Discovery of Biomarkers That Reflect the Intake of Sodium Selenate by Nutritional Proteomics

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Abstract

Selenium offers important health benefits, including the prevention of some types of cancer. The traditional selenium indexes, such as selenium concentration, do not account for the metabolic status of this element regarding its chemoprotective effect. Then, the knowledge of a group of proteins that respond to selenium supplementation could be useful in the assessment of the metabolic status of selenium. The effect of dietary supplementation of rats with sodium-selenate on the blood plasma proteome is investigated. A group composed of six rats is fed a basic diet supplemented with sodium-selenate at 1.9 µg of Selenium per g of food, and a control group is fed a diet that covers the minimum selenium requirements, each for ten weeks. A proteomic approach is used to both quantify the changes in the abundance of some plasmatic proteins and to identify them. Fibrinogen, apolipoproteins, haptoglobin, and transthyretin changed significantly their abundance due to selenium administration. Those proteins are indirectly related to selenium metabolism. Then, the change in the proteomic profile due to selenium supplementation could probably be considered as a new index to assess the metabolic status of selenium. This index might help in the prevention of some diseases by nutritional diagnosis and, consequently, the adequate dietary recommendation.

Introduction

An adequate selenium status is related to the prevention of various forms of cancer, such as colon cancer (1,2), breast cancer (3), skin carcinoma (4), prostate cancer (5), and lung cancer (6,7). The mechanism of chemoprotection against cancer by selenium is not fully understood. It is believed that selenoproteins and some selenium compounds of low molecular weight would have an important role (8). It has been reported that chemoprotection through selenium takes place when supranutritional doses of this element are administered (9).

Selenium bioavailability in mammals is usually determined by measuring the selenium concentration in blood, by determining the activity of glutathione peroxidase in erythrocytes (4) and also by assessing the abundance of selenoprotein P in blood plasma (10). However, it was demonstrated that the chemoprotective capacity of selenium has no relationship with glutathione peroxidase activity or with selenium concentration in blood or tissues (11). Even so, selenoprotein P may be too sensitive to even changes in selenium status, and then its use as a standard would not be recommendable (12). Although the previously mentioned methods for the assessment of selenium status count on general approval, recent findings about the health benefits of selenium have stimulated studies on alternative methods to detect the intake of this element regarding its chemoprotective effect against cancer.

In this work, we investigated the effect of dietary supplementation with sodium-selenate on the proteome of the blood plasma of rats. The aim of this study was to determine whether the intake of sodium-selenate in a supranutritional dose is reflected as differences in the abundance of some plasmatic proteins in comparison to a control group. A nutriproteomics approach was used to determine changes in the abundance of proteins found in the blood plasma of rats and to identify those proteins.

Experimental

Experimental design and statistical analysis

The dose of selenium used in this study was selected on the basis that chemoprotection takes place when supranutritional doses of selenium are fed (9). A dose of 0.15 µg selenium per g of food is the minimum recommended dose and 2.0 mg selenium per g of food is the lower toxicity limit (13). Then, in order to avoid toxic effects on the animals, 1.9 mg selenium per g of food was chosen. The extent of the feeding period was ten weeks because Finley et al. (1) found that chemoprotection takes place in that period of time.

Plasma samples were collected, pooled, and analyzed by 2D gel electrophoresis. The normalized spot volume (relative abundance) of each of the 21 protein spots was determined densitometrically in quadruplicate from the gel images. Statistically significant differences (Student's t-test at a 95% confidence interval) in the relative abundance of proteins were obtained by comparing the normalized volumes of the spots corresponding to the experimental group and to the control group. Statistical analysis was carried out using Statgraphics 5.1 software. Finally, protein identification was performed by mass spectrometry analysis.

Animals

An experimental group of six Wistar rats, 21 days old, was fed a Torula yeast-based diet (Dyets Inc., Bethlehem, PA) supplemented with 1.9 mg selenium per g of food as sodium selenate

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during a feeding period of 10 weeks. A control group consisting of six rats was fed a basic diet supplemented with 0.15 mg selenium per g of food of sodium selenate, which covers the minimum requirements of selenium. Animals were maintained at 20ºC, 12 h/12 h day/night cycles in stainless steel cages with free access to demineralized water and the corresponding diet.

Plasma samples

After the experimental feeding period, blood from each one of the rats was collected by cardiac puncture using standard protocols (14). Collection was made in heparin tubes (BD Biosciences, San Jose, CA). The tubes were inverted up and down ten times and immersed in an ice bath. The tubes were centrifuged at 1300 xg for 10 min at 4ºC. Plasma was separated from the solids. Supernatant was transferred to a new centrifuge tube and centrifuged at 2400 RCF for 15 min at 4ºC in order to discard microplatelets. Plasma samples were kept at –80ºC until analysis.

Sample preparation

Plasma samples collected from the six rats of each group were pooled in order to normalize the variations among the animals. After that, the pooled samples were depleted of albumin using the Qproteome Murine Albumin Depletion Kit (QIAGEN GmbH, Hilden, Germany) according to the instructions of the manufacturer. Albumin depletion is frequently used in proteomics studies and shows high reproducibility (15). Protein concentration in plasma was determined according to the Bradford method using BSA as protein standard. The albumin-depleted protein solutions were freeze-dried and kept at –20°C until electrophoretic analysis.

2D gel electrophoresis

Proteins in the albumin-depleted plasma were analyzed by 2D gel electrophoresis using the method of Toledo et al. (16), which is based on that described by O'Farrell (17). Briefly, 300 mg of protein from the albumin-depleted plasma were resuspended in 50 mL of lysis buffer (9.5 M urea, 2% Triton X-100, 1.6 % ampholytes 4–7 range, 0.4 % ampholytes 3–10 range, and 5% β-mercaptoethanol), incubated at room temperature for 15 min, and loaded onto lab-made first dimension gels (115 mm height and 3 mm i.d. capillary tubes). A 4.0–7.0 pH gradient was used. Gel prefocusing was carried out according to the following program: 200 V for 15 min, 300 V for 15 min, and 400 V for 15 min. Isoelectric focusing (IEF) was performed at 400 V for 20 h to complete 8000 Vh. After IEF, the gels were extruded and equilibrated immediately in 2 mL of equilibration solution (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.0625 M Tris-HCl pH 6.8) for 10 min. Vertical SDS- PAGE was run with lab-made homogeneous acrylamide gel (11.5% acrylamide; 180 mm height and 120 mm width), at a constant voltage of 50 V for 16 h. Gels were soaked in a solution of 25% methanol and 7.5% acetic acid for 30 min, stained in Coomassie Brilliant Blue R-250 for 12 h (0.1% Coomassie blue R250, 25% methanol, 7.5% acetic acid), and destained in a solution of 25% methanol and 7.5% acetic acid. Coomassie Blue staining is the most reliable quantitative protein staining method, and it is widely used in proteomics studies (18). All chemicals were analytical-grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Image analysis

Image acquisition was performed with an ImageScanner II device (GE Healthcare, Uppsala, Sweden). Intensity calibration was carried out using an intensity step wedge prior to the image capture. The Total Lab v 2.01 software was used for image analysis. Spots were automatically detected and matched. Each spot volume was determined densitometrically and processed by background subtraction. Spot volumes of all gels were normalized by dividing the raw quantity of each spot by the total quantity of all valid spots in that gel, as recommended in literature (19,20).

Protein identification by mass spectrometry analysis

Protein spots were excised directly from the gels and analyzed by MALDI-TOF at the University of Colorado Health Sciences Centre (UCHSC, Denver, CO). Gel bands were cut into small pieces to enhance cleaning of bands and trypsin absorption. Bands were manually digested using the standard Proteomics Core protocol (21,22). Samples were digested overnight with modified porcine trypsin at room temperature. Digest solution was spotted on a MALDI target with alpha-cyano-4-hydroxycinnamic acid for sample co-crystallization. Samples were analyzed using MALDI–TOF mass spectrometry in a Voyager DE-STR system (PerSeptive Biosystems Inc., Framingham, MA). Data was calibrated, deisotoped, and centroided, and a peak list was generated. Peak lists were searched using the MASCOT search engine (http://www.matrix science.com) against the nrNCBI database v20070204/Rodent subset.

Results and Discussion

A representative 2D gel image of the blood plasma of the control group is shown in Figure 1. The identities of the protein spots determined by mass spectrometry are shown in Table I and refer to the spots highlighted in Figure 1. The analysis of the changes in the relative abundance of plasmatic proteins is shown in Figure 2. The relative abundance of individual protein spots was compared between the animals fed the diet supplemented with sodium-selenate and the control group. Figure 2 shows that the protein spots numbers 1–4, 8, 9, 12, 20, and 21 showed a lower abundance as compared to the control group. Simultaneously, the protein spots 7, 10, 11, 13, 15, 16, and 19 were more abundant in the sodium-selenate supplemented group. The statistical analysis of the previously mentioned differences is shown in Table II.

Differences in the abundance of some plasmatic proteins were observed between the group fed the sodium selenate–supplemented diet and the control group. A statistically significant increase in the abundance of some plasmatic proteins was observed in rats that were fed the selenium-supplemented diet in comparison to the control group. Those proteins were fibrinogen (spot 7), α-1-antitrypsin-precursor (spot 10), cyclin H (spot 11), apolipoprotein E (spot 13), haptoglobin (spot 15), transthyretin (spot 16), and α-1-antitrypsin (spot 19). On the other hand, there was a significant decrease in the abundance of fibrinogen (spots 5 and 6), apolipoprotein A-I (spot 9), apolipoprotein A-IV (spot 17), and α-1-antitrypsin (spot 20).

We propose that the change in the relative abundance of the

proteins mentioned earlier might constitute a particular proteomic pattern, which could be considered as indicative of the intake of a supranutritional dose of selenium delivered as sodium selenate. The proteins apolipoprotein-E, transthyretin, fibrinogen, and haptoglobin, which were modulated by sodium selenate, would have an indirect biological relation with selenium metabolism, and then those proteins could probably belong to a specific proteomic pattern indicative of selenium status.

Apolipoprotein E is a secreted protein that mediates the binding, internalization, and catabolism of lipoprotein particles. It serves as ligand for the LDL receptor and for the specific apo-E receptor of hepatic tissues. Selenium deficiency has been associated with an increased level of apolipoprotein E in rat plasma, although the mechanism by which selenium deficiency affects lipoprotein metabolism is poorly understood (23). Such an effect has been attributed to housekeeping selenoproteins, which would have a role in the regulation of the biosynthesis and metabolism of lipoproteins (24).

Transthyretin is a hormone-binding protein, rich in aromatic amino acids, that transports thyroxin from the blood stream to the brain. It is synthesized in the liver and in the choroid plexus (25). This protein is involved in the metabolism of the thyroid hormone, and selenium seems to play a major regulatory function on thyroid hormone homeostasis (26). Such metabolic control is performed through the antioxidant activity of several selenoenzymes that are synthesized in the thyroid gland (27). Both apolipoprotein E and transthyretin are related to selenium metabolism and, thus, their increase in abundance in association with selenium supplementation may well have a biological explanation.

Table I. Mass Spectrometry Analysis

Spot Nr.*	Protein	Sequence coverage (%)	Score†	Expect Value
1	Hepatic nuclear factor 6 (HNF6)	97	26	$3.70E^{-02}$
2	Contrapsin-like protease inhibitor (CPi-21)	64	184	$6.50E^{-14}$
3	Alpha-1-antitrypsin (isoform 1)	66	191	$1.30E^{-14}$
4	Apolipoprotein A-IV	83	338	$2.00E^{-29}$
5	Fibrinogen (isoform 1)	71	220	$1.60E^{-17}$
6	Fibrinogen (isoform 2)	62	174	$6.50E^{-13}$
7	Fibrinogen (isoform 3)	58	135	$5.20E^{-09}$
8	Apolipoprotein A-I	78	155	$5.20E^{-11}$
9	Apolipoprotein A-I	83	207	$3.30E^{-16}$
10	Alpha-1-antitrypsin precursor	63	213	$8.20E^{-17}$
11	Cyclin H	34	52	$9.20E^{-01}$
12	Immunoglobulin light chain	64	101	$1.30E^{-05}$
13	Apolipoprotein E (isoform 1)	46	81	$1.20E^{-03}$
14	Apolipoprotein E (isoform 2)	71	119	$2.10E^{-07}$
15	Haptoglobin	34	84	$6.50E^{-4}$
16	Transthyretin	89	106	$4.10E^{-06}$
17	Apolipoprotein A-IV	33	133	$8.20E^{-09}$
18	Zinc finger protein 108	27	60	$1.60E^{-01}$
19	Alpha-1-antitrypsin (isoform 2)	66	290	$1.60E^{-24}$
20	Alpha-1-antitrypsin (isoform 3)	56	273	$8.20E^{-23}$
21	Gelsolin	60	233	$8.20E^{-19}$

* Spot Nr = number assigned to each spot in Figure 2.
† Score and Expect Value = parameters given by the Mascot search engine; a high score and a low expect value represent a low probability that the search result is a random result (i.e., low probability that the result is a false positive).

Haptoglobin is a secreted protein synthesized in liver. It binds free haemoglobin that is released from erythrocytes and inhibits its oxidative activity. It also prevents iron loss through the kidneys and protects these organs from the damage caused by hemoglobin (28). This protein is also known as "liver regeneration-related protein" (29). Fibrinogen is a secreted protein, and it has two functions: to yield monomers that polymerize into fibrin and to act as a cofactor in platelet aggregation (30). It has been reported that fibrinogen beta chain is involved in liver regeneration (31). As selenium is metabolized in the liver, the statistical relationship found between selenium supplementation and the abundance of liver-related proteins would have a biological base.

It results lightly striking that the four proteins that were detected as modulated by selenium intake do not correspond to selenoproteins. This could be attributed to the very low concentration of selenoproteins in blood plasma and also to the comparatively low sensitivity of the protein detection method used in this study.

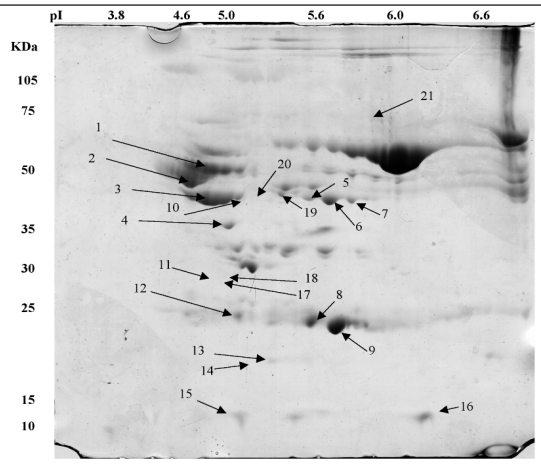

Figure 1. Representative 2D gel image of the control group. For each condition (experimental and control), 2D gels were made in quadruplicate and were stained with Coomassie Brilliant Blue R-250. Each gel was analyzed densitometrically, and the relative abundance of each spot was calculated. Spot numbers are highlighted in the figure. The protein spots were identified by mass spectrometry analysis.

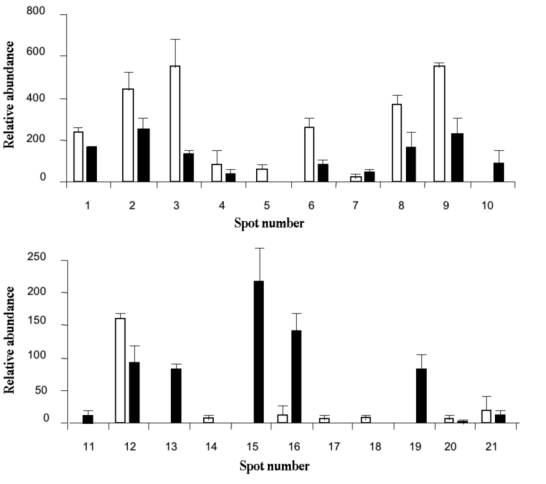

Figure 2. Effect of dietary supplementation with sodium selenate on the relative abundance of some plasmatic proteins. Relative abundance (arbitrary units) of individual protein spots were calculated from four gel replicates. Values are the mean ± standard deviation. Panel A: spots 1–10; panel B: spots 11–21. Control and sodium-selenate-supplemented diet are represented as open and black-filled bars, respectively.

Table II. Statistical Analysis of the Relative Abundance of Proteins

Spot Nr	p-value*	Spot Nr	p-value*
1	0.228	12	0.078
2	0.213	13	n.d.
3	0.149	14	n.d.
4	0.137	15	n.d.
5	n.d. †	16	0.050‡
6	0.005‡	17	n.d.
7	0.002‡	18	n.d.
8	0.129	19	n.d.
9	0.013‡	20	n.d.
10	n.d.	21	0.234
11	n.d.		

* The p-values obtained from the comparison of protein abundances between the two experimental groups and the control group is shown. The average of the relative abundance of each protein spot was calculated from four replicates of 2D gels. A student's t-test was made considering a 95% confidence interval.
† Indicates that the p-value could not be determined because the normalized volume either in the experimental or the control group was zero.
‡ Statistically significant differences.

Conclusion

A group of proteins was found to significantly change their abundance in the blood plasma of rats when a supranutritional dose of selenium supplemented the diet. Despite the fact that those proteins are not selenoproteins, some of them would have an indirect biological relation with selenium metabolism, such as apolipoprotein E and transthyretin. Some liver-related proteins, fibrinogen, and haptoglobin, were also modulated by selenium. Then, given that selenium is metabolized in this organ, the relationship between selenium supplementation and the increased abundance haptoglobin would have biological explanation.

In this way, the proteomic response of rats to dietary supplementation with sodium selenate was partially characterized. The protein profile observed in this work could probably help in defining the metabolic status of selenium in mammals. This pattern could be considered as a new index that might help in the prevention of some diseases by nutritional diagnosis and the consequently adequate dietary recommendation. In addition, this knowledge could probably shed some light on the chemical protection of mammals against some types of cancer in relation to the intake of a selenium compound. Finally, this information could contribute to the elucidation of the complex mechanisms underlying chemoprotection by selenium in mammals, which is poorly understood so far.

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