Original Research

Phospholipid hydroperoxide glutathione peroxidase (Gpx4) is highly regulated in male turkey poults and can be used to determine dietary selenium requirements

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Abstract

The dietary selenium recommendation for turkeys of 0.2 μg Se/g is higher than for many other species. Liver glutathione peroxidase-1 (Gpx1) activity levels determined using hydrogen peroxide (H_2O_2) in previous studies suggest that 0.2 μg Se/g may still be too low and that some of this Gpx1 activity might be due to phospholipid hydroperoxide Gpx (Gpx4). Thus we separated Gpx1 from Gpx4 by chromatography, demonstrated that 47% of the H_2O_2 activity in Se-adequate turkey liver was due to Gpx4, and determined a factor for calculation of each activity. Day-old male poults were fed an Se-deficient torula diet (0.007 μg Se/g) supplemented with graded levels of Se (0–0.5 μg Se/g) for 27 days. Final body weights indicated a minimum Se requirement for growth of 0.05 μg Se/g. The liver had the highest Gpx4 activity in Se-adequate poults, and Gpx4 activity in Se-deficient liver decreased to 5% of Se-adequate levels, with an Se requirement of 0.29 μg Se/g. Liver Gpx1, gizzard Gpx1 and gizzard Gpx4 activities also had Se requirements of 0.28–0.30 μg Se/g, collectively yielding an Se requirement of 0.3 μg Se/g, which is three times higher than the requirements found in comparable rodent studies. We also sequenced partial cDNA clones for turkey Gpx1 (GQ502186) and Gpx4 (GQ502187), and found >60% identity with rodents and humans and >90% identity with chickens. Ribonuclease protection analysis showed that Gpx4 mRNA levels decrease substantially in Se-deficient turkey liver, unlike in rodents. These underlying differences in selenoprotein molecular biology may explain the elevated dietary Se requirements of turkeys.

Keywords: cDNA sequence, glutathione peroxidase, *Meleagris gallopavo*, mRNA, phosphatidylcholine hydroperoxide, RT-PCR

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Introduction

A role for selenium (Se) in turkey health and nutrition emerged after the discovery that Se was an essential nutrient for rodents[1] and chickens.[2] Using a torula yeast-based diet, Walter and Jensen[3] reported in 1963 that low levels of Se (0.1 μg Se/g) did not prevent gizzard myopathy but that 1 μg Se/g provided complete protection. Sulfur amino acids were not protective but ethoxyquin partially reduced incidence. Scott *et al.*[4] fed turkeys low Se diets, and found that supplementation with vitamin E and methionine improved growth, but that supplemental Se was needed for optimum growth. This group found that the Se requirement for complete prevention of gizzard myopathy was 0.18 μg Se/g in diets supplemented with vitamin E and 0.28 μg Se/g in diets not supplemented with vitamin E. They also reported that heart muscle was susceptible to degeneration in Se deficiency, with high mortality beginning on day 16, and with the heart appearing to be the affected organ (60% incidence) resulting in death. Skeletal muscle degeneration was observed histologically but not grossly, with degeneration of the pectoral muscles appearing in the fourth to fifth week.[4] Subsequent studies also reported requirements of 0.20 μg Se/g for prevention of gizzard myopathy[5] and 0.23 μg Se/g to maintain hatchability and reduce chick mortality.[6] The current 1994 National Research Council (NRC) Se requirements of turkeys[7] are 0.2 μg Se/g for all stages from starter to finishing diets, and for breeding and laying hen diets, all based on

prevention of disease. These turkey Se requirements thus are considerably higher than the Se requirement for chickens (0.08 μg Se/g) for prevention of exudative diathesis[2] and for rodents (0.04 μg Se/g) for prevention of liver necrosis.[1]

In 1972, the first molecular role for Se was identified with the discovery that glutathione peroxidase-1 (Gpx1) contains stoichiometric quantities of Se.[8] Following this discovery, Gpx1 assays were used very effectively to establish selenium requirements for livestock and for humans. Interestingly, this has resulted in minimum Se requirements that are essentially identical for most animal species and close to 0.1 μg Se/g (reviewed in references[9,10]). Thus we conducted an initial study with female turkey poults fed an Se-deficient torula yeast diet supplemented with graded levels of Se for 28 days, and found that there was no effect of this Se-deficient diet on growth, but that 0.2 μg Se/g diet was required for plateau levels of liver Se. Liver glutathione peroxidase activity, determined using hydrogen peroxide (H_2O_2) as a substrate, required 0.2 μg Se/g to reach plateau levels, and plasma Gpx3 activity required 0.3 μg Se/g.[11] Recently, a study using corn-soy-based diet also found an Se requirement in male turkey poults of 0.2 μg Se/g to maximize liver Gpx1 activity and 0.3 μg Se/g to maximize plasma Gpx3 activity at 35 days.[12]

Phospholipid hydroperoxide Gpx (Gpx4) was discovered in mammals in 1985 as a second Se-dependent intracellular selenoperoxidase.[13] Gpx4 differs from Gpx1 because it is present as a monomer and because it will reduce bulky hydroperoxide substrates, such as phosphatidylcholine hydroperoxide (PCOOH), which are not substrates for Gpx1.[14] In rodents, Gpx4 activity is far more resistant to Se deficiency as compared with Gpx1 activity,[15] Se requirements based on Gpx4 activity are lower than those based on Gpx1 activity,[16,17] and Gpx4 mRNA levels in rodents are little affected by dietary Se deficiency as compared with Gpx1 mRNA.[16,18] Discovery of additional selenoproteins, such as selenoprotein P, deiodinase, thioredoxin reductase-1, selenoprotein W and Gpx3, provided further biomarkers of Se status that also indicate that the dietary Se requirements in rodents are 0.1 μg Se/g diet or less.[19–23] Thus these studies clearly suggest that minimum dietary Se requirements of turkey poults determined by biochemical biomarkers as well as by clinical (disease-based) biomarkers are considerably higher than for many other species.

The Se in all these selenoproteins is present as selenocysteine (Sec) incorporated in the peptide backbone. A UGA codon (normally a stop codon) in all selenoprotein mRNAs encodes the Sec, the Sec is synthesized from serine[24] and an activated form of Se while it is attached to a unique tRNA, and a unique stem–loop secondary structure, called an SECIS (Sec insertion sequence) must be present in the 3′UTR of the mRNA for Sec to be incorporated at the UGA position.[25,26] Using these molecular biology-based attributes, the complete selenoprotein proteome (selenoproteome) has been identified for humans, rodents and a number of other species.[26] The chicken selenoproteome consists of at least 23 selenoproteins,[27] but the number of selenoproteins in the turkey (*Meleagris gallopavo*) genome is unknown.

Our study with female turkey poults suggested that some of the apparent glutathione peroxidase activity in turkeys might be due to Gpx4,[11] and thus that higher Se requirements in turkeys might be due to this selenoenzyme. To study this hypothesis, we separated turkey Gpx4 from Gpx1 and assayed Gpx4 with different peroxide substrates to measure levels of each separate selenoenzyme activity, and then used these assays to evaluate the dietary Se requirement of male turkey poults. In addition, we isolated partial cDNA clones for turkey Gpx1 and Gpx4, and used these clones to evaluate the level of selenoprotein mRNA transcripts in Se-adequate and Se-deficient liver. These experiments clearly indicate that Gpx4 as well as Gpx1 activities and mRNA levels are highly regulated by Se status in turkeys.

Materials and methods

Reagents

Molecular biology reagents were purchased from Promega (Madison, WI, USA), Invitrogen (Carlsbad, CA, USA) or Sigma (St Louis, MO, USA). All other chemicals were of molecular biology or reagent grade.

Animals and diets

Experiment 1

Male one-day-old poults ($n = 35$, Cuddy Farms, Aurora, MO, USA) were housed in battery brooder cages (5 per pen) with raised wire floors with 24 h lighting in animal quarters in the Animal Sciences Research Center, following the care and treatment protocol approved by the Institutional Animal Care and Use Committee at the University of Missouri. The temperature was maintained at 95°F for the first week, at 90°F for the second week and at 85°F for subsequent weeks. Deionized water was provided in stainless steel troughs and diet in plastic troughs, both *ad libitum*. The basal Se-deficient torula yeast-based diet (0.007 μg Se/g, Supplemental Table 1) was the turkey diet we used previously[11] and contained 30% low-Se torula yeast plus an additional 6.4% crystalline amino acids including 0.93% L-methionine and 150 mg/kg of all-rac-alpha-tocopheryl acetate, to better match the NRC recommendations for protein and amino acids.[7] The poults were allocated randomly to treatment groups, and supplemented with graded levels of Se (0, 0.05, 0.1, 0.2, 0.3, 0.4 or 0.5 μg Se/g) as Na_2SeO_3 (5/treatment). Body weight was measured weekly, and the poults were killed at 27 days.

Experiment 2

Male one-day-old poults ($n = 6$, Con Agra, Neosho, MO, USA) were allocated randomly to two treatment groups, and fed either the Se-deficient (0.008 μg Se/g by analysis) or Se-adequate (0.3 μg Se/g supplemental Se as Na_2SeO_3) diets. The poults were killed at 28 days. All other analyses were conducted as described for experiment 1.

Tissue analysis

At the termination of the study, turkeys were anesthetized with ether and blood was collected in an ethylenediaminetetraacetic acid (EDTA)-coated syringe. Liver was perfused with ice-cold 0.15 mol/L KCl, and then liver, kidney, heart, gizzard, pectoral muscle and testes were collected, rinsed with KCl

and frozen at −80°C until analysis. As described previously,[16,17,21,28–31] whole blood and tissues were subsequently processed to determine tissue selenoenzyme activity and liver Se concentrations. Neutron activation analysis was used to determine liver and diet Se concentrations.[32]

Enzyme activity assays

Tissues were homogenized with a polytron in 9 vol of homogenization buffer (20 mmol/L tris/HCl, pH 7.4, 0.25 mol/L sucrose, 1.1 mmol/L EDTA and 0.1% peroxide-free Triton X100) and centrifuged (10,000 *g*, 15 min) as described previously.[16,29] Testes from each group were pooled and homogenized together. Gpx4 activity was measured by the coupled assay procedure[16] using 78 μmol/L PCOOH, the specific substrate. Gpx3 activity in the plasma (designated as Gpx3) was measured by the coupled assay procedure[33] using 120 μmol/L H_2O_2. Total Gpx activity in tissues was assayed using 120 μmol/L H_2O_2, and Gpx1 specific activity was calculated by subtracting the activity detected with H_2O_2 due to Gpx4 (using the factor of 0.63 EU_{H2O2}/EU_{PCOOH}, determined as described in the section Results) from the total Gpx activity. For all assays, 1 enzyme unit (EU) is the amount of enzyme that will oxidize one micromole of GSH per minute under these conditions. Protein concentration of each sample was determined by the method of Lowry *et al.*[34]

^{75}Se-labeling and chromatography of Gpx1 and Gpx4

A single one-day-old female turkey poult (Cuddy farms, Aurora MO, USA) was fed a standard Se-adequate corn-soybean meal diet (supplemented with 0.3 μg Se/g as Na_2SeO_3) for three weeks, injected intraperitoneally with 200 μCi ^{75}Se (~1000 μCi/μg Se) and killed 24 h later. The liver was perfused with ice-cold 0.15 mol/L KCl and frozen at −80°C.

For analysis, the liver was homogenized in 9 vol of homogenization buffer and centrifuged as described above for enzyme analysis. The resulting supernatant was then centrifuged (105,000 *g*, 60 min, Model L8-70M, rotor 70.1 Ti, Beckman Instruments, Palo Alto, CA, USA) and 8.8 mL of this supernatant was applied to a Sephadex G-150 column (2.6 × 96 cm) at 4°C and eluted with 0.05 mol/L potassium phosphate buffer, pH 7.0, containing 0.3 mol/L NaCl, 2.5 mmol/L EDTA and 0.5 mmol/L GSH as described previously.[24] Six milliliter fractions were collected and assayed for Gpx activity using both H_2O_2 and PCOOH as described above and for ^{75}Se (60% efficiency, Auto-Gamma 5650, Packard Instrument Co, Downers Grove, lL, USA). Cytochrome *c* (11.7 kDa), alpha-chymotrypsinogen (25.7 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68.0 kDa) and gamma-globulin (160.0 kDa) were used as molecular weight standards.

Cloning of turkey Gpx4 and Gpx1 cDNA

Total liver RNA from turkey (*M. gallopavo*, Nicholas white toms, Con Agra, Neosho, MO, USA) was isolated by homogenization in guanidine isothiocyanate buffer as described previously.[35] Total RNA was then used as a template for reverse transcription (RETROscript, Ambion, Austin, TX, USA) to prepare cDNA templates for polymerase chain reaction (PCR) amplification.[36] PCR products were cloned into pGEM-T (Promega), and sequenced at the University of Missouri DNA core. Internal and rapid amplification of cDNA ends (RACE) products from at least two independent reverse transcriptase-PCR (RT-PCR) reactions were cloned and then sequenced in both directions.

For Gpx1, primers were designed based on alignment of cDNA sequences for rat, human, bovine and rabbit nucleotide sequences to identify conserved regions. Partial cDNAs for turkey Gpx1 were cloned by 3′-RACE using primers p160 (5′ cctgcggggcaaggtg 3′ and p148 (5′ tctagacctcaggttttttttttttttttt 3′), followed by a second PCR reaction using primers p164 (5′ ctcggtttcccgtgcaatcag 3′) and p148. This resulted in a 638 bp product that was cloned into pGEM-T and sequenced.

For turkey Gpx4, primers were designed based on a 617 bp chicken Gpx4 EST (AI981938.1, GenBank, Bethesda, MD, USA). Primers p144 (5′ ggtgaattacactcagctcgtcg 3′) and reverse primer p146 (5′ acttagtgaagttccacttgatggcattcc 3′) amplified a 273 bp fragment, which in turn was used to design specific primers to be used for RACE analysis. 3′ RACE using p151 (5′ actcagctcgtcgatctgcacg 3′) and then p144 in combination with p148 amplified a 605 bp product that was homologous to the 3′-end of rat Gpx4. Additional overlapping segments were amplified using additional primers to resolve ambiguous sequence data. 5′-RACE (FirstChoice RLM-RACE, Ambion Inc, Austin, TX, USA) using primer p148 and reverse primers p146 and then p166 (5′ ctccttcagccacttccacagc 3′) amplified a 420 bp product that was cloned into pGEM-T and provided additional 5′ sequence information for turkey GPX4. This resulted in the 818 bp partial sequence for turkey Gpx4.

Ribonuclease protection analysis

Ribonuclease protection analysis (RPA) was conducted as previously described.[29,37] Livers from experiment 2 were homogenized in guanidine isothiocyanate buffer as described previously[35,37] to isolate total RNA for RPA. The 638-bp turkey Gpx1 clone and the 420-bp turkey Gpx4 clone were used as templates for synthesis of the antisense RPA probes, using the appropriate SP6 and T7 promoter in pGEM-T. For glyceraldehyde-3-phosphate dehydrogenase (Gapdh), a turkey Gapdh cDNA sequence (U94327.1, GenBank) was used to design primers p181 (5′ gctgagtatgttgtggagtccac 3′) and p182 (5′ tgccatccctccacagcttcc 3′) which produced a 325-bp cDNA fragment corresponding to nt 1–325 of the 703 nt sequence which was also cloned into pGEM-T. Antisense RPA probes for Gpx1, Gpx4 and Gapdh were produced as described previously[29,37] using 45, 25 and 7.5 μCi [alpha-^{32}P]UTP (3000 Ci/mmol, NEN, Boston, MA, USA), respectively, per reaction according to the manufacturer's protocol (Promega). Protected probe fragments were analyzed by direct imaging of the gel (InstantImager, Packard Instrument Company, Meriden, CT, USA), and were also visualized by autoradiography. For each RNA sample the Gpx1 and Gpx4 mRNA signals were normalized to the Gapdh mRNA signal.

Statistical analysis

Data are presented as means ± SEM; for experiment 1, $n = 5$/treatment; for experiment 2, $n = 3$/treatment. Data in experiment 1 were analyzed by one-way analysis of variance, and differences between means were assessed by Duncan's multiple range analysis ($P < 0.05$), with Kramer's modification for unequal class sizes.[38] When variance equality was significant, as tested by Bartlett's test (alpha = 0.05), significant differences between means were assessed instead by Scheffé's *F*-test. For experiment 2, the unpaired Student's *t*-test was used to compare two treatments. For all tests, $P < 0.05$ was considered significant. The plateau breakpoint for each Se response curve, defined as the intersection of the line tangent to the point of steepest slope and the plateau, was calculated as described previously[17,28,29] using sigmoidal or hyperbolic regression analysis (Sigma Plot, SPSS, Chicago, IL, USA) to estimate the minimum dietary Se necessary to obtain plateau responses.

Results

Gpx4 in turkey liver

Our previous study with female turkey poults suggested that a substantial amount of liver Gpx activity detected using H_2O_2 was due to Gpx4.[11] Thus we subjected liver supernatant from a ^{75}Se-labeled turkey poult to Sephadex G-150 chromatography using a high-salt buffer that we had previously shown would separate the ~80 kDa Gpx1 tetramer from the ~20 kDa Gpx4 monomer.[24] The resulting chromatogram showed four ^{75}Se peaks corresponding to a >200 kDa peak, an 80 kDa peak, an 18 kDa peak and a <5 kDa peak (Figure 1), similar to rat liver chromatograms.[39] Gpx assays with H_2O_2 revealed negligible H_2O_2 activity in the high-MW and low-MW peaks, but substantial H_2O_2 activity in both the 80 kDa Gpx1 peak and the 18 kDa peak; Gpx assays with PCOOH revealed that only the 18 kDa peak contained PCOOH activity and thus contained Gpx4. The five

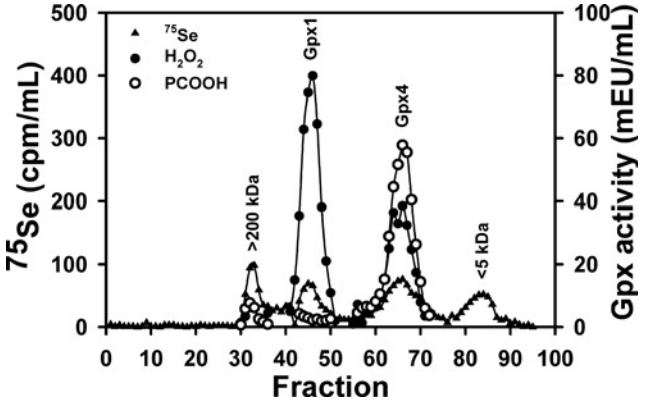

Figure 1 Analysis of ^{75}Se-labeling of proteins in turkey liver supernatant using gel filtration chromatography. Liver supernatant from a three-week-old Se-adequate (0.3 μg Se/g diet) female turkey, injected with 200 μCi [^{75}Se]selenite and killed 24 h later, was subjected to Sephadex G-150 chromatography as described previously.[24,39] Each 6-mL fraction was counted for ^{75}Se, and assayed for Gpx activity using H_2O_2 and PCOOH as described in the text. Chromatography resulted in four ^{75}Se peaks: >200 kDa; 80 kDa Gpx1; 18 kDa Gpx4; and <5 kDa

Gpx4 peak fractions with the highest PCOOH activity had 0.63 ± 0.09 EU when assayed with H_2O_2 for every 1.00 EU assayed with PCOOH. Thus Gpx4 activity determined using PCOOH, which is not a substrate for Gpx1, can be multiplied by this factor, 0.63 EU_{H2O2}/EU_{PCOOH}, to calculate the activity due to Gpx4 that contributes to the total H_2O_2 activity detected in a sample; the difference is activity specifically due to Gpx1. Figure 1 and this factor clearly show that turkey liver has substantial Gpx4 activity, and furthermore, that this level of Gpx4 in turkey liver is responsible for a substantial portion of the apparent 'Gpx1' activity detected when turkey liver is assayed with H_2O_2.

In experiment 1, the poults weighed initially 60.4 ± 1.1 g, and averaged 506 ± 13 g at the end of the study, with an average overall gain of 16.5 ± 0.5 g/day (Supplemental Table 2). There was no significant effect of dietary Se on growth until day 21; at day 27, poults fed the Se-deficient diet weighed 72% of poults fed 0.3 μg Se/g diet. Plotting of individual final weights resulted in a hyperbolic Se response curve with a plateau breakpoint or minimum dietary Se requirement for growth of 0.05 μg Se/g (Figure 2a). In addition, no gross signs of gizzard, heart or muscle myopathy were detected (data not shown).

Liver Se concentrations in poults fed the basal diet were 13% of levels in Se-adequate (0.3 μg Se/g diet) poults, showing that these turkeys were Se deficient (Figure 2b). Se supplementation resulted in a sigmoidal response in liver Se concentration, with a plateau breakpoint at 0.20 μg Se/g diet (Table 1), with liver Se continuing to increase after 0.2 μg Se/g at a rate one-third of the rate before 0.2 μg Se/g. Plasma Gpx3 activity in Se-deficient poults was 15% of levels in Se-adequate poults, and increased sigmoidally with increasing dietary Se to a plateau, with a plateau breakpoint at 0.19 μg Se/g (Figure 2c). Turkey plasma had negligible Gpx4 activity (data not shown).

Tissue Gpx1 and Gpx4 activities

Collected tissues were assayed for Gpx activity with H_2O_2 and PCOOH, and the activity due to Gpx1 and Gpx4 was calculated using the 0.63 EU_{H2O2}/EU_{PCOOH} factor. In Se-adequate poults (0.3 μg Se/g), kidney had the highest Gpx1 activity; heart and gizzard were the next highest with 34% of the activity in the kidney; liver, testes and muscle had 27%, 21% and 5% of the level in the kidney, respectively (Figure 3). For Gpx4 activity, liver had the highest Gpx4 activity, with heart, testes, kidney, gizzard and muscle having 39%, 33%, 26%, 11% and 9% of the level in the liver, respectively.

In Se-adequate turkey liver, chromatography and use of the 0.63 EU_{H2O2}/EU_{PCOOH} factor demonstrated that 47% of the total Gpx activity determined with H_2O_2 was due to Gpx4. These assays similarly found that 31%, 28%, 22%, 7% and 6% of total Gpx activity determined with H_2O_2 was due to Gpx4 in Se-adequate turkey muscle, testes, heart, gizzard and kidney, respectively.

In Se-deficient poults, kidney Gpx1, liver Gpx4 and heart Gpx1 activities all decreased to <10% of Se-adequate levels (Table 1); liver Se and activities of liver Gpx1 and heart

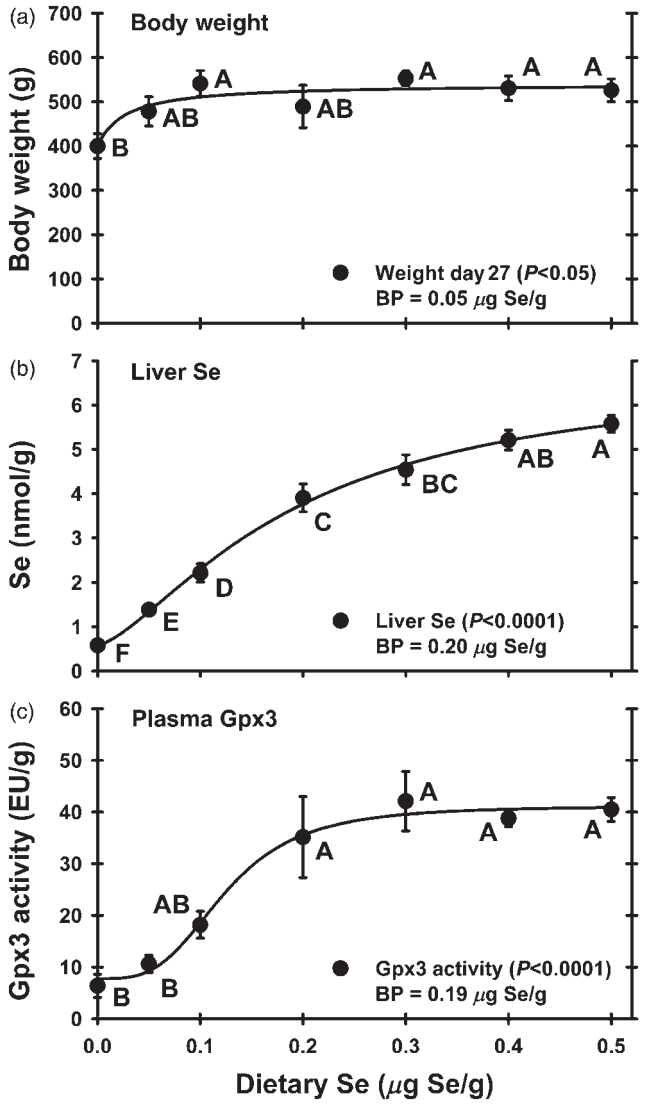

Figure 2 Effect of dietary Se on body weight, liver Se concentration and plasma Gpx3 activity. (a) Body weight at day 27; (b) liver Se concentration; and (c) plasma Gpx3 activity. Values are means ± SEM; $n = 5$/diet for all points except: $n = 4$ for 0 μg Se/g group for liver Se and plasma Gpx3. The level of significance by analysis of variance is indicated in each panel; values with a common letter are not significantly different ($P < 0.05$). The calculated plateau breakpoint (BP) for each Se response curve is also indicated

Gpx4 decreased to 10–20% of Se-adequate levels; activities of muscle Gpx4, muscle Gpx1, kidney Gpx4 and gizzard Gpx1 decreased to 20–30% of Se-adequate levels; activities of gizzard Gpx4 and testes Gpx1 deceased to 50–60% of Se adequate levels; and testes Gpx4 activity only deceased to 73% of Se-adequate levels. Thus there was a considerable range in impact of Se deficiency on Gpx4 and Gpx1 depending on tissue.

The liver had the highest level of Gpx4 activity in any tissue; it was very highly regulated by Se status, and had a plateau breakpoint at 0.29 μg Se/g (Figure 3a). Liver Gpx1 activity was also highly regulated by Se status with a breakpoint at 0.29 μg Se/g, but increased very little between 0 and 0.1 μg Se/g, in contrast to liver Gpx4. The relative levels of Gpx4 and Gpx1 activity in the kidney (Figure 3b) were the opposite of those in the liver, with

Table 1 Selenium requirement hierarchy in male turkey poults

Biomarker	Minimum requirement* (μg Se/g)	Extent of regulation†
Growth	0.05	Low ($P < 0.05$)
Testes Gpx4 activity	0.09	Low‡
Testes Gpx1 activity	0.10	Moderate‡
Heart Gpx4 activity	0.10	High ($P < 0.0001$)
Kidney Gpx4 activity	0.17	High ($P < 0.0001$)
Plasma Gpx3 activity	0.19	High ($P < 0.0001$)
Liver Se concentration	0.20	High ($P < 0.0001$)
Muscle Gpx4 activity	0.23	High ($P < 0.0001$)
Kidney Gpx1 activity	0.24	Very high ($P < 0.0001$)
Heart Gpx1 activity	0.24	Very high ($P < 0.0001$)
Muscle Gpx1 activity	0.25	High ($P < 0.0001$)
Gizzard Gpx4 activity	0.28	Moderate ($P < 0.0001$)
Liver Gpx1 activity	0.29	High ($P < 0.0001$)
Liver Gpx4 activity	0.29	Very high ($P < 0.0001$)
Gizzard Gpx1 activity	0.30	High ($P < 0.0001$)

*Minimum dietary Se requirement for the growing turkey poult as determined for each indicated biomarker. Requirements are the minimum dietary Se necessary for the indicated parameter to reach plateau levels when Se-adequate day-old poults are fed these diets for 27 days, as determined by breakpoint analysis as described in the text

†Susceptibility to Se regulation of the indicated biomarker in Se-deficient tissue in Se-deficient versus Se-adequate (0.30 μg Se/g diet) poults: very high = <10.9% of Se-adequate; high = 11–40.9% of Se-adequate; moderate = 41–70% of Se-adequate; and low = >70% of Se-adequate. These ranges of regulation are the same as applied in previous studies with rodents.[30,31,41] Significance (P value) of regulation is indicated in parentheses

‡P values not determined, as assays for testes conducted on pooled samples

high levels of Gpx1 activity and low levels of Gpx4 activity; the breakpoints for kidney Gpx1 and Gpx4 were 0.24 and 0.17 μg Se/g, respectively. In heart (Figure 3c), the level of dietary Se required to reach the plateau was decidedly different for Gpx1 and Gpx4 activities, with breakpoints of 0.24 for Gpx1 and 0.10 for Gpx4. Gizzard Gpx1 and Gpx4 activity Se response curves (Figure 3d) were similar to those in the kidney, with much higher levels of Gpx1 than Gpx4 activities in Se-adequate poults, and with breakpoints of 0.30 and 0.28 μg Se/g, respectively. Muscle Gpx1 and Gpx4 activities were the lowest of any tissues examined in this study; plateau breakpoints were 0.25 and 0.23 μg Se/g for Gpx 1 and Gpx4 activities, respectively, although muscle Gpx1 activity did not reach a clear plateau in the present study (Figure 3e). Testes Gpx1 and Gpx4 activities both reached clear plateaus with breakpoints at 0.10 and 0.09 μg Se/g, respectively (Figure 3f), with the nominal level of testes Gpx1 activity twice that of Gpx4 activity.

Cloning of turkey Gpx4 and Gpx1 cDNA

To begin to understand why Gpx4 as well as Gpx1 activities decrease dramatically in Se-deficient turkey liver, we isolated partial cDNA clones for these turkey selenoproteins. As shown in Supplemental Figure 1, a 638 nt cDNA sequence (GQ502186, GenBank) was assembled for the 3′ region of turkey Gpx1, corresponding to nt 300–921 of human Gpx1 var 1. The resulting sequence has greater than 60% sequence identity with corresponding portions of human, rat and mouse Gpx1 and 92% sequence identity

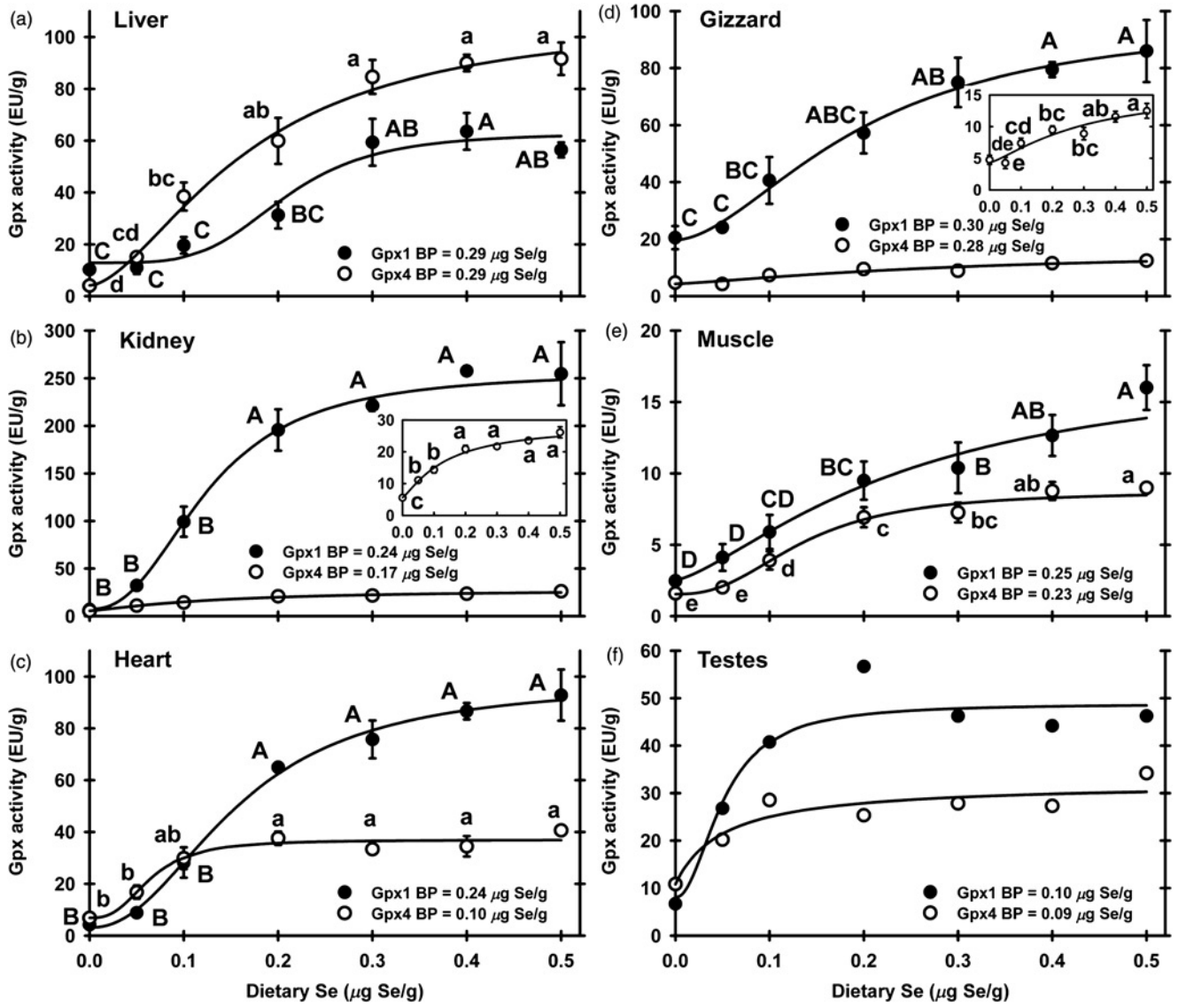

Figure 3 Effect of dietary Se on tissue Gpx1 and Gpx4 activities. (a) Liver; (b) kidney; (c) heart; (d) gizzard; (e) pectoral muscle and (f) testes. Insets in panels (b) and (d) show Gpx4 activities. Values are means ± SEM; $n = 5$/diet for all points except: liver Gpx1, $n = 4$ for 0 and 0.1 μg Se/g groups; liver Gpx4, $n = 4$ for 0 μg Se/g group; kidney Gpx1, $n = 4$ for 0, 0.1 and 0.2 μg Se/g groups; kidney Gpx4, $n = 4$ for 0 and 0.3 μg Se/g group; heart Gpx1 and Gpx4, $n = 4$ for 0 μg Se/g groups; gizzard Gpx1, $n = 4$ for 0, 0.1 and 0.4 μg Se/g groups; gizzard Gpx4, $n = 4$ for 0 μg Se/g group; due to size, testes were pooled for homogenization. $P < 0.0001$ for all enzyme activities by analysis of variance; values with a common letter are not significantly different ($P < 0.05$). The calculated plateau breakpoint (BP) for each Se response curve is also indicated

with chicken Gpx1. Furthermore, the 3′UTR encodes an apparent SECIS element that aligns with the human, rodent and chicken SECIS elements. Translation of the coding region results in a 133 residue sequence aligning with residues 74–203 of human Gpx1p as well as 72–201 of rodent Gpx1p and 64–195 of chicken Gpx1p, with 98% amino acid sequence identity with chicken Gpx1p and 72–75% sequence identity with human and rodent Gpx1p (Supplemental Figure 2a).

For Gpx4, an 818 nt cDNA sequence (GQ502187, GenBank) was assembled for turkey Gpx4, corresponding to nt 135–942 of human Gpx4 var 2 (Supplemental Figure 3). The resulting sequence has greater than 65% sequence identity with corresponding portions of human, rat and mouse Gpx4, and 93% sequence identity with chicken Gpx4. The 3′UTR encodes an apparent SECIS element that aligns with the human, rodent and chicken SECIS elements. Translation of the coding region results in a 188-residue sequence aligning with residues 10–197 of human and rodent Gpx4p and with the 180-residue chicken Gpx4p sequence. Alignment of the protein sequence shows 94% amino acid sequence identity with chicken Gpx4p and 73% sequence identity with human and rodent Gpx4p (Supplemental Figure 2b).

Se regulation of Gpx4 and Gpx1 mRNA levels

In experiment 2, plasma Gpx3, liver Gpx1 and liver Gpx4 activities in Se-deficient poults were 2%, 3% and 6%, respectively, of the activities in Se-adequate poults,

showing that these poults were Se deficient (data not shown); RPA readily detected Gpx1 and Gpx4 as well as Gapdh mRNA in total RNA isolated from turkey liver (Figure 4). Visual inspection clearly showed that both Gpx1 and Gpx4 mRNA were regulated by dietary Se. Counting of bound ^{32}P-probe indicated that Gpx1 mRNA levels in Se-deficient turkey liver decreased to $37 \pm 1\%$ of levels in poults fed 0.3 μg Se/g diet, and Gpx4 mRNA also similarly dropped in Se-deficient liver to $36 \pm 1\%$ of Se-adequate levels. Gapdh mRNA levels were not regulated by dietary Se status ($P = 0.42$, data not shown).

Discussion

Sephadex G-150 chromatography of turkey liver supernatant showed that a substantial amount of the Gpx activity detected with H_2O_2 was due to Gpx4. Using the determined ratio of 0.63 EU_{H2O2}/EU_{PCOOH}, we found that Se-adequate turkey liver had 59.4 EU/g protein of Gpx1 activity and 84.6 EU/g of Gpx4 activity, as compared with levels in rat liver of 800 and 6.6 EU/g for Gpx1 and Gpx4, respectively,[16] using the same assays. Clearly, the relative levels of these two intracellular Gpxs are different in turkey versus rodent liver; this also demonstrates why use of H_2O_2 in the Gpx1 assay of rodent liver (with a ratio of 121:1 for Gpx1:Gpx4 EU/g) detects negligible Gpx4 activity with H_2O_2, but why this assay with turkey liver (with a ratio of 0.70:1 Gpx1:Gpx4 EU/g) detects substantial activity due to Gpx4 and thus overestimates the level of Gpx1 in turkey tissues.

The levels of selenoenzyme activities found in these turkey poults indicate that turkeys have 7%, 53%, 18% and 13% of the Gpx1 levels in the liver, kidney, heart and muscle, respectively, found in rat liver,[16,40] and yet have 12×, 3× and 7× of the Gpx4 levels found in the liver, kidney and heart, respectively, of rats.[16,40] In contrast, turkey testes Gpx1 activity is 1.2× the Gpx1 level in rat testes, but Gpx4 activity is 15% of the Gpx4 level in rat testes. Thus, with the exception of testes, turkeys appear to have higher Gpx4 and lower Gpx1 expression as compared with rodents. The value of this distribution of Gpx activity is unclear, but it does suggest that there was value in collective retention of Gpx activity as relative levels of Gpx family members evolved during the divergence of the avian and mammalian genes.

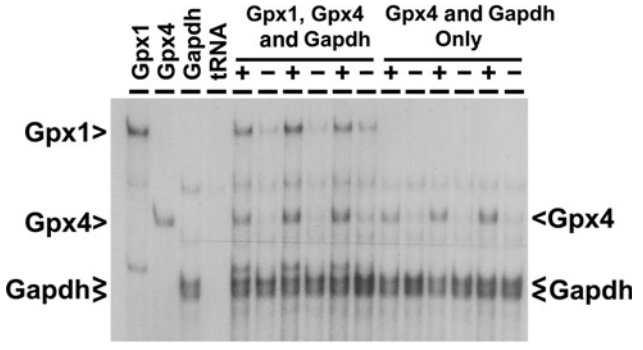

Figure 4 Ribonuclease protection assay (RPA) autoradiogram of Gpx1 and Gpx4 mRNA in the liver. Total RNA (10 μg) isolated from turkey poults fed Se-deficient (−) or Se-adequate (+) diets ($n = 3$/diet) for 28 days, was analyzed for Gpx1, Gpx4 and Gapdh. An Se-adequate RNA sample was hybridized individually with 2× concentration of a single probe (lanes 1–3), and yeast tRNA (lane 4) was analyzed with the probe mixture as a negative control. One set of samples (lanes 5–10) was analyzed with all three probes, and one set (lanes 11–16) was analyzed only with the Gpx4 and Gapdh probes to eliminate smaller fragments protected by the Gpx1 probe

Gpx1 has no activity with the peroxide substrate PCOOH because this bulky substrate cannot enter the constrained active site of this tetrameric enzyme.[14] In contrast, monomeric Gpx4 from rodents has apparent second-order rate constants of 1.9×10^5 and 7.0×10^5 for H_2O_2 and PCOOH, respectively,[14] suggesting under comparable assay conditions, rodent Gpx4 assayed with H_2O_2 should have 27% of the activity determined with PCOOH. To directly determine the Gpx4 and Gpx1 activities in rat testes, we previously purified rat testes Gpx4 and found a ratio of 0.26 ± 0.02 for rat testes Gpx4.[41] The ratio of 0.63 ± 0.09 determined in the present study for turkey liver Gpx4 suggests that the H_2O_2 rate constant for turkey Gpx4, relative to the PCOOH rate constant, is higher versus the constants reported for rodent Gpx4. This higher ratio for the turkey selenoenzymes thus further increases the overestimation of Gpx1 activity in turkey tissues when only H_2O_2 is used as the peroxide substrate.

In these studies with day-old male turkey poults, the minimum dietary Se requirement based on growth was 0.05 μg Se/g. In our previous study with female turkey poults, we found that there was no effect of this Se-deficient diet on growth,[11] suggesting that the slower growth of female poults may result in a lower Se requirement, although a higher initial Se status of the day-old poults could also have contributed to the lack of growth defect in the female poult study. Very recently, Fischer *et al.*[12] conducted a similar study with male poults of a strain representative of today's rapidly growing bird, and with an Se-deficient practical diet based on low-Se soybean meal, wheat and maize; they found no effect of the Se-deficient diet through 14 days, but found significant growth depression by 35 days; reduced growth was prevented by 0.1 μg Se/g diet (as selenate) or higher levels of dietary Se, but supplementation with 0.05 μg Se/g was not included in those studies. Thus with today's genetic stock, the minimum dietary requirement for growth may be higher than the 0.05 μg Se/g found in the present study, at least for male poults.

Supplementation of male poults with graded levels of dietary Se resulted in sigmoidal or hyperbolic increases in tissue selenoenzyme activities up to plateau levels. Breakpoint analyses of these Se-response curves determined that the minimum dietary Se requirements were 0.28–0.30 μg Se/g diet based on Gpx4 and Gpx1 activities in gizzard and liver (Table 1, Figure 3). Thus the current NRC dietary Se requirement of 0.20 μg Se/g for turkeys[7] is clearly not sufficient to maintain gizzard and liver Gpx4 and Gpx1 activities. Slightly lower minimum requirements of 0.17–0.25 μg Se/g were determined in the present studies based on Gpx1 and Gpx4 in kidney and muscle and based on heart Gpx1 activity, plasma Gpx3 activity and liver Se concentration. Similarly, Fischer *et al.*[12] found Se requirements of 0.28–0.3 based on Gpx1 at 14 days,

Gpx3 at 35 days, and liver, plasma and gizzard Se at 35 days, and they found Se requirements of 0.2–0.25 μg Se/g based on plasma Gpx3 at 14 days, liver Gpx1 at 35 days and breast muscle Se at 35 days. Note that these liver Gpx1-based requirements were determined with H_2O_2 alone, and thus represent a requirement for Gpx1 and Gpx4 combined. Collectively, our studies and the studies of Fischer *et al.*[12] show that the minimum dietary Se requirement of the turkey is considerably higher than that determined by similar approaches for rodents[16,17,21,28,31] and most other species,[9,10] and clearly show that the Se requirement at least for the rapidly growing turkey poult is much higher than the current NRC requirement.[7]

Se deficiency in turkeys can result in myopathies of the gizzard, heart and muscle.[3,4] In tissues in the present study, Gpx4 activities fell to 53%, 20% and 22%, respectively, of Se-adequate levels; tissue Gpx1 activities fell to 27%, 6% and 24%, respectively, of Se-adequate levels. In comparison, Gpx1 and Gpx4 in Se-deficient rat liver, the first-affected organ in the rat,[1] decreased to 2% and 34%, respectively,[31] and these levels can fall even lower to 1% and 23%, respectively, in second-generation Se-deficient rats that are otherwise supplemented with sulfur amino acids and vitamin E to prevent impaired growth and liver necrosis.[42] Examination of these relative decreases in Gpx1 and Gpx4 activity, or the nominal levels of Gpx1 and Gpx4 activities in Se-deficient tissues, however, does not suggest either selenoenzyme alone or together as the causative agent(s) responsible for the onset of disease. For instance, gizzard Gpx4 and Gpx1 activities in these Se-deficient poults are much higher than liver levels found in Se-deficient rats.[31,42] Thus it continues to appear that a number of Se-dependent antioxidant proteins with overlapping functions serve to defend these tissues against pro-oxidant species and disease.[9,10] Relative levels of other, non-Se-dependent protective systems are also likely to be contributing factors that determine which organ is first affected as Se deficiency develops.

Lastly, the cDNA sequences reported here for turkey Gpx1 and Gpx4 indicate that the sequence homology at the nucleotide level and at the amino acid level is highly conserved for these selenoproteins, including putative SECIS elements encoded in the 3′UTRs of these mRNAs. Unexpectedly, we found that in Se deficiency, both Gpx4 as well as Gpx1 mRNA levels decrease in Se-deficient turkey liver. This is in contrast to what occurs in rats, where Gpx1 mRNA in liver is very highly regulated, often decreasing to 5–20% of Se-adequate levels but where Gpx4 mRNA level is not regulated by Se status.[16,29,37] In recent rodent studies on Se regulation of transcript levels for the complete selenoproteome, we found that Se deficiency decreases five of the 24 rodent liver selenoproteins to <40% of Se-adequate levels, but that rodent Gpx4 mRNA in the same tissues is not significantly regulated by Se status.[30,31] Thus it appears that Se-adequate turkeys have both higher levels of Gpx4 mRNA and lower levels of Gpx1 mRNA relative to rodents, and that Gpx4 mRNA as well as Gpx1 mRNA levels are regulated by Se status in turkeys. These underlying differences in selenoprotein molecular biology may explain the elevated dietary Se requirements of the turkey.

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