British Journal of Pharmacology (2002) 135, 1415–1424
www.nature.com/bjp

Postnatal changes in β-adrenoceptors in the lung and the effect of hypoxia induced pulmonary hypertension of the newborn

*,[1]Alison A. Hislop, [2]Judith C.W. Mak, [1]David Kelly, [1]Jayne A. Reader, [2]Peter J. Barnes & [1]Sheila G. Haworth

[1]Vascular Biology and Pharmacology Unit, Institute of Child Health, London and [2]Department of Thoracic Medicine, Imperial College School of Medicine, London

1 β-adrenoceptor activation leads to pulmonary vasodilatation. The increase in circulating catecholamines at birth may assist the postnatal fall in vascular resistance by their activation. To study β_1- and β_2-adrenoceptors during postnatal adaptation, we used [^{125}I]-iodocyanopindolol (ICYP) binding to lung membranes and sections to quantify and locate the binding sites in piglets from birth to 14 days of age and compared them with those in adult pigs. In addition, pulmonary hypertension was induced in newborn piglets by hypobaric hypoxia.

2 In lung membranes the equilibrium dissociation constant (K_d) did not change with age for total β-adrenoceptors or for β_2-adrenoceptors, but there was a significant increase in maximum binding sites (B_{max}) between birth and 3 days of age. On tissue sections, B_{max} increased between 3 days and adulthood with no change in K_d.

3 Binding sites of β_1- and β_2-adrenoceptors were localized to the bronchial epithelium, to endothelium of extra- and intra-pulmonary arteries and to lung parenchyma. Total β-adrenoceptor density increased with age at all locations ($P<0.05-0.01$). At birth intrapulmonary arteries showed no binding, β_2-adrenoceptors appeared on day 1 and increased up to 14 days of age. β_1-adrenoceptors appeared by 3 days of age and increased with age.

4 Hypobaric hypoxia from birth led to attenuation in the normal postnatal increase in receptor number, but hypoxia from 3–6 days did not decrease receptor density.

5 The normal postnatal increase in β-adrenoceptors suggests a potential for catecholamine induced dilatation in the lung during adaptation which is attenuated in pulmonary hypertension.
British Journal of Pharmacology (2002) **135,** 1415–1424

Keywords: β-Adrenoceptors; lung development; hypobaric hypoxia; pulmonary arteries; ligand binding studies

Abbreviations: AMP, adenosine-3′,5′-monophosphate; βARs, β-adrenoceptors; B_{max}, maximum binding sites; GMP, guanosine-3′,5′-monophosphate; ICYP, [^{125}I]-iodocyanopindolol; K_d, equilibrium dissociation constant; Tris, tris (hydroxymethyl) aminomethane buffer

Introduction

Birth is characterized by a surge in catecholamine release from the adrenal medulla (Gabriel *et al*., 1983; Kallio *et al*., 1998). This may play a part in the dilatation of both pulmonary arteries and airways during adaptation to extrauterine life by ensuring a sustained fall in pulmonary vascular resistance and optimal aeration of the lung. Dilatation results from the activation of β-adrenoceptors (βARs), which activate adenyl cyclase *via* a stimulatory G protein (G_s) to cause an increase in cyclic AMP (Johnson, 1998). In the lung, β_2ARs predominate over β_1ARs (Barnes, 1995). During development, Schell *et al*. (1992) described an increase in βARs in rat pulmonary arterial and airway smooth muscle cells. It is important to understand the role of βARs in the postnatal adaptive period, when pulmonary vascular resistance decreases rapidly in the normal baby but remains high in cases with persistent pulmonary hypertension. This is a frequent cause of perinatal morbidity and mortality. Catecholamines are used extensively as an inotrope to increase cardiac output in critically ill infants but we do not know what effects these may have on the immature lung.

Previous studies on the porcine lung, which is a suitable model for the human (Haworth & Hislop, 1981), have shown that the rapid vasodilatation seen after birth is related in part to upregulation of nitric oxide synthase. However, it is apparent that this mechanism is not solely responsible for reducing pulmonary vascular resistance after birth (Tulloh *et al*., 1997; Boels *et al*., 1999). The muscarinic receptors and receptors for the peptides calcitonin gene related peptide, vasoactive intestinal peptide and atrial natriuretic peptide showed changes in density during this period (Hislop *et al*., 1998a, b; Matsushita *et al*., 1999). In the present study the density of βARs was studied in the developing porcine lung from birth (5-min-old) to 14 days of age and compared with that in the adult. As a model of human neonatal pulmonary hypertension, piglets were exposed to chronic hypobaric hypoxia. This is an established model of pulmonary hypertension. The animals have right ventricular hypertrophy, an increase in pulmonary arterial smooth muscle, the isolated pulmonary arteries fail to dilate in response to acetylcholine, and the response to exogenous nitric oxide is attenuated.

*Author for correspondence at: Unit of Vascular Biology, Institute of Child Health, 30 Guilford Street, London WC1N 1EH;
E-mail: a.hislop@ich.ucl.ac.uk

Those exposed from birth continue to shunt from right to left through foetal channels and have a systemic arterial oxygen saturation of 71±5% (Tulloh *et al*., 1997). In the present study on both normal and hypoxic piglets, ligand binding was carried out to characterize the number and subtype of the βARs in the membranes of parenchymal lung tissue. In addition, the location and density of βAR subtypes was determined on sections of lung tissue using autoradiography.

Methods

Tissue from normal large white piglets aged less than 5 min (newborn), and 1, 3, 6 and 14 days were studied. Tissue from adult pigs was collected from a local abattoir and frozen within an hour. The piglets were delivered at term and kept with their mother until they were killed with intraperitoneal sodium pentobarbitone. Additionally, some animals were placed in a hypobaric hypoxic chamber for 3 days, to make the animals pulmonary hypertensive (Haworth & Hislop, 1982). The internal temperature was maintained at 29°C and the air pressure maintained at 50.8 kPa. The pressure was returned to normal while cleaning the chamber and feeding the piglets, three times daily for 10–15 min. The animals also had a continuous supply of milk in the chamber. The young animals were kept in accordance with NIH regulations outlined in the '*Guide for the Care and Use of Laboratory Animals*' published by the U.S. National Institutes of Health (Department of Health and Human Services, Publication No. (NIH) 85-23, Revised 1996) and in accordance with British Home Office regulations and the approval of the Animal Ethics Committee of the Institute of Child Health. Two groups of hypertensive animals were studied; those exposed to hypobaric hypoxia from birth until 3 days of age and then killed, and those kept with the sow until 3 days of age and then exposed to hypoxia from 3 to 6 days of age and killed at 6 days. All these animals showed pathological evidence of pulmonary hypertension. They had right ventricular hypertrophy, as shown by a reduction in the ratio of the weight of left ventricle plus septum to right ventricle, and an increase in pulmonary arterial medial hypertrophy assessed by measurement of the percentage wall thickness of peripheral arteries. Data on such animals have been reported previously (Tulloh *et al*., 1997).

Within 1 h of death peripheral, parenchymal lung tissue containing no large airways or arteries was snap frozen in liquid nitrogen and stored at −70°C for use later in the preparation of lung membranes. In addition blocks of lung tissue to include arteries and airways and a block of the main extrapulmonary artery were surrounded by OCT embedding compound (Brights, Cambridge, U.K.) on cork discs and snap frozen in melting isopentane cooled in liquid nitrogen. The blocks were then stored at −70°C. Subsequently, serial 10 μm cryostat sections were cut from each block and thaw mounted on glass slides coated with Vectabond (Vector Laboratories, Peterborough, U.K.). Slides were stored in sealed boxes with desiccant at −70°C.

Receptor binding to lung membranes

Frozen peripheral lung tissue was pulverized with a pestle and mortar in liquid nitrogen and then homogenized in 10 volumes of 0.32 M ice-cold sucrose in 25 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) (Tris) using a Polytron homogenizer (10–15 bursts). After centrifugation at 1000× *g* for 10 min at 4°C the supernatants were centrifuged at 40,000× *g* for 20 min. The resulting pellets were washed once in ice-cold 25 mM Tris buffer and centrifuged at the same speed. The final pellets were resuspended in incubation buffer (25 mM Tris buffer, pH 7.4 at 37°C) and stored as aliquots at −70°C until used in binding assays. Protein concentration was determined according to the method of Lowry *et al*. (1951) using bovine serum albumen as standard.

For saturation studies, the reaction was carried out in triplicate for each sample in a final volume of 0.25 ml of 25 mM Tris buffer (pH 7.4) with 154 mM NaCl, 1.1 mM ascorbic acid containing [^{125}I]-iodocyanopindolol; specific activity 2000 Ci mmol^{-1} ([^{125}I]-ICYP) (Amersham International, Amersham, U.K.) at seven concentrations from 1.25–200 pM and appropriate amounts of membrane protein (approx. 0.075 mg ml^{-1}). Incubations were performed at 37°C for 2 h and terminated by rapid vacuum filtration through GF/C glass fibre filters (Whatman, Clifton, NJ, U.S.A.) pre-soaked in ice-cold buffer using a Brandel cell harvester (model M-24). Each filter was rapidly washed with 3×5 ml ice-cold 25 mM Tris buffer. The filters were counted in an auto gamma counter (model 5550, Packard Instruments). Non-specific binding was defined as the binding in the presence of 200 μM isoprenaline. In order to estimate β_2AR binding, incubation was also carried out in the presence of 0.1 μM CGP 20712A (a selective β_1 antagonist). The maximum binding sites (B_{max}) and the equilibrium dissociation constants (K_d) were determined using the EBDA and LIGAND programmes for each case. The mean and standard error for each age group were compared using ANOVA and Students *t*-test. When making more than one comparison, Bonferroni's correction was used. Differences were considered significant when $P<0.05$.

Receptor binding to lung sections

Slide-mounted sections of lung and main pulmonary artery were brought to room temperature and pre-incubated for 15 min in incubation buffer (25 mM Tris, 154 mM NaCl, 0.25% bovine serum albumen and 1.1 mM ascorbic acid; pH 7.4) at 23°C. They were then incubated with [^{125}I]-ICYP at 37°C for 90 min, which was found to be the optimal incubation time. Non-specific binding was determined by incubating adjacent sections in the presence of 200 μM isoprenaline. To study the β-adrenoceptor subtypes serial sections were incubated with 25 pM [^{125}I]-ICYP in the presence of Tris buffer, 200 μM isoprenaline, 1 μM betaxalol (a selective β_1 antagonist) or 1 μM ICI 118551 (a selective β_2 antagonist). Binding was terminated by washing in buffer at 4°C (two changes in 10 min) and the sections then rinsed in cold distilled water and dried rapidly in cold air. Sections were exposed to Hyperfilm ^{3}H (Amersham U.K.) together with sets of radiolabelled polymer standard sections (American Radiolabeled Chemicals Inc.; St. Louis, MO, U.S.A.) for 38 h at 4°C and then developed in Kodak D19 developer for 5 min at 20°C and fixed in Amfix. These films were used to quantify the density of binding at a magnification of ×30. Sections were then fixed and stained with Miller's elastic and

van Gieson stain or with haematoxylin and eosin stain to identify the structures.

Quantification of the binding sites on autoradiographs

The autoradiographic film images were quantified using a computer assisted image analysis system, the Optilab system (Graftek) for Apple Macintosh. Grey levels of the individual structures within the section were converted to attomoles of bound [^{125}I]-ICYP per unit area (amol mm^{-2}) using a set of calibrated standards for each film. The density of binding was assessed for the extrapulmonary artery, the large intra-pulmonary arteries (>500 μm), airways containing cartilage and the parenchymal region. The latter includes alveoli and alveolar ducts and their accompanying small vessels that cannot be identified individually at this magnification (diameter less than 200 μm). Using adjacent serial sections, specific binding of total βAR and β_1 and β_2 subtypes was obtained by subtracting the non-specific binding (that is, binding in the presence of 200 μM isoprenaline) from total binding in the same area of tissue. The binding sites for total βARs were characterized on the autoradiographs of adult lungs by an association study to give the time course of binding. A saturation study using eight concentration of [^{125}I]-ICYP from 5–200 pM was made on the parenchyma, bronchial epithelium and intra-pulmonary arteries in 3-day-old and adult animals ($n=5$ per age group). The B_{max} was determined and the K_d calculated by Scatchard analysis using the INPLOT programme (version 4.03; GraphPad Software, San Diego, U.S.A.). The values were compared by ANOVA and where appropriate Students t-test.

In order to assess changes in the density of βAR with age and in pulmonary hypertension, the binding sites on parenchyma, airways and arteries were quantified on the autoradiographic film of the tissue sections from each pig. Sections were studied in triplicate for total, both βAR subtypes and non-specific binding. Because of the large number of tissue sections involved it was not possible to incubate the tissue from all animals at the same time. Therefore the results from animals of different ages were compared within each experiment. The density of binding sites measured on the autoradiographs showed the same rank order for different lung structures in all animals of the same age, within each of the binding experiments. Therefore the data from all animals of the same age from all experiments were pooled. The effect of age and exposure to hypobaric hypoxia were compared using ANOVA and where appropriate Students t-test with Bonferroni's correction. $P<0.05$ was considered to be significant.

Results

Effect of age

Binding to lung membranes Saturation isotherm experiments of [^{125}I]-ICYP demonstrated high-affinity and saturable binding best described by a one-site fit over the concentration range of 1.25 to 200 pM at all ages studied (Figure 1). Specific binding was over 80% at all concentrations. The K_d values for total βARs and for β_2ARs showed no significant change with age (Table 1). The B_{max} values were low at birth for both total βARs and β_2ARs. It was significantly increased for both by 3 days of age and remained high. At birth 71% of the total βARs were β_2ARs, while in the adult it was only 54%, suggesting a higher proportion of β_2ARs at birth.

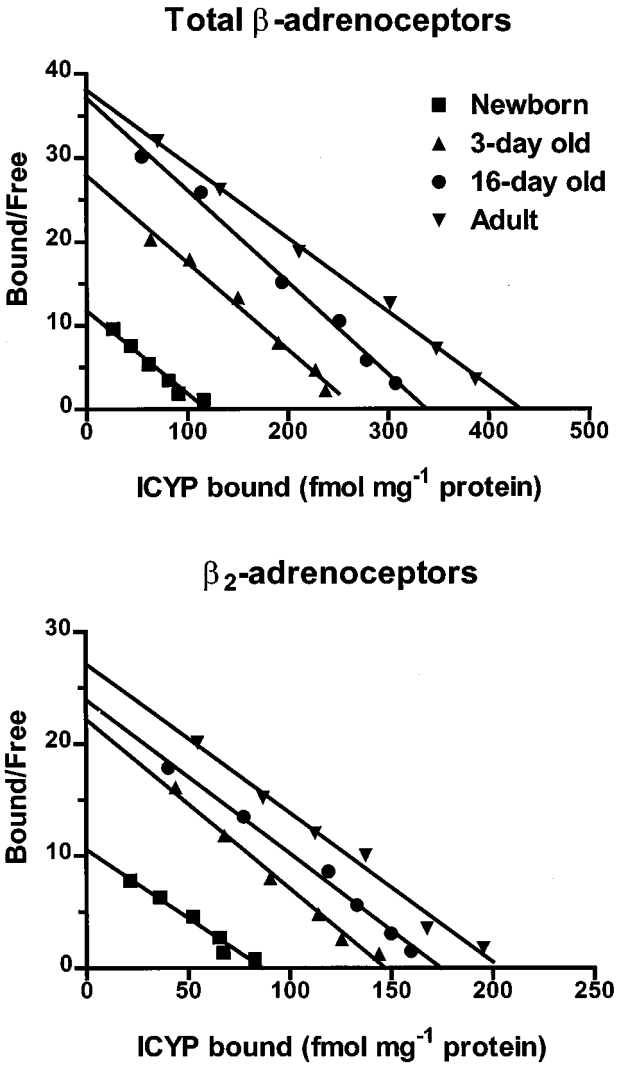

Figure 1 Scatchard plot analysis of [^{125}I]-ICYP binding in newborn, 3-day-old, 14-day-old and adult pig lung membranes. Data represent a typical experiment of one single animal from each group performed in triplicate. Top panel for total β and bottom panel for β_2-adrenoceptors in the presence of CGP 20712A (selective β_1 antagonist).

Binding to tissue sections On the autoradiographic film specific binding sites could be identified on pulmonary arteries and veins, airways and parenchyma (Figures 2–4). For the pulmonary arteries binding was greatest on the endothelium with very low binding on the media. Some binding could be seen on the outside edge of the media in the extrapulmonary artery, which located to nerves and the *vasa vasorum* (Figure 4). For the airways, a high binding density was seen on the epithelium, a low binding density was seen over the smooth muscle cells but it was too low to measure accurately.

Binding of [^{125}I]-ICYP to tissue sections of 3-day-old and adult pigs was saturable and was displaced by incubation with

isoprenaline (Figure 2b). Non-specific binding to the parenchyma was less than 10% of the total. The K_d values for 3-day-old and adult lung structures were between 11.4 and 37.5 pM (Table 2). There was no significant difference between the ages or between structures, possibly because of the wide variation. The B_{max} values for each structure were greater in the adult than at 3 days of age ($P<0.04$ for each structure). The parenchyma had a significantly greater B_{max} than the airway epithelium and artery endothelium at both ages.

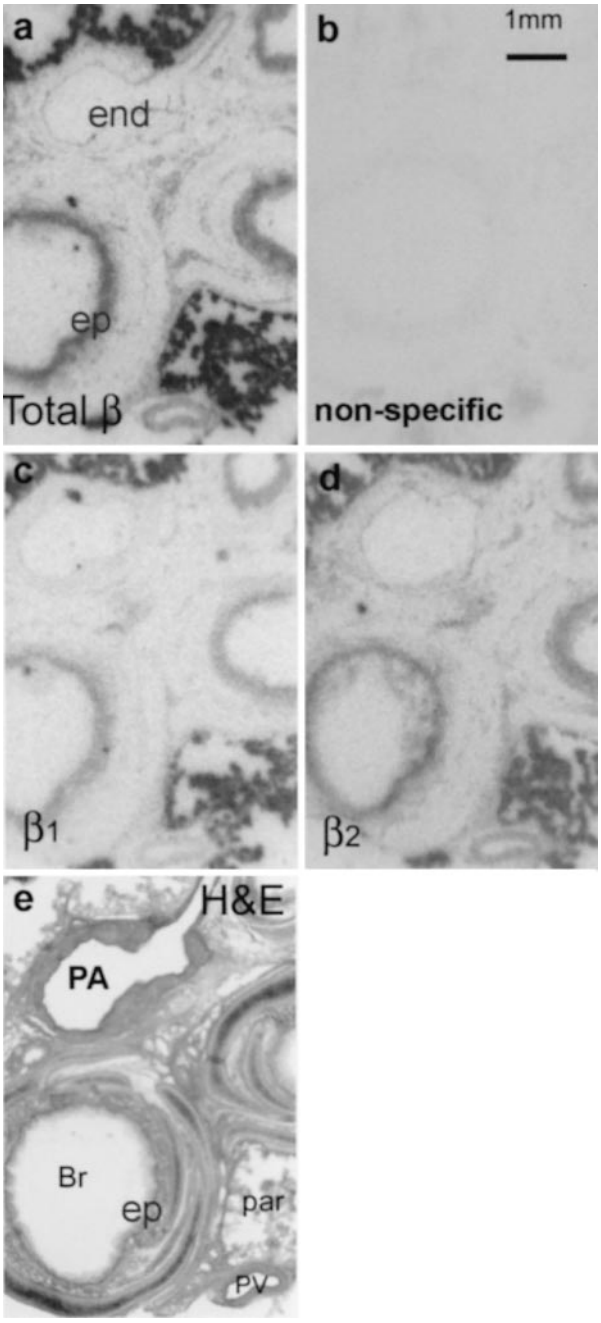

Figure 2 Autoradiographs of lung sections from an adult pig after incubation with [^{125}I]-ICYP showing (a) total binding to βARs in the presence of Tris buffer. (b) Non-specific binding in the presence of excess isoprenaline, (c) β_1-adrenoceptor binding in the presence of ICI 118551, (d) β_2-adrenoceptor binding in the presence of betaxalol and (e) section stained with haematoxylin and eosin (H&E), to show lung structure. Positive binding can be seen on the epithelium (ep) of the bronchi (Br), the endothelium (end) of the pulmonary arteries (PA), wall of the pulmonary vein (PV) and with a very high density of binding on the parenchymal region (par). β_1- and β_2-adrenoceptor binding sites are less than the total but have the same distribution.

At all ages (newborn, 1, 3, 6 and 14 days and adult) the measurable binding density was greatest in the parenchyma, followed in turn by the bronchial epithelium and pulmonary artery endothelium. For all structures the density of binding changed with age (Figure 5). In the newborn animals there was a low density in the parenchyma, which was significantly increased by day 1 ($P<0.01$). There was a continuing increase with age to the adult level, a 6 fold total increase ($P<0.01$ newborn *vs* adult). The density of binding was much less in the bronchial epithelium but also increased significantly between birth and 14 days and adulthood ($P<0.05$), the main increase being between 6 and 14 days. The endothelium of the extrapulmonary artery showed a marked transient increase in binding density at 3 days of age ($P<0.04$ in comparison with 1 and 6 days). At 14 days binding was less than at 3 days but greater than in the newborn ($P<0.05$). In the intrapulmonary arteries there was no specific binding on the endothelium of the newborn animals. Binding appeared by day 1 and there was a significant increase in binding density by 14 days of age ($P<0.01$ compared to 1 day), when the value was not significantly different from that in the adult. The binding density in the intrapulmonary arteries was significantly less than in the extrapulmonary arteries of the same age except in the 14-day-old and adult.

Binding density of βAR subtypes The βAR subtypes were assessed on serial sections after co-incubation with specific β_1AR or β_2AR antagonists. For the parenchyma both β_1ARs and β_2ARs were present at all ages (Figure 6). In the newborn and at day 1 the density of β_2AR binding was greater than β_1AR binding ($P<0.05$). At 3–14 days binding was almost equal and in the adult β_1AR binding was greater than β_2AR ($P<0.05$). The binding density of both subtypes increased with age ($P<0.05$ between newborn and both 14 days and adult). For the airway epithelium, the density of β_2AR binding was greater than that of β_1AR at all ages, and although β_2AR appeared to increase with age neither changed significantly since there was a large standard error. In the endothelium of the extrapulmonary artery at birth only β_2AR binding was present. This increased in density with age and was particularly high at 3 days of age ($P<0.05$ in comparison with 1- and 6-day-old arteries). β_1AR binding was only found on the endothelium from 3 days of age onwards and was always at a lower density than β_2AR binding. It did not increase with age. For the intrapulmonary arteries there was no binding of either β_1ARs or β_2ARs at birth. β_2ARs appeared by 24 h and β_1ARs by 3 days. The binding density was low, but it could be accurately measured because of the distinct line of binding on the endothelium. There was a significant increase between 1 and 14 days ($P<0.05$) in the density of β_2ARs, while the density of β_1ARs increased from 3 days to adult ($P<0.04$).

Effect of chronic hypoxia and pulmonary hypertension

The K_d value of the lung membranes was not altered by the presence of pulmonary hypertension in the piglets exposed

Table 1 Characteristics of [^{125}]-CYP binding on membranes of porcine lung parenchyma

	Number of animals	*Newborn*	*3 day*	*14 day*	*Adult*	*0–3 day H*	*3–6 day H*
		4	7	4	4	6	4
βAR	K_d> (pM)	9.5	7.1	6.7	8.9	7.1	4.5
	95% CI	5.2–13.8	4.9–8.9	4.4–7.9	7.1–10.6	4.4–7.9	3.6–5.4
	B_{max} (fmol mg^{-1} protein)	74 (14)	203 (28)**	271 (32)**	290 (53)**	95 (21)*	153 (26)
β_2AR	K_d (pM)	7.1	5.5	6.3	7.8	6.2	5.1
	95% CI	5.7–8.6	3.8–7.0	4.5–7.9	4.1–6.5	4.1–6.4	2.3–5.7
	B_{max} (fmol mg^{-1} protein)	53 (11)	119 (15)**	134 (14)**	157 (14)**	59 (18)*	103 (13)

Mean values and (standard deviation) are shown. CI, confidence interval; H, Hypoxia exposure; *$P<0.01$ in comparison with 3 day control; **$P<0.01$ in comparison with newborn.

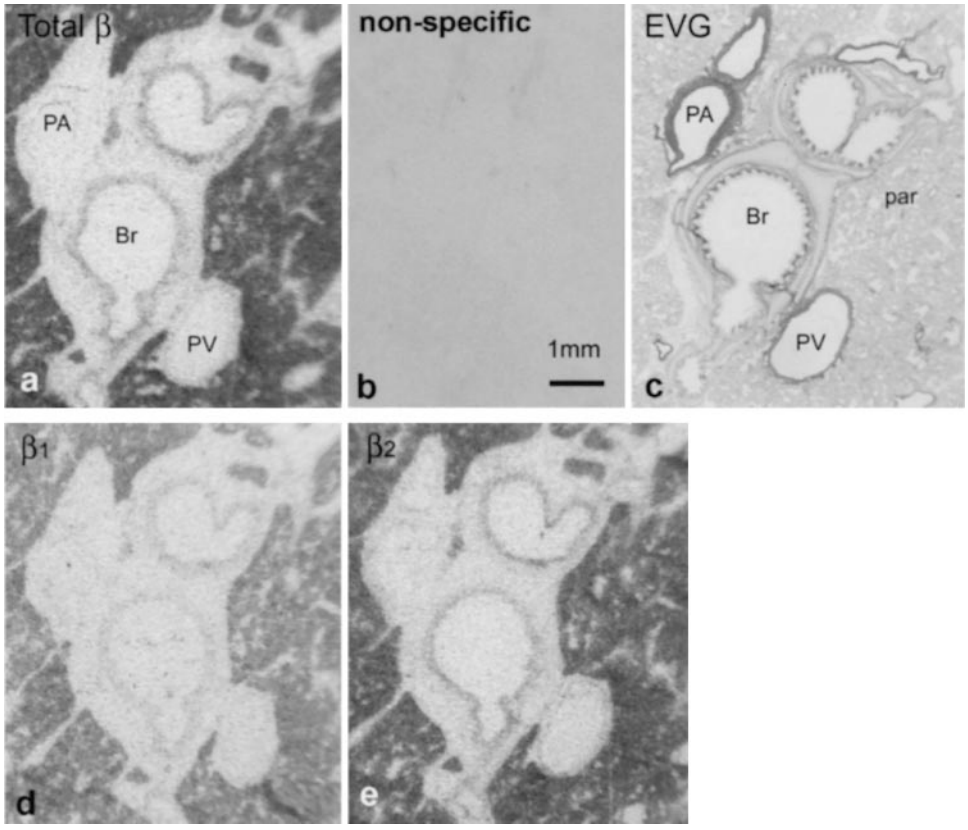

Figure 3 Autoradiographs of serial lung sections from a newborn piglet after incubation with [^{125}I]-ICYP showing (a) total binding to βARs in the presence of Tris buffer, (b) non-specific binding in the presence of isoprenaline, (c) section stained with Millers Elastic and van Geison stain (EVG) to show lung structures, (d) β_1-adrenoceptor binding in the presence of ICI 118551 and (e) β_2-adrenoceptor binding in the presence of betaxalol. Positive binding can be seen on the epithelium of the bronchi (Br) and with a very high density of binding on the parenchymal region (par). There is generally no binding on the endothelium of the pulmonary arteries or pulmonary vein wall. β_1- and β_2-adrenoceptor binding is less than the total but there is the same distribution.

to hypoxia from birth until 3 days of age (Table 1). However the B_{max} values for both the total βARs and β_2ARs were significantly reduced in these animals when compared to the normal 3-day-old animals, and they were not significantly different to the normal newborn. The binding density of total βARs measured on the lung sections from these piglets was also reduced in comparison with the normal 3-day-old on the bronchial epithelium and the endothelium of both extra and intrapulmonary arteries ($P<0.05$, 0.01 and 0.01 respectively) (Figure 7). The density was not significantly different to that in the newborn for the epithelium and the extrapulmonary arteries (see Figure 5). For both epithelium and endothelium the density of β_2AR binding was also significantly less than the normal at 3 days ($P<0.05$ for all). No β_1ARs were present in the extrapulmonary arteries after 3 days of hypoxia. These are present in the normal 3-day-old but not in the normal newborn (see Figure 6).

Exposure to hypoxia from 3–6 days of age had no effect on the binding in the lung membranes (Table 1) or on the binding density of either total βARs or subtypes on any structure when compared to the 6-day control animals.

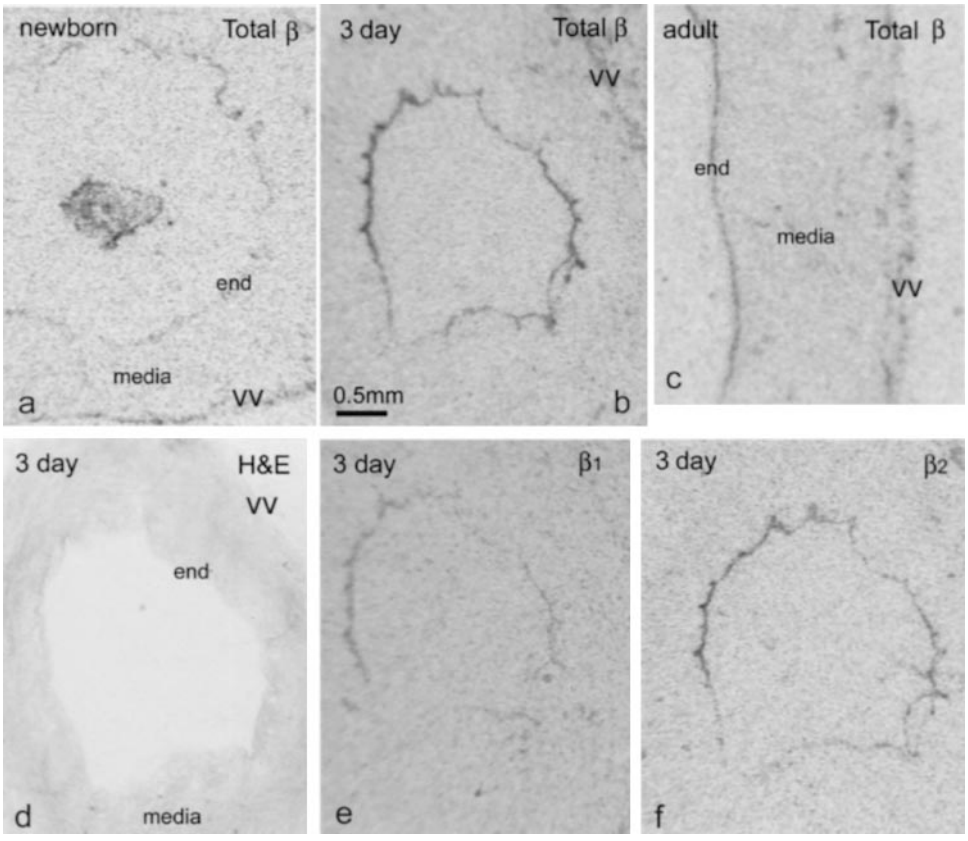

Figure 4 Autoradiographs of main pulmonary arteries after incubation with [^{125}I]-ICYP showing total βAR binding in the presence of Tris buffer in (a) newborn, (b) 3-day-old and (c) adult pigs. (d) is the 3 day pulmonary artery stained with haematoxylin and eosin (H&E). Binding is located to the endothelium (end) and not the media at all ages. Binding at the outer edge of the wall is to the *vasa vasorum* (vv) and nerves. Binding on the endothelium is less in the newborn than at 3 days and in the adult. (e) β_1-adrenoceptor binding in the presence of ICI 118551 at 3 days and (f) β_2-receptor binding in the presence of betaxalol at 3 days. There is a greater density of β_2- than β_1-adrenoceptors, both are less dense than the total binding.

Table 2 Characterization of binding of [^{125}I]-ICYP on porcine lung sections

	3-day-old ($n=5$)	*Adult* ($n=5$)
Parenchyma		
K_d (pM) (CI)	18.03 (11.0–25.1)	11.4 (5.9–16.9)
B_{max} (amol mm^{-2}) (SD)	119.1 (23.9)**	167.3 (27.8)*,**
Bronchial epithelium		
K_d (pM) (CI)	16.9 (12.6–21.3)	20.8 (0.8–40)
B_{max} (amol mm^{-2}) (SD)	10.9 (2.5)	54.9 (22.6)*
Intra-pulmonary arteries		
K_d (pM) (CI)	35.1 (−4.3–74.1)	37.5 (22.6–51.7)
B_{max} (amol mm^{-2}) (SD)	10.7 (7.7)	42 (25)*

SD, Standard deviation; CI, 95% confidence interval; *$P<0.04$ compared with 3-day-old; **$P<0.01$ compared with bronchial smooth muscle and intrapulmonary arteries.

Discussion

We have demonstrated, using a membrane binding technique in porcine lungs, an increase in the number of βAR binding sites (B_{max}) with age, without a change in the dissociation constant. The autoradiographic study confirmed the unchanged affinity and the increase in the density of total binding sites with age. In addition we were able to locate specific binding sites (β_1AR and β_2AR) to the lung parenchyma, bronchial epithelium and sometimes to the endothelium of pulmonary arteries in normal animals and in animals made hypertensive by exposure to hypobaric hypoxia. Of particular interest was the lack of binding sites on the endothelium of the intrapulmonary arteries at birth and their rapid appearance postnatally. The β_2AR binding sites appeared first followed by β_1AR. Maintaining newborn piglets in a hypoxic environment reduced the increase in β_2ARs after birth.

As in the present study previous reports documented the presence of both βAR subtypes in the porcine lung (Goldie *et al*., 1986; McNeel & Mersmann, 1999), and also in the human adult lung parenchyma (Mak *et al*., 1996a). We carried out an autoradiographic study in order to locate the receptors and to determine whether the density or type of βAR varied in the different structures. The highest density of binding was found in the parenchymal region, in which the estimated K_d was similar to that seen in the lung membranes. Measurements of binding density on the autoradiographs showed that the B_{max} for the bronchial epithelium was less than for the parenchyma with more β_2AR binding than β_1AR binding. In human airway epithelium a high density of both βAR types has been described (Carstairs *et al*., 1985; Mak *et al*., 1996a; Davis *et al*., 1990). Few binding studies have previously been made on the pulmonary vasculature. In our porcine study the autoradiographs showed specific pulmonary

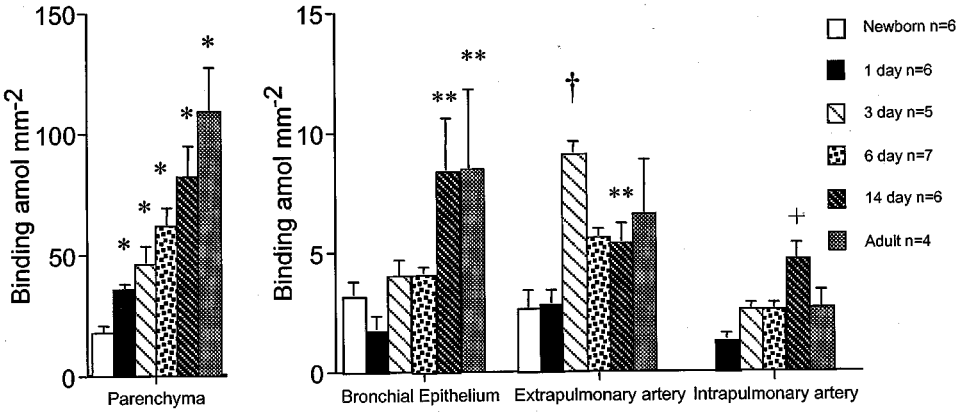

Figure 5 Mean density with standard error of [^{125}I]-ICYP binding to the porcine lung sections in six age groups with the number of animals studied in each group. Values for parenchyma, bronchial epithelium and endothelium of extra- and intrapulmonary arteries are shown. Significant differences after Students' *t*-test with Bonferronis correction. *$P<0.01$ compared with newborn; **$P<0.05$ compared with newborn; †$P<0.04$ compared with 1 day and 6 day; +$P<0.01$ compared with 1 day.

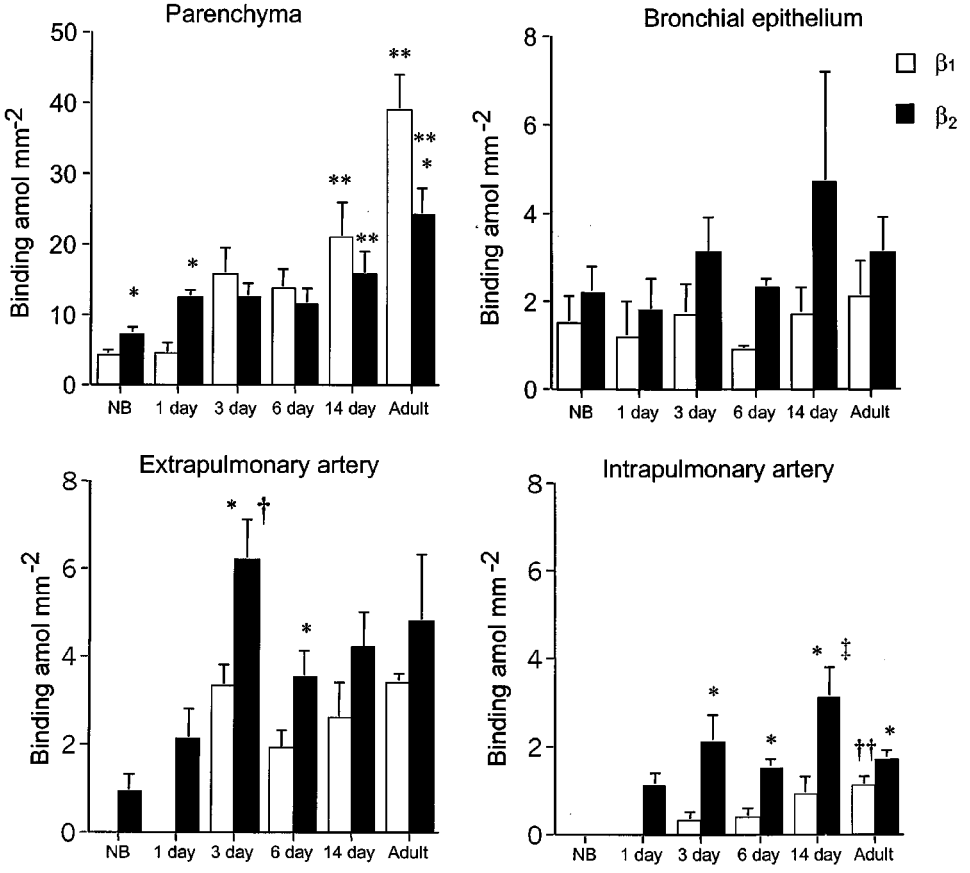

Figure 6 Mean density with standard error of [^{125}I]-ICYP binding of β_1- and β_2-receptor subtypes measured on autoradiographs of porcine lung sections in six age groups. Values for parenchyma, bronchial epithelium and endothelium of extra- and intrapulmonary arteries are shown. The number of animals in each age group is as in Figure 5. Significant differences after Students' *t*-test with Bonferronis correction. *$P<0.05$ β_1-compared with β_2-adrenoceptor; **$P<0.05$ compared with newborn; †$P<0.05$ compared with 1 day and 6 day; ††$P<0.04$ compared with 3 day; ‡$P<0.05$ compared with 1 day; NB, newborn.

arterial endothelial binding of both βAR subtypes. The expression of β_2ARs was greater than β_1ARs, consistent with results on cultured bovine endothelial cells (Ahmad *et al.*, 1990). Mak *et al.* (1996a) found mRNA for both subtypes in human pulmonary arteries. We found that the extrapulmonary arteries showed greater binding than the large intrapulmonary arteries (>500 μm) although the parenchyma showed the greatest binding density. This may be binding to innumerable small resistance pulmonary arteries. Our preliminary studies on isolated porcine pulmonary arteries

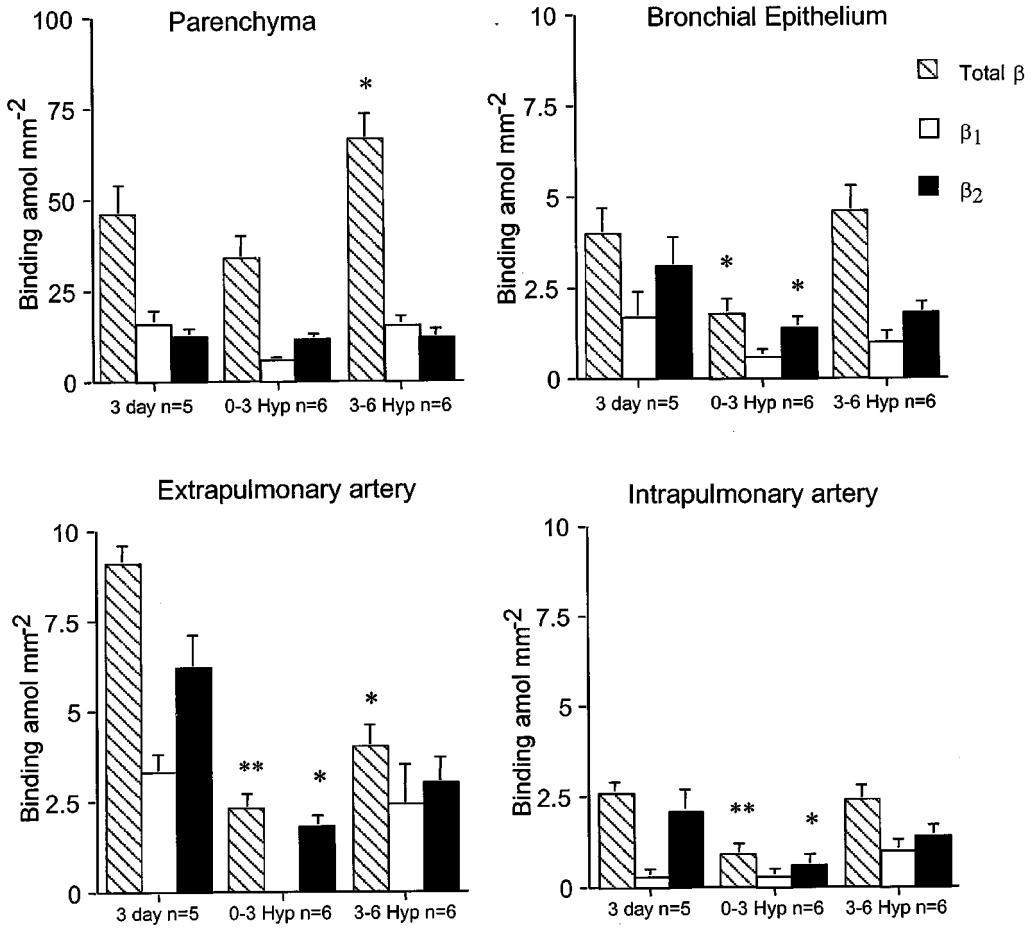

Figure 7 Mean density with standard error of [^{125}I]-ICYP binding of total β binding and the β_1- and β_2-receptor subtypes measured on autoradiographs of porcine lung sections from normal piglets aged 3 days and piglets exposed to hypoxia from birth to 3 days and from 3–6 days of age (Hyp). Values for parenchyma, bronchial epithelium and endothelium of extra- and intrapulmonary arteries are shown. Significant differences after Students' *t*-test with Bonferronis correction. *$P<0.05$ compared with 3 day; **$P<0.01$ compared with 3 day.

show a greater response to isoprenaline in resistance arteries than large intrapulmonary arteries. Studies in the adult rat showed a greater responsiveness to isoprenaline in small resistance arteries ($<300\ \mu$m) than in conduit arteries ($\sim1200\ \mu$m) (Priest *et al*., 1997). There may be a species difference.

Relaxation is generally thought to occur *via* β_2ARs but the relative importance of the β_1 and β_2AR subtype varies with species and structure (Guimaraes & Moura, 2001). In the developing porcine lung the intrapulmonary arteries had relatively few β_1ARs but they did increase with age. In adult rat pulmonary arteries β_1ARs are involved in isoprenaline induced relaxation particularly in the resistance vessels (Priest *et al*., 1997). Arteries of this size would be in the parenchyma of the porcine lung, where in the adult the majority of the receptors were β_1ARs. The relaxant effect of isoprenaline was absent in β_1AR knockout mice in both the femoral and pulmonary arteries (Chruscinski *et al*., 2001) while in aortic and carotid arteries it was only reduced.

Both β_1ARs and β_2ARs were on the endothelium rather than the smooth muscle cells of the porcine pulmonary arteries. This suggests that vasorelaxation may be *via* the action of an endothelium derived relaxant substance. In the large pulmonary arteries of the rat relaxation to isoprenaline *via* β_2ARs is reduced by the nitric oxide (NO) synthase inhibitor L-NMMA, suggesting adrenoceptor stimulated NO release from the endothelium (Priest *et al*., 1997). Isoprenaline increased the nitric oxide synthase and adenyl cyclase activity in human umbilical vein endothelial cells (Ferro *et al*., 1999). Canine pulmonary arteries demonstrate endothelium dependent vasodilatation to isoprenaline *via* the release of NO leading to an increase in cyclic GMP in addition to an endothelium independent increase in cyclic AMP (Yoshida *et al*., 1999). In adult rat pulmonary arteries it appears that ATP-sensitive K^+ channels contribute to the isoprenaline induced relaxation of the smooth muscle cells (Sheridan *et al*., 1997).

There has been relatively little work on βARs in the developing lung. In the present study we found that the affinity of the receptors for [^{125}I]-ICYP in the lung membranes was similar at all ages, however the number of βARs increased with age. Although the B_{max} for the β_2ARs also increased with age the proportion of β_2ARs decreased, suggesting a relative increase in β_1ARs. This was confirmed by the autoradiographic study on the lung parenchyma. In the few published developmental studies the findings varied

according to organ and species. The density of βAR increased in the rabbit heart between the newborn and adult (Schumacher *et al*., 1984), and increased in the rabbit lung after birth in both the bronchial smooth muscle and alveolar wall (Barnes *et al*., 1984). The βAR increased some time after birth in the rat lung, first in the pulmonary arteries and then in the airways (Schell *et al*., 1992). Previous studies on the porcine lung have shown an increase in density of sympathetic nerves throughout the lung during the first two weeks of life (Wharton *et al*., 1988). The presence of βARs on these nerves may help explain some of the increase in binding seen in the present study.

β-adrenoceptor binding increases rapidly after birth in the porcine lung, and the βARs may be particularly important in the newborn period, possibly influencing fluid dynamics and airway and arterial relaxation. As in previous studies, βAR binding was greatest on the parenchyma, particularly on the alveolar epithelial cells. Sodium transport across the alveolar walls increases after β-agonist stimulation leading to clearance of lung fluid (Minakata *et al*., 1998). βAR agonists are also involved in surfactant release and synthesis (Polak *et al*., 1992). The airway relaxant response of rat airways to βAR agonists increases with age (Melville *et al*., 1979), although no direct relationship could be found between the responsiveness to β-agonists and the number of βAR binding sites in rat and guinea-pig trachea (Preuss *et al*., 1999). Foetal pigs of 44–48 days of gestation show a bronchodilator response to β-agonists, suggesting that there are already βARs on the airway smooth muscle (Sparrow *et al*., 1994). This may be an indirect response since we found that the receptors were located on the epithelium rather than the airway smooth muscle. In the porcine pulmonary arteries the increase in βARs occurred rapidly, within the first 3 days of life. The density of other vasodilator receptors changed during the same time period after birth (Hislop *et al*., 1995; 1998a; b; Tulloh *et al*., 1997). Such changes in receptor expression may lead to, or result from, the postnatal increase in pulmonary blood flow.

The mechanism responsible for increasing βAR density after birth are unknown. βAR mRNA in lung membranes is higher at birth than at 3 days (Mak *et al*., 1996b), suggesting transcriptional regulation after birth possibly as a response to the postnatal increase in blood flow. Birth leads to an increase in circulating catecholamines and this might also be related to the increase in binding sites (Wang *et al*., 1999).

Glucocorticoids, which also increase at this time (Le Cozler *et al*., 1999), increase the number of βARs in developing rabbits (Barnes *et al*., 1984) and increase expression of β_2ARs in human peripheral lung *in vitro* (Mak *et al*., 1995). In adult rats dexamethasone increased the lung βAR mRNA rapidly and receptor density increased after 3 days (McGraw *et al*., 2000). Dexamethasone increased the density of β_2ARs on cultured bovine tracheal smooth muscle cells with an increase of G_s protein and a 1.8-fold increase in cyclic AMP production (Kalavantavanich & Schramm, 2000). Similarly studies on rat tracheal cell lines showed an increase in β_2AR and β_2AR-luciferase fusion gene expression in response to dexamethasone (Cao *et al*., 2000).

The effect of pulmonary hypertension on the appearance of the βARs was studied by exposure of piglets to hypobaric hypoxia from birth. There was no change in the affinity of receptor sites in lung membranes, but the number of binding sites after 3 days hypoxia was less than the normal aged 3 days and similar to the newborn lung suggesting a failure of the normal increase. The density of β_2ARs on the endothelium of the intrapulmonary arteries after 3 days hypoxia was reduced, suggesting that the ability to vasodilate may be impaired. A decrease in the number of endothelial endothelin-1, vasodilator receptors has also been described in hypoxia exposed piglets of this age (Noguchi *et al*., 1997) and the endothelium dependent response to acetylcholine is abolished (Tulloh *et al*., 1997). Hypoxic exposure from 3–6 days did not lead to a loss of βARs, either number or type, though endothelium dependent relaxation in response to acetylcholine and calcium ionophore are impaired (Tulloh *et al*., 1997). By contrast, acute hypoxia reduced relaxation to isoprenaline in adult rat pulmonary artery rings while there was no effect on acetylcholine or sodium nitroprusside induced relaxation (McIntyre *et al*., 1995). In adult rats, exposure to 10% oxygen for 28 days also reduced the relaxant response to isoprenaline although activation by forskolin was enhanced suggesting an upregulation of adenyl cyclase but a decrease in receptors (Priest *et al*., 1998). A decrease in βAR density was found after 7 days of hypoxic exposure in adult rats (Shaul *et al*., 1990).

Our results suggest that βARs have a functional role during early lung development. They are thought to act mainly as modulators of bronchoconstriction in the adult lung. In the newborn period in addition to this they may modulate the comparatively high level of hyper-reactivity of the pulmonary vessels characteristic of the transitional newborn lung. Both these effects are likely to be attenuated in neonatal pulmonary hypertension.

This work was funded by the British Heart Foundation and the Imperial College Initiative Award, U.K. to J.C.W. Mak.

References

AHMAD, S., CHRETIEN, P., DANIEL, E.E. & SHEN, S.H. (1990). Characterization of beta adrenoceptors on cultured endothelial cells by radioligand binding. *Life Sci.,* **47,** 2365–2370.

BARNES, P., JACOBS, M. & ROBERTS, J.M. (1984). Glucocorticoids preferentially increase foetal alveolar β- adrenoceptors: autoradiographic evidence. *Ped. Res.,* **18,** 1191–1194.

BARNES, P.J. (1995). Beta-adrenergic receptors and their regulation. *Am. J. Respir. Crit. Care Med.,* **152,** 838–860.

BOELS, P.J., DEUTSCH, J., GAO, B. & HAWORTH, S.G. (1999). Maturation of the bradykinin-response in resistance and conduit pulmonary arteries. *Cardiovasc. Res.,* **44,** 416–428.

CAO, W., MCGRAW, D.W., LEE, T.T., DICKER-BROWN, A., HILLER, F.C., CORNETT, L.E. & JONES, A.M. (2000). Expression of functional β_2-adrenergic receptors in a rat airway epithelial cell line (SPOC1) and cell density-dependent induction by glucocorticoids. *Exp. Lung Res.,* **26,** 421–435.

CARSTAIRS, J.R., NIMMO, A.J. & BARNES, P.J. (1985). Autoradiographic visualization of beta-adrenoceptor subtypes in human lung. *Am. Rev. Respir. Dis.,* **132,** 541–547.

CHRUSCINSKI, A., BREDE, M.E., MEINEL, L., LOHSE, M.J., KOBILKA, B.K. & HEIN, L. (2001). Differential distribution of β-adrenergic receptor subtypes in blood vessels of knockout mice lacking β_1- or β_2-adrenergic receptors. *Mol. Pharmacol.,* **60,** 955 – 962.

DAVIS, P.B., SILSKI, C.L., KERCSMAR, C.M. & INFELD, M. (1990). Beta-adrenergic receptors on human tracheal epithelial cells in primary culture. *Am. J. Physiol.,* **258,** C71 – C76.

FERRO, A., QUEEN, L.R., PRIEST, R.M., XU, B., RITTER, J.M., POSTON, L. & WARD, J.P. (1999). Activation of nitric oxide synthase by beta 2-adrenoceptors in human vein endothelium in vitro. *Br. J. Pharmacol.,* **126,** 1872 – 1880.

GABRIEL, M., HUNNEMAN, D.H. & GAHR, M. (1983). Plasma levels of catecholamine metabolites in the newborn period. *Biol. Neonate,* **44,** 203 – 209.

GOLDIE, R.G., PAPADIMITRIOU, J.M., PATERSON, J.W., RIGBY, P.J. & SPINA, D. (1986). Autoradiographic localization of β-adrenoceptors in pig lung using [^{125}1]-iodocyanopindolol. *Br. J. Pharmacol.,* **88,** 621 – 628.

GUIMARAES, S. & MOURA, D. (2001). Vascular adrenoceptors: an update. *Pharmacol. Rev.,* **53,** 319 – 356.

HAWORTH, S.G. & HISLOP, A.A. (1981). Adaptation of the pulmonary circulation to extra-uterine life in the pig and its relevance to the human infant. *Cardiovasc. Res.,* **15,** 108 – 119.

HAWORTH, S.G. & HISLOP, A.A. (1982). Effect of hypoxia on adaptation of the pulmonary circulation to extra-uterine life in the pig. *Cardiovasc. Res.,* **16,** 293 – 303.

HISLOP, A.A., BOELS, P.J., DEUTSCH, J., POLAK, J.M. & HAWORTH, S.G. (1998a). Developmental changes in binding sites and reactivity for CGRP and VIP in porcine pulmonary arteries. *Peptides,* **19,** 703 – 714.

HISLOP, A.A., MAK, J.C.W., READER, J.A., BARNES, P.J. & HAWORTH, S.G. (1998b). Muscarinic receptor subtypes in the porcine lung during postnatal development. *Eur. J. Pharmacol.,* **359,** 211 – 221.

HISLOP, A.A., ZHAO, Y.D., SPRINGALL, D.R., POLAK, J.M. & HAWORTH, S.G. (1995). Postnatal changes in endothelin-1 binding in porcine pulmonary vessels and airways. *Am. J. Respir. Cell Mol. Biol.,* **12,** 557 – 566.

JOHNSON, M. (1998). The β-Adrenoceptor. *Am. J. Respir. Crit. Care Med.,* **158,** S146 – S153.

KALAVANTAVANICH, K. & SCHRAMM, C.M. (2000). Dexamethasone potentiates high-affinity beta-agonist binding and g(s)alpha protein expression in airway smooth muscle. *Am. J. Physiol.,* **278,** L1101 – L1106.

KALLIO, J., KARLSSON, R., TOPPARI, J., HELMINEN, T., SCHEININ, M. & KERO, P. (1998). Antenatal dexamethasone treatment decreases plasma catecholamine levels in preterm infants. *Ped. Res.,* **43,** 801 – 807.

LE COZLER, Y., BEAUMAL, V., NEIL, M., DAVID, C. & DOURMAD, J.Y. (1999). Changes in the concentrations of glucose, non-esterified fatty acids, urea, insulin, cortisol and some mineral elements in the plasma of the primiparous sow before, during and after induced parturition. *Reprod. Nutr. Dev.,* **39,** 161 – 169.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265 – 275.

MAK, J.C., NISHIKAWA, M. & BARNES, P.J. (1995). Glucocorticosteroids increase beta 2-adrenergic receptor transcription in human lung. *Am. J. Physiol.,* **268,** L41 – L46.

MAK, J.C., NISHIKAWA, M., HADDAD, E.B., KWON, O.J., HIRST, S.J., TWORT, C.H. & BARNES, P.J. (1996a). Localisation and expression of beta-adrenoceptor subtype mRNAs human lung. *Eur. J. Pharmacol.,* **302,** 215 – 221.

MAK, J.C.W., ROFFEL, A.F., HISLOP, A.A., READER, J.A., HAWORTH, S.G. & BARNES, P.J. (1996b). $Beta_2$ adrenergic receptor expression in developing porcine lung: correlation with mRNA levels. *Am. J. Respir. Crit. Care Med.,* **153,** A233.

MATSUSHITA, T., HISLOP, A.A., BOELS, P.J., DEUTSCH, J. & HAWORTH, S.G. (1999). Changes in ANP responsiveness of normal and hypertensive porcine intrapulmonary arteries during maturation. *Ped. Res.,* **46,** 411 – 418.

MCGRAW, D.W., FORBES, S.L., MAK, J.C.W., WITTE, D.P., CARRIGAN, P.E., LEIKAUF, G.D. & LIGGETT, S.B. (2000). Transgenic overexpression of beta 2-adrenergic receptors in airway epithelial cells decreases bronchoconstriction. *Am. J. Physiol.,* **279,** L379 – L389.

MCNEEL, R.L. & MERSMANN, H.J. (1999). Distribution and quantification of beta1-, beta2-, and beta3-adrenergic receptor subtype transcripts in porcine tissues. *J. Anim. Sci.,* **77,** 611 – 621.

MCINTYRE, JR, R.C., BANERJEE, A., HAHN, A.R., AGRAFOJO, J. & FULLERTON, D.A. (1995). Selective inhibition of cyclic adenosine monophosphate-mediated pulmonary vasodilation by acute hypoxia. *Surgery,* **117,** 3-4-318.

MELVILLE, G.N., IRAVANI, J. & MINNIS, J. (1979). Airway response to adrenergic agonists and antagonists in 3 day to 2 year old rats. *Respiration,* **38,** 74 – 80.

MINAKATA, Y., SUZUKI, S., GRYGORCZYKM, C., DAGENAIS, A. & BERTHIAUME, Y. (1998). Impact of β-adrenergic agonist on Na^+ channel and Na^+-K^+-ATPase expression in alveolar type II cells. *Am. J. Physiol.,* **275,** L414 – L422.

NOGUCHI, Y., HISLOP, A.A. & HAWORTH, S.G. (1997). Influence of hypoxia on endothelin-1 binding sites in neonatal porcine pulmonary vasculature. *Am. J. Physiol.,* **272,** H669 – H678.

POLAK, J.M., KNIGHT, M.E., ANDRESEN, T.L. & DESENA, C. (1992). Effects of hyperoxia and beta-adrenergic stimulation on pulmonary surfactant in neonatal rabbits. *Exp. Lung Res.,* **18,** 373 – 384.

PREUSS, J.M.H., RIGBY, P.J. & GOLDIE, R.G. (1999). The influence of animal age on β-adrenoceptor density and function in tracheal airway smooth muscle. *Naunyn-Schmiedebergs-Arch-Pharmacol.,* **360,** 171 – 178.

PRIEST, R.M., HUCKS, D. & WARD, J.P. (1997). Noradrenaline, beta-adrenoceptors mediated vasorelaxation and nitric oxide in large and small pulmonary arteries of the rat. *Br. J. Pharmacol.,* **122,** 1375 – 1384.

PRIEST, R.M., ROBERTSON, T.P., LEACH, R.M. & WARD, J.P. (1998). Membrane potential-dependent and -independent vasodilation in small pulmonary arteries from chroniclly hypoxic rats. *J. Pharmacol. Exp. Ther.,* **285,** 975 – 982.

SCHELL, D.N., DURHAM, D., MURPHREE, S.S., MUNTZ, K.H. & SHAUL, P.W. (1992). Ontogeny of beta-adrenergic receptors in pulmonary arterial smooth muscle, bronchial smooth muscle, and alveolar lining cells in the rat. *Am. J. Respir. Cell Mol. Biol.,* **7,** 317 – 324.

SCHUMACHER, W., MIRKIN, B.L. & SHEPPARD, J.R. (1984). Biological maturation and beta-adrenergic effectors:development beta-adrenergic receptors in rabbit heart. *Mol. Cell Biochem.,* **58,** 173 – 181.

SHAUL, P.W., MUNTZ, K.H., DEBELTZ, D. & BUJA, L.M. (1990). Effects of prolonged hypoxia on adenylate cyclase activity and beta-adrenergic receptors in pulmonary and systemic arteries of the rat. *Circ. Res.,* **66,** 1526 – 1534.

SHERIDAN, B.C., MCINTYRE, JRR, C., MELDRUM, D.R. & FULLERTON, D.A. (1997). K_{ATP} channels contribute to beta- and adenosine receptor-mediated pulmonary vasorelaxation. *Am. J. Physiol.,* **273,** L950 – L956.

SPARROW, M.P., WARWICK, S.P. & MITCHELL, H.W. (1994). Foetal airway motor tone of prenatal lung development of the pig. *Eur. Respir. J.,* **7,** 1416 – 1424.

TULLOH, R.M.R., HISLOP, A.A., BOELS, P.J., DEUTSCH, J. & HAWORTH, S.G. (1997). Chronic hypoxia inhibits postnatal maturation of porcine intrapulmonary artery relaxation. *Am. J. Physiol.,* **272,** H2436 – H2445.

WANG, L., ZHANG, W. & ZHAO, Y. (1999). The study of maternal and fetal plasma catecholamines levels during pregnancy and delivery. *J. Perinat. Med.,* **27,** 195 – 198.

WHARTON, J., HAWORTH, S.G. & POLAK, J.M. (1988). Postnatal development of the innervation and paraganglia in the porcine pulmonary arterial bed. *J. Pathol.,* **154,** 19 – 27.

YOSHIDA, K., FLAVAHAN, N.A., HORIBE, M., SMEDIRA, N.G. & MURRAY, P.A. (1999). Endothelial defect mediates attenuated vasorelaxant response to isoproterenol after lung transplantation. *Am. J. Physiol.,* **276,** H159 – H166.

(Received October 22, 2001
Revised December 27, 2001
Accepted January 7, 2002)