Characterisation of the β adrenergic response cascade in fetal guinea pig lung

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Abstract

Background – Suitable models for the study of lung development are needed. The suitability of the guinea pig for studying the role of the β adrenergic response cascade in fetal lung development has been evaluated.

Methods – Radioligand binding assays with iodine-125 labelled iodopindolol were performed to identify and characterise the β adrenergic receptors. To demonstrate that these receptors were functional, isoprenaline and forskolin stimulated generation of cyclic AMP (cAMP) in the lung tissue was quantitated by radioimmunoassay.

Results – The concentration of β receptors increased with gestational age from 23 fmol/mg at 35 days to 140 fmol/mg at 64 days. Competition binding studies were consistent with a predominance of β_2 receptors. The ability of isoprenaline to stimulate cAMP generation was greater during the saccular phase than during the canalicular phase of lung development. Incorporation of tritium labelled choline into phosphatidylcholine increased significantly between the canalicular and saccular phases.

Conclusions – The β adrenergic response cascade in fetal guinea pig lung exhibits similar characteristics to those previously described in fetal human lung and is therefore a good model in which to study the effects of β agonists on fetal lung development.

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Inadequate lung development in many premature babies leads to respiratory distress syndrome, a disorder characterised by surfactant deficiency, excess alveolar and interstitial lung water, and morphological immaturity of the lung.¹ There is clinical and experimental evidence which indicates that surfactant synthesis and secretion are mediated, in part, by β adrenergic responses.^{2,3} Further studies may identify the mechanisms by which the β adrenergic response cascade mediates surfactant synthesis and secretion and, perhaps, uptake and utilisation of exogenous surfactant, but appropriate experimental models are needed. Owing to ethical considerations fetal human lung of appropriate gestational ages is not always readily available, necessitating the use of another animal model. Although rabbits and rats are commonly used in studies of lung development, we felt that fetal guinea pigs

would be a more suitable animal model for several reasons. Firstly, the guinea pig has a longer gestation (67-69 days) than rabbits and rats and is also relatively mature at the time of delivery.⁴ Secondly, the proportion of time spent in the different stages of morphological maturation of the lung and the timing of these stages in relation to delivery in the guinea pig⁵ are more similar to those in the human than those in the other small mammals. For example, the saccular stage of lung development begins by 50 days (75% of gestation) in the guinea pig⁵ and by 28 weeks (70% of gestation) in the human fetus,⁶ whereas the rabbit and rat only reach the saccular stage by 90-95% of gestation.⁷ In addition, in both the guinea pig⁸ and the human⁶ alveolarisation of the lung is well established before term, and surfactant synthesis⁹ and induction of antioxidant enzymes¹⁰ occur relatively late in gestation in both. The purpose of this study was to characterise β adrenergic responses in fetal guinea pig lung in order to assess the appropriateness of this model to study the involvement of the β adrenergic response cascade in lung development.

Methods

ANIMALS

All animal experiments were approved by the Animal Care Committee of the University of Ottawa and conformed with the guidelines outlined by the Canadian Council for Animal Care.¹¹

Following a one week acclimatisation timed pregnant 3-4 month old Hartley guinea pigs (Charles River Breeders, Quebec, Canada) were sacrificed by intracardiac injection of sodium pentobarbitone. The fetuses were delivered by hysterotomy and were immediately euthanised with intraperitoneal injection of sodium pentobarbitone if they showed any signs of life. The fetuses were weighed and their gestational age was assigned using the table of Draper¹² which reports average fetal weight as a function of gestational age. The fetal lungs were excised under sterile conditions and placed in 50 mmol/l Tris/1 mmol/l EDTA.

MEMBRANE PREPARATION

Because β adrenergic receptors are found in cell membranes, membrane particulates were prepared from the excised lungs; the lungs were minced in 50 mmol/l Tris/1 mmol/l EDTA and filtered through cheesecloth before centrifugation at 1200g for 15 minutes. The supernatant was then centrifuged at 29 000g

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for 15 minutes and the resulting pellet was washed three times in 50 mmol/l Tris/4 mmol/l MgCl_2 as previously described.¹³ The protein concentrations of the particulates were determined according to the method of Bradford¹⁴ with bovine serum albumin as the standard. The membrane particulates were frozen in a dry ice/ethanol bath and stored at -70°C until assayed.

RADIOLIGAND BINDING ASSAYS

There were no previous data on β adrenergic receptors in fetal guinea pig lung so the experimental conditions were first validated by a series of studies designed to assess the optimal incubation time, protein concentration, conditions of the incubation and wash buffer, and subcellular fraction to be assayed. For saturation analysis of radioligand binding experiments the incubation time must be long enough to ensure that a steady state is reached, but not so long that any decrease in binding occurs. This time required to reach steady state between the radioligand and the β receptors was determined by incubating 200 pmol/l iodine-125 labelled iodopindolol with membrane particulates (20 μg protein) in a buffer of 50 mmol/l Tris/4 mmol/l MgCl_2 (pH 7.4), 1 mmol/l ascorbic acid, and either 0.1 mmol/l HCl for total binding or 0.1 mmol/l isoprenaline in 0.1 mmol/l HCl for non-specific binding, at 30°C for 1–90 minutes. At the prescribed times the incubation was stopped by the addition of 5 ml ice cold 50 mmol/l Tris/4 mmol/l MgCl_2 ; bound and free ligand were separated by filtration over Whatman GF/C filters. The filters were washed three times and the radioactivity on the filters was counted to determine the specific radioligand binding over time. Apparent steady state between the β receptors and ^{125}I -iodopindolol was reached by 30 minutes as shown by constant specific binding beyond this time. All subsequent experiments used an incubation time of 45 minutes to ensure that steady state conditions existed.

The optimal protein concentration to perform saturation analysis studies was determined by incubating ^{125}I -iodopindolol (200 pmol/l) with membrane particulates (0–60 μg protein) as described above. From these experiments it was determined that to ensure optimal detection of the β receptors and less than 20% non-specific binding a minimum of 20 μg membrane protein was required.

Using similar methods specific radioligand binding was determined at incubation temperatures of 4°C , 22°C , 30°C , and 37°C ; maximal specific radioligand binding was determined to be at 30°C so this temperature was used in subsequent experiments. Similarly, a total of three washes with ice cold buffer, rather than room temperature or 37°C buffer, was found to be optimal and was used in subsequent experiments.

To determine which subcellular fraction of fetal guinea pig lung contained the largest concentration of β receptors, the amount of total, non-specific and specific binding of ^{125}I -iodopindolol to the β receptors was monitored

as described above in the following fractions obtained from differential centrifugation: (1) 1200g pellet (15 minutes at 4°C); (2) 29 000g pellet (15 minutes at 4°C); (3) 100 000g supernatant (60 minutes at 4°C). The largest β receptor concentration was located in the 29 000g pellet so this protein was used for the radioligand binding analyses.

Having thus optimised the assay conditions, saturation analysis experiments were performed to determine the concentration of β adrenergic receptors. In these assays duplicate samples of each membrane particulate (20 μg protein) were incubated at 30°C for 45 minutes in 0.125 ml buffer containing ^{125}I -iodopindolol (0–500 pmol/l) as described above.

The dissociation constant (K_d), which describes the affinity of a ligand for its receptor, was determined from the initial saturation analysis experiments and validated kinetically.¹⁵ For these kinetic experiments, k_{off} was determined by incubating ^{125}I -iodopindolol (500 pmol/l) with membrane particulate (20 μg protein) in 50 mmol/l Tris/4 mmol/l MgCl_2 at pH 7.4 for 30 minutes at 30°C . At 30 minutes the assay volume was increased 100 fold with 50 mmol/l Tris/4 mmol/l MgCl_2 at pH 7.4 and the incubation continued for an additional 60 minutes. Specific radioligand binding was monitored as a function of time before and after the dilution of the reaction mixture using the radioligand binding assay already described. K_{on} was calculated as outlined by Roberts.¹⁵

Since β receptors exhibit a higher affinity for the S isomer of a ligand than the R isomer, fetal guinea pig lung preparations were examined for stereoselectivity. Membrane particulates (20 μg protein) were incubated as described above with ^{125}I -iodopindolol (200 pmol/l) and either S or R propranolol in decreasing concentrations (1 nmol/l to 10 $\mu\text{mol/l}$). The specific binding of ^{125}I -iodopindolol to the β receptors was monitored by radioligand binding assay described above.

Beta receptor subtype can be determined by agonist competition assays or by competition assays with specific β_1 or β_2 antagonists. For these assays membrane particulates (20 μg protein) were incubated with ^{125}I -iodopindolol (200 pmol/l) in the presence or absence of β agonists (isoprenaline, adrenaline, or noradrenaline), or a selective β_1 antagonist (atenolol, 1 pmol/l–10 $\mu\text{mol/l}$), or a specific β_2 antagonist (ICI 118 551, 1 pmol/l–10 $\mu\text{mol/l}$, a gift from Imperial Chemical Industries) for 45 minutes at 30°C . The radioligand binding was monitored with the assay described above.

CAMP GENERATION

To be important in fetal lung development, any β receptors that are identified by radioligand binding assays must be functional. To assess this we studied the ability of isoprenaline (0–50 $\mu\text{mol/l}$) and forskolin (0–500 $\mu\text{mol/l}$) to stimulate cAMP generation in fetal guinea pig lung of various gestational ages. Approximately 25 mg of lung tissue were incubated in a 0.25 ml volume of 5 mmol/l Tris and 0.9%

sodium chloride (pH 7.4), 1 mmol/l 3-isobutyl-1-methylxanthine (a non-specific phosphodiesterase inhibitor), and 0.1 mmol/l ascorbic acid, with or without isoprenaline or forskolin, at 37°C for 10 minutes. The reaction was stopped by adding 0.25 ml of 10% trichloroacetic acid. The sample was then homogenised and centrifuged. An ether extraction (5 volumes ether:1 volume sample) was performed three times on the supernatant of each sample. The sample was evaporated to dryness under a stream of air in a 70°C dry bath. This cAMP extract was reconstituted in 0.5 ml of 50 mmol/l sodium acetate (pH 4). Cyclic AMP was then determined by a commercially available radioimmunoassay kit (NEN-Dupont). Under our assay conditions the recovery of cAMP (50, 25, 10, 5, 1 pmol) was found to be consistently greater than 80%. The variability in recovering 10 pmol cAMP/mg wet weight of lung tissue was 10%. The detection limit of this assay was 1 pmol cAMP/mg wet weight and there was a 9% variability in recovering this amount of cAMP.

CHOLINE INCORPORATION

Finally we wanted to begin to correlate changes in the β receptors with evidence of other events in the lung that are felt to be mediated, at least in part, through the β adrenergic response cascade. As a potential measure of surfactant synthesis, therefore, incorporation of tritium-labelled choline into phosphatidylcholine was measured.¹⁶ Fresh tissue was chopped into 1 mm cubes and incubated on a rocking platform at 37°C in Waymouth MB752/1 culture medium (Sigma) with 5 μ Ci/ml of ³H-choline chloride in an atmosphere of 5% CO₂/95% air. After four hours the tissue was washed in 0.9% NaCl and homogenised using a Teflon homogeniser. The phospholipids were extracted from the homogenate in a 1:1 chloroform:methanol solution as described by Folch *et al*¹⁷ and separated by thin layer chromatography (TLC) on silica coated TLC plates.¹⁸ The TLC plates were stained in a solution of cupric acetate in sulphuric acid/ethanol and dried for 30 minutes at 125°C.¹⁸ The plates were scraped and the relative amount of radioactivity in the phosphatidylcholine band was determined by liquid scintillation counting.

DATA ANALYSIS

The saturation analysis data were analysed by the equilibrium binding data analysis (EBDA) program (Elsevier Biosoft) on an IBM compatible desk top computer. Competition data were analysed by the GraphPad InPlot program which provides the best fit final estimates of β adrenergic receptor concentration (β_{max}) and dissociation constant (K_d) from a least squares analysis of different site models. Results for receptor concentrations in the different subcellular fractions, the cAMP stimulation, and choline incorporation data were analysed by an analysis of variance followed by Scheffe's post hoc test.¹⁹ The data for total β

receptor concentrations and K_d at different gestational ages were analysed using a simple linear regression. Statistical significance was defined as $p < 0.05$. Data throughout are presented as mean (SE).

Results

Saturation analyses at all gestational ages tested (40–64 days) were linear, indicating a single class of saturable, high affinity binding sites (fig 1); unfortunately there was insufficient tissue from lungs of fetuses < 40 days of gestation to do full saturation analyses even when whole litters were pooled. In two fetuses (59 days and 60 days) the dissociation constants (K_d) determined from saturation analysis were confirmed kinetically and were very similar – for example, at 60 days $K_d = 37.9$ pmol/l from saturation analysis and kinetically $k_{on} = 1.33 \times 10^{-9}$ mol/l/min, $k_{off} = 0.042$ /min, therefore $k_{off}/k_{on} = 31.6$ pmol/l = K_d ; younger fetuses never had sufficient tissue to perform both sets of experiments simultaneously so we could not validate other saturation analyses kinetically.

Figure 1 A typical Scatchard plot of ¹²⁵I-iodopindolol specific binding to the β adrenergic receptors in a 60 day gestation fetal guinea pig lung from saturation analysis experiments.

Figure 2 Binding curves for ¹²⁵I-iodopindolol (200 pmol/l) competing with various adrenergic receptor ligands in the presence of different concentrations of isoprenaline ($\square$), adrenaline ($\blacksquare$), noradrenaline ($\triangle$), atenolol ($\blacktriangle$), and phentolamine ($\diamond$).

Figure 3 Representative binding curves for ^{125}I -iodopindolol (200 pmol/l) competing with ICI 118 551 in membranes from guinea pig lung of different gestational ages. Analysis of these competition curves and seven others not shown showed that the percentages of β_1 and β_2 receptors and the inhibition constants (K_i) at the gestational ages tested were not different.

Figure 4 Changes in the concentration of β adrenergic receptors with gestational age in fetal guinea pig lung ($r=0.862$, $p<0.0001$). The concentration of β adrenergic receptors was determined by full saturation analysis in 13 specimens and by one point analysis using 500 pmol/l ^{125}I -iodopindolol as previously described³² in six specimens because of insufficient tissue to perform full analysis.

Figure 5 Mean (SE) cAMP generation in fetal guinea pig lung during the canalicular and saccular phases of lung development ($n=4$). The amount of cAMP stimulated above basal levels is indicated on the vertical axis. * $p<0.05$ between cAMP generated during the canalicular and saccular phases.

The β receptors we identified demonstrated stereoselectivity for the R and S isomers of propranolol; the pharmacologically active S isomer exhibited a higher affinity for the β receptors ($\text{EC}_{50}=2.5 \times 10^{-8}$ mol/l) than the R isomer ($\text{EC}_{50}=2 \times 10^{-6}$ mol/l).

As an index of β receptor subtypes, competition assays were performed to monitor the ability of various adrenergic agents to compete with ^{125}I -iodopindolol. The order of potency of the agonist affinities (isoprenaline > adrenaline > noradrenaline) was indicative of a predominance of β_2 receptors (fig 2). Confirmation of this observation by competition assays with ICI 118 551, a specific β_2 antagonist, showed that β_2 receptors predominated throughout the time points of gestation studied (41–67 days). Approximately 65% (95% CI 57% to 74%) of the receptors were β_2 and 35% (95% CI 26% to 43%) were β_1 ($n=11$); this did not change with gestation (fig 3). The inhibition constant (K_i) for the high and low affinity sites determined from displacement of ^{125}I -iodopindolol binding by ICI 118 551 were 7 nmol/l (95% CI 1.08 to 23.56 nmol/l) and 1.04 $\mu\text{mol/l}$ (95% CI 0.33 to 3.25 $\mu\text{mol/l}$). The K_i for displacement of iodopindolol binding by atenolol was 17 $\mu\text{mol/l}$ (95% CI 6.9 to 42.2 $\mu\text{mol/l}$). These values are consistent with those previously described for human fetal lung.¹³

The concentration of total β receptors progressively increased from 23 fmol/mg at 35 days of gestation to 140 fmol/mg at 64 days gestation (fig 4). The mean (SE) K_d did not change with gestational age (110 (9.7) pmol/l, range 37–178 pmol/l, $n=13$) ($r=0.004$, $p>0.05$).

To determine whether these receptors were functional, the ability of β agonists to stimulate cAMP was examined. In the saccular phase of lung development (>50 days) isoprenaline stimulated 50% more cAMP than was present in the basal state (fig 5). There was no significant stimulation of cAMP by isoprenaline in the canalicular phase of lung development (40–49 days) (fig 5). No gestational differences were observed with forskolin stimulated cAMP generation (fig 5). Mean (SE) basal levels of cAMP were 8.1 (0.86) pmol/mg wet weight of tissue and did not change with gestation.

Finally, incorporation of ^3H -choline into phosphatidylcholine, a potential measure of surfactant synthesis, was greater in the saccular (58.65 (6.3) cpm/mg tissue) than in the canalicular (34.6 (2) cpm/mg tissue) phase of lung development ($p<0.005$).

Discussion

Neonatal respiratory distress syndrome is a disease that is characterised by anatomical immaturity of the lung, excess lung water, and surfactant deficiency. The resorption of lung water and synthesis and secretion of surfactant phospholipids and surfactant associated proteins are mediated, in part, by β adrenergic responses.^{2,3,20–24} Beta adrenergic receptors have been identified in fetal rabbit and rat lung,^{2,3} as well as in human fetal lung from

early in the second trimester of gestation.^{13,25} Human fetal lung is the ideal model for studying the role of the β adrenergic response cascade in lung development; however, ethical considerations limit the availability of gestation-appropriate samples. Fetal rabbit lung is one of the most commonly used models in which to study β adrenergic responses in fetal lung development, despite the predominance of β_1 receptors²⁶ in contrast to the predominance of β_2 receptors in human fetal lung.¹³ Fetal rat lung has also been used. It has similar proportions of β receptor subtypes²⁷ to those found in humans, but the proportion of time spent in each of the various stages of lung development in the fetal rat is quite different from that in human fetal lung.⁷ The β adrenergic response cascade in fetal guinea pig lung has not been previously reported. However, the relative proportion of β_1 to β_2 receptors in the adult guinea pig is similar to that in the human lung^{28,29} and the concentration of β receptors, especially in the alveolar and bronchial wall epithelium, increases from the newborn to the adult.³⁰

Our studies show that characteristic β adrenergic receptors are present in fetal guinea pig lung. Binding of ¹²⁵I-iodopindolol to β receptors in fetal guinea pig lung is saturable and of high affinity; these findings are similar to those in fetal human lung.¹³ The concentration of β adrenergic receptors increases with gestational age in fetal guinea pig lung in a similar way to fetal rabbit,^{3,26} fetal rat,^{3,27} and fetal human lung.¹³ The order of potency of agonist affinities and competition studies with a specific β_2 antagonist indicates a predominance of β_2 receptors similar to that in fetal human lung¹³ and fetal rat lung,²⁷ but unlike fetal rabbit lung.²⁶

To determine whether the β adrenergic receptors were functional we examined the ability of β agonists to stimulate cAMP and found that there was significant isoprenaline stimulated cAMP generation in the saccular phase of lung development. In contrast, there was no difference detected in the ability of forskolin – a direct activator of adenylate cyclase – to stimulate cAMP generation in the saccular phase compared with the canaliculal phase of lung development. Since β receptors are linked to the catalytic moiety of adenylate cyclase via guanine nucleotide regulatory proteins (G proteins), it is possible that there is a gestational change in the amount or function of these G proteins.

The synthesis and secretion of both surfactant phospholipids^{20–22} and surfactant associated proteins^{23,24} are controlled in part by β adrenergic mechanisms. In our studies we found gestational changes in the ability of isoprenaline to stimulate cAMP in fetal guinea pig lung. Interestingly, the gestational age pattern for isoprenaline stimulation of cAMP was associated with a similar pattern for choline incorporation into phosphatidylcholine, which could indicate increased synthesis of either surfactant phospholipids or membrane phospholipids. It is not known whether similar associations exist in fetal human lung between

the canaliculal and saccular phases of lung development, nor whether this is important in the preparation for extrauterine life. In fetal rabbits administration of an irreversible β adrenergic antagonist (bromacetylalprenolol-methane) during the saccular phase of lung development decreases both the number of receptor binding sites and the phospholipid content of lung lavage fluid.³¹ We have not yet performed experiments to determine if blocking the β receptors in fetal guinea pig lung has any effects on surfactant production.

In conclusion, the β adrenergic response cascade in fetal guinea pig lung exhibits similar characteristics to those previously described in fetal human lung. This, combined with several other aspects of fetal guinea pig development, indicate that fetal guinea pig lung is a better small animal model to study the role of the β adrenergic response cascade in lung development and adaptation to extrauterine life. Even in this time of successful surfactant therapy for respiratory distress syndrome there is still much to learn about the mechanisms of surfactant synthesis, release, and recycling in the newborn. Current evidence points to a role for β adrenergic responses in these functions; an appropriate animal model will help us to understand how better to use these responses in the prevention and treatment of neonatal respiratory distress syndrome.

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