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# Pulmonary endothelium dependent vasodilation emerges after birth in mice

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**Abstract**

At birth, with the first breath, pulmonary vessels undergo profound adaptive processes. A failure in the ability of pulmonary vessels to adapt at birth results in persistent pulmonary hypertension of the new born. The mechanisms regulating pulmonary adaptation at birth are still unclear. Progress in this area is slow, not least because genetically modified mice have not yet been used to address questions in this area of research, principally because pulmonary vessels in new born mice are very small making the study of dilator response *in vitro* difficult. In the current study we have used precision cut lung slices to characterise the functional vasomotor changes in lung vessels of new born mice (1–4 days old), 8–15 day old mice or adult mice. The internal luminal area of pulmonary artery and airways was measured digitally. Vasoconstriction and vasodilatation were expressed as the percentage change in internal luminal area compared with the control area. The thromboxane $A_2$ mimetic U-46619 ($3\times10^{-7}$ M) caused a significant vasoconstriction in vessels of all groups. However, acetylcholine ($3\times10^{-5}$ M) induced arterial dilation only in the 8–15 days, and adult groups. By contrast, sodium nitroprusside, which acts independently of the endothelium, was an effective vasodilator in lung vessels from neonates. These data are the first to characterise the development of endothelium dependent vasodilatation in lung after birth in mice. This approach can be used with genetically modified mice, which is important to further our understanding of the physiology in this area.


*Keywords:* Neonate; Endothelium and pulmonary artery

## 1. Introduction

During fetal life, pulmonary vascular resistance is high causing the pulmonary circulation to receive only 5 to 10% of cardiac output; consequently, most of the oxygenated blood of the right ventricle goes to the aorta *via* the *ductus arteriosus*. Structurally the arteries of man and mice are relatively thick walled as they develop and grow *in utero* (Hall et al., 2000; Stanford et al., 2005). As the gestation advances, pulmonary artery pressure and blood flow increase gradually. The increase in pulmonary vascular tonus occurs mainly at the end of the gestation and seems to be modulated by low oxygen tension, low baseline production of vasodilators such as prostacyclin and nitric oxide (NO), increase in production of vasoconstrictors such as endothelin-1 and leukotrienes, and altered reaction of smooth muscle cells's myogenic tone (Abman and Stenmark, 1992; Ivy et al., 2004). The mechanisms that contribute to alterations in pulmonary vascular response during development are still unknown. We do know however, that at birth and with the first breath, profound changes occur in the airways and vessels of the lung and post-uterine pulmonary development continues from that. In pigs (Arrigoni et al., 1999) and sheep (Gao et al., 1995) the pulmonary blood vessels acquire the ability to release vasodilator hormones *post partum*. In line with this, levels of vasoactive enzymes, including nitric oxide synthase (Hislop et al., 1995; Arrigoni et al., 2002), cyclo-oxygenase (Brannon et al., 1994; Jun et al., 1998, 1999) and heam-oxygenase-1 (Stanford et al., 2005) are increasingly expressed in pulmonary endothelium after birth. If these processes fail the pulmonary vasculature remains in an immature, fetal, state and persistent pulmonary hypertension of the new born ensues (Rosenzweig et al., 2004).

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The basic physiological mechanisms which regulate pulmonary adaptation at birth are still unclear. Importantly, because the vessels are so small, it has not previously been technically possible to study how vasoactive pathways mature in pulmonary vessels in the mouse at birth. We have therefore not been able to take advantage of cutting edge technology and genetically modified animals in our research of this area using *in vitro* approaches. We have recently shown that remodelling of pulmonary vessels in mice continues for at least 5 days after birth (Stanford et al., 2005), although we were not able (for the reasons above) to monitor changes in vasomotor response in new born mice. In the current study we have used precision cut lung slices viewed using image capture technology to study how the vasomotor function of pulmonary vessels change after birth. The technology we describe has been used previously to study responses in adult rat lungs (Parrish et al., 1995; Martin et al., 1996, 2000; Moreno et al., 2006), but has not been applied to new born responses. This approach also allows for lung tissue to be maintained in culture for up to 3 days, providing an experimental window of opportunity for gene manipulation *in vitro*. The information presented here will allow us to utilise new technologies, including genetically modified mice to further understand how pulmonary adaptation at birth is regulated and how those systems may fail.

## 2. Materials and methods

### 2.1. Preparation of lung slices

New born mice (ranging from 1–4 days old), mice at 8–15 days old or adult mice were humanely sacrificed with $CO_2$ in accordance with The European Community guidelines for the use of experimental animals. The trachea was cannulated and the animals were exsanguinated by abdominal aorta rupture. A small vertical cut into the diaphragm was performed to induce lung collapse and instillation of 1 ml of 2% w/v agarose (Type IXA, low melting point agarose) solution into the airways. After allowing the agarose gel to set at 4 °C, lobes were separated and embedded externally in agarose using a tissue embedding unit (TSE systems). Tissue slices (250 μm) were then prepared using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA). Slices were incubated overnight on a rotating platform (1 r.p.m.) housed in a humidified incubator (37 °C, 5% $CO_2$− 95% air) in 12 well plates containing 1 ml of Dulbecco's modified Eagle's Medium (DMEM) medium supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin and 4 mM L-glutamine and 2.5 μg/ml amphotericin B. Solution were changed every hour during the first 4 h as previuosly described (Moreno et al., 2006).

### 2.2. Image acquisition

Incubation and observation of slices was carried on in a warmed (37 °C) incubator chamber (PCLS-Bath Type 847, Hugo Sachs elektronik, Harvard Apparatus). The internal luminal area of the pulmonary arteries and accompanying airways were visualized using a video camera mounted on a microscope (Nikon SMZ-U). The internal luminal area of the pulmonary artery or airway before addition of the thromboxane analogue 9,11-Dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46619) was defined as 100%. Vasoconstriction and vasodilatation were expressed as the percentage decrease or increase in internal luminal area. Acetylcholine was used to determine endothelium dependent dilation and sodium nitroprusside was used to determine endothelium-independent responses.

To distinguish arteries from veins, we used criteria similar to those described previously by Shi et al. (1997): 1) The arteries usually accompanied airways, whereas veins were at a distance from them, and 2) arterial walls had a thick media and their inner lining was slightly wrinkled, whereas veins were thinner and wrinkles were inconspicuous (Fig. 1; shows artery and airway).

### 2.3. Experimental design

After preincubation for 5 min with 0.5 ml of Dulbecco's modified Eagle's Medium (DMEM), the first image was captured ("baseline image"). Then, the liquid was removed and fresh medium added containing an estimated $EC_{80}$ concentration of U46619, ($3\times10^{-7}$ M) (Held et al., 1999; Martin et al., 2000). The slice image was recorded every 15 s for 6 min. Cumulative addition of acetylcholine ($10^{-6}$–$3\times10^{-5}$) was used to determine endothelium dependent dilation and sodium nitroprusside ($10^{-5}$ M) was used to determine smooth muscle responsiveness independent of the endothelium at the end of the protocol. All drugs were purchased from Sigma Gilligham, Dorset, UK. Acetylcholine and sodium nitroprusside were freshly prepared

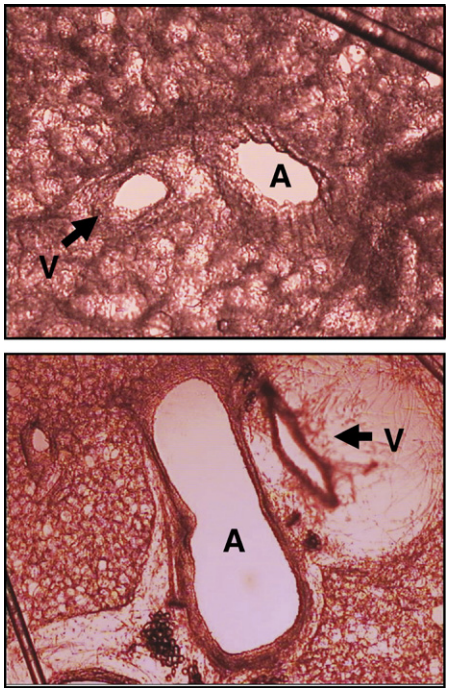


Fig. 1. Gross morphology of lung slices. The upper panel shows tissue from a new born (1 day old mouse) and the lower panel from an adult mouse. Slides are representative of sections studied from the animals in each studied group. Original magnifications, ×75 (upper panel) and ×37.5 (lower panel). A, airway and V, pulmonary artery.

each day in aqueous and ethanol solutions, respectively. U46619 was prepared in high concentration "stock" solution dissolved in ethanol and was stored at −80 °C until used.

### 2.4. Image analysis

The images were captured and analysed using an image analysis program (ZEISS KS 300 3.0, from Image Associates, UK). The luminal area was taken from the limit between endothelial luminal borders and was quantified after setting the appropriate threshold value.

Baseline measurements were defined as 100%. Vasoconstriction and vasodilatation were expressed as the percentage of internal luminal area (ILA) compared with the control measurement using the following equation: Response = ILA after drug/baseline ILA × 100. Thus a 0% response indicated complete luminal closure and 100% indicated no effect.

### 2.5. Analysis of data

The data represents the mean ± S.E.M.for measurements made from six animals for each age group. The starting internal luminal area was taken as 100% and responses to constrictors or dilators calculated as a percentage of this. For responses to U46619 data was compared to baseline measurements using the one-sample *t*-test for normalised data. For response to acetylcholine added in the presence of U46619, or sodium nitroprusside, added in the presence of acetylcholine, data was compared by one-way analysis of variance with Bonferroni's *post-hoc* test for multiple comparisons and differences between groups were considered significant when $P<0.05$.

## 3. Results

### 3.1. Gross morphology of lung slices from newborn and adult mice

Lung slices from new born mice displayed the predicted 'immature' phenotype where arteries were thick walled with narrow lumena and the airspaces were saccules rather than alveoli and appeared thicker walled (Fig. 1). Sections from adult lungs displayed the predicted phenotype with thin walled small alveoli and arteries with relatively large lumena (Fig. 1).

### 3.2. Changing response with age of the pulmonary arteries to U46619, acetylcholine and sodium nitroprusside

The thromboxane mimetic U46619 ($3\times10^{-7}$ M) contracted the pulmonary artery in lung slices from each age group of mice (contracted to 46.31 ± 3.0%, 1–4 days; 41.82 ± 2.2%, 8–15 days; 58.64 ± 2.3%, adult). In lung slices from 8–15 day old mice or from adult mice the cumulative addition of acetylcholine ($10^{-6}$–$3\times10^{-5}$), induced a concentration dependent vasodilator response in tissue precontracted with U46619 (Fig. 2). By contrast, in lung slices from new born mice (1–4 days), acetylcholine did not induce vasodilatation at any concentration tested (Fig. 2). In lung slices from mice of all age groups 100% vasodilatation was achieved in U46619 precontracted vessels when the endothelium-independent dilator, sodium nitroprusside ($10^{-5}$ M) was added to the tissue (Fig. 2).

### 3.3. Responses of airways after birth to U46619 and acetylcholine

Similar to the observations made with pulmonary vessels, U46619 ($3\times10^{-7}$ M) contracted airways in lung slices from each age group of mouse (contracted by 67.93 ± 2.0%, 1–4 day old mice; 51.12 ± 4.7%, 8–15 day old mice; 53.17 ± 2.1%, adult mice). By contrast to results observed in pulmonary vessels, and

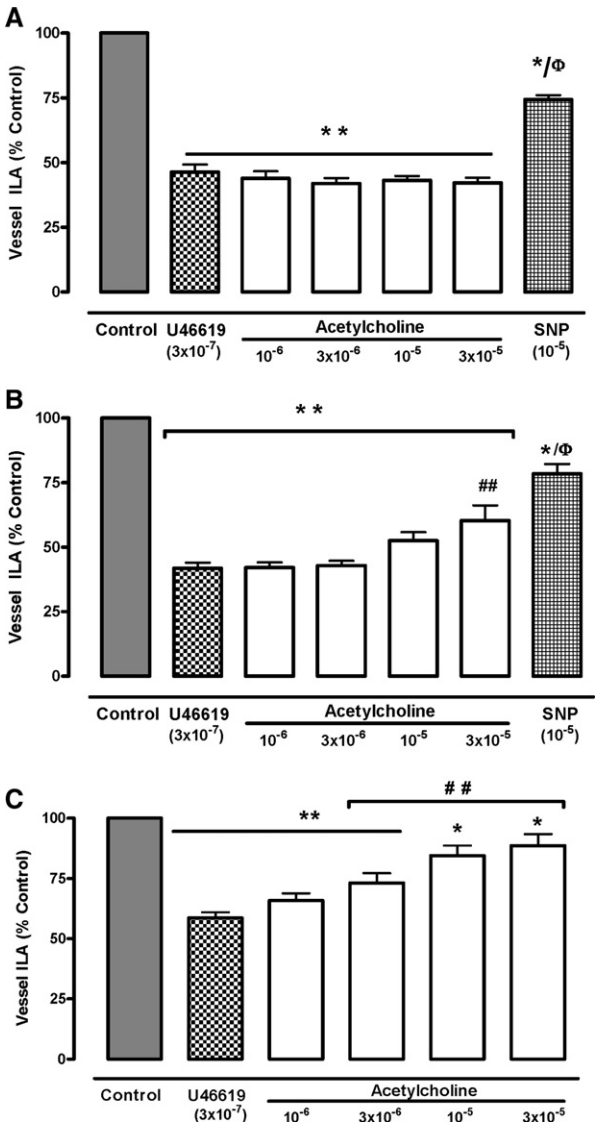


Fig. 2. Effect of U46619, acetylcholine (ACh) and sodium nitroprusside (SNP) on the internal luminal area (ILA) of pulmonary vessels in tissue from new born mice ranging from 1–4 days after birth (panel A), from 8–15 days after birth (panel B) and adult mice (panel C). Data where expressed in % ± S.E.M., of baseline area in DMEM (Control) ($n=6$–7). Significant differences are denoted by * and ** where $P<0.05$ or $P<0.01$, respectively, when compared with Control, $^{\#}$ and $\Phi$ where $P<0.05$ when compared to responses in the presence of U46619 and acetylcholine respectively.

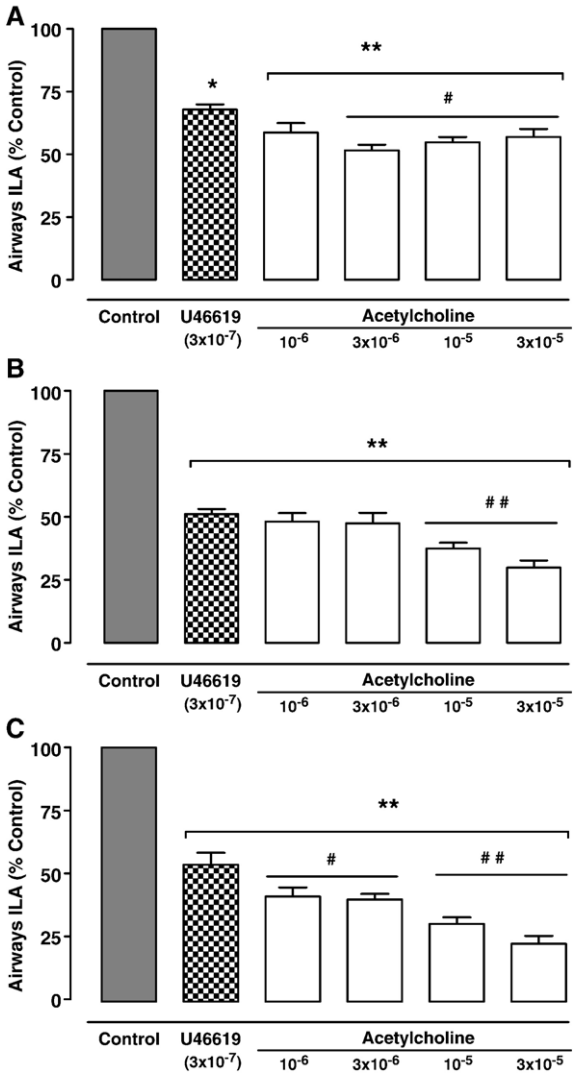


Fig. 3. Effect of U46619 and acetylcholine (ACh) on the internal luminal area (ILA) of airways in tissue from new born mice ranging from 1–4 days after birth (panel A), from 8–15 days after birth (panel B) and adult mice (panel C). Data where expressed in %±S.E.M.of baseline area (n=6). Significant differences are denoted by * and ** where $P<0.05$ or $P<0.01$, respectively, when compared with Control, and # and ## where $P<0.05$ or $P<0.01$ when compared to responses in the presence of U46619 and acetylcholine respectively.

as expected acetylcholine ($10^{-6}$ to $3\times10^{-5}$ M) did not dilate airways, but instead, further contracted them, in tissue from all age groups of mice (Fig. 3).

## 4. Discussion

The endothelium, which lines every blood vessel, releases a number of vasoactive mediators, including the dilator gas NO, on demand. However, in the lung, the endothelium and blood vessels continue to develop at and after birth in a number of mammals including man. Importantly, mammals are not generally born with a fully functional endothelium in pulmonary vessels. Instead, over the first few days and weeks of breathing air the synthetic properties of the endothelium mature. If these normal physiological processes fail the pulmonary vessels remain immature and pulmonary hypertension occurs (Hislop, 2002). This process has been documented in man, sheep, pigs and rats, but we still are very unclear about what cellular and molecular events that regulate this physiological process (Gao et al., 1995; Arrigoni et al., 2002; Hislop, 2002). The adaptation of the endothelium at birth has been difficult to study functionally in the mouse because the vessels are so small that conventional bioassay approaches are inadequate. Here we report, for the first time, how responses can be studied in precision cut lung slices with imaging technology and that the vasodilator properties of the endothelium mature in the mouse lung, as they do in other species.

We found that vessels and airways in the lung contracted to the thromboxane mimetic, U46619, in tissue from all age groups of mice. This suggests that thromboxane receptors (TP) and related downstream pathways are intact at birth. This observation in mice is consistent with our previous work in piglets published by Arrigoni et al. (1999). In the current study with mice, we found that in precontracted lung vessels, acetylcholine induced vasodilator responses in adult lung slices and in those from 8–15 day old animals, but not in tissue from the new born age group. Acetylcholine is an endothelium dependent dilator agent (Furchgott and Zawadzki, 1980). In the absence of a functional endothelium acetylcholine can either have no effect, or produce small contractions of a vessel. Our observations here then, where acetylcholine is causing vasodilatation, can only mean that the secretory function of the endothelium is in place by 8 days. We know that our observation, that there is no relaxation at 1–4 days cannot be explained by any structural immaturity in the underlying vascular smooth muscle because the endothelium-independent dilator, sodium nitroprusside, fully reversed the contraction induced by U46619 in vessels from newborn mice. We cannot conclude definitely, however, which hormone is responsible for the dilation we see. Endothelium dependent dilation in response to acetylcholine can be mediated by NO, prostacyclin or endothelial derived hyperpolarising factor (EDHF), or a mixture of these three pathways. The nature of the dilator response in mouse vessels will be the subject of subsequent manuscripts.

Interestingly, we also noted that, using precision cut lung slices, we could monitor changes in airway response simultaneously with those in the vessels. We found that, similarly to the vascular responses, airways contracted to U46619. However, by contrast to the blood vessel, as predicted acetylcholine did not dilate airways, instead it caused a further bronchoconstrictor response. Acetylcholine is a classical muscarinic agonist which induces bronchoconstriction by an action on m2 and m3 muscarinic receptors. The ability of acetylcholine to constrict airway, but dilate the adjoining vessel is a useful confirmation marker of correct identification of the structures.

In conclusion then, we have used precision cut lung slices viewed using image capture technology to study how the vasomotor function of pulmonary vessels change after birth. The technology we describe also allows for lung tissue to be maintained in culture for up to three days, providing an experimental window of opportunity for gene manipulation *in vitro*. The information presented here will allow our group and others to utilise new technologies, including genetically modified mice to further understand how pulmonary adaptation at birth is regulated and how those systems may fail.

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