AMPK interacts with β-catenin in the regulation of hepatocellular carcinoma cell proliferation and survival with selenium treatment

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Abstract. Selenium has received much attention as an anticancer agent, although the mechanisms of action underlying its pro-apoptotic properties remain unclear. Tumors that respond well to antioxidant treatments, such as hepatocellular carcinoma (HCC), may benefit from treatment with selenium as this compound also has antioxidant properties. Furthermore, a major oncogenic driver in HCC is the nuclear transcription co-activator, β-catenin. In the present study, we examined the mechanism by which selenium reduces survival of HCC cells, and whether this was associated with modulation of the β-catenin pathway. Hep3B cell lines and cancer cell xenografted animals were treated with selenium, and apoptotic events or signals such as AMPK, β-catenin and GSK3β were determined. Further interactions among β-catenin, glycogen synthase kinase 3β (GSK3β), and AMPK were explored by applying AMPK small interfering RNA (siRNA) or GSK3β siRNA with western blotting or immunofluorescence microscopic observation. Selenium activated AMPK, which in turn suppressed β-catenin. Selenium induced the translocation of AMPK into the nucleus and prevented the accumulation of β-catenin therein. Upon inactivation of AMPK by AMPK siRNA, selenium no longer modulated β-catenin, implying that AMPK is an upstream signal for β-catenin. We found that the binding between AMPK and β-catenin occurs in the cytosolic fraction, and therefore concluded that the cancer cell antiproliferative effects of selenium are mediated by a GSK3β-independent AMPK/β-catenin pathway, although AMPK-mediated GSK3β regulation was also observed. We primarily discovered that AMPK is a crucial regulator initiating selenium-induced inhibition of β-catenin expression. Taken together, these novel findings help to illuminate the molecular mechanisms underlying the anticancer effect of selenium and highlight the regulation of β-catenin by selenium.

Introduction

Selenium compounds have received a great deal of attention in cancer treatment and chemoprevention. The possible selective inhibitory effect of selenium on tumor cells makes selenium a promising candidate for controlling tumorigenesis (1). Despite this potential, little scientific evidence exists to describe the exact mechanism underlying the anticancer effect of selenium (2). A variety of genetic studies have revealed that cancer cell proliferation, invasion and metastasis can be suppressed through at least three possible areas of modulation, including the cell cycle and apoptosis, signaling pathways and target genes (3-5). Human clinical trials have revealed dose-limiting toxicity when selenium compounds are administered at doses of up to 0.8 mg/day, and at the maximum dose, plasma concentrations reached 601 μg selenium (6).

One of the promising target molecules of selenium is β-catenin. β-catenin is a transcription factor that plays a pivotal role in cells, regulating a large set of genes involved in cell development, differentiation, growth and metastasis (7). The canonical β-catenin pathway begins with the stabilization of β-catenin. In the inactivated state of the Wnt ligand, a low plasma level of β-catenin is maintained and controlled by the activity of a multiprotein destruction complex that targets β-catenin for ubiquitination and proteolytic degradation (8). Upon binding to Wnt ligands, β-catenin inhibits the formation of the multienzyme complex, and phosphorylated β-catenin is translocated into the nucleus to regulate expression of certain genes such as c-myc, c-Jun and cyclin D1 (9-11). Inappropriate regulation of...
the Wnt/β-catenin pathway is associated with hepatocellular carcinoma tumorigenesis (12). Several studies have shown that the tumor-suppressing inactivated form of β-catenin occurs concomitantly with the activation of 5' adenosine monophosphate-activated protein kinase (AMPK) (13,14).

In the present study, we explored crosstalk between β-catenin and AMPK to elucidate the molecular basis of selenium-induced cancer cell control. AMPK was found to be a crucial regulator initiating selenium-induced inhibition of insulin-like growth factor 1 (IGF-1)-stimulated β-catenin expression. We also discovered that selenium inhibits phosphorylation of glycogen synthase kinase 3β (GSK3β) at Ser9 and β-catenin at Ser552, and the selenium-induced activation of AMPK led to the attenuated nuclear localization of β-catenin.

Materials and methods

Cells and reagents. The Hep3B hepatocellular carcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA). EGCG, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were obtained from Sigma (St. Louis, MO, USA). Compound C was purchased from Calbiochem (San Diego, CA, USA). Monoclonal antibodies specific for p-AMPKα (Thr172), AMPKα1, p-GSK3β (Ser9), GSK3β, p-β-catenin (Ser33/37), p-β-catenin (Ser552) and β-catenin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against lamin B1 was purchased from Santa Cruz (San Diego, CA, USA) and the antibody against lamin B1 was specific for p-AMPKα (13,14). Antibody against lamin B1 was purchased from Santa Cruz (San Diego, CA, USA) and the antibody against lamin B1 was specific for p-AMPKα (13,14). Monoclonal antibodies specific for p-AMPKα (Thr172), AMPKα1, p-GSK3β (Ser9), GSK3β, p-β-catenin (Ser33/37), p-β-catenin (Ser552) and β-catenin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against lamin B1 was purchased from Santa Cruz (San Diego, CA, USA) and the antibody against lamin B1 was specific for p-AMPKα (13,14).

Cytoplasmic and nuclear fractionation. Cells were seeded into six-well plates and treated with test compounds. Cytoplasmic and nuclear proteins were extracted using ProteoExtract® Subcellular Proteome Extraction kit (Calbiochem) and subjected to western blot analysis with specific antibodies. The proteins were then visualized by enhanced chemiluminescence (Intron, Kyunggi, Korea) and detected using a LAS4000 chemiluminescence detection system (Fuji, Tokyo, Japan).

Immunofluorescence staining. The cells were seeded into a 12-well plate with cover glasses. Following treatment at the indicated time and dose, the cells were fixed in 3.7% formaldehyde for 20 min at room temperature (RT) and were permeabilized in 0.2% Triton X-100 for 20 min at RT. Then cells were blocked with 1% bovine serum albumin for 1 h. Next, the cells were incubated overnight with primary antibody against either AMPKα1 or β-catenin. After washing, the cells were incubated with Alexa546-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-mouse IgG (both from Molecular Probes, Eugene, OR, USA) for 1 h at RT. Next, cell nuclei were stained with 10 µM Hoechst 33342 for 10 min and then observed with a confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Immunohistochemistry. Tumor specimens from mice were fixed in 10% formaldehyde, embedded in paraffin and

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Immunohistochemistry. Tumor specimens from mice were fixed in 10% formaldehyde, embedded in paraffin and
sectioned into 5-µm thick slices. Sections were depara-
fined with xylene and dehydrated with 98% ethanol. Serial
sections were stained using standard immunoperoxidase
techniques with primary antibodies against β-catenin (1:50)
and p-AMPKα1 (1:50). For epitope retrieval, specimens were
microwave-treated for 25 min before incubation with primary
antibodies. Pre-immune serum was used as a negative control
for immunostaining, and positive-staining was visualized with
diaminobenzidine, followed by a light counter-staining with
hematoxylin. All findings were evaluated by a pathologist
blinded to the treatment conditions, and samples were evalu-
ated on the basis of stain intensity and percentage of reactive
cells. Images of representative results were recorded.

Statistical analysis. Cell viability and tumor volume data
were statistically analyzed using an unpaired t-test (SPSS,
Inc., Chicago, IL, USA). P<0.05 was considered to indicate a
statistically significant result.

Results

Selenium-induced inhibition of IGF-1-stimulated β-catenin is
associated with activation of AMPK. A large body of evidence
suggests that β-catenin is often aberrantly overexpressed in
hepatocellular carcinoma. IGF-1 has recently been identified
as being capable of increasing β-catenin expression and its
transcriptional activity via phosphorylation of GSK3β (10).
We examined this effect of IGF-1 on hepatocellular carcinoma
growth through regulation of the GSK3β/β-catenin survival
pathway. Our results showed that IGF-1 effectively increased
cell growth in a time-dependent manner (Fig. 1A). We analyzed
the molecular changes in the control and selenium-treated
cells. The phosphorylation of β-catenin on the Ser552 residue
and GSK3β were significantly increased, and phosphoryla-
tion of β-catenin on the Ser33/37 residue was significantly
decreased in a time-dependent manner (Fig. 1B). To examine
whether selenium exerts inhibitory effects on β-catenin and
GSK3β, we analyzed changes in their phosphorylation and
expression. Selenium at concentrations of >500 µM effectively
inhibited IGF-1-stimulated β-catenin and phosphorylation of
GSK3β and enhanced AMPK phosphorylation (Fig. 2A). We
investigated the effects of selenium on the β-catenin trans-
location from the cytosol to the nucleus. We discovered that
selenium decreased IGF-1-increased β-catenin translocation
in the nucleus at 6 h, whereas no marked difference occurred
in the expression of β-catenin in the cytosol (Fig. 2C). To
analyze the localization pattern of β-catenin in this system
further, we immunostained β-catenin and detected it using
fluorescence microscopy. Consistent with western blot results,
β-catenin decreased in the nucleus after selenium treatment
for 6 h (Fig. 2B). Taken together, we inferred that selenium
may redistribute IGF-1-increased β-catenin protein in the
cytoplasm and nuclei.

Selenium-activated AMPK directly suppresses β-catenin.
We investigated whether selenium-reduced β-catenin expres-
sion in Hep3B cells is associated with the activation of
AMPK. We examined the effects of selenium on the activity
of β-catenin in AMPK or GSK3β siRNA-transfected Hep3B
cells. To examine whether selenium-reduced β-catenin levels
are AMPK dependent, we determined the effects of sele-
nium on β-catenin after knockdown of AMPK using siRNA
in Hep3B cells. Selenium did not regulate β-catenin and
GSK3β in the absence of AMPK. However, selenium regu-
lated β-catenin in the absence of GSK3β (Fig. 3A). β-catenin
was regulated not only by the GSK3β signaling pathway
but also by the PI3K/Akt pathway. Thus, selenium regulates
β-catenin via a GSK3β-independent pathway. Furthermore,
the immunostaining results showed that selenium had no
effect on β-catenin localization in AMPK-transfected Hep3B
cells (Fig. 3B).

Next, we examined the direct relationship of AMPK/β-
catenin using immunoprecipitates. Co-immunoprecipitation/
western blot experiments performed with Hep3B cells showed
identical results (Fig. 3C). To further characterize the specificity
of the β-catenin-AMPK interaction, we performed additional
experiments with antibodies specific for β-catenin and AMPK
isoforms in the lysate of untreated or selenium-treated cells.
Treatments with selenium led to the appearance of β-catenin
or AMPK in the Hep3B cell lysate (Fig. 3C). These results
suggest that selenium directly regulates the interaction
between β-catenin and AMPK.
Figure 2. Selenium acts as a negative regulator of β-catenin translocation into nucleus. (A) Hep3B cells were serum-starved for 12 h, pre-treated with IGF-1 (100 ng/ml) for 30 min, and treated with selenium (0.25-1 mM) for 24 h. Total proteins were subjected to western blotting. (B) Cells were fractionated with cytosol and nucleus. Then, cells were treated with selenium 0.5 mM for 6 h and the changes of indicated protein levels were determined by western blot analysis. (C) Cells were treated with 0.5 mM of selenium for 6 h. After treatment, cells were fixed, permeabilized and stained for β-catenin (red). Cell nuclei were stained with Hoechst 33342 dye (blue).

Figure 3. Selenium regulates β-catenin activity in an AMPK-dependent manner. (A) Cells were transfected with AMPKα1 or GSK3β siRNA using DharmaFECT transfection reagent and treated with 0.5 mM of selenium for 6 h. Then, protein levels of p-GSK3β, GSK3β and β-catenin were examined by western blot analysis. (B) Cells were transfected with AMPKα1 siRNA using DharmaFECT transfection reagent and treated with 0.5 mM of selenium for 6 h. Then, protein expression of AMPKα1 and β-catenin were examined by immunofluorescence. (C) Cells were treated with 0.5 mM of selenium for 6 h. Then proteins were fractionated with AMPKα1 or β-catenin using specific antibodies and the protein levels of AMPKα1 or β-catenin were examined by western blot analysis. (D) Cells were fractionated with cytosol and nucleus. Then, cells were treated with selenium 0.5 mM for 6 h. Then, proteins were fractionated with AMPKα1 using specific antibodies and the protein levels of AMPKα1 or β-catenin were examined by western blot analysis.
Selenium suppresses cell proliferation and induces apoptosis through AMPK activation in vitro. To examine whether selenium exerts anticancer activity in Hep3B cells, we examined the effect of selenium on cell proliferation and apoptosis. Selenium effectively inhibited cell growth in a dose-dependent manner (Fig. 4A) and induced apoptosis, as measured by Annexin V/PI and Hoechst 33342 staining (Fig. 4B).

Numerous studies have identified AMPK as a central factor inducing apoptosis in various cancer cells. Our prior study together with that of others has also implicated AMPK as a key regulator of selenium-induced apoptosis in cancer cells (15). To further validate whether AMPK inhibition by compound C is associated with cell proliferation and apoptosis of hepatocellular carcinoma cells. As shown in Fig. 4C, inhibition of AMPK by compound C treatment abolished the growth-stimulatory effects of IGF-1. Furthermore, fluorescence-activated cell sorter results revealed that the apoptotic rate in compound C-treated cells declined from 73.61% to 22.71% after treatment compared with that in selenium-treated cells in the control group (Fig. 4D).

All of these data argue for the critical involvement of AMPK in selenium-induced apoptosis in Hep3B cells.

Selenium inhibits the GSK3β/β-catenin survival pathway in hepatocellular carcinoma xenograft tumors. We built an in vivo hepatocarcinoma xenograft tumor model by inoculating Hep3B cells into male nude mice subcutaneously. We primarily discovered that treatment with selenium (3 mg/kg · day) markedly attenuated tumor growth without adverse effects on body weight and activity compared with that in the control group (Fig. 5A). We analyzed the molecular changes in the control and selenium-treated cells. The phosphorylation of GSK3β and expression of β-catenin were significantly suppressed, but the phosphorylation of AMPK was significantly increased in the selenium treatment group relative to that in the control group (Fig. 5B).

Immunohistochemical analysis confirmed that the control cells had high levels of β-catenin, and the selenium-treated cells had reduced β-catenin levels. Furthermore, tumors in the selenium treatment group showed strongly increased p-AMPK levels (Fig. 5C).

Discussion

Selenium affects numerous intracellular targets, making selenium compounds desirable chemotherapeutic and chemopreventive agents. Studies in both in vitro and in vivo models have suggested that selenium suppresses components of IGF-1-induced β-catenin (15). The objective of the present
study was to investigate the inhibitory effects of selenium on β-catenin or GSK3β through the activation of AMPK in hepatocarcinoma cells and xenograft tumors. Prior *in vitro* and *in vivo* models have suggested that selenium suppresses IGF-induced β-catenin through activation of AMPK, which in turn may suppress cell proliferation and induce apoptosis. Selenium is a nonmetallic trace element that is essential for human health; selenium supplementation also appears to work as an anti-carcinogenic agent (16).

The anticancer activity of selenium has been attributed to various mechanisms, such as mitogen-activated protein kinase suppression or modulation of Akt, mammalian target of rapamycin or β-catenin (17-19). Further, selenium has been shown to inhibit β-catenin accumulation in the nucleus. In colon cancer models, selenium treatment resulted in JNK suppression and subsequent inhibition of β-catenin (20). Our previous studies have revealed that the apoptotic effect of selenium is dependent on the AMPK-regulated extracellular signal-regulated kinase/cyclooxygenase-2 pathway as well as the AMPK/Akt mammalian target of rapamycin pathway (15,21).

Since abnormal β-catenin activation in many human malignancies is well documented and overexpressed β-catenin may have oncogenic effects in hepatocellular carcinoma (22), the interaction between β-catenin and AMPK may represent an important mechanism for the regulation of β-catenin signaling pathways with selenium in cancer. Knockdown of AMPK using AMPK siRNA increased β-catenin in contrast with results after selenium treatment. Importantly, the inhibition of AMPK allows the increases in β-catenin as well as in GSK3β and Akt that can be encountered in advanced hepatocellular carcinoma cells. The present study did not reveal the exact mode of the regulation of β-catenin activity by AMPK, although immunoprecipitation studies have shown that the
binding between AMPK and β-catenin occurs in cytoplasm, and fluorescent microscopic examinations have pointed out that AMPK is responsible for the inhibition of β-catenin into the nucleus. Our data show that selenium increases AMPK via phosphorylation of AMPK Thr172, which in turn leads to the inhibition of β-catenin, indicating that selenium is capable of suppressing β-catenin function via an AMPK-dependent pathway. In addition to β-catenin suppression by selenium-induced AMPK activation, we observed that activated AMPK regulates GSK3β. We used inhibitors of AMPK and GSK3β to determine whether the capability of AMPK to regulate β-catenin was required for selenium to exert its antiproliferative functions. When AMPK was inhibited by AMPK siRNA, selenium treatment failed to decrease β-catenin, indicating that AMPK is required for selenium regulation of β-catenin. By contrast, GSK3β siRNA-mediated inhibition of GSK3β did not affect the capability of selenium to inhibit β-catenin, indicating that direct inhibition of β-catenin by selenium may occur without the involvement of GSK3β.

GSK3β/β-catenin is known to promote tumor growth through its function in the tumor microenvironment, but its exact method for converting normal cells into cancerous cells remains undefined. β-catenin is a transcription factor that plays a pivotal role in cells, regulating a large set of genes involved in cell development, differentiation, growth and metastasis. The β-catenin pathway seems to play a critical role against hepatocellular carcinoma, possibly through alteration at multiple levels, including mutation of β-catenin or its upstream or downstream regulators/effectors such as GSK3β, Akt and T-cell factor/lymphoid enhancer binding factor (23,24).

In the present study, we elucidated that selenium downregulates the β-catenin survival pathway through activation of AMPK in hepatocellular carcinoma cells and xenograft tumor models. We primarily discovered that selenium could inhibit β-catenin at Ser552 and GSK3β at Ser9 in Hep3B cells; however, the attenuation of nuclear localization of β-catenin occurred only under the activation of AMPK. Taken together, these findings help illuminate the molecular mechanisms underlying the anticancer effects of selenium and highlight the regulation of β-catenin through an AMPK-dependent pathway.

References