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Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice

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Abstract

Background: Selenium (Se) exerts its biological activity largely via selenoproteins, which are key enzymes for maintaining the cellular redox homeostasis. However, besides these beneficial effects there is also evidence that an oversupply of Se might increase the risk towards developing metabolic disorders. To address this in more detail, we directly compared effects of feeding distinct Se compounds and concentrations on hepatic metabolism and expression profiles of mice.

Methods: Male C57BL6/J mice received either a selenium-deficient diet or diets enriched with adequate or high doses of selenite, selenate or selenomethionine for 20 weeks. Subsequently, metabolic parameters, enzymatic activities and expression levels of hepatic selenoproteins, Nrf2 targets, and additional redox-sensitive proteins were analyzed. Furthermore, 2D-DIGE-based proteomic profiling revealed Se compound-specific differentially expressed proteins.

Results: Whereas heterogeneous effects between high concentrations of the Se compounds were observed with regard to body weight and metabolic activities, selenoproteins were only marginally increased by high Se concentrations in comparison to the respective adequate feeding. In particular the high-SeMet group showed a unique response compromising higher hepatic Se levels in comparison to all other groups. Accordingly, hepatic glutathione (GSH) levels, glutathione S-transferase (GST) activity, and GSTpi1 expression were comparably high in the high-SeMet and Se-deficient group, indicating that compound-specific effects of high doses appear to be independent of selenoproteins.

Conclusions: Not only the nature, but also the concentration of Se compounds differentially affect biological processes.

General significance: Thus, it is important to consider Se compound-specific effects when supplementing with selenium.

Keywords: Selenium, liver, redox status, energy metabolism
Abbreviations:
AKT, protein kinase B; ARE, anti-oxidant response elements; CCB, colloidal Coomassie Blue; DIGE, difference gel electrophoresis; ERK, extracellular regulated kinase; Fasn, fatty acid synthase; Gck, glucokinase; Gpx/GPx, glutathione peroxidase; GS, glutathione synthetase; G6pc, glucose-6-phosphatase; GSH, glutathione; GST, glutathione S-transferase; Hprt, hypoxanthine guanine ribosyl transferase; Keap1, Kelch-like ECH-associated protein 1; NQO1, NAPH dehydrogenase 1; Nrf2, NF-E2-related factor 2; Pdha, hepatic pyruvate dehydrogenase; PTEN, phosphate and tensin homolog; PTP, protein tyrosine phosphatase; RPL13a, ribosomal protein L13a; Se, selenium; SeMet, selenomethionine; SOD, superoxide dismutase; TG, triglyceride; Txnrd/TrxR, thioredoxin reductase
1 Introduction

During the last two decades the essential trace element selenium (Se) has gained considerable attention as a potential cancer preventive agent [1] leading to a marketing boom of Se supplements [2]. Se is an integral part of selenoproteins, which contain the redox-active amino acid selenocysteine (SeCys) in their catalytically active center [3,4]. The best characterized selenoproteins like glutathione peroxidases (GPx) and thioredoxin reductases (TrxR) play key roles in detoxifying hydrogen peroxide, lipid peroxides and in the reduction of cellular disulfides generated by interactions of free radicals and other oxygen-derived species as part of the normal cellular metabolism. Further selenoproteins are involved in the modulation of the immune response, inflammatory processes as well as chemoprevention [5]. Insufficient Se intake results in a rapid and selective downregulation of several selenoproteins like the ubiquitously expressed GPx1, which impairs the anti-oxidant capacity of a cell. In contrast, high Se concentrations might directly affect the cellular redox balance by inducing the formation of thiol-reactive selenocompounds and by generating oxygen-free radicals [6]. This pro-oxidant effect is supposed to differ depending on the used selenocompound; e.g. selenite, selenocystine, methylessenimic acid, and methylselenocysteine are known to modulate the cellular redox state and are therefore termed “redox active selenocompounds” [7]. Thus, the correlation of the cellular redox status with the Se supply follows a U-shape curve. Based on this strong relationship, it has been shown that the Se status also directly modulates the expression of non-selenoproteins via e.g. redox-sensitive transcription factors like the NF-E2-related factor 2 (Nrf2) [8]. Under physiological conditions, the redox-sensitive Kelch-like ECH-associated protein 1 (Keap1) restraints Nrf2 in the cytosol, whereas oxidative stress disrupts the Keap1/Nrf2 complex leading to the nuclear translocation of Nrf2. This results in the activation of genes carrying anti-oxidant response elements (ARE) within their promoters like glutathione S-transferases (GST) and enzymes of the glutathione (GSH) biosynthesis pathway [9]. Furthermore, the modification of redox-sensitive phosphatases is supposed to be the cause of insulin-mimetic and anti-diabetic properties of supranutritional Se concentrations [10–12], but also insulin-antagonistic and even pro-diabetic effects of an over-supply of Se compounds have been observed and discussed [13–15]. Upon binding to its receptor, insulin initiates a signaling cascade, which is accompanied by a burst of hydrogen peroxide (H$_2$O$_2$) that acts as a second messenger by modifying redox-sensitive cysteine residues [10,16]. It has been demonstrated that the activity of the protein tyrosine phosphatase 1B (PTP1B), which is a negative regulator of the insulin signaling pathway, could be modulated via Se availability in
a redox-dependent manner [17]. Phosphorylation of protein kinase B (AKT) links the insulin signaling cascade to the energy metabolism. Supra-nutritional intake of Se led to an increased AKT phosphorylation in visceral adipose tissues of pigs [18] and to a delayed insulin-induced phosphorylation of AKT and forkhead box protein 1a/3 (FoxO1a/3) in myocytes [19]. Mice overexpressing GPx1 develop insulin resistance, hyperlipidemia and obesity [20], whereas mice with decreased GPx1 activity and consequently increased cellular levels of reactive oxygen species (ROS) are more sensitive to insulin [21]. In this context it is discussed that enhanced levels of anti-oxidant enzymes may lead to an “over-scavenging” of H₂O₂, which is involved in and necessary for redox signaling pathways including the insulin signaling cascade [22] thereby resulting in the deregulation of glycolysis, gluconeogenesis and lipogenesis. Regarding the insulin-mimetic, anti-diabetic or even pro-diabetic effects of Se supply a recently published Cochrane review discussed the inconsistency of results obtained from different trials [23]. A possible explanation for such discrepant data could be the use of different Se compounds.

The current idea is that selenomethionine (SeMet), the major form of Se in foods, is more faster absorbed in the small intestine when compared to inorganic forms, such as selenate and selenite [24–26]. Furthermore, SeMet does not only provide Se for selenoprotein synthesis, but can also be non-specifically incorporated into proteins instead of methionine [27]. This allows Se to be stored in the organism and to be released by normal protein turn-over [28], but can also give rise to toxic effects once functional methionine is substituted in enzymes. The inorganic Se compound selenate shares a sodium-dependent transport system with sulfates, whereas selenite is mainly absorbed by passive diffusion [29,30]. Under conditions of an increased availability of inorganic selenocompounds an enhanced Se excretion in form of methylated selenocompounds or selenosugars occurs, while SeMet is slowly metabolized and rather accumulates in the body. Furthermore, selenite is supposed to have direct effects on the cellular redox status, which in contrast has not been attributed to SeMet.

Based on this information the present study aimed to directly compare the effects of long-term feeding with different Se compounds and doses on metabolic health of mice. Therefore, parameters of the insulin-regulated energy metabolism were analyzed and correlated to the hepatic selenoprotein expression and the anti-oxidant capacity of the liver. In addition, hepatic Nrf2 target gene expression was analyzed in response to different Se compounds and concentrations. New Se-sensitive proteins are identified using a proteome approach followed by mass spectrometry.
2 Material and Methods

2.1 Animals and diets
Four-week old healthy male C57BL/6J mice (Harlan Laboratories, Netherlands) kept under conventional conditions (room temperature 22±1°C, humidity 50±10 %) with free access to their respective diets and autoclaved tap water were used. The experiments were approved by the ethical committee of the Martin Luther University Halle-Wittenberg (42502-2-1187MLU). Mice were randomly assigned to seven experimental groups of 8 individuals/group. The groups were fed with diets supplemented either with adequate (ad; 150 µg Se/kg diet) or supra-nutritive (high, hi; 750 µg Se/kg diet) Se concentrations in form of selenite, selenate and SeMet, respectively, whereas the control group received a Se-deficient diet (-Se). The diets were based on torula yeast and Se-deficient wheat and with the exception of the Se contents composed according to the National Research Council (NRC) recommendations for rodents (Table S1). Se content of the diets was confirmed by ICP-MS (-Se < 20; ad-selenite 165±1.9; hi-selenite 768±3.1; ad-selenate 157±1.9; hi-selenate 762±2.3; ad-SeMet 161±2.7; hi-SeMet 765±3.4 µg Se/kg diet). After the 20 weeks feeding period and 4 hours of feeding deprivation, mice were decapitated under CO₂ anesthesia. Blood was collected in heparinized tubes and centrifuged (15 min, 4°C, 2.000 x g). Plasma was stored at -80°C until further analysis. Liver tissue samples were excised, snap frozen in liquid nitrogen, and stored at -80°C until further use.

2.2 Determination of plasma glucose and insulin concentrations
Glucose concentrations were measured in plasma samples collected from tail veins using a glucometer according to the manufacturer´s instructions. Plasma insulin levels were assayed using a mouse insulin ELISA kit (Mercodia, Uppsala, Sweden). The HOMA-IR was calculated using glucose and insulin concentrations obtained after 4 hours of food withdrawal using the following formula: plasma glucose [mg/dl] x fasting insulin [ng/ml]/405 [31].

2.3 Determination of triglyceride concentrations in plasma
The total triglyceride (TG) content of plasma samples was determined using a commercially available kit (Fluitest TG, Analyticon Diagnostics, Lichtenfels, Germany) according to the manufacturer’s instructions.

2.4 Se analysis in liver tissues using inductively coupled plasma mass spectrometry (ICP-MS)
The digestion of the samples for ICP-MS studies was performed on an Advanced Microwave Digestion System (ETHOS 1, Milestone, Italy) using an HPR-1000/10S high pressure
segmented rotor. Determination of the Se content in liver tissues of mice was performed by ICP-MS using the Thermo Scientific iCAP Qc ICP-MS (Thermo Scientific, Bremen, Germany) with the operational software Qtgra. For $^{80}$Se determination the instrument was adjusted for optimum performance in gas H$_2$/He mixture in Collision Cell Technology (CCT) mode using the supplied auto-tune protocols (Thermo Scientific, Bremen, Germany) [32].

2.5 Real-time RT-PCR
RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method [33] and then subjected to PCR analysis as recently described [34]. Briefly, 1 µg RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit and Oligo dT primers (Thermo Scientific) according to the manufacturer’s protocol. Real-time PCR was performed using SYBR Green I as fluorescent reporter. Target-specific primers (Table S2) were designed using the program Primer3 [35] and validated on agarose gels. Amplification data were analyzed with the Rotor-Gene 6000™ series software (Qiagen, Hilden, Germany) using the method according to Pfaffl [36]. Amplifications of Ribosomal Protein L13a (Rpl13a) and hypoxanthine guanine ribosyl transferase (Hprt) were used for normalization. The data are represented as relative mRNA expression levels as x-fold of the Se-deficient group.

2.6 Preparation of tissue homogenates
For analysis of hepatic GPx, TR, NADPH quinone dehydrogenase 1 (NQO1), and GST activity liver tissue lysates were prepared in Tris buffer (100 mM Tris, 300 mM KCl, 0.1 % Triton X-100, pH 7.6; Calbiochem® protease inhibitor cocktail II (Merck Millipore, Darmstadt Germany)) using a tissue lyser (Qiagen). Lysates for the determination of hepatic superoxide dismutase (SOD) activity were prepared in 0.05 M potassium phosphate buffer containing protease inhibitor (ROCHE, Basel Switzerland). For Western blot analyses tissue lysates were prepared in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5 % DOC, 1 % NP-40, 0.1 % SDS) containing protease and phosphatase inhibitors (HALT™, Thermo Scientific). All lysates were centrifuged for 30 min at 14,000 x g and 4°C. Supernatants were stored at -80 °C until further analyses.

2.7 Western blot analysis
Western blot analysis was performed as recently described [37]. For immune detection the primary (monoclonal or polyclonal) antibodies, purchased form Cell Signaling Technology (Danvers, MA, USA), were anti-phospho-AKT$^{Ser473}$, anti-phospho-AKT$^{Thr308}$, anti-AKT (#9916); anti-phospho-ERK1/2 (#4370), anti-phospho-PTEN$^{Ser380/Thr382/383}$ (#9549), anti-
GAPDH (#2118). The secondary HRP-coupled anti-rabbit immunoglobulins (#7074) were purchased from Cell Signaling Technology.

2.8 Determination of enzyme activities
Total GPx activity was measured in plasma and liver samples according to the method of Lawrence and Burk [38], which was modified for 96-well microtiter plates as recently described using H$_2$O$_2$ as substrate [39]. TrxR catalyze the formation of 5’-thionitrobenzene (TNB) by using the substrate 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB). The consumption of NADPH was spectrophotometrically determined at 412 nm for 2 min at 25°C as previously described [40]. Total SOD activity was determined according to the method of Marklund and Marklund [41]. For determination of MnSOD activity, 1 mM KCN was added to the assay buffer, which selectively inhibits the Cu/ZnSOD. NQO1 activity was determined as previously described [8]. GST activity was spectrophotometrically measured using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of reduced glutathione according to the protocol of Habig and co-authors [42]. The native activity of PTP was determined by analyzing paranitrophenyl phosphate hydrolysis, which could be monitored at 410 nm [43].

2.9 GSH assay
Total GSH (reduced and oxidized) concentrations were determined in plasma and liver homogenates according to a standard procedure coupled to GSH reductase and DTNB [44]. The concentrations were calculated using a GSSG standard curve ranging from 1 to 10 nM GSH equivalent/ml.

2.10 Specific labeling of reduced cysteine residues
Labeling of reduced cysteine residues was performed using the thiol reactive S-300 fluorescence Dye (NH DyeAgnostics, Halle, Germany) according to the manufacturer’s protocol with the exception that the reducing step with TCEP prior to the labeling procedure was omitted in order to label only reduced cysteine residues. Lysates of liver tissues were prepared under nitrogen overlay in 7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris (pH 7.5) using a grinding kit (Readyprep mini grinders, Bio-Rad Laboratories GmbH, Munich, Germany). 5 µg of total protein were labeled with S-300 at 37°C for 1 hour. The reaction was stopped by adding one volume of the labeling buffer containing 2 % DTT. After one-dimensional SDS PAGE separation, gels were scanned using a FUJI FLA-500 scanner (FujiFilm GmbH, Düsseldorf, Germany) and total fluorescence per lane was quantified and
normalized to post fluorescence scanning performed on colloidal Coomassie Blue staining (CCB, Applichem, Darmstadt, Germany) [45].

2.11 Proteome analysis by difference gel electrophoresis (DIGE) and mass spectrometry

Four biological liver samples from each group were 3-times washed in cold PBS, subsequently grinded (Readyprep mini grinders, Bio-Rad Laboratories GmbH, Munich, Germany) and solved in DIGE lysis buffer (30 mM Tris, 7 M urea (Applichem, Darmstadt, Germany), 2 M thiourea (Sigma, Deisenhofen, Germany), 4 % (w/v) CHAPS (Applichem), pH 8.5). After sonification and centrifugation (13,000 x g, 90 min, 15°C) supernatants were collected and stored at -80°C before protein concentrations were determined as previously described [46]. DIGE analysis was performed using the minimal labeling approach according to the manufacturer’s instructions (NH DyeAGNOSTICS) with the exception that 25 µg of protein were labeled with 100 pmol G-dye. Isoelectric focusing using Immobiline DryStrips pH 3-10 NL (18 cm, GE Healthcare) and second dimension SDS separation were done as previously described [47]. After separation the gels were subsequently washed in distilled water, fixed with 10 % acetic acid (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) and 40 % EtOH (Sigma-Aldrich Chemie GmbH, Munich, Germany) followed by a wash in distilled water. Gels were scanned as previously described [48] and gel image analysis was carried out with the Delta2D software package (Decodon GmbH, Greifswald, Germany) according to the manufacturer’s guidelines. Protein spots were considered as differentially expressed if a 1.5-fold altered expression level was observed. In addition, the spots of interest were statistically analyzed with the Student’s t test and only those selected for mass spectrometric identification with a p value < 0.05. For mass spectrometric (MS) analysis a preparative gel was loaded with an amount of 500 µg protein and spots of interest were subsequently picked, in gel digested (DigestPro, Intavis, Cologne, Germany) and further analyzed by MS using the matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (ultrafleXtreme™, Bruker Daltonics Inc., Bremen, Germany) [49]. The PMF dataset analysis was performed using the MASCOT software package (Matrix Science, Dauhaim, USA). In addition the functional classification of the target structures was based on literature backsearches thereby relying on Pubmed entries.

2.12 Statistical analysis

Mean values were calculated from results of 8 animals/group or 4 animals/group in the case of the proteome analyses and given as means ± their standard error of mean (S.E.M.). SPSS 20 software was used to analyze significant differences within the groups, therefore one-way
ANOVA was implemented after asserting the normality of distribution (Shapiro-Wilk test) and the homogeneity of variance (Levene test). If variances were homogeneous the least significant (LSD) test was used, otherwise the Games-Howell test was employed. Differences between the groups were considered to be significantly different at p < 0.05.

3 Results

3.1 A high SeMet intake resulted in lower body weight gain without affecting feed intake

Prior to the experiment the mean body weights of all mice were comparable between the seven groups. After 20 weeks of intervention mean body weights of the groups treated with hi-selenite (p = 0.037) or hi-selenate (p = 0.017) were significantly higher when compared to the Se-deficient group, while the mean body weight of the hi-SeMet group was lower compared to both the hi-selenate (p = 0.024) and hi-selenite (p = 0.052) groups. The low body weight of the Se-deficient group was accompanied by a lower total food intake when compared to Se-supplemented groups (Table 1). Total food intake within the Se-supplemented mice did not differ, but the feed conversion ratio (FCR; mg body weight gain/g food intake) was significantly higher in the groups with hi-selenite (p = 0.036) and hi-selenate (p = 0.042) supplementation when compared to the hi-SeMet group and did not differ between the other groups (Table 1).

3.2 Selenium status after feeding different Se compounds and concentrations

After 20 weeks of intervention hepatic Se concentrations were significantly higher in all Se supplemented groups when compared to the Se-deficient group (p < 0.001). Highest Se levels were observed in mice treated with hi-SeMet when compared to the other Se-supplemented groups (p < 0.01). Additionally there was a trend for increased hepatic Se concentrations in the groups fed with diets containing hi-selenite (p = 0.071) and hi-selenate (p = 0.052) when compared to the corresponding adequately fed groups (Figure 1). Plasmatic GPx activity dramatically decreased in the Se-deficient group (p<0.001), whereas hi-SeMet supplementation resulted in the highest plasma GPx activities (Figure 1). The mRNA expression of Gpx1, the most abundant selenoprotein in the liver, was dramatically reduced under Se deficiency (p < 0.001), while Se supplementation increased its mRNA levels. Also Txnrd1 transcription levels were decreased under Se deficiency, while no changes in the expression pattern of both genes were found within the Se-supplemented groups (Table S3). The mRNA expression pattern of Gpx1 and Txnrd1 was confirmed at their activity levels.
(Figure 1). Both hepatic GPx and TrxR activities dramatically decreased in the Se-deficient group (p < 0.001). Within the supplemented groups, small differences between hi-selenate and hi-SeMet supplementation and the respective adequately fed groups were observed.

3.3 Up-regulation of anti-oxidant Nrf2 target genes during Se-deficiency
Since previous studies have shown that both low and high Se intake can enhance the activity of the redox-sensitive transcription factor Nrf2 [50], the activity of NQO1, one of the best characterized Nrf2 target genes, was analyzed. NQO1 activity was increased in the Se-deficient group (p < 0.001) in comparison to the other feeding groups, while no changes were detected between the Se-supplemented groups (Figure 2). Next to NQO1 also anti-oxidant enzymes are regulated via Nrf2, including MnSOD and several GSTs. While the MnSOD activity (Figure 2) showed the same pattern as the NQO1 activity, total GST activity was differently modulated. Total GST activity was also highest in the Se-deficient group in comparison to the other groups, but the hi-SeMet group showed an intermediate response being significantly higher as all other supplemented groups (p < 0.001, Figure 2). In order to determine the GST isoform responsible for this effect, mRNA expression levels of several isoforms were analyzed (Table S3). The most obvious candidates were GSTa4, GSTm1 and GSTpi1, also known to be regulated via Nrf2. GSTa4 and GSTm1 expression was down-regulated in all supplemented groups. However, in the hi-SeMet group only GSTpi1 expression did not significantly differ from the –Se group (Figure 2) as shown for total GST activity. The mRNA expression pattern of GSTm1 and GSTpi1 was confirmed at the protein level (Table 2).

3.4 Impact of distinct Se compounds and Se concentrations on hepatic proteomic profiles
To gain further insights in the hepatic effects of feeding different Se compounds and concentrations, DIGE-based proteomic analyses were performed as an untargeted approach (Figure 3). The consensus map across all seven experimental groups (4 mice/group, n = 28) was comprised of 821 distinct spots. Based on the average spot intensity within a given group the respective profiles were analyzed. Spots of interest were defined as regulated when the ratio with the Se-deficient group exceeded the factor 1.5, and when the corresponding t-test was defined as p < 0.05 (Table 2). In total 54 differentially expressed protein spots could be identified, which were subjected to MALDI-TOF-MS resulting in the identification of 28 protein species. Four protein spots contained two or more protein identities and were therefore excluded from further analysis. The identified proteins were grouped according to their cellular function(s) (Table 2). Two of the proteins identified are related to inflammation or
immune modulatory processes. Nine of the proteins are involved in cell cycle, cell development and tumor metabolism. Six of the proteins are related to the intermediary metabolism and metabolic processes and three of the proteins exhibit other biological functions. These include tropomyosin beta chain, which is part of the cytoskeleton, brefeldin A-inhibited guanine nucleotide exchange protein 2, which promotes guanine-nucleotide exchange and is involved in protein transport processes and limbin, which modulates the hedgehog signaling pathway. Fourteen of the proteins were up-regulated (Table 2, indicated in red) and seven down-regulated (Table 2, indicated in green) in response to supplementation with one or several Se compounds when compared to the Se-deficient group. Out of the seven down-regulated proteins, GSTm1 and GSTpi1 were both represented in two independent protein spots (Figure 3) suggesting not only a differential expression, but also post-translational modification(s) (PTMs). The other consistently down-regulated proteins (ferritin light chain 1 (FTL1), limbin and L-aspartate dehydrogenase) might reflect the Nrf2 response as known for FTL1 [51]. Interestingly, two proteins, brefeldin A-inhibited guanine nucleotide exchange protein 2 and carbamoyl-phosphate synthase, were down-regulated in the hi-selenite and hi-selenate groups, but up-regulated in the hi-SeMet group only. The underlying mechanism of this regulation pattern might be comparable to that controlling the GSTpi1 expression. Furthermore, three proteins (tropomyosin beta chain, membrane-associated progesterone receptor component 1, and NACHT, LRR and PYD domains-containing protein 6) showed an inverse expression pattern being up-regulated only in the hi-selenite group and down-regulated upon SeMet feeding. However, it is noteworthy that twelve proteins were consistently up-regulated in most of the feeding groups in comparison to Se deficiency (Figure 3).

3.5 GSH levels and mRNA expression of key enzymes involved in GSH homeostasis
To test whether Nrf2 activation during Se deficiency also affected glutathione (GSH) homeostasis, plasma and hepatic levels of GSH were determined in all experimental groups. All Se-supplemented groups exhibited markedly lower plasma GSH concentrations in comparison to the Se-deficient group (Figure 4). In liver tissues an inverse pattern was detected with the lowest GSH levels in the Se-deficient group, whereas all Se-supplemented groups, except for the hi-SeMet group, showed increased GSH levels. GSH levels in the hi-SeMet group were comparable to the Se-deficient group (Figure 4). In order to study the molecular mechanisms underlying these effects, the mRNA expression levels of enzymes involved in GSH biosynthesis, reduction of GSSG, and GSH export were analyzed by RT-PCR (Table 3). These include the \(\gamma\)-glutamylcysteine ligase (GCL), composed of a catalytic
(GCLc) and a modifier (GCLm) subunit, the GSH synthetase (GS) catalyzing the second step of GSH biosynthesis, the glutathione reductase (GR), and Mrp4, the main exporter of hepatic GSH into the plasma. With the exception of GS all enzymes are regulated by Nrf2. As expected GCLc, GR, and Mrp4 mRNA expression was elevated under Se-deficiency in comparison to Se-supplemented groups, while no differences were detected amongst Se-supplemented groups. The strongest up-regulation in the –Se group with a 5-fold change was observed for Mrp4 (Figure 4). The transcription of GCLm and GS was not or only marginally altered across the experimental groups. Thus, low GSH levels in plasma appear to be mediated by the reduced release of hepatic GSH due to the strong reduction in Mrp4 expression.

3.6 Modulation of the oxidation status of proteins by Se supplementation

To determine whether differences in hepatic GSH levels had an impact on the overall oxidative state of the hepatic proteome, protein lysates from each experimental group were individually labeled with a fluorescent dye specifically targeting free (reduced) thiol residues prior to their subsequent separation on a one-dimensional (1-DE) gel. As shown in Figure 5, the overall oxidative state of liver proteins was specifically altered in the hi-selenite and hi-SeMet groups. The hi-selenite group showed a decreased fluorescence labeling efficiency when compared to the Se-deficient group suggesting that these proteins were more oxidized. In contrast, the samples from the hi-SeMet group demonstrated a more intense staining pattern indicating more free (reduced) thiols when compared to the Se-deficient (p = 0.048), hi-selenite (p = 0.001) and hi-selenate (p = 0.037) groups.

3.7 Effects of Se supplementation on the expression and activation of enzymes related to energy metabolism

Due to the effect of high-SeMet feeding on weight gain (Table 1) metabolic parameters after feeding different Se compounds and concentrations were characterized. Therefore, triglycerides (TG), fasting glucose, and fasting insulin levels were determined in plasma and the HOMA-IR score was calculated (Table 4). Plasma TG levels were significantly increased under supplementation with either selenite or selenate when compared to the Se-deficient group. Lowest TG concentrations were observed in mice of the hi-SeMet group in comparison to hi-selenite (p < 0.001), hi-selenate (p < 0.001) and ad-SeMet (p = 0.018). All Se-supplemented groups had higher glucose levels when compared to the Se-deficient group without differences within the supplemented groups. There was a trend for increased insulin levels in the hi-selenate group when compared to ad-selenate (p = 0.069) and hi-SeMet (p =
0.059) and even a significant effect in comparison to the hi-selenite group (p = 0.033). The resulting HOMA-IR score was also increased in the hi-selenate group when compared to the ad-selenate (p = 0.069), hi-selenite (p = 0.021) and hi-SeMet (p = 0.032) groups. In order to gain further insights into the molecular mechanisms leading to the observed metabolic changes, the transcriptional profiles of selected genes involved in glucose metabolism and fatty acid synthesis were determined in liver tissues (Table 4). To understand the increase in plasma glucose levels the mRNA expression of two glycolytic enzymes glucokinase (Gck) and pyruvate dehydrogenase (Pdha1), and of the gluconeogenic enzyme glucose-6-phosphatase (G6pc) was analyzed. Pdha1 was slightly decreased in the hi-SeMet group and unaffected in all other groups. In contrast, Gck was increased across all Se-supplemented groups when compared to the Se-deficient group, but stayed unaffected in the hi-SeMet group. Thus, up-regulation of Gck might explain the increased plasma glucose levels in most of the supplemented groups, but does not provide an explanation for higher glucose levels in the hi-SeMet group. In addition, transcript levels of G6pc were down-regulated in the hi-SeMet group indicating that gluconeogenesis was also not enhanced under these conditions. The triglyceride levels in plasma were up-regulated upon Se supplementation, but down-regulated in the hi-SeMet group. A similar expression pattern was observed for the fatty acid synthase (Fasn) and also mRNA levels of acetyl-CoA carboxylase (Acaca) tended to be affected into the same direction (Table 4). The correlation analysis of hepatic Fasn transcript levels and plasma TG levels defined a Pearson coefficient of 0.512 (p < 0.001). Phosphorylation of AKT at serine 473 and threonine 308 residues plays a key role in mediating insulin signaling thereby linking the insulin signaling cascade to the energy metabolism. In liver tissues, the threonine phosphorylation of AKT remained unchanged in response to Se supplementation, whereas an increased phosphorylation of AKT$^{\text{Ser473}}$ was found in the hi-selenate (p = 0.009) and ad-SeMet groups. In the hi-SeMet group, AKT$^{\text{Ser473}}$ phosphorylation levels were comparable to that of the Se-deficient group. However, the phosphorylation pattern of PTEN and ERK1/2 remained unchanged (Figure 6). Since PTP1B is involved in AKT signaling and has been reported to be regulated by Se [43], the PTP activity was determined in hepatic tissues of mice in response to Se supplementation (Figure 6). There were no obvious differences in PTP activity between all feeding groups, only the PTP activity of the hi-selenate group was reduced in comparison to the ad-selenate (p = 0.038) and hi-SeMet (p = 0.042) groups.
4 Discussion

The current study aimed to directly compare effects of long-term feeding with three different Se compounds supplied at different concentrations. Previous feeding studies mostly either focused on one Se compound or only considered one concentration. As expected, the adequate concentration of all three Se compounds was sufficient to maximize hepatic selenoprotein expression as indicated by GPx and TrxR activities (Figure 1) with very marginal additional effects of the respective supplemented diets. While hepatic Se levels were already saturated upon feeding adequate amounts of the inorganic Se compounds selenite and selenate, a SeMet supplementation further increased the hepatic Se content. SeMet differs from the other two Se compounds in that it can directly substitute for Met and thus can be non-specifically incorporated into proteins. It is discussed that SeMet acts as an unregulated Se storage, but SeMet might also have direct catalytic properties e.g. by removal of peroxides and protein oxidation products [52,53]. Although these potential features have not yet been shown in vivo, they might explain why the hi-SeMet group had less oxidized proteins (Figure 5) and most often exerts distinct effects than the other supplemented groups.

Next to selenoproteins Nrf2 targets are regulated in response to the Se status [54,55]. Already in 1978 an enhanced GST activity during Se deficiency providing a “selenium-independent GPx activity” was described [56] suggesting that this up-regulation of mainly anti-oxidant Nrf2 target genes is an approach to compensate for the loss of the selenium-dependent GPxs and TRs. A similar compensation was also observed in our study, since there was no difference in the amount of oxidized proteins between mice with adequate or deficient Se diets, independent of the Se compound (Figure 5). In contrast to all Nrf2 target genes analyzed with equally low expression levels in all supplemented groups, GSTpi1 showed another expression pattern. In line with total GST activity, GSTpi1 was also up-regulated in the hi-SeMet group reaching comparable levels as the –Se group (Figure 2). Based on the higher Se content in the hi-SeMet group this effect appears to be independent from selenoproteins, but rather mediated by SeMet itself. Using rat hepatoma cells, a previous study has shown an increased GST activity in the presence of high concentrations of Se compounds like selenocysteine Se-conjugates, which was specifically mediated by the isoforms GSTa2 and GSTpi1 [57]. The authors suggested that the β-lyase-mediated generation of selenols is essential for GST induction. SeMet can also be a source for selenocysteine and selenols after being metabolized via the trans-sulfuration pathway. Interestingly, the proteome analysis identified additional proteins with a comparable expression pattern as GSTpi1, namely limbin, ARFGEF2, and CPS1. Further experiments are
needed to clarify whether these proteins are regulated via the same mechanism in response to hi-SeMet feeding. An additional characteristic feature of the hi-SeMet group was the low hepatic GSH content, which was also low in the –Se group, but up-regulated in all other supplemented groups (Figure 4). A putative accumulation of hepatic GSH levels due to the strong down-regulation of the GSH exporter Mrp4 should affect all supplemented groups to a similar extend. Thus, reduced GSH levels in both the hi-SeMet and –Se groups might be caused by a higher GSH consumption via GSTs. To clarify this point, more specific analysis including also the GSH/GSSG ratio need to be performed in the future.

In contrast to the hi-SeMet group, the most specific characteristic of the hi-selenite group was the increase in oxidized proteins only identified in this group. At the same time Nrf2 expression was not induced in this group indicating that the more oxidized conditions are not compensated by Nrf2. Thus, the hi-selenite induced redox shift might affect different pathways than the one induced by Se deficiency. Three proteins were only up-regulated in the hi-selenite group, namely TPM2, PGRMC1, and NLRP6. Interestingly, NLRP6 is part of the intestinal inflammasome, which is essential for mediating interactions between the host and the intestinal microbiota. Inflammasome deficient mice have a higher risk to develop hepatic steatosis and non-alcoholic liver disease (NAFLD) [58], a condition associated with higher levels of oxidative stress. The second protein related to inflammatory processes was fibrinogen gamma chain (FGG) (Table 2), which also showed the highest fold-change in the hi-selenite group. FGG is up-regulated in hepatoma cells with nutrient overload as an in vitro model for NAFLD [59]. In a DSS-induced murine colitis model, high selenite feeding during acute colitis worsens the course of the disease, while this effect was not detected in the presence of high concentrations of SeMet [60]. These results indicate that high doses of selenite specifically modulate the inflammatory response, but underlying mechanisms need to be further studied.

In addition to redox-modulated effects, the impact of different Se compounds on the energy metabolism was analyzed within this study. At the end of the experiment lower body weights were observed under Se-deficient conditions in comparison to Se supplementation. This observation is in accordance with previous studies performed in rats and chicken [61, 62] and can be largely explained by a lower total feed intake in the -Se group. The hi-SeMet group also had an impaired weight gain, which could not be explained by a reduced total feed intake (Table 1). As previously described, blood glucose as well as plasma TG levels were lower in the -Se group [61]. However, the plasma TG content was also reduced in the high-SeMet group and thus could provide an explanation for the lower weight gain of this group. Thus,
SeMet supplementation mimics symptoms also observed under methionine restriction [63–66], such as reduced growth, but enhanced feed intake and reduced hepatic GSH levels. Therefore, it cannot yet be excluded that the effects observed in this study might at least partially result from an interference of SeMet with the methionine metabolism.

Plasma insulin levels were only enhanced in the hi-selenate group. Previously, selenate has been reported to exhibit insulin-mimetic properties [62,67,68]. The liver is the key organ for controlling the insulin-mediated metabolism. In the current study an enhanced phosphorylation of AKT$^{\text{Ser473}}$ was found in the hi-selenate group, which appears to be the consequence of increased plasma insulin levels, even though the phosphorylation pattern of other insulin-related kinases like ERK1/2 remained unaffected. In addition, the PTP activity was decreased in the hi-selenate group in comparison to ad-selenate feeding, which has been previously shown using leptin receptor deficient db/db mouse, which spontaneously develop obesity and type 2 diabetes [12]. It has been postulated that selenite, the cellular metabolite of selenate, and not selenate itself modifies and thereby inactivates PTPs directly [67]. However, in the current study feeding selenite neither had an effect on PTP activity nor on AKT phosphorylation. In contrast to Mueller and co-authors [12] also no decreased PTP activity was detected in the Se-deficient group. Next to the hi-selenate group the ad-SeMet group showed comparable effects with more AKT$^{\text{Ser473}}$ phosphorylation and equally low PTP activity, but under this condition insulin levels were unmodified. Still, the comparable effects of feeding hi-selenate and ad-SeMet might indicate that Se-Met is a more potent modulator of the metabolic state than selenate. PTPs, in particular PTP1B, counteract the insulin-regulated metabolism by blocking the insulin-stimulated tyrosine phosphorylation of the insulin receptor [69]. PTP1B is known to be a redox-sensitive protein [70] although we did not find a correlation between a high redox status and low PTP activity. In the hi-selenate and ad-SeMet group with the lowest PTP activity no changes in oxidized proteins were detected.

The specific phosphorylation of AKT$^{\text{Ser473}}$ mediated by mTOR2 increased glycolysis and lipogenesis in mice [71]. In our study higher plasma TG levels were detected under hi-selenate supplementation (hi pAKT status) when compared to levels detected in response to hi-SeMet supplementation (lower pAKT status). This was further accompanied by an increased mRNA expression of FAS in the hi-selenate group and decreased levels in the hi-SeMet group. In addition, the mRNA expression of the glycolytic enzymes glucokinase (GK) and pyruvate dehydrogenase (PDH) significantly differed between these two experimental groups indicating a lower glycolysis rate in the hi-SeMet group in comparison to hi-selenate. Moreover, the proteomic profiling of liver tissue specimens led to the identification of several
proteins, which are involved in the intermediary metabolism. One of those was the putative glycerol kinase 5 (GK5), which is involved in the biosynthesis of TG by synthesizing glycerol-3-phosphate from glycerol and ATP. GK5 was up-regulated in the hi-selenite and in both selenate groups when compared to the Se-deficient group.

5 Concluding remarks
The present study provides evidence that the effects of high concentrations of different Se compounds markedly differ from each other, which might be due to the fact that higher concentrations are not predominantly needed for selenoprotein synthesis. Only high levels of SeMet further increase the hepatic Se status indicating that this Se compound or its metabolites might have effects independent of selenoproteins, which might be mediated by the unspecific incorporation into proteins instead of methionine. In addition, Se oversupply affected the expression and activity of enzymes involved in the regulation of the energy metabolism, but the underlying mechanisms of the different Se compounds have to be clarified in the future. Thus, it is important to consider compound-specific effects when supplementing with selenium. Based on this study selenite and selenate might have advantages over supplementing with SeMet.

Funding
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6 References


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[70] A. Salmeen, J.N. Andersen, M.P. Myers, T.C. Meng, J.A. Hinks, N.K. Tonks, D. Barford, Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-

Figure legends

Figure 1. Hepatic Se status following long-term supplementation with different Se compounds and Se concentrations.
(A) Se concentrations were measured by ICP-MS, (B) plasma GPx, (C) total hepatic GPx and (D) total hepatic TrxR activities were spectrophotometrically determined as described in the method section. Values are given as means ± S.E.M (n = 8). Significant differences were calculated by one-way ANOVA. * p < 0.05 vs. –Se. ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet). DM, dry matter.

Figure 2. Enzyme activities and mRNA expression of Nrf2 target genes following long-term supplementation with different Se compounds and Se concentrations.
(A) NQO1, (B) MnSOD and (C) total GST activity; (D) GSTa4, (E) GSTm1 and (F) GSTpi1 mRNA expression in liver tissues of mice fed with different Se compounds and Se concentrations for 20 weeks. Enzyme activities were spectrophotometrically determined as described in the methods section. mRNA levels were analyzed by qPCR, normalized to the reference genes Rpl13a and Hprt and depicted in relation to the Se-deficient group (-Se). Values are given as means ± S.E.M. (n = 8). Significant differences were calculated by one-way ANOVA. * p < 0.05 vs. –Se.ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).

Figure 3. Proteomic profiling of hepatic tissue samples
(A) Representative spot pattern of a hepatic proteome (hi-selenate) following separation by 2D-DIGE. Differentially expressed proteins which were identified by MALDI-TOF-MS as described in the material section are marked with numbers (1-24, see also Table 2). (B) Heatmap and cluster analysis regarding the expression profiles of the identified proteins. (C) Representative inserts showing the mapping of GST spots.

Figure 4. GSH levels in plasma and liver tissues and hepatic Mrp4 mRNA expression.
GSH levels in (A) plasma and (B) liver tissue lysates of mice were spectrophotometrically determined as described in the method section. In (C) mRNA expression levels of hepatic Mrp4 were determined by qPCR as described in the method section. Changes are depicted in relation to Se-deficient mice (-Se). Values are given as means ± S.E.M. (n = 8). Significant differences were calculated by one-way ANOVA. * p < 0.05 vs. –Se. ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).
Figure 5. Oxidative status of hepatic proteomes
(A) The level of reduced hepatic proteins was analyzed by trapping the free thiols with a specific thiol-reactive dye (S-300 dye) prior to separation by SDS-PAGE (n = 3). The overall fluorescence signal of each lane (A, upper panel) was normalized to the respective post-stained Coomassie intensity signal (A, lower panel). (B) Quantification of the protein redox state in relation to the –Se group. Data are given as means ± S.E.M. (n = 3). Significant differences were calculated by one-way ANOVA. * p < 0.05 vs. –Se. ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).

Figure 6. Phosphorylation pattern and activity of key enzymes involved in the insulin-regulated energy metabolism.
(A) Representative phosphorylation status of protein kinases in liver tissues following the 20 week feeding period were determined by Western blot analysis as described in the method section. (B) Quantification of the AKT<sup>Ser473</sup> phosphorylation status across the various experimental groups in relation to the –Se group. (C) Total PTP activity was determined under non-reducing conditions as described in the method section. Data are given as means ± S.E.M. Significant differences were calculated by one-way ANOVA. * p < 0.05 vs. –Se. ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet); ns, not significant.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Table 1. Body weights (bw), feed intake, and feed conversion ratio (FCR) of mice fed with different Se compounds and Se concentrations.

Values are given as means ± S.E.M (n=8). Different small letters within a line indicate significant differences within the Se supplemented groups, levels marked with * indicate significant differences compared to the –Se group (p<0.05; one-way ANOVA). ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet)

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<td>-Se</td>
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<td>hi</td>
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<td>body weight (g)</td>
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<td>final</td>
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<td>total weight gain</td>
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<td>21.6 ± 1.10**</td>
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<td>total feed intake (g)</td>
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<td>total FCR (mg bw/g feed)</td>
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Table 2. Differentially expressed hepatic proteins in response to Se treatment

Values are given as means (n=4) in relation to Se-deficient (-Se) mice. Red/green filled boxes indicate significant upregulated/downregulated hepatic proteins in relation to the –Se group.

Levels marked with * indicate significant differences of hi Se intake to the ad Se intake within one indicated Se compound (p<0.05; one-way ANOVA). ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).
Table 3. Influence of different Se derivatives on the gene expression of enzymes involved in GSH biosynthesis

Values are given as means ± S.E.M (n=8) in relation to Se-deficient (-Se) mice. Levels marked with * indicate significant differences compared to the –Se group (p<0.05; one-way ANOVA). ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).

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<tr>
<th>Gene name</th>
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<td></td>
<td>ad</td>
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<td>hi</td>
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<td>γ-glutamylcysteine ligase, modifier</td>
<td>1.00 ± 0.07</td>
<td>0.86 ± 0.06</td>
<td>0.78 ± 0.05*</td>
<td>0.75 ± 0.09*</td>
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<tr>
<td>γ-glutamylcysteine ligase, catalytic</td>
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<td>0.99 ± 0.11</td>
<td>0.91 ± 0.06</td>
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<td>Glutathione synthetase</td>
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<td>Glutathione reductase</td>
<td>1.00 ± 0.08</td>
<td>0.97 ± 0.06</td>
<td>0.83 ± 0.08</td>
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Table 4. Influence of different Se compounds and Se concentrations on parameters of the hepatic energy metabolism and mRNA expression of metabolic proteins

Values are given as means ± S.E.M (n=8) in relation to Se-deficient (-Se) mice. Different small letters within a line indicate significant differences within the Se supplemented groups, levels marked with * indicate significant differences compared to the –Se group (p<0.05; one-way ANOVA). ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).

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<td><strong>Plasma triglycerides (mg/dl)</strong></td>
<td>70.7 ± 2.41</td>
<td>93.5 ± 7.00*</td>
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<td><strong>Plasma glucose (mmol/l)</strong></td>
<td>8.68 ± 0.16</td>
<td>9.71 ± 0.53</td>
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<td><strong>Plasma insulin [µU/ml]</strong></td>
<td>70.0 ± 5.79</td>
<td>64.0 ± 5.62</td>
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<td><strong>HOMA-IR</strong></td>
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<td>1.20 ± 0.10</td>
<td>1.04 ± 0.10*</td>
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<table>
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<td>pyruvate dehydrogenase</td>
<td>1.00 ± 0.04</td>
<td>0.98 ± 0.05</td>
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<td>0.86 ± 0.06</td>
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<table>
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<td>1.00 ± 0.06</td>
<td>1.19 ± 0.10</td>
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<td>fatty acid synthase</td>
<td>1.00 ± 0.14</td>
<td>1.33 ± 0.18</td>
<td>1.18 ± 0.13*</td>
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Highlights:

- Side by side comparison of inorganic versus organic selenocompounds reveals that in particular an oversupply with selenomethionine, rather than the other selenocompounds affects distinct biological processes
- Selenomethionine supplementation results in fundamental changes of the GSH metabolism and oxidation status of proteins
- Treatment with different selenocompounds results in an altered protein expression pattern in liver as defined by 2D-DIGE.