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Regulation of the insulin antagonistic protein tyrosine phosphatase 1B by dietary Se studied in growing rats

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a key enzyme in the counterregulation of insulin signaling, and its physiological modulation depends on H_2O_2 and glutathione (GSH). Se via GSH peroxidases (GPxs) and its specific metabolism is involved in the removal of H_2O_2 and in the regulation of GSH metabolism. Recent results from animal trials and epidemiological studies with humans have shown that a high GPx1 activity or a permanent surplus of Se may promote the development of obesity and diabetes. Our nutrition physiological study with 7×7 growing rats was carried out to examine if PTP1B is modulated by Se supplements and, thus, may represent one trigger mediating these undesirable metabolic effects of Se. One group of rats was fed an Se-deficient diet for 8 weeks. The diets of the other six groups contained Se as selenite or selenate according to the recommendations (0.20 mg/kg diet) and at two supranutritional levels (1.00 and 2.00 mg/kg diet). All Se-supplemented animals featured a significantly higher body weight (6–14%) compared to their Se-deficient companions. Expression and activity of GPx1 in the liver of Se supplemented animals was 10- and 70-fold higher compared to Se deficiency. The detailed study of PTP1B regulation using an enzymatic assay and Western Blot analysis with an antibody against protein glutathionylation revealed that PTP1B was significantly up-regulated by both a maximization of GPx1 activity and by increasing dietary Se supply, reducing its inhibition via glutathionylation. Selenate effected a stronger PTP activation compared to selenite. In conclusion, our results suggest that the modulation of PTP1B activity may represent one plausible mechanism by which a long-term intake of Se supplements exceeding the requirements can promote the development of obesity and diabetes and needs further intensive investigation.

Keywords: Selenium; Selenite; Selenate; PTP1B; Glutathionylation; Diabetes; Insulin resistance

1. Introduction

In industrial countries, the number of patients suffering from obesity, insulin-resistant diabetes and the metabolic syndrome is permanently increasing [1,2]. Concomitantly in the fortification of foodstuffs derived from plants and animals with vitamins, minerals and trace elements including Se by fertilization and animal nutrition is widely practised [3–6]. Thus, consumers on the one hand take additional amounts of these substances unknowingly in their daily diet. On the other hand, a wide choice of vitamin and mineral supplements, originally designed to treat and prevent deficiency syndromes, are freely available in supermarkets [7]. Consumers frequently misuse these supplements in the belief that these will protect them from cancer [8] and other diseases of civilization or just improve their general health [7]. Evidence-based knowledge from epidemiological studies or intervention trials about the actual benefits, however, is missing for most substances. Data from recent literature have increasingly reported on the inefficacy or even adverse effects of vitamin and trace element supplements on health [9–12]. The benefits and risks of Se supplements for the

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prevention of obesity and Type II diabetes are currently also the subject of controversial discussion. Antidiabetic effects of Se were reported for animal models and tissue cultures, but they are restricted to very high selenate doses (Se oxidation state +VI), and their safety for humans has not been tested as yet [13,14]. Oral selenite application, however, failed to ameliorate diabetes [14,15]. The opposite effect of Se with regard to the development of obesity and insulin-resistant diabetes has been shown in transgenic mice overexpressing the selenoenzyme glutathione peroxidase (GPx) 1 [16]. Very recent results from two US studies with humans [Third National Health and Nutrition Examination Survey (NHANES) III and Nutritional Prevention of Cancer (NPC) trial] [17,18] independently have found a distinct positive correlation between serum Se concentration and cases of Type II diabetes. Data analysis in the NHANES III trial (8876 participants) was based on conventional nutrition, whereas the NPC trial was a randomized study in which 600 participants were supplemented with 200 μg Se daily in order to test the influence of the trace element on cancer progression. Another 602 participants of the control group received a placebo for 7.7 years. The data of the NHANES III trial revealed that Se status in US adults is distinctly higher compared to other populations. After subdivision of the data to quintiles, a 1.57-fold higher diabetes prevalence could be found in the top quintile (serum Se ≥137.7 μg/L) compared to the bottom quintile (serum Se ≤111.6 μg/L). The results from the NPC trial were even more pronounced compared to the NHANES III trial. The median value of serum Se concentration from those Se-supplemented participants and from the placebo group was 113.4 μg/L. The risk of developing diabetes in the residual group with a serum Se concentration beyond the median (>113.4 μg/L) was 2.5-fold higher compared to the residual group with a serum Se concentration below the median (≤113.4 μg/L). After subdivision of the data into tertiles, the risk of developing diabetes was even 2.7-fold higher in the top tertile (serum Se >121.6 μg/L) compared to the bottom tertile (serum Se ≤105.2 μg/L). In recent years, a number of studies have focused on the insulin antagonistic protein tyrosine phosphatase 1B (PTP1B) as a molecular target for the treatment of obesity and insulin resistant diabetes [19]. In studies with humans [20] and in animal models, it could be shown that PTP1B deficiency, obtained by a lowered expression [21–24] or biochemical enzyme inhibition through selenate and vanadium compounds [14,25,26], protects from obesity and insulin resistance, whereas high PTP1B activities can accelerate these diseases. In contrast to PTP1B regulation by exogenously applied agents, the enzyme underlies a physiological regulation via oxidation of the active site cysteine residue Cys215 (Fig. 1, upper part). In the presence of H_2O_2, initially, a reversibly oxidized sulphenic acid intermediate is formed [27,28], the further oxidation of which can be prevented by the formation of a cyclic sulphenyl amide [29,30], followed by the reaction with glutathione (GSH)/oxidized GSH (GSSG) to a mixed disulfide with Cys-215, termed *glutathionylation* [27,28]. The activity of reversibly oxidized PTP1B and of the glutathionylated enzyme can be partially recovered by the addition of dithiothreitol (DTT) or thiol-transferase [27,28]. The direct reaction of the reduced Cys-215 SH group in the presence of high GSSG concentrations (>25 mM) may also lead to glutathionylated PTP1B [29]. Besides, its generation as a coproduct of the respiratory chain and from the activity of some oxidoreductases H_2O_2 is also produced in mammalian tissues after insulin binding to its receptor, presumably for the differential regulation of PTP activity [32,33].

The aim of our physiological trial with growing rats was therefore to examine if PTP1B is modulated by Se supplements and, thus, may represent one trigger mediating undesirable metabolic effects of Se, since Se influences both intracellular H_2O_2 and GSH/GSSG concentration, which, in turn, are the very metabolites critical to physiological PTP1B regulation (Fig. 1, lower part).

2. Material and methods

2.1. Feeding trial with healthy growing rats

Forty-nine healthy growing male albino rats from the institute's own strain HK51 were randomly assigned to seven experimental groups of seven animals each. The Se-deficient basal diet (Group 0 Se; <0.02 mg Se/kg diet) was based on Torula yeast (30% Torula yeast, 5% cellulose, 5% glucose, 5% sucrose, 5% soybean oil, 0.6% DL-methionine, 0.05% tryptophan, 3.5% mineral premix, 1.0% vitamin premix, 0.2% choline chloride, 44.65% maize starch) and composed according to the National Research Council recommendations for laboratory rats [34]. The diets for groups 0.2 selenite, 0.2 selenate (recommended dietary level), 1.0 selenite, 1.0 selenate, 2.0 selenite and 2.0 selenate (supranutritional levels) were supplemented with either sodium selenite or sodium selenate to obtain final Se concentrations of 0.2, 1.0, and 2.0 mg Se/kg diet. The animals were kept individually and had ad libitum access to the diet and bidistilled water. After 8 weeks, the rats were decapitated under anaesthesia, and the livers were excised and stored at −80°C until further analysis.

Additionally, one group of seven weaned rats was killed before starting the feeding trial to analyze the initial Se status of the experimental animals.

The protocol of the animal study was approved by the Regional Council of Giessen.

2.2. Determination of Se concentration

Se concentration in the diets, the livers and plasma was determined by hydride generation atomic absorption spectrometry as reported previously [35]. Certified samples from the "National Institute of Standard and Technology" (soft winter wheat flour, NIST No. 8438), (bovine liver, NIST No. 1577b) and from "Medichem" (control serum, Metalle S) served as reference material for Se determination in the different matrices.

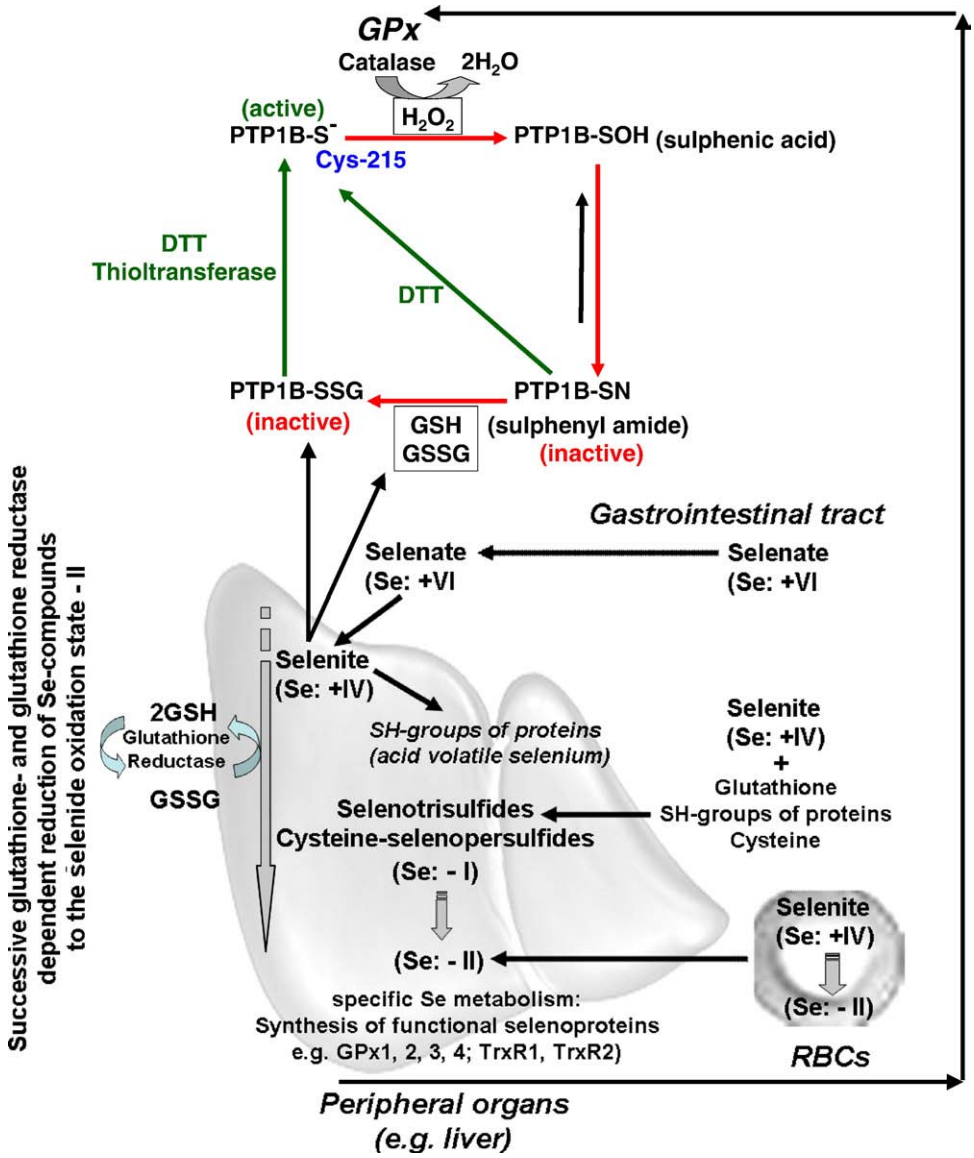


Fig. 1. Current understanding of physiological PTP1B regulation and interfaces with mammalian metabolism of inorganic Se compounds representing the basis for the experimental design of the present study (according to Refs. [14,26–31]) (detailed explanations for the single pathways with reference to the figure are given in the text).

2.3. Liver GPx1 and plasma GPx3

GPx 1 and 3 were measured in the 10,000×*g* cytosolic supernatant of 1:10 (w/v) liver homogenates prepared in 50 mM phosphate buffer or undiluted plasma by the indirect spectrophotometric procedure coupled to GSH reductase and NADPH consumption [36]. One unit of GPx1 or 3 activity was defined as one micromole NADPH oxidized per minute and normalized to 1 mg protein.

2.4. Total GSH and GSSG

The concentration of total GSH (GSH+reduced GSSG) and GSSG in rat liver was analyzed in the 10,000×*g* cytosolic supernatant according to the standard protocol coupled to GSH reductase and DTNB [37]. Sample concentrations were calculated from a standard curve prepared with pure GSSG (concentration range: 0–0.066 μmol GSSG/ml).

2.5. Catalase

Catalase activity was measured spectrophotometrically in crude liver homogenates (1:10 w/v) prepared in 50 mM phosphate buffer [38]. The method is based on the peroxidative activity of catalase to transform methanol and H_2O_2 into formaldehyde. The formaldehyde produced is measured spectrophotometrically at 550 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which, upon oxidation, changes from colorless to a purple color. Catalase activity of the samples

was calculated from a standard curve prepared with formaldehyde (concentration range: 75–1200 μM). One unit of catalase activity was defined as the amount of the enzyme that will cause the formation of 1.0 nmol formaldehyde per minute at room temperature and normalized to 1 mg protein.

2.6. Superoxide dismutase

For the differentiated measurement of superoxide dismutase (SOD) activity (distinguishing between total SOD, mitochondrial Mn SOD and cytosolic Cu/Zn SOD), 1:5 (w/v) crude liver homogenates were prepared in 50 mM Tris succinate buffer (pH 8.2). Subsequently, total SOD activity was assayed by the indirect photometric standard procedure measuring the inhibitory effect of SOD on pyrogallol (1,2,3-trihydroxybenzol) autoxidation [39,40]. The change in absorption caused by the formation of the yellow pyrogallol oxidation product, purpurogallin, was read for 3 min at 420 nm. For each determination, a 100% value without liver homogenate was carried out. According to the original method, one unit of SOD activity was defined as the 50% inhibition of pyrogallol autoxidation by the samples. For the differentiated measurement of SOD activity distinguishing between total SOD, mitochondrial Mn SOD and cytosolic Cu/Zn SOD, the abovementioned measurement was repeated in 50 mM Tris succinate buffer, containing additionally 1 mM potassium cyanide. Cyanide ions selectively inhibit cytosolic Cu/Zn SOD and consequently allow the assay of Mn SOD. Activity of Cu/Zn SOD was calculated from the difference of total SOD and Mn SOD. SOD activities were normalized to 1 mg of protein.

2.7. Liver PTP activity under native and reducing conditions

Differentiated measurement of PTP was carried out using a modified protocol based on paranitrophenyl phosphate (pNPP) hydrolysis [41,42].

2.7.1. Step 1

For the analysis of PTP activity, 1:5 (w/v) liver homogenates were prepared under nitrogen gassing in a nonreducing HEPES buffer [50 mmol/L HEPES, 50 mmol/L NaCl, 1 mmol/L EDTA and 0.1 mmol/L phenylmethylsulphonylfluoride (PMSF), pH 7.4]. Cytosol was obtained by centrifugation at 10,000×*g* for 30 min at 2°C and brought to a final dilution of 1:25 (w/v).

2.7.2. Step 2

Diluted liver cytosol 10 μl was preincubated at 25°C in 240 μl of the DTT-free HEPES buffer for 3 minutes. Then, 250 μl HEPES buffer containing 20 mmol/L pNPP was added and further incubated for 5 min. The reaction was terminated by the addition of 500 μl 2 M NaOH and absorption was read in a spectrophotometer (Beckmann DU 50) at 410 nm. A blank without cytosol was carried out. Native PTP activity was calculated using an extinction coefficient of 0.0166 $\mu M^{-1} \times cm^{-1}$ for the paranitrophenolate ion and normalized to 1 mg protein. To determine the percentage of glutathionylation, reversible by DTT, enzymatic measurement was repeated as described, but a HEPES buffer containing 2.5 mmol/L DTT was used.

2.8. Western blot analysis of PTP1B glutathionylation

For analysis of PTP1B glutathionylation, 1:10 (w/v) liver homogenates were prepared in a nonreducing radioimmuno precipitation assay (RIPA) lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mM PMSF, 1 mM EDTA, 1.0 % sodium desoxycholate, 0.1 % sodium dodecyl sulphate (SDS) and 1% TritonX-100 (pH 7.4)]. After centrifugation (10,000×*g*, 30 min, 2°C), the cytosol was diluted to 1:50 (w/v). Forty micrograms of protein was separated according to the standard method [43] but under nonreducing conditions on 15% SDS–polyacrylamide gels (50 mA, 4°C, 2h). Separated proteins were transferred onto a PVDF membrane (PALL Biotrace 0.45 μm) by semidry blotting [25 min at constant 6V (~60 mA)]. After blocking membranes overnight at 4°C in TBST (20 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% Tween 20, pH 7.6) containing 5% nonfat dry milk and 0.2% bovine serum albumin, analysis was continued by a 2-h incubation with the anti-GSH antibody (Virogen 101-A-100) in TBS (1:1500) buffer and a 1-h incubation with the secondary antibody (1:3000) linked to alkaline phosphatase (goat antimouse IgG−h+I). Membranes were stained in a reaction buffer (0.1 mol/L Tris, 0.1 mol/L NaCl, 0.05 mol/L $MgCl_2$) containing 0.00375% nitro-blue tetrazolium and 0.0025% 5-bromo-4-chloro-3-indoylphosphate. Optical density of the ~50 kDA PTP1B band was evaluated (Gene Tools, Syngene) on scanned membranes (CanoScan LiDe 500F). To examine the in vitro effect of selenite and selenate on PTP1B glutathionylation, a pooled liver sample from the Se-deficient group was homogenized 1:5 (w/v) in Tris-HCl buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mM phenylmethylsulphonylfluoride (PMSF) 1 mM EDTA, pH 7.4], and cytosol was prepared as described above. Cytosol 50 μl was incubated with 50 μl of selenite or selenate solutions, obtaining final Se concentrations of 25, 50, 75 and 100 μmol/L for 10 min. Then, 400 μl of the RIPA buffer was added [final dilution 1:50 (w/v)], and an aliquot was subjected to Western blot analysis, as described.

2.9. Liver triglyceride concentration

Liver lipids were extracted with a hexane:isopropanol (3:2) mixture containing 0.005% butylated hydroxytoluene, as described previously [14].

Subsequently triglyceride concentration in the lipid extracts was determined with a test kit from Biocon, Bangalore, India (FluitestTG). The accuracy of the method was checked with Qualitrol.

2.10. Protein content

The protein content of liver cytosol, including samples for Western blotting, was determined using a standard method [44].

2.11. GPx1 and PTP1B mRNA expression

RNA isolation was carried out using the acid guanidine thiocyanate phenol chloroform method [45]. Reverse transcription of RNA followed by polymerase chain reactions (PCRs) for the examination of GPx1 and PTP1B expression in the liver was carried out as described in detail previously [14]. Using 2.5 μg of cDNA (obtained from reverse transcribed RNA), the PCRs for the amplification of specific fragments within the open reading frame (ORF) of GPx1, PTP1B and β-actin (control) were carried out in a reaction volume of 50 μl with a standard program for the single cycles.

Gene (Gene bank accession number)	Amplified region within the ORF	Forward primer (Tm)	Reverse Primer (Tm)
Rat GPx1 (NM 030826_1)	116–503 (396 bp)	Tcattgagaatgtcgcgtct (55.3°C)	Tttgagaagttcctggtggg (57.3°C)
Rat PTP1B (NM 012637_1)	279–728 (449)	Gcacttctgggagatggtgt (59.4°C)	Aagaggaaagacccgtcctc (59.4°C)
Rat β-actin (NM 031144_1)	301–696 (395)	Tgttaccaactgggacgaca (59.4°C)	Tctcagctgtggtggtgaag (59.4°C)

The standard program was as follows: initial denaturation: (95°C: 3 min) 1×; amplification cycles: [denaturation: 95°C: 45 s; annealing: primer-specific temperature: 40 s; extension: 72°C: 55 s] *x*-times, final extension: (72°C: 5 min) 1×.

2.12. Statistical analysis

A one-way analysis of variance was performed using SPSS 14.0 for Windows. If homogeneity of variance was given, the least significant difference (LSD) test was used to examine differences of means, if not, the Games Howell test was utilized. Values in the tables are given as mean (M)±S.D. or M±S.E.M. and include 3 repetitions for each parameter. Detailed information, error probabilities and tests used are indicated in the table legends. Correlation and regression analyses were also performed with "SPSS 14.0 for Windows."

3. Results

3.1. Zootechnical parameters (body weight development, diet consumption, feed conversion) and liver triglyceride concentration

The Se concentration of the Se-deficient basal diet was below our detection limit of 20.0 μg Se/kg. The analysed dietary Se concentrations (μg/kg) for the Se-supplemented groups were in accordance with the amounts scheduled in the experimental design: 0.2 selenite, 201±11.7; 0.2 selenate, 187±9.80; 1.0 selenite, 934±71.4; 1.0 selenate, 961±25.4; 2.0 selenite, 1932±69.7; 2.0 selenate, 1904±77.4.

The initial body weight in all experimental groups did not differ (Table 1). There was also no significant difference between the experimental groups and the initial status group used for the determination of Se status before starting the feeding trial.

At the end of the trial, mean body weights of rats from all Se-supplemented groups were significantly higher compared to Group 0 Se (Table 1). Within the Se-supplemented groups, final body weights in the groups receiving 0.2 mg Se/kg diet were significantly higher compared with the animals supplemented at the second supranutritional level of 2.0 mg/kg diet. Feed intake in Group 0 Se was lowest within all experimental groups but nearly as high as in the groups supplemented with 2.0 mg Se/kg diet. Rats supplemented at the recommended level had a significantly higher feed intake compared to the abovementioned groups. Feed intake in the groups supplemented with 1.0 mg Se/kg

Table 1
Body weight gain, feed intake, feed conversion and liver triglyceride concentration of rats fed diets with different amounts of Se from selenite or selenate compared to their Se deficient companions

Group Parameter	Initial status	0 Se	0.2 mg Se/kg as selenite	0.2 mg Se/kg as selenate	1.0 mg Se/kg as selenite	1.0 mg Se/kg as selenate	2.0 mg Se/kg as selenite	2.0 mg Se/kg as selenate
Initial body weight (g)	62.4±2.38^{a}	61.2±1.07^{a}	60.1±1.21^{a}	60.6±1.20^{a}	60.5±1.18^{a}	60.4±1.15^{a}	60.4±1.19^{a}	60.3±1.22^{a}
Final body weight (g)	n.d.	307±4.29^{a}	352±7.31^{c}	357±7.71^{c}	337±3.44^{bc}	331±4.60^{bc}	327±6.00^{b}	324±5.31^{b}
Total weight gain (g/8 weeks)	n.d.	246±4.60^{a}	292±6.30^{c}	296±6.70^{c}	276±3.34^{bc}	271±4.36^{bc}	267±6.30^{b}	264±5.72^{b}
Total feed intake (g/8 weeks)	n.d.	983±13.3^{a}	1089±23.6^{c}	1125±24.5^{c}	1028±10.2^{bc}	1024±20.6^{bc}	1013±10.2^{ab}	987±11.9^{ab}
Feed efficiency ratio (g feed intake/g body weight gain)	n.d.	4.01±0.10^{b}	3.72±0.05^{a}	3.74±0.03^{a}	3.72±0.04^{a}	3.79±0.01^{a}	3.80±0.08^{a}	3.82±0.07^{a}
Liver triglycerides (mg/g fresh matter)	n.d.	14.2±0.97^{a}	19.7±2.20^{b}	21.8±1.22^{bc}	27.7±1.15^{d}	25.6±1.38^{c}	25.9±2.04^{c}	27.5±1.99^{d}
Liver triglycerides (mg/kg body weight)	n.d.	45.5±2.79^{a}	57.2±7.71^{ab}	61.1±2.89^{b}	80.4±4.15^{c}	76.2±3.79^{c}	79.2±6.34^{c}	85.2±6.56^{c}

Data are given as M±S.E.M. Significant differences within a line are indicated by different small letters (n=7 animals per group considered, one outlier for body weight data and feed intake was excluded in Group 0 Se): body weight data (P<.05, LSD test); feed efficiency ratio (P<.05, LSD test); triglyceride data (P<.05, LSD test). Prot., protein; n.d., not determined.

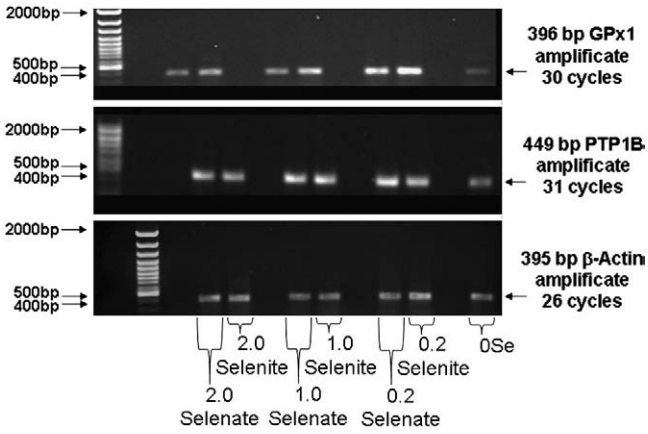


Fig. 2. mRNA expression of GPx1 and PTP1B in the liver of rats fed diets with different Se amounts as selenite or selenate compared to their Se-deficient companions (RNA of 3×2 animals per experimental group was pooled and subjected to cDNA synthesis and gene-specific PCR; the figure shows a representative image of three individual repetitions carried out per cDNA pool).

diet ranged between the intake of the groups supplemented with 0.2 and 2.0 mg Se/kg. Feed conversion (grams of feed intake per grams of body weight gain) was significantly more efficient in all Se-supplemented animals compared to their Se-deficient companions. In contrast to Se deficiency, Se supplementation led to a significant increase in liver triglyceride concentration at all levels investigated. When based on final body weights, average liver triglyceride concentrations in the groups with supranutritional Se supply (1.0 and 2.0 mg Se/kg diet) were 1.76-fold elevated compared to Se deficiency. In the groups with Se supplementation at the recommended level (0.2 mg Se/kg diet), this increase in liver triglyceride concentration was lower and reached a factor of 1.30-fold compared to Se-deficient animals.

3.2. Se status (Se concentration in liver and plasma, GPx1 activity and expression and GPx3 activity), GSH status (liver) and activity of catalase and SOD (liver)

At the end of the trial, the expression of GPx1 mRNA in the liver of selenite- or selenate-supplemented rats at all three levels was 8 to 10-fold higher compared to Se-deficient rats (Fig. 2). At the beginning of the feeding trial, Se status of the rats was still low as indicated by liver Se concentration and GPx1 activity in the initial status group.

The further development of liver Se concentration clearly indicated the Se depletion in Group 0 Se (Table 2). Se supply led to a dose-dependent increase in liver Se content. Thus, in the groups supplemented with 0.2, 1.0, and 2.0 mg Se/kg diet, 65-, 125, and 167-fold higher Se concentrations could be measured compared to Group 0 Se. GPx1 activity in Group 0 Se reached only 1.10–1.27% of the activities measured in the Se-supplemented groups (Table 2). Data indicated that a plateau in GPx1 activity was already achieved with 0.2 mg Se/kg

Table 2
Se status, parameters of the Se- and GSH-dependent redox system in liver and plasma and activities of catalase and SOD in rats fed diets with different selenite or selenate amounts compared to their Se-deficient companions

Group parameter	Initial status	0 Se	0.2 mg Se/kg as selenite	0.2 mg Se/kg as selenate	1.0 mg Se/kg as selenite	1.0 mg Se/kg as selenate	2.0 mg Se/kg as selenite	2.0 mg Se/kg as selenate
Liver								
Se concentration (μg/kg FM)	138±5.47^{a}	18.4±2.00^{b}	1054±73^{c}	1343±104^{d}	2469±123^{e}	2292±130^{e}	3010±113^{f}	3060±180^{f}
GPx1 (mU/mg prot.)	99.8±35.2^{b}	9.96±6.21^{a}	851±93.1^{c}	909±184^{c}	910±87.5^{c}	781±81.1^{c}	812±86.2^{c}	905±134^{c}
Catalase (mU/mg prot.)	n.d.	51.2±10.5^{b}	50.7±10.0^{b}	33.7±4.89^{a}	38.3±7.24^{a}	40.6±14.9^{ab}	37.3±8.59^{a}	35.8±7.75^{a}
Total SOD	n.d.	11.7±0.65^{b}	10.3±1.14^{ab}	10.7±1.08^{ab}	9.52±0.41^{a}	9.45±0.83^{a}	10.0±0.80^{a}	9.20±1.20^{a}
Mn SOD		2.38±0.31^{a}	1.93±0.41^{a}	2.07±0.50^{a}	2.05±0.44^{a}	2.04±0.46^{a}	2.48±0.61^{a}	1.96±0.35^{a}
Cu/Zn SOD (U/mg prot.)		9.30±0.29^{b}	8.37±0.39^{ab}	8.59±0.34^{ab}	7.47±0.23^{a}	7.41±0.28^{a}	7.57±0.37^{a}	7.25±0.41^{a}
Total GSH (nmol/mg prot.)	n.d.	27.9±4.11^{a}	34.5±5.12^{b}	40.1±4.94^{bc}	42.0±5.78^{bc}	37.8±4.51^{b}	45.1±5.25^{c}	47.9±5.06^{c}
GSSG (nmol/mg prot.)	n.d.	1.70±0.26^{a}	13.5±1.66^{b}	11.8±3.21^{b}	14.1±2.17^{b}	12.6±2.04^{b}	15.6±2.35^{b}	14.9±4.40^{b}
% Oxidized of total GSH	n.d.	6.15±0.82^{a}	39.4±2.55^{c}	29.7±8.66^{bc}	33.8±3.93^{bc}	33.9±5.94^{bc}	34.6±2.02^{b}	31.7±9.84^{bc}
Plasma								
Se conc. (mg/L)	110±4.00^{a}	22.9±3.10^{b}	544±20.1^{c}	571±31.8^{c}	648±21.4^{d}	610±44.4^{cd}	706±30.8^{e}	707±37.8^{e}
GPx3 (mU/mg prot.)	31.9±8.48^{a}	1.80±0.85^{b}	119±10.9^{c}	149±22.6^{cd}	161±24.8^{d}	133±14.1^{cd}	137±15.9^{cd}	141±18.0^{cd}

Data are given as M±S.D. Significant differences within a line are indicated by different small letters (n=7 animals per group considered): liver Se (P<.001, LSD test); GPx1 (P<.001, LSD test); catalase (P<.05, LSD test), SOD (P<.05, LSD test); total GSH, GSSG (P<.05, LSD test); % oxidized of total GSH (P<.01, Games Howell test); Se conc. (P<.001, LSD test); GPx3 (P<.001, LSD test).

diet, and additional Se supply neither produced an increase in liver GPx1 nor was there any negative influence on GPx1 activity. Both total GSH and the portion of GSSG were significantly higher in all Se-supplemented groups compared to Group 0 Se, with the highest values for total GSH in Groups 2.0 selenite and 2.0 selenate. The low initial Se status of the rats was also confirmed by plasma Se concentration and GPx3 activity in the initial status group. An effective Se deprivation in the course of the experiment was reflected by a fivefold lower plasma Se concentration in Group 0 Se, compared with the initial status group. The augmentation of dietary Se supply to the recommended level and to supranutritional concentrations led to a dose-dependent increase in plasma Se, which was, however, not as distinctive as in the livers. GPx3 activity in Se-deficient animals decreased to 5.64% of the initial status group. Compared to the Se-supplemented groups, GPx3 activity in Group 0 Se fell to a range from 1.11% to 1.50%. As found for liver GPx1, Se supplementation to supranutritional levels (1.0 and 2.0 mg Se/kg diet) neither increased GPx3 activity nor did it show a negative influence on the activity of the enzyme. At the end of the trial, liver catalase activity representing a microsomal H_2O_2-detoxifying enzyme was significantly higher in the Se-deficient group compared to the Se-supplemented groups, with the exception of Group 0.2 selenite. This result presumably indicates a compensatory mechanism for general cellular H_2O_2 detoxification due to Se deficiency. As higher dietary Se concentrations may induce oxidative stress via the generation of superoxide radicals during their metabolism, a differentiated activity assay was also carried out for SOD. Total SOD activity as well as cytosolic Cu/Zn SOD reached comparable values in the livers of the Se-deficient group and in Groups 0.2 Selenite and 0.2 Selenate supplemented at the recommended level (Table 2). In the groups with supranutritional Se supply, total SOD and Cu/Zn SOD were even significantly lower compared to the Se-deficient animals, and thus, oxidative stress by means of the increased Se supplementation could be excluded. Mitochondrial Mn SOD remained uninfluenced by Se supplementation compared to Se deficiency.

3.3. Regulation of liver PTP1B

The expression of PTP1B mRNA was 2.5- to 3.5-fold reduced in Group 0 Se compared to rats with Se supplementation as selenite or selenate at all dietary levels examined (Fig. 2).

Native liver PTP activity measured without DTT was 1.48- to 3.68-fold higher in rats fed Se-supplemented diets compared to their Se-deficient littermates (Table 3). The lowest activity difference in comparison to Group 0 Se was achieved in Group 0.2 selenite. From Group 0.2 selenate onward, the difference in PTP activity compared to Group 0 Se was distinctly higher. The highest native PTP activity was reached in Group 2.0 selenate. PTP measurement with DTT addition increased PTP activity in all groups, indicating the regeneration of the PTP enzyme inhibited by reversible glutathionylation. PTP activity measured with DTT addition was still the lowest in Group 0 Se compared to all groups with Se supply, but the factors for activity difference were diminished and ranged only from 1.18- to 1.37-fold. The remaining difference in PTP activity can be explained by a higher mRNA expression due to Se supplementation (Fig. 2). The highest percentage of glutathionylation and, therefore, inactivation of PTPs was measured in Group 0 Se (Table 3). An increase in dietary Se concentration led to a dose-dependent loss of PTP glutathionylation. A comparison of the groups supplemented with selenite and selenate at the same dietary level (0.2, 1.0 and 2.0) revealed a significantly lower PTP glutathionylation for selenate supplementation in each case (Table 3).

The coherence between liver and plasma Se concentration (resulting from dietary Se supplementation at different levels) and the Se compound used (selenite or selenate) and PTP activity as well as PTP glutathionylation was demonstrated by correlation- and regression-analyses (Fig. 3A–D). A highly positive correlation between liver Se concentration and native PTP activity could be demonstrated for selenite (r=0.88; P<.001) and selenate (r=0.91; P<.001), whereas the correlation between liver Se concentration and glutathionylation was strongly inverse for both Se compounds (selenite: r=−0.87; P<.001; selenate: r=

Table 3
PTP activity under native and reducing conditions and calculated ratio of PTP glutathionylation in the liver of rats fed diets with different selenite or selenate amounts compared to their Se-deficient companions

Group parameter	0 Se	0.2 mg Se/kg as selenite	0.2 mg Se/kg as selenate	1.0 mg Se/kg as selenite	1.0 mg Se/kg as selenate	2.0 mg Se/kg as selenite	2.0 mg Se/kg as selenate
PTP activity, native (U/mg prot.)	0.59±0.13^{a}	0.88±0.16^{b}	1.22±0.18^{cd}	1.15±0.09^{c}	1.41±0.18^{d}	1.66±0.14^{e}	2.18±0.29^{f}
PTP activity, 2.5 mM DTT (U/mg prot.)	1.78±0.08^{a}	2.23±0.21^{bc}	2.21±0.35^{ac}	2.17±0.16^{bc}	2.10±0.26^{ac}	2.34±0.10^{bc}	2.45±0.24^{bc}
PTP glutathionylation (%)	66.7±7.89^{a}	60.7±4.73^{a}	44.4±4.87^{bcd}	46.8±2.23^{bd}	33.27±7.91^{cef}	29.0±4.67^{eg}	10.2±13.9^{fg}

Data are given as M±S.D. Significant differences within a line are indicated by different small letters (n=7 animals per group considered): PTP activity native (P<.01, LSD test); PTP activity, 2.5 mM DTT (P<.01, LSD test); PTP glutathionylation in % (P<.05, Games Howell test).

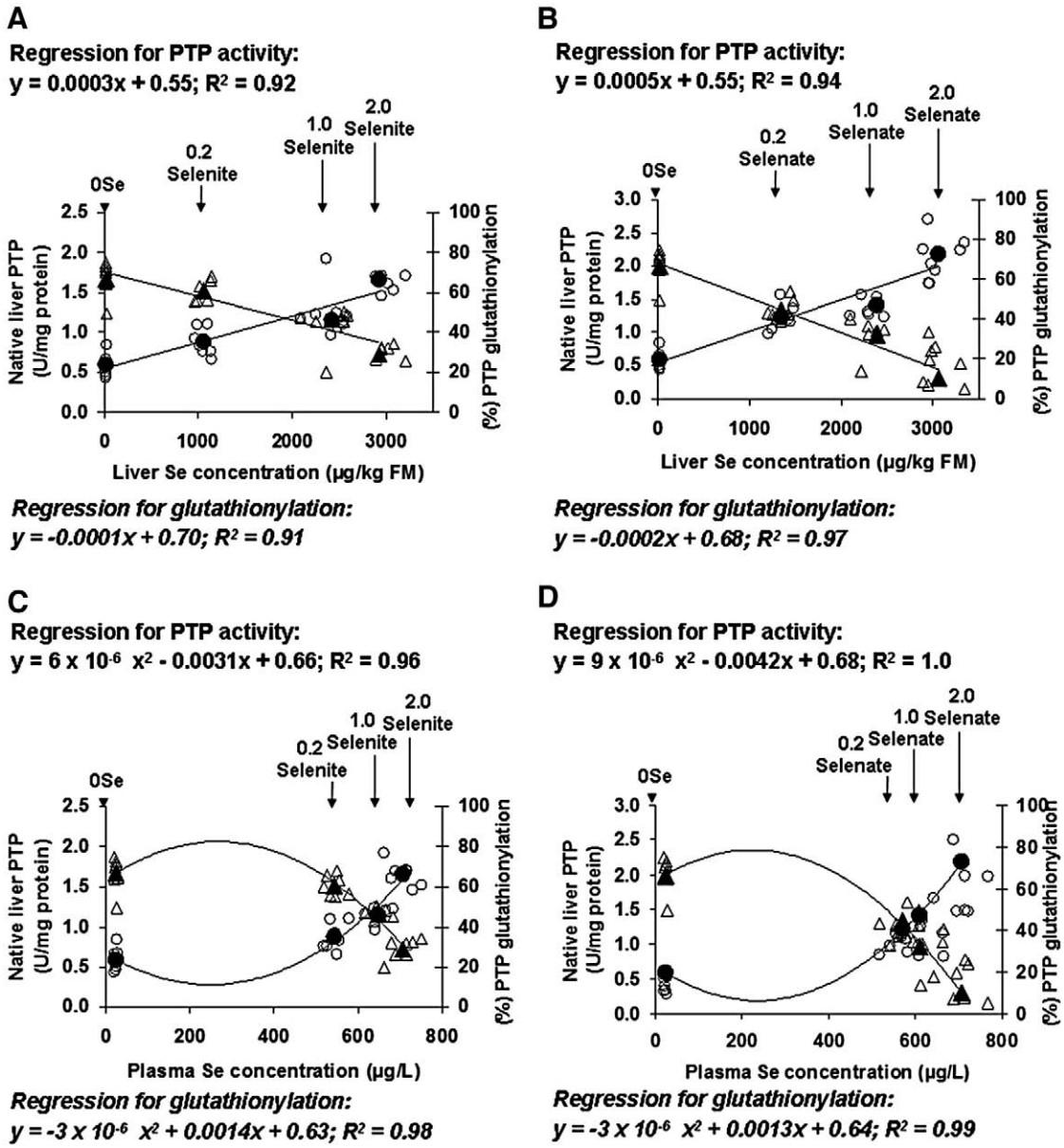


Fig. 3. Regression analyses between liver Se concentration (A and B) or plasma Se concentration (C and D) and native liver PTP activity and PTP glutathionylation due to increasing Se supplementation as selenite (Se IV) (A, C) or selenate (Se VI) (B, D) (blank circles and triangles represent pairs of variates for single animals; black-filled circles and triangles represent the means of pairs of variates). (A) Protein samples of n=6 individuals per group were subjected to Western blot analysis; the figure shows representative blots of three independent repetitions. (B) Protein samples of n=6 individuals per group were subjected to Western blot analysis; the figure shows representative blots of three independent repetitions. (C) The figure shows a representative blot of 3 independent repetitions of the in vitro experiment.

−0.88; P<.001). Slopes of linear regression indicated a faster rise in native PTP activity and a more distinct loss of PTP1B glutathionylation due to selenate supply compared to selenite supply (Fig. 3A and B). These results are in accordance with enzymatic PTP measurement where selenate-fed rats showed a higher native PTP activity and a lower PTP glutathionylation compared to selenite-fed rats (Table 3). By correlation analyses and square regression, comparable coherences could also be shown for the relationship between plasma Se concentration and liver PTP activity as well as PTP1B glutathionylation (Fig. 3C and D).

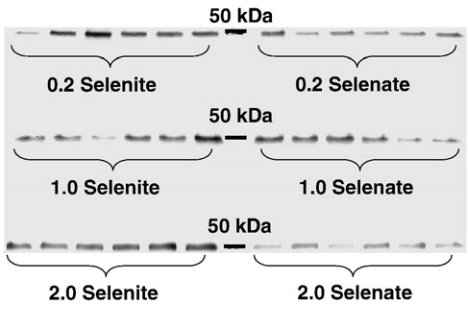
The results obtained by enzymatic measurement of PTP activity could be visualized by Western Blot analysis using an antibody detecting “protein glutathionylation.” PTP1B glutathionylation was 1.5- to 3.5-fold lower in Se-supplemented rats compared to their littermates in Group 0 Se (Fig. 4A). An increase in dietary Se supply led to a decrease in PTP1B glutathionylation. As found for enzymatic PTP measurement, Western blotting of liver cytosol from rats fed

A

Relative OD (Mean ± SD)	Experimental Group		Experimental Group	Relative OD (Mean ± SD)
1,00±0,15	0Se	50 kDa	0.2 Selenite	0,79±0,17
1,00±0,08	0Se	50 kDa	0.2 Selenate	0,41±0,14
1,00±0,09	0Se	50 kDa	1.0 Selenite	0,42±0,15
1,00±0,26	0Se	50 kDa	1.0 Selenate	0,36±0,08
1,00±0,14	0Se	50 kDa	2.0 Selenite	0,39±0,19
1,00±0,30	0Se	50 kDa	2.0 Selenate	0,28±0,09

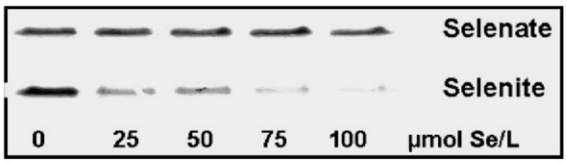
(Protein samples of *n*=6 individuals per group were subjected to Western Blot analysis, the figure shows representative Blots of 3 independent repetitions)

B



(Protein samples of *n*=6 individuals per group were subjected to Western Blot analysis, the figure shows representative Blots of 3 independent repetitions)

C



(The figure shows a representative Blot of 3 independent repetitions of the in vitro experiment)

Fig. 4. (A) PTP1B glutathionylation in liver cytosol of rats fed diets containing different amounts of Se as selenite or selenate in comparison to their companions kept on a Se-deficient diet. (B) Comparison of PTP1B glutathionylation in liver cytosol of rats fed diets containing Se as selenite or selenate at three dietary levels. (C) Glutathionylation of PTP1B after in vitro incubation of liver cytosol with increasing selenite or selenate concentrations, demonstrating that selenate feeding matches in vitro effects of selenite.

selenite vs. rats fed selenate at the same dietary level (0.2, 1.0 and 2.0) revealed a lower PTP1B glutathionylation for animals receiving selenate (Fig. 4B).

4. Discussion

4.1. Influence of Se and its compounds on changes in the Se- and GSH-dependent cellular redox system

The distinct loss of GPx1 and GPx3 activity due to a lack of dietary Se supply indicates the low rank of these selenoproteins and could be confirmed by our data [46]. That an Se supply of 0.2 mg/kg diet meets the requirements of growing rats for an abundant selenoprotein synthesis is also in agreement with the literature. Presumably, a dietary amount of 0.05–0.075 mg Se/kg diet would already be sufficient for a full expression of GPx1 in rats at different physiological states [47,48]. In our study, Se fed as selenite or selenate increased the concentration of total GSH in the liver and, in particular, the concentration of GSSG. Thus, a direct consequence of Se supplementation is a distinct shift in the GSH redox pair to a more oxidized state. This

observation is in accordance with other studies on Se metabolism and has presumably two causes.

Firstly, feeding Se at the recommended dietary amount affects the concentration of GSSG via maximization of GPx1, which forms the oxidized state of this tripeptide.

Secondly, feeding supranutritive Se concentrations leads to a further formation of GSSG since the reduced form of the tripeptide is needed for Se reduction itself [49,50]. The fact that an increase in dietary Se concentration led to a dose-dependent Se storage in the liver [51] but did not augment in additional selenoprotein synthesis merits further investigation into the influence of dispensable Se.

4.2. Influence of Se and its compounds at different dietary levels on the regulation of PTP1B

With regard to the undesirable effects of Se on the development of insulin-resistant diabetes and obesity, our data suggest that a changed physiological regulation of the insulin antagonistic PTP1B by dietary Se and GPx1 may represent one mechanism which could promote insulin resistance and obesity. In contrast to the mouse trial in which GPx1 overexpression promoted the development of obesity and insulin resistance [16], our nutrition physiological trial provides explanations as to how Se supplements exceeding requirement can accelerate these diseases independent of a high GPx1 activity. In the mouse trial, a decreased tyrosine phosphorylation of the β subunit of the insulin receptor and a decreased phosphorylation of the downstream signaling protein AKT at Thr 308 and Ser 473 indicated the increased insulin resistance due to GPx1 overexpression [16]. Our results suggest that the decreased phosphorylation measured in the abovementioned trial reflects more likely an influence of the manipulated Se- and GSH-dependent redox system on PTP1B rather than displaying a direct effect of GPx1 on protein phosphorylation. According to our data and to Fig. 1, a lower dietary Se concentration and the resulting higher peroxide concentration due to a lack of GPx1 activity lead to a higher PTP1B inactivation via glutathionylation. Optimized activities of GPx1 by dietary Se (our present study) or an increase in GPx1 expression (mouse study), however, remove H_2O_2 and disable PTP1B inhibition [29,30,52]. The abovementioned aspects therefore provide a plausible explanation for the development of insulin resistance and obesity due to high GPx1 expression and activity via nutritional Se manipulation. Our data, which showed a slight up-regulation of catalase due to Se deficiency, indicate that this mechanism could not compensate for the distinctly reduced GPx1 activity. Data from a human study support the hypothesis of a coherence between GPx1 activity and an increase in diabetes development by the finding that a high-erythrocyte GPx1 corresponded to an increased prevalence of gestational diabetes [53]. Thus, the predominant mechanism of PTP1B regulation by GPx1 activity seems to consist in a posttranslational modification of the enzyme. Besides this posttranslational regulation, our data also pointed to a slight transcriptional regulation of PTP1B by dietary Se and/or GPx1 activity. An up-regulation of PTP1B expression, and with it, an increase in intrinsic insulin resistance could also be found for mice overexpressing catalase, the second central enzyme in H_2O_2 detoxification [54]. Our data confirm an up-regulation of PTP1B mRNA expression through a high expression of GPx1 as another H_2O_2-detoxifying enzyme [Fig. 2]. In physiological models, like our trial, no overexpression of GPx1 can be obtained by increasing dietary Se concentration beyond requirement. Instead, a physiological model of PTP1B regulation manipulating GPx1 expression and activity via a short-term Se deficiency could be displayed. Our trial also provides information on the influence of Se supply exceeding physiological needs on PTP1B regulation as well as information on the influence of different Se compounds (selenite and selenate) on PTP1B regulation. Since we could show a highly positive correlation between liver and plasma Se concentration and PTP activity (Fig. 3A–D), our data may be helpful for further analysis of recent human studies showing a correlation between serum Se and diabetes prevalence [17,18]. Independent of an optimized GPx1 activity in our trial, an increase in dietary Se concentration led to a dose-dependent increase in PTP activity, corresponding to a loss of PTP1B glutathionylation. Feeding selenate effected a lower PTP1B glutathionylation than feeding selenite (Table 3, Figs. 3, 4B). This effect presumably derives from fundamental differences in mammalian Se metabolism (Figs. 1 and 4). Se from selenite (+IV) and selenate (+VI) is absorbed by different mechanisms [55] Selenite reacts with thiols like GSH prior to its absorption and enters the peripheral organs in the form of selenotrisulfides (oxidation state: −I), or it is reduced in the erythrocytes to the selenide oxidation state −II and delivered to peripheral organs bound to albumin [55,56]. In contrast, unmodified selenate can be detected in the bloodstream and in peripheral tissues [56]. During successive selenate reduction, the thiol-reactive oxidation states (selenite: +IV; selenotrisulfides: −I) can be formed and require GSH for their further reduction to the selenide oxidation state (−II) (Fig. 1). Thus, GSH detraction from glutathionylated proteins could be one mechanism for GSH acquirement. Moreover, our data suggest that in vitro effects of selenite (+IV) match selenate (+VI) feeding. This particular aspect of mammalian Se metabolism could be visualized using an in vitro assay (Fig. 4C). Incubation of liver cytosol from Group 0 Se with increasing selenite or selenate concentrations, representing approximately the Se concentrations in the livers of rats receiving diets with 1.0 and 2.0 mg Se/kg, showed that unreactive selenate (+VI) did not influence PTP1B glutathionylation. In contrast, selenite (+IV), matching selenate feeding, effected a dose-dependent loss of PTP1B glutathionylation (Fig. 4C). Thus, PTP1B regulation by different Se compounds represents a further important finding of our trial, over and above enzyme reactions with H_2O_2, reactive oxygen species and GSH investigated so far [29,52,57]. An important question that needs to be discussed

is how dietary selenite, which enters peripheral organs in less thiol-reactive oxidation states or a fortiori organic Se compounds like selenomethionine, could nevertheless influence protein glutathionylation via the generation of thiol-reactive Se +IV compounds. A possible answer was published very recently [58]. Se from all nutritional compounds must be transformed into the selenide oxidation state −II prior to cotranslational selenocysteine synthesis or excretion via methylated products [58,59]. The mono- and dimethylated Se metabolites (MMSe and DMSe) can again be partially reoxidized to selenite (+IV) and methylseleninic acid representing very thiol-reactive Se compounds [58]. This particular mechanism could therefore represent a plausible explanation of how selenomethionine, which was supplemented in the NPC trial, can also influence the glutathionylation of proteins like PTP1B in a manner similar to selenate during its reduction. Despite a higher GSSG concentration in the livers of Se-supplemented rats in our study, a direct PTP1B glutathionylation in the presence of a high GSSG concentration (>25 mmol/L) [31] could not be confirmed by our data since millimolar GSSG amounts represent a nonphysiological in vitro situation. A manipulation of PTP1B activity influences a number of metabolic processes. Feeding fructose to rats led to a threefold elevated expression and activity of liver PTP1B compared to the control animals [60]. Fructose-fed rats developed severe insulin resistance and fatty livers based on an activation of protein phosphatase 2A, sterol regulatory element binding protein 1-c and fatty acid synthase as the consequence of the increased PTP1B activity. In another rat trial, feeding diets containing 0.30-mg Se/kg diet increased the liver triglyceride content in these animals 1.5-fold in comparison to their Se-deficient companions [61]. Our data could confirm higher triglyceride contents in the livers of Se-supplemented rats (Table 1). Thus, as found in the fructose trial [60], PTP1B activity may similarly represent the link between Se supplementation and peripheral triglyceride synthesis. In a mouse trial, it could be shown that PTP1B-deficient mice had a significantly higher energy expenditure than wild type mice [22]. Despite a reduced feed intake in Groups 2.0 selenite and 2.0 selenate of our trial, possibly deriving from an impaired palatability of high Se diets [62], the feed conversion ratio (grams of feed intake:grams of body weight gain) was, however, significantly improved in all Se-supplemented groups (Table 1). According to the above-mentioned mouse trial [22], the higher feed expense in Group 0 Se could be an indicator for higher energy expenditure due to a reduced PTP1B activity. In human studies and in animal trials, PTP1B was demonstrated as one factor increasing body weight gain and the development of obesity [20–24]. GPx1-overexpressing mice showed a significantly higher body weight and body fat gain [16], whereas mice with a selenoprotein P knockout and consequential lack of peripheral GPx1 synthesis were emaciated [63]. Thus, our physiological study was in line with both trials [16,63], demonstrating that Se supply and high GPx1 activities are involved in body weight gain and in lipid metabolism. An increase in PTP1B activity as the consequence of dispensable Se supply and high GPx1 activities may therefore contribute to peripheral triglyceride synthesis and storage and the development of overweight. A final topic, which should be discussed critically, is how our data, representing a long-term manipulation of Se status (8 weeks), compare to the findings of long-term trials with humans (7.7 years) like the NPC trial [18]. In this context, some essential points should be mentioned.

- Firstly, in contrast to the mouse study in which GPx1 overexpression led to the development of insulin resistance and obesity [16], manipulation of GPx1 by dietary Se, as practiced in our trial, represents a more physiological model, which is therefore probably more comparable to the human data [17,18].
- Secondly, in our trial, the changes in the Se status of the rats, representing a fast-growing animal model, obtained within 8 weeks, were very distinct and can therefore be compared to a longer and less distinct Se manipulation in humans.
- Thirdly, our trial was intended as an animal model to help understand the physiological mechanisms by which a long-term supply with dispensable Se may influence the development of obesity and diabetes with insulin resistance.

5. Conclusion

In view of the critical impact of a permanent dispensable Se supply on the development of diabetes and obesity, our results help to understand physiological mechanisms which may underlie these effects (Fig. 5). Both an optimized activity of GPx1 and dispensable Se effect an up-regulation of the insulin antagonistic PTP1B. An increased PTP1B activity again may promote diabetes by reducing insulin

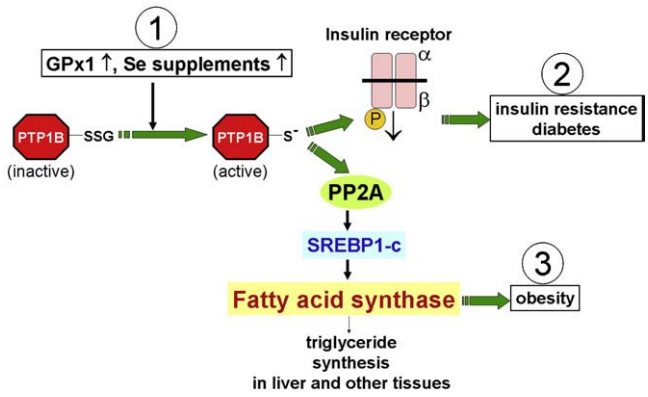


Fig. 5. Molecular pathways by which Se may contribute to the accelerated development of diabetes with insulin resistance and obesity. A high GPx1 activity, as well as a high Se concentration in organs and plasma, leads to an activation of the insulin-antagonistic PTP1B (1) which again can influence insulin resistance (2) and lipid metabolism (3).

sensitivity and contribute to the development of obesity via an increased peripheral triglyceride synthesis and storage. Long-term Se supply above the recommendations may thus be helpful in prostate cancer therapy [64] and with regard to certain toxicological conditions where a decreased phosphorylation of critical signaling proteins due to Se supply is desirable [64,65]. Concerning the development of insulin resistance and obesity, a retardation of phosphorylation signals via an increased PTP1B activity is, however, counterproductive.

In conclusion, our data provide a preliminary basis for future research dealing with the critical functions in metabolic processes of Se supplements exceeding the actually intended effects of this supplement.

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