EFFECTS OF SODIUM SELENITE SUPPLEMENTATION ON PRE β-HIGH-DENSITY LIPOPROTEIN FORMATION-RELATED PROTEINS IN HUMAN PRIMARY HEPATOCYTES

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ABSTRACT

The effects of selenium on preβ-HDL formation were observed in a human primary hepatocyte (Hc cells) under a basal state condition, to represent the human liver in healthy conditions. The Hc cells were cultured in a medium supplemented with 0–10 µM sodium selenite. The effects of sodium selenite supplementation on several target proteins and genes related to preβ-HDL formation were measured by western blot analysis and real-time PCR, respectively. Protein expressions of GPx-1 and apolipoprotein A-I (apoA-I) were upregulated after treatment with sodium selenite. These results were confirmed by increased mRNA expressions of these two genes. The optimum effects of sodium selenite supplementation on protein and mRNA expressions of apoA-I and GPx-1 occurred at 50 nM; however, higher concentrations reduced the effect. In contrast, the expression levels of other preβ-HDL formation-related proteins and mRNA, apolipoprotein A-II (apoA-II) and ATP-binding cassette transporter-1 (ABCA-1), were not significantly affected. These results suggest that sodium selenite supplementation might play a role in preβ-HDL formation in Hc cells under basal state at low doses but not at high doses. Thus, an appropriate dose of selenium supplementation is essential for achieving the therapeutic potential of selenium supplementation for preventing CVD events in healthy individuals.

Keywords: Cardiovascular disease, Glutathione peroxidase, High-density lipoprotein, Selenium

INTRODUCTION

Selenium is an essential trace element to human health, and it is naturally found in grains, cereal, and meat. Selenium deficiency is related to several disorders, including cardiovascular disease (CVD)(Brenneisen et al., 2005). We have previously demonstrated that selenium supplementation (organic forms, inorganic forms, and selenium-enriched foods) can induce apoptosis in cancer cells, suggesting that supplementation is beneficial for prevention and treatment of cancer (Abdulah et al., 2005, 2009). Glutathione peroxidase-1 (GPx-1), a selenium-dependent enzyme, acts as a major intracellular antioxidant by reducing hydrogen peroxide and lipid peroxides; further, it also acts as a peroxynitrite reductase (Blankenberg et al., 2003). A deficiency in GPx-1 activity is associated with the development of cardiovascular events and adverse prognoses in patients with CVD (Blankenberg et al., 2003).

The incidence of CVD remains one of the highest among non-communicable diseases, and CVD is the highest cause of mortality worldwide (WHO, 2011). In a recent report, a low serum concentration of high-density lipoprotein (HDL) was found to be a significant and independent risk factor for CVD (van der Steeg et al., 2008). Apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II) are the major proteins comprising HDL; therefore, apoA levels represent HDL cholesterol levels (Ferrier and Denise, 2011; Kontush and Chapman, 2012). ApoA-I is mainly produced in the liver and small intestine and then secreted into the bloodstream. After acquiring phospholipids and free cholesterol mediated by hepatic and intestinal ATP-binding cassette transporter A1 (ABCA-1), it then becomes nascent preβ-HDL (Ferrier and Denise, 2011; Kontush and Chapman, 2012). Additional phospholipids and free cholesterol from...
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The selenium concentration in the blood is positively correlated with a lower atherogenic index, including higher HDL concentration or apolipoprotein levels (Koyama et al., 1995; Miyazaki et al., 2002; Lacaulstra et al., 2010; Stranges et al., 2011). An in vitro study demonstrated that selenium supplementation increased apoA-I expression in hepatoma cells cultured under lipoprotein saccharide-induced oxidative stress (Stahle et al., 2009). Selenium exists in various chemical forms, and the physiological effects of the different chemical forms vary considerably. Sodium selenite, an inorganic selenium form, is used as a supplement; recently, it is being increasingly consumed by healthy people to delay the onset or progression of age-related degenerative diseases, including CVD (Actis-Goretta et al., 2013). Colpo and colleagues (Colpo et al., 2013) found an increase in the HDL concentration in healthy persons after consuming Brazilian nuts, one of the richest food sources of selenium. In an experimental study, the HDL concentration was increased in male Sprague-Dawley rats fed with a high fat diet containing sodium selenite (Kaur et al., 2009). In contrast, another study showed no beneficial effects of selenium supplementation against CVD (Rees et al., 2013). Therefore, the efficacy of sodium selenite supplementation for preventing CVD remains inconclusive.

Selenium may increase HDL production by inducing the formation of preβ-HDL-related proteins, such as apoA-I, apoA-II, and ABCA-1, through GPx-1 activity. Nuclear factor κB (NF-κB) is a major transcription factor that controls the expression of various genes, and it is primarily involved in immune, inflammatory, and stress responses (Panicker et al., 2010). When the NF-κB signaling cascade is suppressed, apoA and ABCA-1 expression is facilitated (Wang et al., 2006; Stahle et al., 2009). GPx-1 can induce the formation of preβ-HDL-related proteins through several possible mechanisms. First, GPx-1 delays IκB (inhibitor κB) degradation by inhibiting the phosphorylation of IκB (Kretz-Remy and Arrigo, 2001), thereby suppressing the NF-κB cascade. Second, GPx-1 inhibits the production of reactive oxygen species, which activate NF-κB transcription (Makropoulos et al., 1996). Third, GPx-1 directly inhibits the binding of p65 and the transcription factor PPARα, leading to increased availability of PPARα for the transcription of preβ-HDL-related genes (Stahle et al., 2009).

In the present study, we observed the effects of selenium supplementation on the expression of preβ-HDL-related proteins and mRNA in human primary hepatocytes (Hc cells) under basal state conditions. We used non-malignant cells for a better representation of the human liver in healthy conditions, to mimic healthy individuals receiving selenium supplementation in an invivo system. We hypothesized that selenium supplementation would induce GPx-1 expression, which would upregulate apoA and ABCA-1 expressions, suggesting the induction of preβ-HDL formation in the normal human liver.

MATERIALS AND METHODS

CELLCULTURE

The Hc cells were kindly provided by Prof. Takeaki Nagamine, who purchased them from the Applied Cell Biology Research Institute (Kirkland, WA). The cells were maintained as described in a previous study (Hayakawa and Nagamine, 2014) and incubated on dishes coated with type I collagen. For the experiments, cells were seeded at a concentration of 1.5 × 10⁶ cells per 100-mm dish and incubated for 0–72 h. Hc cells were cultured under the same conditions for the selenium-supplemented groups, except that sodium selenite was added to the medium at the specified concentrations.

Before each treatment, we measured the selenium concentration in FBS and determined it to be 257.65 nM. Therefore, the selenium concentration of the culture medium with 10% FBS was 25.76 nM. The concentrations of selenium in the experiments are the supplemented concentrations, in addition to the selenium contained in the FBS; therefore, the culture medium of the control group (0 nM) contained 25.76 nM of selenium from the FBS.

SAMPLE PREPARATION FOR GPX-1 ACTIVITY ASSAY AND WESTERN BLOT ANALYSIS

After incubation in various concentrations of sodium selenite (0, 25, 50, 100, and 200 nM) for 72 h, cells were washed twice with phosphate-buffered saline and harvested; proteins were then extracted using RIPA buffer (Sigma, St. Louis, USA) with 10% protein inhibitor (Sigma, St. Louis, USA). Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad, Tokyo, Japan), following the method described by Lowry (Lowry et al., 1951). The extracted sample was stored at -80°C until the GPx-1 activity assay and western blot analysis were performed.

DETERMINATION OF TOTAL SELENIUM CONCENTRATION

Before treatment with sodium selenite, concentrations of selenium in FBS were measured using a method described previously (Watkinson, 1966). Briefly, a 0.1-mL sample was digested in a heating block with 2 mL acid mixture (nitric acid/perchloric acid at 2:1). The temperature was gradually raised from 50°C to 190°C, and the sample was incubated overnight. Selenate was reduced to selenite using 0.5 mL of 10 N hydrochloric acid at 150°C for 20 min. The selenium concentration was calculated based on fluorometric measurements at an excitation wavelength of 378 nm and an emission wavelength of 525 nm to determine the concentration of piazselenol, which is produced by the reaction of selenite with 2,3-diaminonaphthalene. The accuracy of this analysis was monitored by the measurement of bovine liver SRM 1577b as the reference material (National Institute of Standards and Technology, Gaithersburg, MD).

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Institute of Standards and Technology, MD, USA).

**MTT ASSAY**

Selenium has a very narrow therapeutic dose window (Rayman, 2008); therefore, we confirmed the selenium dose and associated cell viability using a methyl thiazolyltetrazolium assay (MTT) assay, as described previously (Faried et al., 2006). Cell proliferation inhibition analysis was performed in cells in the presence of various concentrations of sodium selenite and different incubation times. Briefly, cells (2 × 10⁴ in 50 μL/well) were plated in 96-well plates. After the initial cell seeding, sodium selenite was added in a concentration range of 0 to 10 μM, and cells were incubated for 24, 48, and 72 h. Then, 10 μL of WST-8 assay cell-counting solution (Dojindo Lab., Tokyo, Japan) was added to each well, and cells were then incubated at 37°C for 3 h. After the addition of 100 μL/well of 1 N HCl, the cell proliferation rate was then determined by measuring the absorbance at a wavelength of 450 nm with a reference wavelength of 650 nm. The absorbance was read using a micro-titer plate reader (Becton-Dickinson, NJ, USA). The overall results were derived from four experiments.

**GPx-1 ACTIVITY ASSAY**

Based on a previous study (Hoefig et al., 2011), we used 72 h as the optimal incubation time for sodium selenite supplementation. The enzymatic activity of GPx-1 was determined from HepG2 homogenates using the method described by Paglia and Valentine (1967). Briefly, the activity was indirectly monitored using spectrophotometric methods by observing the reduction of oxized glutathione to reduced glutathione using nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agent. GPx-1 activity was quantified by measuring the change in NADPH absorbance at 340 nm and expressed as the change in NADPH (Δ mM NADPH) during the unit time (min) and mg protein in the presence of the substrate tert-Butyl hydroperoxide.

**WESTERN BLOTS**

Proteins (30 μg and 40 μg for ABCA-1) were electrophoresed on 5–20% polyacrylamide ready-made gels (Bio-Rad) and transferred onto a polyvinylidenefluoride membrane (Millipore, Massachusetts, USA). HDL formation-related proteins were analyzed using the following antibodies: rabbit polyclonal anti-apoA-I (ab33470; Abcam, Cambridge, MA) at 1:200 dilution, goat polyclonal anti-apoA-II (178464; Calbiochem, Darmstadt, Germany) at 1:1000 dilution, rabbit polyclonal anti-GPx-1 (ab22604; Abcam) at 1:500 dilution, and rabbit polyclonal anti-ABCA-1 (NB400-105; Novus Biological, Littleton, USA) at 1:1000 dilution. Mouse monoclonal anti-GAPDH (MAB374; Abcam) antibody at 1:500 dilution was used as the loading control. Secondary antibodies, namely donkey anti-rabbit IgG (NA934; Amersham, Buckinghamshire, UK), rabbit anti-goat IgG (ab7132; Abcam), or rabbit anti-mouse IgG (ab6728; Abcam), linked to horseradish peroxidase were then applied, and immunoreactive bands were visualized using the prime chemiluminescence assay (ECL; Amersham). Scanning densitometry was performed by Image Quant LAS 4000 (Amersham), and autoradiographs were quantified using the National Institute of Health’s ImageJ software program.

**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS**

After incubation in various concentrations of sodium selenite (0, 25, 50, 100, and 200 nM) for 72 h, total RNA was isolated from HepG2 cells using TRIzol reagent (Invitrogen, CA, USA). RNA was prepared by reverse transcription using oligo-dT and dNTP, and each sample was processed with the RT-PCR kit (TAKARA, Japan). Quantitative real-time-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) according to the manufacturer’s instructions, and then evaluated using the LightCycler 480 Real-Time PCR system (Roche, CA, USA). The expression level of the target gene was normalized against GAPDH mRNA levels. The sequences of primers for quantitative real-time PCR used in this study are listed in Table 1.

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Table 1 - Primers for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Human Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>hABC-A1</td>
<td>GAACCTGGCTGTTTCCATGAT</td>
<td>GATGAGCCAGACTCTTGTG</td>
</tr>
<tr>
<td>hApo-A1</td>
<td>GCCCTGGAGAAACAGCTAAACC</td>
<td>CCAGAAGCTGCTGGGTCA</td>
</tr>
<tr>
<td>hApo-A2</td>
<td>CAAGAAGGCTGGAACACCGAAC</td>
<td>CTGGGTTGGAAGACAATG</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>ACCACATCCATGCCATACCA</td>
<td>TCCACACACCTGTGTGTCA</td>
</tr>
<tr>
<td>hGPx-1</td>
<td>CGCCAAGAAACGAAAGAGATTC</td>
<td>TGAGTGTCATAGGTTCAG</td>
</tr>
</tbody>
</table>

hABC-A1, human ATP-binding cassette transporter-1; hApo-A1, human apolipoprotein A-I; hApo-A2, human apolipoprotein A-II; hGAPDH, human glyceraldehyde-3-phosphatase dehydrogenase; hGPx-1, glutathione peroxidase-1.

**STATISTICAL ANALYSIS**

Statistical analysis was performed using 1-way analysis of variance with Dunnett’s post-hoc multiple comparison tests to compare between “no treatment” as the control group and each experimental group. A p-value < 0.05 was considered statistically significant. The statistical analysis of the data was performed with IBMSPSS (version 20.0 for Windows, IBM, NY, USA).

**RESULTS**

**LOW-DOSE SODIUM SELENITE SUPPLEMENTATION DID NOT INHIBIT CELL PROLIFERATION**

The HepG2 cells showed no inhibition of cell proliferation after 72-h incubation with low doses of sodium selenite; however, inhibition of cell proliferation...
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Began to increase (≥17.3%) at a concentration of 2.5 μM (Figure 1) and reached IC50 at 5μM (Table 2).

Figure 1. Effects of sodium selenite supplementation on inhibition of cell proliferation
Data are presented as mean ± SE and expressed as a percentage (%). The x-axis is presented in logarithmic scale. The results were derived from four experiments (n = 8/group).

Table 2. Summary of the time-resolved IC50 values of sodium selenite determined by MTT assay in Hc cells

<table>
<thead>
<tr>
<th>Incubation times (h)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>48</td>
<td>7.0</td>
</tr>
<tr>
<td>72</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Hc, human primary hepatocytes; IC50, half-maximal inhibitory concentration; MTT assay, methyl thiazolyltetrazolium assay.

SODIUM SELENITE INCREASED GPx-1 ACTIVITY IN Hc CELLS
GPx-1 activity was measured in Hc cells after treatment with 0–200 nM sodium selenite (Figure 2). Sodium selenite supplementation increased GPx-1 activity significantly; this reached saturation levels at a concentration of 50 nM. Thus, GPx-1 activity was dependent on the presence of exogenous sodium selenite.

Figure 2. Effects of sodium selenite supplementation on GPx-1 activity
Data are presented as mean ± SE, n = 5/group. **: p < 0.01 vs. control

EFFECTS OF SODIUM SELENITE SUPPLEMENTATION ON THE EXPRESSION OF PREβ-HDL FORMATION-RELATED TARGET GENES
The mRNA expressions of GPx-1 and several target genes related with preβ-HDL formation (apoA-I, apoA-II, and ABCA-1) were analyzed by real-time PCR (Figure 4). After supplementation with sodium selenite, a significant increase in the mRNA expression of GPx-1 (p<0.01 at 50, 100, and 200 nM) was observed. Similarly, an upregulation in apoA-I mRNA expression was observed at 50 nM concentrations (p<0.05). In contrast, no significant effect of sodium selenite supplementation was noted for ABCA-1 or apoA-II expression.
Results show that sodium selenite supplementation significantly increased the protein and mRNA expressions of apoA-I, suggesting that selenium induced apoA-I expression from the transcription level. Interestingly, the peak of protein and mRNA expressions of GPx-1 and ApoA-I were reached at the same dose of sodium selenite (50 nM). Furthermore, ABCA-1 also showed a trend towards increased expression, although this was not significant. Third, selenium supplementation in the form of sodium selenite did not enhance apoA-I protein or mRNA expression.

In the present study, we used sodium selenite to induce GPx-1 activity and the expressions of related proteins and mRNA. In agreement with previous studies, we demonstrated that sodium selenite increased GPx-1 activity in Hc cells after 72-h incubation, with a dose saturation at 50 nM (Lei et al., 1995; Hoefig et al., 2011). Our results suggested the presence of a time lag between the addition of sodium selenite and the induction of GPx-1 synthesis. Furthermore, supplementation with selenium in the form of sodium selenite to normal hepatocyte cells increased the activities as well as the protein and mRNA expressions of GPx-1, thus protecting cells from potential damage by reactive oxygen species. Sodium selenite, an inorganic form of selenium, is well established as a dietary supplement, and its consumption has increased over the past few years (Rayman, 2008). In healthy people, all dietary requirements can be fulfilled solely from food sources; however, a recent study has reported that the enhancement of certain nutrients, including selenium, is in the tissue relevant (Monsen, 2000). In addition, antioxidant supplementation is recommended for the prevention of oxidative damage in patients with chronic diseases (Actis-Goreta et al., 2004).

Our study showed that sodium selenite supplementation in low doses (<0.31 μM) did not inhibit Hc cell proliferation after 72-h incubation. However, the proliferation of Hc cells was prominently inhibited at sodium selenite concentrations over 2.5 μM. While selenium deficiency has been associated with several disorders, excessive selenium intake can cause selenium toxicity and even death (Rayman, 2008). It is therefore important to be aware of the possible implications of selenium supplementation in healthy people, especially for the prevention of CVD, as it can be beneficial, ineffectual, or even harmful for the human body, depending on the dosage.

In several clinical studies, supplementary selenium intake has been shown to have a positive correlation with HDL concentrations, leading to a beneficial effect in the prevention of CVD (Laclaustra et al., 2010; Stranges et al., 2011). Our study demonstrated that sodium selenite supplementation significantly increased the protein and mRNA expressions of apoA-I. Moreover, an upregulation trend in ABCA-1 protein expression was noted, although this was not statistically significant. These results suggest that selenium supplementation has an effect on preβ-HDL formation, inducing apoA-I expression from the transcription process.

Furthermore, selenium supplementation enhanced GPx-1 and apoA-I expressions in a concentration-dependent manner in Hc cells, with both expressions reaching their peaks at 50 nM. This dose was lower than the value reported in a previous study (Stahle et al. 2009) using a human hepatoma cell line (hepG2), where the GPx-1 activity reached saturation at 100 nM of sodium selenite supplementation. Wilkening et al. (2003) concluded that hepatocytes are the most preferred in vitro models for assessing biotransformation in the human liver and for identifying compounds that are potentially toxic to humans. Considering that selenium has a very narrow therapeutic dose and that excessive selenium consumption can bring on toxicity and even death (Rayman, 2008), the exact concentrations of the supplemented dose of selenium and the type of cells used for examining the effects of selenium supplementation should be carefully considered.

Surprisingly, sodium selenite supplementation did not significantly affect apoA-II expression. This unexpected result suggests that this protein and gene remain need stronger transcriptional inducers, such as oxidative stress. HDL concentrations depend on the following three major factors as follows: (1) production of apoA-I, apoA-II, and ABCA-1 by the liver; (2) the maturation of HDL; and (3) peripheral lipid docking to the liver (Panicker et al., 2010). Our study merely focused on the first factor; therefore, the results do not incorporate all aspects of HDL production. Further in vivo studies are necessary to explore other potential roles of selenium in HDL formation.
CONCLUSION

In Hc cells that represent normal human liver cells, only a low dose of selenium is required to enhance the expressions of GPx-1 and apoA-I, a major component of preβ-HDL. In fact, higher doses of sodium selenite supplementation did not increase these upregulatory effects. Our findings suggest that selenium supplementation would be beneficial in preventing CVD events in healthy humans only when administered at the appropriatedose.

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