Selenoprotein P Is the Major Selenium Transport Protein in Mouse Milk

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Abstract

Selenium is transferred from the mouse dam to its neonate via milk. Milk contains selenium in selenoprotein form as selenoprotein P (Sepp1) and glutathione peroxidase-3 (Gpx3) as well as in non-specific protein form as selenomethionine. Selenium is also present in milk in uncharacterized small-molecule form. We eliminated selenomethionine from the mice in these experiments by feeding a diet that contained sodium selenite as the source of selenium. Selenium-replete dams with deletion of Sepp1 or Gpx3 were studied to assess the effects of these genes on selenium transfer to the neonate. Sepp1 knockout caused a drop in milk selenium to 27% of the value in wild-type milk and a drop in selenium acquisition by the neonates to 35%. In addition to decreasing milk selenium by eliminating Sepp1, deletion of Sepp1 causes a decline in whole-body selenium, which likely also contributes to the decreased transfer of selenium to the neonate. Deletion of Gpx3 did not decrease milk selenium content or neonate selenium acquisition by measurable amounts. Thus, when the dam is fed selenium-adquate diet (0.25 mg selenium/kg diet), milk Sepp1 transfers a large amount of selenium to neonates but the transfer of selenium by Gpx3 is below detection by our methods.

Introduction

Selenium was reported to be a constituent of milk in 1935 [1] and was recognized to be present in milk protein in 1948 [2]. Picciano and colleagues carried out studies in animal and human milk in the 1980s and reported that the selenoprotein glutathione peroxidase (Gpx) was present in milk and that as much as 30% of milk selenium was dialyzable [3,4,5]. Avissar and colleagues later identified human milk Gpx to be Gpx3, the major Gpx form present in plasma, and showed that it accounted for less than 15% of the selenium in human milk [6]. More recently, Schweizer and colleagues presented results indicating that selenoprotein P (Sepp1) is present in mouse milk [7]. Thus, the two plasma selenoproteins, Sepp1 and Gpx3, are present in milk, as are dialyzable forms of selenium.

Many proteins containing selenomethionine–termed selenium-containing proteins–are present in the body and presumably also are present in milk. Selenomethionine is synthesized by plants and incorporated into their proteins. It is a major dietary source of selenium for free-living animals. The amount of selenium consumed in the form of selenomethionine is highly variable and depends largely on the geographic source of the diet. Once ingested, selenomethionine enters the methionine metabolic pool [8] and is incorporated randomly into proteins at methionine positions. Only when selenomethionine is catabolized is selenium released from it and recognized as selenium in the animal. For that reason, studies seeking to elucidate specific selenium metabolic processes–such as the study reported here–usually feed selenium in an inorganic form, which enters the selenium metabolic pool directly. Otherwise measurements of selenium would reflect both the specific and the highly variable non-specific forms of selenium and render results difficult to correlate with specific selenium metabolic processes.

In recent years, mice with deletion of Sepp1 and Gpx3 have been used to study selenium metabolism. Sepp1 is a selenium-rich protein that transports the element from the liver to peripheral tissues [9]. Apolipoprotein E receptor-2 (apoER2) on cells in those tissues binds Sepp1 arriving via the systemic circulation and facilitates its endocytosis [10]. Sepp1 accounts for about two-thirds of plasma selenium in selenium-replete mice. Although it contains up to one-third of mouse plasma selenium, Gpx3 does not appear to have a selenium transport role except in the visceral yolk sac [11]. Small molecule forms account for less than 3% of plasma selenium [12].

The present study used mice with deletion of the genes for Sepp1 and Gpx3 to assess transfer of selenium from the nursing dam to the neonate by these selenoproteins. The results indicate that Sepp1 is important for this transport in selenium-replete mice but that Gpx3 is not.

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The authors confirm that all data underlying the findings are fully available without restriction. All data is contained in supplemental tables.

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Materials and Methods

Animals

Because male Sepp1\(^{-/-}\) mice are sterile, Sepp1\(^{+/+}\) breeding pairs in our colony were mated to produce Sepp1\(^{-/-}\) and Sepp1\(^{+/+}\) mice for study [13]. Homozygote and wild-type breeding pairs produced the Gpx3\(^{-/-}\) and Gpx3\(^{+/+}\) female mice [12]. To produce mice with deletion of both Sepp1 and Gpx3, Sepp1\(^{-/-}\) female mice were mated with Gpx3\(^{-/-}\) male mice. The resulting mice were heterozygotes for both genes and female progeny were mated with Gpx3\(^{-/-}\) male mice to produce breeding pairs of Sepp1\(^{-/-}\)/Gpx3\(^{-/-}\) mice. At weaning Sepp1\(^{-/-}\)/Gpx3\(^{-/-}\) pups were selected for study after their genotype had been verified by PCR [12]. Wild-type mice were C57BL/6 strain.

Weanling mice were fed selenium-deficient Torula yeast-based diet supplemented with 0 or 0.25 mg selenium as sodium selenite per kg diet. The diet was formulated and pelletized to our specifications [14] by Harlan-Teklad (Madison, WI). The mice were housed on aspen shavings and had free access to food and tap water. The light cycle in the room was 14 h on and 10 h off. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Vanderbilt University Institutional Animal Care and Use Committee (Protocol numbers: M/07/304 and M/10/306). All procedures were performed using isoflurane or sodium pentobarbital anesthesia prior to exsanguination, and all efforts were made to minimize suffering.

Neonate experiments

Pregnant Sepp1\(^{-/-}\) mice were fed diet containing 0.25 mg selenium/kg. Neonates were killed by isoflurane inhalation immediately after being taken from the dam 1 or 5 days after birth. A 0.5 cm portion of the tail was taken from neonates for isolation of DNA and genotyping. Each neonate was weighed, frozen in liquid N\(_2\), and stored at \(-80^\circ\) C until being assayed for selenium content.

Milk experiments

Milk was collected from nursing dams between days 10 and 16 of lactation. Neonates were removed from their dam and killed by isoflurane inhalation. Three hours after removal of the neonates, the dam was injected intraperitoneally with 3 units of oxytocin. Thirty to 60 min later, the dam was anesthetized (2 mg pentobarbital intraperitoneally) and milk was collected by application of gentle vacuum to each teat. Following milk collection (0.1–0.3 ml/dam), the dam was killed by isoflurane inhalation. Milk was stored at \(-20^\circ\) C until it was assayed for selenium content. Most individual milk samples were assayed alone but samples with low volume were pooled within experimental groups.

\(^{75}\)Se labeling of milk

Ten \(\mu Ci\) of \(^{75}\)Se-selenite (University of Missouri, Columbia, MO; specific activity \(\sim 1000 \text{ Ci/g}\)) was administered to nursing dams intraperitoneally approximately 24 h prior to milk collection. Milk was collected in PBS and centrifuged at 16,000 g for 15 min to separate the lipid layer, the aqueous layer (whey), and the solids (curd). The whey fraction was transferred to a clean tube. A sample of whey was subjected to SDS-PAGE and the gel was stained with Coomassie blue. The stained 15% acrylamide gel was dried and exposed to Kodak BioMax XAR film.

Selenium analysis

Tissues and milk were predigested in concentrated nitric acid prior to perchloric acid digestion. Fluorometric determination of selenium was carried out by the method of Koh and Benson [15] as modified by Sheehan and Gao [16].

In situ hybridization

Freshly harvested mammary tissue was processed for in situ hybridization detection of Sepp1 and Gpx3 mRNA. A 641 bp mSEPP1 cDNA was prepared by PCR using the plasmid MB23A1, which contains a full-length mSEPP1 insert, and primers 5\' -AGGCAGCTGATACTTGTGTCTTCTGCAGG-3\' and 5\'-AAAGGTGCAAGCCCTT-CACITGTGCTGTGTG-3\' . The PCR product was gel-purified and ligated into the pCR4 Topo plasmid (Invitrogen, Carlsbad, CA). Following transformation into OneShot Top10 cells (Invitrogen, Carlsbad, CA), individual clones were analyzed by PCR to identify the orientation of the mSEPP1 cDNA insert. Clones with opposite insert orientation were used to prepare template DNA by

Figure 1. Selenium-containing proteins in milk whey detected by autoradiogram of an SDS-PAGE gel. Panel A is the autoradiogram of the gel and panel B depicts the gel stained by Coomassie blue. Dams that had been lactating 10–16 d were milked 24 h after administration of a tracer dose of \(^{75}\)SeO\(_3\)\(^2-\) and the whey fraction of the milk was subjected to SDS-PAGE. \(^{75}\)Se-labeled plasma was included as a control. After drying, the gel was exposed to x-ray film. Lane 1 represents plasma (0.5 \(\mu l\) and 321 cpm applied) and lanes 2–5 represent whey (lane 2–1.5 \(\mu l\) and 260 cpm applied; lane 3–2 \(\mu l\) and 124 cpm applied; lane 4–1 \(\mu l\) and 288 cpm applied; lane 5–2 \(\mu l\) and 162 cpm applied). Deletion of selenoprotein genes in the dams is indicated in the lower half of the figure.

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Table 1. Effect of dam genotype on nursing pup weight gain and selenium acquisition from day 1 to day 5 of life.

| Neonate selenium content | Weight gained | Selenium acquired |
|--------------------------|---------------|-------------------|
|                          | day 1 | day 5 | day 1 | day 5 |
| Neonate selenium content | day 1 | day 5 | day 1 | day 5 |
| selenium deficient       | 23   | 32   | 1.3  | 0.1  |
| selenium replete        | 0.4  | 33   | 24   | 2.5  |
| Sepp1                    | 12   | 31   | 6.1  | 24   |
| Sepp1                    | 1.2  | 30   | 1.9  | 7.3  |
| Gpx3                     | 0.9  | 27   | 1.4  | 230  |
| Gpx3                     | 1.4  | 13   | 230  | 360  |

*aSee Table S1 for supporting data.*

bValues are means ± S.D.

cValues were calculated using day 1 and day 5 means.

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PCR, which included the plasmid T7 promoter and the mSepp1 insert. The purified PCR products were used in transcription reactions to prepare sense and antisense digoxigenin-labeled riboprobes as described previously [17]. These were used for in situ hybridization on tissue cryosections using our standard protocol [17].

A 440 bp mouse GPX3 cDNA spanning bases 190–637 in the coding sequence was prepared by PCR using IMAGE clone 441025 as the template (purchased from American Type Culture Collection, Manassas, VA). The PCR product was gel-purified and ligated into the pCR4 plasmid. All subsequent steps were as described for mSepp1.

**Statistical test**

Means were compared using Student’s t-test.

**Results**

The identity of mouse milk proteins that contained selenium was sought. The milk was collected from dams 24 h after they had been injected with a tracer dose of 75SeO42− and whey from that milk was studied with SDS-PAGE autoradiography (Fig. 1A). Whey from C57BL/6 mice (lane 2) contained bands labeled with 75Se that corresponded to Sepp1 and Gpx3 in plasma (lane 1) as demonstrated in a previous publication [12]. No additional 75Se-containing bands were detected in lane 2. Study of whey from Sepp1−/− and Gpx3−/− dams (lanes 3 and 4, respectively) confirmed the identity of the 75Se-labeled bands in lane 2. Lane 5 was loaded with whey from a dam with deletion of both Sepp1 and Gpx3. It contained no 75Se-labeled bands at the positions of Sepp1 or Gpx3. All lanes loaded with whey from dams with selenoprotein genes knocked out (lanes 3–5) contained a faint 75Se band at 13 kDa (Fig. 1A). Figure 1B shows that lanes 3 and 5 were loaded with more protein than the other lanes. More whey was loaded onto those lanes than onto lanes 2 and 4 so that enough 75Se would be present to detect labeled selenoproteins by autoradiography. These results confirm that Sepp1 and Gpx3, the 2 selenoproteins found in plasma, are also present in milk. The 13 kDa 75Se band might represent a selenium-containing protein that became detectable when 75Seavailability increased as a consequence of deletion of Sepp1 and/or Gpx3.

During the week following birth, the mouse is totally dependent on mother’s milk for nourishment. We assessed the effect of maternal dietary selenium on selenium acquisition by neonates for the 4 days of life from day 1 to day 5. Figure 2A shows that neonates of selenium-deficient wild-type dams had much lower whole-body selenium concentrations (12%) than neonates of selenium-replete dams. The average amount of selenium retained by neonates from the milk of selenium-deficient dams over the 4 days was 18% of that retained by neonates of selenium-replete dams (Table 1). Thus, dietary selenium deficiency in the dam sharply reduces the selenium uptake and status of the neonate.

The same protocol was used for experiments with Sepp1−/− and Gpx3−/− dams fed selenium-sufficient diet. It was not possible to study selenium deficiency in Sepp1−/− dams because restricting their selenium intake would have caused severe brain injury and death [14]. Figure 2B shows that neonates of Sepp1−/− dams had 31% of the whole-body selenium concentration of neonates of Sepp1+/− dams, even though all dams had been fed diet supplemented with 0.25 mg selenium/kg. The average amount of selenium retained in the neonates of Sepp1−/− dams was 35% of that retained in the neonates of Sepp1+/− dams (Table 1). Deletion of Gpx3 did not decrease neonate selenium concentration (Fig. 2C) or neonate selenium retention (Table 1). Thus, the
Sepp1+/+ genotype is important for selenium transfer from the selenium-replete dam to the neonate but the Gpx3+/+ genotype is not.

The effect of selenoprotein gene deletion on the selenium content of milk was assessed. Efforts were made to control variations in milk selenium content by studying milk collected between the 10th and 16th days of lactation and by including temporal controls. Comparison of results from Sepp1+/2 and Sepp1+/+ dams indicates that 73% of milk selenium was associated with the Sepp1+/+ genotype (Fig. 3A), while comparison of results from Gpx3+/2 and Gpx3+/+ dams indicates that an insignificant amount of milk selenium was associated with the Gpx3+/+ genotype (Fig. 3B). Thus, the Sepp1+/+ genotype accounts for much more of the selenium in milk from selenium-replete dams than does the Gpx3+/+ genotype.

Because Sepp1 status is important in the transfer of selenium from the dam to the neonate and Gpx3 status is not, in situ hybridization of the lactating mammary gland was carried out at day 13 of lactation. It showed that Sepp1 mRNA was present in the gland (Fig. 4) but that Gpx3 mRNA was not detected (Fig. 5, see also supplemental Fig. 1 for a positive control). This strongly suggests that milk Sepp1 is synthesized by the mammary gland but that little or no Gpx3 is synthesized by it. This finding leaves open the possibility that plasma is the source of milk Gpx3. It also raises the question of how the mammary gland acquires the selenium necessary for Sepp1 synthesis.

The testis exports a large amount of selenium in spermatozoa and acquires selenium to support this export by apoER2-mediated endocytosis of Sepp1 from the systemic circulation [17]. ApoER2 is the only receptor known to facilitate endocytosis of Sepp1 from the systemic circulation and male apoER2−/− mice are sterile. We sought a role for apoER2, a member of the low-density lipoprotein receptor family, in the transfer of selenium from the dam to the neonate. Deletion of maternal apoER2 had no detectable effect on
dam-to-neonate selenium transfer (Fig. 6). Thus, the mechanism of selenium uptake by the mammary gland does not appear to involve apoER2-mediated endocytosis of Sepp1 from maternal plasma. The mechanism by which the mammary gland is supplied with selenium remains unknown.

Discussion

The experiments reported here took advantage of gene deletion techniques to assess the effectiveness of Sepp1 and Gpx3 in transferring selenium from the selenium-replete dam to its nursing neonate. Deletion of Sepp1 reduced selenium transfer to the neonate by 65% while no decreased transfer was detected when Gpx3 was deleted (Fig. 2 & Table 1). Supporting the transfer experiments, analysis of milk revealed that the Sepp1/+ genotype accounted for 73% of the selenium in milk while the amount accounted for by the Gpx3/+ genotype was not statistically significant. Further support for the predominance of Sepp1 over Gpx3 in the transport of selenium by milk was the demonstration that the lactating mammary gland expressed Sepp1 mRNA (Fig. 4) but that Gpx3 mRNA was not detectable in it by in situ hybridization (Fig. 5). Thus, the Sepp1/+ genotype is important for selenium transfer from the selenium-replete dam to the neonate but the Gpx3/+ genotype is not.

It is likely that the maternal Sepp1−/− genotype decreases selenium transfer from dam to nursing pup through two mechanisms. One of the mechanisms is the elimination of selenium-rich Sepp1 from milk as demonstrated in figure 1. The other mechanism is decreasing the whole-body selenium content of the dam [9,11], thereby reducing the amount of maternal selenium available for incorporation into milk. Sepp1 deletion reduces whole-body selenium of non-pregnant female mice by only 21% [11], suggesting that the 73% reduction of milk selenium by Sepp1 deletion is mainly attributable to the absence of Sepp1 from the milk. However, the relative contributions of these mechanisms to the decrease in milk selenium by the Sepp1−/− genotype were not determined directly. Previous work made clear that milk selenium is present in several forms. As confirmed here, the selenoproteins Sepp1 and Gpx3 are among those forms. A large amount of milk selenium—perhaps 30% of it—is present in small-molecule form that has not been characterized [3]. The large fraction of milk selenium in small-molecule form contrasts with the much smaller fraction (less than 3%) of plasma selenium in small-molecule form [12]. The small-molecule forms in plasma include excretory metabolites...
produced by the liver—largely selenosugars and other methylated compounds [18,19]. These excretory forms seem unlikely to account for much of the small-molecule selenium in milk because they are cleared from the blood by the kidneys and appear in the urine.

Little is known about small-molecule transport forms of selenium. At least one small-molecule form in plasma transports selenium to tissues because mice with both Sepp1 and Gpx3 deleted are viable when fed high-selenium diet [12]. This small-molecule transport form in plasma has not yet been characterized and further work is needed to characterize it and the small-molecule selenium in milk.

When present in the diet of lactating individuals, selenomethionine contributes to the selenium in milk. Feeding selenium as selenomethionine to the lactating rat dam results in a higher milk selenium concentration than does feeding selenite [5]. Gel filtration of defatted and dialyzed human milk was shown to have 9 selenium peaks with apparent molecular weights of 8 to 150 kDa [3]. The likely reason that more selenium-containing proteins were detected in human milk than in mouse milk is that humans ingest selenomethionine as their major dietary form of selenium and our mice were fed selenium only as selenite. Selenomethionine is present at methionine positions in many proteins, but selenite, when fed at physiological levels of selenium, does not enter proteins nonspecifically and provides selenium only for selenoproteins. Thus, milk from free-living animals and humans can be expected to contain many proteins that contain selenium in selenomethionine form.

Both Sepp1 and Gpx3 are enzymes with peroxidase activity [20]. It is possible that they have enzymatic functions in milk. They might protect against peroxides in the milk or in the gastrointestinal tract of the neonate. Such a function seems likely
for Gpx3 because it has a selenium transfer function that was found in these studies of selenium-replete mice.

In conclusion, the Sepp1<sup>+/−</sup> genotype is responsible for most of the selenium transfer from the dam to the neonate under selenium-adequate conditions. Sepp1 is synthesized in the mammary gland and the Sepp1<sup>+/−</sup> genotype accounts for most of the selenium in mouse milk. The Gpx3 present in milk accounts for much less selenium than does Sepp1 and, thus, Gpx3 is responsible for very little of the selenium transferred from the dam to the neonate under selenium-adequate conditions. Unidentified small-molecule forms of selenium are present in milk and contribute to the transfer of selenium from the dam to the neonate.

In free-living animals the effect of proteins containing selenomethionine will be superimposed on the effects of Sepp1, Gpx3, and the small-molecule forms of selenium. However, selenoproteins can be expected to maintain their selenium better than selenomethionine-containing proteins under selenium-deficient conditions. Thus, we speculate that Sepp1, and possibly Gpx3, will be of great importance in transferring maternal selenium to the nursing neonate when dietary selenium is limiting in humans and free-living animals.

Supporting Information

Figure S1 This figure shows a tissue, wild-type mouse kidney cortex, that expresses Gpx3 mRNA (stained). It serves as a positive control for figure 5 in the manuscript, which does not detect Gpx3 mRNA in the lactating mammary gland. (TIF)

Table S1 Supporting data for Table 1 and Figure 2. (DOCX)

Table S2 Supporting data for Figure 3. (DOCX)

Table S3 Supporting data for Figure 6. (DOCX)

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Author Contributions

Conceived and designed the experiments: KEH RFB. Performed the experiments: KEH AKM VPW. Analyzed the data: KEH AKM VPW RFB. Contributed reagents/materials/analysis tools: KEH AKM VPW RFB. Contributed to the writing of the manuscript: KEH RFB.

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