B**, results of ad-dn AMPK on ERK phosphorylation or COX-2 expression by the selenate in TPA-pretreated HT-29 cells. **C**, representative photomicrographs of Hoechst 33342-positive HT-29 cells. **D**, effect of ad-dn AMPK on PGE₂ release by selenate in TPA-treated HT-29 cells. *, *P* < 0.05, compared with null cells; **, *P* < 0.05, compared with TPA-treated AMPK WT cells; ***, *P* < 0.05, compared with combination with TPA- and selenate-treated AMPK WT cells.



of ROS in the signaling pathway of the selenate-induced ERK and COX-2 inhibitions, the effect of NAC, a specific ROS scavenger, was examined. NAC treatment almost completely abolished the selenate-generated ROS generation (Fig. 6A) and restored ERK phosphorylation and COX-2 expression, which were suppressed by the selenate supplementation. Total cell populations were shifted from a sub-G₁ to G₁ by NAC treatment (Fig. 6C).

The results strongly support that selenium-induced AMPK activation or suppression of ERK activation and inhibition of COX-2 expression are mediated by ROS.

Selenate also has chemotherapeutic potentials in various other cancer cell types. To establish whether selenium has chemotherapeutic potential in various type of cancer cells, comparison of selenate with clinical therapeutic drugs against

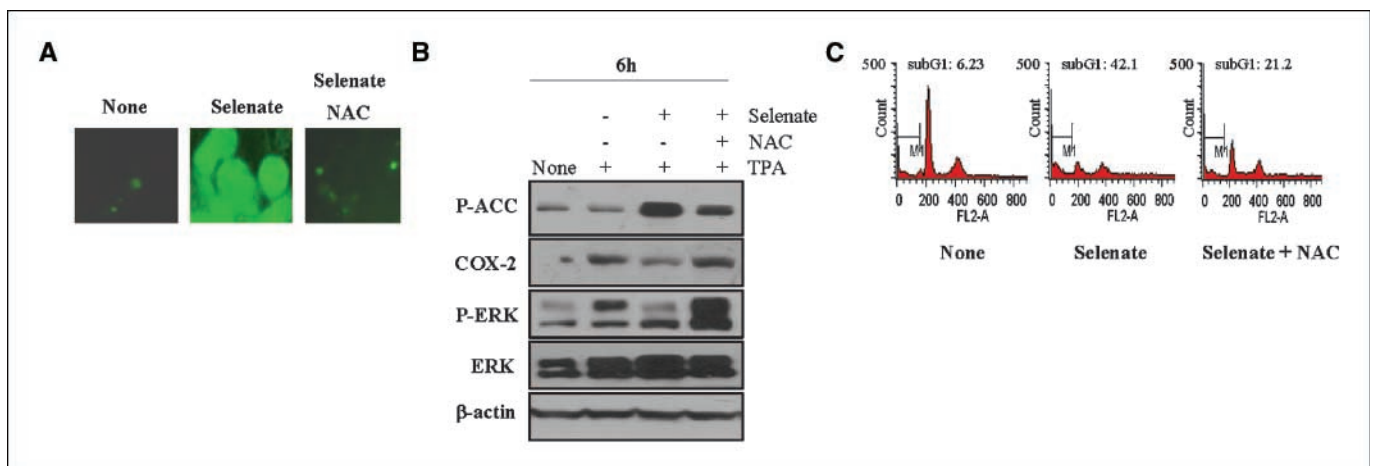


Figure 6. ROS generation by selenate in HT-29 colon cancer cells. **A**, ROS generation by selenate in HT-29 cells was determined by DCFH-DA dye. Cells were pretreated with 5 mmol/L NAC for 30 minutes and then exposed to 250 μmol/L selenate for 6 hours. After additional 30 minutes of incubation in the presence of 10 μmol/L DCFH-DA, changes in fluorescence intensity were measured by fluorescence-activated cell scanning analysis. **B**, effects of NAC on expression of AMPK, COX-2, and ERK by selenate in TPA-pretreated HT-29 cells. Under the identical conditions, the phosphorylation of AMPK and ERK and expression of COX-2 were examined. **C**, effects of NAC on sub-G₁ population by selenate-treated HT-29 cells. Cells were pretreated with 5 mmol/L NAC and then exposed to 250 μmol/L selenate, respectively, and then collected cells were incubated with 10 μmol/L PI dye, and cell death was measured by FACS analysis.

cancer, such as FU and etoposide, was observed by the growth-inhibitory pattern in MCF-7 or HeLa cells as well as in HT-29 cells. The most susceptibility was shown in cells treated with selenate, and the treating with FU or etoposide resulted in comparatively strong resistance of cell apoptosis (Supplementary Fig. S1).

Discussion

We determined the role of selenium in regulating apoptosis, signaling pathways of ERK/COX-2/PGE₂, and overall participation of AMPK in these regulatory processes. Therefore, the activation of AMPK by selenium regulates ERK/COX-2 and PGE₂ decreased after testing the levels of PGE₂ using tumor xenograft mice, *cox-2*^{-/-} cells, and DN AMPK colon cancer cells. The importance of COX-2 in the survival of colon cancer cells was emphasized by several researchers (11–14). We showed that the COX-2 regulation was associated with a reduction of the solid xenograft tumor in selenium-treated nude mice. We also observed that selenate functions as an AMPK activator similar to a synthetic AMPK activator, AICAR. Furthermore, it was shown that AMPK activation by selenium could regulate TPA-induced COX-2 and PGE₂ in cancer cells. Our data clearly showed that inhibition of COX-2 by selenium could be reversed in cells transduced with ad-dn AMPK. Therefore, our results suggest that AMPK may play a critical role in COX-2 regulation and in its target gene expressions under TPA stimulation. It was established that the product of COX-2 pathway or COX-2 itself can enhance cell proliferation and growth of tumor cells (15). In addition, animal and clinical studies suggest that COX-2 up-regulation is a key step in tumorigenesis (16). Furthermore, COX-2 may be important in cancer development by regulating critical features of cancer cell adaptation, such as angiogenesis and migration (17). Evidences from genetic and pharmacologic studies suggest that COX-2 expression is induced at an early stage of tumorigenesis, especially colon cancers (18). There is another implication that COX-2 is not involved in tumor initiation but in the promotion of colorectal adenomas. COX-2 induction is shown to be activated by tyrosine kinases that respond to growth factors, such as mitogen-activated protein kinase (MAPK), including ERK (19). We showed that selenate can inhibit the ERK signaling pathway. A mechanism by which selenium suppresses ERK has been suggested to be a direct action of MAPK (20), but the present study indicates that it might involve a second up-stream regulator, AMPK. AMPK is known as a master negative regulator in protein synthesis of various cells, and it has been considered as a novel therapeutic target for treatments of cancers or other metabolic diseases (21–23). However, the role of AMPK in its growth-inhibitory effect controversially depends on cell types. Cell proliferative effects of the activated AMPK via Akt/mammalian target of rapamycin (mTOR) or phosphatidylinositol 3-kinase signaling were shown in endothelial cells exposed to

ONOO⁻. On the other hand, the inactivation of LKB1 by reactive lipid species was shown to inhibit the phosphorylation of AMPK as well as the downstream TSC-mTOR-S6k cascade in breast cancer cells (24–26).

Selenium compounds used in cancer prevention studies include the inorganic salt selenite and selenate, the organic amino acid derivatives SeMet, selenocystein, and Se-methyl-selenocystein, and synthetic inorganic compounds. Most of the selenium compounds have been shown to reduce DNA adduct formations and mammary tumor incidences (27–29). Selenium can be degraded to hydrogen selenide and incorporated into selenoproteins. The present study shows that selenate could reduce the size of the tumor in nude mice and could inhibit cell growth and COX-2 expression. We showed that selenate abrogates COX-2 expression via ERK pathway in inducing apoptosis of colon cancer cells. Because AMPK was activated in fibroblast cells of COX-2 knockout mouse (*cox-2*^{-/-}) as well as of WT (*cox-2*^{+/+}) by selenate, AMPK is an upstream signal of COX-2. Our data strongly support that AMPK is a negative regulator in cancer development by modulating cancer promotion proteins, such as ERK or COX-2.

The present study provides another important fact that selenium enhances AMPK activity through the generation of ROS. The distinctive generation of ROS by the selenium treatment was abolished completely by NAC, and at the same time, ERK phosphorylation and COX-2 expressions were restored by NAC. In fact, ROS has been implicated in COX-2 regulation (30). Considering the fact that intracellular ROS can be generated by numerous external stimuli and AMPK is highly sensitive to oxidative stress because increased cellular ROS changes AMPK level, at this time AMPK is rapidly activated (31). The involvement of AMPK in the inhibition of tumorigenesis can be expanded beyond the regulation of COX-2. For example, the activation of AMPK by naturally occurring compounds, such as genistein or epigallocatechin gallate, was shown to be mediated through the production of ROS. In general, AMPK has been speculated to be regulated by stress, such as intracellular ROS (32, 33).

We showed that selenium inhibits growth of colon cancer cells *in vivo* and *in vitro* by the activation of AMPK via the downstream COX-2/PGE₂ pathway. An activation of metabolic sensor, such as AMPK, promises to be an effective approach to the prevention and treatment of cancers, especially colon cancers.

Acknowledgments

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