0031-3998/91/2905-0487$03.00/0
PEDIATRIC RESEARCH

Vol. 29, No. 5, 1991
Printed in U.S.A.

Immunohistochemical Study on Perinatal Development of Rat Superoxide Dismutases in Lungs and Kidneys

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ABSTRACT. It has been reported that levels of antioxidant enzymes are low in fetal rat lungs and kidneys, and that they increase rapidly during late gestation. Among the antioxidant enzymes, both copper-zinc and manganese superoxide dismutases (CuZnSOD and MnSOD) are assumed to play a key role in protection against oxidative tissue injury. To determine the nature of the rapid perinatal increase in CuZnSOD and MnSOD, immunoenzyme staining was performed in the lungs and kidneys of fetal (d 18 and 20 of gestation) and neonatal (d 22) rats. The CuZnSOD and MnSOD in the homogenates were assayed by RIA, and they were found to be higher in the neonatal organs than in the respective fetal organs. The neonatal bronchiolar epithelium was stained for both CuZnSOD and MnSOD more intensely than the fetal one. The CuZnSOD staining in the neonatal alveolar wall was more intense than that in the fetal one. There was a significant reactivity for MnSOD in the neonatal, but not in the fetal, alveolar walls. In the kidneys, the reactivities for CuZnSOD and MnSOD were confined to the undifferentiated tubules. Although the tubules were increased in numbers in the neonatal kidneys, the intensity of the staining for both CuZnSOD and MnSOD was unchanged. The histochemical study disclosed that CuZnSOD and MnSOD increased in the kidneys in a manner different from that in the lungs. The low concentration of both CuZnSOD and MnSOD in the fetal lung tissues may contribute to the vulnerability to oxygen toxicity. Such changes in the concentrations in specific tissues were not delineated in the kidneys. (*Pediatr Res* 29: 487–491, 1991)

Abbreviations

CuZnSOD, copper-zinc superoxide dismutase
MnSOD, manganese superoxide dismutase
SOD, superoxide dismutase

In recent years, cell injury mediated by reactive oxygen species has drawn increasing attention from neonatologists. Abrupt increase of tissue oxygen concentration after delivery may induce oxidative injury to vulnerable tissues. There is evidence suggesting that fetal lungs have a low-capacity antioxidant defense system (1–4), and this is postulated to contribute to the development of bronchopulmonary dysplasia in preterm neonates. Superoxide anion radical, which is generated by one electron reduction of molecular oxygen, is considered to be an initiator of free radical chain reactions leading to oxidative cell injury in certain experimental models (5). Thus, SOD (EC 1.15.1.1), a scavenger of superoxide, may play a key role in the protection against free radical injury. Intracellular SOD is found in two forms: CuZnSOD and MnSOD. Using specific RIA (6) for both rat CuZnSOD and MnSOD, we have demonstrated that both SOD increase markedly during the late gestational period (*i.e.* from d 18 to 22 of gestation) in fetal rat lungs and kidneys (7).

The immunoenzyme staining for rat CuZnSOD and MnSOD, which has been developed by us recently (8), revealed that there was a wide range of variability in the expression of both SOD from cell to cell in adult rat tissues. To our knowledge, immunohistochemical localization of CuZnSOD and MnSOD in fetal and neonatal rat tissues has not been reported previously. To further determine the nature of the rapid perinatal increase in SOD, lungs and kidneys of both fetus and neonate were stained for CuZnSOD and MnSOD, and the immunoreactive SOD in these organs were also measured. Our present study revealed that the increase in SOD occurred in certain specific tissues of these rapidly growing organs. Different characteristics of the increase between lungs and kidneys were also shown.

MATERIALS AND METHODS

Animal treatment. Breeding was accomplished by placing male and female Sprague-Dawley rats (Japan SLC Inc., Shizuoka, Japan) together overnight. The midpoint of cohabitation period was considered as the onset of pregnancy. Premature pups were delivered by hysterotomy with the dam under pentobarbital anesthesia (201 μmol/kg; 50 mg/kg) on d 18 and 20 of gestation, and decapitated immediately after the delivery. Neonates were killed within 24 h after delivery under light ether anesthesia. The lungs and kidneys were excised, washed with PBS, and blotted with gauze.

RIA for SOD. The organs were weighed and pooled to obtain at least 80 mg of tissue for each sample, and then kept frozen at −80°C. The tissues were homogenized with 20 times their volume of 10 mM potassium phosphate buffer containing 81 μM digitonin (pH 7.4), and then sonicated on ice for 30 s. The sonicate was centrifuged at 13 000 × *g* for 5 min, and the supernatant was obtained. This served as the sample for RIA. The methods of RIA for rat CuZnSOD and MnSOD have been described (6). The data were expressed as means ± SEM. The statistical significance was determined by the method of least significant difference calculated after one-way analysis of variance.

Immunoenzyme staining for SOD. The specificity of each

Received May 29, 1990; accepted December 17, 1990.
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Supported in part by Grant-in-Aid 63570431 from the Ministry of Education, Science and Culture of Japan.

Table 1. *Immunoreactive SOD in homogenate of lung and kidney**

	Lung			Kidney		
	18 d fetus ($n = 9$)	20 d fetus ($n = 9$)	Neonate ($n = 8$)	18 d fetus ($n = 4$)	20 d fetus ($n = 9$)	Neonate ($n = 8$)
CuZnSOD	1.41 ± 0.06	1.99 ± 0.05†	2.90 ± 0.12‡	1.13 ± 0.07	1.80 ± 0.03†	2.51 ± 0.08‡
MnSOD	77 ± 3	89 ± 12	218 ± 16‡	154 ± 15	364 ± 9†	503 ± 14‡

* Data are means ± SEM. Values are nmol/g wet tissue for CuZnSOD and pmol/g wet tissue for MnSOD.
† $p < 0.001$ (*vs* 18 d).
‡ $p < 0.001$ (*vs* 20 d).

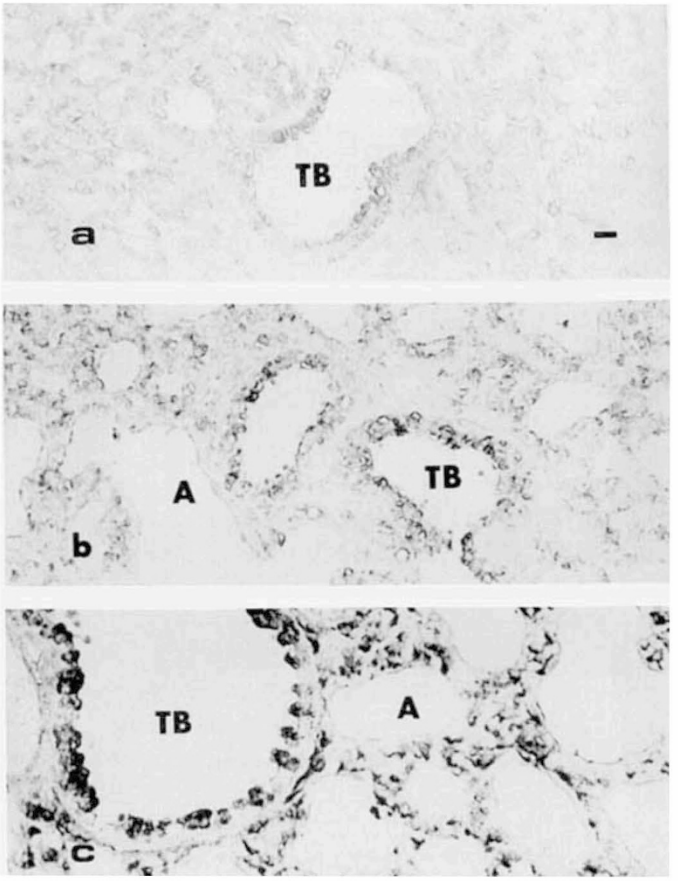

Fig. 1. Photomicrographs of fetal and neonatal rat lungs stained for CuZnSOD. *a*, Fetal lung (d 18); *b*, fetal lung (d 20); and *c*, neonatal lungs. The CuZnSOD reactivity is higher in the neonatal lungs than in the fetal lung. Original magnification ×200. *Bar* indicates 10 μm. *A*, alveolus; TB, terminal bronchiole.

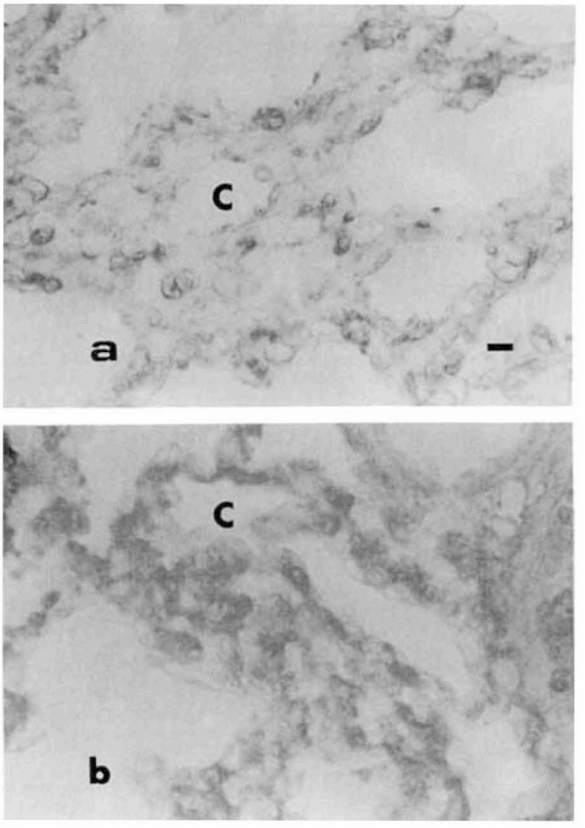

Fig. 2. CuZnSOD staining in lung at high magnification. *a*, Fetal lung (d 20); *b*, neonatal lung. The alveolar wall including capillary endothelium in the neonatal lung is stained more intensely than that in the fetal lung. Original magnification ×400. *Bar* = 5 μm. *C*, capillary endothelium.

antiserum was established when the RIA was developed (6). The immunoenzyme staining for both CuZnSOD and MnSOD was performed as described previously (8). The tissues were fixed with 10% buffered formalin and embedded in paraffin. The specimens were cut into 4-μm thick slices. The tissue sections were washed for 15 min with three changes of PBS between each step. Deparaffinized tissue sections were treated with 0.88 M H_2O_2 for 10 min to inactivate endogenous peroxidase activity. Sections were then treated with 10% normal goat serum for 30 min to block nonspecific binding. The sections were incubated with the diluted antiserum (1:10 000 for anti-CuZnSOD and 1:5000 for anti-MnSOD) overnight at 4°C, then sequentially with 1% anti-rabbit IgG fraction antigen binding fraction labeled with horseradish peroxidase for 60 min, and 0.56 mM diaminobenzidine containing 1.47 mM H_2O_2 for 5 min. Finally, the sections were mounted by Permount (Fisher Scientific Co., Pittsburgh, PA). As controls, tissue sections were treated in the same way, except that antisera were replaced by normal rabbit serum at the same dilutions (8).

Morphometry. Morphometry was performed with an image analyzer (IBAS-2000; Zeiss, Munich, Germany). The sections were observed under a light microscope at a magnification of 200×. Histologic images were transmitted to the image analyzer by a color TV camera (ITC-350M; Ikegami, Tokyo, Japan). The images of the staining profiles were projected on the screen by the specific gray threshold level (9). All images in three visual fields, which were randomly selected, were measured for each sample, and the area percentage was computed.

RESULTS

Immunoreactivity for SOD. Table 1 lists the immunoreactivity for both CuZnSOD and MnSOD in the homogenates of lungs and kidneys. The concentrations of both SOD in each organ were significantly higher in the d 20 fetus than in the d 18 fetus, and also higher in the neonates than in the d 20 fetus, although the difference in the lung MnSOD between d 18 and 20 was not significant. The CuZnSOD level in the lungs was comparable to

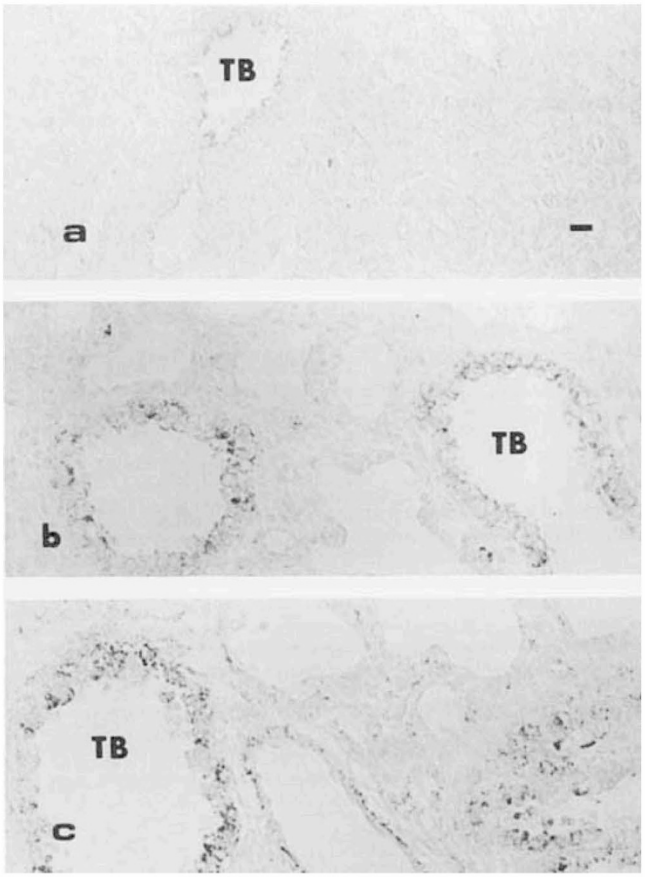

Fig. 3. Fetal and neonatal rat lungs stained for MnSOD. *a*, Fetal lung (d 18); *b*, fetal lung (d 20); and *c*, neonatal lung. In the fetal lungs, positive stainings are found only in the epithelium of the terminal bronchiole (*TB*). Both terminal bronchiole and alveoli are stained in the neonatal lungs. Original magnification ×200. Bar = 10 μm.

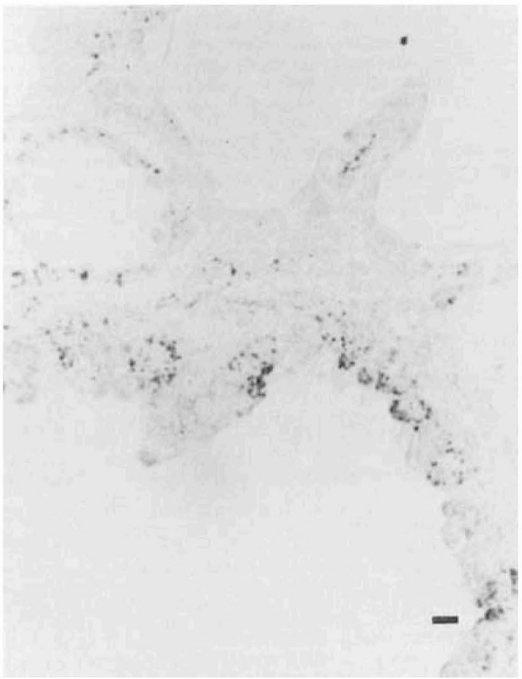

Fig. 4. MnSOD staining in neonatal lung at high magnification. Positive structures are seen both in bronchiole and alveolus. Original magnification ×400. *Bar* = 5 μm.

Table 2. *Area percentage of staining for CuZnSOD and MnSOD in lung and kidney**

	Lung			Kidney		
	18 d fetus	20 d fetus	Neonate	18 d fetus	20 d fetus	Neonate
CuZnSOD	5.7	13.6	20.8	6.7	27.3	45.9
	6.5	14.5	24.2	7.9	27.9	38.5
	8.2	11.3	24.5	6.2	28.6	38.2
MnSOD	0.07	0.37	1.47	14.2	28.4	38.7
	0.05	0.32	1.20	13.4	31.7	32.7
	0.08	0.56	1.82	13.7	31.3	41.6

* The image of the staining was measured, and the area percentage to visual field was computed.

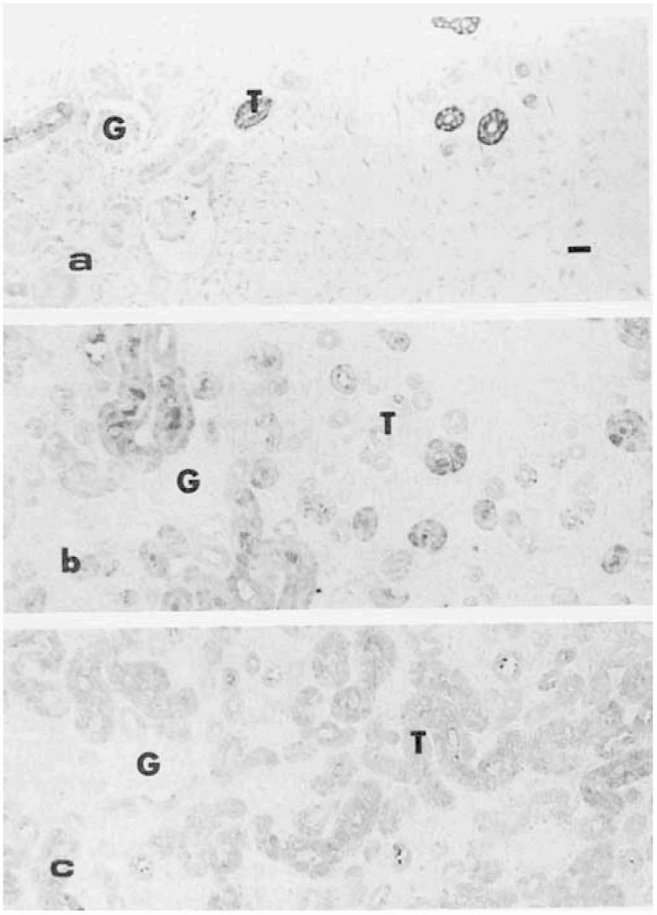

Fig. 5. Fetal and neonatal rat kidneys stained for CuZnSOD. *a*, Fetal kidney (d 18); *b*, fetal kidney (d 20); and *c*, neonatal kidney. Cross-sectional view of juxtamedullary region. Only the renal tubules are stained. The section of the neonatal kidney contains more tubules than that of the fetal kidney. Original magnification ×100. *Bar* = 20 μm. *G*, glomerulus; *T*, tubulus.

that in the kidneys at each time point. The MnSOD represented 12% of the total SOD in the kidneys of the d 18 fetus, and it represented 17% of the total SOD in the kidneys of the d 20 fetus and neonates. The relative concentration of MnSOD in the lungs was much lower than that in the kidneys. The MnSOD represented 4% of the total SOD in the d 20 fetal lungs and, even after a drastic increase, it still represented only 7% of the total SOD in the neonatal lungs.

Immunohistochemical localization of SOD. Positive stainings for CuZnSOD were found only in the bronchial epithelium of the d 18 fetus, but were found in the bronchiolar epithelium and alveolar walls of both the d 20 fetus and neonates (Fig. 1). The staining intensity in the bronchiolar epithelium increased with age. Similarly, the CuZnSOD staining in the alveolar wall, in-

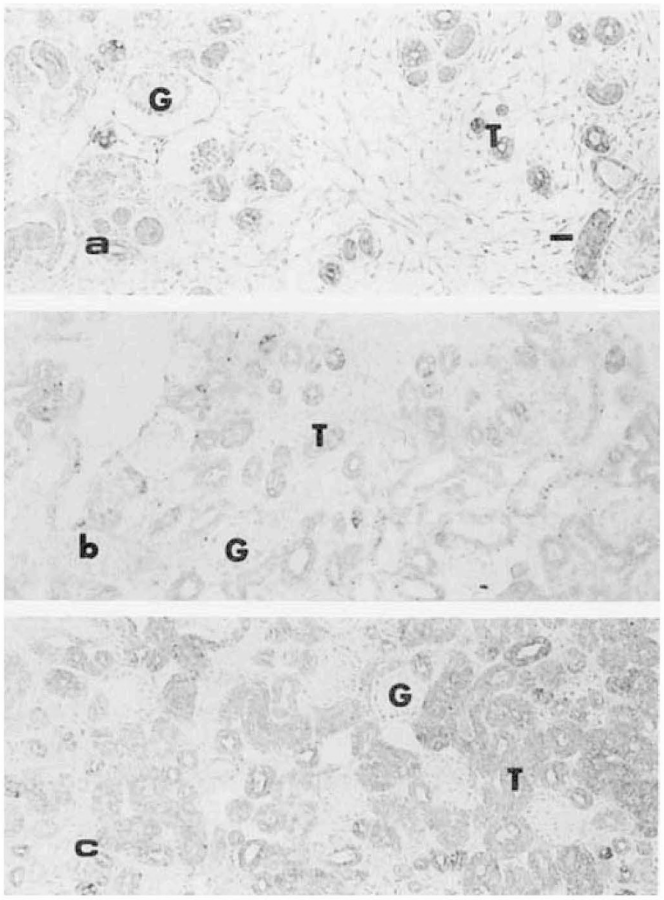

Fig. 6. Fetal and neonatal rat kidneys stained for MnSOD. *a*, Fetal kidney (d 18); *b*, fetal kidney (d 20); and *c*, neonatal kidney. The renal tubules in fetal (d 18 and 20) and neonatal kidneys are stained moderately. Original magnification ×100. *Bar* = 20 μm. *G*, glomerulus, *T*, tubulus.

cluding epithelia and capillary endothelia, also increased during this period (Fig. 2). The MnSOD-positive structures were found in the bronchiolar epithelium of the lungs obtained at each time point. Again, the positive structures increased with age (Fig. 3). There was a significant staining for MnSOD in the neonatal alveolar wall including the epithelium, capillary endothelium, and mesothelium (Fig. 4), but those structures in the fetal lung were not stained for MnSOD. The age-related increase in the staining for each SOD in the lung was clearly demonstrated by morphometric measurement (Table 2). The area percentage of the staining for CuZnSOD was 3-fold higher in the neonatal lung than in the lung of the d 18 fetus. That for MnSOD increased more than 20-fold during this period.

In the fetal and neonatal kidneys, well-developed glomeruli were found mainly in the juxtamedullary region of the cortex. The tubular epithelium was stained for both SOD, but there was no staining in the glomeruli and interstitial space. The renal tubules had not yet been differentiated into proximal and distal types, even in the neonatal kidneys. They were all stained moderately, with equal intensity, for both CuZnSOD and MnSOD (Figs. 5 and 6). As reflected in the morphometric data (Table 2), tubular mass increased rapidly during this period. The area percentage of the staining for CuZnSOD increased almost 3-fold, and that for MnSOD increased approximately 5-fold.

DISCUSSION

There have been studies on the immunolocalization of CuZnSOD in adult rat lungs (8, 10, 11). According to these studies, CuZnSOD reactivity was intense in bronchiolar epithelium and moderate in alveolar wall. Thus, the relative abundance of CuZnSOD in the bronchiolar epithelium of both the fetal and neonatal lungs observed here was similar to the distribution in the adult lungs. The finding that CuZnSOD staining was more intense in the neonatal lung than in the fetal lung was consistent with the increase in the immunoreactive SOD in the lung homogenate during this period.

In our previous study (8), both bronchiolar epithelium and alveolar wall in adult rat were stained for MnSOD moderately, with equal intensity. The lower MnSOD reactivity in the neonatal alveolar wall than in the bronchiole and no reactivity in the fetal alveolar wall observed here indicate that MnSOD accumulates in the alveolar wall later than in the bronchiolar epithelium. The finding that the intensity of the staining increased during late gestation in the whole lung tissues was, again, in keeping with the observed drastic increase in MnSOD measured by the RIA.

A rapid structural development is known to occur in lungs during late gestation: branching and lengthening of terminal tubules, marked widening of the air spaces resulting in the formation of acini with air-blood barriers, and differentiation of alveolar epithelial cells into several types (12). Cellular differentiation may contribute to the increase in both SOD (13). Lung CuZnSOD (14) is reported to be induced by exposure to high concentrations of oxygen. We previously demonstrated that lung SOD increased during late gestation without exposure to oxygen (7). Hass *et al.* (15) measured mRNA in developing rat lung. They suggested that there could be both pretranslational and translational regulation in the induction of CuZnSOD by oxygen exposure. However, the cellular mechanism for the induction still needs to be elucidated.

Hyperoxia increases superoxide production in rat lung mitochondria (16), and is postulated to be a major cause of bronchopulmonary dysplasia (17). Induction of endogenous antioxidant enzymes by the administration of bacterial lipopolysaccharide is reported to protect against oxygen lung injury (18). The lung injury is supposed to be initiated by microvascular endothelial damage (19), leading to the destruction of the alveolar wall. This can be ameliorated by i.v. injection of liposome-entrapped catalase and SOD (20). The low concentration of both SOD in the fetal alveolar wall including capillary endothelium, which is assumed to be particularly vulnerable to oxidant attack, further supports our previous view (7) that preterm lung is vulnerable to oxygen toxicity because of the scarcity of the antioxidant enzymes.

According to previous studies (8, 10) on adult rat kidneys, CuZnSOD reactivity was most intense in proximal tubules, less intense in the rest of the tubules and ducts, and not found in glomeruli. On the other hand, Henle's loops and collecting ducts were more intensely stained for MnSOD than either proximal or distal tubules, and glomeruli were not stained (8). In general, cortex was rich in CuZnSOD and, conversely, medulla was rich in MnSOD (8). It is known that rat kidneys are immature at birth. There is an active nephrogenic zone in which new glomeruli and tubules are still undergoing the phases of development (21). Lack of the differential distribution found in the adult kidney for both SOD was due to the immaturity of the neonatal kidney. However, the finding that the stainings for both SOD were confined to the undifferentiated tubules in the fetal and neonatal kidneys was consistent with the staining profile in adult rat kidneys in which the reactivities were restricted to the tubules and ducts (8).

The observed drastic increase in the immunoreactivities in the kidney homogenates is, most likely, mainly due to the rapid proliferation of the tubular epithelium during this period. There is evidence suggesting that both glomerular and tubular damage can be mediated by reactive oxygen species (22, 23). Tubules are vulnerable to reactive oxygen species, even in the presence of a high concentration of antioxidant enzymes, presumably because the rate of production of reactive oxygen species is high in these

metabolically active sites. Whether or not preterm kidneys are vulnerable to oxidative stress is unknown. In contrast to our present observations that the expression of both SOD in specific tissues was intensified in the lungs during late gestation, the intensity of the staining in the renal tubules was unchanged. This may be related to the finding that the tubules were still undifferentiated even in the neonatal kidneys. Thus, the low levels of both SOD in fetal kidneys may merely reflect the scarcity of tubular mass rather than vulnerability to oxidative stress.

Our present histochemical study disclosed that both CuZnSOD and MnSOD increased in the kidneys in a different manner than they did in the lungs, and this difference could not be depicted by the biochemical study.

Acknowledgment. The authors gratefully acknowledge the technical assistance of Hiromi Horiuchi.

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