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Developmental expression of antioxidant enzymes in guinea pig lung and liver

GUY M. W. RICKETT and FRANK J. KELLY

School of Biochemical and Physiological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, UK

Summary

Antioxidant enzyme activities, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and total glutathione concentration were determined in guinea pig lung and liver over the final period of gestation (days 50–68) and at several ages post-partum. Pulmonary antioxidant capacity increased markedly over the final days of gestation, individual changes ranging from 29 % (glutathione) to 198 % (GSH-Px). Liver antioxidant capacity was always 4-fold to 10-fold greater than that of the lung and exhibited very similar developmental profiles to those observed in the lung. From day 60 gestation to term (68 days), activity of the liver antioxidants increased, ranging from 246 % (CAT) to 610 % (glutathione). A number of antioxidants in both lung and liver exhibited either immediate pre- or post-birth decreases in activity. These falls could not be attributed to the way in which the results were expressed: i.e. they were similar, expressed per unit DNA, per unit protein, or per g wet wt. Following birth, liver antioxidant capacity increased such that the highest enzyme activities or glutathione concentration were recorded at 66 days post-partum. In lung, only Mn-SOD and glutathione exhibited higher levels at 66 days post-partum than at birth.

In combination, these results of pulmonary and hepatic antioxidant enzyme activity indicate that the lung is not unique in acquiring increased antioxidant protection in the final period of gestation. They also suggest that a tissue's antioxidant requirement is dictated more by metabolic rate (hence free radical production) than incident partial pressure of oxygen.

Key words: fetal development, antioxidant enzymes, guinea pig.

Introduction

At birth the fetus leaves a hypoxic uterine environment (pO_2 2.66–3.99 kPa (20–30 mmHg)) to enter a relatively hyperoxic environment (13.3 kPa (100 mmHg)). This transition, involving a five-fold increase in oxygen concentration, is believed to pose a significant oxidative stress on the newborn through the increased production of oxygen free radicals. These partially reduced oxygen intermediates, (i.e. superoxide radical (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH-$)), generated during normal biological reduction of molecular oxygen are capable of various cytoxic effects, e.g. inactivation of sulfhydryl-containing enzymes, interactions with DNA and lipid peroxidation of cellular membranes (Halliwell and Gutteridge, 1984; Slater, 1984). In studies designed to investigate the mechanism of hyperoxic-induced tissue damage in adult animals, Freeman and Crapo (1981) and Freeman *et al.* (1982) found that free radical production (measured as cyanide-insensitive respiration) was increased in lung slices and homogenates exposed to 100 % oxygen. These investigators obtained similar results subsequently utilizing submitochondrial particles (Turrens *et al.* 1982).

Under normal circumstances, tissue oxidative damage is kept to a minimum through the presence of an extensive array of antioxidant defences. These include the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). Over recent years considerable evidence has accumulated to suggest that the fetus prepares for the oxidative stress of birth by rapidly accumulating these antioxidant enzymes in the lung over the final period of gestation (Yam *et al.* 1978; Tanswell and Freeman, 1984; Gerdin *et al.* 1985; Kelly and Rickett, 1987; Sosenko and Frank, 1987). Indeed, in all animal species considered to date, a late gestational accumulation of antioxidant enzymes such as SOD, CAT and GSH-Px has been recorded. This has been considered by some as being a major part of the preparation for a successful transition to a higher oxygen environment at birth (Frank and Sosenko, 1987).

In this study, we investigated the development of the pulmonary antioxidant system in the guinea pig. This mammal has a long gestational period (68 days) and as such probably represents a better mode of human lung development than many other small laboratory animals. Antioxidant enzyme activities (SOD, CAT and GSH-Px) and total glutathione concentration were

determined not only during gestation but also in the early neonatal period and in adolescence. In addition, similar determinations were made in the livers of these animals to determine the pattern of development of the antioxidant defence system of a non-pulmonary tissue.

Materials and methods

Animal operations

Virgin, female Hartley strain guinea pigs (500 g) obtained from our own colony were caged in pairs in a room controlled for temperature (22°C) and light (06:00–20:00 h). Animals had free access to food and water. The oestrus cycle of each animal was established by daily vaginal smears. Timed pregnancies were established by introducing a male into the cage three days prior to the next ovulation. The date of ovulation in the successfully fertilized cycle was taken as gestational day zero. By this method normal gestation ends with birth on day 68.

In those instances where fetal tissues were required, fetuses were delivered by Caesarian section. Operations were performed under halothane anaesthesia maintained with an oxygen: nitrous oxide (4:1) mixture. Following double clamping of the uterine vessels, fetuses were removed individually, rapidly dried of placental fluids, weighed and killed by decapitation prior to the onset of normal breathing. The lungs and livers of these animals were then removed, rinsed in ice-cold saline, blotted dry and weighed. At this point, a 100 mg portion of each tissue was removed and added to 15 vols of ice-cold 0.2 M-perchloric acid for immediate determination of total glutathione concentration (reduced and oxidized) (Griffith, 1985). The remaining tissue was frozen in liquid nitrogen for later analysis of protein, DNA and antioxidant enzyme activities. A number of pregnant dams were allowed to proceed to term and the offspring incorporated in the study at days 2, 5, 10 and 66 postpartum.

Frozen tissue samples (200 mg) in 10 vols of 0.01 M-potassium phosphate, 0.03 M-potassium chloride buffer were homogenized on ice by two 15 s bursts in an Ultraturrax homogeniser (Janke and Kunkel, Staufen, Sweden). The resultant homogenate was then sonicated (MSE Soniprep) at setting 14 by six 10 s bursts. Samples were then removed for DNA (Sterzel *et al.* 1987) and protein analysis (Smith *et al.* 1985). The remaining homogenate was then incubated for 30 min with 1% (v/v) absolute alcohol and mixed with 1% (v/v) Triton X-100. Following centrifugation (10 000 *g*, 5 min), supernatants were removed and frozen (−20°C) for subsequent analysis of antioxidant enzymes. These analysed were performed within two months of sampling, previous studies having shown these enzymes to be stable over this period.

Enzyme analysis

Total and Mn-SOD were determined by the pyrogallol autoxidation method of Marklund (1985). The inhibition of pyrogallol autoxidation by the enzyme was monitored at 420 nm with a reaction rate spectrophotometer (LKB Mk II Kinetic Analyser S, Croydon, Surrey, UK) at 25°C over 2 min. A standard curve was constructed using bovine liver Cu/Zn-SOD (Sigma, Poole, Dorset, UK). Mn-SOD was measured in the presence of 10 mM-CN^- and total SOD in the absence of cyanide. Cu/Zn-SOD was calculated as the difference between total and Mn-SOD activities.

GSH-Px activity was determined by the method of Beutler (1979). In this linked enzyme reaction, oxidized glutathione formed by the action of GSH-Px on lipid hydroperoxide and reduced glutathione is converted back to its reduced form in the presence of glutathione reductase and NADPH. Glutathione is thus maintained at a constant concentration and the reaction followed by the oxidation of NADPH. The reaction was monitored for 2 min at 360 nm using an LKB reaction rate kinetic analyzer at 37°C.

CAT activity was determined using the method of Aebi (1984) in which the initial rate of hydrogen peroxide decomposition is determined. The reaction was followed at 240 nm using a LKB Ultraspec II spectrophotometer. Units of CAT are defined in terms of initial velocity of hydrogen peroxide decomposition per minute and measured against a standard of bovine liver catalase (Sigma, Poole, Dorset, UK).

The following coefficients of variation were determined for each enzyme assay: catalase, 9.2%; total SOD, 11.6%; Mn-SOD, 13%; GSH-Px, 2.7% and GSH, 7.6%.

Results

The pre- and postnatal developmental patterns determined for the three antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase and the non-enzymatic antioxidant glutathione in guinea pig lung and liver are shown in Figs 1–4. Both lung cyanide-sensitive (Cu/Zn-) and cyanide-resistant (Mn-) SOD activities approximately doubled over the final period of gestation (Fig. 1). Following birth, pulmonary Cu/Zn-SOD activity continued to increase until at least 10 days post-partum. Activity of this cytosolic enzyme had however decreased again by 66 days postpartum (Fig. 2). Lung Mn-SOD activity fell between day 65 gestation and a few hours following birth. It continued to decrease until 5 days post-partum. Thereafter, activity of this mitochondrial antioxidant increased 5-fold by 66 days post-partum.

Liver Cu/Zn- and Mn-SOD activities were lowest at the earliest point considered in gestation, day 50. Thereafter, Cu/Zn-SOD increased 10-fold over the final period of gestation, peaking at 291.7 ± 52.1 U mg^{-1} DNA at birth. Following birth here was a sharp drop in activity over the first two days, and thereafter Cu/Zn-SOD activity increased steadily into adulthood (Fig. 1). Liver Mn-SOD activity, although 10-fold lower than the Cu/Zn enzyme followed a very similar pattern of activity changes. This included the prebirth increase in activity, the immediate post birth decrease in activity and subsequently a sustained increase in activity into adulthood (Fig. 1).

Glutathione peroxidase (GSH-Px) activity in fetal lung approximately doubled between day 50 gestation and birth. Over the next 48 h, GSH-Px activity fell 44% before once again increasing steadily into adulthood (Fig. 2). Liver GSH-Px activity was lowest at the earliest time point considered in gestation, 50 days. Between days 60 and 65 of gestation, liver GSH-Px activity increased 4-fold. By 2 days post-partum activity of this antioxidant had fallen considerably, but thereafter activity increased steadily such that by day 66 post-partum GSH-Px activity was approximately that seen just prior to birth (Fig. 2).

Fetal lung catalase activity remained constant between day 50 and day 60 of gestation (Fig. 3). There-

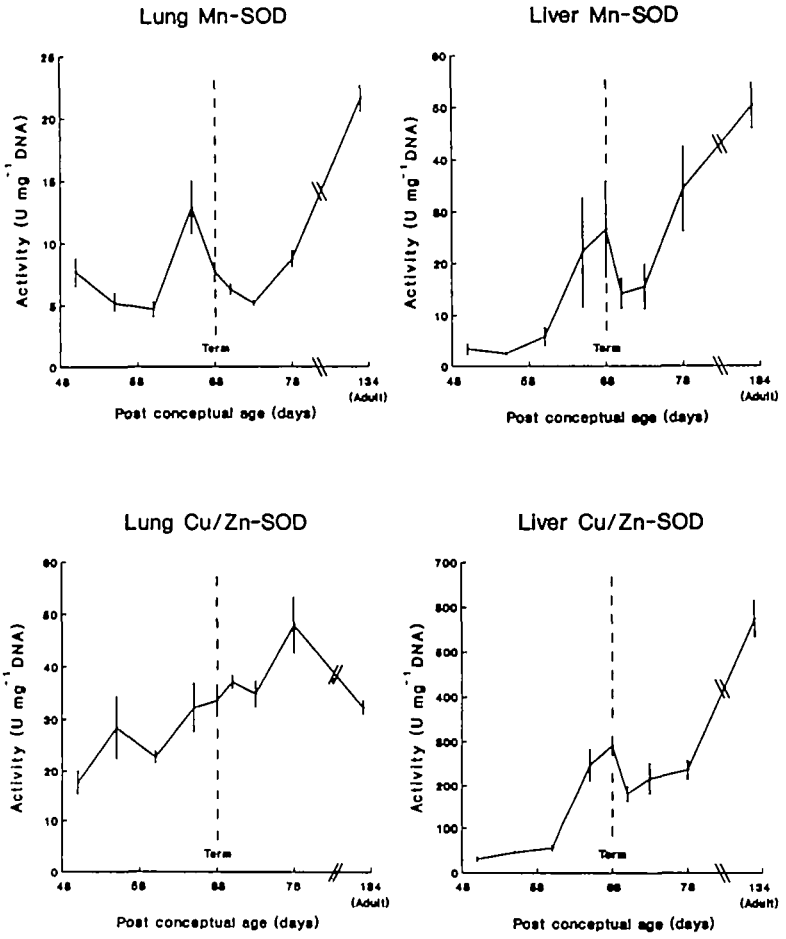

Fig. 1. Lung and liver Mn- and Cu/Zn-SOD developmental profiles. Tissue supernatants (10 000 *g*) were assayed by the method of Marklund (1985) for both cyanide-sensitive (Cu/Zn-SOD) and cyanide-insensitive (Mn-SOD) activity. Results are expressed per unit DNA. Each point represents 5–11 individual animals taken from two or three litters. Error bars represent standard deviations.

after pulmonary CAT activity increased progressively to at least 10 days post-partum, the majority of this increase taking place over the final eight days of gestation. When lung CAT activity was determined in the young adult guinea pigs it was seen to have fallen again to about 60 % of the maximal activity recorded at 10 days post-partum. Liver CAT activity was also lowest at day 50 gestation, the earliest point considered. Thereafter CAT activity increased steadily, increasing 7-fold by birth and then a further 4-fold over birth levels by day 66 post-partum (Fig. 3).

Tissue glutathione concentration was determined as total glutathione, i.e. both oxidized and reduced forms. In both lung and liver, at all ages examined, more than 95 % of the total glutathione was present in the reduced form. In contrast to most lung antioxidant enzymes, pulmonary GSH concentration decreased between days 50 and 60 gestation, falling approximately 37 % (Fig. 4). Thereafter, lung glutathione concentration increased steadily over the remaining period of gestation and throughout the post-birth study period (Fig. 4). Liver glutathione concentration remained

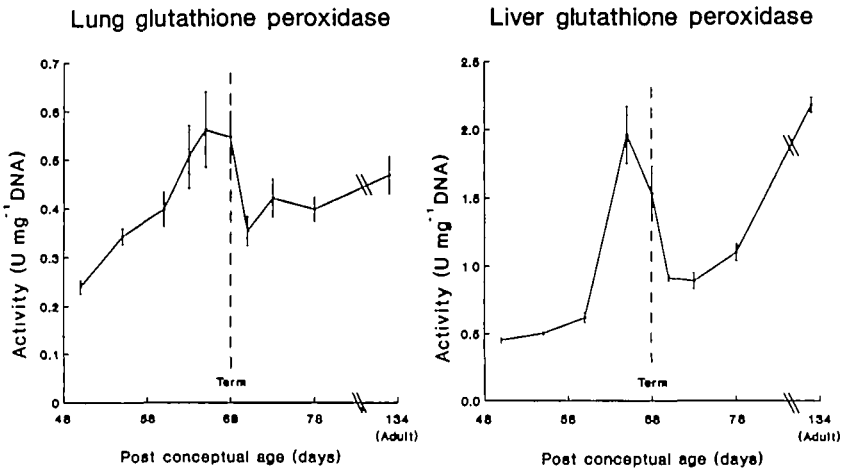

Fig. 2. Lung and liver catalase developmental profiles. Tissue supernatants (10 000 *g*) were assayed for catalase activity by the method of Aebi (1984). Results are expressed as described for Fig. 1.

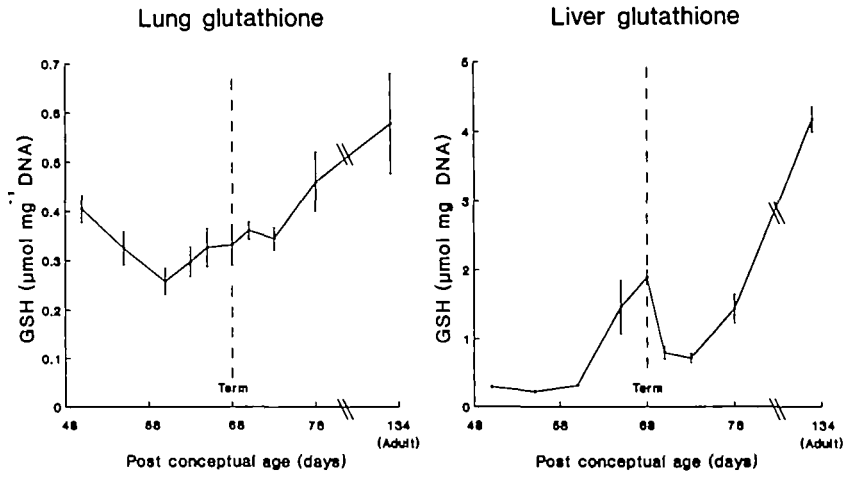

Fig. 3. Lung and liver glutathione peroxidase developmental profiles. Tissue supernatants (10000*g*) were assayed for glutathione peroxidase by the method of Beutler (1979). Results are expressed as described for Fig. 1.

relatively constant until day 60 gestation. Over the final 8 days of gestation, liver glutathione concentration increased 6-fold. Following birth there was again a pronounced fall in antioxidant concentration. This fall plateaued between the 2nd and 5th day post-term and then GSH concentration increased significantly, reaching the maximum value recorded in the 66 days post-term animals.

Determination of pre- and post-birth antioxidant profiles in both lung and liver provided us with the opportunity to compare directly the antioxidant capacity of these two major organs. Lung and liver Mn-SOD activities were approximately equal during fetal life. Following birth until 66 days post-partum liver Mn-SOD activity was approximately twice that found in the lung (Fig. 1). At all points in gestation considered, liver Cu/Zn-SOD activity exceeded that found in the lung. At birth, the activity of Cu/Zn-SOD in the liver was 9-fold greater than that in the lung, and by 66 days post-partum this had increased to approximately 18-fold (Fig. 1). Liver GSH-Px activity was 2–3 fold greater than lung GSH-Px activity at all points in gestation. Following birth, this differential was maintained and by 66 days liver GSH-Px activity exceeded lung activity 5-fold (Fig. 2). At 50 days gestation liver CAT activity had exceeded lung CAT activity 4-fold. By birth this difference had increased to 9-fold. Following birth, lung CAT activity remained relatively static while liver CAT activity increased greatly, making the differential between the two tissues even greater, approximately 50-fold at 66 days post-partum (Fig. 3). Until 60 days gestation, liver and lung total glutathione concentrations were approximately equal. By birth, however, liver glutathione concentration exceeded lung glutathione concentration 6-fold. Following birth a differential was always maintained, such that by 66 days post-partum, liver glutathione concentration was 7-fold greater than lung glutathione concentration (Fig. 4).

Discussion

In recent years, a number of investigations have reported that the activity of pulmonary antioxidant enzymes in the fetus remains low until the last 10–15 % of gestation. This pattern of antioxidant development has been observed in the rat (Tanswell and Freeman, 1984; Gerdin *et al.* 1985; Kelly and Rickett, 1987), guinea pig (Sosenko and Frank, 1987) and rabbit (Frank and Groseclose, 1984). As a result, the general consensus is emerging that the maturation of the pulmonary antioxidant system, much like the surfactant system, is precisely regulated and can be considered as a major component of the general preparation for birth

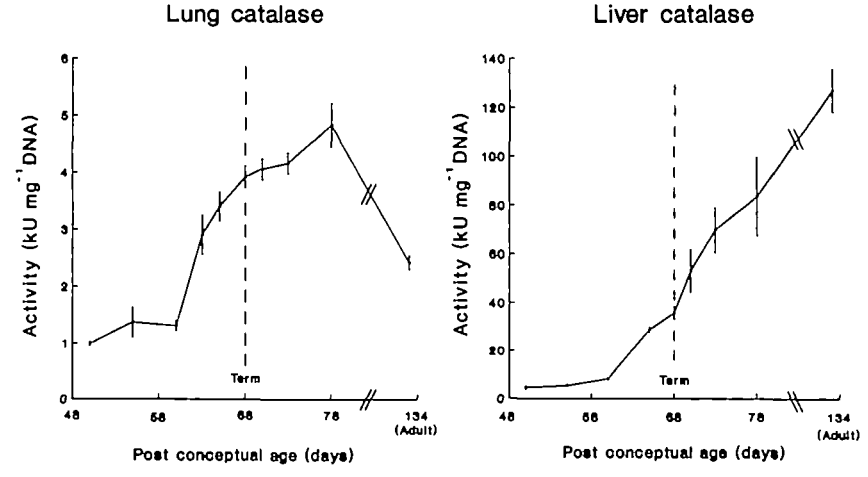

Fig. 4. Lung and liver total glutathione developmental profiles. Tissue supernatants (10000*g*) were assayed for both reduced and oxidized glutathione concentrations by the method of Griffith (1985). Results are expressed as described for Fig. 1.

(Frank and Sosenko, 1987). Indeed our own results reported in this study appear to lend considerable support to this hypothesis. One of the primary aims of this study was to determine if the lung was unique in respect to its late gestational rise in antioxidant enzyme activity. To this end, activities of the four antioxidants considered were also determined in liver from the same animals.

Activities of all four antioxidant enzymes determined in the guinea pig lung increased over the final period of gestation (Figs 1–3). In addition, with the sole exception of pulmonary Mn-SOD activity, the activities of the other three antioxidant enzymes were as great at birth as those recorded 66 days later in young adults. These observations would again agree with current reasoning, which suggests that as the major shift in oxidative stress occurs at birth (with the associated 5-fold increase in pO_2) then the pulmonary antioxidant requirement would be maximal at birth and would not change appreciably thereafter.

The results obtained in liver throw light on two important aspects of tissue antioxidant requirements. First, the absolute enzyme activity for each antioxidant was substantially greater in liver than lung (ranging from a 4-fold to 10-fold difference at term for GSH-Px and CAT respectively). Second, the antioxidant developmental profiles determined for liver were very similar to those observed in lung. Antioxidant activity was generally lowest at the earliest point in gestation considered (50 days). Thereafter, activity increased rapidly to term, at which point it decreased for a period before once again increasing (Figs 1–4). However, these observations in the liver cannot be explained by the current theory, i.e. that it is in preparation for the increased oxidative stress that results from the 5-fold increase in incident oxygen partial pressure that the animal is exposed to at birth. Indeed it is unlikely that the partial pressure of oxygen that the liver is exposed to following birth changes appreciably. Thus the incident oxygen partial pressure (pO_2) is probably not the most important factor in the production of free radical in these tissues (assuming that the tissue activities of these antioxidants reflect a requirement for protection rather than an ability to synthesize them). Instead, the antioxidant complement of a tissue should be related to the oxygen radical production that occurs as a result of the normal aerobic respiration when single electron transfers occur in the respiratory chain. More specifically, the requirement for SOD activity should be directly related to the O_2-production, which in turn is related to the metabolic activity of the tissue rather than the incident oxygen partial pressure.

The metabolic rate and hence O_2 consumption of the liver is considerably higher than most other fetal tissues (Jones and Rolph, 1985). The oxygen free radical flux in this tissue and hence antioxidant requirement is high, explaining the absolute antioxidant activities recorded in the liver. Second, following birth, there is a marked redistribution of blood flow to both lung and liver. Consequently, metabolic activity and free radical production may change significantly. Therefore, the late gestational increases in both lung and liver antioxidant defences are in preparation for this birth-associated event.

In the present study, substantial pre-birth increases in antioxidant capacity were often accompanied by falls in antioxidant activities prior to or just following birth. Similar observations have been reported in only a few previous studies due mainly to the fact that in the majority of studies activity profiles have only been determined up to birth but not subsequently. Frank and Sosenko (1987) reported a fall in guinea pig lung total SOD activity (i.e. both Cu/Zn- and Mn-SOD) between 65 day gestation and term. The present data agree with this finding, and reveal that the decrease is due primarily to a fall in Mn-SOD activity. Tanswell and Freeman (1984) reported developmental profiles for CuZn-SOD and Mn-SOD over late gestation, post birth and into adulthood. Mn-SOD activity fell during the final period of gestation in a similar manner to that described above in the guinea pig lung. The results they obtained in rat lung did differ from those in this study, in that Mn-SOD activity was found to have fallen by adulthood.

Close examination of the results in this study indicate that these apparent falls in antioxidant capacity could not be attributed to the way in which the results were expressed, i.e. per unit DNA. When expressed per unit protein, pre-birth increases in enzyme activity and birth or post-birth decreases in activity were also observed (data not shown). We have taken this as evidence to suggest that the decreases in enzyme activity are real. These changes may represent inactivation of enzyme possibly due to the increased oxidative stress at birth. We have recently shown that acute hyperoxic exposure will lead to a marked inhibition of total protein synthesis in the lung (Kelly, 1988). Alternatively they may arise from a change in the proportion of different cell types in the lung and liver at this time (Owen *et al.* 1977; Snyder *et al.* 1985).

These data support findings in other species that pulmonary antioxidants mature rapidly over the final period of gestation, preparing the fetal lung for an increased free radical flux following birth. The lung is, however, not unique in this respect, as shown by the similar increases in hepatic antioxidant defences over this period. In addition, the substantially higher levels of antioxidants in the liver suggest that tissue antioxidant requirement is dictated more by metabolic rate than incident partial pressure of oxygen.

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