Methylselenol, a Selenium Metabolite, Induces Cell Cycle Arrest in G1 Phase and Apoptosis via the Extracellular-Regulated Kinase 1/2 Pathway and Other Cancer Signaling Genes¹⁻³

Huawei Zeng,⁴ Min Wu,⁵ and James H. Botnen⁴

USDA, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202-9034
and Department of Biochemistry and Molecular Biology, University of North Dakota, Grand Forks, ND 58202

Abstract

Methylselenol has been hypothesized to be a critical selenium (Se) metabolite for anticancer activity in vivo, and our previous study demonstrated that submicromolar methylselenol generated by incubating methionase with seleno-L-methionine inhibits the migration and invasive potential of HT1080 tumor cells. However, little is known about the association between cancer signal pathways and methylselenol's inhibition of tumor cell invasion. In this study, we demonstrated that methylselenol exposure inhibited cell growth and we used a cancer signal pathway-specific array containing 15 different signal transduction pathways involved in oncogenesis to study the effect of methylselenol on cellular signaling. Using real-time RT-PCR, we confirmed that cellular mRNA levels of cyclin-dependent kinase inhibitor 1C (CDKN1C), heme oxygenase 1, platelet/endothelial cell adhesion molecule, and PPARγ genes were upregulated to 2.8- to 5.7-fold of the control. BCL2-related protein A1, hedgehog interacting protein, and p53 target zinc finger protein genes were downregulated to 26-52% of the control, because of methylselenol exposure. These genes are directly related to the regulation of cell cycle and apoptosis. Methylselenol increased apoptotic cells up to 3.4-fold of the control and inhibited the extracellular-regulated kinase 1/2 (ERK1/2) signaling and cellular myelocytomatosis oncogene (c-Myc) expression. Taken together, our studies identify 7 novel methylselenol responsive genes and demonstrate that methylselenol inhibits ERK1/2 pathway activation and c-Myc expression. The regulation of these genes is likely to play a key role in G1 cell cycle arrest and apoptosis, which may contribute to the inhibition of tumor cell invasion. J. Nutr. 139: 1613-1618, 2009.

Introduction

Selenium (Se) is an essential trace element and a substantial body of persuasive evidence indicates that Se plays a role in cancer prevention (1-3). Epidemiological evidence indicates that Se status is inversely associated with cancer risk, and results from some, but not all, nutritional intervention studies show that high Se intakes effectively reduce the risk of mammary, prostate, lung, colon, and liver cancer (4-6). Interest in this area was further stimulated by the finding that supplementation of free-living people with high-Se yeast with predominantly seleno-L-methionine (SeMet)⁶ and trace amounts of other Se forms decreased the overall cancer morbidity by nearly 50% (4). This finding was from the Nutritional Prevention of Cancer (NPC) Trial, a prospective, double-blinded, randomized, placebo-controlled trial involving 1312 patients (4) and led to the design of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (5). However, the recent SELECT data (6) suggest that Se does not help prevent prostate cancer. This is somehow unexpected, because most previous studies found that Se reduced cancer risk (1-5). It has been suggested that

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³ Supplemental Figures 1 and 2 are available with the online posting of this paper at jn.nutrition.org.

⁴ To whom correspondence should be addressed. E-mail: huawei.zeng@ars.usda.gov.

⁵ Abbreviations used: BCL2A1, BCL2-related protein A1; CDKN1C, cyclin-dependent kinase inhibitor 1C; c-Myc, cellular myelocytomatosis oncogene; Cr, threshold cycle; ERK1/2, extracellular-regulated kinase 1/2; FBS, fetal bovine serum; HHIP, hedgehog interacting protein; HMOX1, heme oxygenase 1; MEJase, l-methionine-lyase; MMP, matrix metalloproteinase; NCP, Nutritional Prevention of Cancer; PECAM1, platelet/endothelial cell adhesion molecule; SELECT, Selenium and Vitamin E Cancer Prevention Trial; SeMet, seleno-L-methionine; WIG1, p53 target zinc finger protein.
there are 2 main factors attributing the difference. First, the NPC trial was conducted in men chosen for deficient levels of Se and the benefit was limited to those with the lowest baseline Se levels (4). In contrast, SELECT men generally were replete in Se at baseline (5,6). Second, SeMet given in the SELECT trial may have been less active than Se chemical forms (high-Se yeast) given in the NPC Trial (6).

This underscores the urgency of understanding Se chemical forms, doses, and their molecular targets.

The mechanisms underlying the anticancer activity of Se are not fully understood. In experimental animals, anticarcinogenic effects have been consistently associated with Se at supranutritional intakes (>1 mg/kg diet) that are at least 10 times those required to prevent clinical signs of deficiency and to support near-maximal tissue activities of selenoenzymes (2,7,8). Recent gene therapy data showed that in vivo SeMet treatment of nude mice bearing tumor cells expressing the t-methionine-y-lyase (METase) transgene inhibited tumor growth (9); several lines of evidence have implicated methylselenol as the active in vivo Se metabolite pool for anticarcinogenic effects associated with supplements/high Se intakes (1,2,10). More recently, our study demonstrated that methylselenol inhibits prometastasis pro-matrix metalloprotease (MMP)-2 activation and the invasive potential of HT1080 tumor cells (11). However, much remains to be determined about the effects of methylselenol on tumor-igenic signal transduction.

The biological specificity of methylselenol generation in cell culture media has been well established (12,13). Several recent studies (11,13,14) have shown that in different culture media and cell lines, a low dose of METase or SeMet alone has no detectable biological effects, whereas methylselenol generated by incubating METase with SeMet has been shown to be responsible for anticarcinogenic effects, including regulation of gene expression. Based on our previous data obtained using this experimental model (11), we hypothesize that multiple signaling pathways are involved in methylselenol-induced inhibition of tumor cell invasion. Our present data suggest that methylselenol regulation of the cell cycle and apoptosis may play a role in the inhibition of invasive potential of tumor cells and provide new insights into the mechanisms of the anticancer properties of Se.

Materials and Methods

Cell cultures. HT1080 (passage 19) fibrosarcoma cells were obtained from ATCC and maintained in DMEM (GIBCO Invitrogen) with 10% fetal bovine serum (FBS) (Sigma Chemical) in a humidified chamber at 36.5°C, 5% CO₂ as described previously (11). According to our analysis, DMEM with 10% FBS media contained ~30 nmol/L of Se.

Methylselenol generation. The enzyme (METase) solutions were prepared as previously described (11), divided into aliquots, and stored at −80°C. For cell treatments, the enzyme substrate selenol-t-methionine (SeMet) (1.25–5 μmol/L) (Sigma Chemical) was added to the culture medium and immediately was followed by the addition of METase (40 U/L) for 16 h.

Gene array and real-time RT-PCR assay. Total cellular RNA was isolated from HT1080 cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and the integrity of RNA samples was checked by electrophoresis. Each 2-μg cDNA probe was prepared from total cellular RNA and hybridized to Signal Transduction in Cancer Oligo GEArray membrane (Superarray). These membranes contain 113 oligonucleotide probes representing genes associated with signal transduction pathways involved in oncogenesis. RNA samples were sent to Superarray for hybridization, detection, and data extraction (service contract).

To confirm the data generated by gene array analysis, real-time RT-PCR was conducted using a Quantitect SYBR Green RT-PCR kit (Qiagen) and an Applied Biosystems 7300 Real-Time PCR system. The primers for the different genes studied were designed to include intron spanning when possible. Wig primers (5′-GCT GTC TCT TCG CTC TGT CTA GAA TG-3′; 5′-CTC TTT GCC CGA TGA TCT TCC CTT CG-3′) and β-actin primers (5′-CAT CGA GCA CGG CAT CTC CA-3′; 5′-TAG CAC AGC CTO GAG AAC-3′) were synthesized by Invitrogen. Cyclin-dependent kinase inhibitor 1C (CDKN1C) and early growth response 1 (ERG1) gene primers were purchased from Superarray, BCL2-related protein A1 (BCL2A1), hedgehog interacting protein (HHIP), heme oxygenase 1 (HMOX1), MCL1, plate/endothelial cell adhesion molecule (PECAM1), and PPARγ gene primers were purchased from Qiagen.

Gene expression was quantified using the comparative threshold cycle (ΔΔCT) method (15). ΔΔCT is the fractional cycle number at which the fluorescence passes the threshold. The specific PCR products were examined via 1.9% agarose gel electrophoresis.

The amount of target gene, normalized to an endogenous reference (β-actin) gene, was expressed relative to the control cells (without METase treatment). Thus, for the amount of target gene in HT1080 cells treated with methylselenol, a 1-fold of control change indicated no change, >1-fold of control change indicated upregulation, and <1-fold of control change indicated downregulation.

Apoptosis analysis. A Vybrant Apoptosis Assay kit (Molecular Probes) was used to visualize the apoptotic cells per the manufacturer's instruction. This assay kit detects changes in cell membrane permeability with YO-PRO-1 dye, a green-fluorescent nucleic acid stain that is permeable to apoptotic cells. The images were captured by a Carl-Zeiss LSM 510 Meta Laser Scanning confocal microscope (Carl Zeiss MicroImaging). Differential interference contrast pictures were taken simultaneously. Images were processed using the ImageJ software provided by the manufacturer. Apoptotic cells were quantified using a Guava Nexin kit (Guava Technologies). Briefly, HT1080 cells were trypsinized and suspended in growth media (DMEM with 10% FBS). In the apoptotic cells, molecules of phosphatidylserine are translocated to the outer surface of the cell membrane where Annexin V can readily bind them. Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine, a membrane component normally localized to the internal face of the cell membrane. At least 2000 single-cell events per sample were analyzed by the Guava PCA system.

Western blotting analysis. After methylselenol treatment, adherent cells were scraped, pooled with the detached cells in 5 mL medium, and then these cells were collected by centrifugation at 350 × g for 10 min at 4°C; at least 3 independent experimental cell sample sets were collected. The cell pellet was washed once in ice-cold PBS and lysed in a buffer as previously described (16). After a brief sonication, the cell lysate was centrifuged at 14,000 × g for 30 min at 4°C. The supernatant was designated as whole-cell protein extract and kept at −80°C. The protein concentration was quantified by the Bradford dye-binding assay (Bio-Rad Laboratories). Equal amounts of protein extract (~40 μg) were resolved over 4–20% Tris-glycine gradient gels under denaturing and reducing conditions and electroblotted onto polyvinylidene fluoride membranes (Invitrogen). Membrane blots were blocked in PBS containing 0.05% Tween (v:v) supplemented with 1% (wt:v) nonfat dry milk (Bio-Rad) at 4°C for overnight. Membranes were probed with antibodies against cellular myelocytomatosis oncogene (c-Myc) (Epitomics), and extracellular-regulated kinase 1/2 (ERK1/2) or its corresponding phospho-specific antibody (Cell Signaling) and then incubated with an anti-mouse (1:3000 dilution) horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in blocking solution for 1 h at room temperature. Blots were washed as above and proteins were detected by using an enzymatic chemiluminescence plus kit (Amersham Pharmacia Biotech) with the Molecular Dynamics Image-Quant system.

Statistical analysis. Results are given as means ± SD. Data were analyzed by 1-way ANOVA using Proc Mixed in SAS, version 9.2 (SAS Institute).
that exhibited heterogeneous variances among groups, the experimental differences with a P-value < 0.05 were considered significant. For the real-time RT-PCR data, ANOVA compared the fold change in the text and tables.

Results

Cell growth and cell morphological responses. Cell growth was inhibited to 76.5, 65.3, and 50.1% of the control, respectively, in the cells treated with methylselenol generated by incubating METase (40 U/L) with 1.25, 2.5, or 5 μmol/L SeMet (Fig. 1). Similarly, these cells were increasingly elongated, retracted, and detached from the bottom of the culture flask and many detached cells were not individual cells but appeared as a multi-cell sheet or anoikis (37) when treated with methylselenol generated by incubating 40 U/L METase with jzmol/L SeMet (Fig. 1). Similarly, these cells were increasingly elongated, retracted, and detached from the bottom of the culture flask and many detached cells were not individual cells but appeared as a multi-cell sheet or anoikis (17) when treated with methylselenol (Supplemental Fig. 1).

Cancer signaling gene expression. Signal Transduction in Cancer Oligo GEArray analyses suggested that 9 genes were upregulated or downregulated by ~2-fold in cells treated with methylselenol compared with that of control cells (without METase treatment) (Table 1). To verify these data, a real-time PCR analysis was performed on all 9 genes using 4 different independent RNA sample sets. The mRNA levels of these genes, CDKN1C (5.7-fold of the control), HMOX1 (2.8-fold of the control), PECAM1 (4.2-fold of the control), and PPARG (3.1-fold of the control) were downregulated by methylselenol generated by incubating METase (40 U/L) with 2.5 μmol/L SeMet (Table 1).

Apoptosis. The apoptotic cells were visualized by Vybrant apoptosis assay with green dye staining apoptotic cell. Few apoptotic cells were visualized in the untreated cells (Supplemental Fig. 2A), 5 μmol/L SeMet alone (Supplemental Fig. 2B), or METase (40 U/L) plus its regular substrate l-methionine at 5 μmol/L (Supplemental Fig. 2C). In contrast, when METase (40 U/L) was added into the medium with SeMet (1.25, 2.5, or 5 μmol/L), the apoptotic cells could be detected and apoptosis was generally more apparent with increasing concentrations of SeMet (Supplemental Fig. 2D–F). To measure apoptotic cells, a Guava Nexin kit was used. The apoptotic cells were increased to 3.4-fold of the control (P < 0.001) in the cells treated with methylselenol generated by incubation with 5 μmol/L SeMet.

ERK1/2 signaling and c-Myc expression. Although total ERK1/2 protein expression was not affected, c-Myc expression was decreased to 57.9, 51.9, and 41.4% of the control, respectively, and the active phosphorylated ERK1/2 was decreased to 63.7, 49.8, and 30.9% of the control, respectively, in cells treated with methylselenol generated by incubating METase (40 U/L) with 1.25, 2.5, or 5 μmol/L SeMet (Table 2; Fig. 2).

Discussion

Our previous finding (11) on the inhibitory effect of methylselenol treatment on the migration and invasion of HT1080 tumor cells may explain in part the observation (9) that SeMet treatment of nude mice, bearing tumors cells expressing the METase transgene, significantly inhibits tumor growth and prolongs host survival. Thus, the study of the effects of methylselenol on the gene expression of tumorigenic signaling pathways in tumor cells will further our understanding of the functional roles of methylselenol in invasive cancer cells.

The present experimental setting is similar to our previous report, because the cell proliferation data compare favorably with previously published results (11). The concentrations of the substrate used to generate methylselenol were 1.25, 2.5, or 5 μmol/L SeMet. However, the actual methylselenol concentrations in cell culture media are likely to be much lower than micromolar, because methylselenol is a volatile compound that easily diffuses in the air in the flask and eventually the entire incubator. Compared with our previous data (11), we found that methylselenol at low concentrations had a stronger potential to induce cell cycle arrest than to cause apoptosis. We hypothesized that methylselenol would inhibit tumor cell invasion through the regulation of cancer signal transduction pathways. We used OligoGEArrays of Human Cancer Signal Transduction to profile the expression of 113 genes for studying the activation of 15 different signal transduction pathways involved in oncogenesis and these signaling pathways include mitogen-activated protein

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Gene expression in HT1080 cells incubated with methylselenol, generated by incubating 40 U/L METase with 2.5 μmol/L SeMet, for 16 h</th>
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<tbody>
<tr>
<td>GenBank no.</td>
<td>Gene name</td>
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<tr>
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<td>PPARG</td>
</tr>
<tr>
<td>NM_022470</td>
<td>WIG1</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 3 (array analysis) or 4 (RT-PCR analysis).
2 Asterisks indicate different from control (0 μmol/L SeMet): *P < 0.05; **P < 0.005.

Methylselenol and cancer signaling genes
kinase, wingless-type MMTV integration site family (WNT), Hedgehog, signal transducer and activator of transcription (STAT), stress (DNA damage, p53, hypoxia, heat shock, and p38/JNK), inflammation (Cox-2 and NFκB), survival (NFκB and PI3K/AKT), hormonal (estrogen and androgen), and antiproliferation (TGFβ). We found that methylselenol significantly altered the expression of 7 genes, which are related to the cell cycle and apoptosis (Table 1).

MMP promotes tumor growth, invasion, and metastasis, and our previous data have shown that methylselenol inhibits pro-MMP-2 activation (11). It has been documented that MMP inhibitors modulate the expression or function of proteins involved in the induction of cell cycle arrest and apoptosis (18,19). Thus, our observation that methylselenol altered the gene expression related to cell cycle arrest and apoptosis suggests that methylselenol may play a role similar to that of a MMP inhibitor.

Gene expression analysis showed that 4 genes were upregulated by methylselenol generated by incubating METase (40 U/L) with 2.5 μmol/L SeMet. The mRNA level of cyclin-dependent kinase inhibitor p57kip2 was 5.7-fold of the control. CDKN1C/p57kip2, a candidate tumor suppressor gene, has been implicated in the modulation of cell cycle control, differentiation, apoptosis, tumorigenesis, development, and other cellular events (20,21). Overexpression of p57kip2 in prostate cancer cells significantly suppressed cell proliferation and arrested the cell cycle at the G0-G1 stage by affecting the retinoblastoma protein pathway through CDK4/cyclin D1 and CDK2 complexes (22). This is consistent with the finding that methylselenol inhibited cell growth and induced G1 cell arrest in HT1080 cells (11). Second, the mRNA level of the stress protein HMOX1 was 2.8-fold of the control. HMOX1 can act as a protective enzyme, decreasing the risk of development of some kinds of tumors (23). Third, the mRNA level of the cell adhesion and signaling molecule, PECAM1, was 4.2-fold of the control. PECAM1, a 130-kDa transmembrane glycoprotein, has previously been reported to have cytoprotective effects and to suppress mitochondrial-dependent, Bax-mediated apoptosis (24–26). Fourth, the mRNA level of PPARγ was 3.1-fold of the control in cells treated with methylselenol. PPARγ activation by specific agonists leads to growth inhibition, apoptosis, and differentiation of tumor cells (27). These findings are consistent with the fact that methylselenol inhibited cell growth and induced apoptosis.

In contrast, 3 genes were downregulated by methylselenol. First, the mRNA level of BCL2A1 was 33% of the control. BCL2A1, a member of the BCL-2 protein family, is able to reduce the release of proapoptotic cytochrome c from mitochondria, block caspase activation, and inhibit apoptotic death (28,29). Thus, the reduction of BCL2A1 mRNA is in agreement with the fact that methylselenol induces apoptosis. Second, the mRNA level of HHIP, which encodes a regulatory protein of the hedgehog signaling pathway, was 26% of the control. However, the exact roles of HHIP in the context of tumors remain to be characterized (30–32). Third, p53 target zinc finger protein (Wigi) mRNA level was decreased to 52% of the control. Wigi encodes a p53 target gene that encodes a growth inhibitory zinc finger protein and both overexpression and silencing of Wigi can inhibit cell growth (33).

The above gene array data strongly indicated that cell cycle arrest and apoptosis were the most affected pathways among 15 important cancer signal pathways when HT1080 invasive tumor cells were exposed to methylselenol. These results lead us to think that cell cycle arrest and apoptosis are likely to play a key role in the inhibition of tumor cell invasion. This is consistent with the observation that methylselenol has been shown to block cell cycle progression, induce apoptosis, and regulate gene expression in prostate and mammary cancer cells (10,34,35). Of all methylselenol-responsive genes in the present study, CDKN1C/p57kip2 was the most upregulated gene. It was reported that CDKN1C/p57kip2 suppressed cell proliferation and arrested the cell cycle at the G0-G1 stage (22). Studies have shown that the ERK1/2 pathway is essential for cells to pass the G1 restriction point, and blockage of the ERK pathway induces upregulation of p27kip2, which is closely related to CDKN1C/p57kip2 (36,37). Furthermore, ERK1/2 signaling and induction of c-Myc are both necessary to drive cells from G0 to late G1 phase (38), which is entirely consistent with our present findings that methylselenol inhibited ERK1/2 signaling and c-Myc expression.

<table>
<thead>
<tr>
<th>Met (μmol/L)</th>
<th>Met (μmol/L)</th>
<th>METase (40 U/L)</th>
<th>Western blot intensity units</th>
<th>Total ERK1/2</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
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<td>503947 ± 34212</td>
<td>528214 ± 16975</td>
</tr>
<tr>
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<td>494548 ± 60163</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>+</td>
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<td>495013 ± 6452</td>
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<tr>
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<td>500706 ± 12345</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>+</td>
<td>208809 ± 71925</td>
<td>532377 ± 13082</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 3.
2 Asterisks indicate different from controls (no METase or SeMet). *P < 0.05, **P < 0.005.
The Se concentration of 10 μmol/L represents a physiologically achievable plasma level (39,40). Blood Se concentrations of free-living human populations worldwide range from 0.29 to 40.5 μmol/L, and that of a South Dakota population was 3.24 μmol/L (41). With high Se intake, the extra Se can enrich the methyleneselenol pool by the methylation pathway (2,7). Although no studies definitively determined the physiologic concentration of methyleneselenol, it has been hypothesized that the concentrations of methyleneselenol in a tumor are higher due to the faster blood-borne Se circulation (1). Therefore, the data on methyleneselenol-regulated genes may provide new molecular candidates for future study on methyleneselenol's anticancer action in humans. For example, it is known that high-Se yeast contains not only SeMet but also trace amounts of other Se forms (i.e., Se-methyleneselenocysteine, γ-glutamyl-Se-methyleneselenocysteine, Se-adenosyl selenohomocysteine) (4,42). These trace amounts of other Se forms may partly contribute to the success of high-Se yeast to prevent cancer in the NPC trial (4). Are there synergetic effects of SeMet and other Se forms on the expression of these methyleneselenol-regulated genes? These future studies may further our understanding of the results of the SELECT trial (43).

In summary, our data show that methyleneselenol induces G1-cell cycle arrest and apoptosis via multiple signaling pathways, which may play a key role in methyleneselenol-induced inhibition of cancer cell proliferation and tumor cell invasion.

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Literature Cited


