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Regulation of expression and activity of selenoenzymes by different forms and concentrations of selenium in primary cultured chicken hepatocytes

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The expression and activity of selenoenzymes are regulated by Se. In the present study, the effects of different forms and concentrations of Se on the regulation of glutathione peroxidase (GPx) activity and phospholipid hydroperoxide GPx (GPx4) and type I deiodinase (D1) mRNA levels in chicken hepatocytes were evaluated. Primary cultured chicken hepatocyte monolayers derived from male White Leghorn chickens (aged 30–40 d) were incubated for 24 h with 0 (control), 0·5, 1, 1·5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-selenomethionine (Se-Met), κ-selenocarrageenan (Se-Car) or sodium selenite (Na_2SeO_3). Compared with the control, Se significantly increased GPx activity in all the hepatocytes, but the activity was not increased in the hepatocytes treated with 5 μmol/l of Na_2SeO_3, with maximal effects being observed at 2 μmol/l of Se-Met or Se-Car and at 1·5 μmol/l of Na_2SeO_3, respectively. Significant decreases in GPx4 mRNA levels were observed in all the hepatocytes treated with Se (*v.* control). The D1 mRNA levels were significantly increased in all the groups treated with Se (*v.* control), with maximal effects being observed at 1·5 μmol/l of Se-Met and at 0·5 μmol/l of Se-Car or Na_2SeO_3, respectively. Se-Met at doses of 1·5–5 μmol/l had a greater effect on D1 mRNA than Se-Car and Na_2SeO_3 at equivalent doses. After resulting in a maximal effect, higher Se supplementation led to a dose-dependent reduction in GPx activity and D1 mRNA levels in all the hepatocytes treated with Se. These results suggest that in chicken hepatocytes, the regulations of GPx and D1 by different forms and concentrations of Se vary.

Selenium: Glutathione peroxidase activity: Phospholipid hydroperoxide glutathione peroxidase mRNA: Type I deiodinase mRNA

Se is an essential trace element for animals and humans. Se deficiency is associated with numerous diseases such as Keshan disease and Kashin-Beck disease in humans[1,2], 'white muscle disease' in calves and lambs[3] and exudative diathesis in chicks[4]. Supplementation of Se can enhance resistance to oxidative stress[5], reproductive performance[6,7] and immune function[8–10], improve the yield and quality of meat in broilers[11], and protect against certain types of cancers[12–14].

The physiological functions of Se are mediated through various selenoproteins. Se is incorporated into selenoproteins as selenocysteine (Sec). The Sec residue is located at the active centre of the selenoenzymes, and is encoded by the UGA codon. The UGA codon is normally used as a translation termination codon. Incorporation of Sec into selenoproteins involves a read-through of the UGA codon. Se, a Sec incorporation sequence in the mRNA 3′-untranslated region, a Sec-specific transfer RNA^{Sec} and other translational cofactors are necessary for the read-through of the UGA codon[15–17]. The status of Se affects the stability[18] and translational efficiency of cytoplasmic mRNA of glutathione peroxidase (GPx)[19].

The family of GPx plays an important role in the protection of animals and humans against oxidative stress. Five members of the Se-dependent GPx family have been identified and sequenced in mammals[20]. In poultry, the phospholipid hydroperoxide GPx (GPx4, *EC* 1.11.1.12) gene has been identified and sequenced[21]. Cellular GPx (GPx1, *EC* 1.11.1.9) was first identified as a Se-dependent enzyme, and it can detoxify H_2O_2 and organic peroxides to water and corresponding alcohols using GSH as the hydrogen donor[22]. GPx1 is distributed ubiquitously in various tissues. GPx4 is a membrane-associated GPx which is expressed in various tissues[20]. GPx4 can use phospholipid hydroperoxides as well as H_2O_2 and other lipid hydroperoxides as substrates[22]. Unlike that in mammalian liver, the percentage of GPx4 activity to total GPx activity in poultry liver is high (28 %)[23]. Type I deiodinase (D1), belonging to the Se-dependent iodothyronine deiodinase family, is found predominantly in liver, kidneys and thyroid[24]. D1 can catalyse the production of triiodothyronine from thyroxine by outer-ring deiodination. Triiodothyronine is the most bioactive form of thyroid hormone, which regulates basal metabolism, differentiation and heat production. About 80 % of peripheral triiodothyronine is produced by the catalysis of D1.

Abbreviations: D1, type I deiodinase; GPx, glutathione peroxidase; GPx1, cellular GPx; GPx4, phospholipid hydroperoxide GPx; LDH, lactic dehydrogenase; Sec, selenocysteine; Se-Car, κ-selenocarrageenan; Se-Met, DL-selenomethionine.

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In rats, the mRNA levels and activities of GPx and D1 are highly correlated with the Se status[25]. Previous studies have shown that Se deficiency in the diet leads to significant decreases in the GPx3 activity and triiodothyronine concentration in plasma in chickens, and to a significant increase in thyroxine concentration in plasma[26,27]. Severe deficiency of Se in chickens resulted in depressions in the rate of growth and efficiency of feed utilisation[28]. Contrastingly, supplementation of Se in the diet increased the GPx activity in the plasma and tissues of turkeys, and 0·3 mg Se/kg diet was required for maximal GPx activity in the plasma and liver[29].

Supplementation of Se is a common practice in chicken production in China. Presently, addition of sodium selenite (Na_2SeO_3) to the chicken diets is a prevalent method. Some researchers have suggested that the bioavailability of organic forms of Se (e.g. Se-enriched yeast, which contains selenomethionine (Se-Met)) is higher than that of the inorganic forms of Se such as Na_2SeO_3[30–32]. However, there are only a few studies explaining how the mechanism of organic Se is better than that of inorganic Se in the supplementation of Se in the chicken diet. It is known that the chicken liver is the principal organ involved in the storage and metabolism of Se, and that it can synthesise GPx1, GPx4 and D1. However, little is known about the regulation of the activity and expression of selenoenzymes in the chicken liver, which contribute to the mechanism of action of organic Se in chickens. The purposes of the present study were to evaluate the effects of different forms and concentrations of Se on the regulation of GPx activity and GPx4 and D1 mRNA levels in primary cultured chicken hepatocytes, and to determine the optimal doses of different forms of Se for maximal GPx activity and maximal GPx4 and D1 mRNA levels *in vitro*.

Materials and methods

The experimental use of animals and procedures followed were approved by the Nanjing Agricultural University Animal Care Committee.

Isolation and culture of hepatocytes

Male White Leghorn chickens (aged 30–40 d) were used to obtain hepatocytes. Isolation of hepatocytes was performed using the collagenase perfusion method as described previously[33], with some modifications. Isolated cells were dispersed in 60 ml of serum-free L-15 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0·2 % (w/v) bovine serum albumin and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml amphotericin B) at 4°C. Purification of hepatocytes was performed according to the method described by Wu *et al.*[34]. The viability of the isolated hepatocytes was 92·3 (SD 1·4) % (*n* 3), which was determined by the 0·4 % trypan blue dye exclusion method, and the cell yield from each liver preparation was 4·1 (SD 0·82) $\times 10^8$ hepatocytes (*n* 3). Evaluation of the cells done using light microscopy indicated that about 95 % of the collected cells were hepatocytes.

Culturing of hepatocytes was performed according to the method described by Fujii *et al.*[33], with some modifications. Hepatocytes were seeded into six-well plates at a density of 2×10^6 viable cells per well in 2 ml of L-15 medium (supplemented with 5 % fetal bovine serum, 33 mM-HEPES, 2 mM-L-glutamine, 0·2 % (w/v) bovine serum albumin, 100 nM-dexamethasone, 5 mg/l transferrin, 1 μmol/l insulin and antibiotics, pH 7·65). Cells were cultured at 37°C in a humidified atmosphere. After a plating period of 4 h, cell monolayers were washed twice with Hanks' balanced salt solution, and 3 ml of fresh serum-free L-15 medium were added to each well. Thereafter, the medium was substituted with serum-free L-15 medium every 24 h.

Cell treatments

At 48 h after seeding, the culture medium was removed, and hepatocyte monolayers were washed three times with Hanks' balanced salt solution. Then, hepatocytes were grown in 3 ml of fresh serum-free L-15 medium, and were treated with 0 (control), 0·5, 1, 1·5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-Se-Met (Sigma, St Louis, MO, USA), κ-selenocarrageenan (Se-Car, The First Institute of Oceanography, Qingdao, China) or Na_2SeO_3 (Sigma). Following incubation for 24 h, the culture medium was collected into 2 ml Eppendorf tubes for assay of lactic dehydrogenase (LDH), and the cells were harvested for the analysis of enzyme activity and mRNA. Each treatment was done in six wells (three wells were used for the measurement of GPx activity, and the other wells were used for mRNA analysis) in three separate experiments.

Preparation of hepatocyte lysates

After 24 h of incubation with different forms and concentrations of Se, hepatocyte monolayers were washed three times with ice-cold Ca^{2+}/Mg^{2+}-free PBS, and were 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 ice-cold water, and centrifuged at 12 000 ***g*** for 15 min at 4°C. The supernatants were aliquoted and stored at −20°C for the subsequent analysis of GPx activity.

Analytical methods

Measurement of protein concentration of hepatocyte lysates. Total protein concentration was measured using a Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The results obtained for GPx activity were corrected for total protein concentration.

Measurement of lactic dehydrogenase activity in the incubation medium. Hepatocyte toxicity was analysed by measuring the LDH activity in the incubation medium. The culture medium was collected into 2 ml Eppendorf tubes at the end of the incubation, and was centrifuged at 12 000 ***g*** for 15 min at 4°C. The supernatants were stored at −20°C until analysis (within 3 d). The measurement of LDH activity in the incubation medium was performed as described previously[35]. One unit of enzyme activity was defined as 1 μmol of reduced nicotinamide adenine dinucleotide oxidised per min. The LDH activity in the culture medium was expressed as units per litre. All the samples were measured in duplicate.

Measurement of glutathione peroxidase activity in hepatocyte cytosol. The GPx activity in hepatocyte cytosol was

measured according to the method described by Lei *et al.*[36], using *tert*-butyl hydroperoxide as the peroxide substrate. Briefly, 50 μl of hepatocyte lysate were transferred into 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-NADPH, 2 mM-GSH, 1 mM-NaN_3 and 0·9 IU of glutathione reductase (Sigma)). The reaction mixture was preincubated for 3 min at 25°C, and the reaction was initiated by adding 50 μl of *tert*-butyl hydroperoxide (8 mM). The rate of oxidation of NADPH was monitored using a spectrophotometer at 340 nm for 5 min at 25°C. The non-enzymatic reaction rate was determined by substituting water (serving as the blank) with the hepatocyte lysate, and by recording the decrease in NADPH absorbance. One unit of enzyme activity was defined as 1 μmol of NADPH oxidised per min under these conditions. The GPx activity in hepatocyte cytosol was expressed as mU/mg of total cell protein. All the samples were measured in duplicate.

Measurement of phospholipid hydroperoxide glutathione peroxidase and type I deiodinase mRNA levels by quantitative RT-PCR. After 24 h of 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 before complementary DNA synthesis.

First-strand complementary DNA was synthesised from 2 μg of total RNA using Oligo-dT primers and SuperScript II RT (Invitrogen) according to the manufacturer's instructions. Synthesised complementary DNA was diluted ten times with sterile water, and stored at −20°C before use.

Primer Premier software (PREMIER Biosoft International, Palo Alto, CA, USA) was used to design specific primers for β-actin, GPx4 and D1 based on known chicken sequences (Table 1). Quantitative real-time PCR were performed on an ABI PRISM 7300 Detection System (Applied Biosystems, Foster City, CA, USA) according to the method described by Wu *et al.*[34], with some modifications. The PCR procedure for β-actin, GPx4 and D1 consisted of a step performed at 95°C for 2 min followed by forty cycles at 95°C for 15 s, 58°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 purity of the products. The calculation of the number of copies of each sample was performed from the respective standard curve using the 7300 system software. The ratio of mRNA levels of GPx4 and D1 to that of β-actin internal control was used for statistical comparison of the different treatments.

Statistical analysis

Statistical analysis of the LDH and GPx activities and GPx4 and D1 mRNA levels was performed using the SPSS 11.5 for Windows statistical software package (SPSS, Inc., Cary, NC, USA). When a significant value ($P<0·05$) was obtained using one-way ANOVA, further analysis was done. All the data showed a normal distribution and passed equal variance testing. Differences between means were assessed by Tukey's honestly significant difference test of *post hoc* multiple comparisons. Data were expressed as means and standard deviations. Differences were considered as significant at $P<0·05$.

Results

Effect of selenium supplementation on lactic dehydrogenase release

The effects of different forms and concentrations of Se on the release of LDH into the culture medium are shown in Fig. 1. Compared with the control, Se-Met at doses of 1–3 μmol/l ($P<0·05$) significantly lowered LDH release in the hepatocytes after incubation for 24 h, but at doses of 0·5, 4 or 5 μmol/l ($P>0·05$), it did not lower the LDH release. In the hepatocytes incubated with Se-Car, significantly higher LDH release was observed at doses of 3, 4 or 5 μmol/l ($P<0·05$, *v.* control). The release of LDH was significantly increased in the hepatocytes treated with 2, 3, 4 or 5 μmol/l of Na_2SeO_3 ($P<0·05$, *v.* control). The response occurred in a dose-dependent manner. The release of LDH induced by Na_2SeO_3 at doses of 2, 3, 4 or 5 μmol/l was significantly higher than that induced by Se-Met and Se-Car at equivalent doses, respectively. Furthermore, the release of LDH induced by Se-Car at doses of 3, 4 or 5 μmol/l was significantly higher than that induced by Se-Met at equivalent doses. Cell detachments were not observed during light microscopy in the hepatocytes incubated with Na_2SeO_3 at a dose of 5 μmol/l.

Effect of selenium supplementation on glutathione peroxidase activity

The effects of different forms and concentrations of Se on the GPx activity are shown in Fig. 2. Compared with the control,

Table 1. Primers used for quantitative real-time PCR

Primers	Nucleotide sequence (5′–3′)	Target gene	GenBank accession no.	PCR fragment length (bp)
β-Actin-F (forward)	tca cca cca cag ccg aga ga	β-Actin	NM_205518	72
β-Actin-R (reverse)	cga aat cca gtg cga cgt agc			
GPx4-F (forward)	cat cac caa cgt ggc gtc caa	GPx4	AF498316	92
GPx4-R (reverse)	gca gcc cct tct cag cgt atc			
D1-F (forward)	cgc tat acc aca ggc agt aat g	D1	Y11110	101
D1-R (reverse)	agt tta tcc tgc acc gta ttc t			

GPx4, phospholipid hydroperoxide glutathione peroxidase; D1, type I deiodinase.

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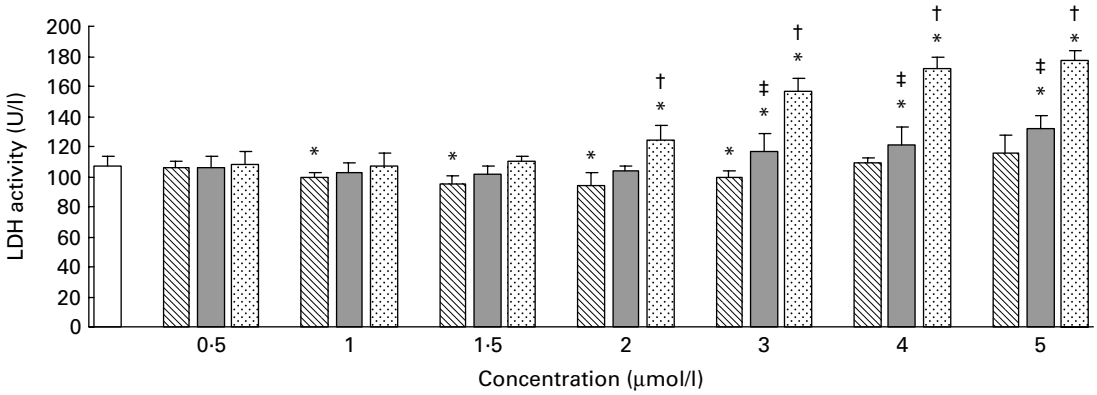

Fig. 1. Effects of different forms and concentrations of Se on lactic dehydrogenase (LDH) release from hepatocytes. The primary cultured chicken hepatocyte monolayers were incubated with 0 (control), 0·5, 1, 1·5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-selenomethionine (Se-Met), κ-selenocarrageenan (Se-Car) or sodium selenite (Na_2SeO_3) for 24 h. The LDH activity in the culture medium was used to evaluate the integrity of cell membrane. Bars represent means and standard deviations of triplicate culture media. * Mean values were significantly different from the control assessed by one-way ANOVA and then by Tukey's multiple comparison test ($P<0·05$). † Mean values were significantly different from Se-Met groups and Se-Car groups at equivalent doses ($P<0·05$). ‡ Mean values were significantly different from Se-Met groups at equivalent doses ($P<0·05$). □, Control; ▨, Se-Met; ■, Se-Car; ⊡, Na_2SeO_3.

Se-Met or Se-Car at doses of 0·5–5 μmol/l and Na_2SeO_3 at doses of 0·5–4 μmol/l significantly increased the GPx activity in the hepatocytes, but Na_2SeO_3 at a dose of 5 μmol/l did not increase the GPx activity ($P<0·05$). In the hepatocytes treated with Se-Met or Se-Car, a significant dose-dependent increase in GPx activity was observed (*v.* control), up to a maximum at a dose of 2 μmol/l. For Na_2SeO_3 supplementation, 1·5 μmol/l was found to be the most effective dose. After resulting in a maximal GPx activity, higher Se supplementation led to a dose-dependent reduction in GPx activity in all the groups treated with Se. The increases in GPx activity induced by Se-Met at doses of 0·5, 1, 2, 3 or 5 μmol/l were significantly higher than those induced by Se-Car and Na_2SeO_3 at equivalent doses ($P<0·05$), respectively. At 1·5 μmol/l of added Se, Na_2SeO_3 had a relatively greater effect on GPx activity than Se-Car ($P<0·05$), but the effect was not greater than that of Se-Met. However, Se-Car at doses of 2, 3, 4 or 5 μmol/l had a relatively greater effect on GPx activity than Na_2SeO_3 at equivalent doses ($P<0·05$). Moreover, 2 μmol/l of Se from Se-Met resulted in the highest GPx activity in all the groups treated with Se.

Effect of selenium supplementation on phospholipid hydroperoxide glutathione peroxidase mRNA levels

The GPx4 mRNA levels measured by quantitative RT-PCR are shown in Fig. 3. Compared with the control, Se-Met, Se-Car or Na_2SeO_3 significantly decreased GPx4 mRNA levels in a dose-dependent manner in all the groups ($P<0·05$). At 0·5–1·5 μmol/l of added Se, the GPx4 mRNA levels were not significantly different between the groups treated with Se-Met, Se-Car or Na_2SeO_3 ($P>0·05$). The GPx4 mRNA levels in the hepatocytes treated with Se-Met at doses of 2–5 μmol/l were higher than those in the hepatocytes treated with Se-Car or Na_2SeO_3 at equivalent doses.

Effect of selenium supplementation on type I deiodinase mRNA levels

The D1 mRNA levels measured by quantitative RT-PCR are shown in Fig. 4. Compared with the control, Se-Met, Se-Car or Na_2SeO_3 significantly increased the D1 mRNA levels in all the groups ($P<0·05$). The maximal D1 mRNA levels

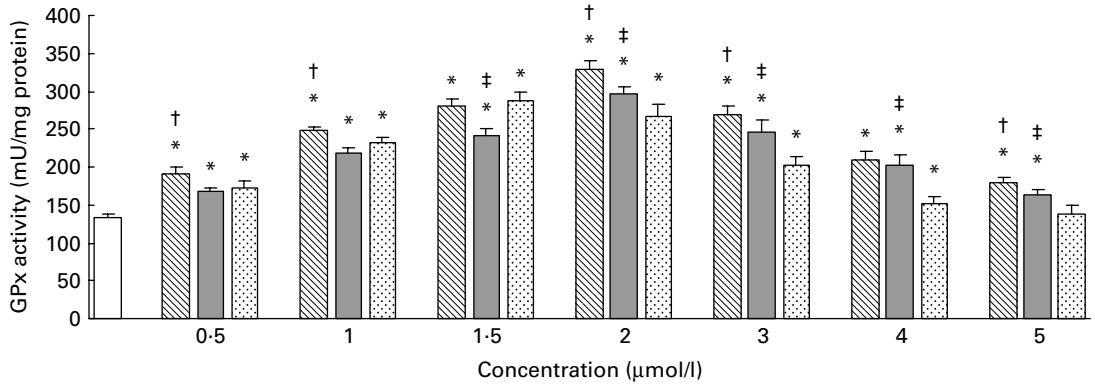

Fig. 2. Effects of different forms and concentrations of Se on glutathione peroxidase (GPx) activity in chicken hepatocytes. The primary cultured chicken hepatocyte monolayers were treated with 0 (control), 0·5, 1, 1·5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-selenomethionine (Se-Met), κ-selenocarrageenan (Se-Car) or sodium selenite (Na_2SeO_3) for 24 h. The GPx activity in hepatocyte cytosol was measured using a spectrophotometric method, and expressed as mU/mg protein. Bars represent means and standard deviations of triplicate cultures. * Mean values were significantly different from the control assessed by one-way ANOVA and then by Tukey's multiple comparison test ($P<0·05$). † Mean values were significantly different from Se-Car groups and Na_2SeO_3 groups at equivalent doses ($P<0·05$). ‡ Mean values were significantly different from Na_2SeO_3 groups at equivalent doses ($P<0·05$). □, Control; ▨, Se-Met; ■, Se-Car; ⊡, Na_2SeO_3.

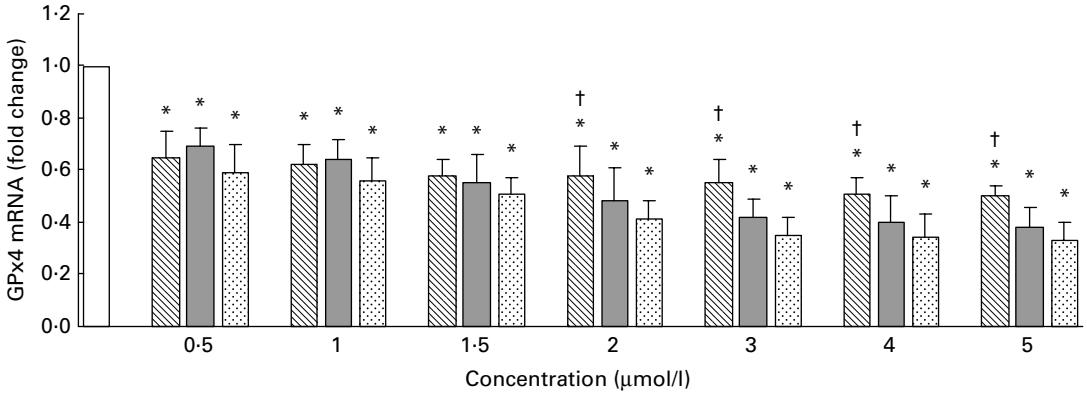

Fig. 3. Effects of different forms and concentrations of Se on phospholipid hydroperoxide glutathione peroxidase (GPx4) mRNA of chicken hepatocytes. The primary cultured chicken hepatocyte monolayers were treated with 0 (control), 0·5, 1, 1·5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-selenomethionine (Se-Met), κ-selenocarrageenan (Se-Car) or sodium selenite (Na_2SeO_3) for 24 h. GPx4 mRNA of chicken hepatocytes was measured by quantitative real-time RT-PCR, and the ratio of mRNA level of GPx4 to that of β-actin internal control was used for statistical comparison. Bars represent means and standard deviations of triplicate cultures. * Mean values were significantly different from the control assessed by one-way ANOVA and then by Tukey's multiple comparison test ($P<0·05$). † Mean values were significantly different from Se-Car groups and Na_2SeO_3 groups at equivalent doses ($P<0·05$). □, Control; ▨, Se-Met; ■, Se-Car; ⊡, Na_2SeO_3.

were observed in the hepatocytes treated with 1·5 μmol/l of Se-Met and 0·5 μmol/l of Se-Car or Na_2SeO_3 (*v.* control). After resulting in a maximal D1 mRNA level, higher Se supplementation led to a dose-dependent reduction in D1 mRNA levels in all the groups treated with Se. The degree of reduction in D1 mRNA levels was relatively small in the groups treated with Se-Met than in the groups treated with Se-Car or Na_2SeO_3. At 0·5 μmol/l of added Se, Se-Car and Na_2SeO_3 had a relatively greater effect on D1 mRNA than Se-Met ($P<0·05$). There were no differences between the hepatocytes treated with Se-Met, Se-Car and Na_2SeO_3 at a dose of 1 μmol/l. At 1·5–5 μmol/l of added Se, however, Se-Met had a relatively greater effect on D1 mRNA than Se-Car and Na_2SeO_3 ($P<0·05$). Moreover, Se-Car at doses of 4 or 5 μmol/l had a relatively greater effect on D1 mRNA than Na_2SeO_3 at equivalent doses ($P<0·05$).

Discussion

The population doubling time of the chicken hepatocytes during the logarithmic phase of growth was 46 h when they were cultured in the serum-free L-15 medium (data not shown).

The compounds of Se are generally classified into inorganic and organic forms. The primary metabolic pathway of Se in animals and cells is the reduction of the elemental form followed by methylation, producing methylselenol, dimethylselenide and trimethylselenonium cation[37,38], which are excreted through lungs or kidneys. Selenide plays an intermediary role in the process of Se metabolism, and is either used for selenoprotein synthesis or used for methylation[38]. There are some differences in the metabolism of inorganic and organic forms of Se. Inorganic forms of Se (e.g. Na_2SeO_3) are reduced

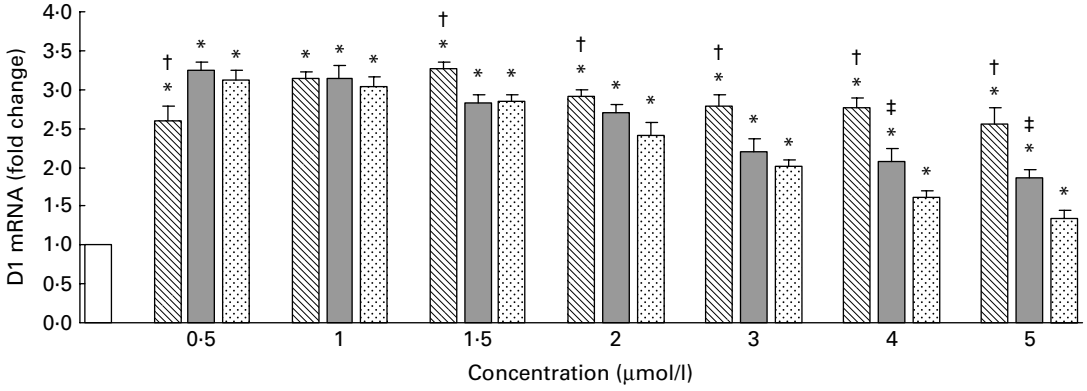

Fig. 4. Effects of different forms and concentrations of Se on type I deiodinase (D1) mRNA of chicken hepatocytes. The primary cultured chicken hepatocyte monolayers were treated with 0 (control), 0·5, 1, 1·5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-selenomethionine (Se-Met), κ-selenocarrageenan (Se-Car) or sodium selenite (Na_2SeO_3) for 24 h. D1 mRNA of chicken hepatocytes was measured by quantitative real-time RT-PCR, and the ratio of mRNA level of D1 to that of β-actin internal control was used for statistical comparison. Bars represent means and standard deviations of triplicate cultures. * Mean values were significantly different from the control assessed by one-way ANOVA and then by Tukey's multiple comparison test ($P<0·05$). † Mean values were significantly different from Se-Car groups and Na_2SeO_3 groups at equivalent doses ($P<0·05$). ‡ Mean values were significantly different from Na_2SeO_3 groups at equivalent doses ($P<0·05$). □, Control; ▨, Se-Met; ■, Se-Car; ⊡, Na_2SeO_3.

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to the selenide state using reducing equivalents from GSH and NADPH. Organic forms of Se (e.g. Se-Met) release Se in the selenide state as a result of catabolism. Moreover, Se-Met can be incorporated non-specifically into proteins as a substitute for sulphur-containing methionine. Greater tissue retention of organic Se than of inorganic Se from Na_2SeO_3 from Se-enriched yeast was observed in broilers[32,39] and swines[40]. The distribution of Se in different organs of laying hens fed Se-supplemented diets varied, and the liver was found to be the principal organ involved in the storage of Se[41]. The results of the present study showed that the regulations of the GPx activity and GPx4 and D1 mRNA levels by different forms of Se varied in primary cultured chicken hepatocytes. This may, in part, be caused by the metabolic differences of these forms of Se in the cultured cells.

Previous studies have suggested that organic forms of Se (e.g. Se-Met) are less toxic than inorganic forms of Se such as Na_2SeO_3[42,43]. LDH is a stable cytosolic enzyme, which becomes extracellular when the cell membrane is damaged. Therefore, the hepatocyte toxicity of the different forms of Se used in the present study can be analysed by measuring the LDH activity in the culture supernatant. The results of the present study are similar to those of our previous report[34], and suggest 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. Earlier research has shown that incubation of isolated rat hepatocytes for 2 h with Se supplied as Na_2SeO_3 at concentrations higher than 6·3 μmol/l led to a decrease in cell viability compared with the controls[44]. Cytotoxicity was not observed in the primary cultured rabbit hepatocytes treated with Na_2SeO_3 at a dose of 100 ng/ml (about 1·27 μmol/l)[45]. The results of the present study were similar to these findings.

Recent research has shown that supplementation with Se supplied as Na_2SeO_3 led to a significant increase in GPx activity in growing male turkey livers, and that 0·3 mg Se/kg diet was required for the maximal GPx activity in the liver[29]. Lei *et al.*[46] also reported that Se supplementation at a dose of 0·2 mg/kg diet was required to support the full expression of GPx1 in pig livers. The results of the study done by Salman *et al.*[47] indicated that the supplementation of organic Se to the diets containing adequate Se increased GPx activity in broiler livers and other tissues. In the present study, significant increases in GPx activity were observed in all the hepatocytes treated with Se except in the hepatocytes treated with 5 μmol/l of Na_2SeO_3. These results are similar to those of our previous research[34]. After resulting in a maximal effect, higher Se supplementation led to a dose-dependent decreasing trend of GPx activity in all the hepatocytes treated with Se. We believe that the decreases in GPx activity are related to the excess supplementation and cytotoxicity of Se. Toxicity associated with Se leads to the inhibition of cell growth[48], DNA fragmentation[49] and reduction in protein synthesis[50]. Ebert *et al.*[51] reported that GPx1 activity was enhanced by 1·8-fold in the bone marrow stromal cells cultured in the presence of 100 nM Se supplied as Na_2SeO_3. Supplementation of Se as Na_2SeO_3 (1 μmol/l) led to significant increases in GPx1 activity in mouse hepatoma cells (about 2-fold), and then GPx1 activity exhibited a decreasing trend at a dose of 2 μmol/l[52]. In the present study, the increases in GPx activity induced by Se-Met at doses of 0·5, 1, 2, 3 or 5 μmol/l were significantly higher than those induced by Se-Car and Na_2SeO_3 at equivalent doses ($P<0·05$). Se-Car at doses of 2, 3, 4 or 5 μmol/l had a relatively greater effect on GPx activity than Na_2SeO_3 at equivalent doses ($P<0·05$). These results are similar to those of our previous report[34], and suggest that different forms and concentrations of Se have varied effects on the regulation of the GPx activity in primary cultured chicken hepatocytes.

Sneddon *et al.*[53] reported that Se supplied as Na_2SeO_3 at a dose of 0·114 μmol/l led to an optimal GPx4 mRNA level in human umbilical vein endothelial cells. In HPL1D cells from human lung peripheral epithelium, a maximal GPx4 mRNA level was observed at 0·1 μmol/l of added Se supplied as Na_2SeO_3[54]. Because the mRNA sequences of GPx1 gene of poultry are not available in the GenBank and the primers of GPx1 cannot be designed, GPx1 mRNA levels in the cultured cells cannot be measured by quantitative RT-PCR. In the present study, significant decreases in GPx4 mRNA levels were observed in all the hepatocytes treated with Se-Met, Se-Car or Na_2SeO_3 compared with those treated with the control ($P<0·05$). These results suggest that the concentration of Se required for the maximal expression of GPx4 mRNA in the primary cultured chicken hepatocytes is relatively low, despite the different forms of Se (speculated $< 0·5$ μmol/l). Using *in vivo* experiments, it was found that Se requirement needed to reach the plateau level of GPx4 mRNA in rat livers was about at 0·033 mg/kg diet[36]. In addition, some researchers believed that Se deficiency had no effect on the expression of GPx4 mRNA in rat livers[19,25,55].

Supplementation of Se as Na_2SeO_3 (0·1 mg/kg diet) led to significant increases in the mRNA level and activity of D1 in rat livers, but Se supplementation above a nutritionally adequate level (2 mg/kg diet) reduced the enzyme activity[56]. The results of the study done by Bermano *et al.*[25] showed that mRNA level and activity of D1 in rat livers required 0·104 mg Se/kg diet to reach plateau levels. However, Sunde *et al.*[55] reported that dietary Se requirement needed to reach the plateau levels of D1 mRNA in the livers of pregnant and lactating rats was less than 0·01 mg/kg diet. In the present study, the maximal D1 mRNA levels were observed in the primary cultured chicken hepatocytes treated with 1·5 μmol/l of Se-Met (about 3·26-fold *v.* control) and 0·5 μmol/l of Se-Car (about 3·24-fold *v.* control) or Na_2SeO_3 (about 3·12-fold *v.* control). These results suggest that concentrations of different forms of Se required for a maximal D1 mRNA level vary in chicken hepatocytes. After resulting in a maximal D1 mRNA level, higher Se supplementation led to a dose-dependent reduction in D1 mRNA levels in all the groups treated with Se, but the degree of reduction in D1 mRNA levels was different between the groups treated with these three forms of Se. As mentioned earlier, the reduction in D1 mRNA levels may be related to excess supplementation and cytotoxicity of Se. At 0·5 μmol/l of added Se, Se-Car and Na_2SeO_3 had a relatively greater effect on D1 mRNA than Se-Met ($P<0·05$), while Se-Met at doses of 1·5–5 μmol/l had a relatively greater effect on D1 mRNA than Se-Car and Na_2SeO_3 at equivalent doses ($P<0·05$). These results suggest that regulations of D1 mRNA levels by different forms and concentrations of Se differ in primary cultured chicken hepatocytes.

In summary, these results demonstrate that supplementation of Se in the culture medium can increase GPx activity and D1 mRNA level in primary cultured chicken hepatocytes, while

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excess supplementation of Se causes cytotoxicity and negative effects. The regulations of GPx activity and D1 mRNA by different forms and concentrations of Se vary in chicken hepatocytes. The concentration of Se required for the maximal expression of D1 mRNA in chicken hepatocytes is higher than that of Se required for the maximal expression of GPx4 mRNA.

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