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Regulation of cellular glutathione peroxidase by different forms and concentrations of selenium in primary cultured bovine hepatocytes

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Abstract

The expression and activity of cellular glutathione peroxidase (GPx1) are regulated by selenium (Se). Generally speaking, organic forms of Se have less toxicity and greater bioavailability compared with inorganic forms. In this study, the effects of different forms and concentrations of Se on the regulation of mRNA level and activity of GPx1 in bovine hepatocytes were evaluated, and the optimal doses of different forms of Se that supported the full expression of GPx1 were determined. Primary cultured bovine hepatocyte monolayers derived from neonatal male Holstein calves (aged 1–2 days) were incubated for 24 h with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/L of Se from DL-selenomethionine (Se-Met), sodium selenite (Na_2SeO_3) or Kappa-selenocarrageenan (Se-Car). Compared with controls, a significantly lower level of release of lactic dehydrogenase (LDH) was observed at 0.5–5 μmol/L of Se-Met, 0.5–1 μmol/L of Na_2SeO_3 and 0.5 μmol/L of Se-Car, but significantly higher LDH release was observed at 2–5 μmol/L of Na_2SeO_3 and 3–5 μmol/L of Se-Car, and the response occurred in a dose-dependent manner. The intracellular content of reduced glutathione in all hepatocytes treated with Se was significantly lower than that of controls. Significant increases in GPx1 mRNA were obtained in all hepatocytes treated with Se, with maximal effects at 3 μmol/L of Se-Met, 1.5 μmol/L of Na_2SeO_3 and 2 μmol/L of Se-Car, respectively. Furthermore, 3 μmol/L of Se from Se-Met resulted in peak levels of GPx1 mRNA. After reaching a maximal level, higher Se supplementation led to a reduction of GPx1 mRNA. The activity of GPx1 showed similar patterns but of lower magnitude. We conclude that (a) the regulation of mRNA level and activity of GPx1 in primary cultured bovine hepatocytes by different forms of Se varies and (b) the optimal doses of Se to support the full expression of GPx1 in bovine hepatocytes when supplied as Se-Met, Na_2SeO_3 and Se-Car are 3, 1.5 and 2 μmol/L, respectively.

Keywords: Se; GPx1; Bovine hepatocytes; Gene expression; Bioavailability

1. Introduction

Selenium (Se) is an essential trace element for animals and humans. Previous data suggest that Se may enhance immune function [1], reproductive performance [2,3] and resistance to oxidative stress [4]. Supplementation of Se can be protective, to some extent, against certain types of cancer [5–8]. Deficiency of Se can be associated with enhanced severity of infectious diseases [1,9] and is linked with numerous diseases such as Keshan disease and Kaschin–Beck disease in humans [10,11], "white muscle disease" in calves and lambs [12] and exudative diathesis in chicks [13].

Cellular glutathione peroxidase (GPx1, E.C. 1.11.1.9), a homotetramer of 84 kDa, was the first Se-dependent enzyme to be identified. The mRNA sequence of GPx1 contains a UGA codon that encodes a selenocysteine (Sec) residue, and this is normally used as a translation termination codon. The Sec residue is located at the active center of GPx1 [14,15]. Supplementation with Se may increase the stability of cytoplasmic GPx1 mRNA [16]. In contrast, Se deficiency decreases the efficiency of incorporation of Sec at the UGA codon site during mRNA translation and results in decay of cytoplasmic GPx1 mRNA, which is mediated by the nonsense codon [17].

Reactive oxygen species (ROS) are generated primarily by electron leakage from mitochondrial electron carriers and enzymes during oxidative phosphorylation in aerobic mammalian cells. If ROS are not removed in a timely

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fashion by an antioxidant enzyme system and overstep physiological concentrations, the cells may encounter oxidative stress, which causes destruction of lipids, proteins and DNA. It is known that GPx, along with superoxide dismutase and catalase, forms the main antioxidant enzyme system in mammals. GPx1 can detoxify hydrogen peroxide to water and a range of organic peroxides to the corresponding alcohols using reduced glutathione (GSH) as the hydrogen donor [15]. Therefore, GPx1 acts as one of the cellular protectors against oxidative damage.

Previous studies have suggested that organic forms of Se [e.g., Se-enriched yeast, which contains selenomethionine (Se-Met)] are less toxic than inorganic forms of Se such as sodium selenite [18–20]. Organic forms of Se have excellent bioavailability and are appropriate for nutritional supplementation [21,22]. Because the expression and activity of GPx1 are highly correlated with the Se status of the animal or cell [23,24], GPx1 has been used as a biochemical indicator of Se status and requirements [25]. Lei et al. [26] reported that Se supplementation at 0.2 mg Se/kg of diet was required to support the full expression of GPx1 in the livers of young pigs. In addition, Sunde et al. [27] showed that maximal activity of GPx1 in the livers of pregnant and lactating rats required 0.075 and 0.1 mg Se/kg of diet, respectively.

The diets of dairy cattle are routinely supplemented with Se. Various inorganic and organic forms of Se are used in many countries. There are no established criteria for the levels of Se that are released from inorganic or organic forms in the rations fed to Chinese dairy cattle. It is known that the mammalian liver is the principal organ involved in storage of Se and that it can synthesize GPx1, GPx4, thioredoxin reductase and deiodinase. However, little is known about the regulation of mRNA level and activity of GPx1 in bovine hepatocytes. The purposes of the present study were to evaluate the effects of different forms and concentrations of Se on the regulation of mRNA level and activity of GPx1 in primary cultured bovine hepatocytes and to determine the optimal doses of different forms of Se for support of the full expression of GPx1 mRNA in vitro.

2. Materials and methods

2.1. Preparation of bovine hepatocytes

Bovine livers were obtained from neonatal male Holstein calves (aged 1–2 days), which were used to produce serum and slaughtered at the local slaughterhouse. The carcass of each calf was skinned and the abdominal cavity was opened by butchers. The caudate process of the liver was excised by a single transverse cut with sterile instruments and placed on a polypropylene tray with the hepatic vein uppermost. There were two large (4–6 mm in diameter) hepatic vessels on the cut surface. Blood was removed from the organ by perfusion with calcium-free cold HEPES buffer (33 mM HEPES, 127.8 mM NaCl, 3.15 mM KCl, 0.7 mM Na_2HPO_4 and 0.6 mM EGTA, pH 7.65) through the hepatic vessels. The liver lobe was then immersed in physiological saline (1–4°C) and transported to the laboratory. The interval from death of the calf to hepatocyte isolation was less than 1 h.

Hepatocyte isolation was performed by the collagenase perfusion method as previously described [28,29], with some modifications. The liver lobe was rewarmed by preperfusion for 10–12 min with the same calcium-free HEPES buffer (37–38°C) but without EGTA. The liver lobe was then perfused with 300 ml of 0.05% (w/v) collagenase type II (Invitrogen, USA) perfusion buffer (33 mM HEPES, 127.8 mM NaCl, 3.15 mM KCl, 0.7 mM Na_2HPO_4 and 3 mM $CaCl_2$, pH 7.65, 37–38°C) using a recirculating system at a flow rate of approximately 120 ml/min for 20–25 min. Digested liver tissue was then transferred to a sterile beaker and the liver capsule was removed. The liver tissue was disrupted gently and the cells were dispersed in 40 ml of serum-free DMEM/Ham F12 medium (Invitrogen) supplemented with 0.2% (w/v) bovine serum albumin (BSA) and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) at 4°C. The cell suspension was filtered once through each mesh (grid size about 150 and 75 μm, respectively) and further diluted with 300 ml of the same serum-free medium. After sedimentation for 10 min at 4°C, the supernatant was discarded. The cells were pelleted by centrifugation (30×*g* and 50×*g* for 3 min each) and then resuspended and washed twice with the same serum-free medium at 4°C. Finally, the isolated cells were resuspended in 40 ml of DMEM/Ham F12 medium containing 15% fetal bovine serum (FBS), 24 mM $NaHCO_3$, 2 mM L-glutamine, 0.2% (w/v) BSA, 100 nM dexamethasone, 5 mg/L transferrin, 1 μmol/L insulin and antibiotics. The viability of isolated hepatocytes was 83.75±1.78% (mean±S.D., n=3) by 0.4% trypan blue dye exclusion, and the cell yield from each liver lobe preparation was $3.34±0.71×10^8$ hepatocytes (mean±S.D., n=3). Evaluation of the cells by light microscopy indicated that about 94% of the collected cells were hepatocytes.

2.2. Hepatocyte cultures and treatments

Hepatocytes were seeded into six-well plates precoated with cattle tail tendon collagen at a density of $1.2×10^6$ viable cells per well in 2 ml of DMEM/Ham F12 medium. Cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere. After an attachment period of 4 h, cell monolayers were washed twice with Hanks' balanced salt solution, and 3 ml of fresh DMEM/Ham F12 medium containing 10% FBS was added to each well. Following culture for 24 h, the culture medium was removed and the cell monolayers were washed three times with Hanks' balanced salt solution. Then, hepatocytes were grown in 3 ml of fresh serum-free DMEM/Ham F12 medium and treated with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/L of Se as DL-Se-Met (Sigma, USA), sodium selenite (Na_2SeO_3, Sigma) or Kappa-selenocarrageenan (Se-Car, First Institute of

Oceanography, China). After 24 h of incubation, the culture medium was collected into 2-ml Eppendorf tubes for assay of lactic dehydrogenase (LDH) and cells were harvested for analysis of mRNA and enzyme activity. Each treatment was done in six wells (three wells for mRNA analysis, the others for measurement of GPx1 activity and GSH concentration) in three separate experiments.

2.3. Preparation of hepatocyte lysates

After 24 h incubation with different forms and concentrations of Se, the cell monolayers were washed three times with ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and harvested by scraping into 1 ml of cold Tris buffer (20 mM Tris–HCl, pH 7.5, 2 mM EDTA and 0.1% peroxide-free Triton X-100). Hepatocyte lysates were then prepared by ultrasonication for 30 s in icy water and centrifuged at 12,000×*g* for 15 min at 4°C. The supernatants were aliquoted and stored at −20°C for subsequent analysis.

2.4. Analytical methods

2.4.1. Determination of protein concentration of hepatocyte lysates

Total protein concentration was determined using a Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China). The enzyme activity results were corrected for total protein concentration.

2.4.2. Determination of LDH activity in the culture medium

Hepatocyte toxicity was analyzed by measuring the activity of LDH in the incubation medium. The culture medium was collected into 2-ml Eppendorf tubes at the end of incubation and centrifuged at 12,000×*g* for 15 min at 4°C. The supernatants were stored at −20°C until analysis. The measurement of LDH activity in the culture medium was performed as previously described [30]. One unit of enzyme activity was defined as 1 μmol of reduced nicotinamide adenine dinucleotide oxidized per minute. The activity of LDH in the culture medium was expressed as units per liter. All samples were measured in duplicate.

2.4.3. Measurement of GSH concentration in hepatocyte cytosol

The concentration of GSH in hepatocyte cytosol was measured according to the method described by Rahman et al. [31]. The method is based upon a reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and GSH. The chromophoric product (5-thio-2-nitrobenzoic acid) possesses a molar absorption at a wavelength of 412 nm that is proportional to the GSH concentration in the sample. The GSH concentrations were calculated from a standard curve prepared with pure GSH standards and were expressed as micromoles of GSH per gram of protein. All samples were assayed in duplicate.

2.4.4. Determination of GPx1 mRNA level by quantitative RT-PCR

After 24 h incubation with different forms and concentrations of Se, the medium was removed and the cell monolayers were washed three times with ice-cold Ca^{2+}/Mg^{2+}-free PBS. Total RNA was isolated from the hepatocyte monolayers using the TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. The dried RNA pellets were resuspended in 40 μl of diethyl-pyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. The total RNA was immediately used or stored at −70°C prior to cDNA synthesis.

First-strand cDNA was synthesized from 2 μg of total RNA using Oligo dT primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Synthesized cDNA was diluted 10 times with sterile water and stored at −20°C before use.

Primer Premier software (PREMIER Biosoft International, USA) was used to design specific primers for GPx1 and β-actin based on known bovine sequences (Table 1). General PCRs were first performed to confirm the specificity of the primers. The PCR products were electrophoresed on 2% agarose gels, extracted, cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced. The plasmids were serially diluted from 10^9 to 10^4 copies and used as templates in quantitative real-time PCRs to establish standard curves. Quantitative real-time PCR was performed on an ABI PRISM 7300 Detection System (Applied Biosystems, USA). Reactions were performed in a 25-μl reaction mixture containing 12.5 μl of 2× SYBR Green I PCR Master Mix (TOYOBO, Japan), 10 μl of either diluted cDNA or plasmid standard, 1 μl of each primer (10 μmol/L) and 0.5 μl of PCR-grade water. The PCR procedure for GPx1 and β-actin consisted of a 95°C step for 2 min followed by 40 cycles consisting of 95°C for 15 s, 62°C for 15 s and 72°C for 30 s. The melting curve analysis showed only one peak for each PCR product. Electrophoresis was performed with the PCR products to verify primer specificity and the purity of the products. The calculation of the number of copies of each sample was performed from the respective standard curve using the

Table 1
Primers used for quantitative real-time PCR

Primers	Sequence (5′–3′)	Target gene	GenBank accession no.	PCR fragment length (bp)
GPx1-F (forward)	ctt gct gct tgg cgg tca	GPx1	X13684	139
GPx1-R (reverse)	agg gga ggc tgg gat gga ta			
β-Actin-F (forward)	tgc ctt ccc aaa agc cac c	β-actin	BC102948	250
β-Actin-R (reverse)	acc tca acc cgc tcc caa			

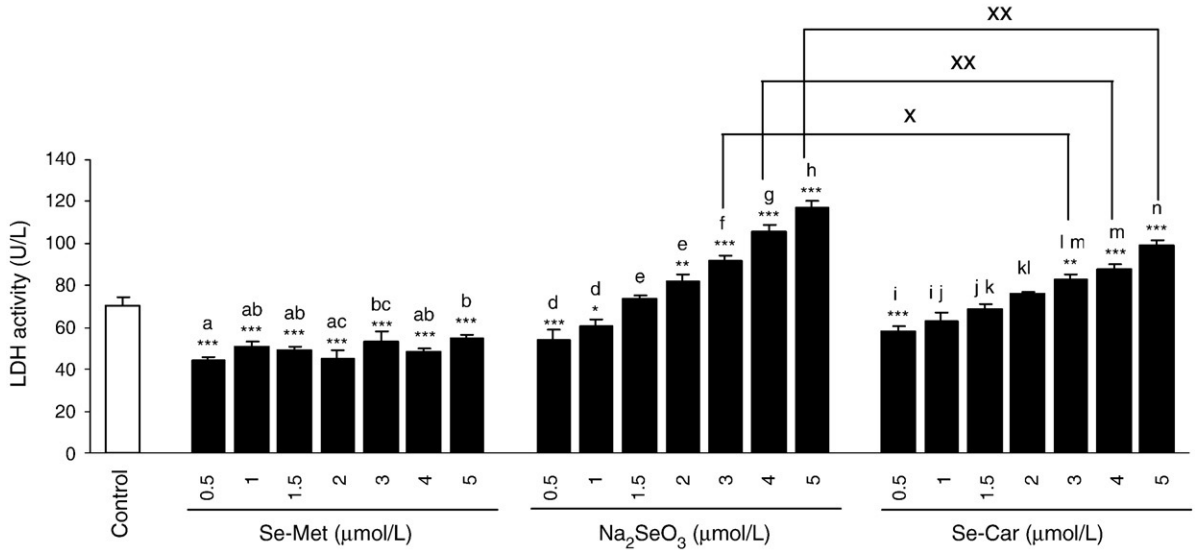

Fig. 1. Effects of different forms and concentrations of Se on LDH release from hepatocytes. The primary cultured bovine hepatocyte monolayers were incubated with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/L of Se as Se-Met, Na_2SeO_3 or Se-Car for 24 h. The activity of LDH in the culture medium was measured to evaluate cell membrane integrity. Bars represent mean±S.D. of triplicate culture media. Bars with "*" are statistically significantly different from control by one-way ANOVA followed by Tukey's multiple comparison test (*P<.05, **P<.01, ***P<.001). Within the groups treated with different levels of Se-Met, Na_2SeO_3 or Se-Car, bars sharing a common letter are not significantly different (P>.05). "x" and "xx" denote significant difference (^{x}P<.05, ^{xx}P<.01) between cells treated with Na_2SeO_3 and Se-Car.

7300 system software. The ratio of the level of GPx1 mRNA to that of the β-actin internal control was used for statistical comparison of the different treatments.

2.4.5. Determination of GPx1 activity in hepatocyte cytosol

The activity of GPx1 in hepatocyte cytosol was measured according to the method described by Lei et al. [32], with *tert*-butyl hydroperoxide (*t*-Bu-OOH) as the peroxide substrate. Briefly, 50 μl of hepatocyte lysate was transferred to a 3-ml quartz cuvette containing 1900 μl of the reaction mixture [50 mM Tris–HCl, pH 7.5, 2 mM EDTA, 0.1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2 mM GSH, 1 mM NaN_3 and 0.9 U of GSH reductase (Sigma)]. The reaction mixture was preincubated for 3 min at 25°C, and the reaction was initiated by adding 50 μl of *t*-Bu-OOH (8 mM). The rate of oxidation of NADPH was monitored in a spectrophotometer at 340 nm for 5 min at 25°C. The nonenzymatic reaction rate was determined by substituting water (serving as the blank) for the hepatocyte lysate and recording the decrease in NADPH absorbance. One unit of enzyme activity was defined as 1 μmol of NADPH oxidized per minute under these conditions. The activity of GPx1 in hepatocyte cytosol was expressed as units per gram of protein. All samples were measured in duplicate.

2.5. Statistical analysis

Statistical analysis of LDH activity, GSH concentration, GPx1 activity and mRNA level was performed using the SPSS 11.5 for Windows statistical software package (SPSS Inc., USA). When a significant value (P<.05) was obtained by one-way analysis of variance (ANOVA), further analysis was done. All data showed a normal distribution and passed equal variance testing. Differences between means were assessed by the Tukey's honestly significant difference test of post hoc multiple comparisons. Data were expressed as mean ±S.D. Differences were considered as significant at P<.05.

3. Results

3.1. LDH release into the culture medium

The effects of different forms and concentrations of Se on release of LDH into the culture medium are shown in Fig. 1. A significant reduction in LDH release was observed for primary cultured bovine hepatocytes incubated with Se-Met at doses of 0.5–5 μmol/L for 24 h (P<.01, vs. control). In the hepatocytes incubated with Na_2SeO_3, significantly lower LDH release was observed at doses of 0.5 (P<.01) and 1 (P<.05) μmol/L (vs. control) and significantly higher LDH release was observed at doses of 2, 3, 4 or 5 μmol/L (P<.01, vs. control); the response occurred in a dose-dependent manner. The release of LDH was significantly reduced by Se-Car at a dose of 0.5 μmol/L (P<.01), but not at doses of 1, 1.5 or 2 μmol/L (vs. control). In contrast, significantly increased release of LDH was measured when hepatocytes were supplemented with 3, 4 or 5 μmol/L of Se-Car (P<.01, vs. control). Furthermore, the release of LDH induced by Na_2SeO_3 at doses of 3, 4 or 5 μmol/L was significantly higher than that induced by Se-Car at equivalent doses. Cell detachments were not observed by light microscopy in the hepatocytes incubated with Na_2SeO_3 at a dose of 5 μmol/L.

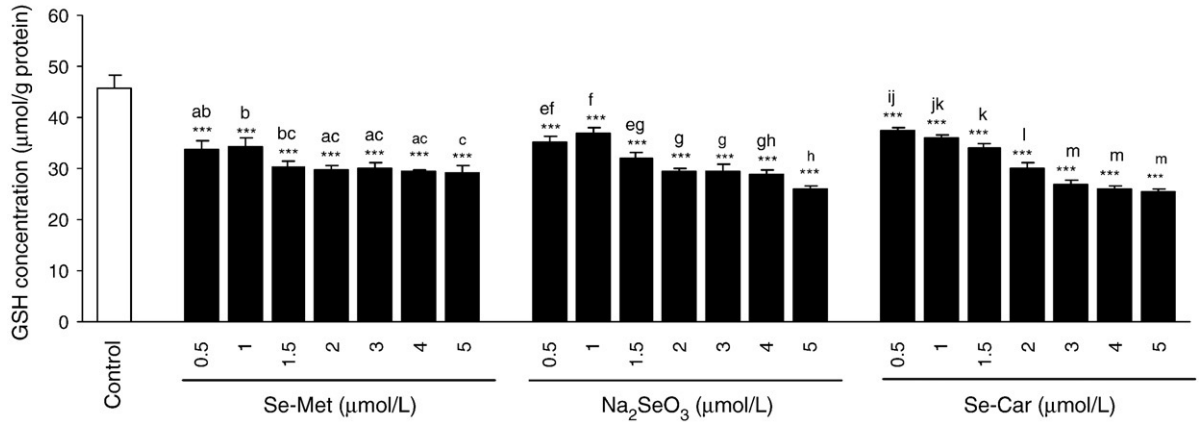

Fig. 2. Effects of different forms and concentrations of Se on GSH concentration in hepatocyte cytosol. Hepatocyte cytosol was obtained from primary cultured bovine hepatocyte monolayers supplemented with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/L of Se as Se-Met, Na_2SeO_3 or Se-Car for 24 h. Total GSH concentration in hepatocyte cytosol was measured by a spectrophotometric method. Bars represent mean±S.D. of triplicate cultures. Bars with "***" are statistically significantly different from control by one-way ANOVA followed by Tukey's multiple comparison test (***P<.001). Within the groups treated with different levels of Se-Met, Na_2SeO_3 or Se-Car, bars sharing a common letter are not significantly different (P>.05).

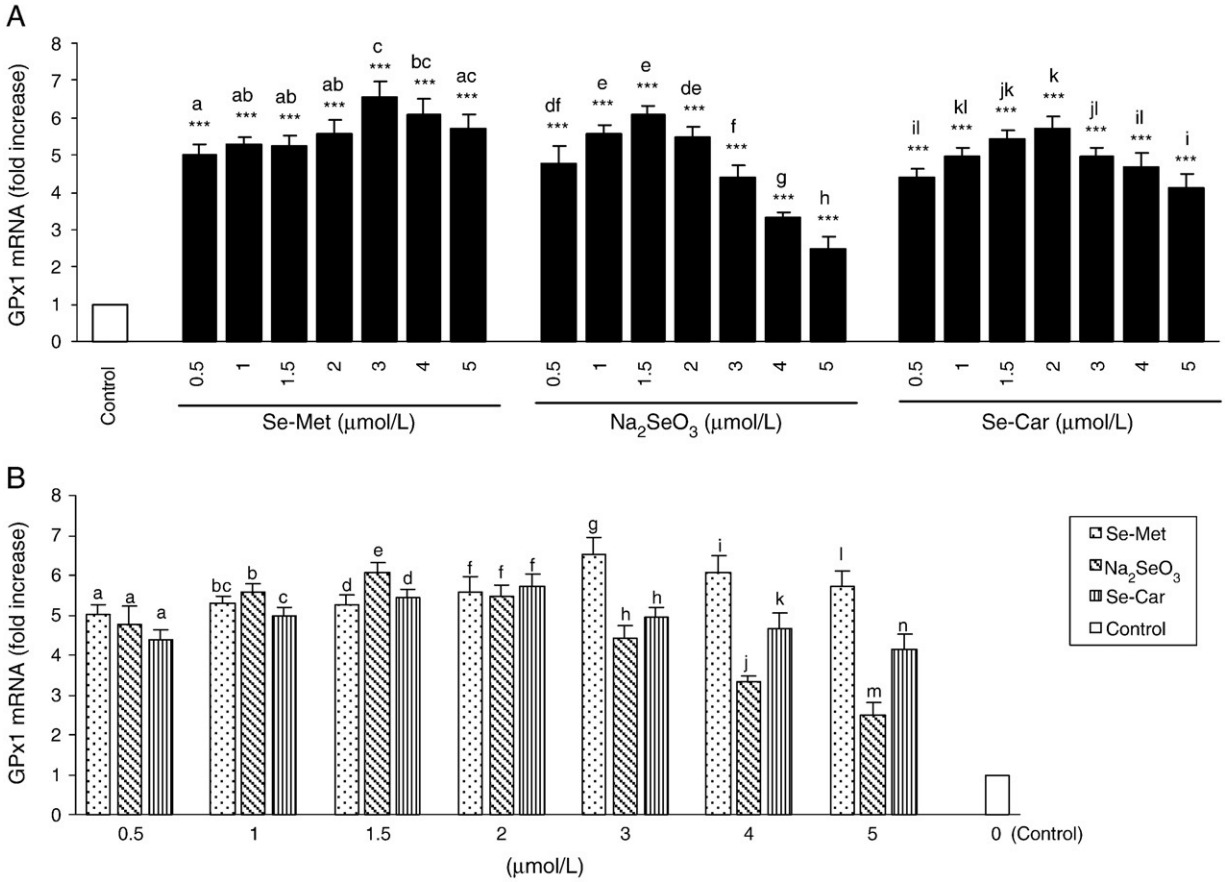

Fig. 3. Effects of different forms and concentrations of Se on GPx1 mRNA in bovine hepatocytes. The primary cultured bovine hepatocyte monolayers were treated with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/L of Se as Se-Met, Na_2SeO_3 or Se-Car for 24 h. GPx1 mRNA in bovine hepatocytes was measured by quantitative real-time RT-PCR, and the ratio of the level of GPx1 mRNA to that of the β-actin internal control was used for statistical comparison. Bars represent mean±S.D. of triplicate cultures. Bars with "***" are statistically significantly different from control by one-way ANOVA followed by Tukey's multiple comparison test (***P<.001). Within the groups treated with different concentrations of Se-Met, Na_2SeO_3 or Se-Car, bars sharing a common letter are not significantly different (P>.05) (A). In the hepatocytes supplemented with Se as Se-Met, Na_2SeO_3 or Se-Car at the same dose, bars sharing a common letter are not significantly different (P>.05) (B).

3.2. Changes in intracellular GSH concentration

The effects of different forms and concentrations of Se on the intracellular concentration of GSH are shown in Fig. 2. In all Se-treated hepatocytes, intracellular concentrations of GSH were significantly lower than control ($P<.001$). Increasing doses of Se-Met in the culture medium (1.5–5 μmol/L) did not lead to further significant reduction of intracellular GSH concentrations. In contrast, intracellular concentrations of GSH in the hepatocytes incubated with increased levels of Na_2SeO_3 or Se-Car were decreased in a dose-dependent manner.

3.3. Effect of Se supplementation on GPx1 mRNA level

The levels of GPx1 mRNA measured by quantitative RT-PCR are shown in Fig. 3. When compared with control, significant increases of GPx1 mRNA were observed in all groups treated with Se-Met, Na_2SeO_3 or Se-Car ($P<.001$) (Fig. 3A). The maximal increases of GPx1 mRNA were observed in the hepatocytes incubated with Se-Met at a dose of 3 μmol/L (about 6.54-fold vs. control), Na_2SeO_3 at a dose of 1.5 μmol/L (about 6.08-fold vs. control) or Se-Car at a dose of 2 μmol/L (about 5.72-fold vs. control) (Fig. 3A). After reaching a maximal level, increased Se supplementation led to a reduction in GPx1 mRNA (Fig. 3A). The degree of reduction of GPx1 mRNA was relatively mild in the groups treated with Se-Met at doses of 4 and 5 μmol/L when compared with the groups treated with Na_2SeO_3 and Se-Car at equivalent doses (Fig. 3B). Na_2SeO_3 had a relatively greater effect on GPx1 mRNA than Se-Met and Se-Car when supplemented at a level equivalent to a dose of Se of 1.5 μmol/L ($P<.05$) (Fig. 3B). Se-Met had greater effects on GPx1 mRNA than did Na_2SeO_3 and Se-Car at doses of 3, 4 and 5 μmol/L ($P<.001$) (Fig. 3B). Furthermore, 3 μmol/L of Se from Se-Met resulted in the highest level of GPx1 mRNA of all the groups treated with Se (Fig. 3B).

3.4. Effect of Se supplementation on GPx1 activity

The effects of different forms and concentrations of Se on the activity of GPx1 are shown in Fig. 4. Compared with control, the supplementation of Se-Met, Na_2SeO_3 or Se-Car

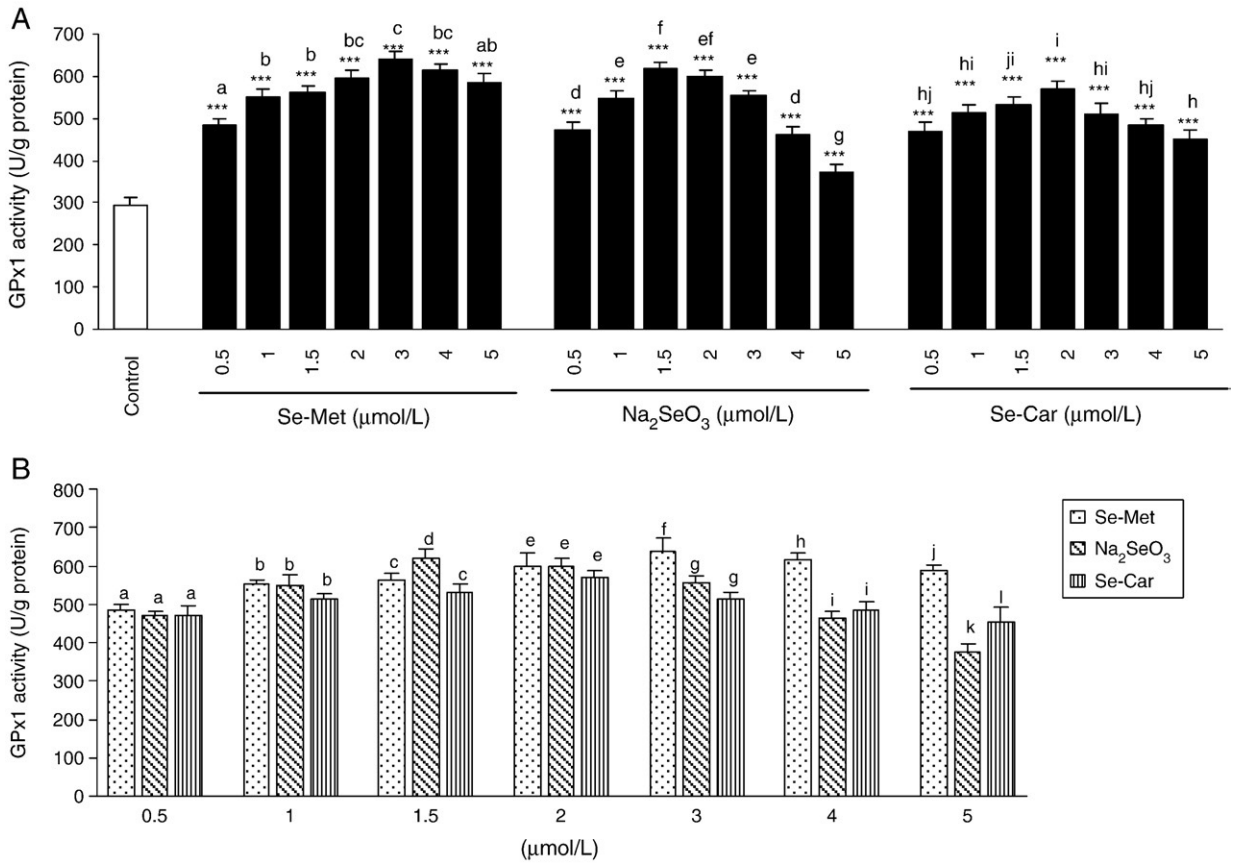

Fig. 4. Effects of different forms and concentrations of Se on GPx1 activity in bovine hepatocytes. The primary cultured bovine hepatocyte monolayers were treated with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/L of Se as Se-Met, Na_2SeO_3 or Se-Car for 24 h. The activity of GPx1 in hepatocyte cytosol was measured using a spectrophotometric method and expressed as units per gram of protein. Bars represent mean±S.D. of triplicate cultures. Bars with "***" are statistically significantly different from control by one-way ANOVA followed by Tukey's multiple comparison test (***$P<.001$). Within the groups treated with different levels of Se-Met, Na_2SeO_3 or Se-Car, bars sharing a common letter are not significantly different ($P>.05$) (A). In the hepatocytes supplemented with the same Se level, bars sharing a common letter are not significantly different ($P>.05$) (B).

led to significant increase in GPx1 activity over the whole dose range ($P<001$) (Fig. 4A). In groups treated with Se-Met, there was a significant dose-dependent increase in GPx1 activity (vs. control), up to a maximum at a dose of 3 μmol/L (Fig. 4A). For Na_2SeO_3 supplementation, 1.5 μmol/L was the most effective dose, and a decreasing trend in GPx1 activity was observed at doses of 1.5–5 μmol/L, in a dose-dependent manner (Fig. 4A). In the hepatocytes treated with Se-Car, the maximal increase of GPx1 activity was observed at a dose of 2 μmol/L, and higher Se-Car supplementation led to a reduction in GPx1 activity (Fig. 4A). At 1.5 μmol/L of added Se, Na_2SeO_3 had a relatively greater effect on the activity of GPx1 than Se-Met and Se-Car ($P<05$) (Fig. 4B). However, Se-Met had greater effects on the activity of GPx1 than Na_2SeO_3 and Se-Car at doses of 3, 4 and 5 μmol/L ($P<001$) (Fig. 4B). Furthermore, 3 μmol/L of Se from Se-Met resulted in the highest level of activity of GPx1 (638.71 U/g protein) of all groups treated with Se (Fig. 4B). The degree of decrease in GPx1 activity was relatively mild in the hepatocytes incubated with Se-Met at doses of 4 and 5 μmol/L when compared with that in the groups treated with Na_2SeO_3 and Se-Car at equivalent doses (Fig. 4B). The activity of GPx1 showed a similar pattern to that of the level of GPx1 mRNA.

4. Discussion

Several chemical forms of Se have been observed in the environment and in diets. Se occurs in the +6 oxidation state as selenate, the +4 oxidation state as selenite, the 0 oxidation state as elemental Se, the −1 oxidation state as selenocystine and the −2 oxidation state as Se-Met. Not all of these forms are metabolized in the same way [33]. The inorganic forms of Se (e.g., sodium selenite) are reduced to the selenide state using reducing equivalents from GSH and NADPH. In contrast, the organic forms of Se (e.g., Se-Met) release Se in the selenide state as a result of catabolism. Se-Met is activated initially by adenosylation and then demethylated and converted to Sec via selenohomocysteine and selenocystathionine in an analogous way to Met, and the Sec formed is degraded further in the liver to serine and selenide [5]. Furthermore, Se-Met can be incorporated nonspecifically into proteins in place of Met. Selenide plays an intermediary role in Se metabolism and is either used for selenoprotein synthesis or methylated to dimethyl selenide and trimethylselenonium ions and exhaled or excreted [5]. Because the metabolic pathways in the body are different for the inorganic and organic forms of Se [33], the absorption and bioavailability may vary. Previous data have shown that selenate has lower biological activity and absorption than selenite [34]. Organic forms of Se possess higher bioavailability [21,22] and lower toxicity compared with inorganic forms of Se [19,20]. The results of this study supported these findings.

LDH is a stable cytosolic enzyme, which becomes extracellular when the cell membrane is damaged. Therefore, the integrity and viability of the cell membrane, and the level of cytotoxicity, can be analyzed by determining the activity of LDH in the culture supernatant. Se is an essential trace element, but it can be toxic at a level not much higher than the beneficial dose. Earlier research has shown that incubation of rat hepatocytes for 2 h with Se as Na_2SeO_3 in concentrations higher than 6.3 μmol/L led to a decrease in cell viability compared with control cells [35]. However, Park and Whanger [36] reported that isolated rat hepatocytes exposed to 200 μmol/L of Se as Na_2SeO_3 for 1 h only showed a 6% increase of LDH activity in the culture medium compared with controls. In the present study, concentrations of Se, supplied as Na_2SeO_3, of greater than 2 μmol/L led to a significant dose-dependent increase in the release of LDH, when compared with controls (Fig. 1). Similar results were obtained in hepatocytes treated with Se-Car when the dose of Se was greater than 3 μmol/L (Fig. 1). A major reason for this difference between experiments may be the duration of incubation. The toxicity of Se is very strongly related to the chemical form. Se from organic forms is less toxic than that from inorganic forms [18–20], and selenite (Se4+) is more toxic than selenate (Se6+) in vitro [37,38]. In this study, treatment with Se-Met (0.5–5 μmol/L), Na_2SeO_3 (0.5–1 μmol/L) or Se-Car (0.5 μmol/L) for 24 h caused a significant decrease in leakage of LDH compared with controls (Fig. 1), which suggests that supplementation of the culture medium with Se enhanced the membrane integrity and antioxidant ability of the cells. The application of 5 μmol/L Se-Met, <2 μmol/L Na_2SeO_3 and <3 μmol/L Se-Car did not result in cytotoxicity. Müller and Pallauf [39] also reported that Se as Na_2SeO_3 at a dose of 100 ng/ml (~1.27 μmol/L) had no cytotoxicity for primary cultured rabbit hepatocytes. The release of LDH induced by Na_2SeO_3 at doses of 3, 4 or 5 μmol/L was significantly higher than that induced by Se-Car at equivalent doses (Fig. 1). These results support the hypothesis that Se-Met is less toxic than Na_2SeO_3 and Se-Car and that Se-Car is slightly less toxic than Na_2SeO_3.

A ubiquitous thiol-containing tripeptide, GSH is one of the most potent cellular antioxidants and is mainly associated with the thiol group of the cysteine residue [40]. It acts as a cosubstrate of GPx and plays an important role in the scavenging of ROS, free radicals and reactive metabolites that may be generated under various conditions. In addition, GSH is an indicator of oxidative stress: its level is often increased in tissues or cells as an adaptive response in increased oxidative stress. It has been reported that deficiency of Se in rats leads to a decrease in hepatic GPx1 activity, in conjunction with an increase in the activity of hepatic glutathione *S*-transferase, and affects GSH metabolism by increasing its synthesis and release in the liver with a concomitant increase of plasma GSH concentration [41]. In the present study, incubation of bovine hepatocytes for 24 h with Se supplied as Se-Met, Na_2SeO_3 or Se-Car at doses of 0.5–5 μmol/L reduced intracellular GSH concentrations

compared with controls (Fig. 2). This observation may be attributable to the fact that Se supplementation of the culture medium enhanced the activity of antioxidant enzymes in the hepatocytes, and thus, oxidative stress was reduced. Previous research has indicated that increasing the Na_2SeO_3 concentration in the culture medium enhanced the activity of GPx1 and decreased the level of intracellular GSH in rabbit hepatocytes [39].

Previous research has also shown that the mRNA level and activity of GPx1 in Se-deficient rat livers were reduced to 6% and 1% of the Se-supplemented levels, respectively [32]. Supplementation with Se at 0.2 mg/kg of the diet was required to support the full expression of GPx1 in pig liver [26]. The maximal GPx1 activity in livers of both pregnant and lactating rats was obtained at 0.075 and 0.1 mg Se/kg of diet, respectively [27]. Using in vitro experiments, the activity of GPx1 was markedly enhanced by increasing the Se concentration in the culture medium of primary cultured rabbit hepatocytes [39], but we speculate that the GPx1 activity did not reach a plateau at 100 ng/ml (about 1.27 μmol/L) of Na_2SeO_3 supplementation. In the present study, the largest increases in the level and activity of GPx1 mRNA were observed in the primary cultured hepatocytes treated with Se-Met at a dose of 3 μmol/L, Na_2SeO_3 at a dose of 1.5 μmol/L or Se-Car at a dose of 2 μmol/L (Figs. 3A and 4A). However, recent results of the study by Romanowska et al. [42] indicated that the mRNA level and activity of GPx1 in HPL1D cells exhibited plateaus at 10 nM Na_2SeO_3 supplementation, with no further change at higher concentrations. Ebert et al. [43] reported that GPx1 activity was enhanced 1.8-fold in bone marrow stromal cells that were cultured in the presence of 100 nM Se supplied as Na_2SeO_3. Incubation of human umbilical vein endothelial cells with 1 μmol/L of Se supplied as Se-Met for 24 h led to a 65% increase in GPx1 activity but did not result in a significant change in the level of GPx1 mRNA [44]. In this study, after reaching the maximal effect, higher Se supplementation led to dose-dependent decreasing trends of the mRNA level and activity of GPx1 in the hepatocytes treated with Se-Met, Na_2SeO_3 or Se-Car (Figs. 3A and 4A). The decreasing trend was mild in hepatocytes treated with Se-Met, moderate in hepatocytes treated with Se-Car and severe in hepatocytes treated with Na_2SeO_3. Recent research has also shown that 1 μmol/L of Se supplied as Na_2SeO_3 significantly increased the activity of GPx1 in mouse hepatoma cells (about 2-fold), and GPx1 activity exhibited a decreasing trend at a dose of 2 μmol/L of Se supplied as Na_2SeO_3 [45]. Cytotoxicity was observed when Se-Car and Na_2SeO_3 were applied at doses over 3 and 2 μmol/L, respectively, while Se-Met, even at a dose of 5 μmol/L, did not show any cytotoxicity (Fig. 1). Thus, we attribute, at least in part, the discrepancy in the finding of a decreasing trend to the following facts: Se-Met is less toxic than Na_2SeO_3 and Se-Car, and Se-Car is slightly less toxic than Na_2SeO_3. Toxicity associated with Se leads to inhibition of cell growth [46], DNA fragmentation [47] and reduction of protein synthesis [38]. In addition, the effects of the three forms of Se on GPx1 activity were not significantly different when supplemented at Se doses of 0.5 and 1 μmol/L in this study (Fig. 4B). The effect of Na_2SeO_3 at a dose of 1.5 μmol/L was significantly higher than that of Se-Met and Se-Car at equivalent doses (Fig. 4B) and reached plateaus. The greatest effects of Se-Met and Se-Car were observed at doses of 3 and 2 μmol/L, respectively. The level of GPx1 mRNA and the activity of GPx1 induced by 3 μmol/L of Se-Met were the highest of those seen in all the groups treated with Se. These results suggest that different forms and doses of Se have varied effects on the regulation of the mRNA level and activity of GPx1.

Taken together, our data demonstrate that supplementation of the culture medium with Se can enhance the mRNA level and activity of GPx1 in bovine hepatocytes significantly. The roles of different forms and concentrations of Se in the regulation of mRNA level and activity of GPx1 differ in cultured cells. The optimal concentrations that support the full expression of GPx1 in bovine hepatocytes of the various forms of Se are also different.

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