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1 **Impact of selenite and selenate on differentially expressed genes in rat liver examined**  
 2 **by microarray analysis**

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30  
 31  
 32 # All authors declare that they have approved the manuscript, and that they have no conflicts  
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36 **SYNOPSIS**

37 Sodium selenite and sodium selenate are approved inorganic Se compounds in human and  
38 animal nutrition serving as precursors for selenoprotein synthesis. In recent years numerous  
39 additional biological effects over and above their functions in selenoproteins have been  
40 reported. For greater insight into these effects our study examined the influence of selenite  
41 and selenate on the differential expression of genes encoding non-selenoproteins in the rat  
42 liver using microarray technology. 5 groups of 9 growing male rats were fed a Se deficient  
43 diet or diets supplemented with 0.20 or 1.0 mg Se/kg as sodium selenite or sodium selenate  
44 for 8 weeks. Genes which were more than 2.5-fold up- or down-regulated by selenite or  
45 selenate compared to Se deficiency were selected. Glutathione peroxidase 1 was up-  
46 regulated 5.5-fold by both Se compounds, whereas glutathione peroxidase 4 was up-  
47 regulated only 1.4-fold. Selenite and selenate down-regulated three phase II enzymes.  
48 Despite the regulation of many other genes in an analogous manner, frequently only  
49 selenate changed the expression of these genes significantly. In particular, genes involved in  
50 the regulation of the cell cycle, apoptosis, intermediary metabolism, and those involved in Se  
51 deficiency disorders were more strongly influenced by selenate. The comparison of selenite-  
52 and selenate-regulated genes revealed that selenate may have additional functions in the  
53 protection of the liver, and that it may be more active in metabolic regulation. In our opinion  
54 the more pronounced influence of selenate compared to selenite on differential gene  
55 expression results from fundamental differences in the metabolism of these two Se  
56 compounds.

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## 81 INTRODUCTION

82 Since the discovery of the essentiality of selenium (Se) five decades ago, this element has  
 83 been increasingly associated with many biological processes [1]. In contrast to other metal  
 84 trace elements which interact with proteins as cofactors, the physiological functions of Se  
 85 derive mainly from the incorporation of Se into the active sites of functional selenoproteins  
 86 via the cotranslational synthesis of selenocysteine [1]. To date the existence of not less than  
 87 33 functional selenoproteins with at least one selenocysteine residue has been reported. The  
 88 best investigated characteristic of Se is its antioxidant role. Six different glutathione  
 89 peroxidases (GPx1 – GPx6) and two thioredoxin reductases (TrxR 1 and TrxR2) contribute  
 90 largely to the maintenance of cellular antioxidant defense. The involvement in thyroid  
 91 metabolism through iodothyronin deiodinase activity represents a further important Se  
 92 dependent target [2]. Under conditions of low Se availability, these selenoproteins selectively  
 93 are down-regulated. Whereas GPx1, exhibiting a high and ubiquitous expression and activity  
 94 in mammalian tissues, and plasma glutathione peroxidase (GPx3) undergo a rapid and  
 95 severe down-regulation, gastrointestinal glutathione peroxidase (GPx2) and  
 96 phospholipidhydroperoxide glutathione peroxidase (GPx4) are far more conserved [3, 4].  
 97 Depending on the animal species, a long term Se deficiency causes liver necrosis and  
 98 myopathies [5-8]. Besides the mechanisms of functional selenoproteins, in recent years  
 99 additional information on Se biochemistry has been obtained. The down-regulation of phase  
 100 II detoxification enzymes such as glutathione-S-transferases and aflatoxin b1 aldehyde  
 101 reductase (Afar) due to adequate Se supply impressively shows the counter regulation of  
 102 cellular stress response [9, 10]. Anti-carcinogenic effects of supranutritive Se dosages,  
 103 investigated in animal trials and tissue culture experiments, may result from the initiation of  
 104 proapoptotic mechanisms by the generation of monomethylselenol and high concentrations  
 105 of reactive oxygen species [11], or from the response of phosphorylation- and signal  
 106 transduction mechanisms [12]. Other hypotheses suggest the reversion of the  
 107 hypermethylated DNA of tumour suppressor genes [13] or the reduction of prostaglandin E2  
 108 and cyclooxygenase 2 via GPx2 activity as being the underlying mechanisms for the  
 109 protective effect of Se against colon cancer [14]. However, the results of large placebo  
 110 controlled human intervention trials did not support uniquely beneficial effects of  
 111 supranutritional Se supplementation in the prevention of several cancers [15, 16]. The  
 112 influence of Se supplementation and selenoprotein activities on obesity, insulin resistance,  
 113 diabetes and hyperlipidaemia has been the subject of recent controversial discussion.  
 114 Whereas only very high supranutritive selenate doses have antidiabetic effects [17], a high  
 115 activity of GPx1 as well as a high Se status derived from the permanent intake of Se  
 116 supplements have lately been reported as being one possible cause of the accelerated  
 117 development of obesity, insulin resistance, diabetes and hyperlipidaemia [18-22]. These  
 118 important results were the outcome of the above mentioned cancer intervention trials and of  
 119 large cross sectional studies [15, 19-21]. Optimum activities of all functional selenoproteins  
 120 can be obtained by a daily Se uptake of 30-70 µg in humans and by a concentration of 0.15-  
 121 0.30 mg Se/kg diet in animals. The inorganic Se compounds sodium selenite (+IV) and  
 122 sodium selenate (+VI) are approved Se additives both in human mineral supplements and in  
 123 animal feed. Both selenite and selenate have a high absorption rate from the small intestine  
 124 [23], but they exhibit some fundamental differences in their absorption mechanism as well as  
 125 in their intermediary metabolism. There is no doubt that selenite and selenate represent  
 126 valuable inorganic Se compounds to ensure adequate selenoprotein synthesis [24, 25].  
 127 Nevertheless, information is lacking whether and in what way selenite and selenate influence  
 128 the expression of genes beyond functional selenoproteins. In this context the present study  
 129 using microarray analysis (MA) as the analytical tool had the following aims:

- 130 ● Data mining of genes regulated differentially by selenite or selenate supplementation  
 131 versus a control group with Se deficiency.
- 132 ● Exploring genes responding in particular to selenite or selenate supplementation by  
 133 direct comparison of the selenite and selenate expression profile.

135 A nutrition trial with healthy growing rats served as the basis for the examination of  
 136 differential gene expression by selenite and selenate.

137

## 138 **MATERIALS AND METHODS**

### 139 **Rats and diet preparation**

140 45 weanling albino rats (HK 51, strain of the Institute of Animal Nutrition and Nutritional  
 141 Physiology) were randomly assigned to 5 groups of 9. The average initial body weight (62.8  
 142  $\pm$  4.0 g) did not vary among groups. The rats were fed a Torula yeast based diet as  
 143 described earlier [30] for 8 weeks. The diet was composed according to the current  
 144 recommendations of the National Research Council (NRC) [26] with the exception of Se. The  
 145 analyzed Se concentration of the basal diet (group 0) was below the detection limit (<0.03  
 146 mg Se/kg diet). Groups I and II were fed diets adequate in Se (0.20 mg Se/kg diet) either  
 147 supplemented with sodium selenite (group I) or sodium selenate (group II). The diets of  
 148 groups III and IV were supplemented with sodium selenite or sodium selenate to a  
 149 supranutritive Se concentration of 1.0 mg Se/kg diet. The rats were kept individually in plastic  
 150 cages with wood shavings as bedding under standard laboratory conditions (22°C, 55%  
 151 humidity, 12h light : dark cycle) and had free access to the diet and deionized water. The  
 152 protocol of the animal experiment was approved by the Regional Council of Giessen and by  
 153 the animal welfare committee of Giessen University. At the end of the trial, the rats were  
 154 anaesthetized in a carbon dioxide atmosphere and subsequently decapitated. The liver was  
 155 removed, frozen in liquid nitrogen, and stored at -80°C until analysis.

156

### 157 **Se analysis in diets and liver**

158 Se concentration in the diets and the livers was determined by hydride generation atomic  
 159 absorption spectrometry (AAS, Unicam PU 9400 X; PU 3960 X) as reported previously [27].  
 160 Certified samples from the National Institute of Standard and Technology (soft winter wheat  
 161 flour, NIST No. 8438 and bovine liver, NIST No. 1577 b) served as reference material for Se  
 162 determination in the different matrices.

163

### 164 **RNA extraction and microarray analysis**

165 The analysis of differentially expressed genes was performed using "10k rat oligoarrays"  
 166 from MWG (Ebersberg, Germany). On one single MA slide, short nucleotide sequences of  
 167 9,715 different rat genes are spotted in duplicate, which allows the co-hybridisation of RNA  
 168 samples from 2 experimental groups on one MA slide. RNA was extracted from the liver of 5  
 169 rats per group by the phenol chloroform guanidine thiocyanate extraction protocol [28],  
 170 further purified by a DNase I digest and pooled. Direct Cy3 or Cy5 labeling of single stranded  
 171 DNA by synthesis from 100  $\mu$ g total RNA, MA hybridization- and scanning, background  
 172 correction, and data analysis up to comparable expression values were performed by MWG-  
 173 BIOTECH AG, Ebersberg, Germany (<http://www.mwg-biotech.com/>). The samples were  
 174 assigned to the 6 microarrays as follows:

175 Microarray 1: Cy3 (group 0), Cy5 (group I); Microarray 2: Cy3 (group 0), Cy5 (group II);  
 176 Microarray 3: Cy3 (group 0), Cy5 (group III); Microarray 4: Cy3 (group 0), Cy5 (group IV);  
 177 Microarray 5: Cy3 (group I), Cy5 (group II); Microarray 6: Cy3 (group III), Cy5 (group IV).

178 For the comparison of the expression data from selenite and selenate treated rats with that of  
 179 their companions on Se deficiency the following criteria were applied:

- 180 ● Only genes with average Cy3 and Cy5 values that were 2-fold above the background  
 181 were used.
- 182 ● Only genes that were at least 2.5-fold up- or down-regulated by selenite or selenate  
 183 treatment were considered.
- 184 ● Only genes regulated in an analogous manner by selenite and selenate were  
 185 considered.
- 186 ● Only genes which fulfilled the statistical assumptions were considered (c.f. statistical  
 187 analysis).

188 Differentially expressed genes were assigned to 5 functional areas and are shown in Table 1.

- 189 Area I: Genes involved in phase I and phase II detoxification  
 190 Area II: Genes involved in the regulation of intermediary metabolism and in the  
 191 regulation of appetite, body weight or other metabolic processes  
 192 Area III: Genes related to the cell cycle, cell development, cell death (apoptosis) or  
 193 tumour metabolism  
 194 Area IV: Genes related to inflammatory or immunomodulatory response  
 195 Area V: Genes with other functions (e.g., hormonal function, receptor function, signal  
 196 transduction)

197 In addition to the examination of the influence of selenite and selenate on differential gene  
 198 expression versus Se deficiency a direct comparison of selenite- and selenate-regulated  
 199 gene expression was carried out.  
 200

201 **Validation of the MA data for selected differentially expressed genes (GPx1, GPx4,  
 202 GSTa2, PTP1B) by two step reverse transcriptase PCR (2-step RT-PCR)**

203 For GPx1, GPx4, GSTa2 and PTP1B the validation of the MA data was carried out using 2-  
 204 step reverse transcriptase PCR (2-step RT-PCR). The expression data were normalized to  $\beta$   
 205 actin expression. The 2-step RT-PCR method applied was elaborated and refined in our  
 206 laboratory, and was described in detail previously [22]. New liver RNA pools were prepared  
 207 for the RT-PCR experiments from the same 5 rats per group to exclude casual and  
 208 systematic errors of the MA expression data. The gene bank accession numbers, the PCR  
 209 primers used, and the length of the amplicates were as follows: Rat GPx1: NM 030826,  
 210 primer forward: TCA TTG AGA ATG TCG CGT CT, primer reverse: CCC ACC AGG AAC  
 211 TTC TCA AA, amplicate length: 388 bp; Rat GPx4: NM: 017165, primer forward: ATG CAC  
 212 GAA TTC TCA GCC AAG, primer reverse: GGC AGG TCC TTC TCT AT, amplicate length:  
 213 461 bp; Rat GSTa2: NM 0170131, primer forward: CAG GAG TGG AGT TTG ATG AGA,  
 214 primer reverse: AGA GGG AAA GAG GTC AGA AG, amplicate length: 463 bp; Rat PTP1B:  
 215 NM 012637, primer forward: GCA CTT CTG GGA GAT GGT GT, primer reverse: AAG AGG  
 216 AAA GAC CCG TCC TC, amplicate length: 449; Rat  $\beta$  actin: NM 0311441, primer forward:  
 217 TGT TAC CAA CTG GGA CGA CA, primer reverse: TCT CAG CTG TGG TGG TGA AG,  
 218 amplicate length: 396 bp.  
 219

220 **Determination of GPx1 and GPx4 activity**

221 The activities of GPx1 and GPx4 were determined spectrophotometrically (Beckmann DU  
 222 50) recording glutathione dependent peroxide reduction coupled to glutathione reductase  
 223 and NADPH oxidation at 340 nm [29]. For GPx1 activity, the diluted cytosolic supernatants of  
 224 rat liver homogenates served as sample material. For GPx4 activity, crude homogenates of  
 225 livers were prepared in a sucrose buffer [30].  $H_2O_2$  served as the substrate for GPx1  
 226 determination, whereas freshly synthesized phosphatidylcholine hydroperoxide (PCOOH)  
 227 was used for measurement of GPx4 activity [30]. One unit of GPx1 and GPx4 activity was  
 228 defined as one micromole NADPH oxidized/min and normalized to 1 mg protein.  
 229

230 **Activity of the phase II detoxification enzymes GSTa2 and epoxide hydrolase**

231 The activity of alpha glutathione-S-transferases to which GSTa2 activity makes a large  
 232 contribution was selectively determined according to a spectrophotometric protocol using the  
 233 specific substrate 7-chloro-4-nitrobenzoic-2-oxa-1,3-diazole (NBD-Cl) [31]. The increase in  
 234 absorption at 419 nm caused by the conjugation of NBD-Cl to 7-glutathionyl-4-nitrobenzoic-  
 235 2-oxa-1,3-diazole (NBD-GSH) was recorded for 3 min. 1 U of alpha glutathione-S-transferase  
 236 activity was defined as the formation of 1  $\mu$ mol of NBD-GSH/min and normalized to 1 mg  
 237 protein.

238 Epoxide hydrolase activity in 1:5 (w/v) crude liver homogenates was measured according to  
 239 a spectrophotometric protocol using styrene oxide as the substrate [42]. In the first reaction  
 240 step, 45  $\mu$ L of the liver homogenates were incubated in 630  $\mu$ L of 50 mM phosphate buffer  
 241 containing 10% DMSO and 10 mmol/L of the substrate styrene oxide for 2 h at 36°C. This  
 242 reaction step allows the formation of the vicinal diol from the epoxide by the activity of  
 243 epoxide hydrolase. Subsequently the samples were treated with 200  $\mu$ L 100 mM sodium  
 244 periodate and 135  $\mu$ L 0.8 M sodium sulphite in order to oxidize the vicinal diol to the

245 corresponding aldehyde and to reduce excess sodium periodate to sodium iodide. 500  $\mu$ L  
 246 supernatant of this reaction were incubated at 70°C with 50  $\mu$ L Schiff's reagent to form a red  
 247 magenta dye. The extinction was read in a spectrophotometer at 560 nm and compared with  
 248 a calibration curve prepared with 1-phenyl-1,2-ethanediol. 1U of epoxide hydrolase was  
 249 defined as the formation of 1  $\mu$ mol vicinal diol/min and related to 1 mg protein.

### 251 **Statistical analysis**

252 Data are given as means $\pm$ standard deviation. For the enzymatic analyses and for the RT-  
 253 PCR data, statistical differences between means ( $P < 0.05$ ) were analysed with "SPSS 14.0  
 254 for Windows" by one way ANOVA after ascertaining the normality of distribution (Shapiro  
 255 Wilk test and Kolmogorov Smirnov test) and homogeneous variances (Levene test).  
 256 Differences between means were analyzed with the LSD test if variances were  
 257 homogeneous or with the Games Howell test if not. Changes in gene expression (MA data)  
 258 were analyzed with Student's t test. At  $P < 0.05$ , expression differences were accepted as  
 259 statistically significant if homogeneous variances and the normality of distribution could be  
 260 ensured. In order to reduce the genes differentially expressed among treatment groups to a  
 261 manageable number for publication only genes which were at least 2.5-fold up-or down-  
 262 regulated by selenite or selenate treatment were considered (Table 1). Only genes which  
 263 were regulated in an analogous manner by selenate at both dietary concentrations or in  
 264 addition by selenite at one dietary concentration are displayed.

## 267 **RESULTS**

### 268 **Effect of Se status on feed intake and live weight**

269 After 8 weeks no significant differences in feed intake and live weight (final average weight  
 270 285.8  $\pm$  7.0 g) were observed between rats receiving a Se deficient diet (0) compared to their  
 271 littermates with Se adequate nutrition (I, II) or supranutritive Se supply (III, IV).

### 273 **Concentration of Se in diets and liver**

274 The basal diet based on Torula yeast was readily accepted by the rats and exhibited a Se  
 275 concentration below the detection limit ( $< 0.03$  mg/kg). Analyzed Se levels of the  
 276 supplemented diets corresponded well with the values scheduled in the experimental design  
 277 (I: 0.21 $\pm$ 0.009 mg/kg fresh matter ( $\sim$ 90% dry matter), II: 0.19 $\pm$ 0.010, III 0.96 $\pm$ 0.071,  
 278 0.85 $\pm$ 0.025). Liver Se concentration in group 0 (21.2 $\pm$ 2.40  $\mu$ g/kg fresh matter) decreased to  
 279 2% of that of rats with adequate Se intake (I: 1044 $\pm$ 88.6, II: 1050 $\pm$ 78.4). Supranutritive Se  
 280 (III: 2233 $\pm$ 197, IV: 2148 $\pm$ 149) again increased liver Se concentration significantly compared  
 281 to groups I and II. At both dietary levels no significant differences existed between selenite  
 282 and selenate regarding their efficacy in influencing liver Se concentration.

### 284 **GPx1- and GPx4 activity and expression**

285 As a sensitive parameter of Se deficiency, GPx1 expression and activity were significantly  
 286 decreased in the Se deficient group 0. (Figure 1 A, C). Independent of the Se compound, the  
 287 supplementation with 0.2 mg Se/kg diet (groups I and II) resulted in a strong increase in  
 288 GPx1 activity (73-fold) which was accompanied by multiplied gene expression in the MA  
 289 experiment ( $\sim$ 7.5-fold). MA data also indicated a marked increase in GPx1 expression ( $\sim$ 6.0-  
 290 fold) for groups III and IV which received diets with supranutritive Se (1.0 mg Se/kg)  
 291 compared to group 0 (Figure 1 A). GPx1 activity did not quite reach the level of the  
 292 adequately supplemented groups (Figure 1 C). The differences in GPx1 mRNA expression,  
 293 as indicated by the MA data, could be confirmed by 2-step RT-PCR analysis (Figure 1 A). In  
 294 contrast only a non significant weak influence of varying Se supplementation on gene  
 295 expression of GPx4 was measured. GPx4 responded to Se deficiency only by a non  
 296 significant minor down-regulation of its mRNA (1.2 to 1.6-fold) and by a 1.5 to 2.0-fold loss of  
 297 activity (Figure 1 B, C). The mRNA expression data obtained by MA analysis could be  
 298 confirmed by 2-step RT-PCR analysis. No influence of the supplemented Se compound  
 299 (selenite or selenate) and of the dietary Se level (0.2 and 1.0 mg/kg) on GPx4 expression  
 300 and activity could be observed.

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301 **General results for differential gene expression with regard to the comparison of**  
 302 **selenite- or selenate feeding versus Se deficiency**

303 Despite no differences between selenite and selenate in the regulation of the selenoproteins  
 304 GPx1 and GPx4, the most challenging and unexpected finding in our study was the fact that  
 305 compared to Se deficiency (group 0) both dietary selenate levels (groups II and IV) had a  
 306 distinctly stronger influence on differential gene expression than selenite (groups I and III).  
 307 Thus with a ratio of 2.5 compared to Se deficiency about 300 genes were significantly  
 308 regulated by selenate feeding, whereas selenite only affected the expression of 43 and 44  
 309 genes, respectively. For both inorganic Se compounds the comparison of the nutritional level  
 310 (0.2 mg/kg) and the supranutritive level (1.0 mg/kg) revealed no significant differences in the  
 311 total number of up- or down-regulated genes.

313 **Specific results for differential gene expression with regard to the comparison of**  
 314 **selenite- or selenate feeding versus Se deficiency after mapping functional areas**

315 After analysis of the general effects of selenite and selenate on differential gene expression  
 316 we mapped genes which were up- or down-regulated in an analogous manner compared to  
 317 Se deficiency at least by selenate feeding (242 genes) into 5 functional areas (c.f. Material  
 318 and Methods). The results of this mapping are shown in Table 1 for 44 representative genes.  
 319 By displaying the regulation ratio as well as the significance level also for the selenite groups  
 320 I and III, Table 1 clearly shows that selenate feeding (groups II and IV) compared to Se  
 321 deficiency (group 0) influences the differential expression of genes in all functional areas  
 322 investigated much more distinctly and significantly than selenite feeding. To confirm the  
 323 results of the MA analysis we validated the expression data for 3 representative target genes  
 324 (GST2a, area I; epoxide hydrolase, area I; PTP1B, area II) by 2-step RT-PCR and/or  
 325 enzymatic analysis in addition to GPx1- and GPx4-expression. One very challenging result of  
 326 our study was the fact that Se strongly influences the regulation of phase I- and phase II-  
 327 detoxification genes (Table 1, area I). Three mRNAs of phase II-detoxifying enzymes,  
 328 namely Aflatoxin B1 aldehyde reductase (Afar), Alpha glutathione-S-transferase (GSTA2)  
 329 and Epoxide hydrolase were distinctly down-regulated by Se feeding (groups I, II, III, and IV)  
 330 compared to Se deficiency (group 0). For these enzymes, selenite feeding was also effective  
 331 at both dietary levels. We could confirm the validity of the MA data by measurement of  
 332 GSTA2 expression and activity (Figure 2 A and B) and Epoxide hydrolase activity Figure 2  
 333 C). Contrary to the down-regulation of phase II-detoxifying enzymes, the phase I-  
 334 haemoprotein cytochrome p450c was up-regulated by selenate.

335 In particular by selenate feeding, numerous genes controlling intermediary nutrient  
 336 metabolism, the regulation of appetite and body weight and other metabolic processes  
 337 (Table 1, area II) were differentially regulated compared to Se deficiency. Although selenite  
 338 feeding influenced the expression of these genes into the same direction, the strength of  
 339 regulation was not significant in most cases. In the case of Heme oxygenase 3 and  
 340 Transferrin receptor, two genes of iron metabolism were distinctly affected in their expression  
 341 by selenate feeding. The distinct up-regulation of Agouti related protein precursor, Hypocretin  
 342 orexin receptor 1, Neuropeptide y precursor, and Ob-receptor gene related protein suggests  
 343 the involvement of Se in central processes of appetite- and body weight-regulation. Moreover  
 344 a distinct influence of Se on carbohydrate- and lipid metabolism was indicated by the  
 345 regulation of Acyl-coenzyme A:cholesterol acyltransferase 1, Fructose-6-phosphate-2-  
 346 kinase, and Phosphorylase-b-kinase. A strong influence of selenate was also observed for  
 347 the regulation of genes involved in metabolic signal transduction pathways (80-k  
 348 diacylglycerol kinase, and Protein tyrosine phosphatase 1B), the regulation of vascular tone  
 349 (Arginase II) and even in folate metabolism (Gamma glutamyl hydrolase precursor). In area II  
 350 the validity of the MA data was confirmed by the examination of the expression and the  
 351 activity of the insulin antagonistic PTP1B (Figure 3). Thus Se feeding with both selenite and  
 352 selenate led to a 1.7 to 2.8-fold up-regulation of PTP1B mRNA, accompanied by a ~3.0-fold  
 353 increase in enzyme activity (Figure 3).

354 In area III (Table 1) a number of apoptosis promoting genes such as BCL2-antagonist,  
 355 Dermatan sulfate proteoglycan II, FOS related antigen, Mast cell protease 1, p58, and

356 Rapamycin- and FKBP 12 target 1 were distinctly up-regulated by selenate. p58 was also  
 357 strongly affected by selenite feeding.

358 In contrast, other regulated genes in area III (NAPOR-3, Granzyme k) indicated an  
 359 antiapoptotic role of Se. Moreover, in area III the regulation of genes participating in growth  
 360 and differentiation of various tumours (ADAMTS-1, PKCQ interacting protein picot, and  
 361 Proliferin related protein) indicate a tumour-protective function of Se. The differential  
 362 regulation of numerous genes related to inflammatory or immunomodulatory reactions (area  
 363 IV) as well as of genes with other functions, such as structural protein function, hormonal  
 364 function, receptor function, a function in signal transduction or a function as an ion channel  
 365 (area V) clearly indicated biological functions of Se beyond its role in functional  
 366 selenoproteins. Whereas the regulated genes of area IV indicate an important target function  
 367 of Se in the modulation of immune response, numerous regulated genes of area V may give  
 368 some new insight into the beneficial effects of Se in the prevention of Se deficiency diseases,  
 369 such as muscular dystrophy and liver necrosis. Differentially expressed genes indicating  
 370 such a response are e.g., Calcium transporting ATPase Serca 1a, Cardiac ankyrin repeat  
 371 protein, Mu-calpain large subunit, Tissue inhibitor of metalloproteinase 3, Titin, and Cyclic-  
 372 3',5'-monophosphate response element binding protein 1.

373  
 374 **Results for differential gene expression with regard to the direct comparison of**  
 375 **selenite and selenate feeding**

376 At a ratio of 2.5 ( $P < 0.05$ ) a total number of 18 genes (3 down-regulated and 15 up-regulated)  
 377 were found as being differentially expressed by selenate and selenite feeding. Table 2 shows  
 378 a selection of 11 differentially expressed genes from this comparison. As can be seen from  
 379 Table 2 the final ratios originate from different regulatory effects (for details see table  
 380 legend).

381 The regulation of the 5-hydroxytryptamine serotonin receptor 6 and the Growth hormone-  
 382 releasing hormone receptor beta indicates a more pronounced influence of selenate in liver  
 383 fatty acid- and sterol metabolism as well as in central mechanisms of body weight regulation  
 384 compared to selenite. The Alphas crystalline-related protein (Heat shock protein 20) and its  
 385 precursor the golgi associated protein Gcp 360 which are down-regulated during the  
 386 development of hepatocellular carcinoma showed a higher expression due to selenate  
 387 feeding. Both genes were also found as being more highly expressed by selenate in the  
 388 expression profile versus Se deficiency (c.f. Table 1). The differential regulation of Ly-49.9  
 389 antigen and the inhibitory natural killer cell receptor Nkr-p1b point to a higher influence of  
 390 selenate in modulating T cell response compared to selenite. The Aldo-keto reductase akr1b-  
 391 7 which was up-regulated by selenate compared to selenite plays an important role in lipid  
 392 detoxification, in particular in the small intestine. Titin which increases the elasticity of  
 393 cardiomyocytes showed a significantly up-regulation due to selenate feeding. Titin was also  
 394 found as being more highly expressed by selenate in the expression profile versus Se  
 395 deficiency (c.f. Table 1).

396  
 397 Additional information on functions of the regulated genes in Table 1 and on other selected  
 398 genes of the functional areas I to V are shown in Tables 1 to 5 as online supporting material.  
 399 Moreover the full microarray data sets are available as online supporting material. The  
 400 assignment of the samples to the 6 microarrays can be seen from the first sheet in the Excel  
 401 file containing the data of the microarrays 1 to 3. The second Excel file contains the original  
 402 data of the microarrays 4 to 6. The red marked columns I and L are the background-  
 403 corrected expression data for the single genes which can be used to compare gene  
 404 expression.

405  
 406 **DISCUSSION**

407 In recent years specific knowledge of the biological effects of Se beyond its role in functional  
 408 selenoproteins has advanced. Both positive and critical functions of Se with regard to  
 409 diseases of the Western World such as cancer, cardiac diseases, obesity, insulin resistance,  
 410 diabetes and hyperlipidaemia have been investigated. To obtain information on cellular  
 411 mechanisms underlying such specific biological effects of Se we used MA scanning as the

412 analytical tool. Moreover, we examined the effectiveness of the two inorganic  
 413 selenocompounds selenite (Se oxidation state +IV) and selenate (Se oxidation state +VI) for  
 414 differential gene expression. In order to produce as distinct effects for Se as possible, we  
 415 compared the gene expression of Se supplemented rats with that of their companions during  
 416 Se deficiency. In addition a direct comparison of selenite- and selenate-regulated genes was  
 417 carried out. With regard to the regulation of functional selenoproteins our data (Table 3) are  
 418 in accordance with very recent findings on the influence of Se status on selenoprotein mRNA  
 419 levels in mice and rats [33].  
 420 The pronounced loss of GPx1 mRNA expression and activity due to Se deficiency and a  
 421 maximization of both parameters by Se supply at the recommended dietary level (0.2 mg  
 422 Se/kg as selenite or selenate, groups I and II) and at a moderate supranutritive supply (1.0  
 423 mg Se/kg as selenite or selenate) confirm the low rank of GPx1 in the hierarchy of  
 424 selenoproteins. Conversely the high rank of GPx4 was confirmed by only a small loss of  
 425 mRNA expression and enzyme activity as well as a low P value for regulation (Table 3) due  
 426 to a lack in dietary Se supply. Our results moreover suggest that selenite and selenate are  
 427 similar in their effectiveness in influencing the expression and activity of GPx1, GPx4 and the  
 428 expression of further selenoproteins.  
 429 The counter-regulation of GPx1 and the phase II detoxification enzymes GSTa2, epoxide  
 430 hydrolase and Afar (Table 1, area I) was another central finding of our study. In the literature  
 431 a few reports describe similar effects of Se on these so called xenobiotica metabolizing  
 432 enzymes. Thus in a rat trial an increased aflatoxin toxicity due to Se supplementation was  
 433 observed, while a moderate Se deficiency effected a distinctly better aflatoxin detoxification  
 434 based on the up-regulation of the assigned Aflatoxin-B1-aldehyde reductase (Afar) and  
 435 additionally of GSTA2 [9]. Other studies reported an up-regulation of several glutathione-S-  
 436 transferases and of Epoxide hydrolase in rat liver by Se deficiency. However, recent  
 437 consolidated findings have shown the underlying mechanism for this phenomenon. In Se  
 438 deficiency, the transcription of phase II detoxification enzymes increases via the activation of  
 439 the Antioxidant Response Element (ARE), a DNA structure located upstream the coding  
 440 DNA sequence of these enzymes. ARE activation is sensitive to changes in cellular redox  
 441 status, an increase in peroxide tone, and to interaction with the transcription factor NF-E2-  
 442 related factor 2 (Nrf2). Different glutathione-S-transferases, various aldo-keto-reductases,  
 443 such as Afar, and epoxide hydrolases possess an ARE. In our study the up-regulation of  
 444 phase II detoxification enzymes by Se deficiency and vice versa the down-regulation with  
 445 sufficient Se supply were supported by Heme oxygenase 3 regulation, representing another  
 446 enzyme with an ARE in its DNA. That heme oxygenases respond to Se status is confirmed  
 447 by the results of other trials, in which an up-regulation of heme oxygenase 1 due to a lack of  
 448 Se supply was reported [10].  
 449 However, a very important and unexpected result of our study was the finding that selenate  
 450 feeding related to Se deficiency had a distinctly stronger influence on differential gene  
 451 expression than selenite.  
 452 Fundamental differences in the intestinal absorption pathways, in the blood transport to  
 453 peripheral organs, and in the subsequent intermediary metabolism of selenite (Se oxidation  
 454 state +IV) and selenate (Se oxidation state +VI) may provide one plausible explanation for  
 455 this effect [23, 34]. In peripheral organs the absorption products from dietary selenite  
 456 (oxidation states 0 and -I) need far fewer reduction steps than selenate (oxidation state +VI).  
 457 In Figure 4 A, B and C possible chemical reactions of selenite and selenate are summarized  
 458 [17, 35, 36] which may explain the increased thiol reactivity of dietary selenate in peripheral  
 459 tissues and represent one essential reason for its greater potential in influencing differential  
 460 gene expression. The metabolic fates of different Se compounds still need intensive  
 461 investigation using Se species analysis and protein mass spectrometry. Antidiabetic and  
 462 insulin sensitizing effects of Se, restricted exclusively to the intermittent application of very  
 463 high selenate doses, are based on the inhibition of Protein tyrosine phosphatase 1B  
 464 (PTP1B). According to Figure 4 B and C selenious acid formation and superoxide anion  
 465 generation during the peripheral reduction of larger selenate quantities provide a good  
 466 example of the high reactivity of oral selenate towards cysteine residues in proteins such as  
 467 PTP1B. For the interpretation of future data from Se studies, one should consider that orally

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468 applied selenate mimics properties of selenite applied in cell cultures and in other in vitro  
 469 studies [11, 17, 23].  
 470 In our present MA experiment we have analyzed a distinct up-regulation of PTP1B mRNA  
 471 due to Se supplementation at the recommended dietary level and at a slightly supranutritive  
 472 concentration. PTP1B has recently been discussed as being a promising target in the  
 473 treatment of obesity, insulin resistance and diabetes. Moreover, the enzyme is involved in  
 474 growth and development of mammary tumours and in the activation of lipogenesis. Via the  
 475 reduction of tyrosine phosphorylation of key signalling proteins such as IRS1 and Akt, an  
 476 increased expression and activity of PTP1B may force the development of insulin resistance,  
 477 obesity, hyperlipidemia, and diabetes [37, 38]. It may also be critical with regard to the  
 478 growth of mammary cancer [39]. In contrast the reduced Akt phosphorylation mediated by  
 479 increased PTP1B activity through dietary Se supplementation has been investigated as one  
 480 mechanism explaining possible beneficial Se effects in prostate cancer treatment [12]. With  
 481 regard to metabolic disorders recent large cross sectional studies and Se intervention trials  
 482 support the PTP1B hypothesis by the finding that a significant positive correlation exists  
 483 between Se status and the incidence of type II diabetes and increased blood lipids [15, 19-  
 484 21]. In this context the detailed regulation of PTP1B by dietary Se has recently been  
 485 investigated by our group [22].  
 486 The regulation of other genes by selenate feeding (Table 1, area II) in particular indicates  
 487 additional pathways by which Se may promote the development of insulin resistance,  
 488 obesity, hyperlipidaemia and diabetes beside PTP1B regulation. Both the down-regulation of  
 489 80-k diacylglycerolkinase and the up-regulation of the Agouti related protein precursor, the  
 490 Hypocretin orexin receptor and of the Neuropeptide y precursor open perspectives for future  
 491 research concerning the role of Se in metabolic diseases [15, 18-21].  
 492 As mentioned above our data for the comparison of Se supplementation versus Se  
 493 deficiency suggest a possible preventive mechanism of Se with regard to prostate cancer.  
 494 Similarly the regulation of genes involved in growth and differentiation of mammary cancer  
 495 and lung cancer (ADAMTS-1), of colorectal cancer (PKCQ-interacting protein Picot), as well  
 496 as of various cancer types (Proliferin related protein) indicated a cancer protective function of  
 497 Se (Table 1, area III) [40-42]. However these effects did not increase by increasing Se  
 498 supplementation from the recommended dietary amount to the supranutritive level. The  
 499 comparison of selenite- and selenate-regulated genes indicated a possible additional role of  
 500 selenate with regard to the prevention of hepatocellular cancer (Alphab crystalline-related  
 501 protein = heat shock protein 20, Table 2) [43].  
 502 Large human intervention trials carried out to investigate the preventive role of long term Se  
 503 supplement use in cancer prevention revealed inconsistent results. Thus in the Nutritional  
 504 Prevention of Cancer (NPC) study in which 1,312 high-risk dermatology patients received  
 505 200 µg Se/day in the form of selenized yeast or a placebo (matched control), a decreased  
 506 risk of lung, colon, prostate, and total cancers was obtained by Se intervention, but an  
 507 increased risk of nonmelanoma skin cancer. In a smaller subtrial of the NPC study in which  
 508 the participants received even 400 µg Se/day the cancer protective effect of the 200 µg dose  
 509 was lost [16]. In contrast the Selenium and Vitamin E Cancer Prevention Trial (SELECT) in  
 510 which healthy men were supplemented either with 200 µg Se/day in the form of  
 511 selenomethionine or with vitamin E (400 I.U./day) or with a combination of both supplements  
 512 compared to a placebo group was abandoned prematurely in autumn 2008 since the Se  
 513 supplemented participants showed a 5% higher incidence of prostate cancer [15]. Due to the  
 514 fact that tumour cells frequently possess a changed apoptosis program, the long term use of  
 515 supranutritional Se supplements in cancer prevention and treatment should be reconsidered  
 516 as well. This position is corroborated by our microarray data indicating both pro- and anti-  
 517 apoptotic features of Se by modulation of the expression of NAPOR 3, BCL2-antagonist 1,  
 518 FOS related antigen, Granzyme k, Mast cell protease, and p58 (Table 1, area III) [44-49].  
 519 Very recent findings suggest that Se supplementation for cancer prevention is of particular  
 520 interest for humans with selenoprotein P polymorphisms since this genetic constellation may  
 521 reduce Se transfer to the peripheral organs and the subsequent sufficient synthesis of  
 522 antioxidant selenoproteins [50].

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523 From the comparison of gene expression related to Se deficiency, our data suggest that Se  
 524 and, in particular, selenate is effective in influencing many other genes in the mammalian  
 525 organism. Thus the regulation of genes that are also expressed in the liver and have specific  
 526 functions for the integrity of the heart muscle and the skeletal muscle (e.g., Cardiac ankyrin  
 527 repeat protein, Fibroblast Growth factor 13, Glial cell line-derived neurotrophic factor receptor  
 528 beta, Mu-calpain large subunit, Tissue inhibitor of metalloproteinase 3, Titin, and Wingless-  
 529 type MMTV integration site family) [51-56] may help to gain new insight into the beneficial  
 530 effects of Se in the prevention of muscular dystrophy (Table 1, area V). Similarly, the  
 531 established effectiveness of Se in preventing liver necrosis is supported by the up-regulation  
 532 of Alpha 7 c integrin, Mitogen activated protein kinase 12 ZPK, Plectin 1 (Table 1, area III)  
 533 and of Cyclic-Adenosine-3',5'-monophosphate response element binding protein 1 (Table 1,  
 534 area V) [57-60].

535 The direct comparison of selenite- and selenate regulated genes revealed additional effects  
 536 of selenate e.g. in liver fatty acid- and sterol metabolism and in central mechanisms of body  
 537 weight regulation (5-hydroxytryptamine serotonin receptor 6 and growth hormone-releasing  
 538 hormone receptor beta) [61], in the protection against hepatocellular carcinoma (Alphab  
 539 crystalline-related protein=heat shock protein 20), in the modulation of T cell response (Ly-  
 540 49.9 antigen and the inhibitory natural killer cell receptor Nkr-p1b), and in the protection  
 541 against lipid toxicity (aldo-keto reductase akr1b-7) [43, 62]

542  
 543 In summary our study on the effects of the two common inorganic Se compounds selenite  
 544 and selenate on the differential expression of genes other than selenoproteins showed the  
 545 following:

- 546 ● Compared to Se deficiency as a control, selenite (Se oxidation state +IV) and selenate  
 547 (Se oxidation state +IV) resembled each other in their effectiveness for selenoprotein  
 548 synthesis. Both Se compounds could be demonstrated to be equally effective in the  
 549 down-regulation of not less than 3 enzymes of phase II detoxification and of a further  
 550 gene with an ARE in its DNA (Afar, epoxide hydrolase, GSTa2, heme oxygenase 3). This  
 551 result demonstrates mechanisms of counter-regulation of antioxidant enzymes.
- 552 ● Compared to Se deficiency selenate has a distinctly stronger influence than selenite on  
 553 differential gene expression in various functional areas. This distinct difference, so far  
 554 unknown, may result from fundamental differences in the absorption and the intermediary  
 555 metabolism of both Se compounds. This remarkable difference for both Se compounds  
 556 should be considered in future investigations into Se biochemistry.
- 557 ● The direct comparison of selenite- and selenate regulated genes revealed additional  
 558 functions of selenate in liver fatty acid- and sterol metabolism and in central mechanisms  
 559 of body weight regulation, in the protection against hepatocellular carcinoma, in the  
 560 modulation of T cell response, and in the protection against lipid toxicity (aldo-keto  
 561 reductase akr1b-7).
- 562 ● In view of the distinct influence of Se on differential gene expression, our data could  
 563 contribute to an improved understanding of mechanisms by which Se may influence  
 564 diseases of the Western world. Other genes differentially regulated by selenate provided  
 565 deeper insight into the beneficial effects of Se in preventing Se deficiency symptoms  
 566 such as muscular dystrophy and liver necrosis.

567  
 568 We conclude that future studies on the pathways of Se in the treatment of diseases  
 569 should consider the particular effects of the Se compound used, and that the compilation  
 570 of comparative differential gene expression profiles for further selenium compounds  
 571 would be of interest.  
 572

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**Table 1** Genes from various functional areas (I-V) differentially regulated in rat liver by feeding selenite and selenate at two dietary levels (0.2 and 1.0 mg/kg) versus Se deficiency

Gene Bank Assoc Number	Gene name	Selenite0.2 (P value)	Selenite1.0 (P value)	Selenate0.2 (P value)	Selenate1.0 (P value)
<b>Area I: Genes related to phase I- or phase II detoxification</b>					
NM013215	Aflatoxin B1 Aldehyde Reductase	4.07↓ (<0.001)	3.14↓ (<0.001)	4.10↓ (<0.001)	3.25↓ (<0.001)
NM012540	Cytochrome P450	1.85↑ (0.434)	1.84↑ (0.209)	2.99↑ (0.022)	2.70↑ (0.030)
M26125	Epoxide Hydrolase	3.13↓ (<0.001)	2.43↓ (<0.001)	2.69↓ (<0.001)	1.74↓ (0.001)
NM017013	Glutathione-S-Transferase A2	2.49↓ (0.003)	2.50↓ (0.002)	2.28↓ (0.001)	2.52↓ (0.001)
<b>Area II: Genes involved in the regulation of intermediary metabolism, the regulation of appetite and body weight or other metabolic processes</b>					
NM031118	Acyl Coenzyme A : Cholesterol Acyltransferase 1	2.37↑* (0.149)	3.52↑* (0.092)	3.44↑ (0.018)	3.00↑ (0.040)
AF206017	Agouti Related Protein Precursor	1.95↑* (0.208)	3.57↑ (0.002)	6.17↑ (0.017)	3.00↑ (0.002)
NM019168	Arginase II	1.10↑* (0.860)	5.88↑ (0.046)	3.14↑ (0.014)	3.25↑ (0.006)
S49760	Diacylglycerol Kinase, 80k-DG Kinase	1.07↓* (0.876)	1.52↓* (0.289)	2.39↓ (0.014)	3.03↓ (0.008)
NM019333	Fructose-6-Phosphate-2-Kinase	3.27↑* (0.176)	2.74↑* (0.161)	3.41↑ (0.012)	4.43↑ (0.005)
AF058787	Heme Oxygenase 3	1.16↓* (0.706)	1.28↓* (0.445)	2.55↓ (<0.001)	3.30↓ (<0.001)
NM024365	5-hydroxytryptamine serotonin receptor 6	2.05↑* (0.214)	1.10↑* (0.888)	4.15↑ (0.005)	7.13↑ (<0.001)
NM013064	Hypocretin Orexin Receptor 1	1.61↑* (0.549)	1.90↑* (0.385)	3.32↑ (0.008)	3.36↑ (0.008)
NM012614	Neuropeptide Y Precursor	2.33↑* (0.321)	1.81↑* (0.213)	3.30↑ (0.004)	3.70↑ (0.002)
NM020099	Ob Receptor Gene Related Protein	2.49↑* (0.297)	2.18↑* (0.377)	3.00↑ (0.046)	3.96↑ (0.019)
NM022626	Phosphorylase B Kinase Alpha Subunit	3.25↑* (0.151)	3.35↑* (0.071)	3.60↑ (0.027)	5.48↑ (0.005)
NM012637	Protein Tyrosine Phosphatase 1B	1.84↑* (0.003)	1.60↑* (0.007)	3.14↑ (0.024)	2.53↑ (<0.001)
M58040	Transferrin Receptor	3.40↑* (0.087)	2.53↑* (0.169)	3.84↑ (0.003)	4.03↑ (0.003)
<b>Area III: Genes related to the cell cycle, cell development, apoptosis or tumour metabolism</b>					
NM024400	ADAMTS-1	1.32↓* (0.330)	1.75↓* (0.055)	2.48↓ (0.006)	2.50↓ (0.006)
X74294	Alpha 7c Integrin	3.11↑* (0.025)	3.33↑* (0.055)	4.50↑ (0.002)	4.11↑ (0.003)
D29960 (Table 2)	Alphab crystallin-related protein	1.90↑ (0.219)	1.59↑ (0.453)	5.85↑ (0.002)	4.10↑ (0.005)
D25543 (Table 2)	Rat gcp360	1.59↑ (0.498)	2.26↑ (0.178)	4.67↑ (<0.001)	5.64↑ (<0.001)
AF090695	NAPOR-3	1.08↓* (0.839)	1.03↓* (0.924)	2.34↓ (0.019)	2.54↓ (0.017)

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612 **Table 1 Continued**

Gene Bank Assoc Number	Gene name	Selenite0.2 (P value)	Selenite1.0 (P value)	Selenate0.2 (P value)	Selenate1.0 (P value)
<b>Area III: Genes related to the cell cycle, cell development, apoptosis or tumour metabolism</b>					
NM053812	BCL 2 Antagonist 1	1.37↑* (0.517)	1.72↑* (0.349)	2.81↑ (0.011)	3.16↑ (0.010)
Z12298	Dermatan Sulfate Proteoglycan II	2.87↑* (0.225)	2.57↑* (0.284)	3.98↑ (0.009)	3.63↑ (0.011)
NM030585	FOS Related Antigen	3.51↑* (0.122)	3.27↑* (0.186)	6.54↑ (0.009)	4.67↑ (0.004)
NM017119	Granzyme K	1.79↓* (0.071)	1.13↓* (0.731)	2.48↓ (0.005)	2.46↓ (0.009)
NM017145	Mast Cell Protease 1	1.77↑* (0.488)	2.47↑* (0.274)	3.72↑ (0.013)	2.73↑ (0.040)
NM013055	Mitogen Activated Protein Kinase 12 ZPK	2.32↑* (0.351)	1.99↑* (0.357)	4.03↑ (0.050)	3.48↑ (0.040)
U15425	P58	1.42↑* (0.625)	1.81↑* (0.087)	2.35↑ (0.007)	2.09↑ (0.036)
NM032614	PKCQ Interacting Protein Picot	1.21↓* (0.582)	1.03↓* (0.914)	2.30↓ (0.006)	2.08↓ (0.008)
U96275	Plectin 1	2.00↑* (0.296)	3.28↑* (0.070)	5.14↑ ( $<0.001$ )	5.54↑ ( $<0.001$ )
NM053364	Proliferin Related Protein	2.33↑* (0.275)	2.68↑* (0.228)	4.15↑ (0.002)	3.61↑ (0.002)
NM019906	Rapamycin And FKBP12 Target-1	2.43↑* (0.095)	2.95↑* (0.174)	3.84↑ (0.010)	3.84↑ ( $<0.001$ )
<b>Area IV: Genes related to inflammatory or immunomodulatory response</b>					
NM021583	Ind. Prostaglandine Synthase	1.73↑* (0.314)	2.64↑* (0.074)	3.33↑ (0.023)	4.58↑ ( $<0.001$ )
NM057193	Interleukin 10 Receptor	1.94↑* (0.220)	2.67↑* (0.065)	2.81↑ (0.010)	2.84↑ (0.009)
NM020542	Macrophage Inflamm. Protein 1 Alpha Rec.	1.48↓* (0.299)	1.33↓* (0.485)	3.17↓ (0.004)	3.86↓ (0.003)
M34097	Rat Natural Killer Cell Protease 1	1.87↑* (0.334)	2.40↑* (0.018)	2.59↑ (0.005)	3.75↑ (0.004)
<b>Area V: Genes with other functions (hormonal function, receptor function, signal transduction)</b>					
NM013220	Cardiac Ankyrin Repeat Protein	2.00↓* (0.115)	1.46↓* (0.319)	3.05↓ (0.043)	2.92↓ (0.045)
S78641	Cycl. Adenosine 3',5'- Monophosphate Resp. Elem. Binding Protein	1.86↑* (0.421)	1.53↑* (0.451)	3.29↑ (0.018)	2.58↑ (0.020)
NM053428	Fibroblast Growth Factor 13	2.80↑* (0.129)	3.14↑* (0.078)	4.63↑ ( $<0.001$ )	4.09↑ ( $<0.001$ )
NM012750	Glial Cell Line Der. Neurotrophic Factor Rec. Beta	1.57↑* (0.524)	2.41↑* (0.332)	3.88↑ (0.006)	3.66↑ (0.019)
NM019152	MU Calpain Large Subunit	1.53↑* (0.430)	2.69↑* (0.227)	4.00↑ (0.013)	4.55↑ (0.006)
NM012886	Tissue Inhibitor of Metalloproteinase 3	1.90↑* (0.358)	4.44↑ (0.046)	3.76↑ (0.011)	2.68↑ (0.045)
AJ401157 (Table 2)	Titin	1.00↑* (0.999)	1.53↑* (0.204)	2.72↑ (0.016)	3.62↑ (0.012)
NM053402	Wingless Type MMTV Integration Site Family	2.91↑* (0.188)	6.16↑ (0.011)	3.51↑ (0.039)	5.44↑ (0.008)

613 \* indicate that selenite supplementation regulated most genes into the same direction as selenate  
 614 supplementation (up↑/down↓), but that these effects were not significant (P<0.05); genes occurring  
 615 additionally in Table 2 are highlighted on a grey background and a reference remark to Table 2 is given

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617**Table 2** Selection of genes differentially regulated in rat liver comparing selenate feeding with selenite feeding at both dietary levels (0.2 and 1.0 mg/kg)

Gene Bank Assoc Number	Gene name	Selenate vs. Selenite [both levels] (P value) [Selenate 0.2 vs. Selenite 0.2] [Selenate 1.0 vs. Selenite 1.0]	Selenite 0.2 vs. 0Se (P value)	Selenite 1.0 vs. 0Se (P value)	Selenate 0.2 vs. 0Se (P value)	Selenate 1.0 vs. 0Se (P value)
NM012850	Putative growth horm. releasing hormone receptor-related protein	<b>3.22↓</b> (0.005) [2.51↓] [4.03↓]	<b>1.11↓</b> (0.628)	<b>1.27↑</b> (0.525)	<b>2.80↓</b> (0.020)	<b>3.17↓</b> (0.014)
** U56936	Nkr-p1b	<b>2.42↓</b> (0.007) [4.00↓] [1.69↓]	<b>1.59↑</b> (0.522)	<b>1.46↑</b> (0.601)	<b>2.51↓</b> (0.523)	<b>1.14↓</b> (0.886)
* AJ401157 (Table 1)	Titin	<b>2.50↑</b> (0.013) [2.72↑] [2.36↑]	<b>1.00↑</b> (0.999)	<b>1.53↑</b> (0.204)	<b>2.72↑</b> (0.016)	<b>3.61↑</b> (0.012)
* D25543 (Table 1)	Rat gcp360	<b>2.67↑</b> (0.004) [2.92↑] [1.74↑]	<b>1.59↑</b> (0.498)	<b>2.26↑</b> (0.178)	<b>4.67↑</b> ( $<0.001$ )	<b>5.64↑</b> ( $<0.001$ )
AF100789	G protein-coupled receptor gpr55	<b>2,74↑</b> (0.002) [3.59↑] [2.24↑]	<b>1.37↓</b> (0.501)	<b>1.27↑</b> (0.639)	<b>2.61↑</b> (0.015)	<b>2.86↑</b> (0.008)
* D29960 (Table 1)	Alphab crystallin-related protein	<b>2.85↑</b> (0.004) [3.07↑] [2.57↑]	<b>1.90↑</b> (0.219)	<b>1.59↑</b> (0.453)	<b>5.85↑</b> (0.002)	<b>4.10↑</b> (0.005)
S64315	Metabotropic glutamate receptor subtype 5b	<b>3.72↑</b> (0.047) [6.82↑] [1.81↑]	<b>1.45↓</b> (0.407)	<b>1.18↑</b> (0.843)	<b>4.68↑</b> (0.006)	<b>2.20↑</b> (0.002)
NM024365	5-hydroxy-tryptamine receptor 6	<b>3.81↑</b> (0.006) [2.02↑] [7.84↑]	<b>2.04↑</b> (0.214)	<b>1.10↓</b> (0.887)	<b>4.14↑</b> (0.005)	<b>7.12↑</b> ( $<0.001$ )
** NM05378 1	Aldose-reductase-like (akr1-b7)	<b>5.14↑</b> (0.014) [4.24↑] [6.20↑]	<b>1.13↓</b> (0.823)	<b>1.36↓</b> (0.637)	<b>3.75↑</b> (0.017)	<b>4.56↑</b> (0.007)
** U56863	Ly-49.9 antigen	<b>7.92↑</b> (0.003) [4.81↑] [15.8↑]	<b>1.49↓</b> (0.296)	<b>3.78↓</b> (0.033)	<b>3.23↑</b> (0.004)	<b>4.16↑</b> (0.020)

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Genes regulated in an analogous manner by selenate and selenite are highlighted with \* and appear on a dark grey background. Moreover a reference to Table 1 is given. Genes regulated in a contrary manner by selenate and selenite are highlighted with \*\* and appear on a light grey background. Genes regulated indifferently by selenite, but consistently by selenate appear on a white background.

622 **Table 3** Hierarchy of functional selenoproteins in rat liver as analyzed by  
 623 microarray analysis

Selenoprotein	Selenite 0.2 vs. 0 Se	Selenate 0.2 vs. 0 Se	Selenite 1.0 vs. 0 Se	Selenate 1.0 vs. 0 Se	Mean P value for extent of regulation
GPx1	6.99	5.48	6.62	5.88	0.0056
SelP	2.06	1.71	2.11	1.94	0.0112
15 kDa Sep	1.15	1.54	1.30	1.32	0.0210
SelW	1.15	1.54	2.18	1.21	0.0230
DIO1	1.57	1.52	1.62	1.24	0.0335
GPx4	1.33	1.24	1.32	1.31	0.0883

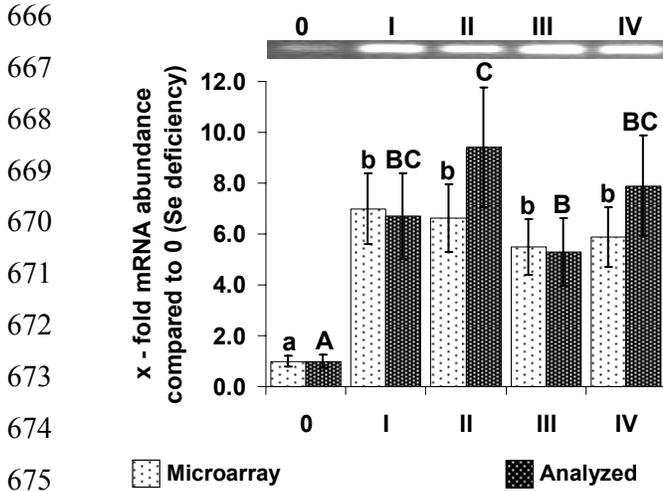
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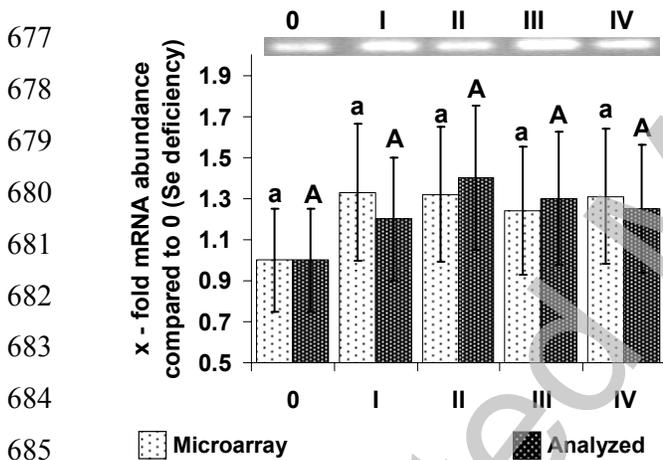
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664 **FIGURE 1**

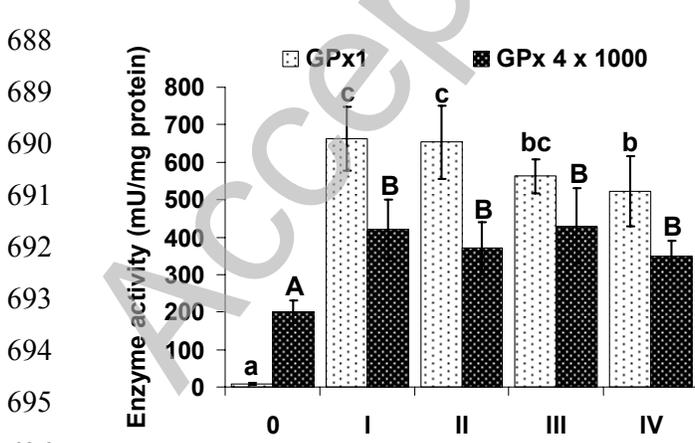
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687 **C**



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697 **Figure 1** **Regulation of GPx1 and GPx4 in rat liver after 8 weeks of Se deficiency**  
 698 **(0) compared to Se supplementation with 0.2 or 1.0 mg Se/kg diet as**  
 699 **selenite (I, III) or selenate (II, IV)**

700 A) mRNA expression of GPx 1 as analyzed by MA analysis and controlled by 2-step RT-  
 701 PCR; B) mRNA expression of GPx 4 as analyzed by MA analysis and controlled by 2-step  
 702 RT-PCR C) GPx1- and GPx4-activity

703 Values are means±SD. Significant differences between means ( $p<0.05$ ) are indicated by  
 704 different small letters for MA expression data (A, B), by different capital letters for 2-step RT-  
 705 PCR (A, B), by different small letters for GPx1 activity and by different capital letters for GPx4  
 706 activity (C).

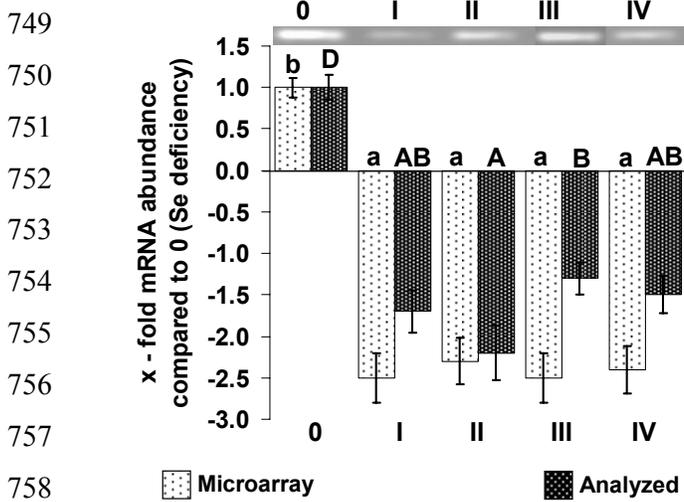
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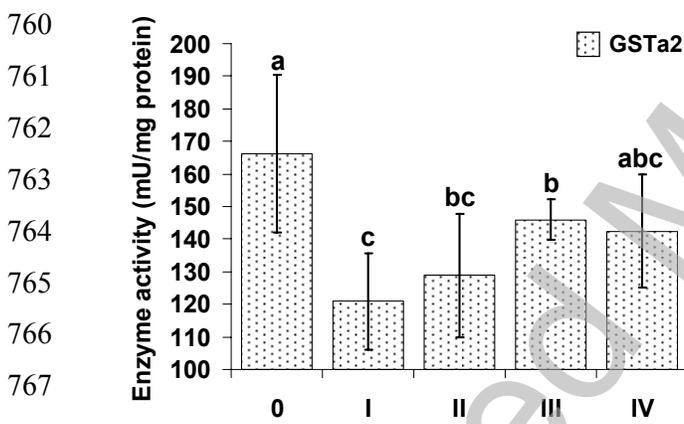
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747 **FIGURE 2**

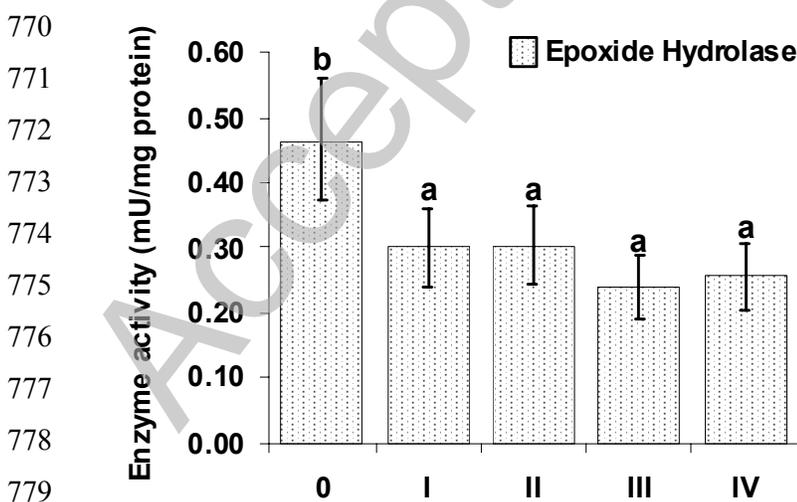
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780 **Figure 2** *Regulation of GSTa2 and epoxide hydrolase in rat liver after 8 weeks of*  
 781 *Se deficiency (0) compared to Se supplementation with 0.2 or 1.0 mg*  
 782 *Se/kg diet as selenite (I, III) or selenate (II, IV)*

783 *A) mRNA expression of GSTa2 as analyzed by MA analysis and controlled by 2-step RT-*  
 784 *PCR B) GSTa2 activity C) epoxide hydrolase activity*

785 *Values are means±SD. Significant differences between means (p<0.05) are indicated by*  
 786 *different small letters for MA expression data for GSTa2 (A), by different capital letters for 2-*  
 787 *step RT-PCR of GSTa2 (A), and by different small letters for GSTa2 and epoxide hydrolase*  
 788 *enzyme activities (B, C).*

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830 **FIGURE 3**

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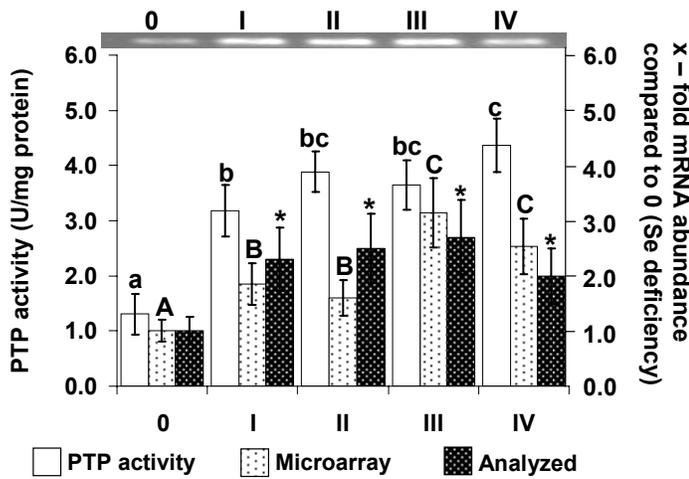
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**Figure 3 Regulation of PTP1B in rat liver after 8 weeks of Se deficiency (0) compared to Se supplementation with 0.2 or 1.0 mg Se/kg diet as selenite (I, III) or selenate (II, IV)**

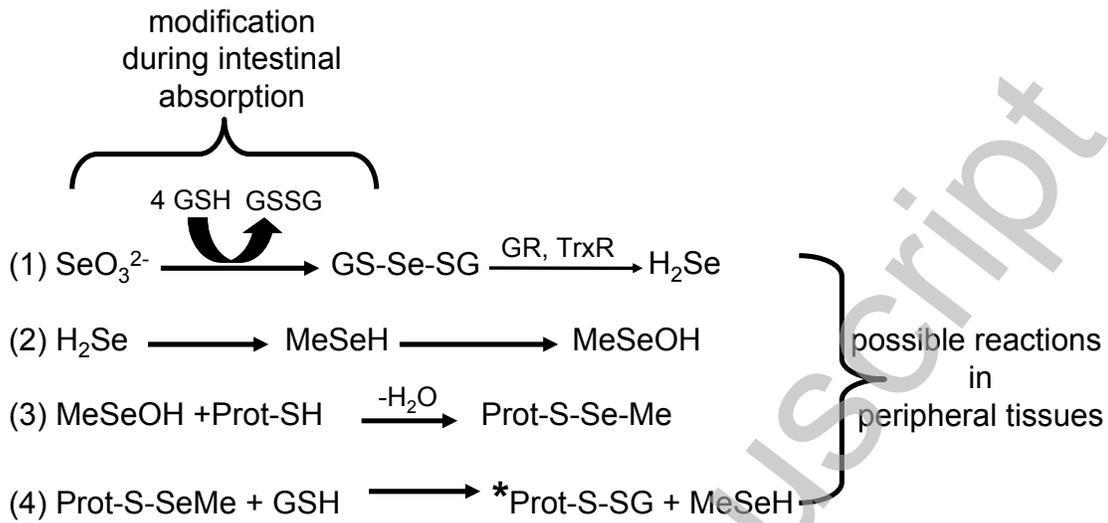
The figure shows PTP1B expression as analyzed by MA analysis and controlled by 2-step RT-PCR as well as PTP enzyme activity  
 Values are means±SD. Significant differences between means ( $p<0.05$ ) are indicated by different small letters for PTP activity, by different capital letters for MA expression data, and by an asterisk for 2-step RT-PCR results.

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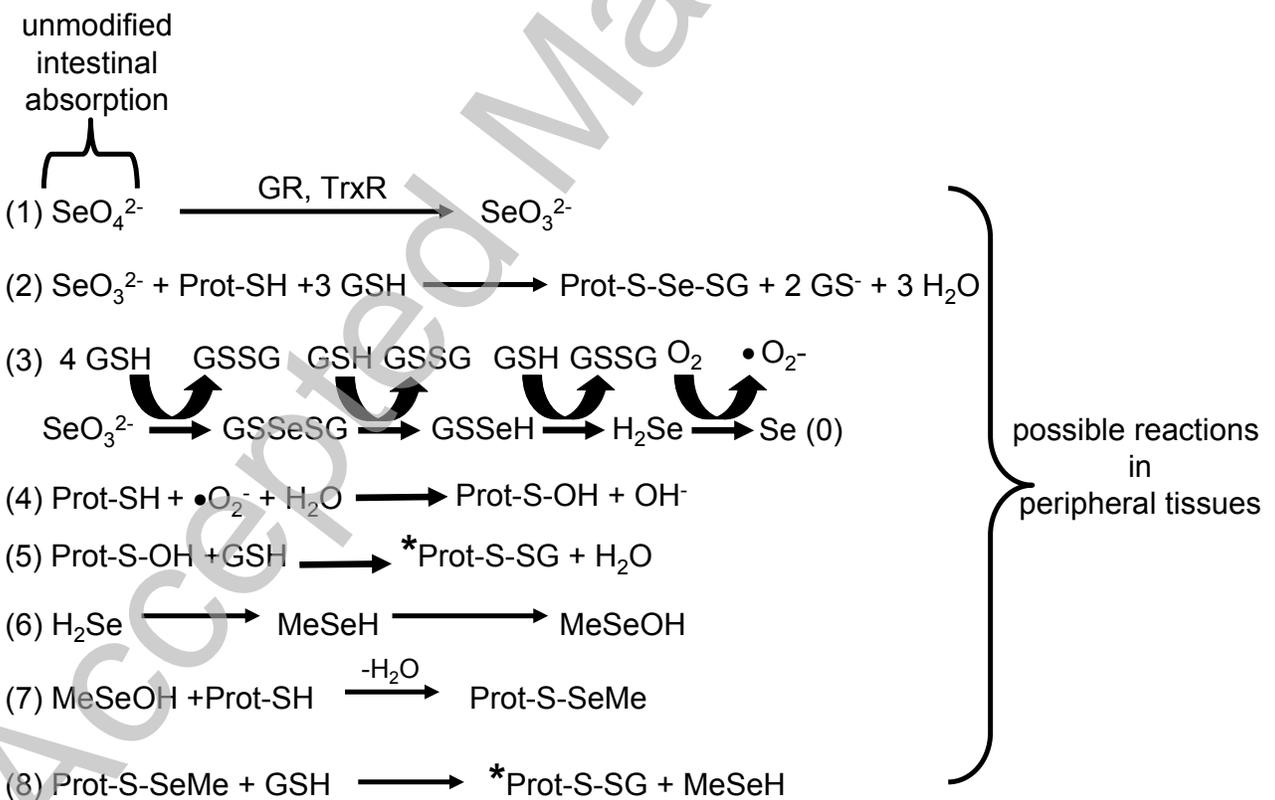
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872 **FIGURE 4**

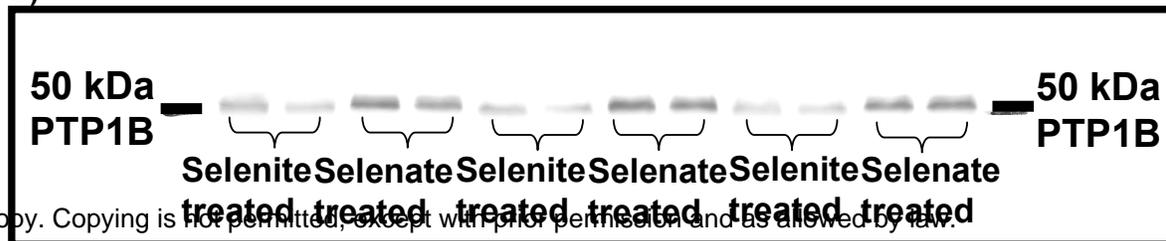
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923 **Figure 4** **Pathways and chemical reactions of selenite and selenate during**  
 924 **absorption and in peripheral tissues, and reactions leading to protein-**  
 925 **thiol-modifications**

926 **A) Selenite pathways:**

927 *(1): Selenite reacts already during its absorption with thiol compounds, such as GSH, to form*  
 928 *selenodiglutathione and selenotrisulfides.*

929 *(1 and 2): Se metabolites delivered to tissues from selenite absorption are*  
 930 *selenodiglutathione, selenotrisulfides or hydrogen selenide.*

931 *(2 and 3): Pathways by which orally administered selenite can modify cysteine residues in*  
 932 *other proteins and thereby lead to changes in differential gene expression derive from the*  
 933 *formation of methylselenol from methylselenide, which again can be produced from hydrogen*  
 934 *selenide, obtained from the entire reduction of selenite.*

935 *(3): Methylselenol may modify cysteine residues in proteins and change the activity of these*  
 936 *proteins via thiol modifications such as glutathionylation by methylselenolate release in the*  
 937 *presence of glutathione.*

938 **B) Selenate pathways:**

939 *(1): Selenate may exert a stronger influence on differential gene expression starting with its*  
 940 *unmodified uptake through the small intestine into the organism. During its reduction in*  
 941 *peripheral organs the following reactions with cysteine containing proteins are feasible:*

942 *(1): In the first reaction step the unreactive selenate (oxidation state +VI) is reduced in a GR*  
 943 *or TrxR dependent reaction to the highly thiol reactive selenite oxidation state +IV.*

944 *(2): In the further course selenite (selenious acid) can modify protein thiol groups to*  
 945 *selenodiglutathione intermediates.*

946 *(3): Excess selenite (selenious acid) reacts with GSH to form hydrogen selenide. In the*  
 947 *further course of the reaction in the presence of oxygen, superoxide anion radicals ( $O_2^{\cdot-}$ ) and*  
 948 *elemental Se can be formed.*

949 *(4): The superoxide radical again can attack cysteine residues in proteins and oxidize them*  
 950 *to sulfenic acid derivatives.*

951 *(5): In the presence of glutathione the sulfenic acid can react to a reversibly inactivated*  
 952 *(glutathionylated) protein-thiol compound.*

953 *The pathways (6), (7), and (8) in selenate metabolism, starting from hydrogen selenide are*  
 954 *identical with the pathways (2), (3), and (4) in selenite metabolism.*

955 *\*in Figure 4 A and B indicate that in selenite metabolism only one pathway leads to the*  
 956 *formation of glutathionylated (inactivated) protein intermediates with SH-group containing*  
 957 *proteins, whereas this modification can be formed by two pathways during selenate*  
 958 *metabolism.*

959 **C) Evidence for the higher thiol reactivity of selenate compared to selenite from**  
 960 **diabetic dbdb mice, treated with supranutritional selenite- and selenate doses**

961 The higher protein glutathionylation effected by orally applied selenate compared to selenite,  
 962 marked with\* in Figure 4 A and B can clearly be proven by Western Blot Analysis using an  
 963 "anti glutathione antibody". The Western Blot shows a distinctly higher glutathionylation of  
 964 PTP1B, a protein with a catalytically active cystein SH-residue, in dbdb mice treated with  
 965 supranutritional selenate compared to their selenite treated companions [unpublished results  
 966 from 17]. Thus this reaction is clear proof of the higher peripheral thiol reactivity of selenate.

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