Marginal selenium deficiency down-regulates inflammation-related genes in splenic leukocytes of the mouse[☆]

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Abstract

Moderate selenium deficiency may lead to an impaired capacity to cope with health challenges. Functional effects of suboptimal selenium intake are not fully known, and biomarkers for an insufficient selenium supply are inadequate. We therefore fed mice diets of moderately deficient or adequate selenium intake for 6 weeks. Changes in global gene expression were monitored by microarray analysis in splenic leukocytes. Genes for four selenoproteins, *Sepw1*, *Gpx1*, *Selh* and *Sep15*, were the most significantly down-regulated in moderate selenium deficiency, and this was confirmed by quantitative polymerase chain reaction (qPCR). Classification of significantly affected genes revealed that processes related to inflammation, heme biosynthesis, DNA replication and transcription, cell cycle and transport were affected by selenium restriction. Down-regulation by moderate selenium deficiency of specific genes involved in inflammation and heme biosynthesis was confirmed by qPCR. Myeloperoxidase and lysozyme activities were decreased in selenium-restricted leukocytes, providing evidence for functional consequences. Genes for 31 nuclear factor (NF)-κB targets were down-regulated in moderate selenium deficiency, indicating an impaired NF-κB signaling. Together, the observed changes point to a disturbance in inflammatory response. The selenoproteins found here to be sensitive to selenium intake in murine leukocytes might also be useful as biomarkers for a moderate selenium deficiency in humans.

1. Introduction

Selenium is an essential micronutrient with various functions which are mainly exerted by selenoproteins containing selenium in the form of selenocysteine. There are only 25 genes for selenoproteins in humans and 24 in mice [1]. While members of the enzyme families of thioredoxin reductases (TrxR), glutathione peroxidases (GPx) and deiodinases (DIO) are well characterized to be involved in cellular redox homeostasis and thyroid hormone metabolism, respectively, functions of the other selenoproteins are still not fully elucidated. Until now, a putative role for the remaining selenoproteins in redox regulation seems to prevail, which can be anticipated from a

thioredoxin-like structure identified in SelH, SelM, SelT, SelV, SelW and Sep15. In addition, many selenoproteins are localized in the endoplasmatic reticulum (ER), including SelS, Sep15, SelK, SelM and SelT (reviewed in [2]).

Beneficial effects of selenium/selenoproteins have been proposed in the prevention of cancer development [3], in male reproduction [4] and in the onset of AIDS in HIV-positive patients [5,6]. An emerging field is the role of selenium and selenoproteins in inflammation [7,8], but underlying molecular mechanisms are still unclear. It is known that a severe selenium deficiency together with a coxsackie B virus infection results in the Keshan disease, an endemic cardiomyopathy [9]. Although this disease is not prevalent in countries where a severe selenium deficiency does not exist, a suboptimal selenium supply still persists in Europe, and this might affect immune function [10]. Critically ill patients, e.g., those suffering from sepsis, exhibit decreased plasma selenium levels, which are negatively correlated with their chance of survival [11]. Selenium supplementation of these patients reduced mortality in several clinical studies [12,13].

In vitro studies on the effect of selenium deficiency on inflammatory parameters have given apparently conflicting results, and this may be due partly to differences between cell lines: for example, on the one hand, the macrophage-like cell line RAW 264.7 showed a

higher lipopolysaccharide (LPS)-induced expression of proinflammatory mediators, such as tumor necrosis factor (TNF) α [14], as well as a higher production of prostaglandins (PG) such as PGE$_2$ [15] in selenium deficiency. TNFα is a known inducer of adhesion molecules like vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1, which are required for monocyte adhesion to endothelial cells and subsequent tissue penetration, a process counteracted by selenium supplementation [16]. In addition, VCAM1 expression is decreased in cells overexpressing the selenoprotein GPx4 [17]. The increase in LPS-induced PGE$_2$ production depends on cyclooxygenase 2 (COX-2), which is down-regulated by selenium supply [14] and GPx2 [18]. Under these conditions, metabolism of arachidonic acid is shifted from PGE$_2$ production to the synthesis of 15-deoxy-PGJ$_2$ [19], an anti-inflammatory prostaglandin [20]. On the other hand, the LPS-induced release of TNFα, interleukin (IL)-1 and IL-4 was increased in selenium-supplemented J774.1 macrophage-like cells compared to selenium-deficient cells [21].

The spleen, together with lymph nodes and mucosal-associated lymphoid tissues, comprises the majority of peripheral immune tissues. It is involved in blood filtering, iron recycling, antibody production and both innate and adaptive immunity [22]. In addition, it serves as a reservoir for monocytes and platelets. After splenectomy, the risk of developing sepsis is 10- to 20-fold increased [23]. The sensitivity of the spleen to selenium supply has been indicated by several studies. Feeding a high-selenium diet (15 mg/kg diet) inhibited proliferation and increased apoptosis in chicken splenocytes [24]. Similarly, splenocyte numbers were reduced upon feeding a diet excessive in selenium (2 ppm selenomethionine). However, the same effects were observed after feeding a selenium-deficient (0.02 ppm selenomethionine) diet [25], underlining the requirement for a balanced selenium supply.

Most of the studies investigating selenium effects focus on deficient or supranutritional selenium levels. There are only a few studies dealing with consequences of a moderate selenium deficiency comparable with that which could arise due to normal variations in nutritional habits or seasonal food choice. These studies focused on the impact of a moderate selenium deficiency on antigen-specific CD4$^+$ T-cell responses and on allergic airway inflammation, respectively [26,27]. In our previous study, global gene expression under a moderate selenium deficiency compared to selenium-adequacy was analyzed in the colon of mice [28]. Four selenoproteins (*Gpx1*, *Sepw1*, *Selh*, *Selm*) most sensitively responding to the selenium status by changes in mRNA expression were found. In addition, pathways of protein biosynthesis, inflammation and Wnt signaling were significantly affected [28]. In the current study, splenic leukocytes from these mice were used to analyze the impact of selenium on global gene expression. The results from microarray and quantitative polymerase chain reaction (qPCR) measurements show that the small change in selenium intake influenced expression of specific selenoproteins and genes involved in the inflammatory response and heme biosynthesis. Mouse data roughly correlate with human data from the SELGEN study [29]. Since blood cells are relatively easily available from humans, the findings might be useful to identify reasonable biomarkers for the selenium status in humans.

2. Methods and materials

2.1. Animal experiment

Leukocytes were obtained from the animals treated as reported previously [28]. Briefly, groups of 12 male C57BL/6J mice (3–4 weeks of age) were fed either a selenium-deficient (0.086 mg Se/kg) or a selenium-adequate diet (0.15 mg Se/kg; representing the estimated nutrient requirements of mice [30]) produced by mixing selenomethionine (Acros, Geel, Belgium) into the deficient diet (Altromin, Lage, Germany). After a 6-week feeding period, animals were anesthetized with isoflurane, and blood was withdrawn with heparinized capillaries by puncture of the retroorbital plexus. Plasma was obtained after centrifugation of the blood for 10 min (3000g, 4°C) and was stored at −80°C. Anesthetized animals were sacrificed by cervical dislocation. The animal study was approved by the Governmental Animal Ethics Committee (MLUV 32-44457141).

2.2. Isolation of splenic leukocytes

Spleens were removed aseptically, placed on a sterile microscope slide and crushed with the end of a 6-ml syringe plunger. Released cells were diluted in 5 ml of cell culture medium (RPMI with 5% fetal calf serum; Gibco, Karlsruhe, Germany). Clumps were dispersed by drawing and expelling the suspension repeatedly through a 6-ml syringe with a 20-gauge needle. A 100-μm mesh was used to remove clumps and particles and to receive a single cell suspension. The mesh was rinsed with 5 ml RPMI. After centrifugation (5 min, 200g) and washing with 10 ml RPMI, erythrocytes were lysed for 5 min in 5 ml of ammonium chloride lysis buffer (0.15 mol/L NH$_4$Cl, 10 mmol/L KHCO$_3$, 0.1 mmol/L Na$_2$EDTA, pH 7.4). Thereafter, 15 ml of balanced salt solution containing 0.2% bovine serum albumin was added, and the suspension was centrifuged for 5 min at 200g. The pellet was suspended in 1 ml phosphate-buffered saline (PBS) and transferred into a 2-ml tube. Thirty microliters of the suspension was smeared on microscope slides and dried on air for counting. The cells of the remaining suspension were pelleted (5 min, 200g) and washed with 1 ml PBS. The pellet was frozen at −80°C until RNA isolation. As determined with May–Grünwald–Giemsa staining, the leukocyte population was independent of the selenium status and on average was composed of 79% lymphocytes, 18% granulocytes and 2% monocytes. In addition, spleen weight was unaffected by the selenium status.

2.3. RNA isolation

Whole cell pellets of splenic leukocytes were suspended in 800 μl of cold Trizol (Invitrogen, Karlsruhe, Germany) and homogenized with a TissueLyser (Qiagen, Hilden, Germany) for 2×2 min at 30 Hz. Total RNA was isolated using the Trizol protocol according to the manufacturer's instructions and RNeasy mini columns (Qiagen). RNA quality was checked using a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany). RNA samples subsequently used for microarray analysis had an RNA integrity number>9.0.

2.4. Microarray and pathway analysis

The microarray analysis using Mouse 44K microarrays (G4122A; Agilent Technologies, Inc., Santa Clara, CA, USA) was done according to the published protocol [31] as described [28]. The RNA of 12 animals per group was individually hybridized to microarrays using a reference pool design. Significantly regulated genes were identified with Student's t test without (P<.05) or with false discovery rate (FDR) analysis at a cutoff of 0.05 according to the Benjamini–Hochberg procedure [32]. Indicated genes were classified manually based on gene ontology (GO) terms for the process and function categories which are additionally provided as Supplementary Table S1. The visualization and analysis tool, PathVisio, was used to perform pathway analysis [33]. In this analysis, the WikiPathways curation analysis pathway collection for mice [34] and the mouse gene database version 20100601 were used. Pathways were ranked based on a z-score.

2.5. Quantitative real-time PCR

RNA (3 μg) was reversely transcribed with 150 fmol oligo(dT)15 primers and 180 U Moloney murine leukemia virus reverse transcriptase (Promega) in a total volume of 45 μl. Real-time PCRs (Mx3005P QPCR System; Stratagene, Amsterdam, the Netherlands) were performed in triplicates with 1 μl of twofold-diluted cDNA in 25-μl reaction mixtures using SYBR Green I (Molecular Probes, Eugene, OR, USA) as fluorescent reporter. The annealing temperature was 60°C for all PCRs. PCR products were quantified with a standard curve ranging from 10^4 to 10^9 copies of each amplicon. Primers (Table 1, Sigma-Aldrich; for selenoproteins, see Ref. [28]) were designed to be specific for cDNA by placing at least one primer onto an exon/intron boundary with PerlPrimer v1.1.14. The mean of the selenium-insensitive reference genes *Rpl13a* and *Hprt1* [28] was used for normalization [35].

2.6. Preparation of protein lysates

Leukocyte pellets were suspended in 250 μl homogenization buffer (100 mmol/L Tris–HCl, 300 mmol/L KCl and 0.1% Triton X-100, pH 7.6) containing 2 μl of protease inhibitor cocktail III (Calbiochem, Bad Soden, Germany) and homogenized with a TissueLyser (Qiagen) for 2×2 min at 30 Hz. All lysates were centrifuged for 15 min at 20,000g and 4°C before protein content was estimated according to Bradford [36]. Samples were stored at −80°C.

2.7. GPx activity

GPx activity was measured in the glutathione reductase-coupled test optimized for tissue samples [37]. A total of 2.5 μl plasma or 4 μl leukocyte lysate was measured in a total volume of 250 μl in a 96-well microtiter plate absorbance reader (Synergy 2;

Biotek Instruments GmbH, Bad Friedrichshall, Germany). The absorbance reader corrects the different filling levels to a light path length of 1 cm. In this way, activity can be calculated according to Lambert–Beer's law. GPx activity was estimated with 50 µmol/L H_2O_2 as substrate and expressed as mU/mg protein for leukocytes and mU/ml plasma, respectively. One unit (U) was defined as consumption of 1 µmol nicotinamide adenine dinucleotide phosphate (NADPH) per minute.

2.8. TrxR activity

TrxR activity was estimated by the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoic acid (TNB) by NADPH [38]. Ten microliters of leukocyte lysate was mixed with 100-µl reaction mixture containing 100 mmol/L KPi, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4, and 15 µl DTNB (50 mmol/L in DMSO) in a 96-well plate. The reaction was started by adding 25 µl of 2 mmol/L NADPH. TNB production was monitored in a plate reader (Synergy 2; Biotek Instruments GmbH, Bad Friedrichshall, Germany) at 412 nm at 25°C. TrxR-independent TNB formation, determined in the absence of NADPH, was subtracted, and data were expressed as mU/mg protein. One unit is defined as the consumption of 1 µmol NADPH, i.e., production of 2 µmol of TNB (extinction coefficient=13.6 $(mmol/L)^{-1} \cdot cm^{-1}$ per min).

2.9. Myeloperoxidase activity

Myeloperoxidase (MPO) activity of leukocyte homogenates was determined as previously described [39] with some modifications. Briefly, 40 µl of eightfold-diluted leukocyte sample was added to 190 µl reagent buffer (0.1 mol/L sodium citrate buffer, pH 5.5 containing 0.125% Triton X-100 and 0.8 mmol/L o-dianisidine dihydrochloride added from an 80-mmol/L stock solution in DMSO) into a 96-well plate. Each sample was measured twice, with and without the MPO inhibitor sodium azide (0.01%). Reaction was started by injecting 10 µl of 2 mmol/L H_2O_2 (final concentration: 100 µmol/L) per well. Absorbance was measured using a microtiter plate absorbance reader (Synergy 2; Biotek Instruments GmbH) at 460 nm. Production of oxidized o-dianisidine is equivalent with H_2O_2 consumption and was determined using an extinction coefficient of 11.3 $(mmol/L)^{-1} \cdot cm^{-1}$ [40]. One unit (U) was defined as consumption of 1 µmol H_2O_2 per minute.

2.10. Lysozyme activity

The lysoplate assay was performed as described [41] with a final concentration of 50 mg of lyophilized *Micrococcus lysodeikticus* in 100 ml of 1% agarose in 0.15 mol/L sodium phosphate buffer, pH 6.6. A total of 25 µl of undiluted sample was transferred to filter paper positioned on the agar plate. The zones of lysis were measured after 18 h of incubation at 25°C using Quantity One software (BioRad, Munich, Germany). The values were converted to µg of lysozyme per mg protein by reference to a standard curve.

2.11. Plasma selenium level

Selenium content of the plasma was measured fluorometrically as previously described [28].

2.12. Statistical analysis

Comparing two groups, significant differences were calculated by an unpaired Student's *t* test. A *P* value of <.05 was regarded as statistically significant. Fold-changes (FCs) are calculated as mean of selenium restricted/selenium adequate.

3. Results

3.1. Plasma selenium concentration and GPx activity as well as GPx and TrxR activity in splenic leukocytes were down-regulated by marginal selenium deficiency

Plasma selenium levels (Fig. 1A) and plasma GPx activity (Fig. 1B) mainly representing activity of GPx3 were drastically reduced upon feeding the selenium-poor diet. Together with liver and colon GPx activity measured previously in these mice [28], it is clear that feeding a diet containing half the estimated nutrient requirements of mice [30] already substantially reduces classical markers for the selenium status. In contrast, in leukocytes, the drop in GPx activity to 76% was less pronounced (Fig. 1C). Compared to the GPx activity in selenium-adequate colon (150 mU/mg protein), leukocyte GPx activity was relatively high (400 mU/mg protein), but still below the activity in the liver (750 mU/mg protein) [28]. Also, leukocytic TrxR activity was only marginally down-regulated to 88% by feeding a selenium-poor diet (Fig. 1D). Mean levels of TrxR activity

were approximately 10 mU/mg protein, which is comparable to activities measured in selenium-adequate colon (authors' unpublished observations).

3.2. Marginal selenium deficiency decreases the expression of a subset of selenoproteins in splenic leukocytes

Microarray analysis revealed that 18 out of 24 murine selenoproteins are expressed in leukocytes (Table 2). *Gpx3*, *Selv*, *Seln* and the genes of the three DIO were not detectable in any dietary group. To validate the microarray data, all 18 selenoproteins were analyzed by qPCR. In both microarray analysis and qPCR, *Sepw1*, *Gpx1*, *Selh* and *Sep15* were the most significantly down-regulated selenoprotein genes under selenium restriction. The decrease in *Gpx4* was statistically significant when estimated by microarray analysis and close to significance in the qPCR assay. *Selk*, *Selm* and *Selt* were significantly down-regulated in qPCR analyses only. *Gpx1* is the selenoprotein with the highest mRNA expression level in the leukocytes samples analyzed (Fig. S1A), followed by *Gpx4*, *Sepx1*, *Sep15*, *Sepw1* and *Sepp1*. This selenoprotein expression pattern highly correlates with the published expression pattern for T-cells isolated from lymph node, spleen and thymus [42].

3.3. Inflammatory response and heme biosynthesis are preferentially affected by selenium restriction

According to microarray analysis, 16,866 transcripts were expressed twofold above the background in leukocytes from mice of different selenium status. Using the statistical criterion FDR with a cutoff of <0.05, only the expression of *Sepw1* (FC: 0.66; FDR: 0.024) and *Eif4e3* (FC: 0.83; FDR: 0.025) was significantly decreased under selenium restriction, which was confirmed by qPCR. Based on a less stringent statistical criterion, a *P* value<.05, 1319 genes were significantly changed, 762 were down-regulated under selenium restriction, while 557 were up-regulated.

Table 1
Primer sequences (5′→3′)

| Gene name	Acc. number	Primer sequence	Product
Camp	NM_009921.1	fwd TATGTGGCAAGGCAGAGCGG rev CTGTGCACCAGGCTCGTTACAG	139 bp
Ccna2	NM_009828.2	fwd GTTTGATAGATGCTGACCCGTACC rev CCAATGACTCAGGCCAGCTC	110 bp
Chi3l3	NM_009892.1	fwd ATGGCCTCAACCTGGACTGG rev AGCCTTGGAATGTCTTTCTCCACA	136 bp
Ctsg	NM_007800.1	fwd TGAGGCAGGGAAGATCATTGGA rev CACCAGAAACCCTCCACAAGCA	114 bp
Fech	NM_007998.4	fwd TCCAGAGGAGAAGAGAAGCGAG rev GTACTGGACCAACCTTGGACTG	181 bp
Hmbs	NM_001110251.1	fwd AAAGATGGGCAACTGTACCTGAC rev TTACGGGCAGTGATTCCAACCAG	150 bp
Ltf	NM_008522.3	fwd TCTCTGTGCCCTGTGTATTGGT rev TTCTCAGCCAGACACCTTAAAGCC	107 bp
Lyz1/2	NM_013590.3	fwd TTCGAGCATGGGTGGCATGG rev GGCTGCAGTAGAAGCACACC	100 bp
Mpo	NM_010824.1	fwd GGCCTCCCAGGATACAATGC rev ACACCGCCCATCCAGATGTC	157 bp
Ngp	NM_008694.2	fwd AGAGACAGCCTAAAGACTGCGAC rev TTCTTGGGTATCCTCTCGACTGC	136 bp
Prdx2	NM_011563.5	fwd GGACTACAGAGGGAAGTACGTGG rev GCAGCCTAGCTTTCGGAAGTC	117 bp
S100A8	NM_013650.2	fwd TGAGTGTCCTCAGTTTGTGCAG rev CTACTCCTTGTGGCTGTCTTTGTG	151 bp
Reference genes:			
Hprt1	NM_013556	fwd GCAGTCCCAGCGTCGTG rev GGCCTCCCATCTCCTTCAT	168 bp
Rpl13a	NM_009438	fwd GTTCGGCTGAAGCCTACCAG rev TTCCGTAACCTCAAGATCTGCT	157 bp
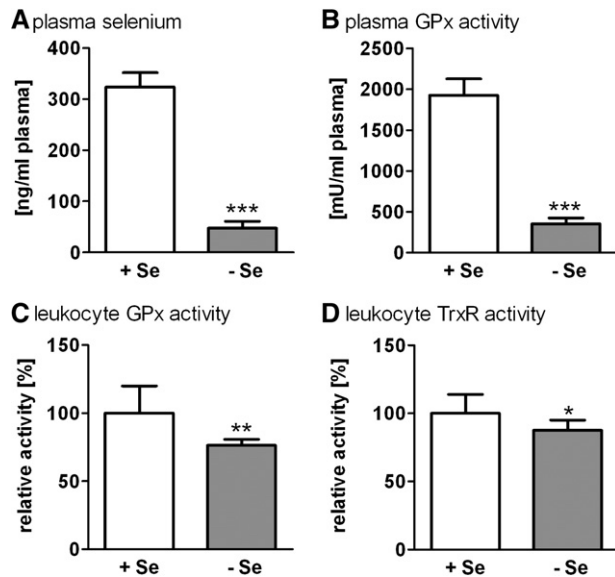
Fig. 1. Plasma selenium concentration (A), GPx activity in plasma (B) and splenic leukocytes (C) and TrxR activity in splenic leukocytes (D) decrease by feeding a marginal selenium-deficient diet. +Se: mice fed a selenium-adequate diet (0.15 mg/kg diet); −Se: mice fed a marginal selenium-deficient diet (0.086 mg/kg diet). Values are means of 12 animals per group for assays measured in plasma and 10 animals for GPx and TrxR activities (with +Se set to 1) measured in triplicate±S.D. *P<.05, **P<.01 and ***P<.001 vs. the selenium-adequate feeding using Student's t test.

A subgroup of 83 genes (Table 3) was selected from these corresponding to genes with a P value<.05 and an absolute FC>1.4 and classified at the basis of gene ontology terms (see "Methods and materials"). Out of these 83 genes, only one gene was up-regulated on the selenium-poor diet; the remainder were down-regulated. The likely explanation for this phenomenon is that, in the underlying data set, absolute FCs of down-regulated genes are higher than those of up-regulated genes. From the selenoproteins, only Sepw1 and Gpx1 showed up in the list because the FCs of the others were too small to fall into the selected range. The classification revealed a consistent down-regulation under selenium restriction of genes involved in inflammatory response, cell cycle, transport, heme biosynthesis and DNA binding (Table 3). The largest group was inflammatory response

including 25.3% of all genes, followed by cell cycle with 14.5%. Pathway analysis of the whole data set based on a P value<.05 and an absolute FC>1.2 using PathVisio partially confirmed these results, with the heme biosynthesis pathway being the most strikingly affected with a z-score of 10. The whole pathway consists of nine proteins, and gene expression of six of them was significantly reduced under selenium restriction (Fig. S2). Affected genes were Alas2, Hmbs, Cpox and Fech (listed in Table 3) and, additionally, uroporphyrinogen decarboxylase (Urod, FC: 0.72; P value=.014) and protoporphyrinogen oxidase (Ppox, FC: 0.81; P value=.013). The heme biosynthesis pathway was followed by the pathways for DNA replication (z-score=8) and cell cycle (z-score=7.9). According to pathway analysis, pathways representative for inflammation were not affected by the selenium status.

Fold-changes of genes for core enzymes of the heme biosynthesis pathway, Hmbs and Fech, and of inflammation-related genes (Mpo, Ltf, Ctsg, S100a8, Camp, Ngp, Prdx2, Lyz2 and Chi3l3) were confirmed by qPCR. As a representative of the cell cycle group, cyclin A2 was chosen for qPCR analysis (Table 3). Representative genes linked to inflammation were generally expressed higher than other genes analyzed in leukocytes; the highest expression levels were observed for Ngp and S100A8 (Fig. S1B). Mutual confirmation of gene expression data by two techniques demonstrated the validity of the data.

3.4. Reduced mRNA expression correlated with a decreased activity of MPO and lysozyme

To test whether the change in mRNA levels influences functionality, enzyme activity was tested for MPO and lysozyme. In both cases, the reduced mRNA expression in mice fed the selenium-restricted diet was found to be correlated with lower enzymatic activity (Fig. 2). This effect did not reach statistical significance, probably due to the high variance of the enzyme activities in animals of the selenium-adequate group.

3.5. Nuclear factor-κB target gene expression is repressed under marginal selenium deficiency

For further analysis of the classified genes, a common transcription factor was searched to explain their selenium-dependent regulation. Twelve of the classified genes (Table 3, gene names in

Table 2
Leukocyte selenoprotein genes responding to selenium supply as measured by microarray analysis and qPCR

Gene name	Description	Acc. number	Microarray (n=12)		qPCR (n=12)	
			P value	FC	P value	FC
Sepw1	Selenoprotein W	NM_009156	2.66×10^{-6}	0.66	9.11×10^{-8}	0.68
Gpx1	Glutathione peroxidase 1	NM_008160	.000081	0.63	.006	0.6
Selh (2700094K13Rik)	Selenoprotein H	NM_001037279	.0005	0.81	2.34×10^{-6}	0.83
Sep15	Selenoprotein 15	NM_053102	.007	0.85	.006	0.55
Gpx4	Glutathione peroxidase 4	NM_001037741	.024	0.87	.055	0.87
Sepp1	Selenoprotein P	NM_001042614	.054	0.84	.185	0.9
Gpx2	Glutathione peroxidase 2	NM_030677	.068	1.14	.484	0.95
Sepx1 (MsrB1)	Selenoprotein X	NM_013759	.227	0.86	.093	0.75
Seli (D5Wsu178e)	Selenoprotein I	NM_027652	.377	0.96	.191	0.91
Selt (2810407C02Rik)	Selenoprotein T	NM_001040396	.399	0.94	.006	0.86
Txnrd1	Thioredoxin reductase 1	NM_001042523	.598	1.03	.184	0.93
Sephs2	Selenophosphate synthetase 2	NM_009266	.662	0.97	.368	0.95
Txnrd2	Thioredoxin reductase 2	NM_013711	.680	0.98	.056	0.83
Sels (H47)	Selenoprotein S	NM_024439	.693	1.02	.093	0.89
Txnrd3	Thioredoxin reductase 3	NM_153162	.695	1.03	.277	0.93
Selk	Selenoprotein K	NM_019979	.751	1.02	.005	0.46
Selm	Selenoprotein M	NM_053267	.845	0.99	.007	0.67
Selo (1300018J18Rik)	Selenoprotein O	NM_027905	.979	1.00	.868	1.01

Genes were sorted by the P value measured in microarray analysis. FC=fold change in relation to the adequate diet.

Table 3
Classification of 83 genes differentially expressed under marginal selenium deficiency

Gene name	Description	Acc. number	Entrez_ID	Microarray		PCR	
				P value	FC	P value	FC
Selenoproteins (2.4%)							
Gpx1	Glutathione peroxidase 1	NM_008160	14775	.000081	0.63	.0057	0.60
Sepw1	Selenoprotein W, muscle 1	NM_009156	20364	2.66×10^{-6}	0.66	9.11×10^{-8}	0.68
Inflammatory response (25.3%)							
Anxa1	Annexin A1	NM_010730	16952	.009	0.57		
C3	Complement component 3	NM_009778	12266	.035	0.65		
Camp	Cathelicidin antimicrobial peptide	NM_009921	12796	.010	0.47	.030	0.40
Chi3l3	Chitinase 3-like 3	NM_009892	12655	.014	0.52	.067	0.43
Chi3l4	Chitinase 3-like 4	NM_145126	104183	.023	0.60		
Ctse	Cathepsin E	NM_007799	13034	.009	0.59		
Ctsg	Cathepsin G	NM_007800	13035	.005	0.64	.013	0.44
H2-DMb1	Histocompatibility 2, class II, locus Mb1	NM_010387	14999	.0004	1.40		
Ifitm6	Interferon induced transmembrane protein 6	NM_001033632	213002	.017	0.64		
Ltf	Lactotransferrin	NM_008522	17002	.005	0.44	.038	0.38
Lyz2	Lysozyme	NM_017372	17105	.014	0.63	.082	0.66
Lzp-s	P lysozyme structural	NM_013590	17110	.030	0.66		
March8	Membrane-associated ring finger (C3HC4) 8	NM_027920	71779	.005	0.62		
Mpo	Myeloperoxidase	NM_010824	17523	.004	0.45	.024	0.48
Ngp	Neutrophilic granule protein	NM_008694	18054	.013	0.49	.059	0.47
Prdx2	Peroxiredoxin 2	NM_011563	21672	.012	0.68	.009	0.46
Rsad2	Radical S-adenosyl methionine domain containing 2	NM_021384	58185	.011	0.64		
S100a8	S100 calcium binding protein A8 (calgranulin A)	NM_013650	20201	.009	0.47	.145	0.57
S100a9	S100 calcium binding protein A9 (calgranulin B)	NM_009114	20202	.044	0.65		
Sphk1	Sphingosine kinase 1	NM_025367	20698	.023	0.69		
Wdnm1-like	Westmead DMBA8 nonmetastatic cDNA 1 like	NM_183249	66107	.035	0.58		
Cell cycle/proliferation (14.5%)							
Birc5	Baculoviral IAP repeat-containing 5 (survivin)	NM_001012273	11799	.010	0.61		
Ccna2	Cyclin A2	NM_009828	12428	.003	0.60	.037	0.59
Ccnb2	Cyclin B2	NM_007630	12442	.020	0.69		
Cdca3	Cell division cycle associated 3	NM_013538	14793	.015	0.68		
Cdca8	Cell division cycle associated 8	NM_026560	52276	.004	0.67		
Cdkn3	Cyclin-dependent kinase inhibitor 3	BC049694	72391	.048	0.71		
Cks2	CDC28 protein kinase regulatory subunit 2	NM_025415	66197	.010	0.68		
Gmnn	Geminin	NM_020567	57441	.014	0.68		
Mki67	Ki-67	X82786	17345	.003	0.60		
Plk1	Polo-like kinase 1	NM_011121	18817	.001	0.68		
Rrm2	Ribonucleotide reductase M2	NM_009104	20135	.015	0.65		
Tyms	Thymidylate synthase	NM_021288	22171	.008	0.66		
Transport (13.3%)							
Ank1	Ankyrin 1, erythroid	NM_031158	11733	.005	0.59		
Aqp1	Aquaporin 1	NM_007472	11826	.006	0.55		
Rhd	Rh blood group, D antigen	NM_011270	19746	.003	0.49		
Slc16a1	Solute carrier family 16, member 1	NM_009196	20501	.006	0.69		
Slc25a37	Solute carrier family 25, member 37	NM_026331	67712	.004	0.62		
Slc38a5	Solute carrier family 38, member 5	NM_172479	209837	.024	0.64		
Slc43a1	Solute carrier family 43, member 1	BC053747	72401	.040	0.71		
Slc43a3	Solute carrier family 43, member 3	NM_021398	58207	.007	0.71		
Slc4a1	Solute carrier family 4 (anion exchanger), member 1	NM_011403	20533	.002	0.48		
Slc6a9	Solute carrier family 6, member 9	NM_008135	14664	.003	0.68		
Tspo2	Benzodiazapine receptor, peripheral-like 1	NM_027292	70026	.004	0.68		
Heme biosynthesis/hemopoiesis (12%)							
Alas2	Aminolevulinic acid synthase 2, erythroid	NM_009653	11656	.042	0.70		
Blvrb	Biliverdin reductase B	NM_144923	233016	.035	0.71		
Cpox	Coproporphyrinogen oxidase	NM_007757	12892	.023	0.64		
Eraf	Erythroid associated factor	NM_133245	170812	.019	0.56		
Fech	Ferrochelatase	NM_007998	14151	.017	0.68	.077	0.57
Hba-a1	Alpha-globin	M10466	15122	.044	0.65		
Hbb-b1	Hemoglobin, beta adult major chain	NM_008220	15129	.012	0.58		
Hebp1	Heme binding protein 1	NM_013546	15199	.035	0.71		
Hmbs	Hydroxymethylbilane synthase	NM_013551	15288	.011	0.59	.028	0.50
Tal1	T-cell acute lymphocytic leukemia 1	NM_011527	21349	.043	0.65		
DNA binding (12%)							
Gfi1b	Growth factor independent 1B	NM_008114	14582	.026	0.69		
H2afj	H2A histone family, member J	NM_177688	232440	.006	0.67		
Hist1h1b	Histone cluster 1, H1b	NM_020034	56702	.014	0.70		
Hist1h2aa	Histone cluster 1, H2aa	NM_175658	319163	.006	0.64		
Hist1h2af	Histone cluster 1, H2af	NM_175661	319173	.006	0.65		
Hist1h4d	Histone cluster 1, H4d	NM_175654	319156	.004	0.67		
Hist2h2ab	Histone cluster 2, H2ab	NM_178213.3	-	.012	0.68		
Hist3h2a	Histone cluster 3, H2a	NM_178218	319162	.004	0.70		
Hmgb2	High mobility group box 2	NM_008252	97165	.001	0.64		
Hist1h2ao	Similar to histone 2a	BC090402	665433	.008	0.62		
Others (20.5%)							

Table 3 (*continued*)

Gene name	Description	Acc. number	Entrez_ID	Microarray		PCR	
				P value	FC	P value	FC
Apol2	Adult male corpora quadrigemina	AK046043	239552	.038	0.70		
Butr1	Butyrophilin related 1	NM_138678	192194	.021	0.69		
C1qdc2	C1q domain containing 2	NM_026125	67389	.009	0.60		
Car2	Carbonic anhydrase 2	NM_009801	12349	.006	0.51		
Ckap4	Cytoskeleton-associated protein 4	NM_175451	216197	.022	0.68		
Clec5a	C-type lectin domain family 5, member a	NM_001038604	23845	.036	0.69		
Epor	Erythropoietin receptor	NM_010149	13857	.008	0.63		
Ermap	Erythroblast membrane-associated protein	NM_013848	27028	.027	0.66		
Hemgn	Hemogen	NM_053149	93966	.036	0.58		
Kel	Kell blood group	NM_032540	23925	.010	0.63		
Metap2	Methionine aminopeptidase 2	NM_019648	56307	.005	0.71		
mICA	Murine inhibitor of carbonic anhydrase	NM_027918	71775	.020	0.68		
Npsr1	Neuropeptide S receptor 1	NM_175678	319239	.009	0.67		
St3gal5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	NM_011375	20454	.021	0.65		
Stmn1	Stathmin 1	NM_019641	16765	.003	0.68		
Trim10	Tripartite motif protein 10	NM_011280	19824	.006	0.69		
Tspan33	Tetraspanin 33	NM_146173	232670	.014	0.64		

Genes were selected according to the following criterion: P value<.05 and absolute FC>1.4. Genes printed in italic are known NF-κB target genes.

italic) were revealed to be nuclear factor (NF)-κB target genes. NF-κB is a key player in inflammation and known to be influenced by selenium. Therefore, the complete microarray expression data set was analyzed for NF-κB target genes. Out of more than 100 genes for known NF-κB targets (listed in Ref. [43] and under www.nf-kb.org) expressed in the leukocyte data set, 40 were significantly regulated by the selenium status (Table 4). Thirty-one genes were down-regulated under marginal selenium deficiency, which account for 77.5%. In addition, the NF-κB target gene *Sels* [44] was unaffected by the selenium status in the microarray, but was also down-regulated in the qPCR analysis (Table 2).

4. Discussion

Feeding a moderate selenium-deficient diet to mice resulted in a consistent down-regulation of the plasma selenium level and GPx activity. As previously reported, GPx activity was also strongly reduced in the liver and colon of these animals [28]. In addition, GPx (Fig. 1C) and TrxR (Fig. 1D) activity was lower in splenic leukocytes, but the fall in activity was less than that in other tissues, which was already shown for CD4$^+$ T-cells isolated from murine spleen [26]. Accordingly, FCs of selenoprotein mRNA levels in leukocytes were smaller than in the colon (see Ref. [28]), but statistically significant, and the affected selenoproteins *Sepw1*, *Gpx1* and *Selh* were the three most selenium-sensitive selenoproteins in both colon and leukocytes [28] (Table 2). In addition, both microarray and qPCR analyses showed that *Sep15* expression was highly affected by selenium intake in leukocytes. In comparison, down-regulation of *Sep15* by selenium restriction was less in the colon [28]. Granulocytes, which represent 18% of the analyzed leukocytes, may substantially contribute to the stronger down-regulation in selenium-poor leukocytes since Sep15 is the major selenoprotein expressed in this subpopulation [45] and was found to be also highly expressed in leukocytes analyzed here (Fig. S1). Although expression of seleno-proteins K, M and T did not appear to be significantly altered in the microarray analysis, all three genes were significantly down-regulated in marginal selenium deficiency when assessed by qPCR, with *Selk* showing the largest FC of all selenoproteins (Table 2). Interestingly, microarray analysis of human lymphocytes after supplementation with 100 μg sodium selenite daily for 6 weeks resulted in up-regulated mRNA expression of both *Selk* and *Sep15* [29]. Thus, it appears that, in leukocytes, mRNA expression of *Sepw1*, *Gpx1*, *Selh*, *Selk* and *Sep15* is sensitive to selenium supply.

The response of selenoprotein expression to selenium intake is known to be tissue specific [46,47]. In a recent study, a panel of molecular biomarkers was analyzed by multiple regression analysis against selenium level and GPx activity in liver and kidney of rats [48]. Hepatic mRNA expression of *Gpx1*, *Sepw1*, *Selh*, *Selk* and *Sep15* was the most significantly correlated with both the selenium level and GPx activity, whereas the ranking in the kidney was *Sepw1*, *Selk*, *Selh*, *Gpx1* and *Gpx3* against the selenium level and *Sepw1*, *Gpx1*, *Selh*, *Txnrd1* and *Selk* against GPx activity. Taking these data together with the present results suggests that *Sepw1*, *Gpx1* and *Selh* represent the most sensitive biomarkers for a moderate selenium deficiency that are common to several tissues, at least in rodents.
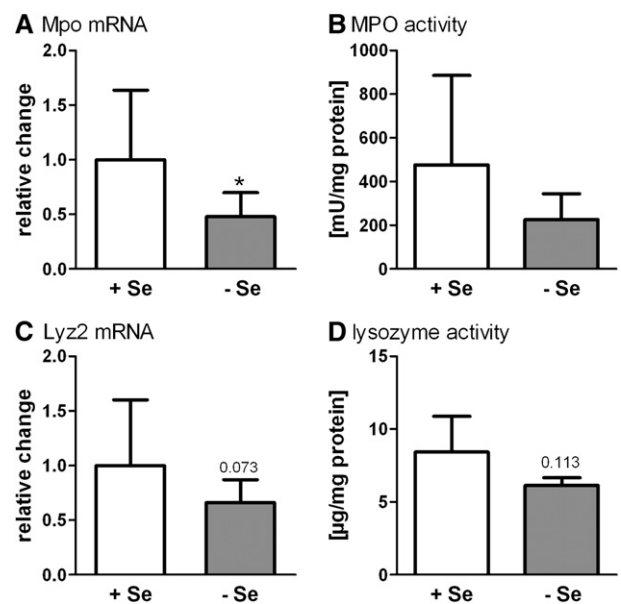

Fig. 2. mRNA expression and activity of MPO and lysozyme (Lyz2) are down-regulated in response to selenium restriction in splenic leukocytes. mRNA expression of *Mpo* (A) and *Lyz2* (C) was measured in 12 animals per group, while MPO activity (B) and the amount of active lysozyme (D) were measured in five animals per group. Gene expression was analyzed by qPCR and normalized to the mean of the reference genes *Hprt1* and *Rpl13a*. +Se: mice fed a selenium-adequate diet (0.15 mg/kg diet); −Se: mice fed a marginal selenium-deficient diet (0.086 mg/kg diet). Values are means±S.D. *P<.05 vs. selenium-adequate feeding using Student's t test.

Table 4
NF-κB target genes differentially expressed in splenic leukocytes

Gene name	Description	Acc. number	Microarray	
			P value	FC
Down-regulated (77.5%)				
Abcb4	ATP-binding cassette, sub-family B	NM_008830	.007	0.72
Birc5	Baculoviral IAP repeat-containing 5 (survivin)	NM_001012273	.010	0.61
Brca2	Breast cancer 2	NM_009765	.015	0.80
C3	Complement component 3	NM_009778	.035	0.65
Camp	Cathelicidin antimicrobial peptide	NM_009921	.010	0.47
Ccl9	Chemokine (C-C motif) ligand 9	NM_011338	.045	0.81
Ccnb2	Cyclin B2	NM_007630	.020	0.69
Cd44	CD44 antigen	NM_009851	.021	0.85
Cdk6	Cyclin-dependent kinase 6	AK030810	.014	0.83
Cks1b	CDC28 protein kinase 1b	NM_016904	.028	0.76
Ctsb	Cathepsin B	NM_007798	.003	0.79
G6pdx	Glucose-6-phosphate dehydrogenase X-linked	NM_008062	.043	0.81
Gclc	Glutamate-cysteine ligase, catalytic subunit	NM_010295	.006	0.78
Hmgb1	High mobility group box 1	BC064790	.014	0.87
Hmgb2	High mobility group box 2	NM_008252	.001	0.64
Hsp90aa1	Heat shock protein 90kDa alpha (cytosolic)	NM_010480	.015	0.81
Kel	Kell blood group	NM_032540	.010	0.63
Lcn2	Lipocalin 2	NM_008491	.041	0.73
Ltf	Lactotransferrin	NM_008522	.005	0.44
Lyz2	Lysozyme	NM_017372	.014	0.63
Myb	Myeloblastosis oncogene	NM_010848	.028	0.83
Pgk1	Phosphoglycerate kinase 1	NM_008828	.009	0.88
Pigf	Phosphatidylinositol glycan anchor biosynthesis, class F	NM_008838	.031	0.95
Plk1	Polo-like kinase 1	NM_011121	.001	0.68
Ppih	Peptidyl prolyl isomerase H	NM_028677	.045	0.90
Prdx2	Peroxiredoxin 2	NM_011563	.012	0.68
S100a8	S100 calcium binding protein A8 (calgranulin A)	NM_013650	.009	0.47
S100a9	S100 calcium binding protein A9 (calgranulin B)	NM_009114	.044	0.65
Slc16a1	Solute carrier family 16 (monocarboxylic acid transporters), member 1	NM_009196	.006	0.69
Top2a	Topoisomerase (DNA) II alpha	NM_011623	.014	0.82
Vcam1	Vascular cell adhesion molecule 1	NM_011693	.009	0.75
Up-regulated (22.5%)				
Adora2a	Adenosine A2a receptor	BC110692	.049	1.10
Afp	Alpha fetoprotein	NM_007423	.001	1.17
Ahctf1	AT hook containing transcription factor 1	NM_026375	.040	1.17
Egr1	Early growth response 1	NM_007913	.034	1.17
Fos	FBJ osteosarcoma oncogene	NM_010234	.035	1.27
Hmgn1	High mobility group nucleosomal binding domain 1	NM_008251	.041	1.21
Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	NM_019408	.019	1.16
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	AK029399	.024	1.16
Rag1	Recombination activating gene 1	NM_009019	.049	1.21

Selenium supplementation in the SELGEN study among others resulted in an up-regulation of the protein biosynthesis network and translation-elongation termination pathways [29]. In the present study, genes for ribosomal proteins, translation elongation and translation initiation factors were mostly down-regulated by moderate selenium deficiency, which reflects their up-regulation upon selenium supplementation in the SELGEN study (Supplementary Table S2). These pathways did not show up in selenium-regulated groups here collected by strict restrictions (Table 3). More interestingly, immune T-cell receptor signaling was up-regulated by selenium in human lymphocytes [29], correlating with the down-regulation of the inflammatory response in the present murine study (Table 3). Therefore, a more detailed comparison of significantly regulated genes in the human and the murine study was performed.

mRNA of *Ltf* (FC: 1.3; *P* value=.0035), *Anxa1* (FC: 1.09; *P* value=.041), *S100a8* (FC: 1.22; *P* value=.034) and *Camp* (FC: 1.29; *P* value=.0013) was up-regulated with selenium supplementation in humans [29] and down-regulated in moderate selenium deficiency in mice (Table 3). Thus, changes observed in the murine data set may also be relevant for humans.

Further pathways strikingly affected by the selenium status are heme biosynthesis and inflammatory response (Table 3). All genes linked to inflammation and confirmed by qPCR were involved in host defense and inflammatory response (Supplementary Table S1). MPO, which is exclusively expressed in neutrophiles and monocytes and for which the heme center of the enzyme is indispensable [49], contributes substantially to the antimicrobial system by generating reactive oxidants like HOCl and radical species [50]. In human neutrophils, MPO levels account for 2%–5% of total cellular protein [51], which explains the need of a high heme biosynthesis rate. Down-regulation of *Mpo* expression in selenium-poor leukocytes was linked to reduced heme biosynthesis and to reduced mRNA expression of the nonheme iron-binding protein lactotransferrin (Table 3). We speculate that these data reflect a link between selenium intake and iron metabolism.

MPO-derived oxidants not only are functional in antimicrobial and cytotoxic effects, but also are involved in signaling and the modulation of the immune response. HOCl has been reported to activate NF-κB [52], which might explain the inhibition of NF-κB target gene expression (Table 4) in parallel to the reduction of MPO activity (Fig. 2) under selenium restriction. However, in contrast to this first study, subsequent studies revealed that most of the HOCl-derived chloramines act as NF-κB inhibitors (reviewed in Ref. [53]). Also, the impact of the selenium status on the activity of the NF-κB pathway is ambivalent. NF-κB activation is inhibited by selenium supplementation as a result of an increased expression of GPx isoenzymes [54] or by direct overexpression of GPx1 [55] or GPx4 [56]. As often not expected, high doses of selenium also promote prooxidant effects, which are mostly attributed to selenium deficiency. Until today, the redox regulation of NF-κB is still a complex issue which is still controversially discussed [57,58]. Consistent down-regulation of NF-κB targets shown here suggests an inhibition of the NF-κB pathway under moderate selenium deficiency.

Down-regulation of inflammation-dependent genes and NF-κB targets under marginal selenium deficiency would result in a reduced capability to deal with infections, which coincidences with results obtained in severe selenium deficiency mouse models. For example, the response of splenic natural killer cells to *Listeria monocytogenes* infection was significantly reduced in severe selenium deficiency compared to selenium-adequate mice [59]. Selenium-supplemented CD4$^+$ T-cells had a higher antigen-specific response and T-cell receptor signaling compared to cells analyzed in poorly or adequately fed mice [26]. T-cells without selenoprotein expression (knockout of the selenoprotein specific tRNA) showed poorly raised serum levels of antigen-specific antibodies after immunization with different antigens [42]. Loss of selenoproteins under marginal selenium deficiency might therefore also be linked to a defective T-cell-mediated immune response.

In conclusion, the current data provide evidence for the suitability of the selenoproteins *Sepw1*, *Gpx1*, *Selh*, *Selk* and *Sep15* as biomarkers for a marginal selenium deficiency in murine as well as in human leukocytes. In addition, inhibition of the inflammatory response under marginal selenium deficiency correlated with an up-regulation observed in selenium-supplemented human subjects. The correlations between selenium-dependent changes in gene expression in human and murine leukocytes make the murine data a reasonable basis to identify selenium-dependent biomarkers in humans.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.06.011.

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