Expression of Copper/Zinc Superoxide Dismutase and Glutathione Peroxidase in Organs of Developing Mouse Embryos, Fetuses, and Neonates

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ABSTRACT. The rise in antioxidant enzyme activity in the lungs of late-gestation fetuses is thought to be caused by the preparation of the pulmonary antioxidant system for birth. However, recent data have shown that such a rise also occurs in the livers of late-gestation fetuses. Consequently, this surge cannot solely be ascribed to the preparation of the pulmonary antioxidant system for birth. In this study we examine the expression of copper/zinc superoxide dismutase (Sod1) and glutathione peroxidase (Gpx1) in various organs of late-gestational mouse fetuses. Furthermore, we compare the expression of these genes in organs of fetuses, neonates, and adult mice. These studies were carried out to investigate whether the change in mRNA levels for these two genes is related to a developmental change in oxidant stress. Our data demonstrate that an increase in both Sod1 and Gpx1 mRNA occurs in lungs and livers of late-gestational mouse fetuses. The brain demonstrates an increase in Sod1 expression at or around the time of birth, the kidney shows an elevation in Gpx1 mRNA levels, and the heart fails to demonstrate a surge in both Sod1 and Gpx1 mRNA levels. Our data show that the liver is the organ with the highest levels of Sod1 and Gpx1 mRNA in embryos and neonates (immediately after birth). In the adult, the liver has the highest levels of Sod1 mRNA and the spleen the highest level of Gpx1 mRNA. These data suggest that the levels of Sod1 and Gpx1 mRNA are unrelated to oxygen consumption and to oxygen tension exposure of individual organs and do not necessarily appear to occur in the lung solely in preparation for birth. The reasons for the increase in antioxidant enzyme(s) mRNA levels in late gestation are more complex and may involve other factors. (*Pediatr Res* 35: 188–196, 1994)

Abbreviations

Sod, superoxide dismutase
Sod1, copper/zinc superoxide dismutase
Gpx1, glutathione peroxidase

hydrogen peroxide, and the highly reactive hydroxyl radical, which will interact with macromolecules in their immediate vicinity causing damage to lipids and DNA (1). Cells have developed antioxidant defenses to protect against such noxious moieties. Important are the scavenging enzymes such as Sod (EC 1.15.1.1), Gpx1 (EC 1.11.1.9), and catalase (EC 1.11.1.6). Eukaryotic cells have a secretory or extracellular form of Sod and two kinds of intracellular Sod enzymes: a ubiquitous predominant copper/zinc-containing enzyme (Sod1), which is mainly cytosolic in location (2), and a manganese-containing enzyme (Sod2), which is found in the mitochondria (3). Sod catalyzes the conversion of the superoxide radical to hydrogen peroxide. Hydrogen peroxide in turn is catalyzed to water by Gpx1 or catalase (4). Consequently, it is the concerted action of all the antioxidant enzymes that enables the cell to function in an otherwise noxious environment.

The birth of a fetus results in a transition from an hypoxic environment *in utero* [PO_2 2.66–3.99 kPa (20–30 mm Hg)] to a relatively hyperoxic environment [13.3 kPa (100 mm Hg)] (5). Thus, the birth of a fetus involves a significant oxidative stress that can result in the generation of toxic reactive oxygen species (5). Indeed, numerous studies have demonstrated a surge in the activity of pulmonary antioxidant enzymes in the fetus during late gestational stages (5–10). The reasons for such an increase have been proposed to be the preparation of the pulmonary system for the acute change in oxygen concentration that occurs at birth. However, this increase in the activity of the antioxidant enzymes in late gestation may be the result of other factors. Such a possibility has been suggested by Rickett and Kelly (5), who have shown that a rise in the activity of antioxidant enzymes also occurs in the livers of guinea pig fetuses during late gestation. No data are currently available on the expression of antioxidant enzymes in other organs of late-gestational mammalian fetuses. To gain further insight into the significance of the rise in antioxidant enzyme activity in organs of late-gestational fetuses and neonates and to examine how widespread this phenomenon is, we investigated the expression of the Sod1 and Gpx1 genes in a variety of murine embryonic, fetal, and neonatal organs. Specifically, this study investigated whether a change in mRNA levels for these two genes was related to a developmental change in oxidant stress.

MATERIALS AND METHODS

Animals. Female mice were mated overnight and checked the next day for the presence of vaginal plugs (11). This day was designated d 1 of gestation. Pregnant mice were killed by cervical dislocation on specified days. Neonatal mice were killed on d 1 and 7 after birth, respectively. Between 8 to 25 whole embryos and individual organs were pooled for each specific time point.

Aerobic cells are exposed to harmful reactive oxygen species that are generated during the process of oxidative metabolism. These reactive oxygen species include the superoxide radical,

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Each of the organs and tissues was carefully dissected from the mice, ensuring that no contamination from other organs or tissue resulted. Furthermore, individual organs were rinsed in PBS, pH 7.0, several times. These organs were immediately frozen in liquid nitrogen for RNA analysis. These experiments were approved by the Animal Ethics Committee of Monash University.

RNA extraction. Total cellular RNA was extracted according to the method of Chomczynski and Sacchi (12). Twenty- μ g aliquots of RNA were lyophilized for Northern analysis, and 20-, 10-, 5-, and 2.5- μ g aliquots were used for dot-blot analysis.

Cloning and sequencing of murine Sod1 cDNA. Total cellular RNA from 1-d neonatal murine brains was used for cDNA synthesis in the following reaction: 10 μ g of RNA were mixed with 20 pM of an oligo dT₍₁₅₎ adaptor containing a *NotI* restriction site (shown in bold) (5'-[T]₁₅CGCCGGCGCTTAAGGCC-3'). The RNA plus added oligonucleotide was heated to 65°C and thereafter slowly cooled to 42°C. For the cDNA reaction the following were added and incubated at 42°C for 1 h: 1.0 mM deoxyribonucleoside triphosphate, 50 mM Tris-HCl (pH 8.3), 6 mM MgCl₂, 10 mM DTT, 35 IU RNasin, and 20 IU Moloney murine leukemia virus reverse transcriptase. An oligonucleotide (5'-CTTCTCGTCTAGATCTCTCTG-3') homologous to the most 5' sequence of a murine Sod1 cDNA (13) was synthesized. Two base changes (underlined) were made to create an additional *XbaI* cloning site (shown in bold). This oligonucleotide, together with the oligo dT₍₁₅₎ adaptor described above, was used in the polymerase chain reaction to amplify a murine Sod1 cDNA from the neonatal brain cDNA library generated above. The polymerase chain reaction was performed as follows: 20 pM of each oligonucleotide was added to 0.5 μ g cDNA in 10 mM Tris-HCl (pH 8.3), 1.25 mM deoxyribonucleoside triphosphate, 0.1% Triton-X100, 1.5 mM MgCl₂, and 5 IU Taq polymerase (Promega, Madison, WI); 35 cycles of 1 min at 95°C, 1 min at 65°C, and 2 min at 72°C. The 600-bp polymerase chain reaction product was excised and purified using GeneClean (Bio-101, CA). The purified DNA was restricted with *NotI* and *XbaI* and cloned into pGEM-9Zf(-). The nucleotide sequence was determined by the dideoxynucleoside chain-termination method (14) using universal forward and reverse primers to sequence from both ends of the insert. The chemical reaction products were electrophoresed on urea/polyacrylamide (30:1) gels. Sequence data were analyzed using PC/Gene Software (IntelliGenetics, Inc., Mountain View, CA).

Northern and dot-blot analysis. The techniques used for gel electrophoresis, Northern transfer, and dot-blot analysis have been previously described (11). The murine Sod1 probe (613 bp) was released from pGEM-9Zf(-) after restriction with *XbaI* and *NotI*. Human catalase (plasmid pCAT10) was obtained from the American Type Culture Collection (Rockville, MD). Murine β -actin and murine Gpx1 were gifts from Drs. T. Papas and I. Chambers, respectively. An oligonucleotide homologous to an 18S ribosomal rat sequence (5'-CGGCATGTATTAGCTCTAGAATTACCACAG-3') was synthesized. Hybridization probes were prepared and labeled as previously described (11). The 18S oligonucleotide was end-labeled using polynucleotide kinase (Promega) and [³²P]ATP (Amersham, Buckinghamshire, UK). Northern filters were prehybridized, and they were hybridized with Sod1, Gpx1, and β -actin probes as previously described (11). The 18S ribosomal oligonucleotide probe was hybridized in molar excess. These filters were washed in 0.1 $\times$ sodium chloride, sodium phosphate, EDTA at 65°C for 15 min. Prehybridization conditions and hybridization with an 18S oligonucleotide were as follows: prehybridization was for 2–4 h in 50% formamide, 2.5 $\times$ sodium chloride, sodium citrate, 5 $\times$ Denhardt's solution, 25 mM H₂PO₄ (pH 7.4), 0.05 mg/mL salmon sperm DNA, and 0.05% SDS, and hybridization occurred for 14–16 h at 37°C in the same solution. These filters were washed in 1 $\times$ sodium chloride, sodium phosphate, EDTA, 0.1% SDS at 40°C for 15 min. Autoradiography was performed using intensifying screens at -70°C and Fuji x-ray RX medical film.

Northern blots were first hybridized with Sod1, the probe removed (11), and then sequentially hybridized with the Gpx1 probe, the β -actin probe, and the 18S oligonucleotide. Before rehybridization of filters, autoradiography was carried out to confirm that no residual signal remained. The transcript size(s) for each of the genes was confirmed on Northern blots relative to 28S and 18S RNA and was also approximated by the use of size markers as previously described (11). Northern-blot and dot-blot autoradiograms were scanned (for quantitation) using an Ultrascan XL LKB densitometer (Bromma, Sweden). Laser densitometry for all of the autoradiograms were conducted on lighter exposures of the filters where the signal density or radioactivity was still in the linear range. Care was also taken to pass the scanning beam in a path where nonspecific signals were not present and thus did not interfere with the quantitation. Sod1 and Gpx1 expression was corrected for RNA loading and is shown relative to β -actin and 18S ribosomal RNA. The mRNA levels of each gene are expressed as a ratio of the area under the curve for the signal of that particular gene over the area under the curve for β -actin, and this ratio was corrected relative to 18S ribosomal RNA to correct for slight differences in RNA loading between different lanes. All the data in this study were similar when corrected relative to β -actin as compared with that corrected relative to β -actin and subsequently to 18S ribosomal RNA. It is also important to note that rehybridization of the filters with another housekeeping gene (GAPDH) showed that the levels of β -actin and GAPDH followed one another extremely closely, and both were significantly different from that of 18S ribosomal RNA (data not shown). One lane on each filter was loaded with 15-d embryo total RNA—this served as a control and facilitated comparison across different filters by normalization of each filter relative to this sample when required.

Tissue preparation and enzymatic assays. Approximately 10 organs were pooled for each time point. Tissues were homogenized (0.5 g/mL wet wt) in 65 mM phosphate buffer containing 1% Triton-X 100 and centrifuged at 105 000 $\times$ g for 1 h. The supernatant was used to measure both Sod1 and Gpx1 activity.

Sod activity was assayed according to Elroy-Stein *et al.* (15). To measure total Sod activity, we added 250 μ L of supernatant to 25 μ L of xanthine (1.142 mg/mL), 25 μ L of hydroxylammonium chloride, 125 μ L of H₂O, and 75 μ L of xanthine oxidase (0.1 IU/mL). The mixture was incubated at 25°C for 20 min. Thereafter, 0.5 mL of sulfonic acid (3.3 mg/mL) and 0.5 mL of α -naphthylamine (1 ng/mL) were added and further incubated at room temperature for 20 min. The absorbance was measured at 530 nm. The addition of 125 μ L of KCN (4 mM) in place of H₂O in the above reaction specifically inhibits Sod1 activity. Thus subtraction of the Sod activity remaining after KCN treatment (*i.e.* Sod2) from the total Sod activity gives the Sod1 activity of the sample. Sod1 activity is expressed as IU/mg tissue.

Gpx1 activity was assayed according to the method of Lawrence and Burk (16). Briefly, 800 μ L of the following solution was prepared for each sample and incubated at room temperature for 5 min: 62.5 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN₃, 0.2 mM β -NADPH, 1 IU glutathione reductase, and 0.31 mM reduced glutathione. Thereafter, 0.1 mL of 0.25 mM H₂O₂ was added and further incubated at room temperature for 5 min, and the reaction was commenced by the addition of 0.1 mL of supernatant. The change in absorbance was measured at 340 nm for 5 min. Gpx1 activity is expressed as μ mol NADPH oxidized/min/mg tissue.

RESULTS

Cloning and Sequencing of Murine cDNA for Sod1. The Sod1 cDNA clone isolated contains 53 bp 5' from the ATG start site, 462 bp coding sequence and 75 bp 3' of the stop codon. A coding region of 462 bp is consistent with other published sequences for murine Sod1 cDNA (13, 17). Our sequence is identical to that of Bewley (17), including the atypical polyade-

nylation sequence (ATTAAA), with the exception that they have an additional A inserted at position 486 and that we find a T instead of an A at position 499. Our cDNA clone is identical to that of Getzoff *et al.* (13) with regard to these two differences. However, we have one difference in our sequence compared with Getzoff *et al.* (13), namely, we do not find a G inserted between position 470 and 471. In this instance our sequence is the same as that of Bewley (17). These differences in our sequence from that of Bewley (17) and Getzoff *et al.* (13) do not affect the amino acid sequence of the clone because they occur in the 3'-untranslated region. Northern analysis of murine tissue showed that our cDNA probe detected one transcript of approximately 0.7kb as expected (18).

Developmental Expression of Sod1 and Gpx1 in Organs and Amnion of Mouse Fetuses. Lung. Sod1 and Gpx1 expression increases sharply before or around the time of birth (Figs. 1 and 2a). This increase is manifested by the 5- and 7-fold higher levels of Sod1 and Gpx1 mRNA, respectively, in the lungs of 1-d neonatal mice as compared with those of 19-d fetal mice. Thereafter, in 7-d neonatal mice, a decline in the mRNA levels for Sod1 and Gpx1 occur. The profiles of Sod1 and Gpx1 mRNA are similar in late gestation and neonatal development. These profiles for Sod1 and Gpx1 mRNA were similar irrespective of whether the Sod1 and Gpx1 mRNA levels were expressed relative to β -actin or β -actin and 18S ribosomal RNA or 18S ribosomal RNA only (data not shown). However, in the liver the data is significantly different when expressed relative to β -actin (or GAPDH) or 18S ribosomal RNA (see below).

The mRNA levels for Sod1 and Gpx1 were also examined at time periods earlier than d 17 (*viz.* d 14 to 16). Curiously, the Sod1 mRNA levels were relatively high in these earlier periods (Fig. 3). Indeed, the Sod1 mRNA levels in 15-d lungs were higher than at any other time in the gestational and neonatal periods. Gpx1 mRNA levels were also higher in 15-d and 16-d lungs than at any other stage in development.

Liver. Sod1 and Gpx1 expression relative to β -actin increases from d 17 through d 19 (approximately 20- and 3-fold increase, respectively) (Figs. 1 and 2b). Thereafter, Sod1 and Gpx1 mRNA levels show a marked decline at or around the time of birth. This decline is still evident on d 7 of neonatal life. The profiles of

Sod1 and Gpx1 mRNA levels in the livers of mice at various stages of development are also similar.

With regard to the expression of Sod1 and Gpx1 relative to 18S ribosomal RNA, if the data are analyzed in this manner, a different pattern of expression is obtained from that described in the preceding paragraph. The levels of Sod1 and Gpx1 mRNA are greatly elevated between d 19 of gestation and the first d of neonatal life (Fig. 4). Gpx1 mRNA levels start off at very high on d 17, are reduced some 6-fold on d 18 and d 19, and thereafter surge at or around the time of birth. On the first day of neonatal life Gpx1 mRNA levels are approximately 6.5-fold higher than that of 19-d fetal livers. Sod1 mRNA levels also show low levels of expression on d 17 to 19 of gestation and then an approximately 4-fold elevation between d 19 and the first d of neonatal life.

With regard to Sod1 and Gpx1 activity, this experiment was undertaken for two reasons: 1) to investigate the correlation of mRNA levels for Sod1 and Gpx1 with enzyme activity; and thereby 2) to determine which of the two methods of mRNA analysis above was more accurate, especially because the liver was the only organ investigated that showed a difference in Sod1 and Gpx1 mRNA profiles. This was dependent on whether the data was corrected relative to 18S ribosomal RNA only or relative to a housekeeping gene (β -actin or GAPDH) and 18S ribosomal RNA (or to a housekeeping gene only because no differences in the patterns for β -actin/GAPDH or 18S ribosomal RNA were obtained).

The enzyme activity for Sod1 and Gpx1 was similar (Fig. 5a) to the mRNA profiles for these two enzymes as demonstrated in the earlier discussion regarding expression relative to β -actin [*i.e.* the data corrected relative to β -actin and 18S ribosomal RNA (Fig. 2b)] (or either β -actin or GAPDH alone, data not shown). These data demonstrate that the activity of Sod1 and Gpx1 are elevated in late gestation at or around the time of birth. Furthermore, the mRNA profile for Sod1 and Gpx1 correlate well with the activity for these two enzymes in the time periods investigated.

Brain. With regard to mRNA profiles, an increase in Sod1 mRNA, and not Gpx1 mRNA, occurs in the brain of late-gestation and neonatal mouse fetuses (Figs. 6 and 2c). An approximately 3-fold increase in Sod1 mRNA levels occurs between

Fig. 1. Northern-blot analysis of total RNA from organs of 17-d mouse fetuses through to 7-d neonates. Filters were hybridized with a probe for Sod1, stripped, and sequentially rehybridized with Gpx1, β -actin, and 18S ribosomal RNA probes. Numbers represent the following: 17, 17th d of gestation; 18, 18th d of gestation; 19, 19th d of gestation; 1N, 1-d neonatal mice; 7N, 7-d neonatal mice.

Fig. 2. Graphic representation of the quantitative expression of Sod1 and Gpx1 (relative to β -actin and 18S ribosomal RNA) in the various organs of 17-d mouse fetuses through to 7-d neonates. The organs analyzed were the lung, liver, brain, kidney, heart, and stomach. The Northern and dot blots shown in Figures 1 and 6 were scanned with a laser densitometer, corrected for RNA loading, and shown relative to β -actin and 18S ribosomal RNA. Note: These profiles are similar if the data are corrected relative to β -actin only [or GAPDH (data not shown)] and 18S ribosomal RNA only [except the liver (see text)].

Fig. 3. Sod1 and Gpx1 mRNA levels in lungs of mice between d 14 to 19 of gestation and 1-d and 7-d neonates. The mRNA levels are shown relative to β -actin and 18S ribosomal RNA. Northern blots shown in Figure 1 (d 17 onward) and data not shown for d 14 to 16 were densitometrically scanned and corrected for β -actin and 18S ribosomal RNA.

d 17 and d 19 of gestation. Sod1 mRNA levels continue to rise into the neonatal stages of development (a further 2-fold increase occurs). Gpx1 mRNA levels, on the other hand, do not demonstrate a similar increase (if at all) in mRNA levels in late gestation. These levels remain relatively unchanged during late development and after birth.

The activity of Sod1 and Gpx1 (Fig. 5b) correlated well with the mRNA profiles of the two genes (Fig. 2c). Sod1 activity was higher on d 19 of gestation as compared with earlier periods of development, and the Sod1 activity was even higher in 7-d neonatal brains. Gpx1 activity, like that of the mRNA levels,

Fig. 4. The Sod1 and Gpx1 mRNA profile of the liver when the data in Figure 1 is corrected relative to 18S ribosomal RNA. Note: The liver is the only organ that showed a difference in Sod1 and Gpx1 mRNA profiles when the data were corrected relative to β -actin (or GAPDH) and 18S ribosomal RNA as compared with that relative to 18S only.

was relatively unchanged in late development and after birth. These data, together with those for the liver, provide further evidence for a good correlation between mRNA profiles and enzyme activity. Consequently, mRNA levels only were subsequently studied.

Kidney. Gpx1 mRNA levels, and not Sod1, increase in mouse kidneys before or around the time of birth (Figs. 1 and 2d). The mRNA levels for Sod1 and Gpx1 are high in 17-d fetal kidneys. Thereafter, the levels of Sod1 and Gpx1 mRNA show a decline in 18-d fetal kidneys. The Sod1 mRNA levels demonstrate a slight elevation on d 19 as compared with d 18. In 1- and 7-d

Fig. 5. Sod1 and Gpx1 activity in the liver (a) and brain (b) of 17-d to 19-d mouse fetuses and 1-d and 7-d neonatal mice. The Sod1 activity is expressed as IU/mg tissue and the Gpx1 activity as μ mol NADPH/min/mg tissue.

neonatal mice the Sod1 mRNA levels are similar to d 18 levels. Gpx1 mRNA levels, on the other hand, decline on d 19 of gestation as compared with that of d 18. Thereafter, the levels of Gpx1 mRNA show an increase, which persists into neonatal development.

Heart. Sod1 and Gpx1 mRNA levels fail to demonstrate a late fetal surge in the heart (Figs. 1 and 2e). Sod1 mRNA levels are essentially unchanged throughout this period and into the first day of neonatal development. However, a sharp rise (a 4-fold increase) in Sod1 mRNA levels occurs in the heart of 7-d neonatal mice as compared with that of 1-d neonatal mice. Gpx1, on the other hand, is expressed at high levels in 17-d fetuses, and these levels decline on d 18 and 19. Thereafter, Gpx1 demonstrates a modest (1.2-fold) increase in mRNA levels just before or at birth. A further 2-fold increase in Gpx1 mRNA levels occurs in 7-d neonatal mice as compared with 1-d neonatal mice.

Stomach. Sod1 mRNA levels, and not those of Gpx1, show an elevation at or around the time of birth. A steady increase in Sod1 expression occurs before (approximately 6-fold increase) and after birth (a further 1.6-fold increase) (Figs. 1 and 2f). A 2-fold rise in Gpx1 expression occurs on d 18 of gestation as compared with d 17. Thereafter, Gpx1 mRNA levels are similar

Fig. 6. Northern- and dot-blot analysis of total RNA from the brains of 17-d fetuses through to 7-d neonates. Northern blots (A) were first hybridized with Sod1, stripped, and rehybridized with β -actin. The filters degenerated after the β -actin hybridizations, and hence the analysis was also conducted on dot blots. Dot blots (B) were hybridized with Sod1, stripped, and rehybridized sequentially with Gpx1 and β -actin.

until d 1 of neonatal development. However, an approximately 2.5-fold increase in Gpx1 mRNA occurs in 7-d neonatal stomachs as compared with those of 1-d neonates.

Amnion. The Northern blots in Figure 7a show the expression of Sod1 and Gpx1 in the amnion of different aged conceptuses. Sod1 expression progressively increases in the amnion of d 10 (the earliest stage investigated) to d 14 conceptuses (Fig. 7b). The increase over this period is approximately 5-fold. The levels of Sod1 mRNA decline after d 14 of embryogenesis, as evidenced by the 3-fold lower levels of Sod1 mRNA in 16-d amnions. Gpx1 expression remains relatively constant in the amnion on d 10 and d 11 of gestation. On d 12, however, an approximately 3-fold increase occurs in Gpx1 mRNA levels. The levels of Gpx1 mRNA decline in 13-d amnions and are still lower in 14-d and 16-d amnions as compared with those of 13-d conceptuses.

Comparison of Sod1 and Gpx1 Expression in Organs of Different Aged Fetuses. Having compared the expression of Sod1 and Gpx1 mRNA levels in individual organs of fetuses on different days of late-gestational development, we compared the expression of Sod1 and Gpx1 in the different organs of fetal, neonatal, and adult mice at specific stages of development. These investigations were conducted with a view of gaining further insight into the reasons for the modulations of Sod1 and Gpx1 mRNA levels.

Day 14 fetuses. The liver expresses the highest levels of both Sod1 and Gpx1 in 14-d murine fetuses (Figs. 8 and 9a). The descending order for Sod1 expression is as follows: liver > heart > kidney > brain > intestine > stomach > lung. The order for Gpx1 expression is as follows: liver > kidney > heart > lung > brain > intestine > stomach.

Fig. 7. The expression of Sod1, Gpx1, and β -actin in the amnions of 10-d, 11-d, 12-d, 13-d, 14-d, and 16-d conceptuses. Northern-blot filters (a) of total RNA from the amnions were hybridized with the Sod1 probe, stripped, and sequentially hybridized with Gpx1, β -actin, and 18S ribosomal probes. Each filter contained a track loaded with total RNA from a 15-d embryo (15E), which served as an internal control and facilitated comparison across filters. Graphic representation (b) of the quantitative expression of Sod1 and Gpx1 (relative to β -actin and 18S ribosomal RNA) in the amnions shown in a. The Northern blots shown in a were scanned with a laser densitometer, corrected for RNA loading, and shown relative to β -actin and 18S ribosomal RNA.

Day 16 fetuses. The liver is still the organ expressing the highest levels of Sod1 mRNA in 16-d murine fetuses (Figs. 8 and 9b). The order of Sod1 expression in organs of 16-d murine fetuses is as follows: liver > intestine > heart > kidney > lung > stomach > brain. The order of Gpx1 mRNA levels in these fetuses is as follows: heart > liver > lung > kidney > intestine > brain > stomach.

Day 1 neonates. The liver is also the organ with the highest

mRNA levels for Sod1 immediately after birth (Figs. 8 and 9c). Sod1 mRNA levels in the liver are approximately 1.5-fold greater than the kidney, which expressed the second highest amount of Sod1. The level of Sod1 in the heart is similar to that in the kidney. The heart is followed by (in descending order of expression) stomach, lung, brain, and intestine. The d 1 neonatal liver also has the greatest amount of Gpx1 mRNA. This amount is approximately 2-fold greater than the heart, which is followed by the kidney, lung, stomach, intestine, and brain.

Day 7 neonates. The quantitative pattern of Sod1 and Gpx1 expression changes in the organs of 7-d neonatal mice (Figs. 8 and 9d). At this stage of development, the organ with the highest mRNA levels for Sod1 is the heart, followed by thymus > brain > liver > lung > stomach > spleen > kidney. The organ with the highest mRNA for Gpx1 is the spleen. The levels of Gpx1 mRNA in the spleen are approximately 3-fold higher than those of the liver (the organ with the second highest level of Gpx1 mRNA). The liver is followed by the heart > thymus > lung > stomach > brain > kidney.

Adult organs. At this stage of development, the liver is the organ with the highest levels of Sod1 mRNA among the seven adult organs studied (Figs. 8 and 9e). This level is approximately 2-fold higher than that of the spleen, which is the organ with the second highest levels of Sod1 mRNA. The other organs have relatively similar levels of Sod1 mRNA with the order as follows: lung > kidney > brain > heart > ovary. The adult spleen is the organ with the highest levels of Gpx1 mRNA. This level is approximately 7.5-fold greater than the level of Gpx1 mRNA seen in the liver (the organ with the second highest level of Gpx1 mRNA). The remaining organs follow as listed: kidney (with approximately a 4-fold greater Gpx1 expression) > lung > heart > ovary > brain. The levels of mRNA for Sod1 and Gpx1 are generally higher in adult organs than in neonatal and embryonic organs, with Gpx1 levels in the brain being the exception.

DISCUSSION

We have shown the following in this study: 1) Sod1 and Gpx1 mRNA levels are quantitatively regulated in a temporal and tissue-specific manner during later fetal development; 2) a surge in expression of both Sod1 and Gpx1 mRNA levels occurs just before or at birth in the lung and stomach and in late gestation in the liver (both mRNA levels and enzyme activity); 3) an elevation in Sod1 occurs before or at birth in the brain; 4) the kidney shows an elevation in Gpx1 mRNA levels at or around the time of birth; 5) the heart does not demonstrate a surge at birth in the expression of the antioxidant enzymes studied; 6) the expression of Sod1 and Gpx1 mRNA progressively increases in the amnion until d 14 and d 13 of gestation, respectively, after

Fig. 8. Northern-blot filters of total RNA from organs of 14-d and 16-d mouse fetuses, 1-d and 7-d neonatal mice, and adult mice. Filters were hybridized with a probe for Sod1, stripped, and sequentially rehybridized with Gpx1, β -actin, and 18S ribosomal RNA probes. 15E, 15-d embryo; Br, brain; Li, liver; St, stomach; He, heart; In, intestine; Lu, lung; Ki, kidney; Th, thymus; Sp, spleen; Ov, ovary.

Fig. 9. Graphic representation of the quantitative expression of Sod1 and Gpx1 (relative to β -actin and 18S ribosomal RNA) in the various organs of 14-d (a) and 16-d (b) mouse fetuses, 1-d (c) and 7-d (d) neonatal mice, and adult (e) mice. The Northern blots shown in Figure 8 were scanned with a laser densitometer, corrected for RNA loading, and shown relative to β -actin and 18S ribosomal RNA. The data for the liver and kidney of 14-d fetuses are shown even though they are not shown in Figure 8.

which a decline occurs in expression of these genes; 7) of the organs studied, the liver is the one with the highest levels of both Sod1 and Gpx1 mRNA levels during murine embryogenesis and immediately after birth; it is also the organ with the highest expression of Sod1 mRNA in the adult; and 8) the spleen is the organ with the highest levels of Gpx1 mRNA in 7-d neonatal and adult mice. These findings are relevant to an analysis of the reason for the surge in antioxidant enzymes in late gestation or at birth, and to the levels of antioxidant gene product in different organs of embryonic, neonatal, and adult mice.

Previous studies have mainly focused on the activity of antioxidant enzymes in the lung in late gestation or at birth. These studies (5–9, 19) have demonstrated that in a variety of animal species (guinea pig, rabbit, hamster, and rat) a surge in pulmonary antioxidant enzyme activity occurs in late gestation before birth. These findings have been ascribed to the preparation of the pulmonary antioxidant system, in a manner analogous to the surfactant system (5), for the process of birth. During the birth process the fetus will move from a relatively hypoxic environment to an hyperoxic environment where an approximately 5-fold elevation in oxygen concentration occurs (5). Thus, the surge in antioxidant enzyme expression may be beneficial in protecting the newborn from oxygen toxicity, especially because an equivalent increase in inspiratory oxygen concentration is lethal to adults (20).

It cannot necessarily be assumed, however, that the surge in antioxidant expression that occurs in late fetal development in other animal species occurs in the mouse. It has been reported

that such a surge fails to occur in the human lung (21). Furthermore, we have been unable to find data examining the expression of Sod1 and Gpx1 in late-gestational mouse fetuses. Only one study has examined the embryonic expression of Sod1 and Gpx1 in the mouse liver and the remaining carcass (22). These workers report an increase in the expression of Sod1 and Gpx1 in the liver and remaining carcass during development. They have not, however, studied the expression of antioxidant enzymes in other individual organs. Thus, our data of increasing Sod1 and Gpx1 mRNA levels in the mouse lung at or just before birth demonstrates that the mouse is similar in this regard to other animal species. Furthermore, our data would appear to support the hypothesis that the pulmonary antioxidant system undergoes a preparation for birth. However, the process of birth cannot be the sole reason for the surge in antioxidant mRNA levels because we find that during organogenesis (d 14–16) the mRNA levels for Sod1 are even higher than those at or around the time of birth. Nevertheless, if the transition from an hypoxic to hyperoxic environment is a trigger for the surge in the expression of lung antioxidant enzymes during late fetal development, then this surge should occur in all aerobic organisms where such a transition occurs. In this context, it remains to be explained why such a surge in antioxidant enzymes fails to occur in human fetuses. This explanation is especially important because it has been proposed that the failure to detoxify reactive oxygen species might be related to aspects of the oxidative-induced morbidity and mortality (e.g. bronchopulmonary dysplasia) that occurs in preterm human infants (23).

It has also been argued by others that the surge in expression of antioxidant enzymes in organs of late-gestation fetuses cannot solely be assigned to the preparation of the pulmonary antioxidant system for birth (5). This proposal was based on the finding that a similar surge in the expression of antioxidant enzymes also occurs in the livers of late-gestation guinea pig fetuses (5). The liver, unlike the lung, is not directly exposed to higher oxygen concentrations at birth. Our data in this study confirm and extend the finding of Rickett and Kelly (5) by showing that the surge in the levels of both Sod1 and Gpx1 occurs in the livers of mouse fetuses in late gestation, albeit that the levels for both these enzymes decline after birth in the early neonatal period. It may be that the elevation of expression of antioxidant enzymes in the liver, and the finding that the expression of the antioxidant enzymes is relatively high in adult livers, is related to the rate of metabolism of the organ rather than to the concentration of oxygen *per se*. Higher rates of metabolism may result in the generation of reactive oxygen species. It is also interesting that the expression of Sod1 and Gpx1 increases in the amnion during the period of organogenesis in the mouse. The amnion is a membrane that surrounds the fetus. Consequently, the elevation of expression of antioxidant enzymes in both the liver and the amnion may afford protection against the generation of reactive oxygen species in the maternal compartment.

The extension of studies on the expression of antioxidant enzymes to other organs may be useful for evaluating the purpose of the surge in antioxidant expression in organ(s) of late-gestation fetuses. The data in this study demonstrate that the elevation in expression of both Sod1 and Gpx1 antioxidant enzymes occurs in the liver, lung, and stomach. An elevation in Sod1 expression, but not Gpx1, occurs in the brain of fetuses in late gestation, at birth, or both. However, the heart fails to show an appreciable elevation of both Sod1 and Gpx1 expression in late gestation or at birth. Yet the oxygen consumption of the heart in fetal lamb development during late gestation is far greater than that of other organs (8.0 mL/100 g of tissue). Indeed, the order of oxygen consumption by organs in late gestation lamb fetuses is heart (8.0 mL/100 g) > brain (4.0 mL/100 g) = liver > kidney (2.6 mL/100 g) > lung (0.6 mL/100 g) > intestine (0.4 mL/100 g) = skin, bone, and muscle (24). Thus, the data suggest that the increase in expression of antioxidant enzymes in late gestation or at birth in organs of the mouse may not correlate with the

oxygen tension or oxygen consumption of organs [if this finding is similar to that in the lamb, which it is presumed to be (25)]. In addition, the finding that Sod1 and Gpx1 mRNA levels are not temporally up-regulated in all tissues in a similar fashion further argues against the idea that the up-regulation of the antioxidant genes occurs in response to the increased oxygen consumption of specific tissues. These findings are further supported by a comparison of the relative levels of Sod1 and/or Gpx1 mRNA in different organs of the adult. The brain is the organ that consumes approximately 25% of the circulating oxygen from the blood (26), whereas the lung, of the internal organs, is exposed to the highest concentration of oxygen (27). Yet both the lung and the brain have much lower mRNA levels for the antioxidant enzymes than the liver in embryonic, neonatal, and adult mice. Thus, the organs with the highest oxygen consumption or oxygen tension do not have the highest expression of antioxidant enzymes.

It may be that the antioxidant enzymes have other functions in addition to preparation of the pulmonary system for birth and the protection of organs from free oxygen radicals. For instance, by altering the activities of Sod1 and/or Gpx1 the formation or persistence of the superoxide radical, hydrogen peroxide, or hydroxyl radical molecules may be favored. It has recently been shown that the exposure of cells to increasing amounts of hydrogen peroxide increases the binding of the NF- κ B transcription factor (28). Indeed, it has been proposed that hydrogen peroxide (and possibly other free radicals) may be second-messenger molecules affecting the proliferation and differentiation of cells (28, 29). Thus, it is conceivable that the expression of Sod1, Gpx1, or both may also be influenced by the need for molecules resulting from the antioxidant pathway to carry out its other functions. In this regard, it is also interesting that the activity of Sod increased during differentiation of the slime mould *Physarum polycephalum* (30). Furthermore, augmentation of Sod1 via liposomes into undifferentiated *P. polycephalum* and Friend erythroleukemia cells induced the differentiation of these cells (31, 32). In addition, it has been shown that the cellular environment becomes progressively more oxidized during embryonic development in the housefly (33). Oberley *et al.* (34, 35) have also shown that the expression of antioxidant enzymes change in different areas of the developing Syrian hamster kidney and in organs where self-renewal occurs, thus suggesting an important role for these enzymes in cell differentiation during development and self-renewal. Thus, all these data are suggestive of other functions for these enzymes, which may influence the levels of Sod1 and Gpx1 in organs of the developing mouse embryo or fetus.

The molecular mechanisms whereby the expression of Sod1 and Gpx1 are up-regulated in the lung and liver of late-gestation fetuses remains to be explained. Our data demonstrate, however, that such an up-regulation occurs at the mRNA level of both genes. Whether the increase in Sod1 and Gpx1 mRNA is due to increased transcription of both genes or due to increased mRNA stability remains to be explained. In addition, it is important that further studies on establishing the expression patterns of Sod1 and Gpx1 during murine embryo development should be pursued using *in situ* hybridization analysis, immunohistochemistry, or both, so that differential expression by specific cell types within organs are identified.

In conclusion, this study has shown that a surge in Sod1 and Gpx1 expression occurs in late-gestation murine fetuses in some but not all organs. The lung, liver, and stomach are organs where such a surge occurs. In the other organs, either an elevation in Sod1 or Gpx1 expression alone occurs (*e.g.* brain and kidney respectively) or no elevation in both Sod1 and Gpx1 occurs (*e.g.* the heart). The data in this study suggest that the mRNA level of Sod1 and Gpx1 expression is unrelated to the oxygen consumption and to oxygen tension exposure in individual organs of late-gestational mouse fetuses and is thus suggestive of other functions for these enzymes.

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Announcement

Paediatric Week Holland

The Paediatric Week Holland will be organized in Rotterdam, The Netherlands from June 29 to July 6, 1994. From July 3 through July 6, a combined meeting will be held of the European Society for Paediatric Research, the European Paediatric Respiratory Society, the European Respiratory Society-Paediatric Assembly, the European Society of Paediatric Allergy/Clinical Immunology, the European Society for Paediatric Infectious Diseases, the European Society of Paediatric Intensive Care, and the European Society for Paediatric Hematology and Immunology. From June 29 through July 2, the British Association of Paediatric Surgeons will meet, and the Dutch Society of Psychosocial Consultation and Paediatrics will meet from July 3 through July 5. Postgraduate courses will be organized in the overlapping weekend. *For further information, please contact: Pieter J. J. Sauer, M. D., c/o Erasmus University Rotterdam, P.O. Box 1738, NL-3000 DR Rotterdam, The Netherlands.*