IJFANS
International Journal of Food
And Nutritional Sciences

Volume 3, Issue 6, Oct-Dec 2014, www.ijfans.com e-ISSN: 2320-7876

INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES

IMPACT FACTOR ~ 1.021

Official Journal of IIFANS

INTERNATIONAL JOURNAL OF FOOD
AND NUTRITIONAL SCIENCES

e-ISSN 2320 –7876 *www.ijfans.com*

Vol.3, Iss.6, Oct-Dec 2014

Research Paper ***Open Access***

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

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Received on: 26th October, 2014 **Accepted on: 6th December, 2014**

ABSTRACT

The effects of selenium onpreβ-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 pre β-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 forachieving 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 anessential trace element to human health, and it isnaturally found in grains, cereal, and meat. Selenium deficiencyis 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 peroxideand 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 thehighest 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 isthen secreted into the blood stream.After acquiring phospholipids and free cholesterol mediated by hepatic and intestinal ATP-binding cassette transporter A1 (ABCA-1),it then becomes nascentpre β-HDL (Ferrier and Denise, 2011; Kontush and Chapman, 2012).Additional phospholipids and free cholesterol from extrahepatic tissue are then necessary for the transformation to mature HDL particles (Kontush and Chapman, 2012).

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; Laclaustra *et al*., 2010; Stranges *et al*., 2011).An *in vitro* study demonstrated that selenium supplementation increased apoA-I expressionin 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 asupplement;recently, it is being increasingly consumed by healthy peopleto delay the onset or progression ofage-relateddegenerative diseases, including CVD(Actis-Goretta *et al*., 2004).Colpo and colleagues(Colpo *et al*., 2013) found an increase in the HDL concentrationin healthy persons after consumingBrazilian nuts,one of the richestfood 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 and Bansal, 2009). In contrast,anotherstudyshowed no beneficial effects of selenium supplementation against CVD(Rees *et al*., 2013).Therefore, the efficacy of sodium selenitesupplementation for preventing CVDremains 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 I-κB(inhibitor κB) degradation by inhibiting the phosphorylation of I-κB (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 inhibitsthe binding of p65 and the transcription factor PPARα, leading to increased availability of PPARα for the transcription of pre-βHDL-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 (Hccells) 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 inan *invitro* system. We hypothesized that selenium supplementation would induce GPx-1expression, which would upregulateapoA 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 ondishes coated with type I collagen. For the experiments, cells were seeded at a concentration of 1.5×10^6 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, 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 × 10^4in 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, 10μL 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 Hccell homogenates using the method described by Pagliaand Valentine (1967). Briefly, the activity was indirectly monitored using spectrophotometric methods by observing the reduction of oxidized 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 (∆ 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 polyvinylidenedifluoride 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 polyclonalanti-ABCA-1 (NB400-105; Novus Biological, Littleton, USA) at 1:1000 dilution. Mouse monoclonal anti-GAPDH (MAB374; Abcam) antibody at 1:1000 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 Hc cells using TRIzol reagent (Invitrogen, CA, USA). RNA was prepared by reverse transcription usingoligo-dT and dNTP, and each sample was processed withthe 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 thenevaluated 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.

Table 1- Primers for quantitative real-time PCR

	Forward	Reverse
hABCA-1	GAACTGGCTGTGTTCCATGAT	GATGAGCCAGACTTCTGTTGC
hApoA-I	GCCTTGGGAAAACAGCTAAACC	CCAGAACTGCTGGGTCACA
hApoA-II	CAAGAAGGCTGGAACGGAAC	CTGGGGTTGGAAGACAATGG
hGAPDH	ACCACATCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
hGPx-1	CGCCAAGAACGAAGAGATTC	TCGATGTCAATGGTCTGGAA

hABCA-1, human ATP-binding cassette transporter-1; hApoA-I, human apolipoprotein A-I; hApoA-II, 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 Hc cells showed no inhibition of cell proliferation after 72-hincubation withlow doses of sodium selenite; however, inhibition of cellproliferation began to increase (≥17.3%) at a concentration of 2.5 µM (Figure1) and reachedIC$_{50}$at 5µM (Table 2).

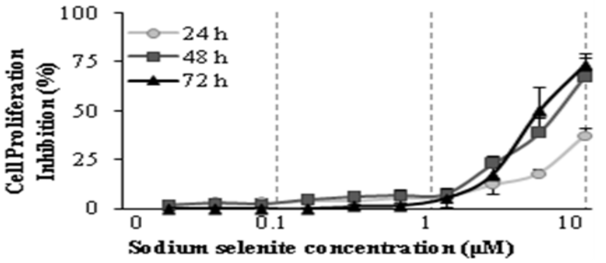

Figure 1. Effects of sodium selenite supplementation on inhibition of cell proliferation
Data are presented as mean ± SE and expressed as a percentage (%). The x-axis is presented in logarithmic scale. The results were derived from four experiments (n = 8/group).

Table 2. Summary of the time-resolved IC$_{50}$ values of sodium selenite determined by MTT assay in Hc cells

Incubation times (h)	IC$_{50}$ (µM)
24	-
48	7.0
72	5.0

Hc, human primary hepatocytes; IC$_{50}$, half-maximal inhibitory concentration; MTT assay, methyl thiazolyltetrazolium assay.

SODIUM SELENITE INCREASED GPx-1 ACTIVITYIN 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 onthe presence of exogenoussodium selenite.

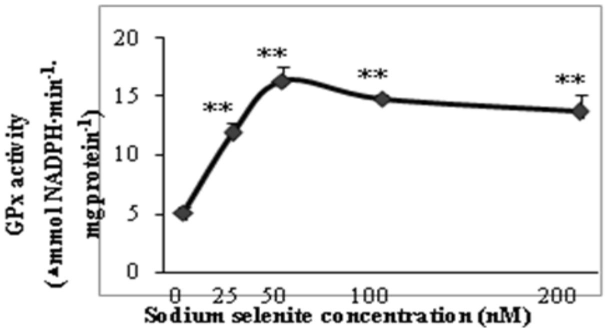

Figure 2- Effects of sodium selenite supplementation on GPx-1 activity
Data are presented as mean ± SE, n = 5/group. **: *p* < 0.01 vs. control

EFFECTS OF SODIUM SELENITE SUPPLEMENTATION ON PREβ-HDL FORMATION-RELATED PROTEINS

Next, we observed the effects of sodium selenitesupplementation on the expression of GPx-1 and preβ-HDL formation-related proteins (apoA-I, apoA-II, and ABCA-1) in a dose range of 0 to 200 nM by western blotting and subsequent quantification by Image J (Figure 3). Sodium selenite supplementation significantly increased the protein expression of GPx-1(p<0.01 at 50nM, and p<0.05 at 100 nM)and apoA-I(p<0.05 at 50nM).These two protein expressions peakedat a dose of 50 nM. The expression of ABCA-1also showed a tendency to increase although this was not significant. In contrast, selenium supplementation had no effects on apoA-II expression.

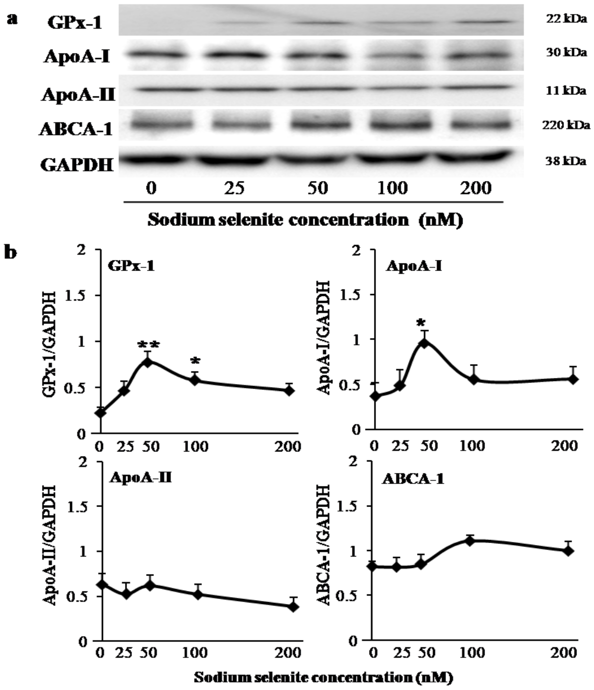

Figure 3. (a) Western blot analyses of GPx-1, apoA-I, apoA-II, and ABCA-1 of Hc cells after treatment with sodium selenite. (b) Quantification of western blot analyses using ImageJ software
(a) GAPDH expression was used as a reference. (b) Data are presented as mean ± SE with n = 6/group. * : *p* < 0.05 vs. control. **: *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-1and 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 upregulationin apoA-I mRNA expression was observed at 50 nMconcentrations (p<0.05). In contrast, no significant effectof sodium selenite supplementation was noted for ABCA-1or apoA-II expression.

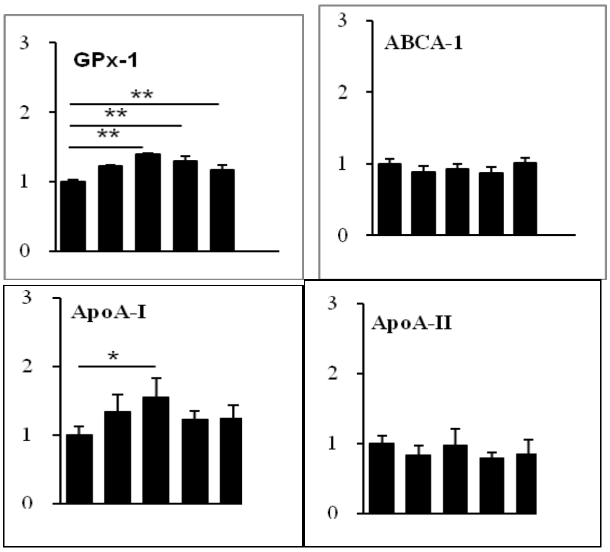

Figure 4. mRNA Expressions of GPx-1, apoA-I, apoA-II, and ABCA-1 in Hc cells after treatment with sodium selenite as analyzed by real-time PCR
Data are presented as mean ± SE (n = 5/group). *: *p* < 0.05 vs. control. **: *p* < 0.01 vs. control.

DISCUSSION

In this study, we demonstrated that in Hc cells, GPx-1 activity and protein and mRNA expression were dependent on the presence of exogenous sodium selenite. Second, sodium selenite supplementation significantly increased 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 increasedexpression, although this was not significant. Third, selenium supplementation in the form of sodium selenite did not enhance apoA-IIprotein or mRNA expression.

In the present study, weused sodium selenite to induce GPx-1 activity and the expressions ofrelated proteins and mRNA.In agreement with previous studies, we demonstrated that sodium selenite increased GPx-1 activity in Hc cellsafter72-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 increasedthe activityas well as the proteinand 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 increasedover the past few years (Rayman, 2008). In healthy people, all dietary requirements can be fulfilledsolely from food sources; however, a recent study has reported thatthe enhancement of certain nutrients, including selenium, in the tissuesis important (Monsen, 2000).In addition, antioxidant supplementation is recommended for the prevention of oxidative damage in patients with chronic diseases (Actis-Goretta *et al*., 2004).

Our study showed that sodium selenitesupplementation in low doses (<0.31 μM) did not inhibit H ccell proliferation after72-h incubation. However, the proliferation of Hc cells was prominently inhibitedat 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, especiallyfor the prevention of CVD,as it can bebeneficial,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 selenitesupplementation significantly increased the protein and mRNA expressions of apoA-I. Moreover, an upregulationtrend 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 100nM 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 doseof selenium and the type of cells used for examining the effects of selenium supplementation should becarefully considered.

Surprisingly, sodium selenite supplementation did not significantly affect apoA-II expression. This unexpected result suggests that this protein and genemay 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-1bythe 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 incorporateall 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 suggestthat selenium supplementation would be beneficial in preventing CVD events in healthy humansonly when administered at the appropriatedose.

ACKNOWLEDGEMENTS

This study was supported by the GP 2012 grant (Gunma University, Japan). The authors would like to express their gratitude to Prof. Takeaki Nagamine for his kind gift of the Hc cells, and to ChihoYoshizawa and Miki Matsui for technical assistance.

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