Expression of Manganese Superoxide Dismutase in Ovine Kidney Cortex during Development

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ABSTRACT. Manganese superoxide dismutase (MnSOD) is one of the main antioxidant enzymes in mammalian tissue. Previous studies have shown that the activity of MnSOD increases in the rat kidney during development. To define further the developmental change in MnSOD activity and better understand some of the steps involved in the control of MnSOD expression during kidney development, we measured MnSOD messenger RNA and enzyme activity in the ovine kidney cortex during fetal life, in the newborn period, and in adults. MnSOD mRNA and enzyme activity were detected at 0.65 gestation. Hybridization of the Northern blot with a human MnSOD cDNA probe showed evidence of two transcripts of 4.0 and 1.5 kb, respectively. There was a significant increase with age of MnSOD activity and MnSOD mRNA ($p < 0.0001$). The abundance of each MnSOD transcript significantly increased with age ($p < 0.001$). In the fetuses, both transcripts increase in parallel; in newborns and adults the 1.5-kb increase was significantly greater than the 4.0-kb increase. Enzyme activity and mRNA were strongly correlated ($r = 0.89$, $p < 0.0001$). These data indicate that the expression of MnSOD is developmentally regulated in the ovine kidney cortex. This increase seems to be dependent largely on pretranslational events. (*Pediatr Res* 35: 41–44, 1994)

Abbreviations

SOD, superoxide dismutase
MnSOD, manganese superoxide dismutase
CuZnSOD, copper and zinc superoxide dismutase
NBT, nitroblue tetrazolium
G, gestation

Superoxide dismutases are enzymes that catalyze the dismutation of superoxide radicals into oxygen and hydrogen peroxide. In eukaryotic cells two forms of SOD are found, one containing copper and zinc (CuZnSOD) in the cytosol and one containing manganese (MnSOD) in the mitochondrial matrix. Inefficient detoxification of superoxide radicals has been associated with the etiology of various diseases in adults and premature infants (1–3). Studies in several experimental animal models, including sheep, have shown that antioxidant enzymes are weakly expressed in fetal lung until late in gestation. It has been suggested that infants born prematurely would be at increased risk for oxidative stress because of the reduced levels of lung antioxidant activity (4–6).

Recent data indicate that the activity of antioxidant enzymes is developmentally regulated in the kidney as it is in the lung. MnSOD activity has been shown to increase in guinea pig and rat kidney from fetal to the adult period (2, 7). In addition, MnSOD has been also detected by immunohistochemistry in kidney cortex and medulla in the Syrian hamster and the staining increases progressively during development (8).

Therefore, to understand better the mechanisms of MnSOD expression in the developing kidney, we measured MnSOD activity and mRNA levels in ovine kidney cortex in early gestation (0.65 G) and late gestation fetuses (0.96 G), newborn lambs and adult sheep. We hypothesized that parallel increases in mRNA and enzyme activity would occur as maturation progressed.

MATERIALS AND METHODS

Animals. Ewes with known single insemination dates in which pregnancy was confirmed at 50 d of gestation by ultrasound, and nonpregnant adult animals, were obtained from a local supplier. Newborn lambs were obtained from pregnant ewes brought to the vivarium at term and allowed to deliver naturally. Pregnant ewes were anesthetized with ketamine 10 mg/kg. The fetuses were delivered through an abdominal hysterotomy, given a lethal dose of pentobarbital and the kidneys were removed. Samples of kidney cortex were obtained from 2 female and 4 male fetuses at 92–94 days of gestation (early gestation, EG) and from 3 female and 3 male fetuses at 138–142 days of gestation (late gestation, LG). Tissues were collected similarly from 2 female and 3 male newborn lambs (0.4–2 days old) and 4 female nonpregnant sheep. Slices of kidney cortex were obtained by radial slicing, including inner and outer cortex, immediately frozen in liquid nitrogen and stored at -80°C until processed. All procedures were approved by our Institutional Animal Care and Use Committee.

MnSOD activity assay. The SOD activity was determined by the NBT method as previously described (9). Briefly, tissue was homogenized on ice (100 g/L) in 50 mM phosphate buffer (pH 7.8) with a tissue homogenizer (Tekmar Co., Cincinnati, OH) by three bursts of 30 sec each. The homogenate was then sonicated on ice with a sonic dismembrator (Fisher Scientific, Pittsburgh, PA; model 550) for three cycles of 30 sec each. Protein content was determined by the method of Bradford (10). Tissue samples containing from 0 to 500 μg of protein were incubated with 10^{-4} M xanthine, 5×10^{-5} M NBT, 1 unit of catalase, and 1 mM diethylenetriaminepentaacetic acid. As soon as xanthine oxidase (10^{-3} units) was added to the mixture, the rate of reaction was monitored by following the changes in absorbance of the mixture with a spectrophotometer at 560 nm (Shimadzu UV-Vis spectrophotometer, Shimadzu Scientific Instruments, Columbia, MD). Endogenous xanthine oxidase inhibitory activity was measured in a representative sample from each tissue by following the conversion of xanthine to uric acid at 290 nm. The sensitivity of

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this assay was such that 1 unit of SOD activity equalled 15 ± 3 ng of pure bovine liver SOD. To specifically measure the activity of MnSOD, 5×10^{-3} M of sodium cyanide (NaCN) was added to the incubation mixture for 30 min to inhibit CuZnSOD. In the tissues studied no appreciable reduction of NBT by endogenous reductase occurred and the activity of xanthine oxidase remained unchanged during the course of the assay.

Total RNA extraction. The total RNA was extracted from fetal, newborn, and adult kidneys using a modified acid-guanidinium procedure (11). Tissue slices were homogenized (100 g/L) in RNA extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M β -mercaptoethanol) with polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) at setting 6 for 30 s. After homogenization, 50 μ L of 2 M sodium acetate, pH 4.0, 500 μ L H₂O-saturated phenol, and 100 μ L of chloroform-isoamyl alcohol (49:1) were added. After this the samples were centrifuged for 3 min at $14\,000 \times g$ and 1 volume of ice-cold isopropyl alcohol was added to the aqueous phase. After incubation at -80°C for 15 min, samples were centrifuged for 10 min. The resulting pellets were dissolved in 500 μ L of RNA extraction buffer, reprecipitated with the same volume of isopropyl alcohol for 15 min at -80°C , and centrifuged for 10 min. The pellets were then washed twice with cold 75% ethanol, dried, and resuspended in water that has been treated with diethyl pyrocarbonate. The RNA concentration was determined by absorbance at 260 nm.

Northern blot analysis. Total RNA (20 μ g) from each animal was separated by electrophoresis through formaldehyde-agarose (1.2%) gels in 10 mM 3-N-morpholine propanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate, and transferred to nylon membrane (Zeta-probe, Bio-Rad) with $20\times$ SSC. An RNA ladder (0.25–9.5 kb, Bethesda Research Laboratories) was electrophoresed with the samples, stained with ethidium bromide, and photographed for use as a size marker. The nylon membrane was irradiated with ultraviolet light for 90 s to link the nucleic acids to the membrane irreversibly. The membrane was prehybridized for 4 h at 65°C with 0.5 M sodium phosphate buffer (pH 7) containing 7% SDS, 1 mM EDTA, denatured salmon sperm DNA (100 mg/L), and tRNA (10 mg/L) and then hybridized with the same buffer with the addition of 2×10^6 cpm/mL ^{32}P MnSOD cDNA probe. After overnight hybridization, the filter was washed at 65°C five times for 10 min each in a buffer containing 0.1 M EDTA, 1% SDS, and 40 mM NaH₂PO₄, pH 6.8 (12). After autoradiography, the MnSOD probe was stripped and the filter was rehybridized with the ^{32}P β -actin probe (4×10^6 cpm/mL) for control.

Probe preparation. The isolation of full-length cDNA for human MnSOD has been described previously (13). The cDNA probe was labelled with the random priming procedure of Feinberg and Vogelstein (14).

A 770-bp cDNA from chicken β -actin was obtained from Oncor, Inc., and labeled with the random priming method (14).

Data analysis. After autoradiography the intensity of the bands on all Northern blot autoradiograms was measured by optical densitometry recorded on a laser densitometer. Two measurements of signal intensity at different exposure times were obtained for each sample. To correct for any variation in RNA blotted on the nylon, the densitometric signals obtained from the β -actin were quantified and results from the MnSOD signal were expressed relative to the β -actin signal. In previous experiments we demonstrated that β -actin does not change significantly in the ovine kidney cortex during development and in the adult (15). Duplicate samples of total RNA from each animal were analyzed for MnSOD and β -actin results were expressed as the average of the two measurements. Data were analyzed by analysis of variance after logarithmic transformation. Differences among groups were evaluated with Newman-Keuls test. A significance level of 0.05 was used to reject the null hypothesis in all cases. MnSOD activity in the kidney cortex was measured from each animal and expressed as units per milligram of total protein.

Data were analyzed by analysis of variance as described for the mRNA. Regression analysis was carried out to determine whether there was a relationship between MnSOD mRNA and enzyme activity.

RESULTS

The activity of MnSOD was detected in the ovine kidney cortex by 90 d of gestation (0.65 G). Figure 1 shows the MnSOD activity in the ovine kidney cortex from the animals at each gestational age. There was a significant increase in MnSOD activity from early fetal life to the adult period ($F = 29.5$, $p < 0.0001$). The values of MnSOD activity were lowest in early gestation fetuses (30 ± 2.3 units/mg) and increased similarly in the late gestation fetuses (42.3 ± 0.22 units/mg) and newborn lambs (47.5 ± 4.8 units/mg) ($p < 0.005$). Levels in the adult were higher than those observed in fetuses and newborn (99.5 ± 18.5 units/mg) ($p < 0.0001$).

To evaluate whether the increase in MnSOD activity was associated with an increase in MnSOD mRNA, the level of MnSOD mRNA was measured in the ovine kidney cortex in the same group of animals. Northern analysis showed the presence of two transcripts of 4.0 and 1.5 kb in the ovine kidney cortex after hybridization with a human MnSOD cDNA (Fig. 2).

Relative amounts of MnSOD mRNA were quantitated by the Northern blot technique. Figure 3 shows the relative levels of MnSOD mRNA (1.5 + 4.0 kb/ β -actin) observed in each group. MnSOD mRNA levels significantly increased with age ($F = 20.2$, $p < 0.0001$). MnSOD mRNA levels were lowest in early gestation fetuses (0.36 ± 0.118), increased 2-fold in the late gestation fetuses (0.635 ± 0.02) ($p < 0.05$), 3-fold in the newborn lambs (1.173 ± 0.306) ($p < 0.01$), and 7-fold in the adult (2.52 ± 1.13) ($p < 0.001$). No significant changes in β -actin were observed in the ovine kidney cortex from the same groups of animals.

To determine whether there was a relationship between MnSOD mRNA and enzyme activity, regression analysis was performed. Figure 4 shows the correlation between mRNA and enzyme activity. Levels of MnSOD mRNA (1.5 + 4.0 kb) and enzyme activity were strongly correlated ($r = 0.9$, $p < 0.0001$). To determine whether the increase in MnSOD was due to a parallel increase of both transcripts, we have quantitated each transcript separately in each animal. Relative levels of the 1.5 and 4.0 kb transcripts are represented in Figure 5. Both transcripts increase with age ($p < 0.001$). During fetal life the two transcripts increase in parallel and the ratios between 1.5 and 4.0 kb transcripts did not change (1.5/4.0 kb was 1.1 ± 0.12 and 1.1 ± 0.17 at 0.65 G and 0.95 G, respectively). In the newborn and adult sheep, the increase in the 1.5-kb mRNA was greater than the 4-kb mRNA increase, thus the 1.5/4.0 kb ratios were higher

Fig. 1. MnSOD activity (units per milligram of protein). Values represent mean $\pm$ SE of levels in early gestation (EG) fetuses ($n = 4$), late gestation (LG) fetuses ($n = 6$), newborn lambs (NL) ($n = 5$), and adult sheep (A) ($n = 4$). Bars identified by different letters are significantly different.

Fig. 2. *Upper panel*: Northern blot analysis of total RNA from ovine kidney cortex. The lane contained 20 μ g of total RNA from a late gestation (LG) fetus. Hybridization with a human MnSOD cDNA probe shows evidence of two transcripts of 4.0 and 1.5 kb, respectively (*upper panel*). The same filter was washed and rehybridized with a β -actin cDNA probe (2.0 kb) (*lower panel*).

Fig. 3. Relative levels of MnSOD mRNA evaluated by Northern blot technique and densitometric analysis of the autoradiograms. Values represent mean $\pm$ SE of the ratio of MnSOD (1.5 + 4.0 kb) to β -actin densitometric units from early gestation (EG) fetuses ($n = 6$), late gestation (LG) fetuses ($n = 6$), newborn lambs (NL) ($n = 5$), and adult sheep (A) ($n = 3$). Different letters above the bars indicate that values are significantly different ($a < b < c < d$).

than those observed in the fetuses (1.9 ± 0.2 and 1.9 ± 0.5 , $p < 0.005$). Enzyme activity correlated well with each single transcript ($r = 0.9$, $p < 0.0001$ for both transcripts).

DISCUSSION

The antioxidant system may play an important role for the fetus during the perinatal transition to an oxygen rich environment. MnSOD represents the primary antioxidant enzyme in the mitochondria (16).

This study is the first report of the pattern of expression of MnSOD gene (mRNA level and enzyme activity) in the ovine kidney cortex during development. Our results demonstrate that MnSOD gene is expressed in the kidney cortex by 0.65 gestation and that levels progressively increase during development. The

Fig. 4. Correlation between MnSOD mRNA (1.5 + 4.0 kb) and MnSOD activity in 18 animals ($r = 0.89$, $p < 0.0001$).

Fig. 5. Quantitative analysis of the 1.5-kb and 4.0-kb mRNA species. Both transcripts increase with age ($p < 0.0001$ and $p < 0.001$, respectively). In newborn lambs and adult sheep the ratios between 1.5 and 4.0 kb transcript are higher than those observed in fetuses ($p < 0.005$).

increased expression of MnSOD transcripts is associated with an increase in MnSOD activity. The mRNA levels strongly correlate with enzyme activity. These findings suggest that in the ovine kidney cortex the expression of MnSOD is developmentally regulated and that from fetus to adulthood the control of steady-state levels of MnSOD activity is largely dependent on the regulation of MnSOD mRNA levels. A similar mechanism of pretranslational control has been indicated for the CuZnSOD and for many other enzymatic activities (5- α -reductase, sucrose isomaltase, amylase, argininosuccinatelyase, catalase, and lactate-phlorizin hydrolase) during development (17-21).

The newborn tissues are exposed to the damaging effect of free oxygen radicals, due to the significant rise in PO_2 occurring at birth. Oxidant-mediated injury has been suggested as an important mechanism of renal failure in postischemic reperfusion and tubular necrosis (2, 22). *In vitro* studies have shown that increased levels of MnSOD activity in cultured cells significantly improved cell survival after exposure to high concentrations of O_2 (23, 24). Thus, the increase in expression of MnSOD observed as gestation progresses may confer to the kidney cortex of mature fetuses increased protection against free oxygen radicals and may be important for the survival of the fetus in the extrauterine environment. At present, factors involved in the developmental increase in MnSOD activity in the kidney are unknown. The observed increase in the perinatal period is temporarily correlated with significant hormonal changes such as the increase in plasma cortisol level. In the lung glucocorticoid hormones increase antioxidant activity along with the increase in the surfactant (25). It is possible that similar factors are acting on the MnSOD expression in the kidney. However, the progressive increase in MnSOD expression observed from newborn to adulthood sug-

gests that in the extrauterine environment other factors beside hormonal may be important.

MnSOD mRNA in the ovine kidney is represented by two transcripts of 1.5 and 4.0 kb, respectively. Transcripts of the same size have been observed in ovine lung tissue (G. M. R. Carbone and J. C. Rose, unpublished data). Multiple transcripts for the MnSOD have been observed in human and several animal species including rats, mice, cows, and pigs (26). It has been indicated that the sequences at the 3'-untranslated region account for the differences in transcript size (1). In the rat all the transcripts are fully processed (27); however, it is still not known whether they are all efficiently translated to protein. The 3'-untranslated region could be biologically active in regulating the expression of MnSOD gene (1).

Our data have shown that both mRNA species increase in the kidney from fetal life to adulthood; however, whereas during fetal life the relative abundance of each transcript is similar, in the newborn and adult the 1.5-kb transcript is approximately 2 times more abundant than the larger 4.0-kb transcript. These findings indicate that there are age-related differences in the regulation of the two mRNA species. The variation in transcript abundance may result from differences in production or stability of each mRNA. The biological significance of the increase in the abundance of one transcript over the other is not clear at present. Our data indicate that in basal conditions both transcripts may be translated to protein because the enzyme activity is well correlated with the combination of both mRNA species and with each individual transcript. However, it is possible that with stimulation the release of active enzyme is achieved primarily by translation of the smaller transcript (28). If this is the case, newborns and adult animals would have greater ability to mount an antioxidant response than fetuses. Further studies are necessary to verify this hypothesis.

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