Gestational Changes in Pulmonary Converting Enzyme Activity in the Fetal Rabbit

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SUMMARY Changes in angiotensin-converting enzyme were measured in the lungs of fetal rabbits isolated and perfused in situ at varying ages from 22 days gestation to 7 days of age under controlled conditions of flow, pH, and temperature. Enzyme activity was assessed by infusing bradykinin or angiotensin I in Krebs-Henseleit solution and measuring residual peptide in the effluent by radioimmunoassay. The levels of substrate studied were below those required for enzyme saturation. Lungs of 22 day gestation fetuses removed only one-third of either peptide. The activity at term and in neonatal life resulted in more than 80% peptide removal. The time of the greatest rise in the percent substrate cleared occurs earlier than the time of the greatest increase in lung and body weight. The lower percentage of substrate cleared in early gestation appears to result in part from a limited surface area for enzyme activity in the primitive fetal pulmonary microvascular bed, since morphological studies with fluorescein-tagged anticonverting enzyme antibody demonstrated the presence of enzyme in the lung as early as 17 days of gestation. Electron micrographs of the pulmonary endothelial cell surface reveal that the degree of surface infolding and hence surface area increases with gestation. The higher percentage of substrate cleared in later gestation closely parallels the structural and ultrastructural development of the vascular bed. The presence of converting enzyme in the placenta by the second third of gestation and the large size of the placenta suggest that this organ may be a major locus of converting enzyme activity in the fetus.

IMMATURITY of the lung and the consequent predisposition to neonatal respiratory distress syndrome (RDS) is a great risk to the survival of the prematurely born infant. In view of the role of the lungs in the clearance, activation, and release of vasoactive substances, we hypothesized that immaturity of these functions prior to term birth is important in the pathogenesis of RDS, especially in the disorders of circulatory regulation and water balance seen in this disease. Converting enzyme, a peptidylpeptide hydrolase (EC 3.4.15.1) located on the plasma membrane of endothelial cells, degrades bradykinin (BK) and activates angiotensin I (A I) to angiotensin II (A II). Both peptides have been assigned important roles in neonatal circulatory adaptations and transvascular fluid balance.

Studies in the fetal lamb have suggested a deficiency in converting enzyme in the fetus, but others have shown that high levels of A II circulate in prematurely born animals. Because of this apparent paradox, we measured the time course of maturation of converting enzyme activity in the lungs of fetal rabbits during the last third of gestation, and related this to the distribution of the enzyme as previously determined by immunofluorescence and to the complexity of the endothelial cell surface as revealed by electron micrographic techniques.

Methods

One hundred and one fetal New Zealand white rabbits, ranging in weight from 4 to 60 g, were obtained from 34 timed pregnancies and were studied at 22, 24, 25, 26, 28, 29, and 30 days of gestation. The fetal rabbits were delivered by hysterotomy following induction of maternal anesthesia with intravenous pentobarbital (25 mg/kg). They were prevented from breathing by placing a fluid-filled latex glove-tip over the head; their lungs remained fluid-filled throughout the study. Also, eight rabbits with a mean weight of 120 g were studied 1 week following normal term delivery. The lungs of these rabbits were ventilated with 5% CO₂ in room air using a Harvard small animal ventilator.

The ductus arteriosus was ligated through a midsternal incision, and Silastic catheters, i.d. 0.02 inch, were passed through a right ventriculotomy into the pulmonary artery and through a left ventriculotomy into the left atrium. Figure 1 shows the system used for perfusing the lungs with either BK (Bachem Corp.) or A I (New England Nuclear) in Krebs-Henseleit solution. Intravascular pressures...
were measured at 22, 26, and 29 days gestation (six fetuses at each gestation), from Statham 23Db strain gauges attached to side arms of the catheters and continuously recorded. For these studies, the flow rate was 100 ml/kg per min.

To determine whether differences in converting enzyme activity with gestation could be attributed to enzyme saturation, each gestational age group was subjected to the following experimental conditions:

**Series A**
- **Varied flow rate:**
  - 10 mg/kg per min
  - 100 ml/kg per min
  - 200 ml/kg per min
- **Constant substrate concentration:**
  - \([BK] = 1 \mu g/ml\)
  - \([AI] = 1 \mu g/ml\)

**Series B**
- **Constant flow rate:**
  - 100 ml/kg per min
- **Varied substrate concentration:**
  - \([BK] = 0.2, 1.0, \text{and} 4.4 \mu g/ml\)
  - \([AI] = 0.2, 1.0, \text{and} 4.0\)

Thus, fetuses in each gestational age group were studied under conditions of high and low flow rate and high and low substrate concentration. (Bradykinin studies at 24 and 28 days were done only at a flow rate of 100 ml/kg per min and a substrate concentration of 1 \mu g/ml.) By this experimental design, comparable data on the effect of variations in flow and variations in substrate concentrations were generated for each gestational age group. The gas tensions with which the perfusate was equilibrated and the flow rates perfusing the lung were selected to approximate conditions which would exist during the transitional period following birth and not those in utero. The pH was 7.40 ± 0.02 (mean ± sd) and the Po2 was 113 ± 2 mm Hg in both the perfusate and the effluent.

The pulmonary venous effluent was collected over ice and immediately frozen at −70°C until assay for residual AI or BK. Specimen collection was delayed until the effluent was blood free. Stability of the preparation was determined by visual inspection for the development of edema and the postperfusion determination of wet-to-dry weight ratios of the lungs; fluid was not allowed to escape from the airways prior to the wet weight measurement. Only samples from grossly nonedematous lungs are included. The wet-to-dry lung weight ratios of the perfused lungs were 9.5 ± 0.85, 11.1 ± 0.48, and 8.8 ± 2.38 at 22, 26, and 29 days of gestation, respectively, and were not significantly different by the t-test of unpaired data from the littermate unperfused control lungs which were 10.3 ± 1.40, 8.9 ± 0.52, and 9.9 ± 0.54 at the same ages (P > 0.1). Although the perfused lungs did not have significantly more fluid than the controls, we cannot...
exclude the possibility of small amounts of interstitial edema not detectable by the wet-to-dry ratio. Approximately 90% of attempted perfusions were stable for the 30 minutes of perfusion time. Each preparation was perfused at one concentration of peptide and at one flow rate.

To confirm the specificity of this preparation for converting enzyme, a separate series of 12 experiments was performed in which the fetuses were treated with SQ 20881, a specific inhibitor of converting enzyme. Six fetuses of 22 days gestation and six fetuses of 29 days gestation were perfused with either BK or A I, plus SQ 20881, 6 µg/ml. In these fetuses, we used flow rates of 100 ml/kg per min. In each fetus, the pre- and postpulmonary concentrations of substrate were equal; for all experiments the mean perfusate substrate concentration was 1.018 ± 0.025 µg/ml and the mean effluent concentration was 1.014 ± 0.021 µg/ml. This indicates that converting enzyme was completely inhibited by SQ 20881 at each gestation studied, and further, that no peptide was lost by diffusion into fetal lung tissue.

The method of Goodfriend and Odya11 was used for bradykinin radioimmunoassay. Diluted specimens were incubated with 0.01 M sodium phosphate buffer (containing 0.1% lsozyme and 0.01 M 1,10-phenanthroline), 125I-tyr-5-bradykinin as the trace and anti-BK antibody at a final dilution of 1:20,000. Following overnight incubation at 4°C, bound peptide was separated from free by precipitation with an equal volume of saturated ammonium sulfate solution, and counted in a well-type gamma counter. The standard deviation of replicates from 10 standard curves was 0.24% at the zero point. The sensitivity of the assay defined as the lowest concentration of standard which produced a bound fraction significantly different from no added BK, was 20 pg/ml. Over the range of 10–300 pg/ml, the mean coefficient of variation of 25 samples assayed in duplicate was 7.20 ± 0.95%. The anti-angiotensin antibody, 125I-labeled A I (New England Nuclear), and diluted specimens were incubated overnight in phosphate buffer, pH 7.2, and the unbound fraction was separated with dextrancoated charcoal. The limit of detectability of the A I radioimmunoassay, defined as the concentration of A I equivalent to twice the standard deviation of the zero binding point, is approximately 20 pg/ml. The standard deviation of replicates from eight standard curves for zero binding was 0.59% over the range of 0.01 to 1.0 ng/ml. The mean coefficient of variation of 20 samples assayed in duplicate was 13.3 ± 2.1%.

Activity of the lung enzyme, defined as the percent substrate cleared, was assessed by calculation of the percentage of BK and A I cleared by a single passage through the lungs, using the following equations:

\[
\begin{align*}
\text{A I cleared (ng/min)} & \times 100 = \\
\text{A I presented (ng/min)} - \text{[A I]} \times 100 &= \\
\text{[A I]} \times (Q) &= \\
\text{% substrate cleared} &= \\
\end{align*}
\]

where [A I] = substrate concentration (ng/ml) in perfusate (S) or effluent (E), and Q = flow (ml/min). Each gestational age was analyzed for differences in substrate conversion when compared to the preceding gestational age. Statistical analysis was performed by the t-test of unpaired data; only P values less than 0.01 are reported as significant.

Tissue blocks of less than 1 mm³ were taken from the lungs for electron microscopy; they were fixed in 2.5% glutaraldehyde in 0.1 m phosphate buffer and postfixed in Dalton’s fixative. Dehydration was carried out in graded concentrations of ethanol and the tissue blocks were embedded in Spurr's medium. Thin sections were cut on a Porter-Blum ultramicrotome, placed on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined at 60 kV in an AEI 6B electron microscope.

**Results**

We examined separately the effect of varying flow and of varying substrate concentration on percent substrate cleared using the same range of experimental conditions at each gestational age. Figure 2 shows that large variations in the amount of substrate presented to the lung, whether achieved by varying flow or substrate concentration, had no effect on the percent of BK degraded; rather, the percent of BK degraded was a reproducible function of gestational age. This figure also demonstrates that the amounts of substrate presented to the lung in these experiments were below the levels required to saturate pulmonary converting enzyme.

Because there was no difference in the percent substrate cleared regardless of the flow rates or substrate concentrations used (Fig. 2), all the data for each gestational age group were pooled. The effect of gestational age on BK clearance by the lung is shown in Figure 3. At 22–23 days gestation, one-third of the peptide is cleared, with significant increases developing throughout the last third of gestation, reaching 88% or close to neonatal levels at term. Note that the mean value at each gestational age was obtained from individual experiments in which the flow rates and substrate concentration were varied over comparable ranges, as described in Methods. Similar results were obtained by studying A I clearance at each gestational age (Fig. 4). With both substrates, the greatest rise in percent substrate cleared does not occur at the time...
of greatest increases in either lung weight or body weight.

The mean perfusion pressures measured during sample collection at 22, 26, and 29 days were 9.4 ± 4.9 mm Hg, 9.9 ± 6.2 mm Hg, and 8.6 ± 6.5 mm Hg, respectively. The differences are not significant. Pressures tended to be lower in those fetuses perfused with BK, but the differences were not significant. We examined whether the variation in perfusion pressure within a given gestational age affected the percentage clearance of substrate: there was no correlation between perfusion pressure and percentage clearance. Further, as stated above, the wide range of flow rates chosen for these experiments did not affect the amount of substrate cleared at a given gestational age.

**Discussion**

These results indicate (1) there is a rise in the activity of converting enzyme for both of its substrates in the fetal rabbit from very low levels to those approaching neonatal levels over the last third of gestation, and (2) the time of the greatest...
increase in substrate clearance over the last third of gestation does not occur at the same time as the greatest increase in growth as reflected in lung dry weight or body weight.

The isolated perfused lung model is a conventional method for the study of converting enzyme in adult animal lungs11 which we have applied to the fetal lung. We saw no difference in the percent substrate cleared between those fetuses delivered at the beginning of an experiment (less than 30 minutes of exposure to pentobarbital) and those delivered later (4 hours or more of exposure to pentobarbital). For this reason, we do not think that the anesthetic agent had any effect on our results. We assumed that the clearance of A I reflects its conversion to A II. Previous studies in adult animals12 have uniformly demonstrated quantitative recovery of A I after passage through the lungs as the sum of A I plus A II, with no nonspecific degradation. The observation that all enzyme activity measured by this method was inhibited with SQ 20881 confirms the specificity of the preparation for converting enzyme. Furthermore, since A I conversion and BK degradation are functions of the same enzyme, the similar rates of increase in the percent substrate cleared over gestation when measured with either substrate indicates A I conversion to A II and not nonspecific degradation.

We cannot attribute the low levels of substrate clearance in early gestation to a developmental delay in the presence of the enzyme, since it has been shown10 that immunoreactive converting enzyme is present as early as in the second third of gestation. Although biochemical activity is decreased at 22 days gestation, persistent substrate clearance could always be demonstrated at this age. Indeed, both by criteria of location and substrate specificity, the enzyme appears to be distributed and functions much as in the adult lung.

The decreased ability of the fetal lungs to clear these peptides is unlikely to represent saturation of limited amounts of enzyme by excess substrate. We know from experiments of Fanburg and Glazier,12 using an adult dog preparation similar to our fetal model, that the percent of A I converted to A II decreases as the amount of substrate increases above that required to saturate the enzyme. Since in fact, 100-fold variations in the amount of substrate presented to the lung in our experiments (Fig. 2) had no effect on the percent degraded, the levels used must have been below those required for enzyme saturation.

There are at least two possible explanations for our findings. First, alterations in the velocity of substrate hydrolysis or affinity of the enzyme for the substrate may occur with gestational age. The range of substrate concentrations used did not allow us to calculate V_{max} and K_{m} because saturation was not approached. We believe that it is physiologically more pertinent to be working with levels of substrate well below saturation, since this permits assessment of the degree to which substrate clearance is impaired in prematurely born animals. Second, the enzyme may be present in an active form on the endothelial surface, but the vascular surface area available for conversion is limited. The observation that the recruitment of the vascular bed by increasing left atrial pressure results in an increase in A I conversion12 suggests that at least under some experimental conditions enzyme activity varies directly with vascular surface area. In the primitive vascular bed of the fetal animal, the surface available for conversion also may be limited relative to the volume of the vessel, thus permitting substrate to pass through the lung intact by streaming past the enzyme on the limited vascular surface.

The fluorescein-tagged antibody to converting enzyme demarcates the vascular surface available for A I and BK clearance. By this technique it can be seen that the lungs of the 17-day gestation fetus contain converting enzyme, but the lung at this time contains relatively few vessels;10 hence, the vascular surface is minimal and converting enzyme activity is expected to be low. From 22 to 26 days of gestation, a time of minimal increase in lung weight, the percent substrate cleared is rapidly increasing. At this same time, the immunofluorescence preparation of the lung demonstrates that substantial remodeling of the vascular bed is occurring with extensive capillary development. Increasing converting enzyme activity parallels this increased surface area.

A second way in which vascular surface for conversion can increase is suggested by the ultrastructural studies of the endothelial cell surface by Ryan et al.13 They described numerous surface projections and indentations (“caveolae intracellulares”) that are densely lined with converting enzyme. Thus, the amount of surface area with enzyme on it is increased by the complexity of these specialized surface adaptations. Although our methods did not permit quantification of these structures, comparison of electron micrographs of the surface of a cell taken from a 19-day gestation with that from a 26-day gestation suggests an appreciable increase in the degree of surface adaptation in the older fetus (Fig. 5): there are many more caveolae and the endothelial projections are increased in length. The scant number of caveolae in primitive vessels lined by relatively smooth-surfaced endothelial cells is one factor that could result in decreased converting enzyme activity in the younger fetus by minimizing the surface area exposed to the vascular lumen. The apparent correlation between increased converting enzyme activity and the degree of cell surface complexity will need to be confirmed by morphometric techniques.

These studies confirm and extend previous studies of converting enzyme activity in the fetus in which activity was assessed in fetal lambs by bioassay.8 In those studies, the decrease in blood pressure in response to prepuberal injections of BK
FIGURE 5 Rabbit lung capillaries at 19 days (upper panel) and at 26 days (lower panel) of gestation reveal a change from rare caveolae and cytoplasmic projections at the end of the second third of gestation to several of these structures as the lung matures. C = caveolae and P = cytoplasmic projections; 26,000x.

was only slightly less than aortic injections, suggesting decreased pulmonary clearance of BK. Similarly, one premature fetus showed no apparent conversion of A I to A II using blood pressure response bioassay. Our data indicate that converting enzyme is present in the fetus but that there is decreased conversion of its substrates, A I and BK, probably because of morphological immaturity of the pulmonary capillary bed and the endothelial surface.

Studies in human newborn infants have shown that umbilical venous A II levels are higher than those in the umbilical arteries. Using converting enzyme as a marker for the vascular bed, Wigger and Stalcup have shown that the placenta contains converting enzyme as early as the second third of gestation. Because the mass of the placenta approaches that of the total fetus at this age, the total amount of converting enzyme must be large relative to other vascular beds of the fetus. This raises the possibility that the placenta may be a major site of angiotensin conversion in utero. Although the amount of converting enzyme activity in any particular vascular bed appears limited by the state of its microvascular development, the total amount of enzyme present in early gestation taken together with the markedly elevated levels of plasma renin activity found in the normal fetus may account for the levels of A II described in fetal animals under unstressed conditions. However, after birth and the loss of placental converting enzyme, a mature pulmonary vascular bed may be necessary for adequate metabolism of vasoactive peptides, especially when high pulmonary vascular resistance leads to shunting of some substrate away from the lung through patent fetal channels. Thus, our observation that the fetal lung did not metabolize amounts of substrate known to occur in neonatal life has particular relevance to the prematurely born infant. Since inability to convert A I to A II or to inactivate BK would be expected to foster hypotension, the prematurely born animal could be severely handicapped at times of activation of the kallikrein-kinin system such as occurs at birth, or in situations of hypotensive stress.

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