ACCELERATED PUBLICATION

Efficient selenium transfer from mother to offspring in selenoprotein-P-deficient mice enables dose-dependent rescue of phenotypes associated with selenium deficiency

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Mice deficient in selenoprotein P exhibit a disturbed selenium distribution and reduced activities of other selenoenzymes and display defects in growth and motor co-ordination. We have normalized selenoenzyme activities and rescued the phenotype of mutant mice by supplementing their nursing mothers with sodium selenite. Our results indicate that selenium from inorganic sources can be transferred efficiently via mother's milk to the developing offspring in a form that is both highly bioavailable by target tissues and yet sufficiently safe to prevent overdosages.

Key words: glutathione peroxidase, growth, lactation, selenium, selenoprotein P, transport.

INTRODUCTION

Selenium (Se) is an essential trace element that is required for normal growth and development [1–3]. It is present in the rare amino acid selenocysteine (Sec), which is co-translationally incorporated into certain proteins. Thus Sec represents the 21st proteinogenic residue not included in the classical genetic code. In eukaryotes, co-translational insertion of Sec is directed by an in-frame opal stop codon (UGA) in combination with specific stem-loop structures within the 3′-untranslated region of transcripts and a unique set of accessory factors [4]. Selenoenzymes that have been identified as containing Sec as part of their active centre [5] are the family of glutathione peroxidases (GPxs) [6], the thyroid hormone de-iodinases [7], the thioredoxin reductase isoenzymes [8] and methionine-R-sulphoxide reductase [9], all catalysing redox reactions. Taking advantage of the conserved features present in selenoprotein-encoding genes and transcripts, mathematical algorithms were developed and employed successfully to predict the human selenoproteome to comprise 25 individual members [10], the majority of which still lack a clearly defined function.

The most peculiar selenoprotein is selenoprotein P (SePP). It represents a plasma protein that carries more than 50 % of plasma Se [11] and contains up to ten Sec residues per molecule in humans and rodents. Accordingly, a transport role for SePP was proposed [12]. In addition, it was described as a scavenger for peroxynitrite [13], a lipid-hydroperoxide peroxidase [14] or an important component in mercury-detoxification processes [15]. Recently, we have established SePP-knockout mice ($SePP^{-/-}$), which display a complex phenotype characterized developmentally by significant growth retardation, neurologically by seizures and a movement disorder, and biochemically by a severely disturbed Se distribution within the body [16]. An independent study published contemporarily reported similar findings [17]. The defects observed seem to result from an impairment of the liver to dispose intracellular Se stores efficiently in the form of secreted SePP into the bloodstream and from an insufficient supply to other organs of this organified form of the essential trace element. Since the synthesis of selenoproteins depends essentially on Sec-loaded $tRNA^{Sec}$ (Sec-specific tRNA) molecules [18], disturbances of intracellular Se levels are mirrored in drastically altered expression of selenoproteins in a tissue-specific manner, similar to dietary Se restriction [19]. In an attempt to characterize how developing organisms can overcome severe Se deficiency in the absence of SePP, we have offered supplemental sodium selenite to nursing mice and monitored the emerging health effects in the offspring.

MATERIALS AND METHODS

Animals

Pups lacking SePP ($SePP^{-/-}$) were generated by mating heterozygous ($SePP^{+/-}$) mice. The animals were maintained in a 12 h:12 h light/dark cycle with free access to food and water as described in [16]. Standard lab chow with 0.24 p.p.m. Se (Altromin, Lage, Germany) was used. Sodium selenite (Sigma, Munich, Germany) was added directly to the drinking water at the indicated concentrations [1 μM sodium selenite corresponds to 0.08 p.p.m. Se (w/w)]. Newborn mice were marked with stamp pad ink (Laco Office Products, Sottrum, Germany). Body masses were determined twice a week, blind to genotypes which were determined after the experiment by the PCR-based methodology described in [16]. No behavioural impairments were noted under the selenite supplementation conditions. Blood was taken by exsanguination, treated with heparin and centrifuged at 12 000 ***g*** at 4 °C for 10 min. Subsequently, plasma was removed and frozen at − 80 °C. Kidneys were removed and immediately frozen in liquid nitrogen. Animal experimentation was approved by the animal welfare committee of the local governmental authorities in Berlin, Germany.

Abbreviations used: GPx, glutathione peroxidase; pGPx, plasma GPx; Sec, selenocysteine; SePP, selenoprotein P; TCA, trichloroacetic acid; $tRNA^{Sec}$, Sec-specific tRNA.

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Behavioural testing

The movement disorder of SePP-deficient mice was quantified using the rotarod device (Ugo Basile, Comerio, Italy). This test analyses motor co-ordination or fast fatigue by placing the animals on a rotating rod and monitoring the time they manage to run in balance. After 2 min of accommodation at 4 rounds per minute (rpm), mice were tested five times in acceleration mode (linear acceleration from 4 rpm to 40 rpm in 5 min). Between each trial, mice were given at least 3 min to rest and recover. The retention time on the rotating rod was measured for 6 to 16 animals per genotype.

Se determination

Se measurements were performed by a fluorimetric assay [20] and validated as described in [16]. Over the last 2 years, inter-assay variation was $< 6\,\%$ and intra-assay variation was $< 5\,\%$. The limit of detection was 15 μg/l, equalling 1.5 ng in the test volume of 100 μl. Human pooled serum with Se determined at 79 μg/l in relation to a seronorm serum standard (Sero AS, Billingstad, Norway) was used as a control within and between the measurements.

TCA (trichloroacetic acid) precipitation of milk

Mouse milk was mixed with an equal volume of 10 % (v/v) TCA, incubated on ice for 20 min and centrifuged at 4 °C and 14 000 ***g*** for 15 min. Milk fat accumulated at the top of the clear aqueous phase. The precipitate contained all protein-bound Se. Using the same procedure, TCA-treatment of mouse sera led to precipitation efficiencies of $100 \pm 3\,\%$ of serum Se.

Western blot analysis

An antiserum was developed in rabbits directed towards a synthetic peptide (H_2N-SKPSENQQPGPSETAC-COOH) from mouse SePP. This antiserum was used directly without further purification at 1:5000 dilution. The pGPx (plasma GPx) antiserum was kindly provided by Professor Brigelius-Flohé, German Institute of Nutrition, Potsdam, Germany, and used at 1:2000 dilution. Secondary horseradish peroxidase-conjugated antibody (Dako, Copenhagen, Denmark) was used at 1:2000 dilution and bands were detected using an enhanced chemiluminescence (ECL®)-based detection system (Amersham Biosciences) in combination with regular X-ray films (X-Omat™, Kodak).

Enzymic assay

The enzymic activity of GPx was determined with mouse plasma samples by a coupled test procedure as described in [16]. NADPH consumption by glutathione reductase was monitored at 340 nm as a measure of the GSSG formation catalysed by GPx. Enzymic activity was recorded at 37 °C in a buffer containing 0.02 M potassium phosphate, pH 7.0, 0.6 mM EDTA, 0.15 mM NADPH, 2 mM GSH and 4 units of glutathione reductase (Calbiochem, Darmstadt, Germany). The reaction was started by the addition of t-butylhydroperoxide (0.1 mM final concentration) as substrate. Background NADPH consumption was determined in the presence of 100 mM mercaptosuccinate and subtracted. Under our experimental conditions, GPx activity in plasma of wild-type mice was 18.9 ± 2.1 nmol of NADPH/min per ml.

Northern blot analysis

Kidney was homogenized and used to isolate total RNA according to standard procedures (Trifast, PeqLab, Nürnberg, Germany). cDNA fragments encompassing the complete reading frame of murine pGPx were amplified by PCR, subcloned into pGEM-T vectors (Promega, Mannheim, Germany) and verified by DNA-sequencing (BigDye Termination Sequencing Kit; Applied Biosystems, Warrington, U.K.). The pGPx-specific cDNA insert was prepared by restriction digests, followed by preparative gel electrophoresis, and was subsequently radioactively labelled with [α-^{32}P]dCTP (NEN DuPont, Köln, Germany) using a random priming kit (High Prime, Roche Diagnostics GmbH, Mannheim, Germany). Hybridizations were conducted essentially as described in [21] and specific signals were quantified employing a phosphoimager (Cyclone, PerkinElmer, Köln, Germany). Signals for ubiquitin were recorded thereafter using the same method and used to calculate relative changes in transcript concentrations.

RESULTS

Rescue of the growth deficit of SePP$^{-/-}$ mice by Se-enriched drinking water

SePP-deficient mice are born normal and cannot be distinguished from their heterozygous or wild-type littermates by phenotypic characteristics alone if their mothers are kept on normal lab chow containing 0.24 p.p.m. Se. Total body mass and mass gain were essentially identical, independent of genotype during the first 2 weeks (Figure 1A). However, around postnatal day 15, when wild-type and SePP$^{+/-}$ mice engage in a growth spurt, SePP$^{-/-}$ mice gain only a little extra mass (Figure 1A) and appear substantially smaller. At this age, however, young mice still depend on their mother's milk. We hypothesized that SePP$^{-/-}$ pups could be treated with Se via their mother, as it is known that milk contains significant amounts of Se-containing compounds [22,23]. Therefore, in an attempt to ameliorate the phenotype of SePP$^{-/-}$ mice at an early stage, we administered 100 μM sodium selenite to their mothers' drinking water starting from conception. At this concentration, selenite supplementation is well-tolerated and does not lead to fatalities [24] or reduced liquid consumption [25]. Nonetheless, this approach should be seen rather as a proof of principle, as this concentration is close to doses reported to be toxic [24]. Still, selenite at 100 μM in drinking water completely abolished the growth defect in SePP$^{-/-}$ mice compared with their wild-type littermates (Figure 1B). However, mass gain of SePP$^{+/+}$ and SePP$^{+/-}$ animals nursed by their mothers consuming this high-selenite-containing drinking water was somewhat lower than in untreated controls (compare Figures 1A and 1B).

Dose-dependent restoration of motor co-ordination by selenite

Unlike the situation with the mass gain, addition of 100 μM selenite did not impact negatively on the motor coordination, since the wild-type, the heterozygotes and the SePP$^{-/-}$ mice performed equally well under the treatment, reaching control values on the rotarod device (Figure 2). In the next experiments, we reduced the selenite concentration in the drinking water by steps of one order of magnitude. Complete rescue of the growth defect was still observed at 10 μM selenite; partial rescue was elicited at 1 μM (Figure 1C). These effects were paralleled by an improvement in motor co-ordination. The mice supplemented with 10 μM or 100 μM selenite did not develop the neurological phenotype characterized by poor performance on the rotating rod (Figure 2), whereas 1 μM selenite was only partially effective with respect to motor co-ordination (results not shown). Thus selenite supplementation of lactating mothers can rescue the early growth defect and poor motor co-ordination of mice lacking SePP.

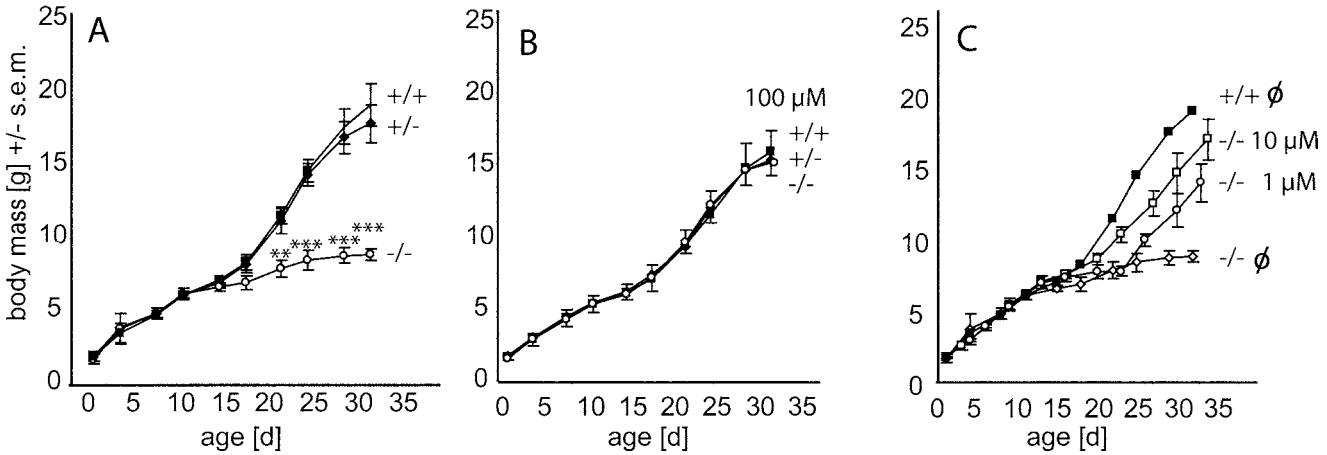

Figure 1 Mass gain during the first weeks of life

Pups from heterozygous breeders (+/+) were labelled individually and body mass was determined twice a week. (**A**) SePP$^{-/-}$ mice (○; $n = 11$) did not engage in the growth spurt normally starting within the third week of life. (**B**) Complete rescue of the growth defect upon supplementation of nursing mothers with drinking water containing 100 μM sodium selenite ($n = 6$–11). (**C**) Dose-dependent rescue of SePP$^{-/-}$ mice is observed at supplementation with 1 μM (○) and 10 μM (□) sodium selenite ($n = 7$ and $n = 9$ respectively). Ø, no supplement; $^{**}P < 0.01$; $^{***}P < 0.001$ [Student's t test compared with wild-type (+/+)].

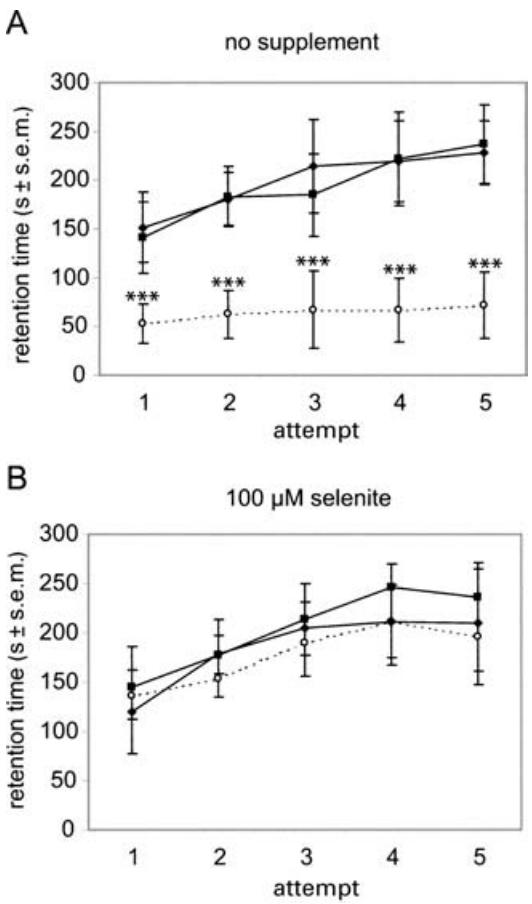

Figure 2 Motor co-ordination analysis

(**A**) SePP$^{-/-}$ mice performed poorly on the accelerating rotarod (open circles; $n = 8$) when the mothers were raised on a regular lab chow. Heterozygous mice (closed diamonds; $n = 16$) cannot be distinguished from their wild-type littermates (closed squares; $n = 9$). (**B**) Complete rescue of motor co-ordination of SePP$^{-/-}$ mice is observed in the same test when mothers received drinking water containing 100 μM sodium selenite ($n = 6$–11). The rotating drum was linearly accelerated from 4 rpm to 40 rpm within 5 min. $^{***}P < 0.001$ (Student's t test compared with wild-type and heterozygote).

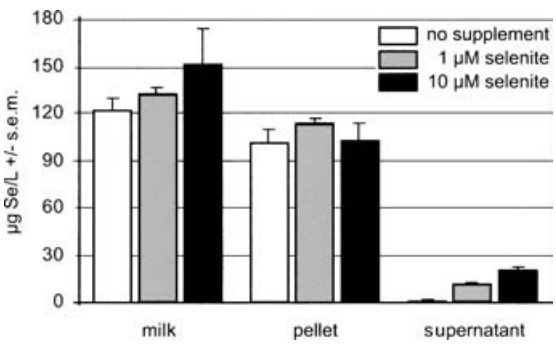

Figure 3 Milk Se content increases upon supplementation of mothers with sodium selenite

After TCA precipitation of milk proteins, Se content was determined in the protein pellet and supernatant. Although the Se content of milk protein did not change upon supplementation, an increase of TCA-precipitation-resistant Se was noted. Results are means ± S.E.M. ($n = 3$).

Effects of Se supplementation on milk and plasma Se

We next studied whether the supplemental Se was passed on from the mothers to the offspring directly as selenite or other inorganic forms, or whether Se was biotransformed into milk selenoproteins. It is known that both pGPx [26] and SePP (M. Scharpf, L. Schomburg, U. Schweizer and J. Köhrle, unpublished work) are present in human milk. Therefore we analysed full milk from dams exposed to sodium selenite for Se content. Figure 3 shows that 10 μM sodium selenite treatment increased milk Se content by approx. 20%. After TCA precipitation, the extra Se was found in the supernatant, indicating that this extra Se was not protein-bound.

The next question we addressed was the extent to which the Se supplementation regimen affected Se status in treated mice. To this end, we measured Se in the plasma of treated and untreated mice at the age of 5 weeks. Plasma Se levels were decreased in SePP$^{+/-}$ mice by 35% as compared with SePP$^{+/+}$ littermates (Figure 4). Accordingly, levels of SePP in plasma were reduced by roughly half in SePP$^{+/-}$ mice as illustrated by a semi-quantitative Western blot analysis (Figure 4, inset) and as reported independently

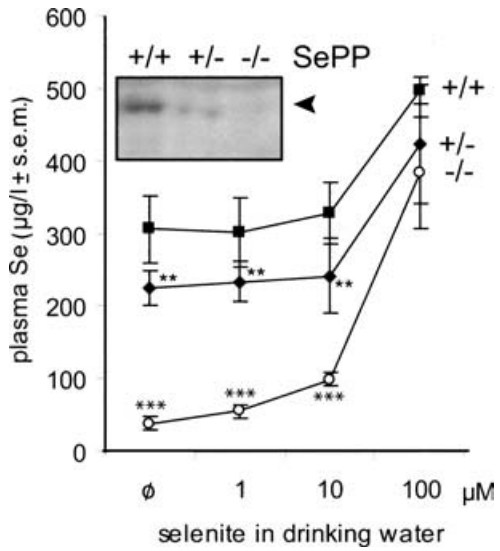

Figure 4 Plasma Se concentration after supplementation regimens

Western blot analysis indicated that plasma Se is mainly controlled by SePP levels (inset). Plasma Se concentrations of wild-type (closed squares; $n = 6$) and heterozygous (closed diamonds; $n = 6$) mice remain constant after sodium selenite supplementation up to 10 μM. Dose-dependent increase of plasma Se is observed in the SePP$^{-/-}$ mice (open circles; $n = 6$). Upon supplementation with 100 μM sodium selenite, plasma Se is elevated to non-physiological levels irrespective of the genotype. $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's t test compared with wild-type). Ø, no selenite supplementation.

by Hill et al. [17]. Drinking water supplemented with 10 μM selenite did not affect Se plasma levels significantly in the SePP$^{+/+}$ and SePP$^{+/-}$ offspring (Figure 4). This indicates that SePP synthesis and SePP plasma levels are at the maximum in Se-replete mice, in accordance with respective observations with humans [27]. In the SePP$^{-/-}$ mice, however, dose-dependent increases in plasma Se levels were observed, reaching 30 % of wild-type levels at 10 μM selenite (Figure 4). At 100 μM supplementation, plasma Se levels were highly increased (+ 25 % in the wild-type) in all genotypes and probably reflect a non-physiological situation. Here, adverse side-effects were already manifest in a decreased body mass (Figure 1B) and Se excretion via the urine at levels exceeding 50 times the control values (results not shown).

Regulation of pGPx expression by Se supplementation

As SePP is absent in SePP$^{-/-}$ mice, increased plasma Se levels cannot be attributed to this protein. The question thus arose whether the increased Se in plasma of treated SePP$^{-/-}$ mice is inorganic in nature, essentially representing the supplemented selenite, or whether it is to be attributed to the second major plasma selenoprotein, i.e. pGPx. Western blot experiments indicate that selenite in the drinking water of the nursing mothers can affect pGPx protein levels in the progeny (Figure 5). Basically, pGPx levels in plasma are significantly lower in SePP$^{-/-}$ mice compared with their wild-type littermates (Figure 5). Although the pGPx levels in the wild-type mice do not readily respond to an increased Se supply, SePP$^{-/-}$ mice normalize their pGPx levels in plasma upon supplementation of their mother's drinking water with 10 μM selenite. Changes in GPx activity in plasma paralleled these alterations in immunoreactive pGPx protein levels (Figure 5A; [17]). In a direct comparison of the supplemented mice, pGPx activities were even normalized in the SePP-deficient mice as compared with wild-type littermates treated equally

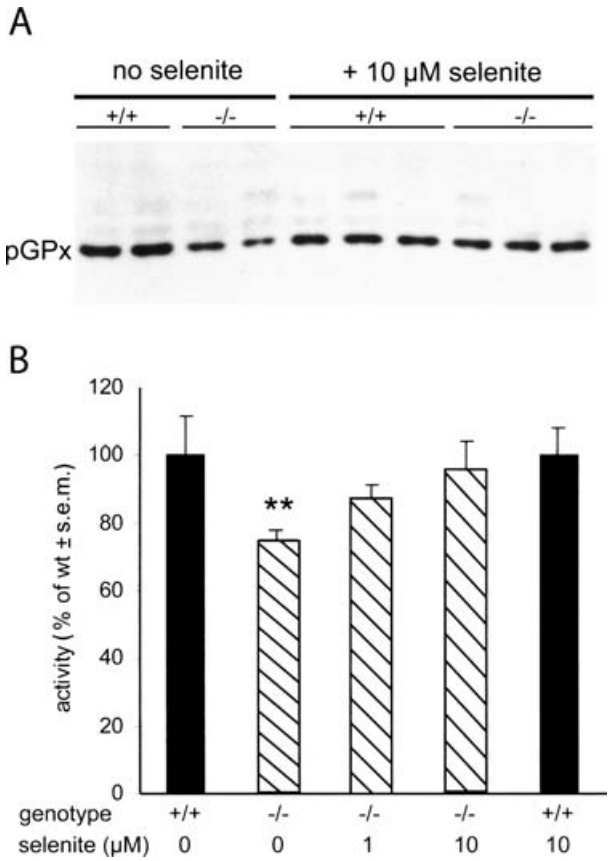

Figure 5 pGPx expression increases in selenite-treated SePP$^{-/-}$ mice

(**A**) Western blot analysis of GPx protein in plasma. pGPx levels are significantly reduced in SePP$^{-/-}$ mice, but can be restored to wild-type levels after supplementing their nursing mothers with 10 μM sodium selenite in drinking water. (**B**) Enzymic activity of GPx in plasma. SePP-deficient mice display significantly reduced activity of pGPx ($^{**}P < 0.01$). Dose-dependent increase of GPx activity is observed in SePP$^{-/-}$ mice upon selenite supplementation. Enzymic activity of GPx in the wild-type (wt) mice is not affected by the increased Se supply ($n = 6$ for each group).

(Figure 5B, right-most two bars). The increased plasma Se level at 100 μM selenite supplementation is, however, not to be attributed to increased pGPx expression, as pGPx activity did not increase significantly beyond wild-type levels at this concentration (results not shown). It is well established that kidney is the main source of pGPx in plasma [28]. The next question was, therefore, whether or not the Se content of kidney is directly related to pGPx plasma levels. We found that Se content and GPx activity were reduced in the kidneys of SePP$^{+/-}$ and SePP$^{-/-}$ mice [16]. In the present paper, we now show that this reduction was normalized by Se supplementation with 10 μM selenite in the water (Table 1). To determine whether or not the altered pGPx levels result from corresponding changes at the transcriptional level, we performed Northern blot experiments. Levels of kidney mRNA encoding pGPx were not decreased in SePP$^{-/-}$ mice as compared with SePP$^{+/+}$ controls (Figure 6). Likewise, Se supplementation did not increase pGPx mRNA levels in kidneys of treated mice irrespective of the genotype. Thus our data suggest that renal Se availability is normalized in SePP$^{-/-}$ mice via the milk from their mothers, who consumed the Se-enriched drinking water. This increased the availability of locally modulated pGPx synthesis in the kidney, most likely by increasing the translational efficiency via the pool of Sec-loaded tRNA^{Sec}.

Table 1 GPx enzyme activity in kidney is reduced in SePP$^{-/-}$ mice

Results are means ± S.E.M.; $n = 6$ for each group; $^{**}P < 0.01$.

	GPx enzyme activity (nmol/mg per min)	
Genotype	No supplement	10 μM selenite
SePP$^{+/+}$	405 ± 24	383 ± 55
SePP$^{+/-}$	334 ± 79	373 ± 48
SePP$^{-/-}$	188 ± 38**	364 ± 68

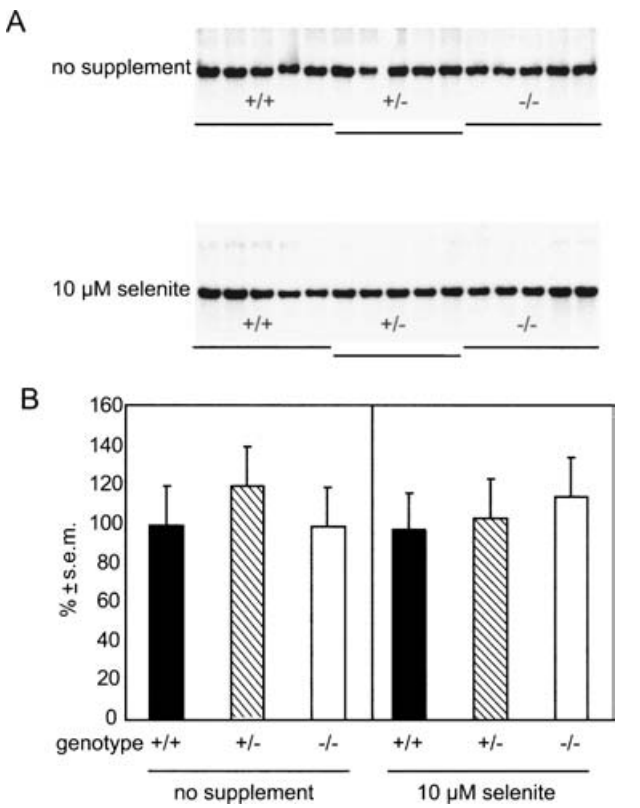

Figure 6 Northern blot analysis of pGPx transcript levels in kidney

(**A**) Steady state transcript levels of pGPx in kidney are not altered by SePP deficiency. In addition, selenite supplementation at 10 μM does not affect mRNA levels of renal pGPx. (**B**) Quantification data represent relative transcription levels corrected for variability in loading via the signals obtained for ubiquitin mRNA as determined by phosphoimager-based analysis.

DISCUSSION

Recently, we have established a transgenic line of mice that lack SePP expression [16]. Since these mice display reduced levels of Se and Se-dependent enzymes in a number of tissues, we have provided evidence that SePP fulfils a crucial function in Se distribution and transport, as suggested by the early works of Motsenbocker and Tappel [12]. In the present paper, we report a crucial role for SePP in early postnatal development. Generally, rats or mice kept on Se-deficient diets display growth retardation and a number of other defects [29,30]. The underlying mechanism for this general phenomenon, and especially the reason why SePP$^{-/-}$ mice do not undergo the characteristic growth spurt usually starting around postnatal day 15, is still subject to investigation. As the growth defect is detected at a time when young mice still depend on their mothers' milk, we decided to analyse early Se supplementation via the mother. Complementary findings, albeit focusing on supplementation strategies after the time of weaning, i.e. when important developmental processes have already been completed, were already reported recently by Hill et al. [17].

We reasoned that the original transport hypothesis [12] combined with the recent data from the SePP-deficient mice [16,17] implies that supplementation with high doses of Se might be suitable to compensate for a lack of SePP expression during development. This rationale is based on the observation that rats raised on an Se-deficient diet for many generations still retain high brain Se levels [31], corroborating well the established hierarchy of the different organs for Se supply [32]. This preferential supply of brain cells was also observed in the heterozygous SePP$^{+/-}$ mice [16], even implying some sort of specific reception. But most importantly, even in the complete absence of SePP, there was still expression of selenoproteins in organs other than the liver in the SePP$^{-/-}$ mice [16,17]. This indicated to us that SePP, although not absolutely essential for Se metabolism at an ample Se supply, greatly facilitates organification, uptake and distribution of this essential trace element within the organism. Accordingly, our results document that Se supplementation is able to dose-dependently rescue growth defects as well as the movement disorder of SePP$^{-/-}$ mice during postnatal development. Interestingly, these health effects can be exerted indirectly by supplementing the nursing mothers that transfer Se to their progeny via breast-feeding. It is noteworthy that, at 10 μM selenite supplementation, plasma Se levels and GPx activities did not increase in the wild-type pups, indicating that the form and concentration of the Se compounds in milk is adequately adjusted and well-tolerated. These findings clearly underscore the crucial role of the mother to provide Se in due amounts and adequate forms to be readily used by the newborn, thereby efficiently avoiding the danger of intoxication/selenosis.

However, at the highest selenite dose tested (100 μM), the pups did not grow as well as non-treated controls, but the differences between the genotypes vanished. These data agree with earlier reports that toxic effects can be exerted at exceedingly high concentrations [24,33]. Humans also contain significant amounts of Se in the mother's milk [26,34]. Inorganic Se supplements are normally not transferred directly into human mother's milk, but appear as an increase in protein-bound Se [35]. The fact that, unlike in (Se-deficient) humans [26], Se supplementation did not increase milk selenoproteins in our mice may be explained with the more-than-sufficient Se content of our mice feed. Similarly, plasma Se of these mice was not increased by supplementation of 1 or 10 μM selenite. On the molecular level, a role for selenoproteins in milk is also suggested by the prolactin-induced up-regulation of a Sec-tRNA-activating factor (Staf), thereby increasing $tRNA^{Sec}$ synthesis in the mammary gland during lactation [36]. Generally, the expression of selenoproteins is dependent on Se supply to maintain a sufficiently high Sec-loaded $tRNA^{Sec}$ pool that enables efficient selenoprotein synthesis [18]. Recently, Hatfield and colleagues [37] have abolished the synthesis of all selenoproteins, including SePP, in the mammary gland, but, surprisingly, reported apparently normal lactation. In the present study, however, we analysed the effects of varying Se levels in milk with a new sensitive biological assay system, i.e. mice lacking SePP, and thus relying on an easily usable ample supply of the trace element. All chosen readouts could be rescued in a dose-dependent manner, including growth, ataxia and renal GPx expression.

Generally, SePP is known to carry 50–60 % of plasma Se [11] and to serve as a readily available Se carrier [38]. In the SePP$^{-/-}$ mice, however, Se plasma levels are reduced by almost 80–90 %, thereby implying that the other major plasma

selenoprotein, pGPx, is also affected by SePP deficiency. Upon Se supplementation up to 10 μM, plasma Se levels increased to approx. 30% of wild-type levels in SePP$^{-/-}$ mice, which corresponds well to the fraction of plasma Se that is normally attributed to pGPx [39]. At the same time, plasma GPx activity returned to normal, depending on the concentration of selenite supplement. The data suggest that, in our SePP-deficient mouse model, we can fine-tune the level of Se deficiency in certain organs and thereby adjust the severity of the phenotypes to be studied. In addition, this model opens the possibility to test the bio-availability of different Se-containing compounds with respect to their transfer from mother to offspring and potency to rescue deficiency effects.

Taken together, our studies again clearly underscore the importance of Se for growth and brain function, emphasize the central role of SePP for efficient Se distribution and usage, and point to an as-yet only poorly appreciated ingredient within the milk that is transferred actively to the progeny. It might be worthwhile to consider the Se status and changes thereof during the periods of breast-feeding in both the mothers and their offspring.

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