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The gastrointestinal microbiota affects the selenium status and selenoprotein expression in mice☆

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Abstract

Colonization of germ-free (GF) mice has been shown to induce the gastrointestinal form of the selenium-dependent glutathione peroxidases, GPx2. Since bacterial colonization of the gastrointestinal tract is associated with stress, we aimed to clarify how bacteria affect selenoprotein expression in unstressed conditions. GF and conventional (CV) FVB/NHan^{TMHsd} mice were fed a selenium-poor (0.086 ppm) or a selenium-adequate (0.15 ppm) diet for 5 weeks starting from weaning. Each group consisted of five animals. Specific glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) expression was measured in plasma, liver and intestinal sections by activity, protein and mRNA level as appropriate. Under selenium-adequate conditions, selenoprotein expression did not differ in GF and CV mice. Under selenium-limiting conditions, however, GF mice generally contained higher GPx and TrxR activities in the intestine and liver, higher GPx1 protein and RNA levels in the liver, higher GPx2 protein levels in the proximal and distal jejunum and colon and higher GPx1 and GPx2 RNA levels in the colon. In addition, higher selenium concentrations were estimated in plasma, liver and cecum. All differences were significant. It is concluded that bacteria may compete with the host for selenium when availability becomes limiting. A variable association with different microorganisms might influence the daily requirement of mice for selenium. Whether the microbiota also affects the human selenoprotein status appears worthy of investigation.
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1. Introduction

The selenoprotein GPx2, the gastrointestinal variant of the glutathione peroxidases (GPxs), is transcriptionally regulated by the Keap1/Nrf2 system [1,2] and, thus, belongs to a complex enzymatic system that is generally considered to protect against inflammatory stimuli and carcinogens [3]. The gene encoding the selenoprotein thioredoxin reductase-1 (TrxR1) is another target of Nrf2 [4,5], whereas a direct link of the remaining 23 mammalian selenoprotein genes to the Keap1/Nrf2 system is not obvious. Epidemiological (reviewed in Ref. [6]) and clinical [7] evidence supports a role of the trace element selenium in preventing carcinogenesis. It therefore appears conceivable that selenium contributes to chemoprevention by supporting the biosynthesis of Nrf2-dependent selenoproteins.

An anticarcinogenic action of GPx2 is supported by inverse genetics. Although an unchallenged knockout of *gpx2* did not yield any obvious phenotype, *gpx2*$^{-/-}$ mice were predisposed to UV-induced squamous cell carcinoma formation [8]. A knockout of both *gpx1* and *gpx2* yielded mice that developed an acute inflammation in the colon and distal ileum at the age of 27–70 days [9] and tumors at the age of 5–9 months [10]. A single allele of *gpx2* was sufficient to prevent the phenotype [9,11]. Interestingly, the phenotype was only observed in conventionally grown animals, whereas their germ-free (GF) counterparts remained asymptomatic for up to 12 months [9]. Colonization of double heterozygous (*gpx1*$^{+/-}$*gpx2*$^{+/-}$) mice with nonpatho-

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genic bacteria led to the induction of GPx2, likely as part of the adaptive response to the bacterial challenge [9]. Also, the up-regulation of GPx2 during colorectal carcinogenesis [12,13] and in Barrett's esophagus [14] has therefore been considered to indicate an anticarcinogenic reaction rather than a manifestation of malignant transformation [15].

Expression of TrxR in the intestinal system has been less well investigated. In particular, the influence of intestinal bacteria on TrxR expression or activity is unknown. Like GPx2, TrxR1 is induced by activators of Nrf2, suggesting a protective role for this selenoprotein as well. However, also, TrxR1 expression is elevated in cancer cell lines [16] and in human gastrointestinal cancer tissue [16,17]. This might reflect the need for an enhanced TrxR-dependent synthesis of deoxyribonucleotides in cancer cells [18,19] but has also been viewed as a regulatory response to counteract malignant transformation since TrxR keeps the tumor suppressor p53 in a functional, that is, reduced state [20]. In this way, TrxR contributes to the p53-mediated induction of either antioxidant enzymes under mild oxidative stress or pro-oxidant, that is, pro-apoptotic, enzymes, under severe oxidative stress [21].

If GPx2 and TrxR are indeed pivotal components of the adaptive system, their activation would require both a suitable stimulus and an adequate selenium supply [22]. We, therefore, intended to clarify how the selenium status affects the selenoprotein biosynthesis in CV and GF mice. In contrast to the presumed increase of GPx2 expression by bacterial colonization, which was expected to be further enhanced by selenium supplementation, a hitherto overlooked phenomenon was observed: The gastrointestinal microbiota appeared to compete for the limited selenium supply and, in consequence, to impair selenoprotein synthesis in a selenium-poor status.

2. Materials and methods

2.1. Animals and feeding protocol

All animal studies were approved by the corresponding ministry of Brandenburg. Male conventional (CV) FVB/NHan^{TMHsd} (FVB/N) mice (3 weeks of age) were obtained from Harlan Winkelmann (Borchen, Germany) and were randomly assigned to one of the experimental diets. Mice were housed under SPF conditions with a 12-h light–dark cycle. GF FVB/N mice were delivered by cesarean section and nursed by GF foster mothers. The mice were maintained in Trexler-type isolators (Metall & Plastic, Radolfzell, Germany) with a sterile water supply at 22±2°C, a relative humidity of 55±5% and a 12-h light–dark cycle (0600/0600 h). Diets were sterilized by gamma radiation (minimum, 25 kGy). The GF status was checked weekly by Gram staining of fecal smears on a glass slide followed by microscopic control and incubation of fecal material in both thioglycollate and Wilkins–Chalgren broth (Oxoid, Basingstoke, UK).

2.2. Diets

The selenium-deficient diet C1045 was obtained from Altromin (Lage, Germany). It is based on the principle of a selenium-deficient diet first described by Burk [23]. It consists of 30% torula yeast, 57% sucrose, 6.7% corn oil, a vitamin mix and a selenium-free mineral mix. According to our analyses, the selenium content of the diet was 0.086 mg/kg (see below). The selenium-adequate diet was produced by mixing selenomethionine (Acros, Geel, Belgium) into the selenium-deficient diet to yield a final selenium content of 0.15 mg/kg. Diets were fed as powder for a period of 5 weeks starting from weaning. Thus, at the end of the experiment, animals were 8 weeks old. All animals had free access to food and drinking water.

2.3. Sample preparation

Anesthetized animals were sacrificed by cervical dislocation. Intestinal contents were thoroughly removed. Tissues were freeze-clamped in liquid nitrogen and stored at −80°C until further analysis. Blood was collected by retroorbital puncture with heparinized capillaries. Plasma was obtained after centrifugation of the blood for 10 min at 3000×*g* and 4°C and was stored at −80°C. For Western blotting and measurement of enzyme activities, tissues were extensively ground in liquid nitrogen. Tissue powder (20–30 mg) was suspended in 500 µl homogenization buffer (100 mmol/L Tris–HCl, 300 mmol/L KCl and 0.1% Triton X-100, pH 7.6) containing 4 µl of protease inhibitor cocktail (Calbiochem, Bad Soden, Germany). Homogenization was achieved with a tissue lyser (Qiagen, Hilden, Germany) for 2×2 min at 30 Hz. Cellular debris were removed by centrifugation at 20,800×*g* for 15 min at 4°C. Protein content was assessed according to Bradford [24].

2.4 GPx and TrxR activity

For estimation of enzyme activities, tissue samples were diluted 100-fold (liver), 50-fold (plasma) and 10-fold (intestinal sections) with homogenization buffer. GPx activity was measured in the glutathione reductase-coupled test [25]. In brief, 890 µl assay reagent consisting of 100 mmol/L Tris, 5 mmol/L EDTA, 1 mmol/L NaN_3, 3 mmol/L GSH, 0.1 mmol/L NADPH, 0.1% Triton X-100 and 600 mU glutathione reductase (Sigma, Taufkirchen, Germany) was added to 100 µl sample and incubated for 10 min at 37°C. The reaction was started by addition of H_2O_2 (10 µl; final concentration, 50 µmol/L). The consumption of NADPH (ε=6.3 $(mmol/L)^{-1} \times cm^{-1}$) was monitored for 5 min at 340 nm. Consumption of 1 µmol NADPH/min was taken as 1 U. GPx levels were expressed as milliunits per milligram of protein.

TrxR activity was estimated according to Gromer et al. [26] with slight modifications. Briefly, 100 µl of the diluted tissue lysate was mixed with 820 µl TrxR reaction buffer (100 mmol/L potassium phosphate and 2 mmol/L EDTA, pH 7.4) and 30 µl DTNB (100 mmol/L in DMSO). The reaction was started with 50 µl 4 mmol/L NADPH in TrxR

reaction buffer. For subtraction of TrxR-independent conversion of DTNB to 2-nitro-5-thiobenzoate (TNB), samples were also measured without NADPH. One enzyme unit is defined as the NADPH-dependent production of 2 μmol of TNB 13.6 $(mmol/L)^{-1} \times cm^{-1}$ per minute.

2.5. Western blots

SDS-PAGE and Western blotting were performed as previously described [27]. Briefly, 30 μg protein per lane was loaded to a 12.5% polyacrylamide gel and the electrophoresis was run with 15 mA in 25 mmol/L Tris buffer, containing 192 mmol/L glycine and 3.4 mmol/L SDS, pH 8.5. SDS gels were blotted to nitrocellulose (2 h, 1.2 mA/cm^2, 4°C). Prior to antibody addition, blots were blocked in 5% nonfat dry milk in Tris-buffered saline (50 mmol/L Tris and 150 mmol/L NaCl, pH 7.5, containing 0.1% Tween 20) for 1 h at room temperature. Primary antibodies were rabbit anti-GPx2 [28], sheep anti-GPx1 (Abcam, Cambridge, UK) and rabbit anti-β-actin (ab 8227, Abcam). Staining conditions were 4°C overnight (GPx2) and 2 h at room temperature (GPx1 and β-actin). Secondary antibodies (1 h at room temperature) were peroxidase-conjugated anti-rabbit or anti-sheep IgG, respectively (Chemicon, Hofheim, Germany). After staining of GPx1 or GPx2, blots were treated for β-actin staining with Restore Western Blot Stripping buffer (Perbio Science Deutschland GmbH, Bonn, Germany). Detection was achieved in a Fuji LAS3000-CCD system with SuperSignal West Dura (Perbio) as substrate. Protein bands were quantified with the Aida/2D Densitometry 4.0 software (Raytest, Straubenhardt, Germany). GPx signals were normalized to β-actin.

2.6. RNA isolation and quantitative real-time PCR

Tissue was grounded thoroughly under liquid nitrogen. Tissue powder (20–30 mg) was suspended in 800 μl of cold Trizol (Invitrogen, Karlsruhe, Germany). Homogenization was achieved with a tissue lyser (Qiagen) for 2×2 min at 30 Hz. RNA was isolated following the Trizol protocol according to the manufacturer's instructions. Afterwards, genomic DNA was digested with 10 U RQ1 DNase (Promega, Mannheim, Germany) and RNA was cleaned up with a phenol–chloroform extraction. RNA concentrations were measured with a Nanodrop 1000 (Peqlab Biotechnologie GmbH, Erlangen, Germany).

RNA (3 μg) was reversely transcribed with 150 fmol oligo(dT)15 primers and 180 U Moloney murine leukemia virus reverse transcriptase (Promega) in a total volume of 45 μl. For real-time PCR, cDNA was diluted 10-fold. Real-time PCRs (Mx3005P QPCR System, Stratagene, Amsterdam, the Netherlands) were performed in triplicate with 1 μl of the diluted cDNA in 25 μl reaction mixtures using SYBR Green I (Molecular Probes, Eugene, OR) as fluorescent reporter. The annealing temperature was 60°C for all PCR reactions. PCR products were relatively quantified with a standard curve ranging from 1×10^4 to 1×10^9 copies of each amplicon. Primers were designed to be specific for cDNA by placing at least one primer onto an exon/intron boundary with PerlPrimer v1.1.14 (http://perlprimer.sourceforge.net). For primer sequences, see Table 1. For normalization, the mean of the reference genes Hprt and Rpl13a was calculated and used as normalization factor [29].

2.7. Selenium content in plasma, diet and tissues

The selenium content in biological materials was measured fluorimetrically as originally described [30] and modified [31]. Thirty to forty milligrams of tissue powder, 70 mg of the respective diet or 30 μl of plasma was diluted with 100 μl of water and digested with 500 μl acid mixture ($HNO_3/HClO_4$, 4:1, v/v) for 30 min at 100°C, 30 min at 120°C and 120 min at 190°C in a Thermostat VLM 4.0 (VLM GmbH, Leopoldshöhe, Germany). During the first two heating steps, vials were opened twice to allow gases to evaporate. After cooling to room temperature overnight, 500 μl HCl was added and the sample was heated for 15 min at 90°C and for 45 min at 150°C. Thereafter, samples were cooled to room temperature and 2 ml of EDTA (2.5 mmol/L, pH 8.0) and 500 μl of 6.3 mM 2,3-diaminonaphthalene in 0.1 mol/L HCl were added. After incubation for 30 min at 55°C, samples were extracted with 3 ml of cyclohexane. Fluorescence was measured after 24 h using an AB2 luminescence spectrometer (SLM-AMINCO) (346 nm excitation, 520 nm emission). For quantification, a standard curve with selenium

Table 1
Primer sequences (5′→3′)

Gene	Accession number	Primer sequence	Product
Hprt1	NM_013556	Forward: GCAGTCCCAGCGTCGTG Reverse: GGCCTCCCATCTCCTTCAT	168 bp
Ribosomal protein L13a	NM_009438	Forward: GTTCGGCTGAAGCCTACCAG Reverse: TTCCGTAACCTCAAGATCTGCT	157 bp
GPx1	NM_008160	Forward: GAAGAGATTCTGAATTCCCTCAA Reverse: CACACCAGGAGAATGGCAAGA	256 bp
GPx2	NM_030677	Forward: GTGCTGATTGAGAATGTGGC Reverse: AGGATGCTCGTTCTGCCCA	252 bp
Trspap1	NM_027925.3	Forward: AGCACCATGCAGACATATGAAGAG Reverse: GTGACAGTCCATCAGAGCATCGT	134 bp

standard solutions for atomic absorption spectroscopy (Sigma-Aldrich, Steinheim, Germany) with concentrations between 0 and 450 μg/L was measured in parallel.

2.8. Statistics

Group differences were tested by two-way ANOVA followed by Bonferroni's posttest. Student's *t* test was used to compare differences between group means. The methods used are indicated in figure legends. Each group consisted of five animals.

3. Results

3.1. GPx expression in plasma and liver of GF animals exceeds that of CV animals when selenium supply becomes limiting

Plasma GPx activity represents the activity of the extracellular form (GPx3). It did not differ between CV and GF mice under 'selenium adequacy' (Fig. 1A). Under limited selenium supply, however, plasma GPx activity appeared to be higher in GF animals albeit not significantly.

Also, in the liver, GPx activities were generally higher in the selenium-adequate state than under selenium restriction and even higher in GF animals (Fig. 1B). Under selenium-limiting conditions, a drastically enhanced GPx activity was observed in GF animals compared to CV animals (Fig. 1B).

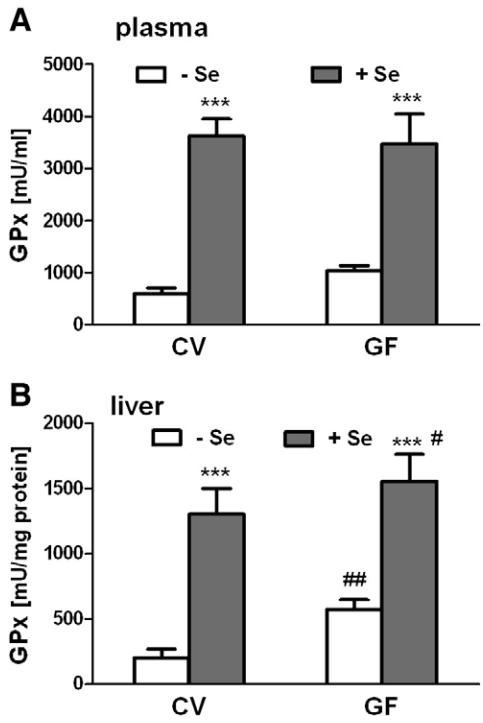

Fig. 1. GPx activity in plasma (A) and liver (B) of selenium-poor (white columns) and selenium-adequate (gray columns) CV and GF mice. Values are means±S.D. (n=5). Significance was analyzed with two-way ANOVA and Bonferroni's posttest. ***P<.001 versus the respective selenium-poor group; $^{\#}P$<.05 versus the selenium-adequate CV group; $^{\#\#}P$<.01 versus the selenium-poor CV group. For further details, see Materials and Methods.

Although total GPx activity was measured in the liver, it might mainly represent GPx1 activity. Selenoproteins are synthesized according to a certain hierarchy, which means that some disappear fast when selenium supply becomes limiting and some remain stable even in pronounced deficiency (reviewed in Ref. [32]). Hierarchy is based on the degree of degradation of mRNA when selenium becomes limited. Due to its low ranking in the hierarchy of selenoproteins, GPx1 is the GPx type that responds most rapidly to changes in selenium availability. GPx4, which is also present in the liver, ranks high in the hierarchy and, thus, is not expected to decline markedly under the moderate selenium-deficient conditions created in this study. GPx2 is reported to be absent from mouse liver [33]. Therefore, changes in GPx activity measured here in the liver essentially represent changes in GPx1.

Western blots confirmed the marginal to absent expression of GPx2 in mouse liver (not shown). GPx1 protein levels, however, perfectly reflect GPx activities: generally higher amounts in mice fed the selenium-adequate diet (Fig. 2A) and significantly higher levels in GF than in CV mice fed the selenium-poor diet (Fig. 2B and C). Also, GPx1 mRNA was higher in GF animals, indicating a higher stability (Fig. 2D).

3.2. GPx2 expression is elevated in the intestine of GF compared to CV mice under restricted selenium supply

3.2.1. Activities

The unexpected higher hepatic GPx activity as well as protein and mRNA levels of GPx1 in GF animals under selenium-limiting conditions led us to test whether the phenomenon could also be observed in other tissues, namely, in the intestinal tract. GPx activities were higher under selenium-adequate than under selenium-limiting conditions in all intestinal sections investigated. Maximum activities were not different between the GF and the CV group (Table 2). However, as in plasma and liver, intestinal GPx activity was higher in selenium-poor GF than in selenium-poor CV animals. This difference was significant in the proximal jejunum (Fig. 3A), cecum (Fig. 3D) and colon (Fig. 3E), whereas the difference in the distal jejunum (Fig. 3B) and ileum (Fig. 3C) was rather marginal.

3.2.2. Protein levels

Western blots revealed a low level of GPx1 protein in all intestinal sections (not shown), indicating that under the experimental conditions used, the low-ranking GPx1 was possibly not optimally synthesized, even under selenium adequacy. Similar conclusions can be drawn from a recent publication [34] showing a reasonable GPx1 expression in mice intestine only when fed a diet containing 2 mg selenium per kilogram in the form of selenite. The diet fed here delivered a substantially lower amount of selenium (0.15 mg/kg) in the form of selenomethionine, which is

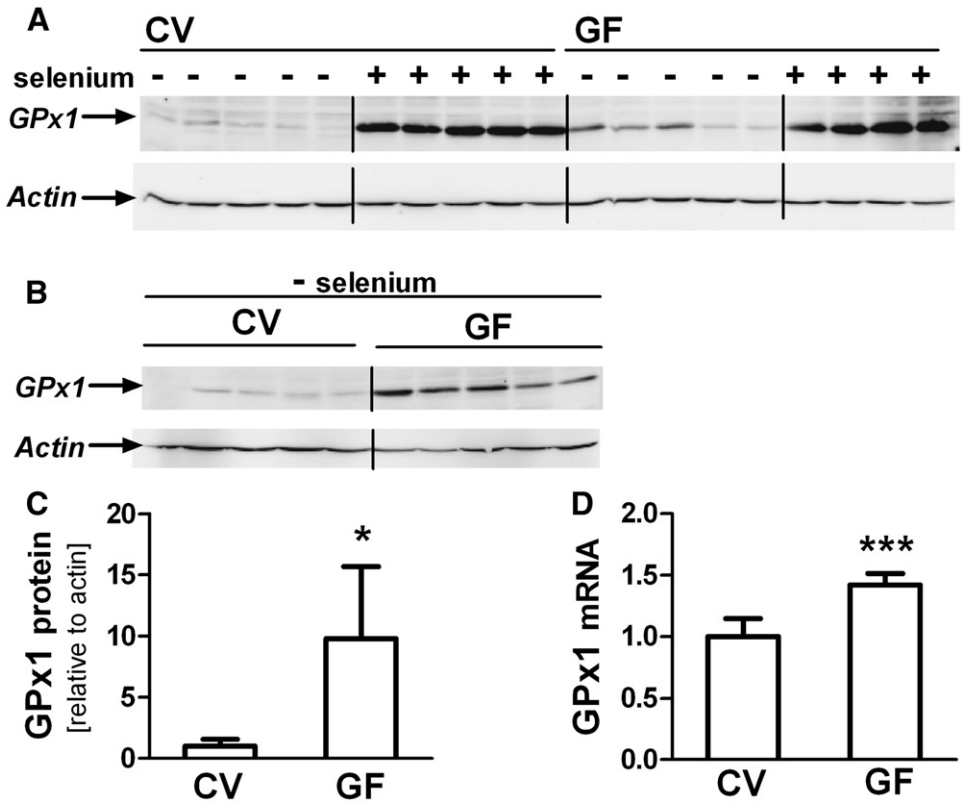

Fig. 2. GPx1 expression in livers of individual animals of each group as measured by Western blotting (A–C) and real-time PCR (D). (A) GPx1 in CV and GF mice fed the Se-poor (−) or the Se-adequate (+) diet. (B) For quantification of the differences in GPx1 expression between CV and GF mice, separate blots were run with samples from Se-poor CV and GF mice and bands made visible by a longer exposition time. (C) Densitometric quantification of the individual bands from Panel B normalized to β-actin. Presented blots are representative of three independent analyses. (D) GPx1 mRNA levels in Se-poor animals as measured by qPCR. mRNA of CV mice was set to 1, and the level of GF mice was calculated in relation to CV. Significance was analyzed by Student's *t* test. **P*<.05 versus CV.

less efficiently used for selenoprotein biosynthesis than selenite. In contrast to GPx1, GPx2 was distinctly expressed. Protein levels of GPx2 in selenium-poor GF were higher than that in CV mice (Fig. 4A). A densitometric quantitation revealed a 3.2-, 15.9-, 2.6-, 3.8- and 3.4-fold expression in the proximal jejunum, distal jejunum, ileum, cecum and colon, respectively. These differences were significant for the proximal jejunum and colon and nearly significant for the distal jejunum (*P*=.074) and the ileum (*P*=.098). With the exception of the distal jejunum, the GPx2 protein levels reflect total GPx activities in the intestinal sections. Therefore, the activity appears to mainly result from GPx2.

GPx activities were generally higher in the liver than in the intestine (compare Figs. 1 and 3 and Table 2), which could probably be due to the expression of different GPx forms in both organs. Since there is no possibility of discriminating between GPx1 and GPx2 activities and since the protein levels of both GPxs had to be measured with different antibodies, the data do not allow any conclusion as to the contribution of the individual GPxs to the total activity in both organs. It can, however, be stated with certainty that GPx2 protein levels are higher in the intestine than in the liver, supporting the postulated role of GPx2 in the maintenance of the proliferative state of intestinal progeny cells [35,36].

3.2.3. RNA levels

To test whether mRNA levels were also affected by the gastrointestinal microbiota in selenium deficiency, we quantified GPx1 and GPx2 RNA via real-time PCR. The colon as a section with the most striking difference in GPx2 expression was chosen for analysis. Although hardly detected at the protein level, GPx1 mRNA was present. The amounts, however, usually were eightfold lower than that for GPx2 (not shown). Nevertheless, a trend towards a higher amount in GF animals under selenium limitation was

Table 2
GPx activities (mU/mg protein) in the intestine of selenium-adequate GF and CV mice

Intestinal section	GF animals	CV animals
Proximal jejunum	168±32.4	212±79.3
Distal jejunum	113±21.8	116±25.5
Ileum	128±16.3	152±21.8
Cecum	162±18.6	145±16.1
Colon	208±20.0	205±20.7

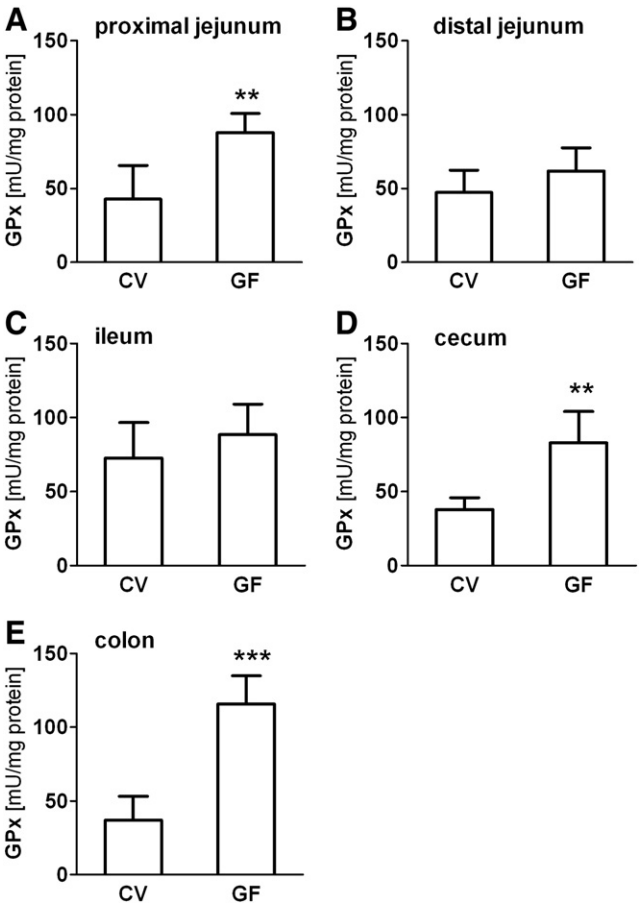

Fig. 3. GPx activity in the proximal jejunum (A), distal jejunum (B), ileum (C), cecum (D) and colon (E) of selenium-deficient CV and GF mice. Values are means±S.D. (n=5). Significance was analyzed using Student's t test. **P<.01, ***P<.001 versus CV animals.

observed for GPx1, whereas the higher level of GPx2 was clearly significant (Fig. 4B).

3.3. TrxR activity under restricted selenium supply is higher in the liver and intestine of GF than that of CV animals

As observed for GPx, TrxR activity was significantly higher under selenium adequacy than under selenium restriction in the liver and all intestinal sections (Table 3, Fig. 5) and did not differ between GF and CV animals.

TrxR activity, which, due to the lack of specific substrates for TrxR1 and TrxR2, represents the total activity, was higher in all intestinal sections than in the liver as observed earlier [34]. As described above for GPx, TrxR activity was higher in selenium-deficient GF than in CV animals (Fig. 5A–F). Due to the lack of a specific antibody for TrxR forms, we were not able to differentiate between TrxR1 and TrxR2. However, TrxR activity correlated with TrxR1 protein levels in the liver and colon from wild-type FVB/N mice (the same strain used here) under different selenium states [34] and TrxR1 RNA was the prominent form in the intestine of C57BL/6 mice [37]. Therefore, it can be assumed that the activities reported here mainly represent TrxR1. TrxR, thus, is another selenoprotein, the level of which decreases to a smaller extent in GF than in CV mice when the selenium supply becomes limited.

3.4. Selenium concentration in plasma, liver and cecum

Despite the identical selenium content in the diet fed to CV and GF animals, the levels of the investigated selenoproteins in GF mice fed the selenium-poor diet generally exceeded those in CV mice. It was, therefore, tested whether the enhanced selenoprotein synthesis in GF animals was reflected by the tissue selenium levels. In accordance with the selenoprotein expression, the selenium content was similar in selenium-adequate mice in plasma and liver (Fig. 6). The selenium content in the cecum was lower in GF mice most probably due to the lack of bacteria. The cecum harbors a large number of bacteria, which might have incorporated a substantial part of ingested selenium, thereby not contributing to selenoprotein synthesis of the host (see Fig. 4A). In animals fed the selenium-poor diet, the selenium content was higher in GF mice, which correlates with the expression of investigated selenoproteins (Fig. 6).

4. Discussion

The study was based on the observation that colonization of previously GF mice with intestinal bacteria induced GPx2 [9]. Up-regulation was explained as a reaction to the stress situation caused by the sudden contact with bacteria and as an indication for an anti-inflammatory function of GPx2. Bacterial colonization of the gastrointestinal tract starts at birth, and a stable bacterial community develops within 3 weeks [38,39]. During this time, GPx2 expression increases with a peak around 5 weeks of age and then declines and stabilizes at a lower level [9]. The transient up-regulation of GPx2 might be considered as response to the inflammatory phase during colonization. Unfortunately, the time course of GPx2 expression has never been monitored in GF animals, and therefore, it cannot be decided whether GPx2 expression is a stress response to bacterial colonization or whether GPx2 represents a marker for mucosa maturation. In the present study, 8-week-old GF mice were compared with mice of the same age associated with bacteria from birth onwards. Thus, a sudden recolonization after a period of life without intestinal bacteria did not take place and the normally colonized mice can be considered stress free at the time of investigation. Therefore, the findings are to be discussed in the context of the response of the host and the microbiota to the selenium supply rather than in the context of an induction of GPx2 — or other selenoproteins — by a sudden colonization with the gastrointestinal microbiota accompanied by an inflammatory response.

Under selenium adequacy, GPx2 as well as TrxR expression in liver and throughout the intestine was similar

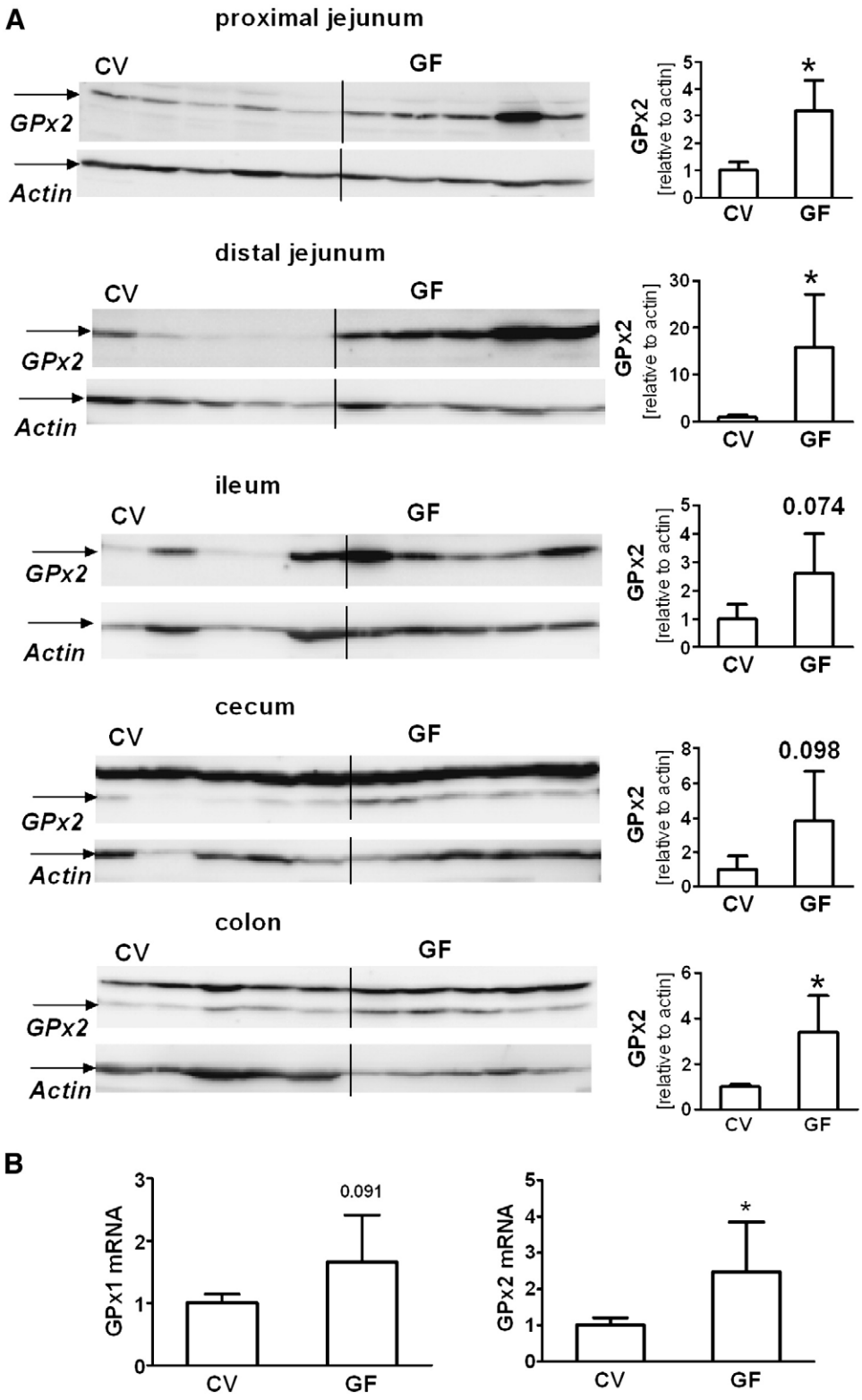

Fig. 4. GPx2 protein and GPx1 and GPx2 RNA levels in the indicated intestinal areas of selenium-deficient CV and GF mice. (A) GPx2 protein levels of individual selenium-deficient mice as measured by Western blotting. Diagrams represent the densitometric quantification of the individual bands normalized to β-actin. *$P<.05$ versus CV animals calculated by Student's t test. In samples of the large intestine (cecum and colon), a distinct band is present above the GPx2-specific one. This band usually shows up, does not respond to selenium and is even present in the cecum and colon of GPx2$^{-/-}$ mice (unpublished observation). Thus, the band is unspecific and not related to GPx2. (B) GPx1 and GPx2 mRNA in the colon of Se-poor CV and GF mice. mRNA was measured by qPCR, and the level of GF mice was expressed in relation to CV mice, which was set to 1.

Table 3
TrxR activities (mU/mg protein) in the liver and intestinal sections of selenium-adequate GF and CV mice

Tissue	GF animals	CV animals
Liver	9.0±1.21	7.8±1.15
Proximal jejunum	17.4±2.77	15.4±1.24
Distal jejunum	15.8±0.85	15.8±1.01
Ileum	15.7±0.40	15.5±1.80
Cecum	12.4±1.25	11.1±0.78
Colon	14.8±1.00	12.2±1.64

in GF and CV mice. As usual, selenoprotein activity was lower under selenium-limiting conditions. What was unexpected, at least regarding GPx2, was the generally lower activity in CV mice compared to GF mice, which, to our knowledge, has never been described before. Activity, protein and mRNA levels correlated with the plasma selenium status, indicating that the selenium supply to tissues was lower in CV animals.

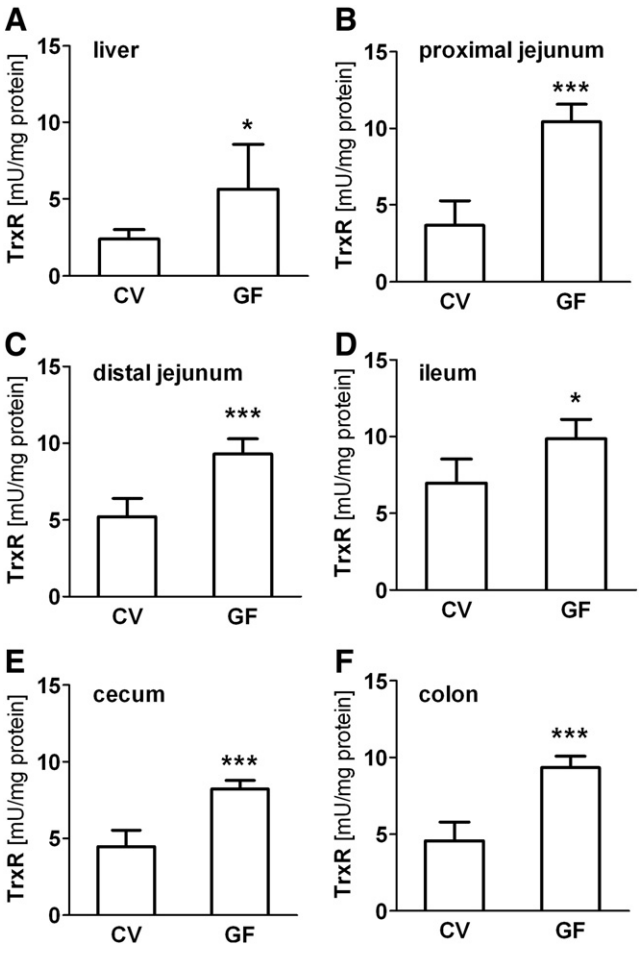

Fig. 5. TrxR activities in the liver (A), proximal jejunum (B), distal jejunum (C), ileum (D), cecum (E) and colon (F) of selenium-deficient CV and GF mice. Values are means±S.D. (n=5). *P<.05, ***P<.001 versus CV animals as analyzed by the t test. For further details, see Materials and Methods.

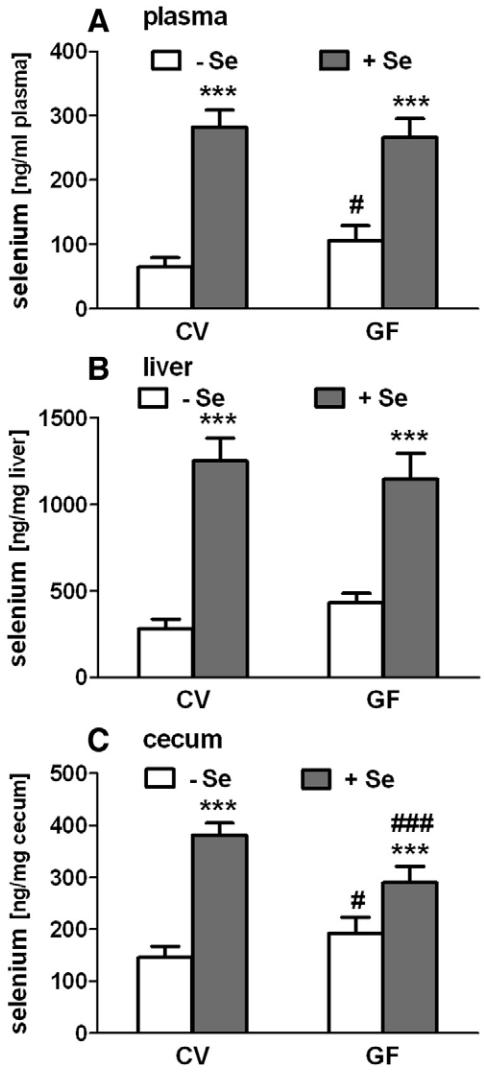

Fig. 6. Selenium concentration in plasma (A), liver (B) and cecum (C) of CV and GF mice fed a selenium-poor (white columns) or a selenium-adequate (gray columns) diet. Values are means±S.D. (n=5). ***P<.001 versus the respective selenium-poor group; $^{\#}P$<.05 and $^{\#\#\#}P$<.001 versus the respective CV group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's posttest.

There are several possibilities to explain these unexpected observations.

4.1. Change in intestinal homeostasis

Bacteria influence the renewal rate of the intestinal mucosa. The time needed for enterocytes to migrate from the crypts to the tips of the villi is twofold longer in GF than in CV animals [40]. In addition, the number of proliferative cells in GF mice is reduced by approximately one third. Altogether, enterocytes of GF animals exist in a mature state for a longer period of time than those of CV animals, which might be reflected by a higher selenoprotein expression. While this might explain the higher levels of selenoproteins in the intestine of GF animals, it does not easily explain higher levels in the liver or plasma.

4.2. Change in intestinal digestion and absorption

The motility of the gastrointestinal tract between CV and GF animals differs [41]. Gastric emptying and speed of transit through the small intestine are slower in GF mice [42], resulting in a more efficient absorption of carbohydrates such as glucose and xylose in GF mice [43]. However, whether bacteria influence the absorption of selenium compounds has never been investigated. Also unclear is the mechanism of selenium absorption, which certainly differs depending on the actual compound. Selenomethionine and selenocysteine appear to be taken up like their corresponding sulfur amino acids. Selenate is absorbed in a carrier-mediated process, which is not inhibited by selenite, indicating different carriers for these molecules. Other dietary selenium compounds have not been investigated in this respect [44]. Whether the microbiota affects the uptake of selenium compounds is not known. In GF animals, however, pancreatic enzymes have a longer survival time since they are not inactivated by bacteria. Accordingly, higher levels of free amino acids are found in the fecal and cecal content of GF rats [45]. Selenoproteins are only present in food of animal origin. The diet used here was selenium deficient due to replacement of casein by torula yeast. The selenium-adequate diet was obtained by adding the required amount of selenium in the form of selenomethionine. Thus, a more efficient proteolysis of putative dietary selenoproteins as selenium source for the high level of selenoproteins here observed in tissues of GF mice does not seem likely.

4.3. Competition between microbiota and the host for selenium

For us, the most plausible explanation for the observed phenomenon is a competition between bacteria and the host for the available selenium. The few reports dealing with this topic are in support of this assumption. Intestinal bacteria, at least in rats, incorporated 40–46% of an orally given dose of selenium in their cells [46]. Although this high amount was obtained by feeding 2 ppm selenium in the form of selenomethionine, which is more than 10 times the dosage fed here, it shows that bacteria do incorporate dietary selenium. Further support for the competition hypothesis comes from a study in which differential gene expressions in colonic epithelial cells of GF mice and previously GF mice after bacterial reconstitution were analyzed by microarrays [47]. Among the genes down-regulated in response to bacterial reconstitution, the one encoding selenoprotein P ranked fifth. Since selenoprotein P is responsible for delivering selenium to organs and tissues [31], the decrease in this selenoprotein will further reduce the selenium supply for the host. Another example of such an element, wherein micronutrient bacteria may compete with the host, is zinc. Under limited zinc supply, zinc contents in plasma and bone were higher in GF than in colonized rats [48]. By increasing the zinc content in the diet, the differences disappeared. These early findings exactly reflect what was observed here with selenium.

A number of bacterial species synthesize selenium-containing proteins. These can be divided into two groups: first, selenoproteins containing selenocysteine like in eukaryotes, and second, proteins containing labile selenium. Synthesis of the first group is dependent on the functional pathway for cotranslational incorporation of selenocysteine into the growing peptide chain. A bioinformatic approach identified the entire selenoprotein sets in all completed bacterial and archaeal genomes [49]. In archaea, formate dehydrogenase, coenzyme F420-reducing hydrogenase subunits and selenophosphate synthetase have been detected in the genera *Methanococcus* and *Methanopyrus* [50,51]. In eubacteria, formate dehydrogenase was found to be present in 37 bacterial genomes; selenophosphate synthetase in 17, for example, in *Actinobacillus pleuropneumoniae*; glycine reductase complexes in 17, for example, in *Clostridium* species; and a selenium-dependent peroxiredoxin in, for example, *Eubacterium acidaminophilum* [51]. Formate dehydrogenases and glycine reductase are present in a large number of bacterial species colonizing the gastrointestinal tract of humans and animals. Examples are *Escherichia coli* and *Clostridium* and *Enterobacterium* species. Since the number of bacterial genome sequences is still limited, it can be expected that the number of selenium-containing enzymes is considerably higher.

The second group of bacterial selenium-containing proteins can have selenium bound to molybdenum, nickel or tungsten. A prototype is nicotinic acid hydroxylase of *Clostridium barkeri* in which selenium is coordinated to a molybdopterin cytosine dinucleotide.[52] The same type of coordination has been found in the xanthine dehydrogenase [53]. Via coordination, selenium can be incorporated into proteins avoiding the complex and energy-consuming mechanism of selenoprotein biosynthesis. In this way, a higher amount of selenium might become protein bound in bacteria than in tissues of the host.

5. Conclusions

Obtained data indicate that GF animals have a lower selenium requirement for selenoprotein biosynthesis than conventionally colonized animals. This conclusion is based on the generally higher expression of the tested selenoproteins in GF compared to CV animals fed the selenium-poor diet and from the tissue selenium levels that correlate with enzyme activities. The data may further indicate that colonized animals have a higher risk of developing a deficiency when the selenium supply becomes limited.

Although the host and the intestinal microbiota mutually benefit from their symbiotic relationship, they might become competitors when the supply of micronutrients becomes limited. Intestinal bacteria might withdraw selenium from the host, which results in a two- to threefold lower level of host selenoproteins under selenium-limiting conditions.

Whether this has unfavorable consequences for humans and animals or whether the daily intake of selenium is adapted also to the requirements of commensal bacteria remains to be investigated. In view of the propagated high intake of probiotics, the selenium metabolism in these organisms should be investigated to respond with a higher intake of selenium if necessary.

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