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Expression pattern of cytosolic glutathione peroxidase (cGPx) mRNA during mouse embryogenesis

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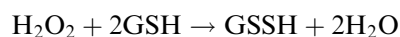
Abstract The selenoprotein cytosolic glutathione peroxidase (cGPx) is ubiquitously distributed in a variety of organs, and its primary function is to protect oxidative damage. To investigate the spatial and temporal expression pattern of cGPx mRNA in embryogenesis, as this has not been studied before, reverse transcription-polymerase chain reaction (RT-PCR) was carried out in a thermal cycler using mouse-specific cGPx primers, and in situ hybridization was performed in whole embryos or embryonic tissues using digoxigenin-labeled mouse cGPx riboprobes. Expression of cGPx mRNA was detected in all the embryos retrieved from embryonic days (EDs) 7.5 to 18.5. On EDs 10.5–12.5, cGPx mRNA was highly expressed in the margin of forelimb and hindlimb buds and dorsally in the cranial neural tube, including the telencephalon, diencephalon, and hindbrain neural tube. On ED 13.5, cGPx mRNA was accumulated especially in vibrissae, forelimb and hindlimb plates, tail, and spinal cord. On EDs 14.5–16.5, cGPx mRNA was found in the developing brain, Rathke's pouch, thymus, lung, and liver. On ED 17.5, the expression of cGPx mRNA was apparent in various tissues such as brain, submandibular gland, vibrissae, heart, lung, liver, stomach, intestine, pancreas, skin, and kidney. In particular, cGPx mRNA was greatly expressed in epithelial

linings and metabolically active sites such as whisker follicles, alveolar epithelium of lung, surface epithelium and glandular region of stomach, skin epithelium, and cortex and tubules of kidney. Overall results indicate that cGPx mRNA is expressed in developing embryos, cell-specifically and tissue-specifically, suggesting that cGPx may function to protect the embryo against reactive oxygen species and/or hydroperoxides massively produced by the intracellular or extracellular environment.

Keywords Selenoprotein · Antioxidant enzyme · Reactive oxygen species · Mouse embryo · In situ hybridization

Introduction

In mammals, reactive oxygen species (ROS) are continuously generated during aerobic tissue metabolism. Excessive generation of ROS in the cells can cause detrimental changes such as lipid peroxidation, DNA breakage, protein degradation, and enzyme inactivation. The ROS can be converted mainly into molecular oxygen and water by antioxidant enzymes and normally are kept at a minimum level in circulating blood and organ tissues (Fridovich and Freeman 1986; Fantel 1996; Gutteridge and Halliwell 2000). There are three main antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). GPx removes H₂O₂ by coupling its reduction to H₂O with oxidation of reduced glutathione (GSH), and it also removes organic hydroperoxides by coupling their reduction to H₂O and an alcohol with oxidation of GSH.



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The family of GPx comprises four distinct mammalian selenoproteins. Classical or cytosolic GPx (cGPx), the first discovered mammalian selenoprotein, is expressed in almost all tissues, including erythrocytes, kidney, liver, heart, lung, brain, lens, and so on (Mills 1957; Flohe et al. 1973; Rotruck et al. 1973; Frampton et al. 1987). Gastrointestinal GPx (GI-GPx) is expressed predominantly in liver, intestine, and colon (Chu and Esworthy 1995). Plasma GPx is detected in milk, plasma, and lung alveolar fluid (Takahashi et al. 1987). Phospholipid hydroperoxide GPx (PHGPx), an intracellular enzyme, can directly reduce lipid hydroperoxides in cell membrane and is highly expressed in testis (Imai and Nakagawa 2003).

Maternal selenium depletion leads to embryonic glutathione deficiency, thereby enhancing teratogenesis and death of embryos by phenytoin (Ozolins et al. 1996). Selenocysteine is incorporated into selenoproteins as directed by an opal nonsense codon in mammals studied (Chambers et al. 1986; Kryukov et al. 1999). A specific tRNA (*tRNA^{sec}*) is involved in the synthesis of selenoprotein. The mouse *tRNA^{sec}* gene (*Trsp*) has been localized to the proximal segment of chromosome 7. Targeting disruption of the *Trsp* causes peri-implantation lethality (Ohama et al. 1994; Bosl et al. 1997). *PHGPx* heterozygous mice appear to be normal even though they have a low level of PHGPx, whereas *PHGPx* homozygous embryos show early embryonic lethality (Imai and Nakagawa 2003; Imai et al. 2003). Therefore, selenoprotein including GPx may be essential for normal process of embryogenesis.

In mammals, birth immediately leads to exposure of the lung to oxygen, which increases the production of ROS. The lung responds to the increased ROS by increasing the activity of antioxidant enzymes. The developmental regulation of SOD enzymes is crucial for adaptation of fetuses to the relatively high oxygen environment after parturition. The level of mRNA and enzymatic activity for SOD slightly increases from birth to adulthood in the lungs of different species (Frank and Groseclose 1984; Hass and Massaro 1987; Yuan et al. 1996). This phenomenon is known as "preparation for birth." However, the increase in the activity of antioxidants does not appear exclusively at the preparation for birth in the lung, as evidenced by its lack of association with oxygen consumption and oxygen tension exposure of individual organs (de Haan et al. 1994).

The embryo undergoes apparent morphological changes during its ontogenesis, and many ROS can be produced in a cell-specific or tissue-specific manner by embryo metabolism associated with its ontogenic development. There is no clear-cut evidence for expression of antioxidants specifically during embryonic development. In the present study, we investigated the spatial and temporal expression of cGPx mRNA in whole embryos and developing organs using reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization analyses.

Materials and methods

Experimental animals

Male and female ICR mice (8–10 weeks old) were purchased from a commercial breeder, Biogenomics (Seoul, Korea). All animals were acclimated in polycarbonate cages for 1 week. They were kept in an environmentally controlled room with a temperature of $21 \pm 2^\circ\text{C}$, relative humidity of $55 \pm 10\%$, frequent ventilation of 10 times per hour, and a 12-h light/dark cycle. The animals were fed standard mouse chow (Samyang, Incheon, Korea) and tap water ad libitum throughout the experimental period. All animal experiments were conducted in compliance with the "Guide for care and use of laboratory animals" from the National Institutes of Health in the USA.

Preparation of embryos

One male and three females were housed in a cage for mating. The successful mating was confirmed by the presence of a copulatory vaginal plug. The day this plug was found was designated embryonic day (ED) 0.5. Under pentobarbital anesthesia, pregnant mice were sacrificed, and embryos were obtained from EDs 7.5 to 18.5. The embryos were used for RT-PCR and whole-mount (embryo) or tissue in situ hybridization analyses.

Total RNA extraction and PCR analysis

Total RNAs were isolated from mouse embryos using TRIzol reagent (Invitrogen, USA) according to the manufacturer's manual. The first-strand cDNA was synthesized using the SuperScript Preamplification system (Invitrogen, USA). To determine the expression pattern of cGPx mRNA in mouse embryos, cDNA was amplified by a thermal cycler (MJ Research, USA) using the primers 5'-tacattgttgagaagtgcg-3' and 5'-gacagcagggttctatgtc-3' (*S₁-AS₂*; 266 bp; Chambers et al. 1986). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used as an internal standard control (Lee et al. 2004). Results were analyzed with an AlphaEase version 5.5 analyzer system (Alpha Innotech, USA).

In situ hybridization

To prepare RNA probes for in situ hybridization, a pGEM-T easy plasmid (Promega, USA) containing a mouse cGPx cDNA clone (accession number: NM_008160, *S₁-AS₂* fragment; 266 bp) was linearized with *Spe* *square* or *Nco* *square* restriction enzyme. Digoxigenin (DIG; Roche, Germany)-labeled sense or antisense riboprobe for cGPx was obtained by in vitro

transcription in the presence of T7 or Sp6 RNA polymerase (Promega, USA) at 37°C for 120 min. In situ hybridization for tissue sections was performed as described previously (Braissnat and Wahli 1998). Tissue sections of mouse embryos (EDs 14.5–18.5) were placed on RNase-free glass slides and deparaffinized. The sections were incubated in phosphate-buffered saline (PBS) containing 10 µg/ml of proteinase K at 37°C for 10 min and treated with 4% paraformaldehyde (PFA) in PBS for 15 min. The hybridization reaction was carried out at 58°C for 17 h with 50 µl of hybridization mix on each section. Signal detection was performed at room temperature for 30 min to 1 day in detection buffer containing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT; DAKO, Japan) solution. Whole mount in situ hybridization was performed as previously described (Correia and Conlon 2001). Mice embryos on EDs 10.5–13.5 were fixed in 4% PFA/PBS overnight and dehydrated in methanol. They were then rehydrated, treated with proteinase K, rinsed, and postfixed with 4% PFA. Hybridization was performed at 68°C overnight. Specimens were rinsed and incubated with alkaline phosphatase-conjugated antidigoxigenin antibody diluted 1:2,000 in a blocking agent (Roche,

Germany). The antibody was detected with BCIP/NBT solution. The reaction was stopped with PBS and Tween (PBT), and the specimens were stored in 80% glycerol in PBT.

Results

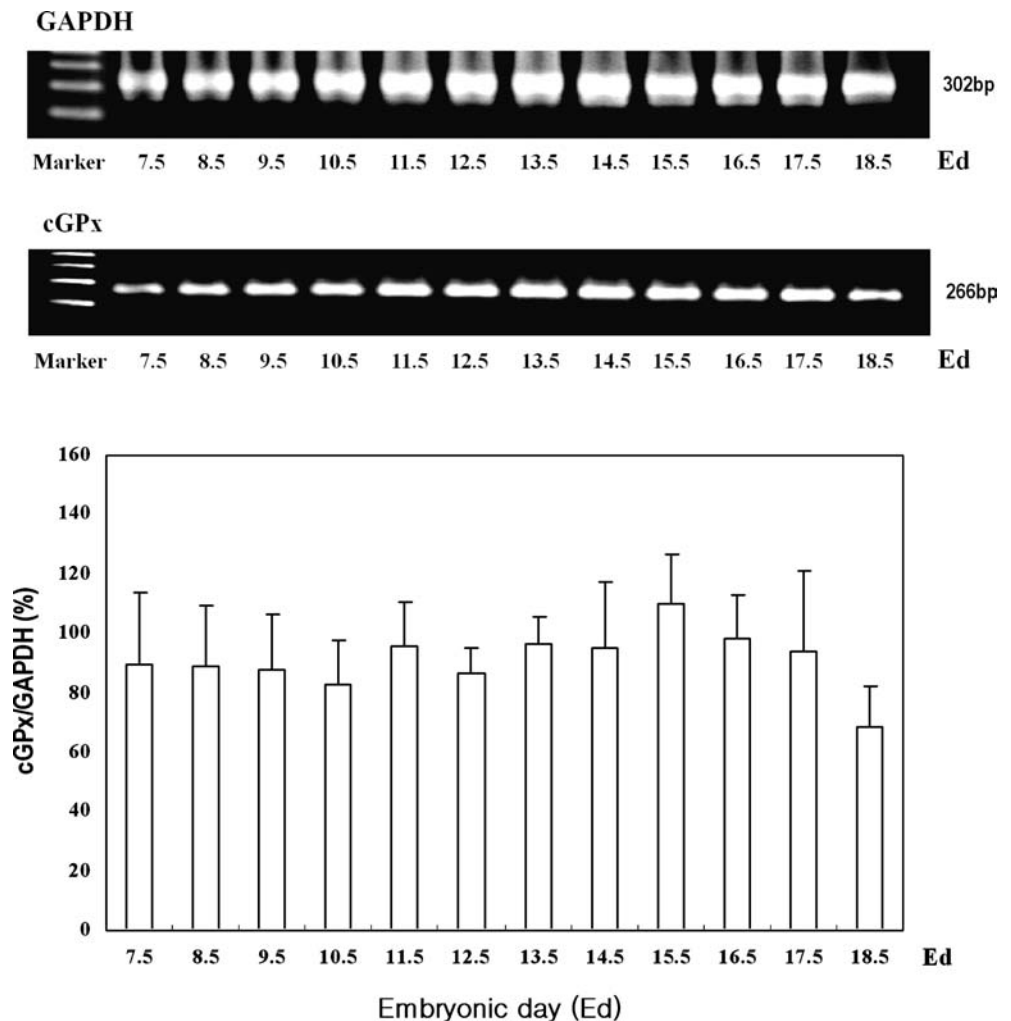
Expression level of cGPx mRNA during embryogenesis

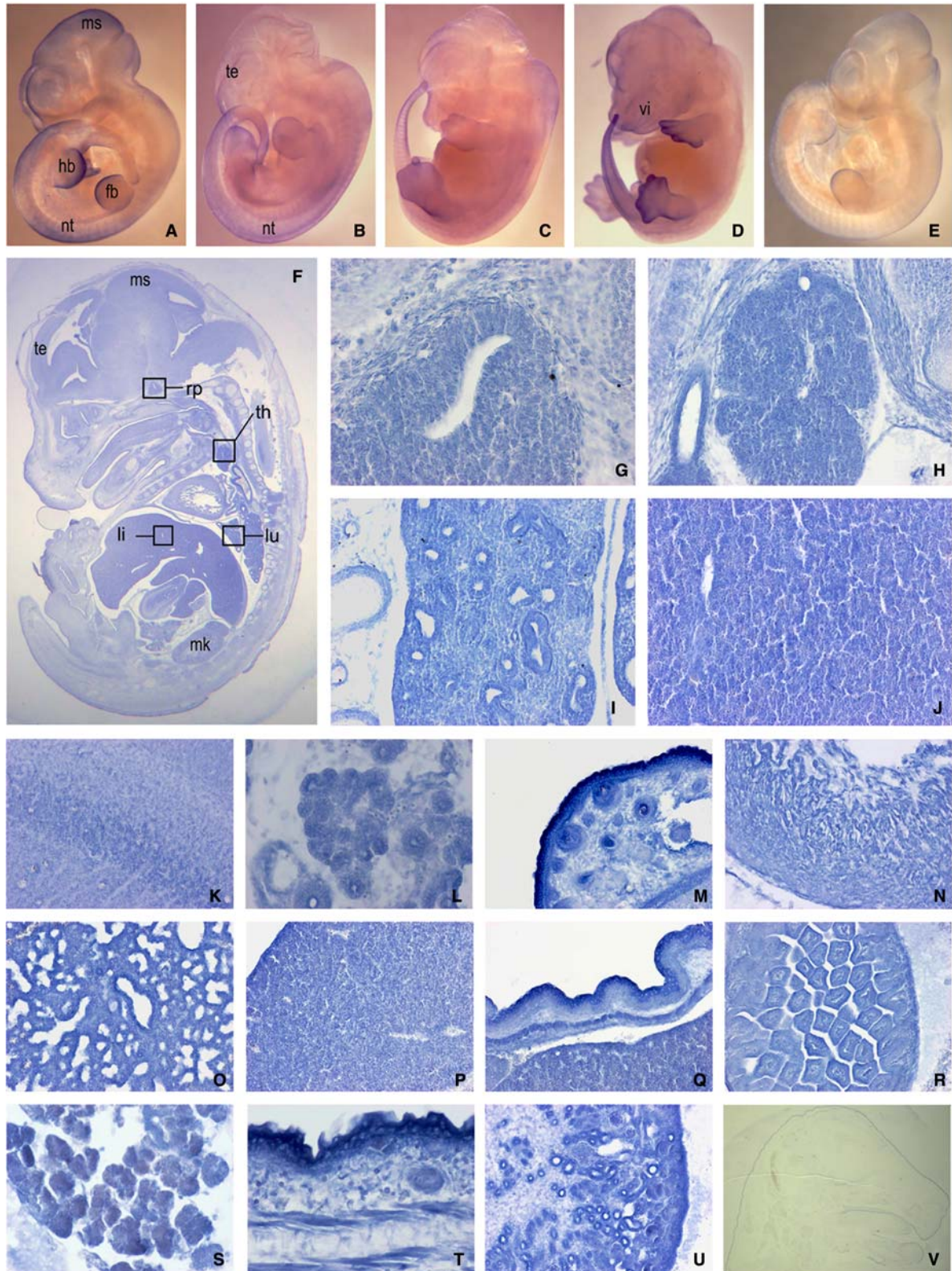
Temporal expression profiles of cGPx were examined by RT-PCR analysis. As shown in Fig. 1, the cGPx transcript was detected throughout all the embryonic stages from EDs 7.5 to 18.5. The cGPx signals peaked at ED 15.5 and slightly decreased thereafter. The primers for mouse GAPDH were used as an internal standard in the PCR analysis.

Localization of cGPx mRNA in whole embryos

The spatiotemporal expression pattern of mouse cGPx mRNA was investigated by whole mount in situ hybridization (Fig. 2a–e). At ED 10.5, cGPx mRNA

Fig. 1 Expression level of cGPx mRNA during mouse embryogenesis. Total RNAs isolated from embryos were reverse-transcribed, and the cDNAs were amplified by PCR using the mouse cGPx or GAPDH primers. The detected signal of cGPx mRNA was normalized using GAPDH mRNA. The expression of the cGPx mRNA appears throughout all the embryonic stages. Each data represent mean \pm SEM from five mouse embryos





was highly expressed in the margin of forelimb and hindlimb buds and dorsally in the cranial neural tube, including the telencephalon, diencephalon, and hind-

brain neural tube (a). At EDs 11.5–12.5, cGPx mRNA was mainly expressed in the margin of forelimb and hindlimb buds, telencephalon, and dorsal neural tube at

Fig. 2 Expression of cGPx mRNA during mouse development. Pictures **a–e** are viewed laterally. **a** Embryonic day (ED) 10.5 embryo showing expression in the margin of forelimb (*fb*) and hindlimb (*hb*) buds, mesencephalon (*ms*), and dorsal neural tube (*nt*). **b, c** EDs 11.5–12.5 embryos showing expression in the margin of forelimb and hindlimb buds, telencephalon (*te*), and dorsal neural tube (*nt*). **d** ED 13.5 embryo showing high expression in vibrissae (*vi*), forelimb and hindlimb plates, tail, and spinal cord. From ED 11.5 to 13.5, the expression of cGPx mRNA does not change dramatically during these stages, but it stabilized, showing the more intense pattern in the limbs. **e** cGPx mRNA is not detected in the ED 10.5 embryo using a DIG-labeled cGPx sense riboprobe. **f–j** Hybridization with a cGPx antisense riboprobe on a sagittal section of mouse embryos at ED 14.5. **f** Overview. Boxes are shown at higher magnification of the Rathke's pouch (**g**), thymus (**h**), lung (**l**), and liver (**j**). **k–u** Hybridization with a cGPx antisense probe on sagittal sections of developing tissues at ED 17.5. Higher magnification of the telencephalon of brain (**k**), submandibular gland (**l**), whisker follicles (**m**), heart (**n**), lung (**o**), liver (**p**), stomach (**q**), intestine (**r**), pancreas (**s**), skin (**t**), and kidney (**u**). (**g**, 200 times; **h–u**, 100 times). **v** Hybridization with a cGPx sense probe on sagittal sections of head part at ED 17.5. (*ms* mesencephalon, *te* telencephalon, *fb* forelimb bud, *hb* hindlimb bud, *nt* neural tube, *vi* vibrissae, *rp* Rathke's pouch, *th* thymus, *li* liver, *lu* lung, *mk* metanephric kidney)

the level of the hindlimbs (b, c). At ED 13.5, cGPx mRNA was highly expressed in vibrissae, forelimb and hindlimb plates, tail, and spinal cord (d). In addition, there were no apparent signals in in situ hybridization analysis using a DIG-labeled cGPx sense probe (e).

Tissue-specific expression of cGPx mRNA in developing embryo

The expression of cGPx mRNA was examined in the developing mouse tissues from EDs 14.5 to 17.5 using in situ hybridization (Fig. 2f–v). At ED 14.5, cGPx mRNA was expressed in the developing brains, including telencephalon, mesencephalon, and Rathke's pouch (f, g). Also, cGPx mRNA was strongly detected in thymus (h), lung (l), and liver (j), whereas it was weakly detected in heart, spinal cord, and metanephric kidney (f). At EDs 15.5–16.5, cGPx mRNA was persistently detected in the same regions as those at ED 14.5 (data not shown). At ED 17.5, the cGPx mRNA expression was detected in various tissues such as brain (k), submandibular gland (l), vibrissae (m), heart (n), lung (o), liver (p), stomach (q), intestine (r), pancreas (s), skin (t), and kidney (u). The expression patterns in the telencephalon of brain (k), heart, and liver were diffusely presented in all compartments. However, cGPx mRNA was greatly expressed in epithelial linings and metabolically active sites such as whisker follicles (m), alveolar epithelium of lung (o), surface epithelium and glandular region of stomach (q), skin epithelium (t), and cortex and tubules of kidney (u). On the other hand, there were no apparent signals on the analysis using DIG-labeled cGPx sense probe (v).

Discussion

ROS can be produced by normal embryo metabolism during embryogenesis (Goto et al. 1993). The production of ROS may interfere with the embryo redox status, thereby causing “oxidative stress,” which may alter essential cellular functions such as gene expression (Wasserman and Fahl 1997). The oxidative stress in embryos is known to stimulate gene expression of anti-oxidant enzymes, which protect early gametes and embryos against ROS damage during embryo development (Bavister and Boatman 1997). Most selenoproteins also participate in scavenging ROS. Previous reports demonstrated that selenoproteins are essential for early embryonic development (Ohama et al. 1994; Bosl et al. 1997). Homozygous mutation of thioredoxin gene in mice caused embryonic death in the preimplantation stage (Matsui et al. 1996). In addition, thioredoxin-2 mutant embryos showed massively increased apoptosis at ED 10.5 and completely disappeared at ED 12.5 from placenta (Nonn et al. 2003). On the other hand, mice inactivated by homologous recombination for GI-GPx and selenoprotein P showed no developmental defects (Esworthy et al. 2001; Hill et al. 2003). Furthermore, deficiencies of extracellular SOD and CuZnSOD did not affect the normal process of mammalian development, whereas MnSOD mutation induced growth retardation in C57BL/6J mice (Carlsson et al. 1995; Reaume et al. 1996; Huang et al. 2001). These findings suggest that the antioxidant enzymes may be regulated in various manners during embryogenesis.

Recent studies have shown that genetic inactivation of cGPx induced by high oxidative stress resulted in growth retardation in adulthood because of reduced mitochondrial energy production, indicating that the expression of cGPx may be essential for normal cellular function (Esposito et al. 2000; Lei 2001). In the present study, cGPx mRNA level was investigated at the ontogenic stage of mouse embryos using semiquantitative RT-PCR analysis. The cGPx mRNA was constantly expressed throughout all the embryonic stages. These findings suggest that the defense system of cGPx is conserved essentially for all steps of embryonic development.

Esposito et al. (2000) reported that X-gal staining of C57BL/6 mouse embryos at day 13.5 revealed *GPx1^{tm2Mgr}* expression in the liver, spinal cord, and eye and a distinctive pattern in brain consistent with brain stem. Analysis of adult tissues revealed the highest levels of expression in liver and kidney cortex. In the present study, cGPx mRNA was highly expressed in vibrissae, forelimb and hindlimb plates, tail, and spinal cord at ED 13.5 and in most organs including liver, kidney, and brain at ED 17.5. These findings indicate that cGPx is expressed tissue-specifically on the ontogenesis of mouse embryos.

The brain has been characterized by comparatively high SOD activity during mouse embryogenesis, but the

cGPx and CAT activities are low throughout development. In adult brain, cGPx activity increases with aging, suggesting an increase in organic hydroperoxide formation during aging (Hussain et al. 1995). Fetal exposure to many substances, including cocaine, phenytoin, calcium channel blockers, and nitric oxide synthase inhibitor, caused transient uteroplacental hypoperfusion and induced malformations of limb and central nervous system (CNS) (Fantel and Person 2002). In the present study, cGPx mRNA was mainly expressed in limb buds, dorsal neural tube, and brain at EDs 10.5–13.5. The expression of cGPx mRNA during the development of limb appeared first in the margin of limb bud and spread out to footplate. The cGPx mRNA expression during the development of brain was first detected in mesencephalon and thereafter observed in all CNS regions. These findings indicate that cGPx may act as an important antioxidant in limb and CNS development.

Lungs are exposed to relatively high oxygen tensions, especially at birth, which causes oxidative injury in the neonate who is more susceptible to oxidative stress than in adult (Araujo et al. 1998). Therefore, cGPx may act as a major protector against ROS massively produced by lung development. In the present study, cGPx mRNA was strongly expressed in developing lung tissues. In addition, the skin becomes the primary outer protective barrier. Ultraviolet (UV) light induces oxygen radicals responsible for adverse effects on the skin, including carcinogenesis. Selenium plays a protective role in UV-A damage of cultured skin fibroblasts (Leccia et al. 1993). The protective effect in skin may be mediated in part by the selenium-dependent synthesis of GPx (Kingsley et al. 1998). These findings indicate that selenium-dependent cGPx may be effective for preventing oxidative damage in embryonal skin. In the present study, cGPx mRNA was significantly expressed in epithelial cells of skin and whisker follicles. The signal was remarkably increased in the skin epithelium between ED 14.5 and ED 17.5.

After ED 14.5, cGPx mRNA expression was detected in various tissues including stomach, intestine, pancreas, kidney, lung, heart, liver, thymus, and Rathke's pouch. The cGPx mRNA was expressed in the glandular region and surface epithelium of stomach and the intestinal villae of small intestine. The intestinal crypt epithelial cells are very sensitive to abdominal and pelvic radiation therapy. The damage in the mucosal epithelium can result in a variety of symptoms, including diarrhea and electrolyte imbalance (Potten and Loeffler 1990). The expression of cGPx mRNA in renal tubules, glomeruli, and cortex was higher than in mesenchyme and medulla. Ischemia/reperfusion induces oxidative stress and consequently damages the proximal convoluted tubules of kidney in rats (Aragno et al. 2003). In this study, cGPx mRNA expression was massively found both in tissues in contact with the external environment and in additional epithelial tissues involved in absorption, transport, and excretion. These data imply that cGPx is

expressed mainly in metabolically active sites. Higher metabolic rates may result in the generation of ROS. cGPx activity was high in the epithelial linings of fetal and neonatal rat tissues (Asayama et al. 1996). These facts could be explained by cGPx being a general anti-oxidant enzyme in embryo.

The spatiotemporal expression of cGPx mRNA was observed in the forelimb and hindlimb buds, CNS, active metabolic tissues, and developing epithelial tissues. These findings are consistent with the hypothesis that cGPx functions to protect the embryo from oxygen radicals and/or hydroperoxides induced by the intracellular or extracellular environment. A specific role of cGPx relative to other antioxidants on the ontogeny of embryos remains to be further studied.

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