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Research paper

# Epigallocatechin-3-gallate enhances key enzymatic activities of hepatic thioredoxin and glutathione systems in selenium-optimal mice but activates hepatic Nrf2 responses in selenium-deficient mice

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ABSTRACT

Selenium participates in the antioxidant defense mainly through a class of selenoproteins, including thioredoxin reductase. Epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active catechin in green tea. Depending upon the dose and biological systems, EGCG may function either as an antioxidant or as an inducer of antioxidant defense via its pro-oxidant action or other unidentified mechanisms. By manipulating the selenium status, the present study investigated the interactions of EGCG with antioxidant defense systems including the thioredoxin system comprising of thioredoxin and thioredoxin reductase, the glutathione system comprising of glutathione and glutathione reductase coupled with glutaredoxin, and the Nrf2 system. In selenium-optimal mice, EGCG increased hepatic activities of thioredoxin reductase, glutathione reductase and glutaredoxin. These effects of EGCG appeared to be not due to overt pro-oxidant action because melatonin, a powerful antioxidant, did not influence the increase. However, in selenium-deficient mice, with low basal levels of thioredoxin reductase 1, the same dose of EGCG did not elevate the above-mentioned enzymes; intriguingly EGCG in turn activated hepatic Nrf2 response, leading to increased heme oxygenase 1 and NAD(P)H:quinone oxidoreductase 1 protein levels and thioredoxin activity. Overall, the present work reveals that EGCG is a robust inducer of the Nrf2 system only in selenium-deficient conditions. Under normal physiological conditions, in selenium-optimal mice, thioredoxin and glutathione systems serve as the first line defense systems against the stress induced by high doses of EGCG, sparing the activation of the Nrf2 system.

## 1. Introduction

Green tea is manufactured from the leaves of *Camellia sinensis* and is a beverage consumed worldwide. It is characterized by the presence of large quantities of catechins. Of these catechins, epigallocatechin-3-gallate (EGCG) constitutes for about 50–75% by weight and is considered to be the most biologically active catechin [1]. EGCG has been reported to possess preventive activities against heart diseases, diabetes and cancer [1]. EGCG acts as an antioxidant or as a pro-oxidant depending upon the dosage and the cellular redox state [2]. The antioxidant functions of EGCG are associated with its quenching effect on reactive oxygen species (ROS) and reactive nitrogen species

*Abbreviations:* ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSO, buthionine sulfoximine; CDNB, 1-chloro-2, 4-dinitrobenzene; ECL, enhanced chemiluminescense; EDTA, ethylene diamine tetraacetic acid; EGCG, epigallocatechin-3-gallate; FoxO, Forkhead box class O; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; γ-H2AX, phosphorylated histone 2AX; 4-HNE, 4-hydroxynonenal; HO1, heme oxygenase 1; $H_2O_2$, hydrogen peroxide; IL-2, interleukin-2; IL-6, interleukin-6; IL-10, interleukin-10; Keap1, kelch-like ECH-associated protein 1; NADHP, nicotinamide-adenine dinucleotide phosphate; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PBS, phosphate buffer solution; Prx, peroxiredoxin; PVDF, polyvinylidene fluoride; qPCR, quantitative polymerase chain reaction; QQQ-MS/MS, triple quadrupole mass spectrometer; RIPA, tissue fluid of fast pyrolysis; RNS, reactive nitrogen species; ROS, reactive oxygen species; Rps6, ribosomal protein S6; RT, room temperature; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; Se, selenium; Sec, selenocysteine; SelP, selenoprotein P; SOD, superoxide dismutase; Sp1/Sp3, specificity protein 1/3; TBS-T, tris-buffered saline with 0.05% Tween 20; Trx, thioredoxin; TrxR, thioredoxin reductase; UPLC, ultra-high performance liquid chromatography

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(RNS) [3]. Alternatively, due to auto-oxidation of the highly active hydroxyl groups and concomitant formation of superoxide anion and hydrogen peroxide [4], EGCG may further modulate antioxidant defense by activating nuclear factor erythroid 2-related factor 2 (Nrf2) that initiates transcription of a series of cytoprotective genes [5,6].

The essential trace element selenium (Se) participates in the antioxidant defense mainly through a class of selenoproteins, which contain selenocysteine (Sec) that is co-translationally incorporated into the growing polypeptide from Sec-tRNA$^{[Ser]Sec}$ provided that the termination codon, UGA, is followed by a Sec insertion sequence (SECIS) element [7]. In humans, 25 selenoproteins have been found and of which glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) are well studied. GPx acts as an antioxidant enzyme by reducing hydroperoxides and hydrogen peroxide using glutathione (GSH) as a reducing agent [8]. TrxR catalyzes the reduction of the disulfide at the active site of thioredoxin (Trx) to maintain cellular redox homeostasis. Trx has a plethora of functions including ROS neutralization and activation of certain redox-sensitive transcription factors [9]. Inadequate Se intake that occurs in many regions of the world reduces selenoprotein biosynthesis and disturbs redox homeostasis [10,11]. Notably, selenoprotein biosynthesis was pronouncedly impaired in the mice fed with Se-free diet for 16 or 22 weeks. In such conditions, the Nrf2 defense pathway was activated, leading to increased expression of heme oxygenase 1 (HO1) and NAD(P)H: quinone oxidoreductase 1 (NQO1) [12]. The response of different selenoproteins to Se deficiency follows a strict hierarchy and some selenoproteins are synthesized at the expense of others. For example, low-ranking GPx1 and selenoprotein P (SelP) are sensitive to Se deficiency, whereas high-ranking TrxR1 is more refractory to Se deficiency [13]. Interestingly, knockout of GPx1 or SelP did not induce hepatic HO1 activity whereas the inhibition of TrxR by aurothioglucose or 1-chloro-2, 4-dinitrobenzene upregulated hepatic HO1 activity in mice [14–16]. These results suggest the importance of TrxR1 in regulating Nrf2 system. TrxR1 reduces the disulfide bonds to free cysteine residuals in Kelch-like ECH-associated protein 1 (Keap1) and keeps them in the reduced state, which arrests Nrf2 in the cytoplasm [17]. Inactivation of TrxR1 leads to disulfide bond formation in Keap1, and consequently Nrf2 is released and enters into the nucleus to drive the transcription of many cytoprotective genes [17]. On the other hand, TrxR1 is also an Nrf2 target gene. Such an intricate interaction between TrxR1 and Nrf2 suggests that TrxR1 is an important regulator of Nrf2 activation [17]. In addition, as a highly conserved housekeeping gene, the core promoter of TrxR1 can also be regulated by other transcription factors unrelated to Nrf2, such as octamer binding protein or specificity proteins 1 and 3 (Sp1/Sp3) [18].

We and others previously showed that Se and dietary phytochemical sulforaphane could synergistically induce TrxR1 through Nrf2 pathway in vitro [19–21]. This suggests that the interaction of dietary constituents could significantly alter the cellular redox traits. Currently, three major antioxidant defense systems have been well characterized: the Trx system comprising of NADPH, Trx and TrxR coupled with peroxiredoxin (Prx); the GSH system comprising of NADPH, GSH, and glutathione reductase (GR) coupled with glutaredoxin (Grx); and the Nrf2 system. In the present study, we investigated the interactions among these antioxidant defense systems after administration of a nontoxic, high-dose of EGCG to mice using Se nutritional status as a perturbator. We demonstrate that, under physiological conditions, preceding the activation of the Nrf2 system, the Trx and GSH systems serve as the first line defense against EGCG-induced stress in Se-optimal mice and that EGCG is a potent inducer of the Nrf2 system in Se-deficient mice.

## 2. Materials and methods

### 2.1. Chemicals and drugs

EGCG ( > 99%) purified from green tea was obtained from Ebeikar Tea & Extracts Co., Ltd. (Hangzhou, China). Melatonin ( > 98%), GR (from *Escherichia coli*), rat TrxR1, β-D-glucuronidase and sulfatase were all obtained from Sigma (St. Louis. MO, USA). ELISA kits for interleukin-2, 6 and 10 (IL-2, IL-6 and IL-10) and 4-hydroxynonenal (4-HNE) were purchased from BD Biosciences (San Jose, CA, USA) and CUSABIO (Wuhan, China), respectively. ECL Plus reagent and PVDF membrane were purchased from Bio-Rad Laboratories, Inc. (USA). The primary antibodies against GPx1/2, Nrf2, HO1, NQO1, phosphorylated histone 2AX (γ-H2AX) and anti-mouse secondary antibody as well as anti-rabbit secondary antibodies were obtained from Santa Cruz (Dallas, TX, USA). The primary antibodies against Histone H3 and β-actin were purchased from Sigma. The primary antibodies against p38 and P-p38 were purchased from Cell Signal Technology, Inc. (USA). TrxR1 primary antibody was kindly provided by Dr. Gary F. Merrill (Department of Biochemistry and Biophysics, Oregon State University). Other chemicals were of the highest grade available.

### 2.2. Animals and treatments

All protocols involving animal experiments complied with the guidelines of the Anhui Agricultural University Committee for the Care and Use of Laboratory Animals. Male Kunming mice (17–19 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (China). The mice were housed in plastic cages with controlled temperature (25 ± 1 °C), humidity (50 ± 10%) and 12 h light/dark cycles. The mice were allowed access to food and water ad libitum. Paired Se-optimal diet (0.18 mg Se/kg) and Se-deficient diet ( < 0.01 mg Se/kg) were provided by Trophic Animal Feed High-Tech Co., Ltd. (Nantong, China). After the mice were fed for 5 weeks, 6 mice were randomly selected from Se-optimal or Se-deficiency group, and were intraperitoneally (i.p.) injected with 70 mg/kg EGCG once daily for 3 consecutive days and then observed for an additional 12 days. The remaining fed mice after 6 weeks were i.p. injected with saline or 45 mg/kg EGCG once daily for 7 consecutive days, and then all the mice were sacrificed 24 h after the last treatment.

Additional animal experiment was designed to investigate the influence of melatonin on EGCG-meditated antioxidant response in Se-optimal mice. Male Kunming mice (20–22 g) were fed with Se-optimal diet and were i.p. injected with saline as control, 45 mg/kg EGCG, 50 mg/kg melatonin or the combination of EGCG and melatonin once daily for 5 consecutive days, and then all mice were sacrificed 24 h after the last treatment.

To determine EGCG concentrations following a single dose of EGCG administration, male Kunming mice (20–22 g) fed with Se-optimal diet were i.p. injected with EGCG at the dose of 45 mg/kg once and were sacrificed at 0.5, 1, 3 and 24 h, respectively. Mice in the control group were sacrificed at 3 h.

### 2.3. Biomarker assessments

Serum was centrifuged at 9,000*g* at 4 °C for 10 min. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). Serum levels of IL-2, IL-6, IL-10 and 4-HNE were determined using ELISA kits indicated above.

Liver tissues were excised and homogenized in ice-cold 0.15 mM, pH 7.2 phosphate buffer solution (PBS) containing 1 mM EDTA. The homogenate was obtained by centrifugation (15,000*g* at 4 °C for 15 min). Protein levels were determined by the Bradford dye-binding assay with bovine serum albumin as the standard.

Both GPx and TrxR activities were determined using the Smith and Levander method with some modifications [22,23]. For measuring GPx activity, a stock mixture composed of 6.5 mM EDTA-$Na_2$, 1.3 mM $NaN_3$, 2.5 mM GSH, 0.5 mM NADPH and 1.7 U/mL GR in 65 mM PBS (pH 7.4) was freshly prepared and kept at 37 °C. Samples (47 μL) were mixed with the stock mixture (250 μL) and 3 μL hydrogen peroxide ($H_2O_2$) as a GPx substrate at a final concentration of 250 μM. The changes in absorbance at 340 nm over time were monitored by a microplate reader at 37 °C. GPx activity was calculated in terms of μmols of NADPH oxidized/min/mL serum or mg protein. For measuring TrxR activity, a stock mixture composed of 10 mM EDTA-$Na_2$, 5 mM dithio-bis-nitrobenzoic acid, 240 μM NADPH and 0.2 mg/mL bovine serum albumin in 100 mM PBS (pH 7.0) was freshly prepared and kept at 37 °C. Then paired samples whose TrxR activities are active or inhibited by auranofin were prepared as follows: 54 μL sample was mixed with 6 μL 5% ethanol or 6 μL 5% ethanol containing 1.47 mM auranofin, at 37 °C for 10 min, respectively. Reaction was started by mixing 250 μL the stock solution with 50 μL sample, and the changes in absorbance at 412 nm over time were monitored by a microplate reader at 37 °C. TrxR activity was calculated by subtracting the slope rate of the reaction in the presence of auranofin from the slope rate of the reaction in the absence of auranofin. The consumption of one molecule of NADPH reduces of one molecule of dithio-bis-nitrobenzoic acid and generates two thio-bis-nitrobenzoic acid anions, which have an extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$ at 412 nm. TrxR activity was thus presented as μmols of NADPH oxidized/min/mg protein.

Se content was assessed using a 2, 3-diaminonaphthalene-based fluorescent method [24,25]. Glutathione S-transferase (GST) activity was assessed using 1-chloro-2, 4-dinitrobenzene (CDNB) and GST activity was calculated as nmol CDNB conjugate formed/min/mg protein [26]. Trx activity was determined using the method of Holmgren and Björnstedt with rat TrxR1 as a Trx reductase, the activity was presented in terms of μmol of NADPH oxidized/min/mg protein [27]. GR activity was measured by the method of Carlberg and Mannervik with oxidized glutathione as a substrate [28], and Grx activity was determined according to the method of Scian and Atkins with 2-hydroxyethyl disulfide as a model substrate [29]. Both GR and Grx activities were presented in terms of nmol of NADPH oxidized/min/mg protein.

To measure plasma EGCG, 100 μL plasma was transferred into an Eppendorf tube containing 20 μL ascorbate-EDTA solution (0.4 M $NaH_2PO_4$ buffer containing 20% ascorbic acid-0.1% EDTA, pH 3.6) and then 10 μL of a mixture of β-D-glucuronidase (250 U) and sulfatase (1 U) was added. The mixture was incubated at 37 °C for 45 min. Following the addition of 1 mL of ethyl acetate, the mixture was vortexed for 3 min and centrifuged at 13,000*g* for 10 min at 4 °C. The upper organic phase was mixed with 10 μL of 0.2% ascorbic acid and then was dried by vacuum centrifugation. The dried solid was dissolved in 100 μL of 10% methyl alcohol aqueous solution and centrifuged at 17,000*g* for 15 min at 4 °C. The resultant supernatant (5 μL) was analyzed using ultra-high performance liquid chromatography (UPLC)-triple quadrupole mass spectrometer (QQQ-MS/MS). Separation was achieved by a Hypersil GOLD column (particle size 1.9 mm; column size 50×2.1 mm) with a guard column (particle size 3 mm; column size 10×2.1 mm) at 35 °C. The mobile phase consisted of (A) 0.05% aqueous formic acid and (B) methanol. The gradient of solvent B was as follows: 0–1 min, 10%; 1–7 min, from 10% to 30%; 7–7.5 min, from 30% to 70%; then kept at 70% to 8 min; 8–8.5 min, from 70% to 10%; then kept at 10% to 10.5 min. The elution rate was 0.3 mL/min. Mass spectra were acquired simultaneously using electrospray ionization in the negative ionization mode over the range of *m/z* 100–1000. The dry gas was set to 6 L/min at 325 °C with a nebulizer pressure of 45 psi. The MRM mode of QQQ-MS/MS was used to detect the target compounds by selected product ions from the parent ions (EGCG/GCG, 457→169). The data of UPLC-QQQ-MS/MS were analyzed by Agilent MassHunter Qualitative Analysis software. To measure hepatic EGCG levels, 0.2*g* liver was homogenized with 1 mL of the ascorbate-EDTA solution and 500 μL homogenate was incubated with 20 μL of the mixture of β-D-glucuronidase and sulfatase at 37 °C for 45 min. Then 200 μL of ethanol was added to precipitate the tissue. Following the addition of 500 μL of methylene chloride and sufficient votex, the mixture was centrifuged at 16,000*g* for 5 min at 4 °C. The upper aqueous phase was extracted with ethyl acetate and the subsequent procedures were carried out in a same manner as the plasma samples.

### 2.4. RNA isolation, cDNA synthesis and Real-time PCR

Total RNA from the liver was isolated with Trizol reagent according to the manufacturer's protocol (Takara Biotechnology, Dalian, China), and cDNA synthesis was carried out with reverse transcriptase containing 100 ng of total RNA, oligo dT primer, and PrimeScript RT Enzyme Mix according to the manufacturer's instructions (RT-for-PCR kit, Takara Biotechnology). Real-time PCR was performed with a CFX System (Bio-Rad) according to the manufacturer's instructions (Takara Biotechnology). GAPDH or ribosomal protein S6 (Rps6) was selected as a housekeeping gene for normalizing all the gene expressions. Primers used for PCR reactions were designed using available gene sequences as shown in Table 1.

### 2.5. Preparation of nuclear fractions

The preparation of nuclear fraction was carried out according to the method of Carmona-Ramírez *et al.* [30]. In brief, liver tissues were washed once with cold PBS and then were lysed on ice with 10-fold volume of cold isotonic buffer A plus protease inhibitor cocktail (Sigma) at a concentration of 1 μg/mL for 10 min. After centrifugation at 500*g* for 5 min, the nuclear pellets were washed for 10 min with cold buffer B and then resuspended in hypertonic cold buffer C plus the protease inhibitor cocktail. Nuclear fractions without cytosol contaminant were validated using trypan blue dye exclusion assay.

**Table 1**
Primer sequences for RT-PCR.

| Genes | Direction | Sequences | Gene Bank |
|---|---|---|---|
| HO1 | Forward | 5′-TCAGAAGGGTCAGGTGTCCAGA-3′ | NM_010442.2 |
| | Reverse | 5′-GCATAGACTGGGTTCTGCTTGTT-3′ | |
| NQO1 | Forward | 5′-GGCGAGAAGAGCCCTGATTG-3′ | NM_008706.5 |
| | Reverse | 5′-GTTCATAGCATAGAGGTCAGATTCG-3′ | |
| p21 | Forward | 5′-GACTTCTCCCATTTCTTAGTAGCAG-3′ | NM_007669.4 |
| | Reverse | 5′-TGACACCCACGGTATTCAACAC-3′ | |
| TrxR1 | Forward | 5′-ACCTGGGCATCCCTGGAGAC-3′ | BC037643.1 |
| | Reverse | 5′-GCACCATTACAGTGACGTCTAAGC-3′ | |
| GAPDH | Forward | 5′-CTTTGGCATTGTGGAAGGGCTC-3′ | AY618199.1 |
| | Reverse | 5′-GCAGGGATGATGTTCTGGGCAG-3′ | |
| Rps6 | Forward | 5′-ACTACTGTGCCTCGTCGGTTGG-3′ | BC092050.1 |
| | Reverse | 5′-TGCTTTGGTCCTGGGCTTCTTAC-3′ | |

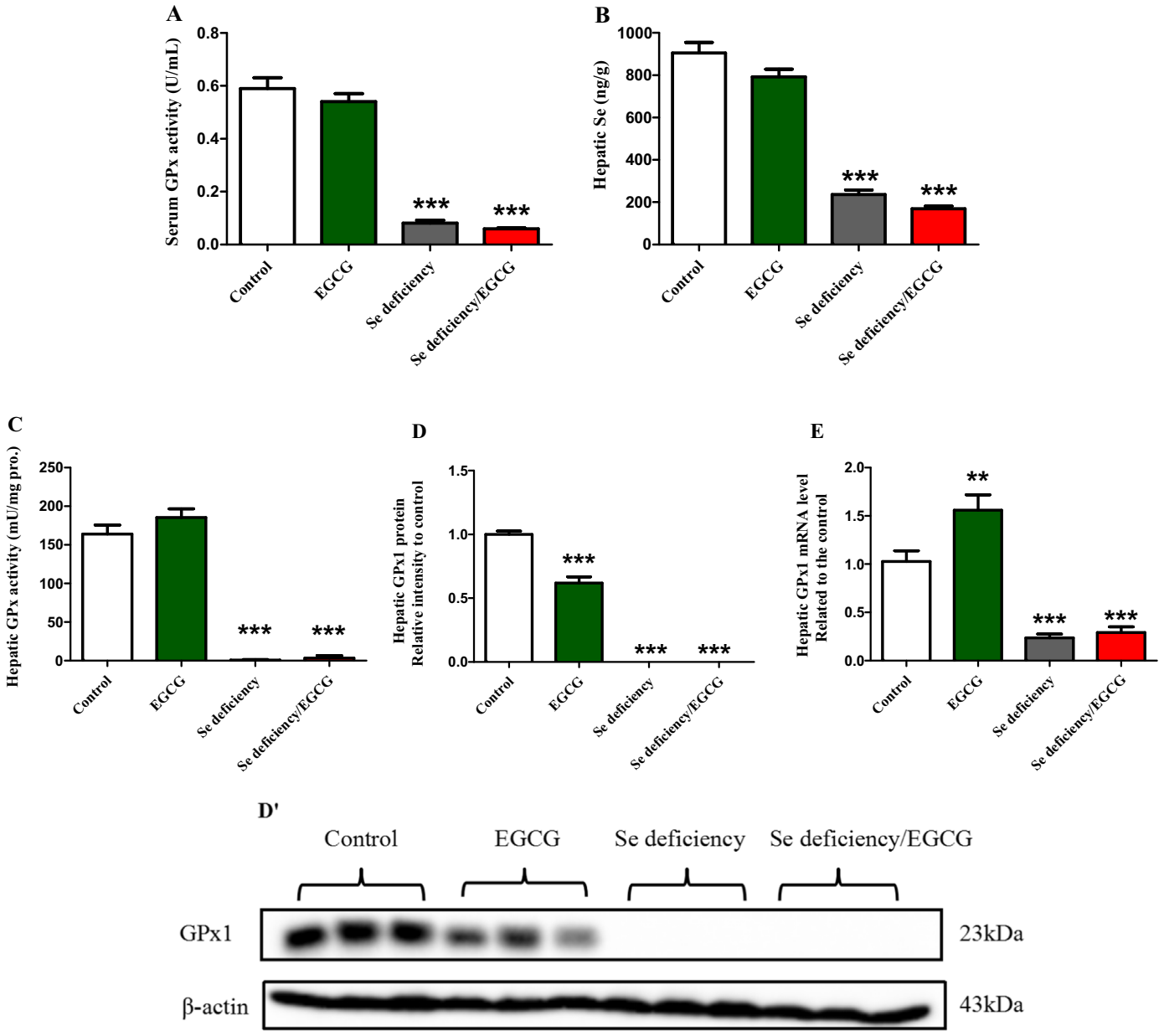


**Fig. 1. Biomarkers of Se status.** Mice were fed with Se-optimal or Se-deficient diet for 7 weeks. During the last 7 days, mice were i.p. injected with saline or 45 mg/kg EGCG once daily. (A) Serum GPx activity. (B) Hepatic Se levers. (C) Hepatic GPx activity. (D) and (D′) Hepatic GPx1 protein. (E) Hepatic GPx1 mRNA. Data are presented as means ± SEM (n=6). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control.

### 2.6. Western blot analysis

The concentrations of total protein extracted with the RIPA reagent were determined by the BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Protein extracts were boiled at 95 °C for 10 min in loading buffer, and then loaded onto 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) for protein separation. Proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% non-fat dried milk in tris buffered saline with 0.05% Tween 20 (TBS-T) for 2 h at room temperature (RT), and then incubated with specific primary antibodies diluted in TBS-T by 200 to 5,000 folds overnight at 4 °C. The membranes were washed four times, each for 10 min with TBS-T and then were incubated with secondary antibody diluted in TBS-T by 2500- or 5000-fold for 1 h at RT. After washing for four times with TBS-T, antibody bindings were detected using the ChemiDoc XRS+ detection system (ECL, Bio-Rad), and the corresponding bands were quantified by densitometry with the Quantity One® Image Analyzer software program (Bio-Rad).

### 2.7. Statistical analysis

The data are presented as means ± SEM. All statistical analyses were performed using GraphPad Software (Prism version 5, San Diego, California). The significances were examined by one-way analysis of variance (ANOVA) *post hoc* Tukey or Dunnett test, or two-way ANOVA *post hoc* Bonferroni test, as appropriate. Log-rank test was used for survival comparison. A *p*-value less than 0.05 was considered statistically significant.

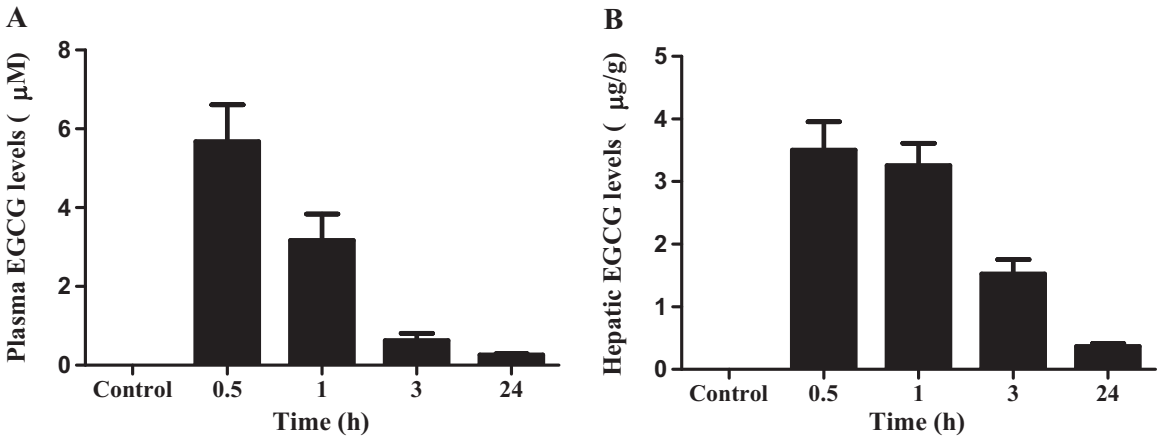


**Fig. 2. Time-dependent changes of EGCG in mice.** Se-adequate mice were i.p. injected with EGCG at the dose of 45 mg/kg once and were sacrificed at the indicating time. Samples were treated with β-D-glucuronidase and sulfatase to release conjugated EGCG, and then EGCG levels were determined using UPLC-QQQ-MS/MS. (A) Plasma EGCG concentration. (B) Hepatic EGCG level. Data are presented as means ± SEM (n=3).

## 3. Results

### 3.1. Selenium status

Dietary Se deficiency can result in significant decrease of Se content and selenoenzyme activities, particularly GPx1 activity in the liver [31,32]. In the present study, the levels of serum and hepatic GPx activity (Fig. 1A,C), hepatic GPx1 protein and mRNA (Fig. 1D,D′,E) as well as hepatic Se (Fig. 1B) significantly decreased in the mice fed with Se-deficient diet, indicating that the Se deficiency model had been successfully established. EGCG decreased the expression of hepatic GPx1 protein but increased the expression of hepatic GPx1 mRNA in the Se-optimal mice (Fig. 1D,D′,E). However, EGCG treatment did not affect the levels of serum or hepatic GPx activity or hepatic Se levels, irrespective of Se status (Fig. 1).

### 3.2. Dynamic change of plasma and hepatic EGCG levels

Following a single i.p. injection of EGCG (45 mg/kg), the plasma and hepatic EGCG levels were 5.7 μM and 3.6 μg/g (8 nmol/g), respectively, at 0.5 h. Then the levels decreased time-dependently over 24 h (Fig. 2).

### 3.3. EGCG enhances hepatic TrxR, GR and Grx activities only in selenium-optimal mice

EGCG significantly induced the mRNA and protein levels of hepatic TrxR1 in the Se-optimal mice (Fig. 3A, B and B′), leading to the augment of TrxR activity (Fig. 3C). As expected, Se deficiency resulted in a significant decrease of hepatic TrxR1 mRNA and protein levels as well as TrxR activity (Fig. 3A–C). Notably, EGCG significantly increased hepatic TrxR activity through inducing TrxR1 mRNA and protein (Fig. 3A–C) as well as hepatic GR and Grx activities in the Se-optimal mice (Fig. 3D and E). However, Se deficiency abolished the induction effects of EGCG on these enzymes (Fig. 3A–E).

### 3.4. Melatonin does not influence the induction of hepatic TrxR, GR and Grx activities by EGCG in selenium-optimal mice

To investigate whether EGCG-induced increases of hepatic TrxR, GR and Grx activities in Se-optimal mice are associated with overt pro-oxidant effects of EGCG, melatonin that can attenuate EGCG-evoked hepatotoxicity [33] was employed. The effects of EGCG on hepatic TrxR1 protein and TrxR, GR and Grx activities in Se-optimal mice seen above were herein recapitulated (Fig. 4A-D). Melatonin did not influence the effects of EGCG on either hepatic TrxR1 protein (Fig. 4A,A′) or TrxR and Grx activities (Fig. 4B,D). Melatonin even modestly enhanced the induction of hepatic GR activity by EGCG, most likely due to an additive effect, since melatonin itself significantly raised hepatic GR activity (Fig. 4C). These results suggest that the induction of key enzymes in the Trx and GSH systems by EGCG probably involves only mild rather than overt pro-oxidant action of EGCG.

### 3.5. Selenium deficiency promotes the elevation of Nrf2 response caused by EGCG

EGCG slightly but not significantly increased the nuclear Nrf2 protein levels in the liver (Fig. 5A) and significantly increased hepatic mRNA levels of NQO1 (Fig. 5B). However, EGCG did not significantly activate hepatic Nrf2 responses since HO1 mRNA levels, HO1 and NQO1 protein levels, and Trx and GST activities were not significantly increased (Fig. 5C-F). Although Se deficiency had a tendency of increasing nucleus Nrf2 protein levels (Fig. 5A), neither mRNA levels nor protein levels of hepatic NQO1 and HO1 were significantly increased in response to Se deficiency (Fig. 5B-D). However, hepatic GST activity was significantly enhanced in Se deficient mice (Fig. 5F), presumably associated with the non-significant increase of Nrf2 protein in the nucleus. In Se-deficient mice, EGCG significantly increased hepatic Nrf2 protein levels in the nucleus and consequently caused a significant induction of both mRNA and protein levels of HO1 and NQO1 as well as a significant increase of Trx activity (Fig. 5). Overall, both EGCG and Se deficiency alone appeared to trigger a modest Nrf2 response as evidenced by NQO1 mRNA induction and GST activity elevation, respectively. Only in Se-deficient conditions, EGCG caused a robust Nrf2 response. Such a unique Nrf2 response appeared to be associated with phosphorylation of p38 mitogen-activated protein kinase, which was increased by 2.2-fold ($p < 0.01$) only in Se-deficient mice when treated with EGCG (Supplementary Fig. 1).

Our previous studies have shown that high-dose EGCG-evoked hepatotoxicity is associated with hepatic Nrf2 activation, whereas EGCG at a non-toxic high dose (45 mg/kg, i.p.) for 5–7 days in Kunming mice, which are also used herein, did not generate a comprehensive Nrf2 response in the liver of Se-adequate mice [33,34]. Therefore, we next investigated whether the robust Nrf2 response as seen in the Se-deficient mice was associated with the EGCG-triggered hepatotoxicity.

### 3.6. Selenium deficiency does not enhance EGCG toxicity

High-dose EGCG could generate excessive amounts of ROS, which might trigger hepatotoxicity and even lethality [35,36]. In the present

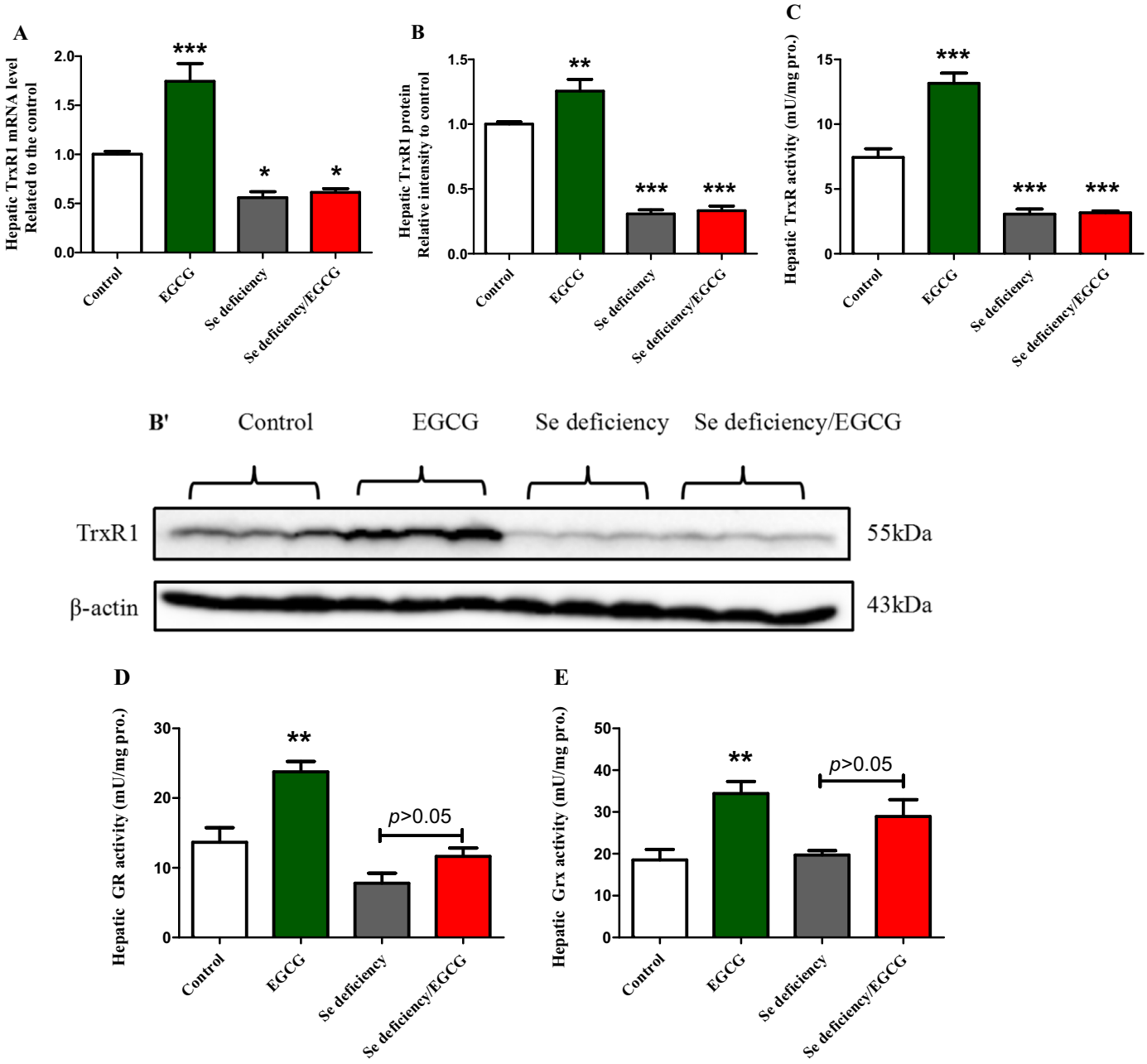


**Fig. 3. Influence of EGCG on hepatic TrxR1, GR and Grx.** Mice were fed with Se-optimal or Se-deficient diet for 7 weeks. During the last 7 days, mice were i.p. injected with saline or 45 mg/kg EGCG once daily. (A) Hepatic TrxR1 mRNA. (B) and (B′) Hepatic TrxR1 protein. (C) Hepatic TrxR activity. (D) Hepatic GR activity. (E) Hepatic Grx activity. Data are presented as means ± SEM (n=6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control.

study, the administration of EGCG (45 mg/kg, i.p.) once daily for 7 days to the Se-optimal mice neither affected body weight (Fig. 6A) nor caused hepatotoxicity as indicated by the normal serum levels of ALT, AST and 4-HNE (Table 2), as well as hepatic γ-H2AX protein (Fig. 6C) whose expression has been demonstrated to be dramatically increased during the EGCG-triggered liver injury [33–36]. The EGCG treatment actually decreased the inflammation as evidenced by the lower levels of serum IL-2 and IL-10 (Table 2). Although no toxic effects were observed, the EGCG dose used was likely the maximum non-toxic dose, because of the modest but significant increase of hepatic p21 mRNA (Fig. 6B). In this context, it is worth noting that we previously found that p21 mRNA levels were increased over one order of magnitude by EGCG at a dose triggering hepatotoxicity (55 mg/kg, i.p., once daily for 5 consecutive days in Kunming mice, the same mouse strain as used herein, our unpublished data). If Se deficiency enhances EGCG toxicity, the maximum non-toxic dose of EGCG is expected to decrease in the Se-deficient mice. However, the Se-deficient mice well tolerated to the maximum non-toxic dose of EGCG (45 mg/kg) indentified in the Se-optimal mice as reflected in body weight (Fig. 6A) and hepatic γ-H2AX protein (Fig. 6C), serum ALT and AST activities, and 4-HNE levels (Table 2). Moreover, Se deficiency did not interfere with the beneficial effect of EGCG on inflammatory markers (Table 2) and even abolished the elevation of p21 mRNA levels (Fig. 6B). These results indicate that Se deficiency does not enhance EGCG toxicity under the experimental conditions used. Our present results suggest that the robust Nrf2 response to EGCG in the Se-deficient mice (Fig. 5) was not related to the EGCG-evoked hepatotoxicity. It is possible that the activation of the Nrf2 cytoprotective enzymes compensated for the deficit in antioxidant defense caused by Se deficiency. Further support for the idea that Se deficiency does not

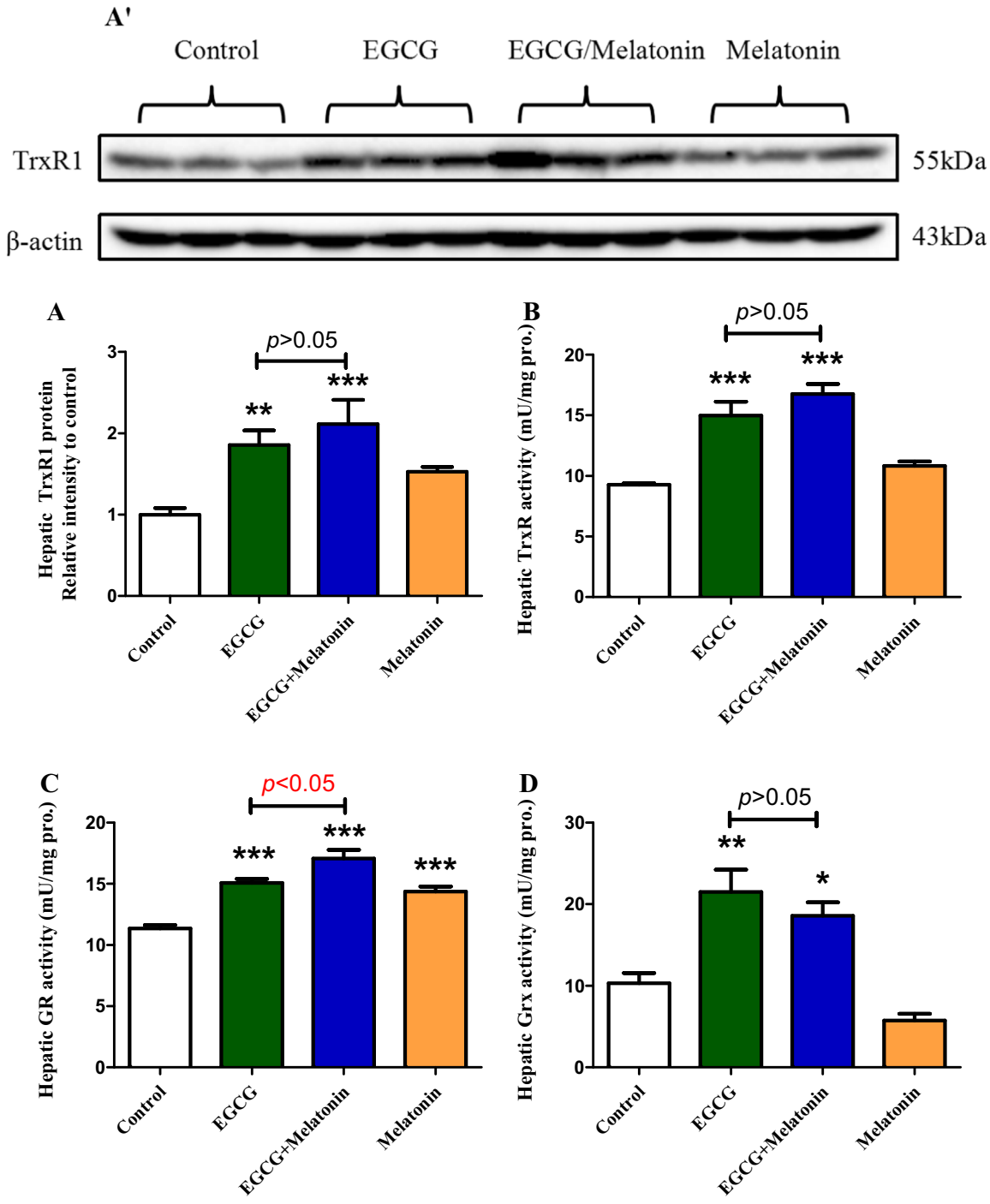


**Fig. 4. Influence of melatonin on EGCG-mediated increase of hepatic TrxR, GR and Grx activities.** Se-optimal mice were i.p. injected with saline, 45 mg/kg EGCG, 50 mg/kg melatonin or the combination of EGCG and melatonin once daily for 5 consecutive days. (A) and (A′) Hepatic TrxR1 protein. (B) Hepatic TrxR activity. (C) Hepatic GR activity. (D) Hepatic Grx activity. Data are presented as means ± SEM (n=6). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. Control.

enhance the toxicity of EGCG was obtained from another experiment using lethal doses of EGCG. Administration of EGCG (70 mg/kg, i.p.) to mice, once daily for 3 days, resulted in the same (50%) mortality in the Se-optimal and Se-deficient mice (Fig. 6D). Moreover, the survival profiles of the two groups showed no significant differences.

## 4. Discussion

Trx and GSH systems are the two well-documented NADPH-dependent disulfide reduction pathways that are essential for maintaining redox homeostasis. In response to an oxidative stress, the Nrf2 pathway is also known to be activated to participate in the maintenance of redox homeostasis. The present study revealed a Se status-dependent switch of hepatic defense pathways in response to EGCG. In Se-optimal mice, EGCG simultaneously enhanced hepatic Trx and GSH systems, whereas in Se-deficient mice, EGCG did not induce these two systems and became a more efficient Nrf2 activator in the liver.

Chemically, EGCG has a strong ROS and RNS scavenging capacity [37]. In addition, EGCG has been shown to increase the activities of Mn-SOD and total SOD, catalase and GR in isolated human neutrophils [38] and to upregulate the expression of Prx6 in murine and human submandibular gland cells [39]. Moreover, green tea can significantly

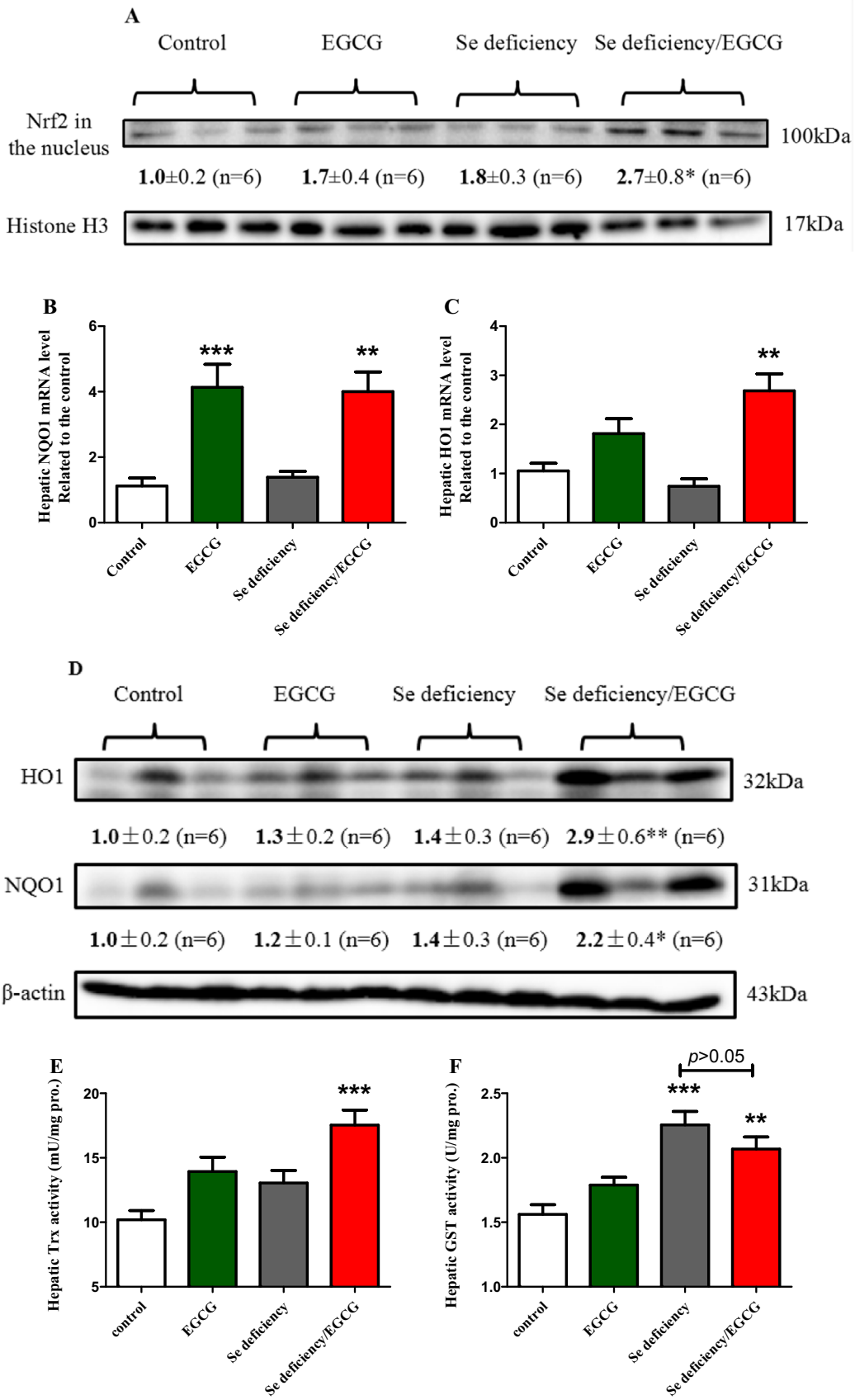


**Fig. 5. Nrf2 response to EGCG in different Se status.** Mice were fed with Se-optimal or Se-deficient diet for 7 weeks. During the last 7 days, mice were i.p. injected with saline or 45 mg/kg EGCG once daily. (A) Hepatic nuclear Nrf2 protein level. (B) Hepatic NQO1 mRNA level. (C) Hepatic HO1 mRNA level. (D) Hepatic proteins of HO1 and NQO1. (E) Hepatic Trx activity. (F) Hepatic GST activity. Data are presented as means ± SEM (n=6). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. control.

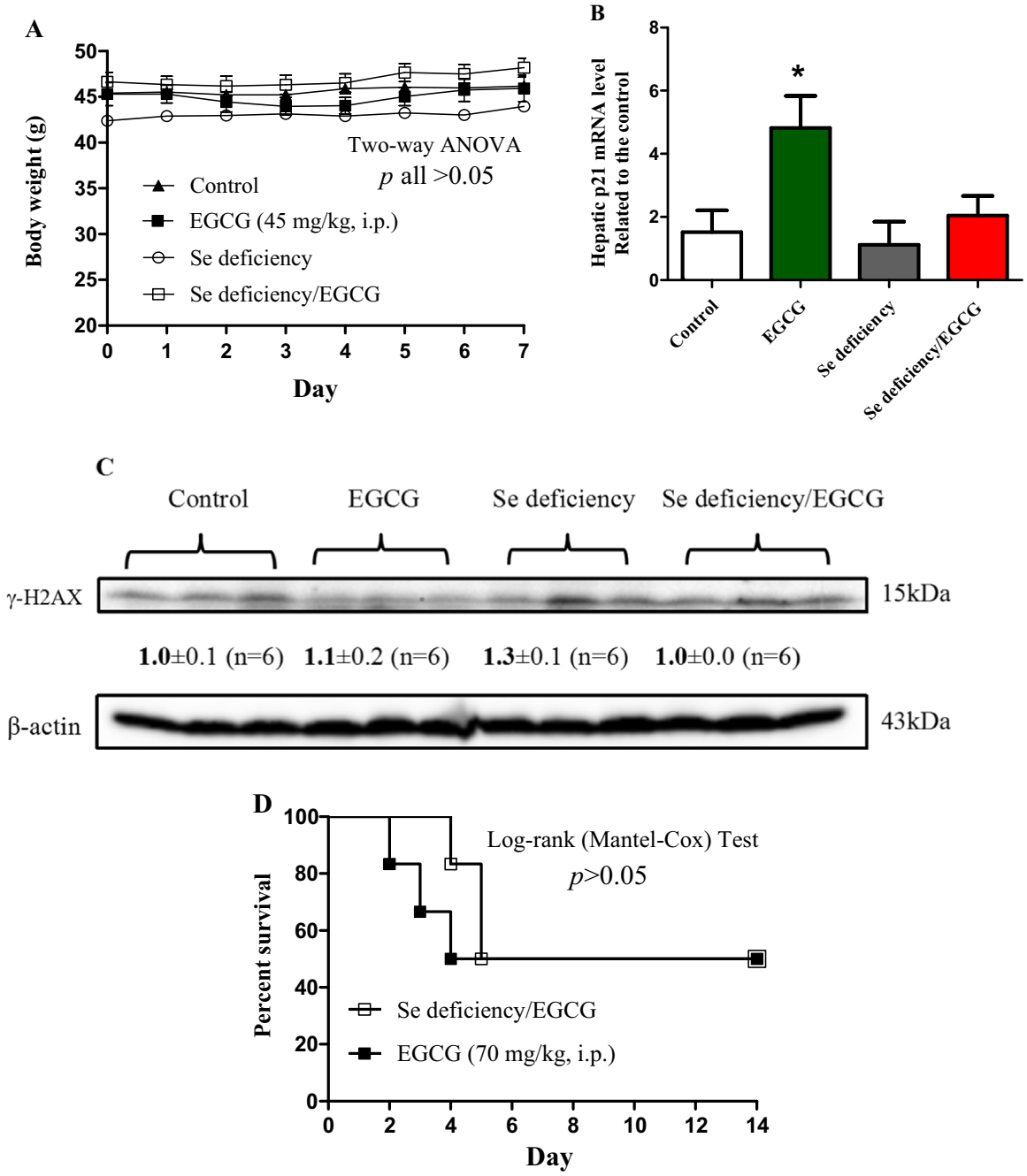


**Fig. 6. Toxic reactions to EGCG in different Se status.** (I) Mice were fed with Se-optimal or Se-deficient diet for 7 weeks. During the last 7 days, mice were i.p. injected with saline or 45 mg/kg EGCG once daily. (A) Body weight. (B) Hepatic p21 mRNA. (C) Hepatic γ-H2AX protein. (II) Mice were fed with Se-optimal or Se-deficient diet for 7 weeks. On the 6th week, mice were i.p. injected with saline or 70 mg/kg EGCG once daily for 3 consecutive days designed as Day 0, Day 1 and Day 2 as shown in panel (D) that presents their survival profiles. Data are presented as means ± SEM (n=6). *$p < 0.05$ vs. control.

increase intestinal, pulmonary and hepatic GPx and catalase activities in mice [40]. Therefore, both the direct ROS/RNS scavenging capacity and the induction of antioxidant enzymes are viewed as antioxidant mechanisms of green tea polyphenols, in particular the well-studied EGCG. Our present study is the first to demonstrate that EGCG can increase hepatic TrxR, GR and Grx activities simultaneously in mice. The Trx and GSH systems provide electrons to ribonucleotide reductase for DNA replication and jointly support a wide range of reductive pathways and protect cells against the oxidative damage. They also serve as a backup system for each other [41]. Thus, only when both systems are inhibited, pronounced tissue damages or cell death would occur. Several lines of observations are consistent with this concept: 1) Buthionine sulfoximine (BSO) (a GSH biosynthesis inhibitor) and auranofin (a potent TrxR inhibitor), when used together, significantly sensitized the response of cancer cells to cytotoxic drugs [42]; 2) The combination of BSO and arsenic trioxide (a TrxR inhibitor) or BSO and nedaplatin (a TrxR inhibitor) synergistically killed cancer cells in vitro or in vivo, respectively [43,44]; 3) Co-treatment of BSO with either ifosfamide or cyclophosphamide (which inactivates TrxR) induced much more severe acute renal failure or heart injury in mice than a single agent alone [45,46]. We propose that the EGCG-induced enhancement of hepatic Trx and GSH systems and their concerted actions is an important mechanism by which EGCG exerts antioxidant effect.

Among the investigated three enzymes whose activities were increased by EGCG, only TrxR1 gene is a well-elucidated Nrf2 target

**Table 2**
Serum biomarkers associated with hepatic damage and inflammation.

| Serum parameters | Control | EGCG | Se deficiency | Se deficiency/ EGCG |
|---|---|---|---|---|
| ALT (U/mL) | 31.3 ± 7.6 | 50.9 ± 11.7 | 28.5 ± 2.4 | 30.6 ± 4.2 |
| AST (U/mL) | 22.2 ± 3.3 | 21.8 ± 3.0 | 17.1 ± 2.6 | 18.4 ± 1.5 |
| IL-2 (pg/mL) | 94.7 ± 12.7 | 37.0 ± 7.3** | 52.8 ± 17.3* | 29.4 ± 1.7** |
| IL-10 (ng/mL) | 3.4 ± 0.3 | 1.5 ± 0.2** | 2.7 ± 0.6 | 1.2 ± 0.1*** |
| IL-6 (ng/mL) | 0.6 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.1 | 0.9 ± 0.1 |
| 4-HNE (ng/mL) | 4.6 ± 0.3 | 4.5 ± 0.4 | 4.4 ± 0.8 | 4.4 ± 0.2 |

Mice were fed with Se-optimal or Se-deficient diet for 7 weeks. During the last 7 days, mice were i.p. injected with saline or 45 mg/kg EGCG once daily. Data are presented as means ± SEM (n=6).
* $p < 0.05$ vs. control.
** $p < 0.01$ vs. control.
*** $p < 0.001$ vs. control.

gene [47]. Melatonin, which is a free radical scavenger and a broad-spectrum antioxidant [48–50], can effectively alleviate high-dose EGCG-triggered oxidative stress and suppress EGCG-induced Nrf2 activation [33,34]. The present result, showing that melatonin did not influence the induction of TrxR1 by EGCG, suggests that overt pro-oxidant action of EGCG is not involved in the EGCG-induced increase of TrxR1. Previous studies demonstrate that inhibition of TrxR1 elicits Nrf2 response [14–16]. TrxR1 can act as a potent regulator and gatekeeper of Nrf2 [17,51]. The mechanisms by which TrxR1 is induced by EGCG are unclear. One possibility is that a mild TrxR1 inhibition by EGCG [52,53] can trigger TrxR1 synthesis through a mild and transient activation of Nrf2, which increases the transcription of TrxR1 [17]. In this scenario, the induced TrxR1 in turn plays a role as a gatekeeper to close the door for overt Nrf2 activation. Another possibility is that EGCG activates the forkhead box class O (FoxO) transcription factors [54]. Upon translocation into the nucleus, FoxO stimulates the transcription of genes coding for a myriad of antioxidant proteins, including members of the superoxide dismutase, Prx, Trx and TrxR families [55,56]. This mechanism may explain why EGCG could increase many antioxidant enzyme activities. Whether FoxOs participate in the EGCG-induced increase of TrxR, GR or Grx activity requires further investigation. Emerging evidence has shown that S-glutathionylation of Keap1 leads to Nrf2 activation [57,58], and this suggests that Grx in conjunction with GSH system can participate in attenuating Nrf2 activation. Based on the above discussion, we propose that, in Se-optimal conditions, high basal TrxR1 and its induction by EGCG as well as the related increase in GR and Grx activities limit Nrf2 activation; whereas in Se-deficient conditions, low basal TrxR1 and its mute response to EGCG as well as the related lack of induction of GR and Grx promote Nrf2 activation. The interactions among these enzyme systems are depicted in Fig. 7.

In vitro experiments have shown that many Nrf2 inducers upregulate TrxR1 mRNA under either Se-deficient or Se-replete conditions [19,59–61]. However, how Se status influences Nrf2 inducer-triggered upregulation of TrxR1 mRNA remains unclear. Some studies found that TrxR1 mRNA was more efficiently induced in Se-replete conditions compared to Se-deficient conditions [19,60], whereas the opposite result was also reported [61]. The present study shows that EGCG induces gene expression of hepatic TrxR1 only in Se-adequate but not in Se-deficient mice. The discrepancy between in vitro and in vivo Se-deficient conditions on TrxR1 mRNA regulated by Nrf2 inducer may be related to the different influences of Se levels on basal TrxR1 mRNA in vitro and in vivo. Se status did not affect basal TrxR1 mRNA levels in vitro [19,60,61]. However, we found that Se deficiency significantly reduced basal TrxR1 mRNA levels in vivo (Fig. 3A), which is similar to the observation reported by others [62]. Under Se-deficient conditions, biosynthesis of TrxR1 is impaired even if EGCG induces TrxR1 mRNA.

We have shown in this study that EGCG did not affect hepatic GPx activity irrespective of Se status (Fig. 1C). Since GPx1 is an important member of GSH system and is one of the most abundant selenoenzymes in the liver, we also investigated the influence of EGCG on GPx1 expression. We found that in the Se-optimal mice, EGCG decreased the expression of hepatic GPx1 protein (Fig. 1D, D′) but increased the expression of hepatic GPx1 mRNA (Fig. 1E). The EGCG-induced elevation of hepatic GPx1 mRNA might be associated with the activation of p53 [63,64]. The activation of p53 by EGCG is suggested by the increase of p21 in the Se-adequate mice (Fig. 6B). The lower expression of GPx1 protein may be due to Se sequestration by highly increased TrxR1 protein [65–69], since TrxR1 is at the top of selenoprotein hierarchy while GPx1 is at the bottom [13]. GPx family comprises 8 members, GPx1-4 and 6 are selenoprotein and GPx5, 7 and 8 are not [70]. EGCG can induce the expression of hepatic GPx2, 3, 5 and 7 mRNA [36]. Thus, reduced hepatic GPx1 protein levels caused by EGCG may not affect total activity of hepatic GPx.

In this study, we used a Se deficiency diet as a tool to study the interactions among the anti-oxidative defense enzyme systems. The serum GPx activity, a commonly used biomarker for Se status, in the mice after Se deprivation in the present study was approximately 14% of Se-adequate mice. This is similar to conditions produced by using a marginal Se deficient diet (50% of the Se-adequate levels) [71]. Overt Se deficiency would markedly induce hepatic Nrf2 response, as was reported previously [12]. Typical Nrf2 response in the liver of the mice post Se deprivation in the present study was not observed. Thus, the present results were probably obtained from an intermediate Se status between suboptimal Se levels and overt Se deficiency. EGCG-induced Nrf2 response in humans with suboptimal Se levels may or may not be as strong as seen in this study. However, the presently observed

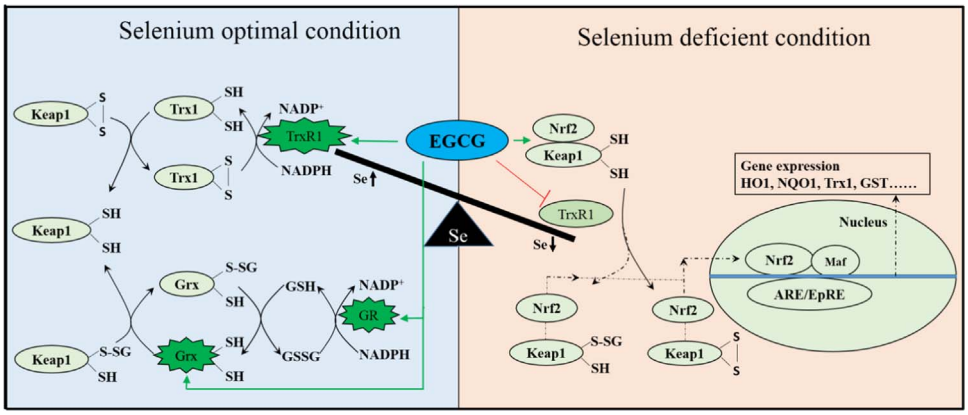


**Fig. 7. A Se status-dependent diverged regulation of cellular defense pathways in response to EGCG.** Formation of disulfide bonds and/or S-glutathionylation in Keap1 activates Nrf2. In Se-optimal condition, high basal TrxR1 and its induction by EGCG as well as related increase in GR and Grx activities limit Nrf2 induction by EGCG (left panel). In Se-deficient condition, low basal TrxR1 and its mute response to EGCG as well as the lack of responses of GR and Grx to EGCG promote Nrf2 activation (right panel).

induction of hepatic TrxR1, GR and Grx by EGCG is expected to similarly occur in Se-adequate humans. Peak plasma concentrations of EGCG in mice and humans following pharmacological oral doses have been reported to be 2–9 μM and 7.5 μM, respectively [72]. In the present study, peak plasma EGCG concentration of mice subjected to single i.p. injection of EGCG (45 mg/kg) was 5.7 μM (Fig. 2A), thus the dose of EGCG employed in this study, although much higher than that resulted from consumption of tea beverages, is similar to the consumption of green tea extract-based dietary supplements (for weight loss), in which EGCG is a major ingredient. Adverse reactions, mainly hepatotoxicity, owing to copious consumption of green tea extracts by some individuals have been observed, but the percentage is rather low [73,74]. It is likely that presently observed responses of anti-oxidative defense systems serve as the protective mechanisms.

Based on the present results, we conclude that in Se sufficiency, the higher basal levels of TrxR1 and the induction of TrxR1, GR and Grx by EGCG would spare Nrf2 from activation by EGCG; whereas in Se deficiency, the Nrf2 system is robustly activated due to the lower basal levels of TrxR1 and the lack of TrxR1, GR and Grx induction by EGCG. Our study is the first demonstration of the interactions of these anti-oxidative defense enzymes in response to the stress of a dietary chemical, EGCG, in animals.

## Conflict of interest

The authors have no conflict of interest to declare.

## Acknowledgements

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## Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.10.009.

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