British Journal of Pharmacology (1998) 123, 712–718

Transition of functional innervation in the developing porcine airway from nitrergic to catecholaminergic

D.R. Connellan & [1]H.W. Mitchell

Department of Physiology, University of Western Australia, Nedlands, Perth, Australia 6907

1 We determined the distribution and chemical nature of the inhibitory neurotransmitter(s) to the airway smooth muscle (ASM) before and after birth.

2 Relaxation responses to electrical field stimulation (EFS) were studied in isovolumic bronchial segments from foetal (approximately 100/115 days gestation) and adult (25 kg) pigs, and in isovolumic tracheal segments from the foetus, and tracheal smooth muscle strips from the adult pig. Preparations were conditioned in low doses of atropine (10^{-7} M) to reduce the effects of excitatory neurotransmission and then exposed to carbachol to produce submaximal muscle tone. Some studies were also carried out on bronchial segments from 4 week old pigs.

3 EFS (65 V, 2 ms, 5–20 Hz for 5 s) produced a TTX-sensitive relaxation in epithelium-intact and epithelium-denuded preparations. In foetal bronchial and tracheal preparations, EFS-induced relaxation was strongly inhibited by N^G-nitro-L-arginine (L-NOARG, 10^{-6} to 10^{-4} M; $P<0.01-0.001$). However, in the adult, only relaxations of the trachea were inhibited by L-NOARG; bronchi were resistant to L-NOARG and also to N-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M). The inhibitory actions of L-NOARG (10^{-6} to 10^{-4} M) were substantially reversed by 10^{-2} M L-arginine. Experiments with bronchial segments from 4 week old pigs showed partial inhibition of relaxations by L-NOARG.

4 The L-NOARG-insensitive relaxations recorded in the adult bronchus were blocked by propranolol (10^{-6} M).

5 The onset of relaxation to EFS was more prompt and the rate of relaxation more rapid in foetal bronchi than in adult bronchi ($P<0.0005$). Maximum relaxation and recovery times were the same.

6 Foetal and adult bronchi were relaxed by sodium nitroprusside (SNP) with similar sensitivity and maximum effect. The rate of relaxation to SNP was not different in the two ages.

7 In the absence of atropine and carbachol, excitatory cholinergic responses to EFS (65 V, 2 ms, 5 Hz for 20 s) were not altered by L-NOARG (10^{-4} M) or L-NAME (10^{-4} M) in the adult bronchus but were modestly increased by L-NOARG in the foetal bronchus ($P<0.01$).

8 The tracheobronchial tree appears functionally innervated by nitrergic input to the smooth muscle before birth. However, at or after 4 weeks of age the inhibitory neural input to the bronchi is catecholaminergic, but it remains nitrergic in the trachea. There is also a weak nitrergic pre- or postsynaptic inhibition of the effects of cholinergic neurotransmission in the foetal bronchus but not in the adult.

Keywords: Nitric oxide; tracheobronchial tree; development; inhibitory non-adrenergic non-cholinergic (NANC); airway smooth muscle

Introduction

The foetal tracheobronchial tree possesses a dense neural network supplying airway smooth muscle (ASM) from early in the first trimester (Sparrow *et al*., 1995). Rhythmic, myogenic contraction/relaxation phases are present in both human and porcine foetal bronchus causing the movement of foetal lung fluid (McCray, 1993; Sparrow *et al*., 1995). Foetal ASM contracts to acetylcholine, histamine, substance P and K^+ depolarizing solution suggesting that the cellular and molecular apparatus required for airway control is functional from early in development (Booth *et al*., 1992; Sparrow *et al*., 1994). Electrical field stimulation (EFS) of the mural nerves to the tracheobronchial tree of pre- and post-natal pigs produces a contraction followed by relaxation. The contractions, which are tetrodotoxin (TTX)-sensitive, are blocked by atropine indicating the presence of cholinergic neurotransmission before and after birth.

The inhibitory transmitter to the porcine adult trachea is non-catecholaminergic (Leff *et al*., 1985) and may be mediated by nitric oxide (NO) (Kannan & Johnson, 1992). However, the composition of the relaxant neurotransmitter to the adult bronchus or to the foetal airway is unknown. Propranolol has no effect on the inhibitory neural responses to the tracheobronchial tree of the foetal pig and only a partial antagonistic effect on central bronchi in the adult (Mitchell *et al*., 1990). As some neural and motor responses of the lung show maturation around birth or during the neonatal period (Waldron *et al*., 1989; Sparrow & Mitchell, 1990; Haxhiu-Proskurica *et al*., 1992; 1993), it is possible that the nerves conferring the strong inhibitory control over the tracheobronchial tree also differ with development.

Studies, primarily with NOsynthase (NOS) inhibitors, suggest that NO is an inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter to the tracheobronchial tree smooth muscle in several postnatal species (Brave *et al*., 1991; Li & Rand, 1991; Kannan & Johnson, 1992; Sekizawa *et al*., 1993; Lei *et al*., 1993; Gao & Vanhoutte, 1993a; Yu *et al*., 1994; Kondo *et al*., 1995; Takahashi *et al*., 1995), and also in adult human airways (Belvisi *et al*., 1992; Bai & Bramley, 1993; Fernandes *et al*., 1994; Ward *et al*., 1995b). NO also modulates

[1] Author for correspondence.

synaptic transmission (Scott & Bennett, 1993), and acetylcholine (ACh) release in rat trachea (Sekizawa *et al*., 1993) and reduces cholinergic and substance P-like neurotransmission in guinea-pig ileum (Gustafsson *et al*., 1990; Wiklund *et al*., 1993).

The purpose of our study was firstly to determine the chemical nature of the inhibitory neurotransmitter to the airways before and after birth. The second aim was to determine whether the inhibitory neurotransmitter is the same in the bronchi and trachea. Neurotransmission was produced by field stimulation of the mural nerves, which causes non-discriminatory release from pre- and postsynaptic nerves. For the most part we studied responses of the smooth muscle *in situ* in segments of airway, in order to maintain much of the architecture of the airway and the neural plexus within.

Methods

Tissue and apparatus

Airways from immature (>900 g foetuses, approximately 100/115 days gestation) and mature (25 kg, approximately 10 weeks, 'adult pig') Landrace pigs were investigated. A limited number of experiments was also carried out on suckling pigs (4 weeks). Postnatal pigs were killed with a humane killer and exsanguinated. Foetal pigs were obtained from the local abattoir. The lungs and trachea were rapidly removed and placed on ice for transport back to the laboratory.

A length of segmental bronchus was removed from the lung by dissecting away the parenchyma and tightly ligating the side branches. In the foetus we used bronchi from a lower lobe and in the adult from either an upper or lower lobe. The bronchial segments were 2.5 cm long – in the adult the i.d. at the distal end was 2.5 mm and it was 1.5 mm in the foetus. Segments spanned a similar region of the bronchial tree in either age group (divisions ~2–10). Tracheal segments, 2.5 cm length, were taken from the caudal region of the foetal trachea. Bronchial and tracheal segments were cannulated at each end and mounted horizontally in a warmed (37°C) bath containing Krebs solution (in mM: NaCl 121, KCl 5.4, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, 3-[N-morpholino]propane-sulphonic acid 5.0, glucose 11.5 and $CaCl_2.2H_2O$ 2.5 gassed with carbogen (5% CO_2 in oxygen)). A pH of 7.2 was obtained with the addition of NaOH (1 M). The lumen was flushed with Krebs solution and the resting transmural pressure was 5 cmH_2O. The adventitia of the airway was also bathed in this solution. Three-way taps were attached to the cannulae at each end of the segment to enable the lumen to be sealed. Changes in lumen pressure in the isovolumic segments were detected with a pressure transducer (Motorola MPX2010GP) and the output was displayed on a chart recorder.

The trachea from adult pigs was too large to mount in this apparatus therefore smooth muscle responses were recorded from transverse strips, 1 × 15 mm, of trachealis muscle and mucosa. Strips were mounted in an organ bath by silk threads at each end. One end was fixed in the bath whilst the other was connected to a force transducer (Ugo Basile Biological Instruments, Varessa, Italy). A passive tension of 1 g was used.

The bronchial and tracheal preparations were first left to equilibrate for 45 min with changes in Krebs solution every 15 min. The tissue was stimulated with acetylcholine to ascertain maximum force generation (ACh; 10^{-2} M (E_{max})). The preparations were electrically field stimulated (EFS; 65 V at source, 2 ms, 5–20 Hz) with a GRASS S44 stimulator via platinum ring electrodes which encircled the preparation.

EFS-induced relaxation

We compared relaxation-responses to electrical field stimulation in bronchial and tracheal preparations from the different aged pigs. Excitatory cholinergic responses to EFS were first abolished by atropine (20 min incubation with 10^{-7} M), the tissue was then washed three times at 10 min intervals. Carbachol (CCh) was added to the bath, to provide active tone, until the preparation contracted to 50% of the maximal contraction to ACh obtained in each tissue (EC_{50}). This required a high dose of CCh (10^{-5} M) to overcome the competitive blockade. Relaxations produced upon EFS were typically stable and repeatable (Figure 1), for at least 1 h. The induced tone was also stable for the duration of the experiment in more than three-quarters of the preparations. In the remainder there was a small rise in tone of some 15%, but without a re-emergence of excitatory responses to EFS.

We investigated the effects of NOS inhibitors N^G-nitro-L-arginine (L-NOARG, 10^{-6}–10^{-4} M) and N-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) on non-adrenergic relaxations evoked by EFS. L-Arginine (10^{-2} M) was used to reverse the effect of the NOS inhibitors. Two protocols were followed to investigate the EFS-induced relaxations. Firstly, airway preparations were stimulated at 2 min intervals (5 s trains of stimulation, 65 V, 2 ms, 10 Hz). After at least 3 stable relaxation-responses had been recorded (control responses) a NOS inhibitor was added to the bath. EFS was continued at

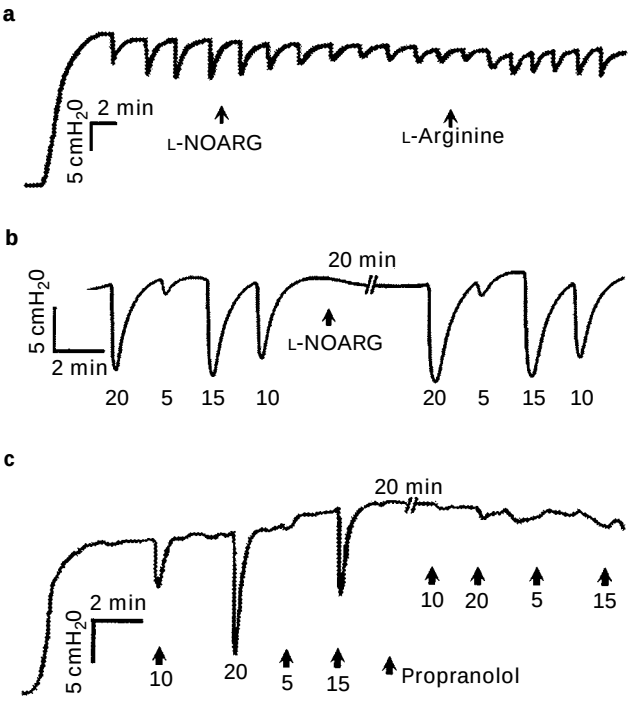

Figure 1 Sample traces of experiments investigating the effect of L-NOARG or propranolol on EFS-induced relaxations (5 s trains of stimulation, 65 V, 2 ms) on atropine-treated foetal (a) and adult (b and c) bronchi. Tone was induced with carbachol. Airway preparations were stimulated at 2 min intervals by a constant frequency of stimulation (10 Hz, trace a), or a range of frequencies (5–20 Hz, traces b and c). After control relaxation-responses to EFS had been recorded, L-NOARG (10^{-6}–10^{-4} M) or propranolol (10^{-6} M) was added. In (a) EFS was continued at 2 min intervals until a maximum effect of L-NOARG (10^{-6} M) was observed (10–20 min), then L-arginine (10^{-2} M) was added to detect reversal of the inhibitory action of L-NOARG. In (b) and (c), EFS was repeated 20 min after the introduction of L-NOARG (10^{-4} M) or propranolol of the bath.

the 2 min intervals over the remainder of the experiment – usually some 30–40 min. In the second protocol, the effect of the NOS inhibitor was studied with the same stimulus parameters as above but over a range of frequencies (5–20 Hz delivered in a random order). The NOS inhibitor was added to the bath after control responses had been recorded and after 20 min exposure the different frequencies were again delivered. An effect of propranolol (10^{-6} M) on EFS-induced relaxations of adult bronchi was also determined by this protocol. Example tracings showing the two protocols are presented in Figure 1. Sodium nitroprusside (SNP) was added cumulatively (10^{-8}–10^{-4} M) to preparations at the end of some experiments.

The kinetics of the EFS-induced relaxations were investigated in foetal and adult bronchi. The following were measured; (a) the time from the start of the train of stimulation until the onset of the relaxation, (b) the time from onset until maximal relaxation, (c) the size of the relaxation and (d) the time taken for the tissue to recover from maximal relaxation to the pre-stimulation tone level.

EFS-induced cholinergic contractions

We also studied the effect of L-NOARG (10^{-4} M), L-NAME (10^{-4} M) or SNP (3×10^{-6} M) on excitatory cholinergic responses obtained in airways in the absence of CCh and without pre-treatment with atropine. Consistent contractile responses were produced to EFS in bronchial tubes of both ages (20 s trains of stimulation delivered every 5 min, 60 V, 2 ms, 5 Hz). After at least three stable contractions were recorded (control responses) a NOS inhibitor or SNP was added to the bath and the stimulations were continued at 5 min intervals until a maximum response was recorded ($\sim$15–20 min).

Drugs and statistics

Drugs used were; acetylcholine chloride (ACh; Sigma), carbamyl choline (carbachol; CCh; Sigma), L-arginine hydrochloride (L-Arg; Sigma), atropine sulphate (Sigma), N-nitro-L-arginine (L-NOARG; Sigma), N-nitro-L-arginine methyl ester (L-NAME; Sigma), sodium nitroprusside (SNP; Eldritch), Tetrodotoxin (TTX; Sigma). All drugs were made up as a stock solution in distilled water, frozen in aliquots and diluted in Krebs solution upon demand.

Data are expressed as means $\pm$ s.e.mean. Differences between means were analysed with a two-tailed ANOVA and a Tukey's *post hoc* test. Values of $P<0.05$ were taken as significant; n = number of animals.

Results

Electrical field stimulation (65 V, 5 Hz, 2 ms) produced contractions that were both atropine and TTX sensitive. In atropine-treated preparations with CCh-induced tone, EFS (65 V, 2 ms, 10 Hz) caused TTX-sensitive relaxations. EFS-induced relaxations were not altered after epithelial removal in either tracheal strips or bronchial segments ($n=4$).

The nature of EFS-induced relaxations

EFS-induced relaxations (5 s trains of stimulation, 10 Hz, at 2 min intervals) of the foetal trachea and bronchus were abolished by L-NOARG (10^{-4} M; $n=5$; $P<0.001$). However, the effect of L-NOARG on relaxations was not reversed by L-arginine. EFS-induced relaxations of foetal airway preparations were also strongly inhibited by lower doses of L-NOARG (10^{-5}–10^{-6} M; $n=4$–6; Figure 1a), and this effect was reversed by L-arginine (10^{-2} M; Figures 1a and 2b). Similarly, tracheal preparations from adult pigs were strongly inhibited by L-NOARG (10^{-6}–10^{-4} M; $n=8$; $P<0.001$; Figure 2a) and substantially reversed by L-arginine (10^{-2} M). In contrast, relaxations in the adult bronchus were unaffected by L-NOARG (to 10^{-4} M), either at 10 Hz stimulation or at lower and higher frequencies (5–20 Hz; $n=4$–8; Figures 1b and 2a). L-NAME was also ineffective against EFS-induced relaxations in the adult bronchus (10^{-4} M; $n=3$). However, adult bronchial relaxations were all but abolished by propanolol (10^{-6} M) over the range of frequencies tested (5–20 Hz; $n=4$; Figure 1c). There were no differences in sensitivity to either L-NOARG or propranolol between airways taken from a lower or upper lobe of the lung.

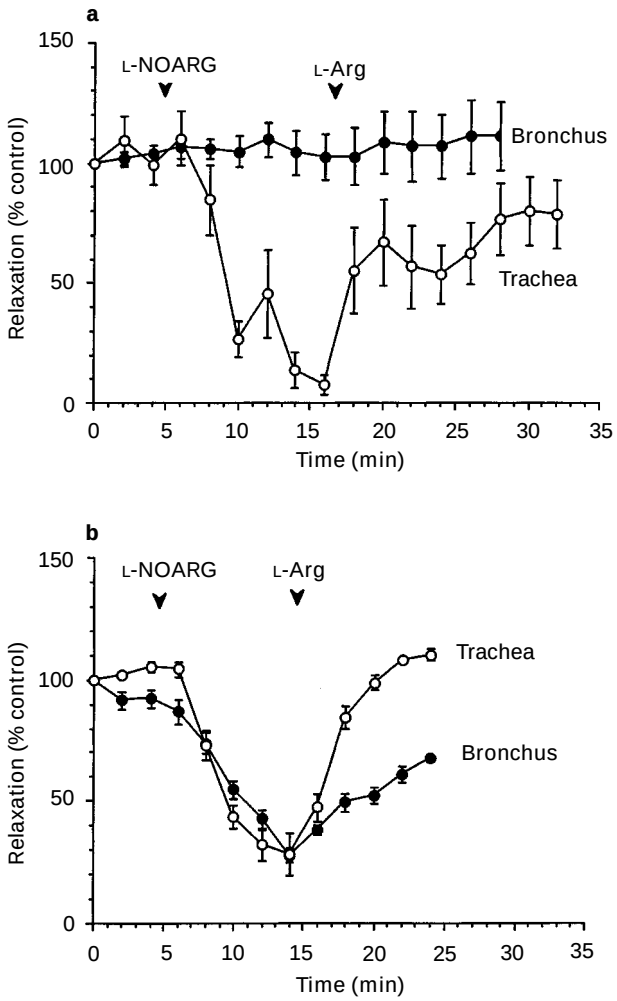

Figure 2 Effect of L-NOARG on EFS-induced relaxations in adult (a; $n=8$) and foetal (b; $n=5$) airways. Atropine-treated and carbachol-contracted airway preparations were stimulated at 2 min intervals as shown in Figure 1a (5 s trains of stimulations, 65 V, 2 ms, 10 Hz). The figure plots of EFS-induced relaxations, expressed as a percentage of the size of relaxations to EFS obtained at the start of the experiment (i.e. control responses), over the course of approximately 30 min. EFS-induced relaxations in the trachea at both ages (a and b), were strongly inhibited by L-NOARG (adult = 10^{-6} M; foetal = 10^{-5} M; $P<0.001$–0.05). Similarly relaxations in foetal bronchus (b) were also inhibited by L-NOARG (10^{-6} M; $P<0.001$–0.05). However, those in adult bronchus (a) were not altered, even by high doses of L-NOARG (10^{-4} M). L-Arginine (L-Arg 10^{-2} M) substantially restored the relaxation responses to EFS.

Four week old pigs were also used to investigate sensitivity of the bronchus to L-NOARG ($n=6$). Half of the 4 week old pig bronchi studied were partially sensitive to L-NOARG (10^{-4} M), reducing the size of the EFS-induced relaxations by approximately 50%, but the other bronchi were fully resistant.

EFS-induced relaxation kinetics

The time course of relaxation responses to EFS (5–20 Hz) was compared in adult and foetal bronchi ($n=4$ each age). In foetal bronchi the time from the start of stimulation until the onset of relaxation remained constant at approximately 2 s, at all frequencies of stimulation. However, in adult bronchi the onset time was reduced with increasing stimulation frequency ($P<0.05$; Figure 3a). The rate of relaxation (i.e. time from onset to maximum relaxation) of foetal bronchus was significantly faster than in the adult at all frequencies ($P<0.0005$; Figure 3b). The sizes of the relaxations were not different in the two ages at any frequency tested (Figure 3c). Similarly, the recovery times at all frequencies were the same in both age groups (Figure 3d).

The relaxant effect of sodium nitroprusside (SNP)

Bronchial and tracheal preparations from all age groups (after atropine and in the presence of CCh; $n=6$ each age) relaxed in a similar dose-dependent manner to SNP (10^{-8}–10^{-4} M; Figure 4). The rate of relaxation to SNP was alike in both adult and foetal bronchi - e.g. 4.5 ± 0.5 s and 6.0 ± 0.9 s to 10^{-6} M SNP, respectively, in adult and foetus.

The effect of L-NOARG and SNP on EFS-induced contractions

In the absence of atropine and CCh, SNP (3×10^{-6} M) reduced the size of EFS-induced contractions to $23.5\pm6.5\%$ of control in the adult bronchus without affecting the resting tone ($P<0.001$; $n=5$; Figure 5).

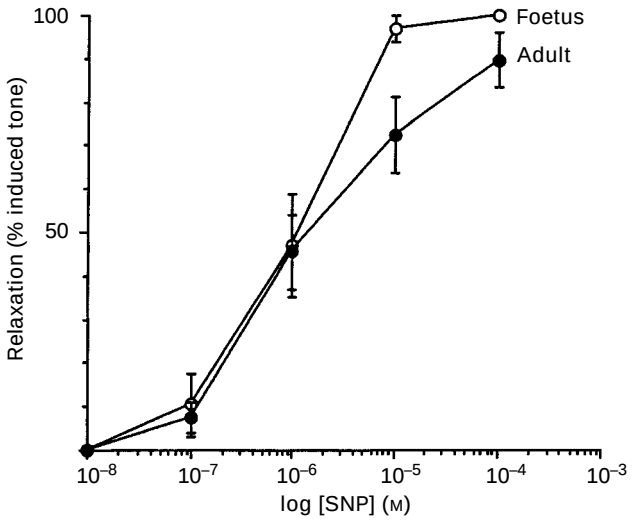

Figure 4 Effect of sodium nitroprusside (SNP) on carbachol-induced tone in atropine-treated foetal and adult bronchus. Relaxation is expressed as a percentage of the initial tone ($n=6$ each age). Vertical lines show s.e.mean.

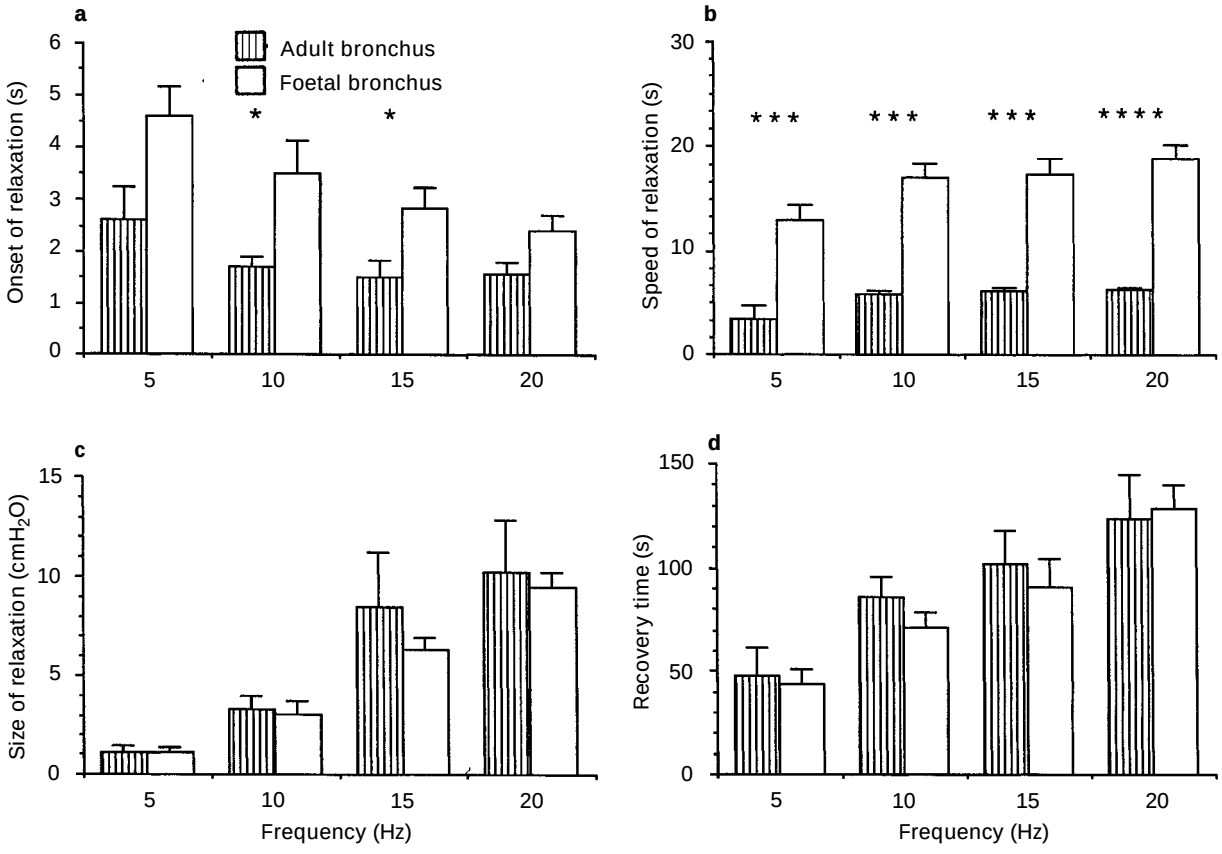

Figure 3 Kinetics of EFS-induced relaxations in both foetal and adult bronchus to a range of stimulus frequencies. (a) Time from the start of field stimulation to the onset of relaxation; (b) time from onset of relaxation to maximum relaxation; (c) size of the relaxation; (d) recovery time from the maximal relaxation. $^*P<0.05$; $^{***}P<0.001$ between ages ($n=4$ each age).

In foetal bronchi L-NOARG (10^{-4} M) increased EFS-induced contraction to $124.8 \pm 8.3\%$ ($n=4$; $P<0.01$) of the control contraction. However, the adult bronchus ($n=5$) was unaffected by either L-NAME (10^{-4} M) or L-NOARG (10^{-4} M; Figure 6). Neither of the NOS antagonists changed the resting tone in adult and foetal bronchial preparations.

Discussion

EFS elicits a muscarinic contractile response in foetal trachea and bronchus (Booth *et al*., 1992; Sparrow *et al*., 1994; 1995) and a strong neural relaxation which is largely NANC in origin (Mitchell *et al*., 1990). In the present study in the late term foetus, inhibitory NANC responses of the trachea and bronchus were all-but abolished by L-NOARG, suggesting that the relaxations might be nitrergic. Similarly, relaxations in adult trachea appeared nitrergic. However, neural relaxations in the adult bronchus were catecholaminergic since they were abolished by propranolol.

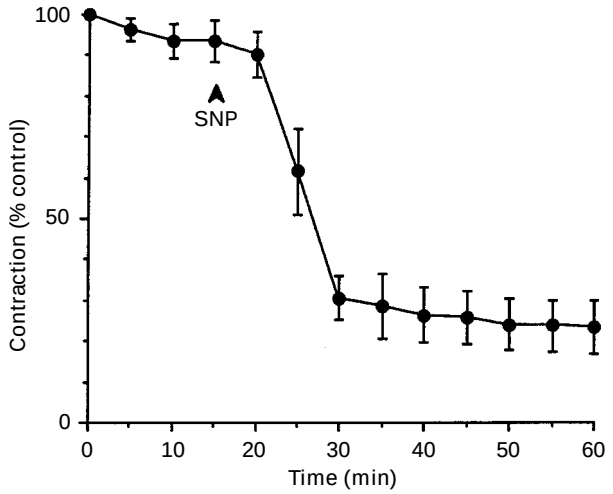

Figure 5 Effect of sodium nitroprusside (SNP; 3×10^{-6} M) on EFS-induced contractions (20 s trains of stimulation delivered every 5 min, 65 V, 2 ms, 5 Hz) in adult bronchi ($n=5$). SNP was added after several control responses had been recorded and the percentage change in the size of these calculated. SNP reduced the size of the EFS-induced contractions to $23.5 \pm 6.5\%$ of the control ($P<0.001$). Vertical lines show s.e.mean.

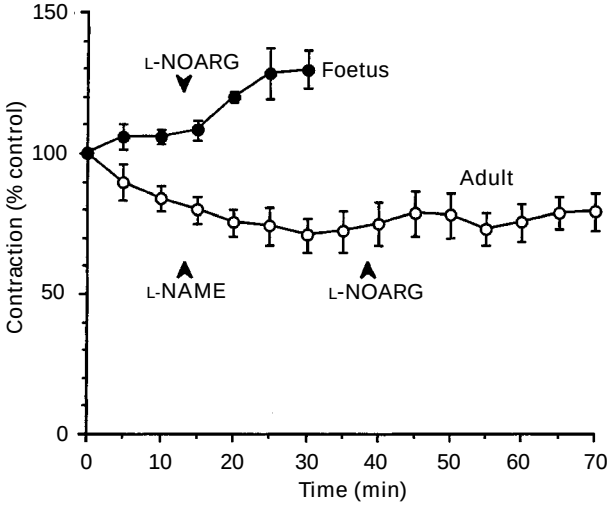

Figure 6 Effect of NOS inhibitors (L-NAME, L-NOARG, 10^{-4} M) on EFS-induced contractions in foetal and adult bronchus (20 s trains of stimulation delivered every 5 min, 65 V, 2 ms, 5 Hz). NOS inhibitors were added and the percentage change in the size of control EFS-induced contractions was calculated. Contractile responses were significantly enhanced to $123.8 \pm 8.3\%$ of control in the foetal bronchus ($P<0.01$), but adult bronchus was unaffected by L-NAME or L-NOARG ($n=5$; $P<0.01-0.05$ between ages). Vertical lines show s.e.mean.

L-NOARG blocked the inhibitory EFS-induced responses by up to 80–90% in each foetal airway preparation. Previous studies showed complete resistance of these tissues to propranolol (Mitchell *et al*., 1990) suggesting little or no contribution from catecholaminergic nerves in trachea or bronchi. In the adult, in contrast, relaxant responses in large central bronchi show partial (25%) sensitivity to propranolol (Mitchell *et al*., 1990), whereas the more peripheral bronchi used in the present study showed strong sensitivity to propranolol. Past and present findings in the adult, therefore, suggest that the extent (or density) of catecholaminergic innervation increases peripherally in the lung, whilst the density of nitrergic innervation decreases.

Our results indicate that the neurotransmitters subserving inhibitory mechanisms change with maturity in the bronchus, but not in the trachea. With such a marked difference between foetal and adult bronchi we also investigated bronchi from 4 week old pigs. Half of these bronchi were partially sensitive to L-NOARG at 10^{-4} M with a reduction in the size of the EFS-induced relaxation, whilst the reminder were insensitive to the NOS inhibitor suggesting that the transition in the neural input was incomplete at this age.

Nitrergic nerves appear to be most sensitive to low frequencies of electrical stimulation (Brave *et al*., 1991; Li & Rand, 1991; Kannan & Johnson 1992; Belvisi *et al*., 1992; Lei *et al*., 1993; Takahashi *et al*., 1995). In our study, 10 Hz produced stable and repeatable inhibitory relaxations in both the foetal and adult tracheobronchial tree. Relaxations were abolished at this frequency in foetal but not in the adult bronchus. To rule out the possibility that the resistance of the adult bronchus to L-NOARG was a result of the stimulation parameter used (10 Hz), we repeated experiments using a wider range of stimulation frequencies (5–20 Hz). However, high concentrations of L-NOARG failed to alter adult bronchial relaxations to EFS at any of these. In order to compare responses across ages, we precontracted tissues to 50% of the ACh E_{max}, as it has been previously found that the level of muscle tone may influence the size of nerve-induced inhibitory relaxations and hence the release of NO (Cohen & Berkowitz, 1974; Vargas *et al*., 1990; Linden *et al*., 1993).

Our proposal for an age-related transition of inhibitory mechanisms in the bronchial tree is supported by a comparison of the kinetics of the EFS-induced relaxation responses in the foetus and adult. The time for onset of the bronchial relaxation after electrical field stimulation was significantly less in the foetal bronchus, compared with the adult, and the rate of relaxation was three times faster. These findings could be explained either by the presence of different neurotransmitters or by differing end organ responses to the putative transmitters. However, airway preparations from both ages relax to β-adrenoceptor agonists and to exogenous NO, suggesting that the differing kinetics of the relaxant response were due to the release of different transmitters.

The bronchial epithelium in other species has been suggested to modulate the responsiveness of airway smooth muscle to contractile and relaxant agonists (Flavahan *et al*., 1985; Panitch *et al*., 1993). In preliminary experiments neither the inhibitory frequency-response curve nor the effect of L-

NOARG (10^{-4} M) on the relaxations were altered by removing some of the epithelium. This is consistent with findings in dog isolated bronchial rings, in which epithelial removal did not influence inhibitory NANC mechanism (Gao & Vanhoutte, 1993b). The lack of an epithelial modulatory effect and the observation that relaxations were TTX-sensitive, confirm that the origin of the mediator responsible for relaxation of the pig airway was the neural plexus to the smooth muscle.

Our demonstration of an age and airway generation-related transition in functional control over smooth muscle tone is supported for immunohistochemical studies suggesting NOS-immunoreactive (NOS-IR) nerves decrease with age in both pig and man (Diaz de Rada *et al*., 1993). Our findings of a predominantly central airway distribution of nitrergic relaxation in the adult is consistent with studies suggesting that NOS-IR nerves are most dense in the central airways of guinea-pig, horse, rat, pig and human respiratory tracts, but absent in the bronchioli (Fischer *et al*., 1993; Kobzik *et al*., 1993; Diaz de Rada *et al*., 1993; Yu *et al*., 1994; Ward *et al*., 1995a). In the cat there is evidence for actions of multiple inhibitory neurotransmitters at different levels of the tracheobronchial tree, with nitrergic mechanisms prominent in the central airway with a non-nitrergic mechanism prominent in bronchi, which is consistent with our findings (Takahashi *et al*., 1995; Kondo *et al*., 1995).

Some studies suggest a prejunctional role for NO, attenuating cholinergic excitatory neurotransmission (Gustafsson *et al*., 1990; Sekizawa *et al*., 1993; Wiklund *et al*., 1993). In our study SNP had a considerable inhibitory effect on EFS-induced contractions in the adult bronchus, suggesting a potential action of NO either via a pre- or postjunctional mechanism. As SNP also exerted a strong relaxant action on carbachol-induced tone (foetal and adult, Figure 4), we cannot clearly distinguish between these two possible mechanisms of action. However, the absence of an effect of NOS inhibitors on EFS-induced contractions in the adult suggests that endogenous NO plays no role in excitatory neurotransmission or transmitter effects at this age. By contrast, EFS-induced contractions in foetal bronchi were modestly augmented by L-NOARG, supporting our proposal of a releasable source of NO in the immature airway. Whether this source is the same as that responsible for the EFS-induced relaxant responses, also shown in the present study, is unclear. As noted above, a direct action of SNP on pig bronchial smooth muscle was demonstrated. We cannot establish whether the effect of the NOS inhibitor on EFS-induced contractions in the foetus is associated only with a postjunctional action of released NO, or whether a prejunctional action is also involved.

At present, suggestions as to the role of such a strong inhibitory input to the immature airway smooth muscle are largely speculative. Airways have now been clearly shown to be controlled by excitatory and inhibitory nerves as early as the first trimester (Booth *et al*., 1992; McCray, 1993; Sparrow *et al*., 1994; 1995). Peristaltic-like airway narrowing in the foetal lung is associated with the movement of the lung fluid (Sparrow *et al*., 1995), which is important for lung growth (Moessinger *et al*., 1990). It is possible that excitatory and inhibitory neural inputs are involved in the coordination of peristaltic-like activity and therefore the development of the pressure gradients, which appear to underly the effect of lung fluid on lung growth. It is unknown why the chemical coding of the transmitter concerned should change after birth.

We suggest that in the foetus the principal neurotransmitter producing relaxation throughout much of the tracheobronchial tree is NO. After birth there is a gradual transition away from NO towards an catecholaminergic pathway of relaxation. However, this transition occurs primarily in the bronchi of the lung and neurotransmission in the trachea remains nitrergic.

Special thanks to K.J. Raynor for assistance with some experiments. The work was supported by the Sudden Infant Death Syndrome (SIDS) Foundation of Western Australia, the National SIDS Council of Australia and the National Health and Medical Research Council of Australia.

References

BAI, T.R. & BRAMLEY, A.M. (1993). Effect of an inhibitor of nitric oxide synthase on neural relaxation of human bronchi. *Am. J. Physiol.,* **264,** L425 – L430.

BELVISI, M.G., STRETTON, C.D., MIURA, M., VERLEDEN, G.M., TADJKARIMI, S., YACOUB, M.H. & BARNES, P.J. (1992). Inhibitory NANC nerves in human tracheal smooth muscle: a quest for the neurotransmitter. *J. Appl. Physiol.,* **73,** 2505 – 2510.

BOOTH, R.J., SPARROW, M.P. & MITCHELL, H.W. (1992). Early maturation of force production in pig tracheal smooth muscle during foetal development. *Am. J. Respir. Cell Mol. Biol.,* **7,** 590 – 597.

BRAVE, S.R., HOBBS, A.J., GIBSON, A. & TUCKER, J.F. (1991). The influence of L-NOARG on field stimulation induced contractions and acetylcholine release in guinea pig isolated tracheal smooth muscle. *Biochem. Biophys. Res. Commun.,* **179,** 1017 – 1022.

COHEN, M.L. & BERKOWITZ, B.A. (1974). Age related changes in vascular responsiveness to cyclic nucleotides and contractile agonists. *J. Pharmacol. Exp. Ther.,* **191,** 147 – 155.

DIAZ DE RADA, O., VILARO, A.C., MONTUENGA, L.M., MARTINEZ, A., SPRINGALL, D.R. & POLAK, J.M. (1993). Nitric oxide synthase-immunoreactive neurons in human and porcine respiratory tract. *Neurosci. Lett.,* **162,** 121 – 124.

FERNANDES, L.B., ELLIS, J.L. & UNDEM, B.J. (1994). Potentiation of nonadrenergic noncholinergic relaxation of human isolated bronchus by selective inhibitors of phosphdiesterase isozymes. *Am. J. Respir. Crit. Care Med.,* **150,** 1384 – 1390.

FISCHER, A., MUNDEL, P., MAYER, B., PREISSLER, U., PHILIPPIN, B. & KUMMER, W. (1993). Nitric oxide synthase in guinea pig lower airway innervation. *Neurosci. Lett.,* **149,** 157 – 160.

FLAVAHAN, N.A., AARHUS, L.L., RIMELE, T.J. & VANHOUTTE, P.M. (1985). Respiratory epithelium inhibits bronchial smooth muscle tone. *J. Appl. Physiol.,* **58,** 834 – 838.

GAO, Y. & VANHOUTTE, P.M. (1993a). Attenuation of contractions to acetylcholine in canine bronchi by an endogenous nitric oxide-like substance. *Br. J. Pharmacol.,* **109,** 887 – 891.

GAO, Y. & VANHOUTTE, P.M. (1993b). Products of cyclooxygenase mediate the responses of the guinea pig trachea to hydrogen peroxide. *J. Appl. Physiol.,* **74,** 2105 – 2111.

GUSTAFSSON, L.E., WIKLUND, C.U., WIKLUND, N.P., PERSSON, M.G. & MONCADA, S. (1990). Modulation of autonomic neuroeffector transmission by nitric oxide in guinea pig ileum. *Biochem. Biophys. Res. Commun.,* **173,** 106 – 110.

HAXHIU-POSKURICA, B., ERSENBERGER, P., HAXHIU, M.A., MILLER, M.J., CATTAROSSI, L. & MARTIN, R.J. (1993). Development of cholinergic innervation and muscarinic receptor subtypes in piglet trachea. *Am. J. Physiol.,* **264,** L606 – L614.

HAXHIU-POSKURICA, B., HAXHIU, M.A., KUMAR, G.K., MILLER, M.J. & MARTIN, R.J. (1992). Tracheal smooth muscle responses to substance P and neurokinin A in the piglet. *J. Appl. Physiol.,* **72,** 1090 – 1095.

KANNAN, M.S. & JOHNSON, D.E. (1992). Nitric oxide mediates the nonadrenergic, noncholinergic relaxation of pig tracheal smooth muscle. *Am. J. Physiol.,* **262,** L511 – L514.

KOBZIK, L., BREDT, D.S., LOWENSTEIN, C.J., DRAZEN, J., GASTON B., SUGARBAKER, D. & STAMLER, J.S. (1993). Nitric oxide synthase in human and rat lung: Immunocytochemical and histochemical localization. *Am. J. Respir. Cell Mol. Biol.,* **9,** 371 – 377.

KONDO, T., KOBAYASHI, I., HIROKAWA, Y., SUDA, S., OHTA, Y. & ARITA, H. (1995). Differences in motor control in the bronchus and extra thoracic trachea. *J. Autonom. Nervous System,* **55,** 1–8.

LEFF, A.R., MUNOZ, N.M., TALLET, J., DAVID, A.C., CAVIGELLI, M.A. & GARRITY, E.R. (1985). Autonomic response characteristics of porcine airway smooth muscle *in vivo*. *J. Appl. Physiol.,* **58,** 1176–1188.

LEI, Y.-H., BARNES, P.J. & ROGERS, D.F. (1993). Regulation of NANC neural bronchoconstriction *in vivo* in the guinea pig: involvement of nitric oxide, vasoactive intestinal peptide and soluble guanylyl cyclase. *Br. J. Pharmacol.,* **108,** 228–235.

LI, C.G. & RAND, M.J. (1991). Evidence that part of the NANC relaxant reponse of guinea pig trachea to electrical field stimulation is mediated by nitric oxide. *Br. J. Pharmacol.,* **102,** 91–94.

LINDEN, A., ULLMAN, A., LOFDAHL, C.-G. & SKOOGH, B.-E. (1993). NANC neural activation in guinea pig bronchi; powerful and frequency dependent stabilising effect on tone. *Br. J. Pharmacol.* **109,** 845–851.

MCCRAY, P.B. (1993). Spontaneous contractility of human fetal airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.,* **8,** 573–580.

MITCHELL, H.W., SPARROW, M.P. & TAGLIAFEERI, R.P. (1990). Inhibitory and excitatory responses to field stimulation in fetal and adult pig airway. *Ped. Res.,* **28,** 69–74.

MOESSINGER, A.C., HARDING, R., ADAMSON, T.M., SINGH, M. & KIU, G.T. (1990). Role of lung fluid volume in growth and maturation of the fetal sheep lung. *J. Clin. Invest.,* **86,** 1270–1277.

PANITCH, H.B., WOLFSON, M.R. & SHAFFER, T.H. (1993). Epithelial modulation of preterm airway smooth muscle contraction. *J. Appl. Physiol.,* **74,** 1437–1443.

SCOTT, T.R.D. & BENNETT, M.R. (1993). The effect of nitric oxide on the efficacy of synaptic transmission through the chick ciliary ganglion. *Br. J. Pharmacol.,* **110,** 627–632.

SEKIZAWA, K., FUKUSHIMA, T., IKARASHI, Y., MARUYAMA, Y. & SASAKI, H. (1993). The role of nitric oxide in cholinergic transmission in rat trachea. *Br. J. Pharmacol.,* **110,** 816–820.

SPARROW, M.P. & MITCHELL, H.W. (1990). Contraction of smooth muscle of pig airway tissues from before birth to maturity. *J. Appl. Physiol.,* **68,** 468–477.

SPARROW, M.P., WARWICK, S.P. & EVERETT, A.W. (1995). Innervation and function of the distal airways in the developing bronchial tree of fetal pig lung. *Am. J. Respir. Cell Mol. Biol.,* **13,** 518–525.

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

TAKAHASHI, N., TANAKA, H., ABDULLAH, N., JING, L., INOUE, R. & ITO, Y. (1995). Regional difference in the distribution of L-NAME-sensitive and -insensitive NANC relaxations in cat airway. *J. Physiol.,* **488,** 709–720.

VARGAS, H.M., IGNARRO, L.J. & CHAUDHURI, G. (1990). Physiological release of nitric oxide is dependent on the level of vascular tone. *Eur. J. Pharmacol.* **190,** 393–397.

WALDRON, M.A., CONNELLY, B.J. & FISHER, J.T. (1989). Non-adrenergic inhibitory innervation to the airways of the new born cat. *J. Appl. Physiol.,* **66,** 1995–2000.

WARD, J.K., BARNES, P.J., SPRINGALL, D.R., ABELLI, L., TADJ-KARIMI, S., YACOUB, M.H., POLAK, J.M. & BELVISI, M.G. (1995a). Distribution of human i-NANC bronchodilator and nitric oxide-immunoreactive nerves. *Am. J. Respir. Cell Mol. Biol.,* **13,** 175–184.

WARD, J.K., BARNES, P.J., TADJKARIMI, S., YACOUB, M.H. & BELVISI, M.G. (1995b). Evidence for the involvement of cGMP in neural bronchodilator responses in human trachea. *J. Physiol.,* **483,** 525–536.

WIKLUND, C.U., OLGART, C., WIKLUND, N.P. & GUSTAFSSON, L.E. (1993). Modulation of cholinergic and substance P-like neurotransmission by nitric oxide in the guinea pig ileum. *Br. J. Pharmacol.,* **110,** 833–839.

YU, M., WANG, Z., ROBINSON, N.E. & LEBLANC, P.H. (1994). Inhibitory nerve distribution and mediation of NANC relaxation by nitric oxide in horse airways. *J. Appl. Physiol.,* **76,** 339–344.

(Received August 20, 1997
Revised October 10, 1997
Accepted October 29, 1997)