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Differential Expression of Glutathione Reductase and Cytosolic Glutathione Peroxidase, GPX1, in Developing Rat Lungs and Kidneys

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A mutant rat GPX1 (a cytosolic predominant form), in which the selenocysteine residue in the catalytic center was replaced by cysteine, was prepared and an antibody against the mutant enzyme was raised. The resultant antibody specifically reacted with rat GPX1 and was, together with the Glutathione reductase (GR) antibody, used in a Western blot analysis and immunohistochemistry experiments. To elucidate the physiological coupling of these enzymes under oxidative stress which accompanies the birth, developmental changes of the protein levels and enzymatic activities of GR and GPX1 were examined for lungs and kidneys from prenatal fetus to adult rats. The expression of GR was already evident at the prenatal stage and remained high in lungs at all stages. However, GR activity in kidneys gradually increased after birth reaching maximal levels at adulthood. An immunohistochemical study showed that GR was strongly bound to the bronchial epithelia in lungs and the epithelial cells of renal tubes. GPX1 was expressed in the renal tube epithelial cells and its level gradually increased after birth in a manner similar to that of GR. The expression of GPX1 in the lungs was, on the other hand, variable and occurred in some alveolar cells and bronchial epithelia only at restricted periods. It preferentially localized in nuclei at a late stage of development. Thus, the expression of the two functionally coupled enzymes via GSH did not appear to coordinate with development, tissue localization or under oxidative stress. Since many gene products show GSH-dependent preoxidase activity, other peroxidase(s) may be induced to compensate for the low GPX1 levels at stages with high GR expression.

Keywords: Glutathione; Glutathione reductase; Glutathione peroxidase; Oxidative stress; Immunohistochemistry

INTRODUCTION

Glutathione reductase (GR) is involved in reducing the oxidized form of glutathione (GSSG) back to the reduced form (GSH) in an NADPH-dependent manner and constitutes a major system for the maintenance of constant levels of GSH in most tissue.[1] GSH plays pleiotropic roles in living organisms, such as maintaining cells in a reduced state, detoxification of xenobiotics and as a source of reducing power for some antioxidative enzyme.[2] Thus, GR indirectly participates in protection of cells against oxidative stress and cytotoxic compounds and the maintenance of redox balance of the cells as well. Significant alterations in GSH levels under various physiological and pathological conditions have been reported.[3]

Glutathione peroxidases (GPXs) reduce various peroxides, which occur in cells using GSH as an electron donor and constitute a large family of enzyme.[4] These enzymes are classified into two groups; one of which contains selenocysteine (Sec) at its active center, while the other does not. At least four isozymes belong to the former group in mammals, and the cytosolic form, GPX1, is widely distributed in tissues and has been the most extensively investigated. GPX1, like other antioxidative enzymes, prevents apoptosis, which is induced by oxidative stress and other stimuli.[5] Since a selenol group of Sec is highly sensitive to oxidative

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modification, it can serve as a target of various chemicals such as nitrogen compounds[6,7] and dicarbonyl compounds.[8]

Although catalase exclusively detoxifies hydrogen peroxide only with no cofactor requirement, GPX requires GSH as a reducing equivalent.[4] Thus, it would be reasonably expected that the expression of GR and GPX would be coordinated, from the morphological and developmental points of views. In terms of oxidative stress, the lung is exposed to high concentrations of oxygen. Also, it is likely that kidney would be exposed to oxidized metabolites including lipid peroxides. Hence, antioxidative enzymes would play an important role in these tissues. Enzymatic activities of GR have been investigated in various tissues under physiological and pathological conditions,[1] but only a few of these reports contain detailed information on the precise distribution of the protein in tissues.[9–11] This is primarily due to the unavailability of a specific antibody. No report has appeared on the distribution of GR in lung and kidney. Although several immunohistochemical studies have been published for GPX1,[9,12,13] again, no reliable antibody against GPX1 is commercially available.[14] Hence, only the enzymatic activities of GR and GPX have been reported in most cases.

To investigate the role of the GSH/GR system, we have recently established an antibody against rat GR using the recombinant GR protein produced in a baculovirus/insect cell system.[15] A polyclonal antibody was also produced by making a recombinant rat GPX1 in which the Sec residue was substituted by Cys. By using these antibodies, we examined the distribution of GR and GPX1 in rat lungs and kidneys and developmental changes in their expression at prenatal and neonatal stages by immunohistochemistry as well as activity and protein assays. The results, however, showed that their expressions were not coordinated despite the expected functional coupling.

MATERIALS AND METHODS

Reagents and Animals

GSH and GSSG were obtained from Boehringer Mannheim and Sigma, respectively. NADPH was obtained from Oriental Yeast Co., Ltd. Other reagents were of the highest grade available. Adult (15–16 weeks old) and newborn Wister rats which were maintained under conventional conditions were used.

Cell Culture

Spodoptera frugiperda (Sf21) cells were maintained at 27°C in Grace's insect cell medium (Gibco/BRL) supplemented with 10% FCS, 3.3 mg/ml yeastolate, 3.3 mg/ml lactalbumin hydrolysate, and 50 μg/ml gentamicin as described[16] with minor modifications.[17]

Cloning and Sequencing of Rat GPX1 cDNA

Full length rat GPX1 cDNA was cloned from a rat liver λZAPII cDNA library (Stratagene) using the partial rat cDNA fragment described previously.[18] The full-length cDNA fragment was digested with *Bam* HI and then ligated to pBluescript (Stratagene). A DSQ 1000 apparatus (Shimadzu) was used for the DNA sequencing.

Site-directed Mutagenesis

CJ236 strain[19] was transformed with this pBluescript which carried the full-length rat GPX1 cDNA. Single stranded, uracyl-containing DNA templates were rescued from the culture medium of the transformed bacteria and used for site-directed mutagenesis as described previously.[20] The mutations were confirmed by DNA sequencing.

Generation of the Recombinant Baculovirus Carrying Rat Mutant GPX1 cDNA

The rat mutant GPX1 cDNA was excised from the resultant plasmid by digestion with *Bam* HI and ligated to the baculovirus transfer vector pVL1393 (Invitrogen). The transfer plasmid (1 μg) which contained the rat mutant GPX1 cDNA was purified by CsCl gradient ultracentrifugation and cotransfected with a baculovirus DNA (0.1 μg Baculogold, Pharmingen) into Sf21 cells using lipofectin (Gibco/BRL). After five days of incubation, the viral supernatant was harvested. The recombinant baculovirus was amplified three times.

Overproduction and Purification of Mutant GPX1 from the Infected Sf21 Cells

Sf21 cells were infected with the recombinant virus at a multiplicity of infection of three and incubated for 2–3 days. Purification of the recombinant mutant GPX1 was performed using two chromatographic procedures. Sf21 cells were harvested 72 h after infection and lysed at 4°C in 10 mM potassium phosphate buffer, pH 7.4 (buffer A). The cells were disintegrated with 20 strokes in a Dounce homogenizer and the lysate was centrifuged at 15,000 rpm for 20 min to remove insoluble materials. The supernatant was loaded onto a DE52 ion-exchanger column (Whatman) which had been pre-equilibrated with buffer A. The pass through fractions were pooled and concentrated on an Amicon membrane (PM-10) under N_2 pressure. The buffer was changed

to 50 mM potassium phosphate, pH 7.4, containing 50 mM NaCl. The fractions from the DE-52 column were subsequently applied to a Sephacryl S-200 (Pharmacia) gel filtration column.

Enzyme Assays

GPX activity was determined according to the method of Lawrence and Burk.[21] One unit is defined as the amount of enzyme which oxidizes 1 μmol of NADPH (corresponding to 2 μmol of GSH) per min. GR activity was assayed spectrophotometrically by measuring the rate of oxidation of NADPH at 340 nm.[1] The reaction mixture consisted of 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 0.1 mM NADPH and 1 mM GSSG, and the decrease in absorbance at 340 nm at 30°C was recorded. One unit of GR activity is defined as the amount of the enzyme that catalyzes the oxidation of 1 μmol of NADPH per min. Assays were performed in duplicate.

Preparation of Antibody to Rat GPX1

One hundred micrograms of the purified rat GPX1 protein was emulsified in Freund's complete adjuvant and injected into a lymph node of a female rabbit. An additional 10 μg of protein, in incomplete adjuvant, was injected subcutaneously at two-weeks intervals.

Preparation of Tissue Homogenates and Protein Assay

Experiments using rats were performed under the protocol approved by the Animal Research Committee, Yamagata University School of Medicine. Rats were killed by decapitation under anesthesia with diethyl ether. Tissues were homogenized in four volumes of PBS containing 10 μg/ml pepstatin, 10 μg/ml leupeptin, 100 μM APMSF and 1 mM benzamidine with a polytron homogenizer. After centrifugation at 10,000*g* for 20 min, the supernatant was collected and held at −20°C. Protein concentrations were determined using a BCA kit (Pierce), with BSA as a standard.

SDS-PAGE and Western Blot Analysis

Protein samples were subjected to 10–12% SDS-PAGE[22] and then transferred to nitrocellulose membranes (Shleicher and Schuell) under semi-dry conditions with the use of a Transfer-blot SD semi-dry transfer cell (Bio-Rad). After blocking by incubation with 5% skimmed milk in TBST (20 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) for 2 h at room temperature, the membranes were incubated with the rabbit antibody to either rat GR (1:1000 dilution)[15] or rat GPX1 antibody (1:500 dilution) for 12 h at 4°C. After washing with TBST, the membranes were incubated with 1:1000 diluted, peroxidase-conjugated goat anti-rabbit IgG (Organon Teknika Corp.) for 1 h. Following washing, the peroxidase activity was determined by the chemiluminescence method using an ECL kit (Amersham).

Immunohistochemistry

The SAB method was employed in these experiments. Briefly, paraffin-embedded tissue sections were treated with goat serum for 10 min to block non-specific binding and were then reacted with the antibodies specific for each enzyme for 60 min with 1:200 dilution. They were reacted sequentially with biotinylated, goat anti-(rabbit IgG), peroxidase-conjugated streptoavidin, and then the chromogen, 3,5-diaminobenzidine for 3 min. Finally, the samples were counterstained with Mayer's hematoxylin for 1 min.

RESULTS

Replacement of Sec to Cys in Rat GPX1 and Production of the Mutant Enzyme in the Baculovirus System

Since GPX1 has Sec in its catalytic center, it was not possible to produce recombinant protein on a large scale using conventional systems such as thioredoxin reductase.[23] Hence, we first replaced the codon for Sec with Cys in the rat GPX1 cDNA by means of site-directed mutagenesis. The mutant cDNA was then subcloned into the transfer vector and subjected to recombination with the baculovirus DNA in Sf21 cells. The mutant GPX1 was produced by infecting the recombinant virus and was then purified by two-step procedures involving a DE52 ion exchange column and a Sephacryl S-200 column (Fig. 1). The purified GPX1 exhibited a major band at 24 kDa and a minor band at 40 kDa, corresponding to the dimer. The specific activity was 15.7 mU/mg protein, which was about 1/2000 of the GPX1 purified from bovine erythrocytes and was virtually consistent with the reported value for the recombinant bovine GPX1 with the same mutation.[24]

Raising Antibody Against GPX1

Although we have recently established the antibody against rat GR,[15] a competent antibody against rat GPX1 is not currently available.[14] We, therefore, raised an antibody by immunizing the purified mutant GPX1 in a rabbit. The resulting antibody reacted with both proteins from the recombinant baculovirus-infected Sf21 cells and the purified

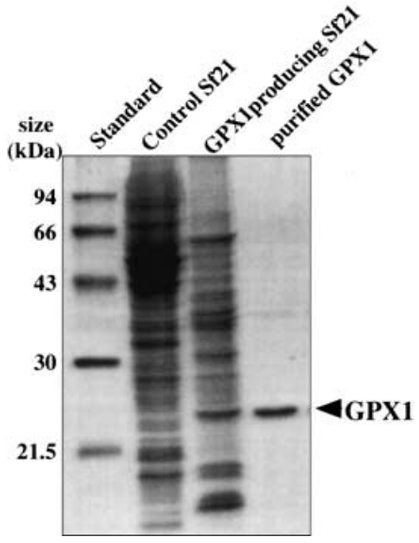


FIGURE 1 Production and purification of mutant rat GPX1 in the baculovirus system. Samples are: molecular size markers (lane 1), 30 μg of total cytosolic proteins from control cells (lane 2), cells infected with the baculovirus-carrying the mutant GPX1 cDNA (lane 3), 2 μg of the purified mutant GPX1 (lane 4). Proteins were detected by staining with Coomassie brilliant blue G250.

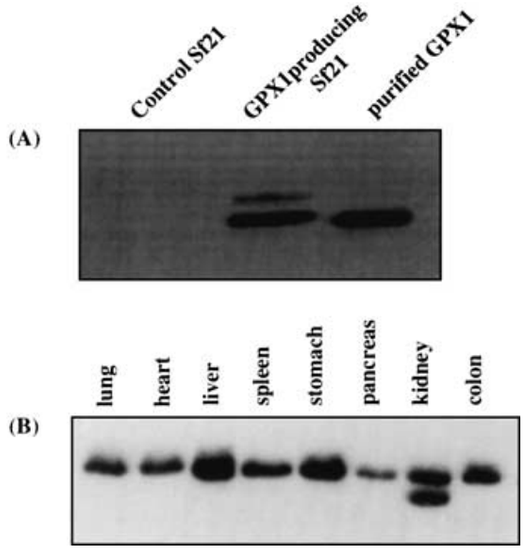


FIGURE 2 Western blots of rat tissues with the anti-GPX1 IgG. (A) The same samples as used in Fig. 1 were subjected to 12% SDS-PAGE and then blotted onto a nitrocellulose membrane. Western blot analysis was performed with 1:500 diluted GPX1 antiserum. (B) 30 μg of cytosolic proteins from the eight indicated tissues were subjected to Western blot analysis. The arrowheads indicate the position of GPX1 with molecular mass of 23 kDa.

protein (Fig. 2A). A minor reactive band with mobility lower than that of the purified protein was observed in the baculovirus-infected cell fraction. This, however, would also be derived from the produced GPX1 because no band was observed in non-infected, control cells.

The antibody to the Western blot was then applied to the analysis of eight tissues in the rat (Fig. 2B). The antibody detected one protein band in seven tissues, but clearly reacted with an extra band in the kidney proteins, which had a higher mobility in the gel. Since purified GPX1 from bovine erythrocytes contained GPX1 with a truncation at the N-terminal sequences,[25] this may also be due to the N-terminal heterogeneity, which appears to have no effect on enzyme activity. In the case of kidney, the two bands appeared to correspond to GPX1. Thus, the antibody was found to be specific to GPX1, and was used, along with anti-rat GR, for the following studies.

Developmental Changes of GR and GPX1 Activities

Since oxidative stress is significantly enhanced in neonates after birth, as the result of direct exposure to air, which contains approximately 20% oxygen, changes in the expression of GR and GPX1 in lungs and kidneys were examined from the fetal prenatal stage to the neonate stage, to determine if they were affected by environmental oxygen conditions. Figure 3 shows the activities of these enzymes at 3 days before birth (E19) to 16 days after birth (P16) as well as for 16 week-old adult rats. In lungs, while GR activities were substantially constant, GPX activities were transiently induced immediately after birth (P0), slightly decreased thereafter (from P3 to P16), and reaching the highest level at the adult stage. In kidneys, both GR and GPX activities gradually increased in course of the developmental periods, with the adult being maximal. The issue of whether the lower level of GR activity of P16, compared to early stages had physiological meaning or not remains unclear.

Developmental Changes of GR and GPX1 Proteins

While GSSG-reducing activity is specific for the GR protein, which is encoded by one gene, many gene products exhibit GSH-dependent peroxidase activities. We, therefore, performed a Western blot analysis in order to investigate the expression of GPX1 as well as GR in the same samples of the developing lungs and kidneys in rats (Fig. 4). Levels of GR protein were virtually consistent with their activities in both lungs and kidneys. While GPX activities roughly matched the levels of GPX1 protein in the lungs, they did not correspond to that in the kidney. This can be attributed to the contribution of other enzymes which also have GPX activity and which are also expressed in kidneys. Moreover, as also seen in Fig. 2B, an additional higher mobility band, which was not seen in lungs or in the fetal or new-born rat

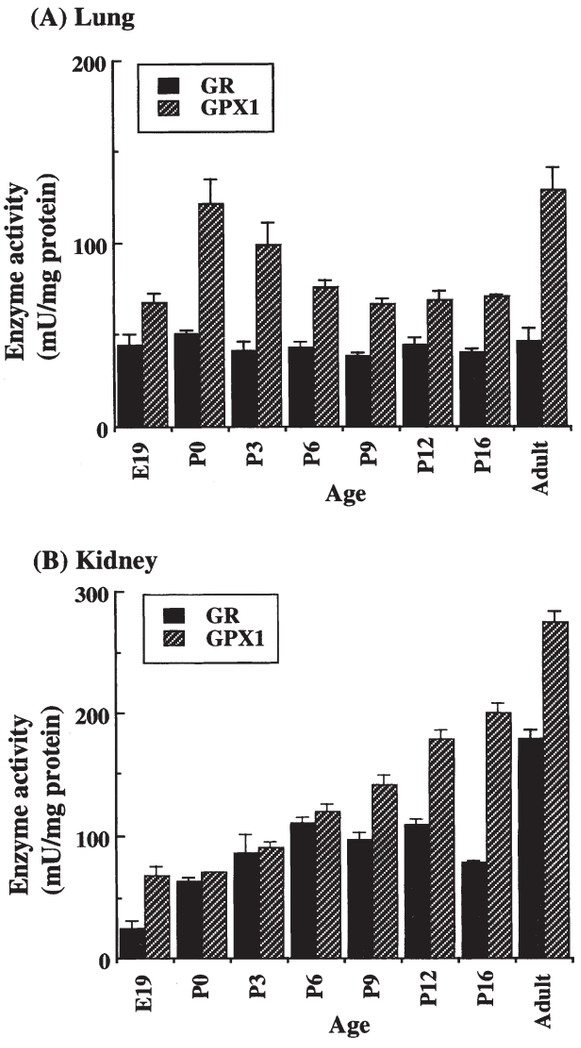


FIGURE 3 Developmental changes of GR and GPX activities in the lungs and the kidneys of the rat. Cytosolic fractions were prepared from three rat lungs (A) and kidneys (B) at the indicated ages. GR (closed columns) and GPX activities (hatched columns) were measured and are presented as mean ± S.D. of triplicate samples.

kidneys, was observed in kidney tissue three days after birth. Changes of the levels of the higher mobility protein were rather matched to GPX activity.

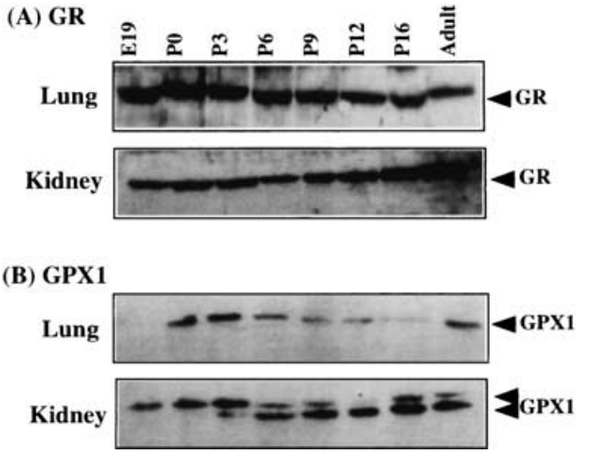


FIGURE 4 Developmental changes of proteins for GR and GPX1 in the lungs and the kidneys of the rat. About 40 μg of tissue homogenates were subjected to Western blot analysis. The nitrocellulose membranes were reacted with 1:1000 diluted GR (A) or GPX1 antisera (B). Arrowheads indicate positions of GR or GPX1.

Localization of GR and GPX1 in Lungs and Kidneys at Various Developmental Stages

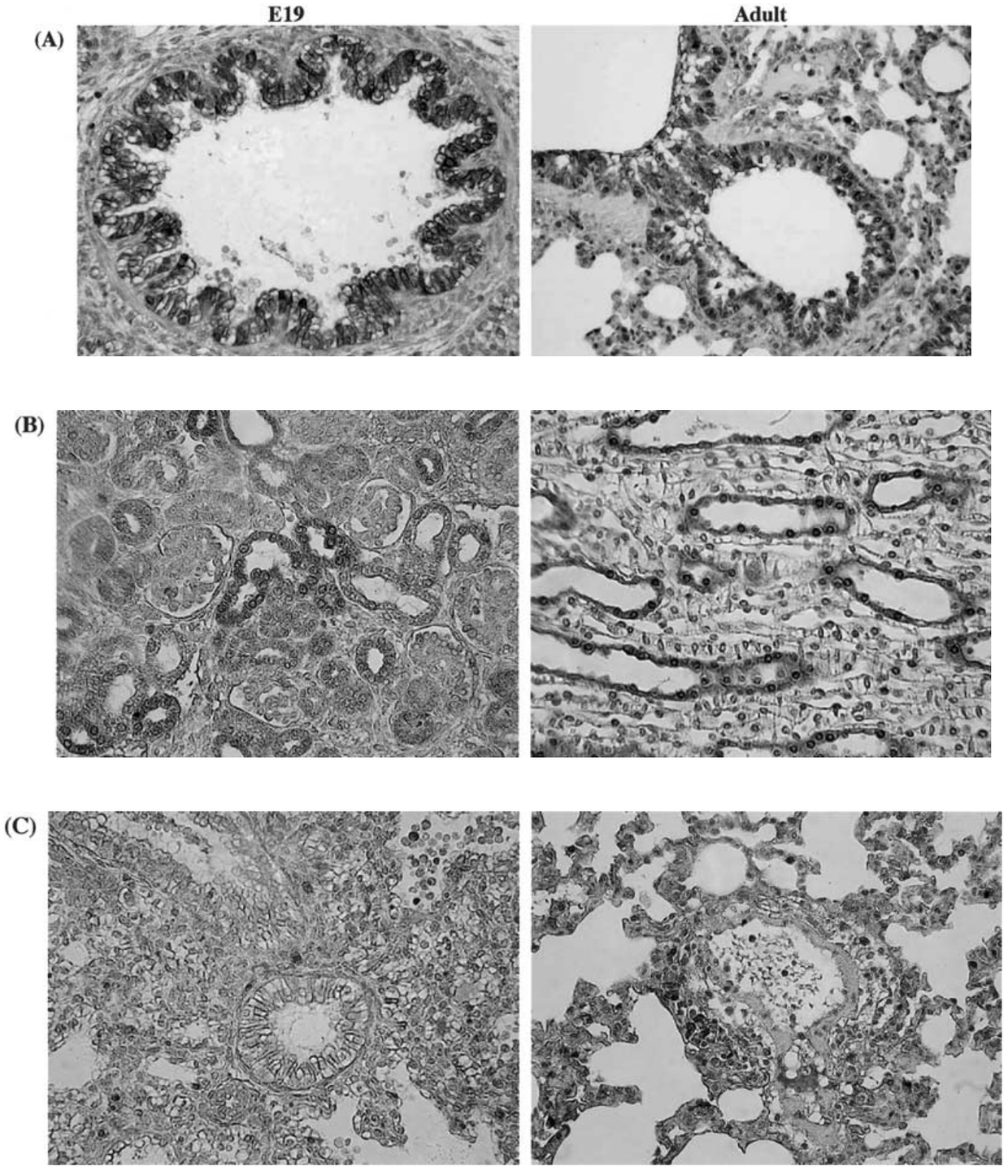
To investigate the localization of GR and GPX1 in these tissues, immunohistochemical examination was performed for rat lungs and kidneys. Typical data are shown in Fig. 5 and the results are summarized in Table I. The distribution of GR is high in the epithelium of bronchi and bronchioles in lungs and to a lesser extent in alveolar cells (Fig. 5A). The expression of GR was already evident at E19 and was maintained at high levels in both lungs and kidneys (Fig. 5B) through this period to adult ages, consistent with the observed.

The expression of GPX1 was, on the other hand, variable compared with that of GR. In lungs, the anti-GPX1 IgG was bound to some alveolar cells (Fig. 5C), but not to bronchial epithelia. The expression of GPX1 was negligible at E19, increased for the period of P3 to P6, and then gradually decreased. This occurred preferentially in nuclei at the later stage

TABLE I GR and GPX1 immunoreactivity in the developing lungs and kidneys of rats

Age		E19	P0	P3	P6	P9	P12	P16	Adult
GR									
Lung	Epithelium	+++	+++	+++	+++	+++	+++	+++	+++
	Alveolus	+	+	+	+	+	+	+	+
Kidney	Tubule	++	++	++	++	++	+++	+++	+++
	Cortex	–	–	+	+	++	++	++	+++
GPX1									
Lung	Epithelium*	–	+	++	++	+	+	+	+
	Alveolus	–	+	+	+	+	+†	+†	++†
Kidney	Tubule	–	+‡	+	+	++	+	+	+++
	Cortex	–	–	–	–	+	++	++	++

*Bronchus epithelium. †Some nuclei. ‡Some tubules.



of this period. In kidneys, the expression of GPX1 was observed in the epithelia of renal tubules and was high around P0, decreased afterwards, and then increased again after P12. At the later stage, not only tubular epithelia, but also the cortical region were strongly stained (Fig. 5D). Thus, more cells began to express GPX1 in the aging kidney and contributed to total GPX activities.

DISCUSSION

The data herein describes changes in enzymatic activities, protein levels and immunohistochemical localization of GR in developing rat lungs and kidneys, and compared their levels with those of GPX1 for the first time. Since oxidative stress in the lung increases markedly after birth, the physiological

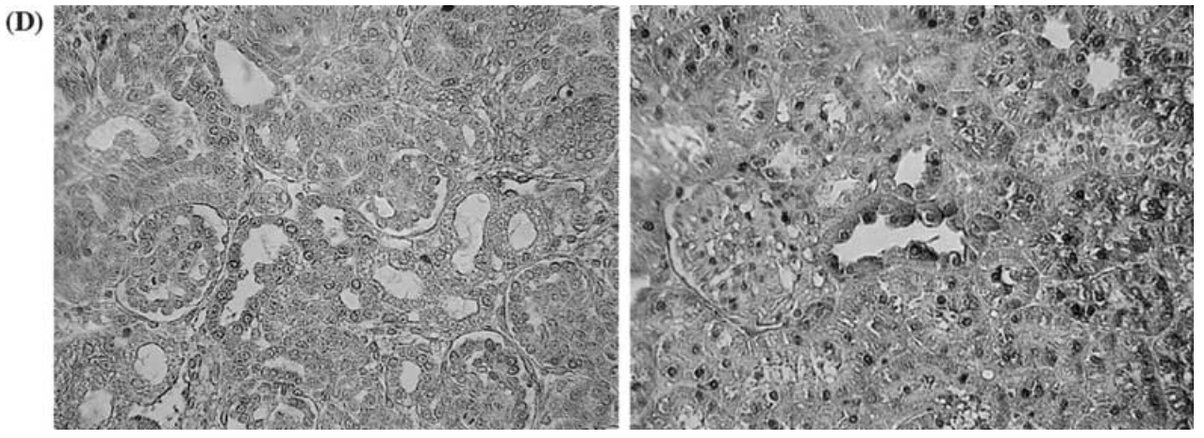


FIGURE 5 Immunostaining of rat lungs and kidneys at different stages of development. Tissue sections, lung (A and C) and kidney (B and D), were reacted with the antibodies specific for GR (A and B) and GPX1 (C and D) with 1:200 dilution. Left and right pictures in each panel represent E19 and adult stages, respectively (× 400).

significance of antioxidative enzymes emerges during this process. On the contrary, kidney is exposed to oxidized metabolites in the late fetal stage. We, thus, speculate that the induction of these enzymes would be expected in these tissues at prenatal stages. Since GSH is required for GPX activity for reducing power, the coordinated expression of GR with GPX activities would be expected. As a result, expression of GR and GPX1 activities and proteins fairly corresponded in kidney. However, the tissue distribution of GR in lung was markedly different from that of GPX1, and the induction of these enzymes during the developmental process also were not coordinated. The increase in the GPX activity seemed to be somewhat coordinated with the lipid peroxide levels which reached a peak at 10 days after birth.[26] The inconsistency between the levels of the GPX1 protein (Fig. 3) and the activity (Fig. 4) would be attributed to the modification of the protein. Since the levels of the higher molecular weight band appeared to correspond to the activity in the kidney, limited proteolysis of the GPX1 protein may enhance the peroxidase activity. It is also possible that other peroxidases may be involved in the activity.

The distribution of GR in the lung substantially matched that of peroxiredoxin VI (Prx VI), which is a recently identified peroxidase and is abundant in the lung and the kidney.[27] Although Prx VI belongs to a family of enzymes which show thioredoxin-dependent peroxidase activity, it actually exerts its activity using GSH as a sole physiological electron donor.[28] Since high concentrations of GSH are detected in lung epithelial lining fluids,[29] reduction of GSSG to GSH would be preformed by the GR which is present in the epithelia of bronchus and bronchiole. This would enable Prx VI, which is also excreted from Clara cells and is present in the epithelial lining fluids in the lung,[30] to detoxify peroxides using GSH as the electron donor.

The expression profile of GR was unexpectedly different from GPX1 from developmental points of view. The expression of GR was high in these tissues at all stages of the developing lungs. However, the expression of GPX1 varied depending on the developmental stages in addition to differences in its intracellular distribution. Since oxidative stress increases markedly after birth, the significant augmentation of GPX1 protein levels suggest that GPX1 minimize the damage from generated ROS in the lung and kidney. Although we examined the expression of a member of the GPX family, namely GPX1, only in this study, other members may be simultaneously expressed coordinately with GR. Recently, GPX1-deficient mouse has been developed by several groups.[31–33] In spite of its predicted physiological significance, the GPX1-deficient mice were totally healthy under laboratory conditions.[31] Hence, compensation of the deficit of the GPX1 activity by other enzyme(s) with a similar activity was presumed. However, the GPX1-deficient mice were hypersensite to paraquat and hydrogen peroxide toxicity[32] and susceptible to viral infection and mutation,[33] suggesting a pivotal role of GPX1 in such situations.

It is also noteworthy that extensive nuclear staining was observed in some cells in the lung, especially at the later stage of developing alveolus. The same phenomena was found in skin (Fuji *et al.*, unpublished observation), but both the underlying mechanism and the physiological significance of this subcellular localization is unknown at present. A similar observation has been reported for this enzyme in Syrian hamster kidneys. This suggests the importance of the detoxification of peroxides in nuclei or an additional function of GPX1 to its well-known antioxidative role. In fact, a novel role was found for

GPX4, which preferentially detoxifies phospholipid hydroperoxides and is highly expressed in the testes. During spermatogenesis, GPX4 functions as a structural protein in the mitochondria in its oxidized inactive form.[34] Multiple functions have also been reported for peroxiredoxins, including Prx I, which is localized in the cytosol and nuclei and inhibits the c-Abl function by binding to its SH3 domain.[35,36] These antioxidative enzymes possess residues which are sensitive to reactive oxygen species and, hence, may have an additional role in the redox regulation of cellular functions.

Acknowledgements

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