Selenium deficiency increases the expression of inducible nitric oxide synthase in RAW 264.7 macrophages: role of nuclear factor-κB in up-regulation

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The inducible isoform of nitric oxide synthase (iNOS) is implicated in atherosclerosis, malignancy, rheumatoid arthritis, tissue and reperfusion injuries. A key determinant of the pro-oxidant versus protective effects of NO is the underlying redox status of the tissue. Selenoenzymes, such as glutathione peroxidases (GPxs) and thioredoxin reductases, are key components of cellular defence and promote optimal antioxidant/oxidant balance. In this study, we have investigated the relationship between Se status, iNOS expression and NO production in Se-deficient and Se-supplemented RAW 264.7 macrophage cell lines. The cellular GPx activity, a measure of Se status, was 17-fold lower in Se-deficient RAW 264.7 cells and the total cellular oxidative tone, as assessed by flow cytometry with 2’,7’-dichlorodihydrofluorescein diacetate, was higher in the Se-deficient cells than the Se-supplemented cells. Upon lipopolysaccharide (LPS) stimulation of these cells in culture, we found significantly higher iNOS transcript and protein expression levels with an increase in NO production in Se-deficient RAW 264.7 cells than the Se-supplemented cells. Electrophoretic mobility-shift assays, nuclear factor-κB (NF-κB)-luciferase reporter assays and Western blot analyses indicate that the increased expression of iNOS in Se deficiency could be due to an increased activation and consequent nuclear localization of the redox-sensitive transcription factor NF-κB. These results suggest an inverse relationship between cellular Se status and iNOS expression in LPS-stimulated RAW 264.7 cells and provide evidence for the beneficial effects of dietary Se supplementation in the prevention and/or treatment of oxidative-stress-mediated inflammatory diseases.

Key words: free radical, nitric oxide (NO), oxidative stress, selenium-dependent glutathione peroxidase.

INTRODUCTION

Selenium is an essential trace element for all mammalian species and functions primarily through selenoenzymes, which contain Se as selenocysteine (SeCys) [1,2]. The most well-characterized selenoenzymes are the Se-dependent glutathione peroxidase (Se-GPx) and thioredoxin reductase families, the activities of which are responsible for the recognition of Se as an important dietary antioxidant [1]. These enzymes are involved in many biochemical processes such as protection against oxidative stress and redox-based regulation of gene expression [2]. Se deficiency results in a significant decrease in Se-GPx, and an increase in reactive oxygen species (ROS) production [1,2]. In addition, inadequate Se nutrition is associated with an increase in reactive nitrogen species (RNS) such as NO and peroxynitrite production, which have been linked to increased risk of such diseases as cardiomyopathy [3], rheumatoid arthritis [4], cancer [5,6], Alzheimer’s disease [7] and multiple sclerosis [8]. Thus cellular Se status plays an important role in the reduction of oxidative stress in the body.

Many immunological cell types, including macrophages, synthesize NO, which regulates their cellular function [9]. In addition, NO has also been found to be a major intercellular messenger involved in such diverse activities as neural signalling and vasorelaxation [10]. Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths and tumour cells [11]. However, owing to its highly reactive nature, NO can also be destructive to the body’s healthy cells when overproduced [12]. Furthermore, NO is rapidly oxidized to RNS, which can S-nitrosate thiols to modify key signalling molecules such as kinases and transcription factors [9].

Nitric oxide synthase (NOS) catalyses the breakdown of L-arginine to NO and citrulline [13]. Three different isoforms of NOS are present in mammals, namely two constitutive enzymes (neuronal NOS, and endothelial NOS) and one inducible enzyme (iNOS) [13]. A variety of stimulants, such as silica, UV light, cytokines and lipopolysaccharide (LPS), are known to up-regulate the expression of iNOS [14]. In addition, there is evidence to show that up-regulation of iNOS expression and nitrite production involve the activation of nuclear factor-κB (NF-κB) and subsequent binding of the κB enhancer elements in the iNOS gene promoter [15,16]. In the present study, we have investigated the relationship between Se status, as an important modulator of cellular oxidative stress, and iNOS expression in response to LPS stimulation in RAW 264.7 cells, a murine macrophage-like cell line. These cells have been previously established as a model for the study of iNOS expression [15,17]. Our studies are based on the hypothesis that inadequate Se status in macrophages leads to an oxidant/antioxidant imbalance, and is responsible for the activation of the redox-active transcription factor NF-κB, which, in turn, induces the expression of iNOS. This is the first report to demonstrate an inverse relationship between Se status and iNOS expression in a whole-cell system.

Abbreviations used: EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H$_2$DCF-DA, 2’,7’-dichlorodihydrofluorescein diacetate; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; RT-PCR, reverse transcriptase PCR; Se-GPx, Se-dependent glutathione peroxidase.

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EXPERIMENTAL

Cell-culture conditions

RAW 264.7 cells, a murine macrophage cell line, were cultured at 37 °C in a humidified 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine, 50 μg/ml gentamicin (all from Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 5% defined fetal bovine serum (Hyclone Laboratories, Logan, UT, U.S.A.). The Se-deficient RAW 264.7 cells were grown under the above conditions and the Se deficiency of cells was documented by the measurement of SeGPx activity. The Se-supplemented RAW 264.7 cells were grown in the same medium with the addition of sodium selenite (Sigma, St. Louis, MO, U.S.A.) to a final concentration of 2 nmol/ml. This concentration was determined to be the highest level tolerated by the RAW 264.7 macrophages without affecting cell viability and growth rates (results not shown).

For luciferase reporter assays involving NF-κB wild-type and truncated-promoter constructs, cells were seeded at 1 x 10⁵ cells/well in a 96-well plate and transfected with 750 ng of plasmid DNA using Superfect (Qiagen, Los Angeles, CA, U.S.A.) for 2 h. Cells were allowed to grow for 72 h prior to stimulation with LPS for 6 h. Following stimulation, cells were washed with PBS containing calcium and magnesium (1 mM each) and overlaid with 100 μl of PBS. Luciferase activity was determined using LucLite Plus Assay kit (Packard, Hartford, CT, U.S.A.).

Cellular Se-GPx activity assay

The harvested cells were washed three times in ice-cold PBS and centrifuged at 500 g for 10 min at 4 °C. Cell pellets were resuspended in mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, U.S.A.) for 30 min on ice and then centrifuged at 10000 g for 15 min. The supernatant was collected and total protein was determined using the BCA (bicinchoninic acid) reagent (Pierce). The cellular Se-GPx activity in Se-deficient and-supplemented RAW 264.7 cell lysates, which were either stimulated with LPS (1 μg/ml) or unstimulated, was measured according to standard methods using H₂O₂ as a substrate [18]. The reaction was initiated by the addition of 1.5 mM H₂O₂ and the oxidation of NADPH was monitored spectrophotometrically at 340 nm. The specific activity is expressed as nmol of NADPH oxidized/min per mg of protein.

Determination of oxidative tone

RAW 264.7 macrophage cells were grown in Se-deficient and Se-supplemented media as described above. For a sensitive measurement of total ROS generation during Se deficiency, cells were harvested, washed once with PBS and incubated with 2.5 μM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA; Molecular Probes, Eugene, OR, U.S.A.) for 15 min at 25 °C. The number of cells exhibiting increased fluorescence of DCF, generated by ROS, was analysed by a Beckman–Coulter XL-MCL single laser flow cytometer. These studies were performed at the Life Science Consortium’s Flow Cytometry Facility, The Pennsylvania State University, University Park, PA, U.S.A.

Quantitative reverse transcriptase PCR (RT-PCR) analyses

Total RNA was isolated from Se-deficient and -supplemented RAW 264.7 cells, stimulated at various times with LPS, using Trizol reagent (Life Technologies) and quantified on the basis of A₂₆₀. The RNA samples were treated with RNase-free DNase for 30 min to avoid any genomic DNA contamination as per the manufacturer’s instructions (Promega, Madison, WI, U.S.A.). Equal amounts of RNA (50 ng) from each of the samples were used in RT-PCR with iNOS-specific sense (5'-AATGGGAACATCGTGCCGACACT-3') and antisense (5'-GCGTGTGTCACAAAGTCTC-3') primers. β-Actin was used as an internal standard with the following sense and antisense primers respectively: 5'-TGAATCTCTGGCCATCCATGAAAC-3' and 5'-TAAACGCGAGTCAATACAGTCCG-3'. The PCR products were analysed on a 2% agarose gel and the DNA bands were quantified on an Eagle Eye system (Stratagene) using the Scion Image software program (Frederick, MD, U.S.A.).

Preparation of nuclear extracts from cells

For the analysis of NF-κB translocation, nuclear extracts were prepared from Se-supplemented and -deficient RAW 264.7 cells (2 x 10⁶ cells) grown in six-well cluster dishes. Fresh medium was added to cells prior to stimulation with LPS (1 μg/ml) for the indicated times. Following stimulation, cells were washed and harvested in ice-cold PBS. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce), according to the manufacturer’s instructions. Total protein was measured using the BCA reagent (Pierce).

Western blot analyses

Samples of 20 μg of total protein or 50 μg of nuclear protein were electrophoresed on a SDS/12.5%, polyacrylamide gel and transblotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.) and blocked for 1 h at 25 °C in 10 mM Tris/HCl, pH 8, containing 150 mM NaCl, 0.1%, Tween-20 and 5% skimmed milk. Detection of iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the whole-cell lysates, obtained with M-PER, was performed after incubation with their specific antibodies for 1 h at 25 °C, while NF-κB was detected in the nuclear fraction after 1 h of incubation with an NF-κB p65-specific antibody at 25 °C. GAPDH was used as a control to normalize protein loading. Rabbit anti-mouse iNOS polyclonal antibody, rabbit polyclonal NF-κB (p65) and mouse anti-GAPDH monoclonal antibody were from Cayman Chemicals (Ann Arbor, MI, U.S.A.), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and Chemicon International (Temecula, CA, U.S.A.), respectively. A horseradish peroxidase-conjugated anti-IgG antibody was used as the secondary antibody. The bands were visualized by enhanced chemiluminescence assay kit (Pierce) according to the manufacturer’s instructions.

Electrophoretic mobility-shift assay (EMSA)

Nuclear proteins (6 μg) were incubated with 15000 c.p.m. of ³²P-labelled NF-κB double-stranded oligonucleotide (Promega), 1 μg of poly(dI-dC) and 1.75 pmol of unlabelled AP-1 double-stranded oligonucleotide (Promega), as a non-specific competitor, for 30 min and subjected to electrophoresis on a 6% polyacrylamide gel under non-denaturing conditions. In addition, EMSA was also performed with the same nuclear extracts and probed with SP1 oligonucleotide as a control, essentially following the same protocol as in the case of NF-κB. To confirm the specificities of each of the probes, unlabelled oligonucleotides (NF-κB and SP1; 3.5 pmol) were used as specific competitors.

NO assay

Aliquots (100 μl) of culture medium were collected from the same cells grown and stimulated with LPS under identical conditions
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as used for Western blot analysis. NO production was measured using the Griess reagent (Sigma) following the manufacturer’s instructions.

Se deficiency increases overall oxidant stress in RAW 264.7 macrophages

Se-deficient and Se-supplemented RAW 264.7 cells were incubated with 2.5 μM H$_2$DCF-DA for 15 min. Cellular fluorescence, an indication of overall oxidative stress, was measured by a Beckman Coulter flow cytometer. Arbitrary cellular fluorescence units for each sample, an average value for three independent experiments, are expressed as means ± S.D. and were analysed by Student’s t test. *P < 0.05.

Generation of luciferase constructs

The wild-type iNOS promoter DNA was PCR amplified from genomic DNA isolated from RAW 264.7 cells. The promoter sequence containing all the cis-acting elements, including the TATA box, was amplified from −1589 to −15, and cloned into pGEM-T (Promega). This sequence contained two NF-κB sites; one from positions −972 to −962 and the other from positions −86 to −76. The wild-type iNOS promoter was subcloned into pGL3-luciferase vector (Promega) linearized with NcoI and XhoI. A truncated mutant, NF-κB80pGL3, was generated by restriction digestion of the wild-type iNOS-pGL3 construct with SacI. NF-κB80pGL3 contains the iNOS promoter sequence from −329 to −15 and is devoid of the NF-κB site at −972 to −962. The luciferase assays were performed as mentioned above.

RESULTS

Se deficiency induces oxidative stress in RAW 264.7 cells

The cellular Se-GPx activity was measured in cell lysates prepared from Se-deficient and -supplemented RAW 264.7 cells, after ten passages in the respective media, to be 4.7 ± 0.34 and 84.2 ± 3.9 nmol of NADPH oxidized/min per mg of protein respectively. The 17-fold difference in Se-GPx activity was stable over 6 months and the Se-GPx activities in Se-deficient and -supplemented RAW 264.7 cells treated with LPS did not change either (results not shown). Furthermore, incubation of RAW 264.7 cells with 2.5 μM H$_2$DCF-DA, a fluorescent label of total oxidative tone, clearly showed that Se-deficient RAW

Figure 1 INOS mRNA (A) and protein (B) levels are increased in Se deficiency

The time course of iNOS expression in Se-deficient and Se-supplemented RAW 264.7 cells was performed by incubating the cells with 1 μg/ml LPS for 0–24 h. (A) Total mRNA was isolated and used in quantitative RT-PCR reactions as described in the Experimental section. A typical gel profile is shown in the left-hand panel. Densitometry of the iNOS and β-actin bands were performed on material from three independent experiments. Data are expressed as means ± S.D. and analysed by Student’s t test (right-hand panel; *P < 0.05). (B) Western immunoblot analysis was performed on the cell lysates (20 μg) with the iNOS-specific antibodies. In order to normalize protein loading, GAPDH was used as an internal control. The results presented are typical of those obtained; they were repeated on at least three separate occasions.
264.7 cells were already under twice the oxidative stress as Se-supplemented cells (Figure 1). Collectively, these data indicate that Se status adversely affects the general oxidative tone of the cells.

Se deficiency increases iNOS expression and NO production in LPS-stimulated RAW 264.7 cells

To determine the effect of Se deficiency on the expression of iNOS, both Se-deficient and -supplemented RAW 264.7 macrophage cells were activated with LPS, and mRNA was analysed by RT-PCR, while protein levels were analysed by Western immunoblot analysis (Figure 2). The RNA samples were processed in RT-PCR reactions for both iNOS and β-actin. The level of iNOS mRNA was compared with the level of β-actin for each time point (Figure 2A). As seen from Figure 2(A), the ratio of iNOS to β-actin was 3-fold higher in Se-deficient cells even before LPS stimulation. Following LPS stimulation, the iNOS transcript levels were found to increase in both cell-types; however, the increase in iNOS mRNA in Se-deficient cells was significantly higher than in Se-supplemented cells. The transcript levels were found to plateau around 6 h post-stimulation in both cell-types (Figure 2A). Furthermore, the increase in iNOS mRNA levels was accompanied by a corresponding increase in iNOS protein levels in Se-deficient cells (Figure 2B). The GAPDH signal, used as an internal standard, was also detected to be at the same level for all samples (Figure 2B).

To demonstrate that the different levels of iNOS protein expression seen by Western blot analysis affected the overall iNOS activity, NO production was analysed in the culture media over a period of 24 h post LPS stimulation. Cells were stimulated with 1 µg/ml LPS and aliquots of the cell culture supernatant were taken at various time points. NO levels increased in culture media of both Se-supplemented and Se-deficient cells following stimulation with LPS (Figure 3); however, the Se-deficient cells exhibited a significant increase in NO production over Se-supplemented cells.

NF-κB is involved in the overexpression of iNOS in LPS-stimulated RAW 264.7 cells during Se deficiency

To gain insight into the potential mechanism by which Se status affects iNOS expression, we investigated the activation of NF-κB in these cell lines. Nuclei were isolated from Se-supplemented and -deficient RAW 264.7 cells at various times post-stimulation with 1 µg/ml LPS. Nuclear extracts were analysed by Western
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This is a representative experiment where both the wild-type and the truncated iNOS promoter/reporter constructs were transfected into Se-deficient and -supplemented RAW 264.7 cells. The cells were stimulated with LPS for 6 h and the luciferase activities were determined as described in the Experimental section.

**DISCUSSION**

In the present study, we provide experimental support for the hypothesis that Se, as an integral part of Se-GPx active site, may exert its chemopreventive effects, in part, by significantly inhibiting the expression of iNOS and subsequent NO production, which has been implicated in the pathophysiology of many disease states. Macrophages express significant amounts of iNOS upon stimulation by a variety of substances, including LPS, a bacterial endotoxin, leading to an increase in NO production [19]. One of the biochemical mechanisms by which NO affects cellular processes is through direct interaction with cellular proteins by nitrosylation and nitrosation reactions. The consequences of protein damage by NO appears to be procarcinogenic, as seen in the case of NO inhibition of human 8-oxodeoxyguanosine DNA glycosylase 1, a DNA repair enzyme, which blocks apoptosis [20]. In addition, there are numerous reports on the NO-induced damage of DNA [20–22]. Such free-radical-mediated cellular events, and the inhibition of repair-protein functions, permit DNA damages to accumulate, leading to the development and progression of inflammatory diseases and cancer. Furthermore, these reports lend credence to the fact that cellular redox status plays a major role in containing the overproduction of free radicals such as NO and other reactive forms derived from it.

In order to establish the role of Se in the modulation of oxidant-stress-related pathways of gene expression, we have developed RAW 264.7 macrophage cell lines grown under Se-deficient and -supplemented conditions. A concentration of 2 mmol/ml sodium selenite is the highest Se level tolerated by the cells without affecting cell viability and growth rate, and it is also at a comparable level with the normal physiological plasma Se concentrations in humans [23]. The specific activity of one of the selenoenzymes, Se-GPx, is considered as a measure of the Se status [2]. In our model system, we have consistently observed a 17-fold decrease in Se-GPx activity with Se-deficient RAW 264.7 cells when compared with those values observed in Se-supplemented cells. In addition, an increase in overall oxidative stress was also observed in Se-deficient cells. Elevated levels of ROS indicated by flow cytometry are considerably higher in Se-deficient cells than in Se-supplemented cells. The results of the experiments performed clearly indicate a compromise in antioxidant defence mechanisms in Se-deficient macrophages, and such an impairment has a regulatory effect on iNOS induction and subsequent NO production.

A significant increase in the expression of iNOS was observed in RAW 264.7 macrophages deficient in Se compared with those cultured in the presence of Se. The levels of iNOS mRNA were stimulated upon LPS addition in both Se-deficient and Se-supplemented cells; however, in the Se-deficient cells, the increase in iNOS transcript levels was significantly higher at 6 h post-stimulation than those found in the Se-supplemented cells. A parallel increase in iNOS protein was also observed and found to be more robust in Se-deficient cells compared with Se-supplemented cells. The increase in iNOS expression was followed by an increase in NO production between Se-deficient and Se-supplemented cells with time post-stimulation. These results are consistent with the results of flow-cytometric experiments in Se-deficient cells that demonstrate an increase in overall levels of ROS to signal the production of pro-inflammatory genes, such as iNOS. The levels of NO produced in Se-deficient cells upon stimulation was significantly higher than in the Se-supplemented cells. However, there was a lag in the production of NO, which is likely due, in part, to its diffusion across the membrane as well as the sensitivity of the assay. The above results clearly suggest that Se status affects the regulation of iNOS expression, most likely through influencing the signalling pathways to iNOS induction.

An imbalance between the cellular antioxidant levels and ROS production leads to oxidative stress. It has been documented...
that ROS mediates the induction by tumour necrosis factor-\(\alpha\), glycated proteins, LPS and interleukin-1 of iNOS [24–27]. In addition, there is ample evidence to suggest that the transcription factor NF-\(\kappa\)B is involved in the regulation of iNOS induction by such stimuli as LPS [28] as well as in the control of the immune system in response to injury and infection [29]. The use of wild-type and mutated iNOS promoter–luciferase constructs in our studies implicating NF-\(\kappa\)B activation by LPS involves this redox-sensitive transcription factor. Furthermore, Western immunoblots and EMSAs also confirm our hypothesis that NF-\(\kappa\)B activation and iNOS expression are synonymous. The extent of activation of NF-\(\kappa\)B is significantly higher and prolonged in the case of Se-deficient cells when compared with Se-supplemented cells.

This is the first report to demonstrate an inverse correlation of iNOS expression and activity with Se status. The increased ROS levels seen in our Se-deficient RAW 264.7 macrophage model cell culture system can be attributed, in part, to significantly lowered levels of antioxidant enzymes such as Se-GPx. In fact, Han et al. [30] have recently reported that \(\text{H}_2\text{O}_2\), among other ROS, is mainly produced upon LPS stimulation of RAW 264.7 macrophages, which participates in the up-regulation of iNOS gene expression via the NF-\(\kappa\)B pathway. It is, therefore, conceivable that in Se deficiency, the overproduction of \(\text{H}_2\text{O}_2\) results in overall cellular oxidative stress, which leads to an increased expression of pro-inflammatory genes such as cyclo-oxygenase-2 [31] and iNOS. We have demonstrated here that the increased oxidative stress in Se-deficient cells triggers the overexpression of iNOS via the activation of the redox transcription factor NF-\(\kappa\)B. Our results are in close agreement with those of Hutter and Greene [32], who have demonstrated that the cellular redox environment plays a singular role in regulating signalling events operating through the control of gene expression by transcription factors, particularly NF-\(\kappa\)B. Se modulates the activity of NF-\(\kappa\)B in LPS-treated human T-cells and lung adenocarcinoma cells via a different mechanism involving the redox state of specific cysteine residues of NF-\(\kappa\)B and selenoproteins, like thioredoxin [16]. However, our studies differ from those of Kim and Stadtman [16] in that we have cultured our cells in Se-deficient media rather than treating just the nuclear fraction with selenite, as reported by them. Furthermore, the cytoprotective role of thioredoxin in modulating the DNA binding of transcription factors such as NF-\(\kappa\)B, AP-1, p53 and PEBP-2 (polymavirus enhancer-binding protein-2) coincides with the increased translocation of this protein into the nucleus upon subjecting the cells to inflammatory stress [33,34]. In addition, the regulatory role of thioredoxin also extends its ability to modulate the kinase pathways upstream of NF-\(\kappa\)B-inducing kinase [35]. However, in Se deficiency, the increased activation and DNA binding of NF-\(\kappa\)B could possibly suggest that other pathways of thioredoxin-independent binding of NF-\(\kappa\)B predominate in the nucleus, since the levels of the thioredoxin and thioredoxin reductase redox couple are likely to be lower than usual. Currently, investigations in our laboratory are focused on the translocation of thioredoxin into the nucleus in Se deficiency and whether this translocation has any role to play in the DNA binding of NF-\(\kappa\)B. Collectively, our results suggest that strategies, such as dietary supplementation of Se, to inhibit NO generation or to scavenge RNS, may prove useful in decreasing the risk of cancer development and chronic inflammatory diseases.

Financial support from the National Institutes of Health PHS grants HL60044 and DK 49122 are gratefully acknowledged. We thank Dr John P. Vanden Heuvel for his valuable suggestions with the RT-PCR experiments.

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Received 11 February 2002/1 May 2002; accepted 13 May 2002
Published as BJ Immediate Publication 13 May 2002, DOI 10.1042/BJ20020256