Consumption of a high-fat diet abrogates inhibitory effects of methylseleninic acid on spontaneous metastasis of Lewis lung carcinoma in mice

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We investigated the effect of dietary supplementation with selenium on spontaneous metastasis of Lewis lung carcinoma in mice fed a high-fat diet. Mice were fed a low-fat diet or that diet modified with 45% of calories from corn oil and supplemented with 0 or 2.5 mg selenium/4029 kcal as methylseleninic acid. After 6 weeks, mice were each injected 2.5 × 10⁶ Lewis lung carcinoma cells subcutaneously. The resulting primary tumor was removed surgically 10 days later; the experiment was terminated after an additional 10 days. High-fat feeding increased pulmonary metastases by 17% compared to the low-fat diet (P < 0.01). Selenium supplementation reduced the metastases by 11% compared to nonsupplemented controls (P < 0.05); the reduction was less for animals fed the high-fat diet (5%) than for those fed the low-fat diet (18%). Supplemental Se lowered plasma concentrations of prostates (urokinase plasminogen activator, P < 0.01; matrix metalloproteinase-9, P < 0.05) and angiogenic factors (vascular endothelial growth factor, P < 0.01; tissue inhibitor of metalloproteinase-1, P < 0.01) compared to nonsupplemented controls. High-fat feeding increased plasma concentrations of adipokines plasminogen activator inhibitor-1, monocyte chemotactic protein-1, tumor necrosis factor-α, and leptin regardless of the level of dietary selenium; supplemental selenium lowered plasma concentrations of plasminogen activator inhibitor-1 (P ≤ 0.05) and monocyte chemotactic protein-1 (P ≤ 0.05) in low-fat fed mice but not in high-fat fed mice. These results indicate that consumption of a high-fat diet abrogated the antimetastatic effects of selenium by increasing the expression of adipose-derived inflammatory cytokines.

Introduction

Selenium (Se) is an essential nutrient, being required for some 25 selenoenzymes (1). Epidemiological studies have shown nutritional Se status to be associated with lower cancer incidence and mortality (2), suggesting that Se may be chemopreventive. This hypothesis is supported by the results from hundreds of studies using animal primary tumor models (3–5). The clinical significance of Se in human cancer prevention has been tested in only a few clinical trials. The Selenium and Vitamin E Cancer Prevention Trial (SELECT) (6) found no reduction in prostate cancer risk by supplementation with Se as selenomethionine. However, because the baseline Se status of the SELECT cohort was comparable to those of the upper tertile (>120 ng/ml) in the Nutrition Prevention of Cancer trial (7) which found cancer risk reduction to be limited to subjects in the lower tertiles (8), the results of SELECT (6) are actually not inconsistent with the results of other Se-intervention trials (e.g., Nutrition Prevention of Cancer trial) (7) that indicate that Se can reduce cancer risk for at least some individuals, e.g., those of relatively low Se status. That many subjects in SELECT were overweight or obese raises the possibility that adiposity may also have contributed to those null findings.

The metabolic basis of the antitumorigenic activity of dietary Se is thought to involve the production of a methylated metabolite, methylselenol (9). In fact, Se-compounds that can serve as proximal precursors to methylselenol [e.g., methylseleninic acid (MSeA), methylselenocysteine] have been found to be more effective in inhibiting tumorigenesis than those metabolized through hydrogen selenide (e.g., selenomethionine, selenite) (10,11).

Few studies have addressed the effects of Se on metastatic cancer; yet those, too, have yielded positive results (12–14). That dietary supplementation with MSeA can reduce spontaneous metastasis of Lewis lung carcinoma (LLC) in mice while supplementation with selenomethionine is ineffective (12) suggests that methylselenol may also have antimetastatic activity. Such activity could be of great practical importance, as recurrent or metastatic cancer, the spread of malignant cells from a primary tumor to different sites of the same organ or to distant organs, directly affects the prognosis and survival of cancer patients.

It is possible that cancer metastasis may be enhanced by obesity, which is a leading risk factor for cancer (15) and is associated with poor prognosis and disease outcome in cancer patients (16,17). In fact, cancer patients who are obese are at a greater risk of recurrence (18,19) with shorter disease-free survival rates than nonobese patients (20). It has been shown enhanced metastasis of B16BL6 melanoma in ob/ob mice (21) and of LLC in C57BL/6 mice fed an obesogenic, high-fat diet (22), but there is no published information regarding whether obesity may affect the antimetastatic activity of dietary Se. Therefore, we conducted the following studies to test the hypothesis that obesity induced by high-fat feeding impairs the antimetastatic activity of Se.

Materials and methods

This investigation was approved by the Animal Care and Use Committee of the United States Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center. The procedures followed the guidelines of the National Institute of Health for the care and use of laboratory animals (23).

Animals and diets

Three-week-old male C57BL/6 mice (Harlan, Madison, WI) were maintained in a pathogen-free room on a 12:12-h light-dark cycle. The temperature of the room was at 22 ± 1°C. Mice were fed diets based on a modified AIN93G diet (24) with soybean oil being replaced with corn oil to provide 15% of energy (hereafter referred to as the low-fat diet) or increased to provide 45% of energy (hereafter referred to as the high-fat diet); each diet was supplemented with 0 or 2.5 mg Se/4029 kcal in the form of MSeA (Sigma-Aldrich, St. Louis, MO) (Table I). The gross energy of each diet (Table I) was determined by oxygen bomb calorimetry (Model 6200, Oxygen Bomb Calorimeter, Parr Instrument, Moline, IL).

LLC cells

LLC cells (provided by Dr. Pnina Brodt, McGill University, Montreal, Quebec, Canada) were cultured with RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere of 5% CO₂ in air at 37°C. Cells used for animal studies were in vitro-selected once (22); they were monitored for phenotype (by microscopic examination of cell morphology), proliferation properties (by growth curve analysis), and metastatic capability (by injecting cells subcutaneously into mice and examining metastatic formation in lungs). Cells were free of mycoplasma by Hoechst DNA staining and direct culture tests performed by ATCC (Manassas, VA). Such assessments showed that cell identity and metastatic behavior were similar to those of original stocks from the institution providing the cell line.

Experimental design

Mice (n = 30 per group) were fed their respective diets for 6 weeks before they were injected subcutaneously with 2.5 × 10⁶ viable LLC cells/mouse into the...
lower dorsal region. The resulting primary tumor was surgically removed 10 days later when it was approximately 1 cm in diameter. Subsequently, mice were maintained on their respective diets for an additional 10 days. Mice fed the low-fat diet but not injected with LLC cells were used as negative controls (n = 12) in comparing plasma concentrations of cytokines, proteases, and angiogenic factors with those low-fat fed mice injected with LLC cells. One week before LLC injection, the composition of fat and lean body mass of conscious, immobilized mice was analyzed using quantitative magnetic resonance (Echo whole-body composition analyzer, Model 100, Echo Medical System, Houston, TX). Food intake (n = 6 per group) was recorded 5 days per week for 3 weeks before the LLC injection; caloric intake was calculated using the gross energy of each diet.

**Primary endpoints**

At termination, mice were injected intraperitoneally with a mixture of ketamine and xylazine, and their lungs were harvested and fixed with Bouin’s solution (Sigma-Aldrich). The number of pulmonary metastases was counted and the cross-sectional area and the average diameter of each metastasis were measured using an ImagePro-Plus software- (Media Cybernetics, Silver Spring, MD) and camera-equipped stereomicroscope. Tumor cross-sectional area was defined as the area measurement that reports the surface area of each metastasis; average diameter was the average length of the diameters measured at two degree intervals joining two outline points and passing through the centroid. Tumor volume was estimated with the assumption that tumors were spherical and calculated using the average diameter measured (20). Plasma and liver were collected and stored at −80°C. Mice with surgically nonremovable primary tumors or mice with tumor recurrence after the surgery were excluded from the study.

**Secondary endpoints**

**Selenium analysis.** Samples of the diets and liver were digested with a mixture of nitric acid, hydrochloric acid, and magnesium nitrate. Digested samples were analyzed by hydride-generation on a Pinnacle 900T Atomic Absorption Spectrometer (Perkin Elmer Pinnacle 900T instrument, Perkin Elmer, Waltham, MA) equipped with automated hydride-generation flow injection system. Plasma Se was analyzed by Graphite Furnace Atomic Absorption on a Pinnacle 900T Atomic Absorption Spectrometer. Results were expressed as mg Se/kg for diet (dry weight; Table I), μmol Se/l for plasma and μmol Se/kg for liver (Table II).

**Cytokines, proteases, and angiogenic factors.** Sandwich enzyme-linked immunosorbent assay kits were used to quantify plasma concentrations of plasminogen activator inhibitor-1 (PAI-1, Molecular Innovations, Novi, MI), urokinase plasminogen activator (uPA, Cell Sciences, Canton, MA), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), leptin, vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinase-1 (TIMP-1), and matrix metalloproteinase-9 (MMP-9; all were from R&D Systems, Minneapolis, MN) following the manufacturers’ protocols. Samples were read within the linear range of the assay, and the accuracy of the analysis was confirmed by the controls provided in each kit.

**Results**

**Growth and body composition**

Mice fed the high-fat diet developed greater body weights than those fed the low-fat diet; the difference was significant (P ≤ 0.01) starting 2 weeks after the initiation of feeding (Figure 1). Selenium supplementation did not alter body weight gains compared to mice fed the low-fat diet (Table I).

**Table I.** Composition of experimental diets

<table>
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<tr>
<th>Ingredient</th>
<th>Low-fat</th>
<th></th>
<th>Low-fat + Se</th>
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<th>High-fat</th>
<th></th>
<th>High-fat + Se</th>
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<td>397.5</td>
<td>1590</td>
<td>33.5</td>
<td>134</td>
<td>33.5</td>
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<td>200</td>
<td>800</td>
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<td>Sucrose</td>
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<tr>
<td>Dextrin</td>
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<td>630</td>
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<td>39</td>
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<td>L-Cystine</td>
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<td>1000</td>
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<td>Se, mg/kg</td>
<td>0.15±0.02</td>
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<td>0.22±0.02</td>
<td>3.00±0.36</td>
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<tr>
<td>Gross energy, kcal/g</td>
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<td>Calculated composition</td>
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<td>Protein, %</td>
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<td>24.1</td>
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<td>Total 18:2n6, %</td>
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<td>4</td>
<td>13.8</td>
<td>13.8</td>
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<td></td>
<td></td>
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<tr>
<td>Total 18:3n3, %</td>
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<td>0.07</td>
<td>0.24</td>
<td>0.24</td>
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*Values are means ± SEM; n = 6 per group for Se analysis, and n = 3 per group for gross energy content.

Table II. Selenium concentrations of plasma and liver from mice fed the low-fat or high-fat diet with or without supplemental Se

<table>
<thead>
<tr>
<th>Se</th>
<th>Low-fat</th>
<th></th>
<th>Low-fat + Se</th>
<th></th>
<th>High-fat</th>
<th></th>
<th>High-fat + Se</th>
<th></th>
<th>Diet</th>
<th></th>
<th>Se</th>
<th></th>
<th>Diet × Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, μmol/l</td>
<td>3.90±0.24</td>
<td>5.72±0.58</td>
<td>4.67±0.24</td>
<td>5.45±0.70</td>
<td>0.61</td>
<td>&lt;0.05</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver, μmol/kg</td>
<td>61.29±4.30</td>
<td>113.09±10.47</td>
<td>84.55±6.43</td>
<td>111.63±5.81</td>
<td>0.14</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*Values are means ± SEM, n = 6 per group.
not affect body weights of low-fat fed mice; however, it was associated with lower body weights in high-fat fed mice (P ≤ 0.05) starting 5 weeks after the initiation of feeding (Figure 1). Mice fed the high-fat diet showed increased body fat mass (30%, P < 0.01; Figure 2A) and reduced relative lean mass (8%, P < 0.01; Figure 2B) compared to those fed the low-fat diet; however, because of their greater body size high-fat fed mice had 9% more lean mass weight (P < 0.01; Figure 2C) than the low-fat controls. Selenium supplementation to either diet did not affect body composition compared to nonsupplemented controls (Figure 2A–C). Mice fed the high-fat diet consumed 6% more total calories than those fed the low-fat diet (P < 0.01; Figure 2D); Se supplementation slightly reduced caloric intake by 4% compared to nonsupplemented controls (P = 0.06; Figure 2D). Supplemental Se increased the Se contents of plasma and liver by 30% (P < 0.05) and 50% (P < 0.01), respectively, compared to nonsupplemented controls (Table II). The level of dietary fat had no effects on plasma or liver Se (Table II).

Primary endpoints
Mice fed the high-fat diet showed 17% more metastases to the lungs than those fed the low-fat diet (P < 0.01; Figure 3A). Selenium s.c. cell injection surgery

Fig. 1. Body weights (n = 30 per group). Two-way ANOVA and Tukey contrasts were performed to test for differences among the groups. Mice fed the high-fat diet were heavier than those fed the low-fat diet, and the difference was significant starting 2 weeks after the initiation of experimental feeding (P < 0.01). Mice supplemented with Se showed lower body weights than controls when fed the high-fat diet; the difference became significant starting 5 weeks after the initiation of feeding (P ≤ 0.05). LF, low-fat diet; LF+Se, low-fat diet with Se supplementation; HF, high-fat diet; HF+Se, high-fat diet with Se supplementation.

Fig. 2. Fat mass:body mass (A), lean mass:body mass (B), lean mass weight (C), and caloric intake (D) of mice fed the low-fat or high-fat diet with or without supplemental Se. Two-way ANOVA was performed to test for differences among the groups. Values are means ± SEM (n = 18 per group for caloric intake). LF, low-fat diet; LF+Se, low-fat diet with Se supplementation; HF, high-fat diet; HF+Se, high-fat diet with Se supplementation.

Fig. 3. Number (A), cross-sectional area (B), and volume of pulmonary metastases (C) of mice fed the low-fat or high-fat diet with or without supplemental Se. Two-way ANOVA was performed to test for differences among the groups. Values are means ± SEM (n = 24–27 per group). LF, low-fat diet; LF+Se, low-fat diet with Se supplementation; HF, high-fat diet; HF+Se, high-fat diet with Se supplementation.
supplementation reduced the number of metastases by an average of 11% compared to nonsupplemented controls ($P < 0.05$; Figure 3A). This effect was greater for mice fed the low-fat diet (18% reduction) than for those fed the high-fat diet (5% reduction). Tumor cross-sectional area and volume were each 35% greater in mice fed the high-fat diet than those fed the low-fat diet ($P < 0.01$; Figure 3B and C); Se supplementation did not affect these two parameters compared to nonsupplemented controls (Figure 3B and C).

Secondary endpoints

The presence of LLC was associated with increases in plasma levels of PAI-1 (77%, $P < 0.01$; Figure 4A), MCP-1 (80%, $P < 0.01$; Figure 4B), and TNF-α (300%, $P < 0.01$; Figure 3C) compared to non–tumor-bearing mice. LLC-bearing mice fed the high-fat diet showed greater increases in plasma concentrations of PAI-1 (170%, $P < 0.01$; Figure 4A), MCP-1 (72%, $P < 0.01$; Figure 4B), and TNF-α (460%, $P < 0.01$; Figure 4C) compared to low-fat fed mice. Mice fed supplemental Se in the low-fat diet showed 31% lower plasma concentrations of both PAI-1 and MCP-1 ($P < 0.05$; Figure 4A and B) compared to nonsupplemented controls; however, supplementation of Se to the high-fat diet had no significant effects on plasma level of any of those cytokines (Figure 4A–C).

Plasma leptin concentration was not affected by the presence of LLC or by dietary Se level (Figure 4D), but it was increased by high-fat feeding (260%, $P < 0.01$; Figure 4D) compared to those fed the low-fat diet.

LLC-bearing mice showed greater plasma concentrations of uPA (1400%, $P < 0.01$; Figure 5A) and MMP-9 (82%, $P < 0.01$; Figure 5B) compared to non–tumor-bearing controls. In LLC-bearing mice, high-fat feeding increased plasma uPA (94%, $P < 0.05$; Figure 5A) and MMP-9 (36%, $P < 0.01$; Figure 5B) compared to low-fat fed controls. Selenium supplementation reduced plasma uPA to the level of non–tumor-bearing controls in mice fed either diet ($P < 0.05$; Figure 5A) and reduced MMP-9 by 20% ($P < 0.05$; Figure 5B) compared to nonsupplemented controls.

The presence of LLC increased plasma concentrations of VEGF (64%, $P < 0.01$; Figure 5C) and TIMP-1 (61%, $P < 0.01$; Figure 5D) compared to non–tumor-bearing mice. High-fat feeding increased plasma levels of both VEGF (39%, $P < 0.01$; Figure 5C) and TIMP-1 (43%, $P < 0.01$; Figure 5D) compared to the low-fat diet; supplemental Se reduced both VEGF (20%, $P < 0.01$; Figure 5C) and TIMP-1 (17%, $P < 0.01$; Figure 5D) compared to their respective nonsupplemented controls.

Discussion

Results from the present study are consistent with our previous report (12) that dietary supplementation with Se as MSeA reduces spontaneous metastasis of LLC. Further, they show that feeding an obesigenic, high-fat diet enhances metastasis and diminishes protective effects of Se.

The form of Se used in the present study, MSeA, is a proximal precursor to methylselenol, the metabolite thought to be inhibitory of primary carcinogenesis (10,11). Because methylselenol can be demethylated to selenide, the obligate intermediate for selenoprotein biosynthesis (27), it can support selenoprotein expression. Unlike selenomethionine, the major food form of Se, it does so without being diverted by nonspecific incorporation into other proteins; this is evidenced by the minimal increases in plasma Se concentrations observed in response to dietary MSeA supplementation. These results demonstrate that MSeA can be an effective chemopreventive or therapeutic agent to reduce the risk of metastatic cancer; further experiments are needed to establish the effective doses in human trials. These results also suggest that the form of Se chosen for SELECT, selenomethionine, may explain, at least partly, those observed null results (6).

Metastasis is a multistep process; its inhibition can occur through effects on dissemination from the primary tumor, intravasation into...
the circulation, arrest in a distant vascular bed, extravasation into the interstitium of a target organ, and proliferation to form metastases in that organ. That Se reduced the number of lung metastases but not their size suggests a role of Se early in the metastatic process of malignant progression.

We found that supplemental Se reduced plasma levels of uPA and MMP-9, two proteases involved in extracellular matrix degradation (28,29), and that reduction was not affected by high-fat feeding, suggesting ways by which Se may inhibit cancer invasion. Elevated plasma uPA and MMP-9 levels in high-fat fed mice indicate the involvement of adiposity in their production, which is suggested by findings that 3T3 preadipocytes can produce uPA (30) and MMP-9 (31) during adipose differentiation.

Selenium would appear to affect angiogenesis by reducing the production of angiogenic factors during LLC aggression. Such reductions were observed in the present study and have been reported previously with other angiogenic factors including fibroblast growth factor basic and platelet-derived growth factor-BB in LLC-bearing mice (12). That these reductions by Se were not affected by high-fat feeding, suggests that the impairment of Se-antimetastasis does not occur at the production of angiogenic factors. In fact, high-fat feeding increased plasma VEGF and TIMP-1 in LLC-bearing mice.

Adipose tissue has been considered an endocrine organ that produces adipokines (adipose-derived inflammatory cytokines), and their production is proportional to adiposity. Consumption of a high-fat diet increased adiposity and plasma levels of PAI-1, MCP-1, and TNF-α in non–tumor-bearing C57BL/6 mice (32), and obesity-enhanced metastasis is known to be accompanied by increases in plasma PAI-1 and MCP-1 (22). That high-fat feeding increased plasma concentrations of PAI-1, MCP-1, TNF-α, and leptin regardless of Se supplementation and that Se reduced plasma proteolytic proteases (uPA, MMP-9) and angiogenic factors (VEGF, TIMP-1) regardless of the type of diet suggest that adipose-derived inflammatory cytokines may be key determinants of the enhanced LLC aggression by high-fat feeding, which may overpower the inhibitory actions of Se on metastasis. This would support the possibility that any antitumorigenic activity of Se may be diminished in obese individuals.

The LLC-associated increases in plasma levels of inflammatory cytokines (PAI-1, MCP-1, TNF-α), proteases (uPA, MMP-9), and angiogenic factors (VEGF, TIMP-1) demonstrate the aggressiveness of this carcinoma model. In clinical practice, high expressions of PAI-1 (33,34), MCP-1 (35,36), TNF-α (37), uPA (34), MMP-9 (29,38), VEGF (38), and TIMP-1 (39) have been associated with regional or distant metastasis and poor prognosis in cancer patients. Elevations of those cytokines (40–42), proteases (43,44), and angiogenic markers (22,45) have been reported in animal models of various malignancies. The present results are consistent with the existing knowledge and prove the usefulness of the LLC model to study spontaneous metastasis.

Significant increases in the number and size of lung metastases by the high-fat diet and significant reduction in pulmonary metastases by Se supplementation indicate that this model is useful to study dietary modification and metastasis. The lack of inhibition of Se on tumor size was consistent with our previous report that Se results in a marginal, but not significant, reduction in lung metastasis size (12). Previous studies showed that Se reduces the size of experimentally induced primary tumors (5,46), indicating it is an action on initiation, promotion, or progression. Clearly, the role of Se on metastatic growth warrants further investigation.

It is likely that the lower caloric intake of mice fed the Se-supplemented high-fat diet caused the lower body weights of that group. However, that effect is unlikely to be related to the reduced inhibition of Se-antimetastasis because metastatic inhibition was observed in low-fat fed Se-supplemented mice that had similar caloric intakes. Further, the lower body weights of those mice did not result in significant changes in body composition or changes in plasma concentrations of adipokines.

We used corn oil as the source of dietary fat, which is comprised of 57% linoleic acid (18:2n6) (25) and providing 25% of total energy in our high-fat diet. This diet was low (0.5% of dietary energy) in α-linolenic acid (18:3n3), which comprises 1% of fatty acids in corn oil (25). It is possible that this fatty acid imbalance (47) may have contributed to the effects observed for the high-fat diet, as α-linolenic acid has been suggested to be anticarcinogenic (48,49). This possibility warrants further investigation.

In summary, the present study demonstrated that consumption of a high-fat diet abrogated anti-metastatic effects of Se, and that this effect is likely to involve adipose-derived inflammatory cytokines. Considering the prevalence of overweight and obesity in the USA (50), this finding may have relevance to achieving the best outcomes of chemoprevention, including that involving Se.

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