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# STUDIES ON COPPER-ZINC SUPEROXIDE DISMUTASE EXPRESSION IN DEVELOPING HUMAN LIVER AND KIDNEY

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CuZn superoxide dismutase levels were found to be high in developing human kidney and liver compared to some other tissues including lung. In kidney, the enzyme was expressed in proximal and distal tubules, loop of Henle and collecting tubules and after 35 weeks of gestation it appeared to be distributed basally in proximal cells and luminally in distal cells. Glomerular structures were generally negative. CuZn superoxide dismutase was widely expressed in developing liver, with hepatocytes and bile duct epithelium demonstrating positivity.

The low level of expression of CuZn superoxide dismutase in the glomerulus compared with the tubules was not expected since intrinsic glomerular cells demonstrate greater production of reactive oxygen species in response to some stimuli than do tubular cells.

Expression of this enzyme may be determined by the need to generate hydrogen peroxide.

KEY WORDS: CuZn superoxide dismutase, human, kidney, liver, development.

## INTRODUCTION

The putative importance of oxygen-derived free radicals in the aetiology of some human diseases has prompted interest in the enzymes responsible for the detoxication of these potentially dangerous species. Various enzymes are involved and though the relative importance of each is unclear, CuZn superoxide dismutase (EC 1.15.1.1), the cytosolic enzyme that catalyses the dismutation of superoxide anion to hydrogen peroxide has attracted particular attention.[1-3]

The importance of CuZn superoxide dismutase is indicated by its almost ubiquitous distribution in aerobes[2] as well as studies showing that the enzyme reduces oxidant toxicity in various tissues including lung[4] kidney[5] and liver.[6] Transfection of mouse cells however, with the gene for the human enzyme results in increased hydrogen peroxide-induced lipid peroxidation rather than membrane protection,[7] suggesting that the enzyme can catalyse a disproportionately large increase in the formation of hydrogen peroxide. These studies have implications for tissue development since at

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least in some species, hydrogen peroxide is an effector of cell differentiation,[8] possibly by stimulating prostaglandin synthesis.[9] Reactive oxygen species for example, increase synthesis of prostaglandin $E_2$ by glomeruli from adult rats[9] and the fetal kidney is a major source of prostaglandins, which are believed to have marked effects on renal morphogenesis.[10]

The developmental expression of CuZn superoxide dismutase is of interest because although the foetus exists in a relatively hypoxic environment, tissue oxygen consumption and, presumably production of free radicals varies considerably.[11] Further, the transition from inter- to extra-uterine life necessitates moving from an environment of low oxygen tension to one that is comparatively hyperoxic and birth might be a stimulus for the expression of free-radical detoxicating enzymes. Whilst animal studies have shown time- and tissue-specific expression of CuZn superoxide dismutase in developing lung, liver and kidney,[12,13] the only data presently available in a human tissue, the lung, show continuous expression of the enzyme during fetal and neonatal life.[14]

Comparing the developmental expression of CuZn superoxide dismutase in human tissues that apparently have different susceptibilities to free radical attack may help elucidate the role of this enzyme. We now describe studies to determine the levels and distribution of CuZn superoxide dismutase in developing kidney, a tissue that in both neonates and adults commonly suffers damage following ischaemia with subsequent reperfusion and liver, a tissue that appears more resistant to this type of attack. Our findings are compared with equivalent data from lung, a tissue whose sensitivity to free radicals has attracted much interest regarding the pathogenesis of bronchopulmonary dysplasia.[14] Since there have been criticisms of the methods used to assay the activity of this enzyme,[15] we have also used a radial immunodiffusion approach to determine tissue levels.

## MATERIALS AND METHODS

### *Patient Samples*

Samples of lung, liver, kidney, adrenal, spleen, diaphragm and heart were obtained within 4 h of death from aborted fetuses (11–24 weeks of gestation) following termination of pregnancy, premature and term infants (25–42 weeks gestation) who died in the neonatal period and infants who suffered sudden infant death syndrome (2–60 weeks of postnatal age). Infants with respiratory distress syndrome, bronchopulmonary dysplasia or clinical or histological evidence of other disease affecting the tissue being studied were excluded. The aims and methods of the study were approved by the Paediatric Reproductive Medicine Ethics of Medical Research Sub-Committee of the Simpson Memorial Maternity Pavilion, Royal Infirmary, Edinburgh.

### *Chemicals*

CuZn superoxide dismutase, purified from human blood[14] was used as standard in radial immunodiffusion studies and to raise a polyclonal anti-serum in sheep.[14] Agarose was obtained from LKB-Pharmacia Ltd., Milton Keynes, U.K. and Gelbond film from FMC Corporation, Marine Colloids Division, Rockland, Maine, U.S.A.

*Determination of CuZn superoxide dismutase levels*

Enzyme levels were determined in tissue 150 000 g supernatants (cytosols)[14] using radical immunodiffusion. Agarose (160 mg) was mixed with 12 ml PBS buffer (Oxoid) and 4 ml polyethylene glycol (12% w/v; mol, wt 6000) and dissolved in a boiling water bath. The solution was cooled to 50°C and 200 μl of anti-human CuZn superoxide dismutase serum was added with mixing. This mixture was poured onto a Gelbond plate, allowed to set and holes were punched in the gel. Samples and enzyme standards (20 μl) were pipetted into the wells and incubated (48 h at 25°C) in a moist chamber. One drop of NaCl (154 mM) was added to each well and filter paper, moistened with NaCl, placed over the gel followed by weighted dry paper towels. After 10 min the papers were removed and the gel immersed in NaCl (25°C) for 15 h. Moistened filter paper followed by dry paper towels were again placed over the gel which was then dried (2 h; 40°C). After cooling, the gel was immersed in Page Blue for 10 min and destained in 3 changes of ethanol: water: glacial acetic acid (3: 8: 1, v/v). CuZn superoxide dismutase concentrations were determined from plots of the square of the ring diameter versus concentration of enzyme standard. The between-batch c.v. of the method was 8%.

*Immunohistochemistry*

Tissue samples were fixed in buffered formalin, processed to paraffin wax and serial sections cut[14] and stained with haemotoxylin and eosin, anti-CuZn superoxide dismutase serum or approprate negative control antiserum using an avidin-biotin complex method.[14,16]

*Analytical*

Total protein concentrations were determined using the bicinchoninic acid method (Sigma Chemical Co., St Louis, Mo 63178, U.S.A.). CuZn superoxide dismutase activities in tissue homogenates were determined by monitoring the reduction of nitroblue tetrazolium by superoxide anion as previously described.[14]

TABLE I

*Levels of CuZn superoxide dismutase in human tissues during development.* The levels of CuZn superoxide dismutase in tissues obtained between 11 and 60 weeks post conception were determined using radial immunodiffusion. Data show levels of the enzyme (μg/mg cytosol protein).

| | mean | S.E.M. | n |
|---|---|---|---|
| Liver | 8.5 | 2.1 | 10 |
| Kidney | 6.2 | 1.1 | 17 |
| Adrenal | 16.1 | 2.9 | 21 |
| Heart | 3.8 | 1.9 | 9 |
| Spleen | 3.3 | 0.70 | 17* |
| Lung | 2.7 | 0.41 | 15 |
| Erythrocytes | 0.86 | 0.06 | 19 |

* Correlation of CuZn superoxide dismutase levels with time in spleen, $r = 0.87$, is highly significant ($p < 0.001$)

## RESULTS

### *Determination of CuZn superoxide dismutase in tissue cytosols*

Table 1 shows the levels of the enzyme in liver and kidney and, for comparison in erythrocytes, lung, spleen, heart and adrenal. Comparison of the 95% confidence intervals for the enzyme levels in tissues showed that mean levels in liver, kidney and adrenal were significantly greater than those in erythrocytes and lung. Enzyme levels in spleen but not other tissues demonstrated a significant increase with time (Table 1).

Enzyme protein determined immunologically was associated with CuZn superoxide dismutase activity; activities in 7 fetal liver and kidney homogenates obtained during the first two trimesters were 5.4 $\pm$ 0.56 (SEM) and 6.3 $\pm$ 0.42 (SEM) U/mg protein respectively.

### *Immunohistochemical studies of CuZn superoxide dismutase distribution in developing kidney*

Sequential 5 $\mu$m sections of kidneys obtained during development were stained with haematoxylin and eosin and examined using light microscopy. Foetal kidneys demonstrated a primitive architecture with nephrons at markedly different stages of development. The newly formed collecting tubules present in the early metanephric kidney were surrounded, at their distal ends, by metanephrogenic condensations of cells. These initially formed into hollow spheres which then folded into S-shaped bodies.

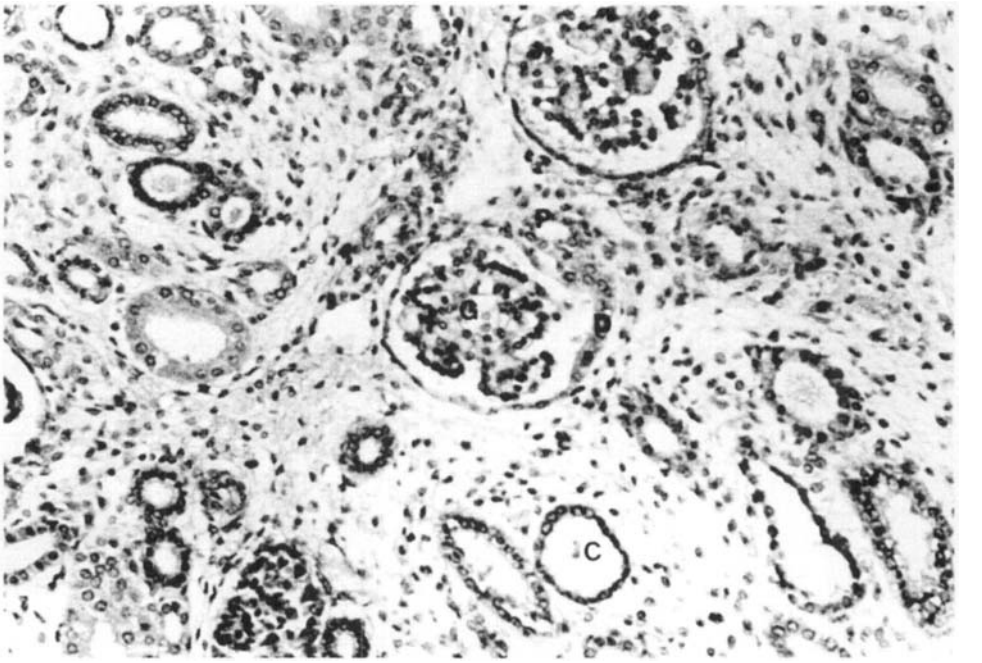
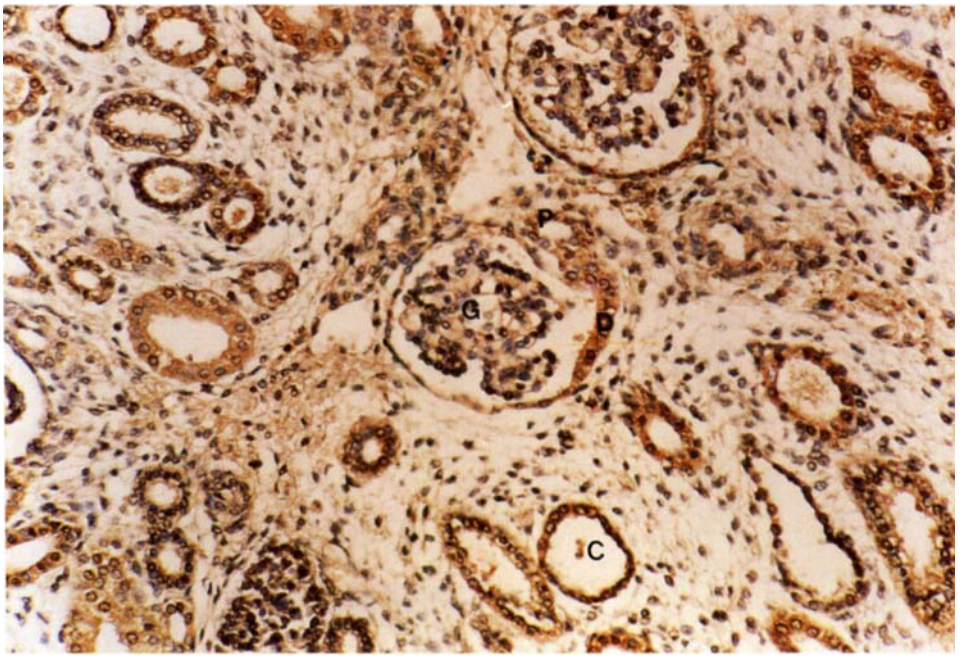
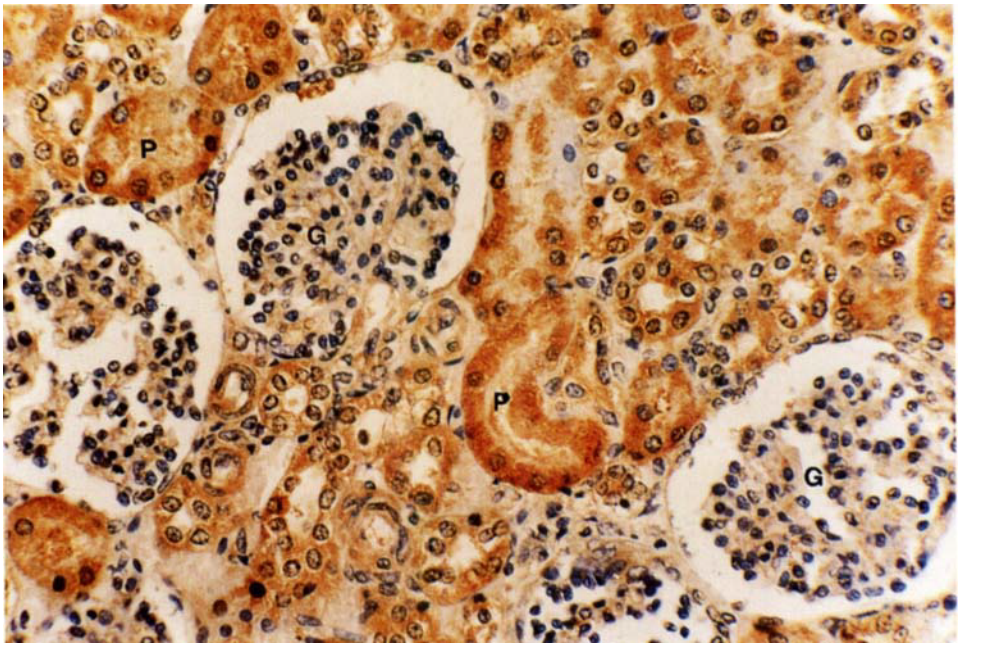



FIGURE 1 (see Color Plate I at the back of this publication). Section of foetal kidney obtained after 13 weeks of gestation showing expression of CuZn superoxide dismutase by primitive glomerulus (G), developing Bowman's capsule (D), origin of proximal tubule (P) and collecting tubule (C). Magnification $\times$ 125.




**Color Plate I (See Figure 1, page 108)**

FIGURE 1 Section of foetal kidney obtained aftger 13 weeks of gestation expression of CuZn superoxide dismutase by primitive glomerulus (G), developing Bowman's capsule (D), origin of proximal tubule (P) and collecting tubule (C). Magnification × 125.




**Color Plate II (See Figure 2, page 109)**

FIGURE 2 Section of kidney obtained 41 weeks postnatal age showing CuZn superoxide dismutase expression in the basal area of the proximal tubule (P). There was only weak expression of the enzyme in the glomerulus (G).

One end of each body thinned to form the primitive Bowman's capsule and glomerulus whilst the other fused with the elongating collecting tubule. Each S-shaped body lengthened and differentiated into the distal and proximal tubules and loop of Henle.

Sections were also stained with anti-CuZn superoxide dismutase serum. In samples obtained before 20 weeks of gestation, metanephrogenic tissue caps, S-shaped bodies and mesenchymal tissue, including blood vessels were negative. The primitive Bowman's capsule comprised flattened cells similar to those found in the adult as well as cuboidal cells that finally differentiate into the tubule; both cell types were positive for CuZn superoxide dismutase. Whilst glomerular structures were generally negative, thin rims of positivity were seen around the capillary loops (Figure 1). Once the primitive nephron began to fuse with the collecting tubule, positivity was seen along its entire length. All proximal and distal convoluted tubules and collecting tubules were positive. Developing urothelium was also positive. After 20 weeks of gestation the nephron was more developed and proximal and distal tubules, loop of Henle and collecting ducts could be distinguished more clearly, these structures were positive for the enzyme.

After 35 weeks of gestation the intracellular distribution of the enzyme in cells from the proximal and distal tubules appeared different. In proximal cells the enzyme was concentrated basally (Figure 2) whilst in some distal cells there was a more luminal distribution. Whilst it is possible that this is an artefactual observation resulting from the 'perinuclear halo', a phenomenon sometimes seen in post mortem material, a luminal distribution of the enzyme was also seen in cells that were not affected by autolytic change.

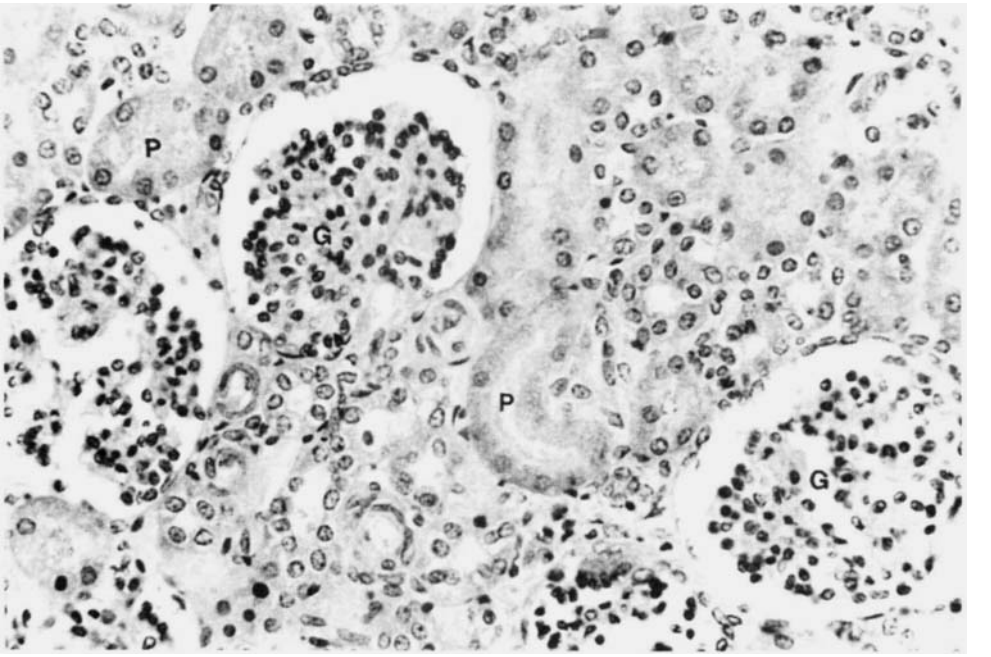



FIGURE 2 (see Color Plate II at the back of this publication). Section of kidney obtained 41 weeks postnatal age showing CuZn superoxide dismutase expression in the basal area of the proximal tubule (P). There was only weak expression of the enzyme in the glomerulus (G).

*Immunohistochemical studies of enzyme distribution in liver*

Examination of haematoxylin and eosin-stained sections of livers obtained between 12 and 24 weeks of gestation showed hepatocytes, large numbers of haematopoietic cells and developing portal tracts. After 24 weeks gestation the numbers of haematopoietic cells fell so that by 40 weeks gestation they were seen only occasionally.

Although the cellular composition of liver changed markedly during development there was no change in the expression of CuZn superoxide dismutase. Hepatocytes and large bile duct epithelium generally demonstrated positivity and there was no preferential periportal or centrilobular distribution of the enzyme. The epithelium of small bile ducts appeared to be negative.

*Discussion*

We have described the distribution of CuZn superoxide dismutase in developing liver and kidney. Both organs contain high levels of the enzyme compared with other tissues examined. Studies of between-tissue differences in adults have shown higher levels of immunoreactive enzyme in human liver and kidney than in heart and lung.[18] Our finding of higher levels of expression in developing liver and kidney is therefore expected as is wide inter-individual variation in enzyme level and activity in human tissues.[17,20] Both enzyme protein and activity were present in samples obtained during the first and second trimesters. Thus while the immunological methods did not differentiate between catalytically active and inactive protein the data suggests that enzyme protein quantified using radial immunodiffusion and visualised by immunohistochemistry is catalytically active. This is in keeping with previous studies showing that enzyme activity in tissues is related to concentration and messenger RNA level.[19]

Studies in rat liver have shown CuZn superoxide dismutase to be predominantly distributed in cytoplasm with smaller amounts of the enzyme being found in nuclei, mitochondria and lysosomes.[21] Our measurements of cytosolic enzyme levels may therefore, somewhat underestimate cellular content but will reflect relative levels of expression in tissues.

This study supports our observation that enzyme is consistently and widely expressed in developing human lung.[14] In some species, superoxide dismutase is not expressed in lung until late in gestation and it has been proposed that in premature animals this organ is consequently vulnerable to free radical attack.[22] These data have been extrapolated to humans and form the basis of the hypothesis that bronchopulmonary dysplasia results, in part, from free radical-induced damage.[23] Whilst recent data show that human lung does not demonstrate a late-foetal surge in expression,[14] significantly lower enzyme levels were found in this organ than in liver or kidney. Since the level of enzyme necessary to cope with the superoxide anion load on particular tissues is not known,[2] it is possible that in mechanically ventilated neonates receiving high concentrations of oxygen the capacity of the tissue to detoxicate the superoxide anion formed as a result of hyperoxia is exceeded and increased formation of hydroxyl radical and tissue damage results. The relative importance of other detoxicating enzymes is unclear although studies by Raes *et al.*[24] indicate the importance of glutathione peroxidase rather than superoxide dismutase in protection of human fibroblasts from reactive oxygen species.

The finding that CuZn superoxide dismutase is consistently and widely expressed

in developing human liver is a further example of the between-species and even strain differences in the patterns of expression of this enzyme during development. Schisler and Singh[13] for example, found increased, decreased and unchanged levels of activity in livers from different strains of mice while Mavelli *et al.*[25] showed a postnatal increase in activity in isolated rat hepatocytes. The level of hepatic expression is compatible with the high level of oxygen consumption demonstrated by the tissue during development and interestingly, adult liver is particularly resistant to the type of free radical attack encountered in ischaemia-reperfusion experiments.[6]

The developing kidney demonstrated similar levels of enzyme expression as liver although its level of oxygen consumption is low. Enzyme was expressed in both differentiated and undifferentiated structures. For example, while the undifferentiated metanephrogenic tissue cap and S-shaped body did not demonstrate positivity, flattened cells in the primitive Bowman's capsule which are probably differentiated as well as the cuboidal cells of the capsule that have yet to differentiate were positive.

Data on the distribution of two enzymes that catalyse the detoxication of oxygen-derived substrates in developing kidney are now available.[26] While neither CuZn superoxide dismutase nor α-set glutathione S-transferases are strongly expressed in the glomerulus, α-set glutathione S-transferases are present in the proximal tubule and CuZn superoxide dismutase along the entire tubule. Further, recent studies by Litwin *et al.*[27] in kidneys from human adults have shown that expression of the peroxisomal enzyme, catalase, is confined to epithelial cells of the proximal tubule. These data were unexpected since the glomerulus is exposed to reactive oxygen species derived from two sources; infiltrating blood cells and resident glomerular cells.[9] Resident cells can be activated by a variety of stimuli including phorbal myristate acetate[9] and Shah and Naum-Bedigian[28] have shown greater production of reactive oxygen species (detected by the chemiluminescence response) by glomerular cells than by tubular cells following stimulation with this agent. Recently, there has been much interest in the role of reactive oxygen species, such as hydrogen peroxide, in stimulating glomerular synthesis of prostaglandins[9] particularly since the prostanoids have marked effects on the growth and maturation of the fetal kidney.[10] It is possible therefore, that the distribution of free radical detoxicating enzymes, particularly the superoxide dismutases, is more determined by the need to generate hydrogen peroxide than to detoxify superoxide anion.



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