ONTOGENIC CHANGES IN PORCINE PULMONARY VASCULAR AND AIRWAY SMOOTH MUSCLE RESPONSIVENESS IN VITRO

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ABSTRACT

A marked decrease in pulmonary vascular resistance occurs in the newborn period, and a decrease in susceptibilty to bronchospasm occurs with maturation in humans. Both of these processes may be related to changes in the responsiveness of the smooth muscle to constricting and relaxing mediators. The goals of these studies were to determine if maturational changes occurred in the responsiveness of airway and vascular porcine smooth muscle in vitro. Studies were performed using pigs ranging in age from newborn to adult. Rings of pulmonary artery with and without endothelium, and tracheal smooth muscle strips or bronchial rings were suspended in organ baths filled with physiological salt solution, maintained at 37^{o}C, and bubbled with 95%O_2 5%CO_2. Tissues were stretched to their optimal length for force development, and then concentration-response curves to a variety of agonists obtained.

Pulmonary arteries from immature pigs were less responsive than those from adult pigs to adrenoreceptor mediated vasoconstriction. An alpha-2 adrenoreceptor mediated release of endothelium-derived relaxing factor was demonstrated in pulmonary arteries from adult pigs but not in those from immature pigs. Arteries from immature pigs were less responsive to relaxation induced by nitric oxide and hypoxia but not by sodium nitroprusside. Bronchial rings from immature pigs produced greater force (normalised to wet weight) in response to acetylcholine or potassium chloride than did rings from adult pigs, although the concentration of acetycholine required to produced half-maximal response (EC50) was similar. Rings from newborn pigs were more sensitive to the bronchodilator ketamine than those from adult pigs, as manifested by a shift in EC50. The relaxant action of ketamine on tracheal smooth muscle was shown to be due to an inhibitory effect on excitation of

the postsynaptic nicotinic receptors of the intramural parasympathetic ganglion and a direct effect on the smooth muscle cell. Nonadrenergic noncholinergic innervation was shown to be present from birth. Hypoxic bronchodilation was seen in bronchial rings from all ages. In conclusion, this study demonstrates age-related changes in the in vitro responsiveness of both pulmonary vascular and airway smooth muscle to contractile and relaxant agonists, responses depending not only upon the agonist used to elicit contraction or relaxation, but also upon the age of the animal studied.

DECLARATION

This thesis has been submitted in partial fulfillment of the degree of Doctor of Medicine at the University of Edinburgh, and does not contain any work which has been previously submitted by myself to any University for any other degree.

The work contained in this thesis is original research undertaken by myself.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
d	day
DMPP	1,1,dimethyl-4-phenyl-piperazinium iodide
DNMMA	N-g-methyl-D-arginine
E	endothelium
GMP	guanosine monophosphate
LNAME	n-w-nitro-L-arginine methyl ester
LNMMA	N-g-methyl-L-arginine
MB	methylene blue
NB	newborn
NE	noradrenaline
NO	nitric oxide
PG	prostaglandin
w	week
4DAMP	4-diphenylacetoxy-N-methyl-piperadine methiodide
5HT	5-hydroxytryptamine

INTRODUCTION

Over the past decade there has been increasing interest in the effect of maturation on the contractile properties of smooth muscle. As the pathways of excitation-contraction coupling in mature smooth muscle become better understood, questions have arisen as to whether the response of smooth muscle to agonists alters with age, and if so what is the mechanism behind this alteration. In this study on porcine pulmonary vascular and airway smooth muscle, the first of these questions will be addressed, and possible mechanisms behind any age-related changes discussed.

1.1 Myosin and actin in smooth muscle

Myosin is an enzymatic structural protein found in all types of muscle (Stull, 1980). It is responsible for the conversion of chemical energy into mechanical work. The myosin acts as an adenosine triphosphatase (ATPase), and when bound to actin cleaves ATP causing release of phosphate. The release of this high energy phosphate is associated with a conformational change in the myosin molecule, the power stroke. The native myosin molecule is a hexamer, a highly asymmetric molecule with molecular weight of 480kDa. (Sobieszek and Bremel, 1975). It consists of 2 heavy chains with a molecular weight of 200kDa (Hartshorne and Gorecka, 1980) and two pairs of regulatory light chains, 20 and 17kDa (Driska and Hartshorne, 1975). The two heavy chains interact to form a coiled alpha helix "tail" at the carboxyl terminal end, while the N terminal forms a globular conformation known as the head region. The ATP hydrolysis site, actin binding site and two pairs of light chains are all found in the head region.

Isoforms of the myosin heavy chain exist. At least two isoforms are known to exist in porcine airway smooth muscle (Mohammad and Sparrow, 1988; Murphy et al, 1991a)

and in vascular smooth muscle an embryonic form may also occur (Kuro-o et al, 1989).

The composition of the myosin light chain in smooth muscle is different from that found in skeletal and cardiac muscle. (Hartshorne, 1987). The myosin light chain 17kDa has been found to exist in two isoforms in porcine aorta (Hasegawa 1988), and there are also two isoforms of the 20kDa light chain (Erdodi et al, 1987).

Smooth muscle actin exists in two forms, smooth alpha and smooth gamma. The distribution of smooth muscle isoactins varies depending upon the muscle, e.g. the ratio of alpha to gamma is 3.2 in pig aorta, and 0.8 in tracheal smooth muscle (Cohen and Murphy, 1978). Beta and alpha non-muscle isoactins are also found in smooth muscle tissues and may reflect non-contractile aspects of smooth muscle function such as proliferation or secretory functions (Fatigati and Murphy, 1984).

The sliding filament theory for muscle contraction was developed to explain skeletal muscle contraction and requires the existence of thick (myosin containing) and thin (actin containing) filaments (Huxley and Hanson, 1954). The regular arrangement of actin and myosin filaments to form sarcomeres does not occur in smooth muscle. Thick filaments do occur in smooth muscle but are longer than in skeletal muscle (2.2±0.14 microns, Ashton et al, 1975 versus 1.5-1.6 microns, Page and Huxley, 1963). Far fewer thick filaments, however, are present in smooth muscle compared with skeletal (Murphy et al, 1974). Despite these differences, the sliding filament theory provides a useful basis for the understanding of filament interaction in smooth muscle as well as skeletal.

1.2 Calcium and contraction

Smooth muscle will contract in response to an increase in cytosolic free calcium, but controversy still exists concerning the exact coupling of calcium to contraction. There are two basic mechanisms by which neurotransmitters and other mediators elevate intracellular calcium. One is by changes in surface membrane potential and activation of voltage dependent calcium channels (electromechanical coupling), and the other by membrane potential independent mechanisms (pharmacomechanical coupling) (Coburn and Baron, 1980). Sobieszek (1977) showed that the calcium sensitivity of actomyosin ATPase activity was associated with the phosphorylation of the 20KDa myosin light chain. This phosphorylation results from the activation of myosin light chain kinase, a calcium and calmodulin dependent enzyme (Kamm and Stull, 1985). Correlations have been shown between myosin light chain phosphorylation and both calcium dependent actin activated myosin ATPase activity (Disalvo et al, 1978) and force development (Driska et al, 1981). It is becoming clearer that the regulation of smooth muscle contraction is more complicated than the model in which myosin light chain phosphorylation acts as a simple switch. Force can be developed in the absence of increases in myosin light chain phosphorylation (Wagner and Ruegg, 1986). Crossbridge cycling rate as measured by ATPase activity can be regulated independently of changes in the myosin light chain phosphorylation (Moreland et al, 1991). Alteration in the activity of myosin light chain kinase, (Jiang et al, 1992), alteration in calcium sensitivity of the contractile apparatus by either inhibition of the myosin light chain phosphatase (Kitazawa et al, 1989) or activation of protein kinase C (Nishimura et al, 1989), and cooperativity between myosin heads (Somlyo et al, 1988) may also play a role.

1.3 Second messenger systems

One of the major mechanisms of signal transduction of pharmacomechanical coupling is by phosphoinositol hydrolysis (Coburn and Baron, 1980). Activation of surface receptors, muscarinic in airway smooth muscle and alpha-1 adrenergic in vascular smooth muscle, results in activation of phospholipase C via an intermediary G protein. Phospholipase C activation converts phosphoinositide 4,5-biphosphate in the cell membrane in to myoinositol 1,4,5,-triphosphate and 1,2 diacylglycerol. Myoinositol 1,4,5,-triphosphate binds to surface receptors on the sarcoplasmic reticulum, thereby releasing calcium. Diacylglycerol activates a membrane associated enzyme, protein kinase C. Protein kinase C may sensitise the contractile proteins to calcium.

Adenyl cyclase is also a membrane associated enzyme and is activated by stimulation of surface receptors, such as the beta-adrenergic receptor in airway smooth muscle, and causes relaxation of the muscle (Torphy and Hay, 1990). Again an intermediary G protein is involved. Activation of adenyl cyclase leads to an increase in cyclic AMP levels, and activation of protein kinase A. Protein kinase phosphorylates regulatory proteins in the cell resulting in relaxation. Activation of alpha-2 adrenoceptors inhibits adenyl cyclase activity and results in a decrease in cyclic AMP levels. Nitric oxide causes both airway and vascular smooth muscle relaxation. It activates soluble guanylate cyclase (Torphy and Hay, 1990). The subsequent increase in cyclic GMP activates a cyclic GMP dependent protein kinase, to increase cytosolic calcium removal, inhibit calcium influx and perhaps alter the sensitivity of the contractile proteins to calcium (Nakatsu and Diamond, 1989).

Alteration in the receptors on the muscle, the secondary messenger system or in the contractile proteins will modify the response of a muscle to a drug. If the

contractile apparatus is fundamentally altered by aging, then most, if not all drugs would be affected. More discrete changes in tissue sensitivity would result from factors modifying one or more components of a specific receptor.

1.4 Changes in pulmonary vascular resistance at birth

In the fetus pulmonary vascular resistance is high, but at the time of birth with the onset of ventilation it falls, and pulmonary blood flow increases eight fold. The exact mechanism of the rapid pulmonary vasodilation is unknown, but may involve structural adaptations, responses to changes in the oxygen environment and or the release of vasoactive mediators. A further reduction in pulmonary vascular resistance occurs over two to six weeks until adult levels are reached (Haworth and Hislop, 1981).

The earliest structural changes seen in the adaptation to extrauterine life occur in the precapillary arteries. Five minutes after birth endothelial cells become thinner and show less overlap, and become orientated along the direction of flow (Hall and Haworth, 1986a). These changes are compatible with the vessels having been stretched at birth. Similar changes also occur in muscular arteries (Haworth et al, 1987). After the first few days of life, connective tissue increases, with increased deposition of the internal elastic lamina (Hall and Haworth, 1986b). Smooth muscle cells increase in size and number and connective tissue is deposited in the media and subendothelium. At birth pulmonary vascular smooth muscle cells are immature with synthetic rather than contractile elements. In the pig the concentration of myofilaments begins to increase at three weeks and continues until the animal is mature at six months of age.

No muscle of bronchial - pulmonary shunt
Closure of major shunts at birth - eg ductus

Marked changes in the oxygen environment of the pulmonary arteries occurs at birth. Ventilation of fetal lungs without increasing pO_2 reduces pulmonary vascular resistance, and a further fall occurs when oxygen is added to the ventilatory gas mixture. (Teitel et al, 1990). When fetal lambs are exposed to hyperbaric oxygen without expansion or ventilation of the lungs pulmonary vascular resistance also falls (Heymann et al, 1969). A variety of mechanisms could be involved. Increased oxygen could directly dilate pulmonary vascular smooth muscle, could result in the release of a substance that actively dilates the pulmonary circulation, could inhibit vasoconstriction or inhibit the production of a substance causing active constriction.

The release of vasoactive substances may also play a role in the transition of the pulmonary circulation. Exogenous prostaglandins particularly prostacyclin cause pulmonary vasodilation, and mechanical ventilation of the lungs leads to prostacyclin release (Gryglewski et al, 1978, Leffler et al 1984a, Leffler et al 1984b). Pretreatment of fetal goats with indomethacin attenuates the fall in pulmonary vascular resistance seen with ventilation (Leffler et al 1978). Such studies indicate that prostacyclins may play some role in the adaptation of the pulmonary circulation.

When fetal lungs are ventilated with air or oxygen bradykinin is released (Heymann et al, 1969) a potent vasoactive peptide which can cause pulmonary vasodilation. The degree to which the vasoactive effects of bradykinin are direct, or mediated by endothelium-derived relaxing factor or prostacyclin is not clearly established.

Finally, the pulmonary vascular endothelium may manifest endocrine or paracrine function with the release of

endothelium-derived relaxing factor, now thought to be nitric oxide.

1.5 Nitric oxide

In 1980 Furchgott published the first article showing that the relaxation of smooth muscle of isolated arteries, induced by acetylcholine, was dependent on the presence of endothelial cell and that the relaxation was mediated by a factor released from these cells on stimulation of their muscarinic receptors (Furchgott and Zawadzki, 1980). A number of other vasodilators including ATP, adenosine diphosphate and bradykinin were subsequently shown to produce relaxation of isolated arteries by an endothelium-dependent mechanism (Furchgott and Vanhoutte, 1989). It was subsequently shown that endothelium-derived relaxing factor caused an increase in cyclic guanosine monophosphate (Rapoport and Murad, 1983) and could activate soluble guanylate cyclase (Forstermann et al, 1986). The suggestion that endothelium-derived relaxing factor was nitric oxide was made by several groups (Ignarro et al, 1987a; Palmer et al 1987).

The source of nitric oxide in endothelial cells is the nitrogen of the guanidino moiety of L-arginine. The enzyme involved in the production of nitric oxide is nitric oxide synthase, a cytosolic enzyme which is NADPH, calcium and calmodulin dependent. Nitric oxide has a short half-life with spontaneous oxidation in the presence of oxygen in aqueous solution.

Haemoglobin and other reduced haemoproteins have a high binding affinity for nitric oxide and thereby inactivate it. Structural analogues of L-arginine have also been developed, and can inhibit nitric oxide formation or release.

Nitric oxide can serve an autocrine function, stimulating cGMP production in vascular endothelial cells, and a

paracrine function by diffusing to nearby vascular smooth muscle cells and causing vasodilation. The physiological stimuli for generation of nitric oxide are not fully understood but pulsatile flow and shear stress may be important (Rubanyi et al 1986).

There are few studies on maturational changes in responsiveness to nitric oxide. Endothelium-derived relaxing factor activity is present in rings of guinea pig artery by one to three days of age (Davidson and Eldemerdash, 1990), but is virtually absent in in vitro preparations of pulmonary arteries from fetal sheep (Abman et al, 1991). Acetylcholine dilates late-gestational fetal sheep pulmonary arteries in vivo, and nitro-L-arginine inhibits both basal and stimulated endothelium-derived relaxing factor activity (Abman et al, 1990). With regard to porcine studies, Zellers and Vanhoutte (1991) studied three, ten and thirty day old pigs, and found that the pulmonary artery relaxation in vitro to acetylcholine was greater in the ten and thirty day old animals compared with the three day old pigs. Liu et al (1992) found no acetylcholine induced relaxation in porcine pulmonary arteries from newborns, but almost 100 percent relaxation by three to ten days of age, declining with increasing age thereafter.

Changes with age in the the endothelial cell receptor type, number or coupling of receptors to production of nitric oxide, or in the capacity of the endothelial cells to produce nitric oxide, or alterations in the underlying responsiveness of the smooth muscle to nitric oxide may result in altered pulmonary reactivity.

1.6 Neural control of airway smooth muscle tone

Autonomic nerves play an important role in the regulation of airway calibre (Laitinen and Laitinen, 1987; Barnes, 1992). Cholinergic innervation provides the predominant bronchoconstricting neural input. Efferent para-

sympathetic innervation to the airways arises from the nucleus of the tenth cranial nerve in the central nervous system and travels to the parasympathetic gangllia immediately proximal to the airway. A short postganglionic fibre completes the pathway.

The sympathetic innervation is sparse in humans, but circulating adrenaline may alter airway tone. Sympathetic fibres arise from the spinal cord and synapse in sympathetic ganglia that are the thoracic sympathetic chain. Many long postganglionic fibres may arise from each ganglionic synapse. Sympathetic nerves innervate the mucous glands and bronchial vasculature but not the airway smooth muscle. Bronchodilatory influences depend upon adrenal secretion of adrenaline.

The efferent fibres of nonadrenergic noncholinergic inhibitory nerves travel in the vagus nerve. The potential importance of nonadrenergic noncholinergic inhibitory activation as a relaxant is unknown. Nitric oxide has recently been proposed as a mediator of the nonadrenergic noncholinergic innervation in the pig (Kannan and Johnson, 1992).

A potential role for bronchoactive peptides released from the sensory endings of C-fibres has been proposed. These substances are the tachykinins and include substance P and the neurokinins.

Alterations in innervation patterns with age, or changes in response to the neurotransmitter due to alteration in receptor number or distribution may occur.

1.7 Developmental changes in airway reactivity

Airway reactivity refers to the magnitude of and threshold for the change in cross-sectional area of the respiratory tree resulting from a stimulus that contracts airway smooth muscle.

Comparisons between newborn and adult airway smooth muscle have utilised both in vivo and in vitro studies of airway smooth muscle. On the basis of the "squeeze" flow volume curves before and after acetylcholine inhalation (Tepper, 1987) it was concluded that infants had an enhanced bronchial responsiveness compared with adults. A similar conclusion was reached based on inhalation of nebulised histamine (LeSouef et al, 1989, Landau et al, 1989). Several factors including laryngeal and upper airway resistance and drug delivery can influence such measurements. In vivo measurement of airway resistance in newborn and adult animals usually involves nerve stimulation. All fibres of the nerve are activated and therefore antagonistic innervations may be recruited if present, and this may influence responsiveness (Waldron et al, 1989). In vivo studies may also assume that the load provided by noncontractile tissue is similar between newborn and adult animals. Bhutani et al (1981) reported that airway compliance is greater in the newborn and preterm rabbit than the adult. The greater compliance of neonatal airways should allow smooth muscle contraction to reduce airway diameter more easily than in the adult. However, the increase in airway resistance seen is less than or only equal to that seen in the adult (Fisher et al, 1990). This indicates an apparent decrease in the responsiveness of the newborn airway smooth muscle.

Much of the in vitro work on mechanics of airway smooth muscle has been performed on the trachea, partly because of the parallel orientation of the muscle fibres. The muscle demonstrates Frank-Starling like length tension characteristics during isometric contraction. This is also true for airway smooth muscle in bronchial rings despite their helical orientation. By contrast with in vivo studies, in vitro investigations suggest airway smooth muscle from neonatal or young animals has an enhanced capability to generate isometric force compared

with the adult (Duncan and Douglas, 1985; Wills and Douglas, 1988; Sparrow and Mitchell, 1990). Reduced isometric force generation in the immature animal compared with the adult has been demonstrated in sheep (Panitch et al, 1989).

Comparison of maximal force values between age groups is constrained by the choice of normalisation factor (Jiang et al, 1991, Stephens et al, 1992). The majority of comparisons are made on the basis of stress, although morphological and biochemical methods of normalisation may prove better. Normalisation of force on the basis of contractile protein has been used in studies of force generation in vascular smooth muscle (Murphy et al, 1974). In airway smooth muscle a similar response to carbachol in adult and fetal pigs was demonstrated when normalised to tissue weight (Mitchell et al, 1990a). The same group (Sparrow and Mitchell, 1990) found that normalisation of maximal force generated by tracheal smooth muscle, on the basis of cross-sectional area of myosin rather than stress altered the rank ordering of maximal isometric response force values among fetal, four week, twenty week and adult pigs. The very small tissue stress generated in the fetal tissue reflected the paucity of myosin heavy chain and smooth muscle present, rather than "less effective" airway smooth muscle. In four week old animals, the force generated was greater than in all other age groups regardless of the normalisation process. Murphy et al (1991a) also reported that the maximal isometric force generated in response to methacholine was greater in airway smooth muscle from two week old pigs compared with ten week old pigs. The decrease in force was independent of estimates of the amount of airway smooth muscle (based on measurements of the force normalised by tissue weight and myosin weight as opposed to cross-sectional area).

Normalisation, even to myosin content is not simple, since some studies have shown an increase in myosin content of pig tracheal smooth muscle with age (Sparrow and Mitchell, 1990) whereas others have shown that the myosin content of the bronchi from ten week old pigs was less than that from two week old pigs (Murphy et al, 1991a). Alteration in the myosin heavy chain isoform is also known to occur in the maturing pig (Sparrow and Mitchell, 1990), and it is not known if the different isoforms are associated with different contractile properties.

Mitchell et al (1990b) demonstrated that physostigmine augmented contraction in airways from ten week old pigs, but not two week old, suggesting that part of the alteration in airway responsivenness was due to age-related differences in acetylcholinesterase activity. This would then suggest that the enhanced reactivity seen in the immature pig airways would be agonist specific. Stimulation of contraction with potassium chloride has produced conflicting results with no change seen in the responsiveness of tracheal smooth muscle of two and ten week old pigs (Murphy et al 1989, 1990b) but a greater response to KCl in airway generations 2-4 in the two week old pig compared with the ten week old pig (Murphy et al 1991b).

Age-related changes in reactivity may also manifest themselves by changes in sensitivity to an agonist as well as in the magnitude of the response. Tracheae from immature guinea pig (Duncan and Douglas, 1985) and cow (Wills and Douglas) are more sensitive to contractile agents than mature animals. Panitch et al (1989) showed the reverse to be true for sheep. No clear trend is apparent for the pig (Sparrow and Mitchell, 1990). Extrapolation of the in vitro findings to in vivo responsiveness rests on the assumption that isometric force is relevant to the narrowing of airways in vivo

(Stephen et al, 1992) and that other antagonist factors are not present.

1.8 Objectives

The overall aim of these studies was to determine if maturation altered the responsiveness of porcine pulmonary and airway smooth muscle. To do this, in vitro isometric contractile properties of pulmonary artery rings, tracheal smooth muscle strips and bronchial rings from pigs of various ages were studied.The specific aims were to determine if age-related changes occurred in:

(1) the response of pulmonary vascular smooth muscle to agonists causing contraction.

(2) the response of pulmonary vascular smooth muscle to agonists causing relaxation.

(3) the release of endothelium-derived relaxing factor.

(4) the response of airway smooth muscle to agonists causing contraction.

(5) the response of airway smooth muscle to agonists causing relaxation.

METHODS

2.1 Preparation and mounting of pulmonary artery rings

2.1.1 Adult pigs

Lungs from freshly killed adult (200lb weight) Large White pigs were transported from a local abbatoir in pregassed (95%O_2 -5%CO_2, Air Products), cold (4^{o}C) physiological salt solution (Appendix). The pulmonary artery was dissected out and all visible connective tissue removed from the artery. The intralobar muscular portion of the artery was then cut in to rings 3-4mm in diameter and 3mm long. In some rings the endothelium was deliberately removed by rubbing the inside of the ring with a pair of watchmakers forceps.

The rings were threaded on to a horizontally orientated fixed position surgical steel rod and on to a metal stirrup attached by a short silk thread (Mersilk 000) to an FT03 Grass force transducer. This transducer was mounted on a micromanipulator to enable the rings to be stretched. Rings were suspended in 25ml jacketed glass organ baths, filled with physiological salt solution, maintained at 37^{o}C and gassed with 95%O_2 5%CO_2. All tubing connections were siliconised plastic. The FT03 tranducer was connected to a calibrated Grass Model 7 Polygraph with flat bed chart recorder. Isometric forces were recorded as a function of time. Rings were allowed to equilibrate for thirty minutes after being placed in the bath prior to the determination of optimal resting force.

2.1.2 Young pigs

Immature animals were anaesthetised with an intraperitoneal injection of pentobarbital and exsanguinated. Animals less than 2 hours of age, three days, ten days, six weeks, ten weeks and fifteen weeks old were studied. The heart and lungs were removed en

bloc and the intralobar pulmonary artery dissected out and mounted as described for the adult. Lungs from newborn animals were obtained from a single litter, all other lungs from at least three litters.

2.1.3 Fetal pigs

The fetuses (three weeks premature) were delivered by caesarian section and killed with an overdose of halothane and nitrous oxide. The heart and lungs were removed en bloc and the intralobar pulmonary artery dissected out and mounted as described for the adult. Fetal lungs were obtained from a single litter.

2.2 Preparation and mounting of airway smooth muscle

2.2.1 Tracheal smooth muscle

Pig lungs from freshly killed adult Large White pigs (200lb weight) were transported from a local abbatoir in pregassed (95%O_2 5%CO_2) cold (4^{o}C) physiological salt solution. The trachea was dissected free from surrounding connective tissue. Tracheal smooth muscle strips were prepared by cutting the ventral aspect of the cartilage rings to expose the luminal surface. The epithelium was then carefully dissected off, revealing the transverse orientation of the muscle fibres. Parallel muscle strips 5-7mmm wide were dissected out and 3.0 silk thread tied to each end of the muscle strip. The silk thread was used to tie the strip to a fixed anchor in the organ bath, and to an FT03 Grass force transducer. This transducer was mounted on a micromanipulator enabling the srtips to be stretched. Muscles were suspended in 25 ml jacketed glass organ baths, filled with physiological salt solution, maintained at 37^{o}C and gassed with 95%O_2 5%CO_2 (Air Products). All tubing connections were siliconised plastic. The FTO3 transducer was connected to a calibrated Grass Model 7 Polygraph with flat bed chart recorder. Isometric forces were recorded as a function of time.

2.2.2 Adult pigs: Bronchial rings

Rings of bronchi, 3-4mm wide, from third and fifth order bronchi were dissected out. The epithelium was removed by carefully rubbing he luminal surface of the rings with a pair of watchmaker forceps. Bronchial preparations were threaded on to a horizontally orientated fixed position surgical steel rod and a metal stirrup attached by a short silk thread to a FTO3 Grass Force transducer. The bronchial rings were suspended in organ baths with the rest of the set-up as described above.

2.2.3 Young pigs

Immature animals were anaesthetised with an intraperitoneal injection of sodium pentobarbital and exsanguinated. Animals aged ten weeks, ten days, three days, and newborn were studied. The heart and lungs were dissected out en bloc and third order bronchial rings dissected out and mounted as for the adult.

2.3 Optimal resting length in vascular smooth muscle

Potassium chloride (KCl) was added to the 25ml organ bath to produce a final concentration of 40mM. The artery was allowed to contract until the force reached a plateau. The artery was then repeatedly washed with physiological salt solution until the initial force was regained and the ring allowed to equilibrate for 15 minutes. The ring was then stretched and stimulated again with KCl. The procedure was repeated until there was no further increase in the KCl generated contraction with stretch. The rings were then deemed to be at their optimal resting length and force.

2.4 Optimal resting length in airway smooth muscle

Muscle were stimulated via two parallel plate electrodes with 25Hz, 0.5ms, 15V pulses for thirty seconds at five minute intervals. Pulses were generated by a Grass S44 square wave generator and were passed through a current

amplifier (Mayo Section of Engineering). The physiological salt solution in which the muscles were bathed was changed every fifteen minutes. The muscles were progressively stretched and electrically stimulated until the force response produced was consistent and maximal. The muscles were then deemed to be at their optimal length and resting force. The response to acetylcholine (10^{-4}M for tracheal strips and 10^{-3}M for bronchial rings) was then determined, and deemed to be maximal. The muscles were then repeatedly washed until resting force was regained. Various experimental protocols (as described below) were then performed.

2.5 Preparation of drugs

All drugs were freshly prepared each day in distilled water. Indomethacin was dissolved in the presence of an equimolar amount of sodium carbonate. UK 14304 was dissolved in 50 microlitres of 0.1M HCl, which in itself had no effect on the pulmonary arteries. Drugs were added in 100 microlitre aliquots and the concentration of drugs are reported as the final molar concentration in the organ bath.

For the preparation of nitric oxide, helium was bubbled through distilled water for three hours to remove dissolved oxygen. Nitric oxide was then bubbled through this solution for one hour to produce a saturated solution of nitric oxide. This was then serially diluted in distilled water pregassed with helium.

2.6 Experimental protocols for vascular smooth muscle

Cumulative concentration-response curves were produced by adding 100 microlitre aliquots of agonist to the bath in half log molar increments, when the response to the previous dose had stabilised.

Some arteries were incubated with inhibitors or antagonists prior to the production of concentration-

response curves. All inhibitors or antagonists were added after optimal resting force had been determined, and were present for thirty minutes prior to and throughout the subsequent concentration-response curve. Any change in baseline force with incubation of the agents was noted.

For relaxant agents, concentration-response curves were performed on arteries at resting force or with agonist-induced, stable increased tone.

2.6.1 Muscarinic receptor mediated responses

Release by acetylcholine of endothelium-derived relaxing factor in adult pig pulmonary arteries

Pulmonary arteries from adult pigs, with and without endothelium, were contracted with phenylephrine to 85±22% and 83±16% respectively of their maximum contraction to phenylephrine. A concentration-response curve to acetylcholine (10^{-9}M to 10^{-4}M in log increments) was then performed.

To determine if relaxations induced by acetylcholine were dependent upon the agonist used to induce tone, the effect of 10^{-6}M acetylcholine was studied in arteries with and without endothelium precontracted with KCl to their EC50, and on arteries with endothelium contracted with $10^{-5.5}$M histamine in the presence of 10^{-5}M indomethacin.

The role of different muscarinic receptors in mediating endothelium-derived relaxing factor release was determined in arteries with endothelium and without endothelium contracted with 10^{-6}M phenylephrine. The effect of 10^{-6}M acetylcholine on these precontracted arteries was studied in the absence of muscarinic receptor antagonists, and in arteries with endothelium in the presence of the M1 receptor antagonist pirenzipine

(10^{-7}M), the M2 receptor antagonist gallamine (10^{-7}M) or the M3 receptor antagonist 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP)(10^{-7}M).

2.6.2 Adrenoreceptor mediated responses

Effect of inhibitors of endothelium-derived relaxing factor on the response to noradrenaline in adult pig pulmonary arteries

Cumulative concentration-response curves to noradrenaline (10^{-9}M to 10^{-4}M) were obtained by adding the drug in half log molar increments. Noradrenaline concentration-response curves were also produced in rings which were incubated with either the cyclooxygenase inhibitor indomethacin (10^{-5}M) or the inhibitors of endothelium-derived relaxing factor, N-g-methyl-L-arginine (LNMMA)($3x10^{-5}$M) and n-w-nitro-L-arginine (LNAME)($3x10^{-6}$M). In other experiments the guanylate cyclase inhibitor methylene blue (10^{-5}M) was added.

Effect of adrenoreceptor antagonists on the response to noradrenaline in adult pig pulmonary arteries

Cumulative concentration-response curves to noradrenaline were obtained in the presence of the following antagonists, the alpha-1 adrenoreceptor antagonist prazosin (10^{-7}M), the alpha-2 adrenoreceptor antagonists yohimbine (10^{-6}M) and tolazoline (10^{-5}M) and the beta adrenoreceptor antagonist propranolol (10^{-6}M).

Concentration-response curves to alpha-1 and alpha-2 adrenoreceptor agonists in adult pig pulmonary arteries

The effect of the alpha-1 adrenoreceptor agonist phenylephrine and the alpha-2 adrenoreceptor agonists clonidine and UK 14304, on arteries at resting force, were assessed by construction of cumulative concentration-response curves (10^{-9} to 10^{-4}M) in half log molar increments.

Effect of inhibitors of endothelium-derived relaxing factor and adrenoreceptor antagonists on the response to noradrenaline in immature pig pulmonary arteries

Pulmonary arteries were studied from pigs aged fifteen weeks, ten weeks, ten days, three days, newborn and fetal pigs. Cumulative concentration-response curves to noradrenaline (10^{-9} to 10^{-4}M) were obtained in the presence of the cyclooxygenase inhibitor indomethacin (10^{-5}M). In arteries from pigs aged fifteen weeks noradrenaline concentration-response curves were also obtained in arteries preincubated for thirty minutes with LNAME ($3X10^{-6}$M), prazosin (10^{-7}M) and yohimbine (10^{-6}M).

2.6.3 Histamine receptor mediated responses

Effect of histamine on adult and immature pig pulmonary arteries

Concentration-response curves to histamine (10^{-9} to 10^{-4}M in half log molar increments) in the presence and absence of indomethacin (10^{-5}M) were constructed in pulmonary arteries from adult pigs. It was not possible to construct cumulative concentration-response curves in pulmonary arteries from neonatal pigs as contractions were not well sustained. Instead the response to a single exposure to 10^{-4}M histamine was compared.

2.6.4 Response to potassium

Concentration-response curves to potassium chloride (10 to 100mM in 10mM increments) were constructed in pulmonary arteries from adult and three day old pigs.

2.6.5 Response to nitric oxide and sodium nitroprusside

Response to nitric oxide in adult and immature pig pulmonary arteries

Pulmonary arteries from adult pigs, with and without endothelium, were contracted with either 10^{-4}M phenylephrine or 80mmol KCl and the effect of $10^{-5.5}$M nitric oxide compared. A non cumulative concentration-response curve to nitric oxide ($10^{-8.5}$ to $10^{-5.5}$M in log molar increments) was performed on arteries with and without endothelium contracted with 10^{-4}M phenylephrine. Pulmonary arteries from neonatal pigs were contracted with 10^{-5}M Prostaglandin $F2_{alpha}$ ($PGF2_{alpha}$) in the presence of 10^{-5}M indomethacin, since they contracted poorly to phenylephrine. A concentration-response curve was then constructed to nitric oxide ($10^{-8.5}$M to $10^{-5.5}$M in log increments).

Response to sodium nitroprusside in adult and immature pig pulmonary arteries

Sodium nitroprusside concentration-response curves (10^{-9}M to 10^{-4}M in log molar increments) were performed on arteries precontracted with phenylephrine. The effect of 10^{-4}M sodium nitroprusside was compared in pulmonary arteries from adult pigs precontracted with 10^{-4}M histamine or 80mmol KCl; and on arteries from fifteen week , 10 week, 10 day, 3 day old pigs precontracted with 10^{-4}M noradrenaline.

2.6.6 Response to oxygen

Effect of hypoxia on the response to noradrenaline in adult pig pulmonary arteries

A concentration-response curve to noradrenaline (10^{-9}M to 10^{-4} in half log increments) was performed on pulmonary arteries with and without endothelium from adult pigs. Instead of the normal gas used to oxygenate the

physiological salt solution the arteries were exposed to physiological salt solution bubbled with 8%O_2 5%CO_2 throughout the experiment.

Effect of hypoxia on the contractile response in adult and immature pig pulmonary arteries

The effect on force of altering the oxygen concentration was studied. Pulmonary arteries from adult, and six week old pigs were contracted with 10^{-4}M phenylephrine, from ten day old and newborn pigs with 10^{-5}M $PGF2_{alpha}$, since the contractile response to adrenergic agents was so diminished in the neonatal age group. When a stable contraction was obtained, the oxygen concentration in the organ bath was reduced by changing the gas bubbling the physiological salt solution from 95%O_2 5%CO_2 (normoxic) to 8%O_2 5%CO_2 (hypoxic). After twenty minutes of bubbling with the hypoxic mix, and when a new stable force plateau had been reached, the gas was switched back to the normoxic mix and after twenty minutes the force plateau measured.

2.7 Experimental protocols for airway smooth muscle

2.7.1 Electric field stimulation

Muscles were electrically stimulated at five minute intervals. The effect of changing frequency (0.25-40Hz, 0.5ms, 15V), changing duration (0.1-5ms, 25Hz, 15V) and changing voltage (1-30V, 0.5ms, 25Hz) was determined. The duration-response curve was repeated after incubation of the muscle with 10^{-5}M tetrodotoxin for thirty minutes. Tetrodotoxin was added to prevent stimulation of intramural parasympathetic ganglia from contributing to the contraction. Tracheal smooth muscle strips, third order and fifth order bronchial rings from adult pigs were studied to determine the influence of airway generation upon response. Third order bronchial rings from ten week old, ten day old, three day old and newborn pigs were studied in order to determine

maturational effects. Results were expressed as a percentage of the maximum response to acetylcholine.

2.7.2 Response to histamine, 5HT, acetylcholine and potassium

The response to various contractile agonists was studied in different airway generations in the adult pig, and in the same airway generation in the developing animal. Cumulative concentration-response curves (10^{-9} to 10^{-4}M in half log increments) to histamine, 5HT and acetylcholine were performed in adult tracheal smooth muscle. Acetylcholine concentration-response curves were also constructed in third and fifth order bronchial rings from adult pigs, and third order bronchial rings from pigs aged ten weeks, ten days, three days and newborn. In some rings acetylcholine concentration-response curves were constructed after incubation for thirty minutes with 10^{-5}M tetrodotoxin. The maximum response to 80mmol KCl was determined in third order bronchial rings from adult, ten day and three day old pigs.

2.7.3 Response to ketamine: Tracheal smooth muscle

In tracheal smooth muscle, the effect of the bronchodilator ketamine on both concentration-response curves to agonists and its effect on precontracted muscles was studied.

<u>Effect of ketamine on concentration-response curves</u>

Six tracheal muscle strips from each of six pigs were studied. Two of the six muscle strips from each pig were incubated with tetrodotoxin (10^{-6}M) for thirty minutes before cumulative concentration-response curves to acetylcholine (10^{-9} to 10^{-4}M in half log molar increments) were obtained. The tetrodotoxin was added to prevent stimulation by acetylcholine of intramural parasympathetic ganglia from contributing to the induced contraction.

In two other muscle strips from each pig frequency response curves to electric field stimulation (0.5 ms, 15 V, 0.25-25 Hz) were obtained. Hexamethonium (10^{-5}M) and propranolol (10^{-6}M) were present in the bath for thirty minutes prior to and during the electric field stimulation to prevent stimulation of nicotinic cholinergic and beta adrenoreceptors, respectively.

In the remaining two muscle strips from each pig, concentration-response curves to 1,1,dimethyl-4-phenyl-piperazinium iodide (DMPP) were obtained by adding DMPP non cumulatively to the bathing solution to give concentrations of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M. DMPP stimulates nicotinic cholinergic receptors of the intramural parasympathetic ganglia. To avoid desensitization DMPP was washed out immediately after the contractile response had reached a maximum. Thirty minutes elapsed between each addition of DMPP.

After these initial measurements the six strips were washed with physiological salt solution until they returned to their resting force. In one of each of the above three pairs of muscle strips 10^{-4}M ketamine (racemate) was added to the baths, the other three strips serving as controls for the effect of time. After thirty minutes a second complete set of measurements in response to acetylcholine, electric field stimulation and DMPP was obtained in all muscle strips.

Effect of ketamine on precontracted muscles

Four muscle strips from each of six pigs were contracted with 4.1 ± 3.4 x 10^{-6}M acetylcholine to 50.4 ± 9.1% of their maximum response to 10^{-4}M acetylcholine. One strip had no further drugs added thereby acting as a time control. A cumulative concentration-response curve to ketamine (10^{-7} to $10^{-3.5}$M) was obtained in the three remaining strips, one strip having been incubated for

thirty minutes with 10^{-6}M tetrodotoxin, another incubated for thirty minutes with 10^{-6}M propranolol.

Four muscle strips from each of six pigs were exposed to 63 mM KCl substituted physiological salt solution (KCl substituted for NaCl on an equimolar basis) to determine the maximal response to KCl. Thereafter the muscle strips were repeatedly washed until they returned to resting force, and were then contracted to 52.9 ± 5.1% of this maximal response (EC50) with 27.2 ± 4.8 mmol isotonic KCl physiological salt solution. In one strip of the four strips from each pig concentration-response curve to ketamine (10^{-7} to $10^{-3.5}$M) was obtained (as for the acetylcholine precontracted tissue), a second muscle strip serving as a time control. The third and fourth muscle strips from each pig were incubated with 10^{-6}M atropine for thirty minutes before contracting them again with KCl (29.8 ± 3.3 mmol) to their EC50 (49.5 ± 11.8% of maximal response). In the third muscle strip a ketamine concentration-response curve was obtained, the fourth muscle strip serving as a time control.

2.7.4 Response to ketamine: Bronchial rings

The effect of ketamine on bronchial rings precontracted with acetylcholine, KCl or histamine was studied. Third order and fifth order bronchial rings from adult pigs and third order bronchial rings from ten week, ten day, three day old and newborn pigs were contracted with acetylcholine to the EC50 of the maximal response to 10^{-3}M acetylcholine. A ketamine concentration-response curve (10^{-7} - $10^{-3.5}$M in half log increments) was constructed. The effect of ketamine in this concentration range was also studied in third order adult bronchial rings contracted with KCl to 55.5±6.7% of their maximal response to acetylcholine. The relaxation induced by $10^{-3.5}$M ketamine on third order adult bronchial rings contracted with 10^{-4}M histamine was also determined.

2.7.5 Influence of epithelium

The influence of the epithelium on the response to acetylcholine, electric field stimulation and ketamine was studied. Rings of third order bronchi from ten week old pigs were prepared in the standard way, with rubbing of the ring to remove epithelium. Other rings were left unrubbed so that the epithelium was intact, and responses in rings with and without epithelium could be compared. The maximal response to 10^{-3}M acetylcholine was determined. A concentration-response curve to acetylcholine (10^{-9}M - 10^{-3}M in half log increments) was constructed or a frequency-response curve to electric field stimulation (0.5 - 40Hz, 0.5ms, 15V) was performed. Other rings were contracted to their EC50 with acetylcholine and a ketamine concentration-response curve (10^{-7}M -$10^{-3.5}$M in half log increments) was constructed.

2.7.6 Nonadrenergic noncholinergic responses

Adult tracheal smooth muscle was contracted to EC50 with acetylcholine and then electrically stimulated wilth either 25Hz or 1Hz (0.5ms,15V) for thirty seconds. The effect of beta blockade on the response was studied by incubating the muscle with 10^{-6}M propranolol for 30 minutes prior to contraction with acetylcholine, and the effect of alpha blockade by preincubation with 10^{-6}M phentolamine. The effect of 25 Hz or 1Hz(0.5ms, 15V) electric field stimulation for thirty seconds was also studied in tracheal smooth muscle strips contracted with 10^{-4}M histamine (preincubated with 10^{-6}M phentolamine and 10^{-6}M atropine) in the presence and absence of 10^{-6}M propranolol.

To determine maturational changes in the nonadrenergic noncholinergic response, the effect of 25HZ, 0.5ms, 15V electric field stimulation for thirty seconds was studied in bronchial rings, contracted to an EC50 with acetylcholine, from adult, ten day, three day old and newborn pigs.

2.7.7 Response to oxygen

Bronchial rings from adult, ten day, three day old and newborn animals were contracted with 10^{-3}M acetylcholine in physiological salt solution gassed with 95%O_2 5%CO_2 ("normoxia"). The physiological salt solution was then bubbled with 8%O_2 5%CO_2 87%N_2 ("hypoxia") for twenty minutes before switching back to 95%O_2 5%CO_2.

2.8 Analysis of chart recordings

2.8.1 Calibration

Prior to each experiment the force transducers for each organ bath and the chart recorder were calibrated with gold weight standards. This allowed subsequent conversion of recording deflections to grams force.

2.8.2 Optimal resting length

Before stretching the artery the chart recorder had zero deflection. Stretching the ring caused an upward deflection on the recorder and subsequent stabilisation at an increased level. The displacement from zero baseline was measured in squares and then converted to grams. The displacement from zero baseline when the response to KCl was consistent and maximal was deemed the optimal force, with the muscle at optimal resting length. A similar procedure was followed for airway smooth muscle contracted with electric field stimulation.

2.8.3 Cumulative concentration-response curves

In cumulative concentration-response curves to contractile agonists, after stabilisation at each given concentration, the displacement of the pen recorder from the position at optimal resting force and length was measured and converted to grams. For cumulative concentration-response curves to relaxant agents the

displacement from the level of induced tone was measured and converted to grams.

2.8.4 Data transformation

For some agonists, contractions were converted to a percentage of the maximal contraction obtained with that agonist. In other cases contractions were expressed as a percentage of the maximum contraction obtained in the muscle. Relaxations were expressed as a percentage of the induced tone.

2.9 Statistics

2.9.1 Vascular smooth muscle

Students t-test (2-tailed) was used to compare the baseline characteristics and responses to various agonists of intact pulmonary artery rings with pulmonary artery rings devoid of endothelium. Responses of the vascular smooth muscle in different age groups were compared using one way analysis of variance with the Bonferroni method of correction for multiple comparisons. Statistical significance was taken as $p<0.05$. In all cases n refers to the number of pigs studied. Results are expressed as mean ± standard deviation. The EC50 was defined as the negative log molar concentration of a drug which produces 50% of the maximal response.

2.9.2 Airway smooth muscle

Student's t-test (2-tailed) was used to compare the responses of bronchial rings with epithelium to those without epithelium. Responses in different age groups to various agonists were compared using one way analysis of variance with the Bonferroni method of correction for multiple comparisons. Statistical significance was taken as $p<0.05$. In all cases n refers to the number of pigs studied. Results are expressed as mean ± standard deviation. The EC50 was defined as the negative log molar concentration of a drug which produces 50% of the maximal response.

In determining the effect of ketamine on tracheal muscle strips, the contractile responses were adjusted for the effect of time according to the formula Ct = (C2/C1)*Ck in which Ct = time adjusted control response from ketamine muscle; C1 = response of control muscle (initial measurement); C2 = response of control muscle (measured in parallel with ketamine muscle); Ck = response of ketamine muscle (initial measurement, i.e., before exposure to ketamine). Similarly in precontracted muscles exposed to varying concentrations of ketamine the time adjusted responses were calculated. Concentration-response curves were compared using repeated measures of analysis of variance.

To localise the effect of ketamine, the concentration of DMPP producing 20% of the maximal contraction to acetylcholine was determined. The contractile response in the presence of ketamine at this concentration of ketamine was determined, and the reduction in contractile response due to ketamine was calculated. The same procedure was performed on the concentration-response curves to acetylcholine and the frequency-response curves to electric field stimulation. Statistical differences in the mean reductions in contractile response to DMPP, acetylcholine and electric field stimulation were by paired two-tailed t-test.

2.10 Histology

At the end of each experiment the arteries were fixed in 2.5% glutaraldehyde, embedded in araldite and cut in one micron sections. They were stained with toluidine blue and examined under light microscopy to determine the absence or presence of endothelium.

Bronchial rings and tracheal smooth muscle strips were similarly fixed and the presence or absence of epithelium noted.

RESULTS

VASCULAR SMOOTH MUSCLE

3.1 Muscarinic receptor mediated responses

Release by acetylcholine of endothelium-derived relaxing factor in adult pig pulmonary arteries

In arteries precontracted with phenylephrine, acetylcholine produced a concentration-dependent relaxation only in arteries with endothelium (Fig. 1). No relaxation was seen in the absence of endothelium. Maximum relaxation was achieved with 10^{-6}M acetylcholine. The effect of this concentration of acetylcholine was compared in arteries precontracted with different agonists. In arteries with endothelium, contracted with 30mmol KCl to 53±13.9% of their maximal response to KCl, 10^{-6}M acetylcholine produced minimal relaxation ($p<0.01$ compared with the relaxant effect of acetylcholine on arteries with endothelium precontracted with phenylephrine (Table 1). No relaxation was seen in arteries without endothelium contracted with KCl to 56±6.6% of their maximum response to KCl. In arteries with endothelium, contracted with $10^{-5.5}$M histamine, 10^{-6}M acetylcholine produced 27.9±6% relaxation (not significantly different from that seen in arteries precontracted with phenylephrine)(Table 1).

Acetylcholine (10^{-6}M) produced relaxation in arteries with endothelium precontracted with phenylephrine (10^{-6}M) but had no effect on arteries without endothelium (Table 1). Neither pirenzipine(10^{-7}M) nor gallamine (10^{-7}M) altered the percentage relaxation from phenylephrine precontraction, induced by 10^{-6}M acetylcholine. The relaxation was significantly reduced ($p<0.05$) by 4-DAMP(10^{-7}M)(Table 2).

Figure 1: Concentration-response curve to acetylcholine

Concentration-response curve to acetylcholine in pulmonary arteries with (o, n=3) and without (●, n=3) endothelium from adult pigs. Responses are expressed as % relaxation from precontraction with phenylephrine. (mean ± S.D.)

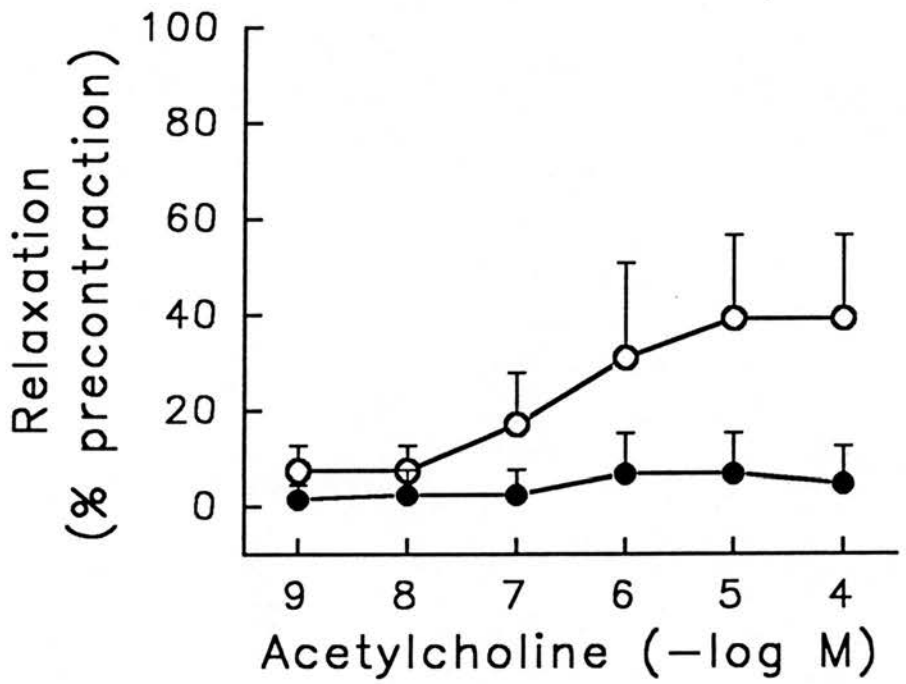

Relaxation
(% precontraction)
100
80
60
40
20
0
9
8
7
6
5
4
Acetylcholine (−log M)

TABLE 1: Effect of acetylcholine (10^{-6}M) on pulmonary artery rings with and without endothelium

Agonist	Contraction (g) + E	Contraction (g) - E	Relaxation + E	Relaxation - E
PE	0.86±0.37	1.78±1.2	38.6±9.4	0
KCl	1.32±0.85	1.47±0.73	3.6±10.7*	0
Histamine	2.3±0.98	-	27.9±6.0	-

Contraction is expressed in grams.

Relaxation is expressed as a % of precontraction of the muscle.

* $p<0.01$ compared with PE (phenylephrine), all n=3.

E = endothelium

TABLE 2: Effect of acetylcholine (10^{-6}M) on intact pulmonary artery rings contracted with phenylephrine in the presence of muscarinic antagonists

	Control	Pirenzipine	Gallamine	4-DAMP
Relaxation (% precontraction)	38.6±9.4	37.5±6.1	42.6±19.1	2.0±3.4*

* $p<0.05$ compared with control, all n=3

3.2 Adrenoreceptor mediated responses

Noradrenaline responses in adult pig pulmonary artery

There was no significant difference in the resting force or maximum contraction of pulmonary arteries with or without endothelium (Table 3).

TABLE 3: Resting tension and maximum contraction in pulmonary arteries with and without endothelium

Agonist	n	Endothelium	Resting force (g)	Maximum contraction (g)
noradrenaline	10	with	1.02±0.2	1.67±1.1
	7	without	1.12±0.4	1.62±0.8
phenylephrine	3	with	1.03±0.9	1.30±0.5
	3	without	1.26±0.9	1.13±0.4
UK14304	6	with	0.7±0.2	0.34±0.4
	7	without	1.04±0.2	0.60±0.5
Clonidine	3	with	0.93±0.5	0
	2	without	1.25±1.0	0.05±0.07

In arteries with endothelium, noradrenaline caused a concentration-dependent increase in tone at low concentrations (Fig. 2a). At high concentrations (10^{-5} to 10^{-4}M) there was a significant endothelium dependent relaxation ($p<0.001$ compared with arteries without endothelium. By contrast, arteries without endothelium showed only a contractile response to noradrenaline. The EC50 value for noradrenaline was similar in tissues with and without endothelium (Table 4). The cyclooxygenase inhibitor indomethacin (10^{-5}M) did not alter the EC50 in arteries with, or without endothelium, and did not inhibit the endothelium-dependent relaxations to noradrenaline (Table 4).

<u>Effect of inhibitors of endothelium-derived relaxing factor on the response to noradrenaline in adult pig pulmonary artery</u> (Table 4)

Incubation with either methylene blue (10^{-5} M)(Fig. 2b) or methylene blue in combination with indomethacin (10^{-5}M) tended to reduce but did not abolish the relaxation to noradrenaline in arteries with endothelium (%

contraction $p<0.05$ compared with arteries without endothelium).

TABLE 4: EC50 for agonists with and without inhibitors of endothelium-derived relaxing factor; and response to 10^{-4}M noradrenaline as a percentage of maximum response to noradrenaline

E	+	+	+	-	-	-
Agonist	n	EC50	%Contr	n	EC50	%Contr
NE	10	6.4±0.3	76.2±14.5	7	6.7±0.5	99±0.9
NE & indo	10	6.5±0.3	69.9±20.2	7	6.8±0.2	96±6.3
NE & MB	7	6.8±0.3#	88.0±3.2	9	6.7±0.2	98±2.6
NE&MB&Indo	7	6.7±0.2#	88.0±5.0	7	6.6±0.3	97±2.6
NE &LNMMA	6	6.6±0.4	89.5±9.1	6	6.4±0.4	98±2.6
NE &DNMMA	5	6.7±0.1	62.6±12.5	5	6.6±0.3	92±5.2
NE &LNAME	6	6.3±0.3	100#	6	6.5±0.3	99±0.3

# $p<0.05$ compared with NE control

Abbreviations:
%Contr % contraction
NE noradrenaline
MB methylene blue
Indo indomethacin
+ endothelium present
- endothelium absent

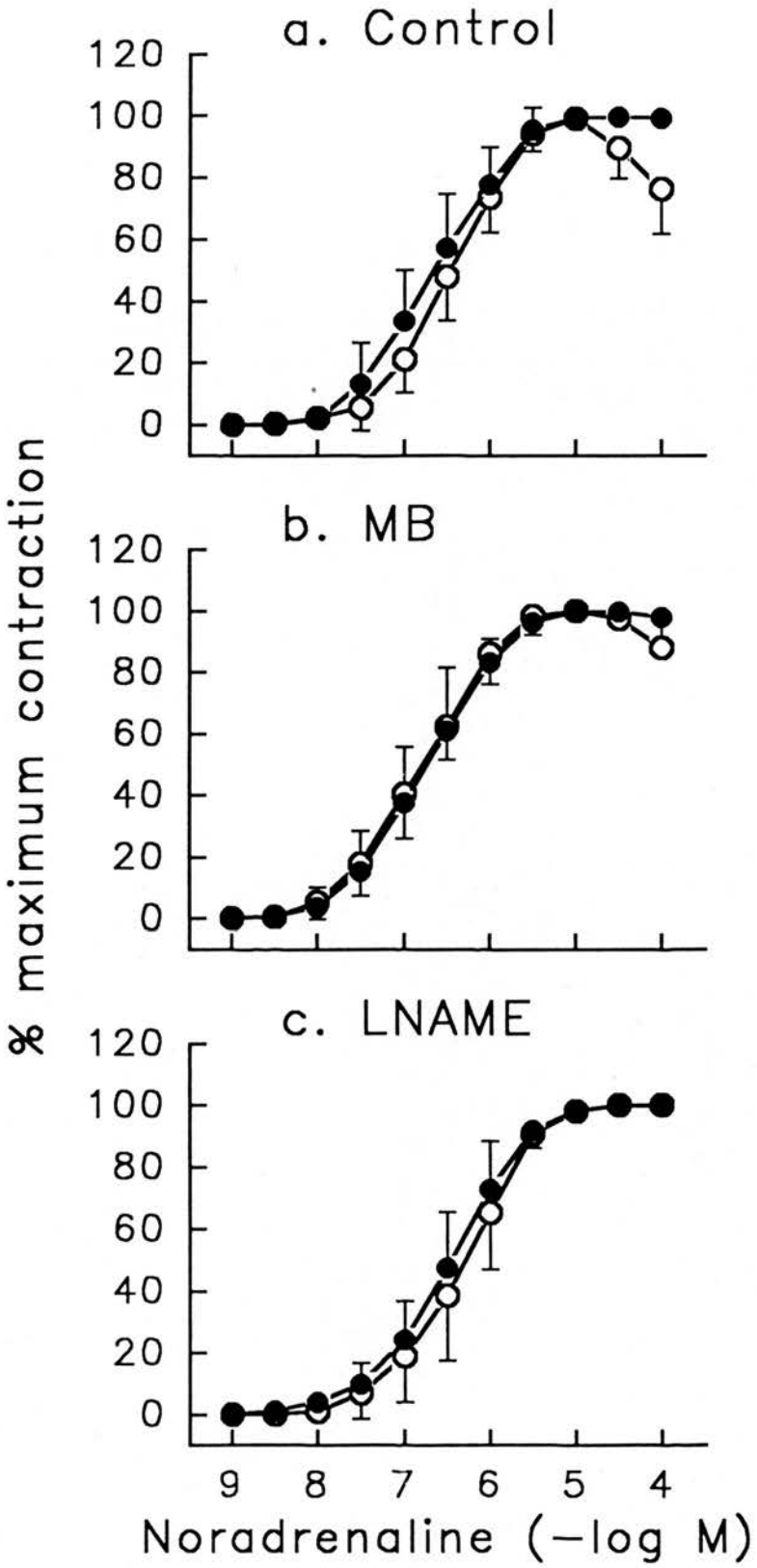
Neither LNMMA ($3x10^{-5}$M) nor DNMMA affected the relaxation that occurred at high concentrations of noradrenaline in arteries with endothelium (Table 4). However, LNAME ($3x10^{-6}$M) completely abolished the response (Fig. 2c). None of these agents had any effect on the response to noradrenaline in arteries without endothelium.

Figure 2: Effect of antagonists of endothelium-derived relaxing factor on the concentration response curve to noradrenaline in pulmonary arteries from adult pigs

Figure 2a: Concentration-response curve to noradrenaline in pulmonary arteries with (o, n=10) and without (●, n=7) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 2b: Concentration-response curve to noradrenaline in the presence of 10^{-5}M methylene blue, in pulmonary arteries with (o, n=7) and without (●, n=9) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 2c: Concentration-response curve to noradrenaline in the presence of $3x10^{-6}$M LNAME, in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).


a. Control
b. MB
c. LNAME
% maximum contraction
120
100
80
60
40
20
0
9
8
7
6
5
4
Noradrenaline (−log M)

Effect of adrenoreceptor antagonists on the response to noradrenaline in adult pig pulmonary artery

The beta-adrenoreceptor antagonist, propranolol (10^{-6}M), did not affect either the EC50 value for noradrenaline or the maximal response to noradrenaline in arteries with or without endothelium (Fig. 3a) (Table 5). In addition, there was no effect of propranolol on the endothelium-dependent relaxation to noradrenaline.

TABLE 5: EC50 for adrenoreceptor agonists with and without antagonists, and response to 10^{-4}M noradrenaline as a percentage of maximal contraction

E	+	+	+	-	-	-
agonist	n	EC50	%contr	n	EC50	%contr
NE	10	6.4±0.3	76.2±14.5	7	6.7±0.5	99±2.8
NE & prop	5	6.6±0.3	68.8±10.7	5	6.9±0.1	98.4±1.5
NE & tolaz	6	5.5±0.1**	100#	6	5.7±0.1**	100
NE & yohimbine	7	5.2±0.3**	100#	6	5.3±0.2**	100
NE & prazosin	6	5.5±0.5**	84.9±5.5	6	5.5±0.4**	100
PE	3	6.2±0.2	-	3	6.3±0.1	-
UK14304	6	6.8±0.3*	-	7	6.5±0.4	-

% contraction in comparison with NE control: # $p<0.05$

EC50 in comparison with NE control: * $p<0.05$, ** $p<0.01$

Abbreviations: NE noradrenaline
tolaz tolazoline
prop propranolol
E endothelium

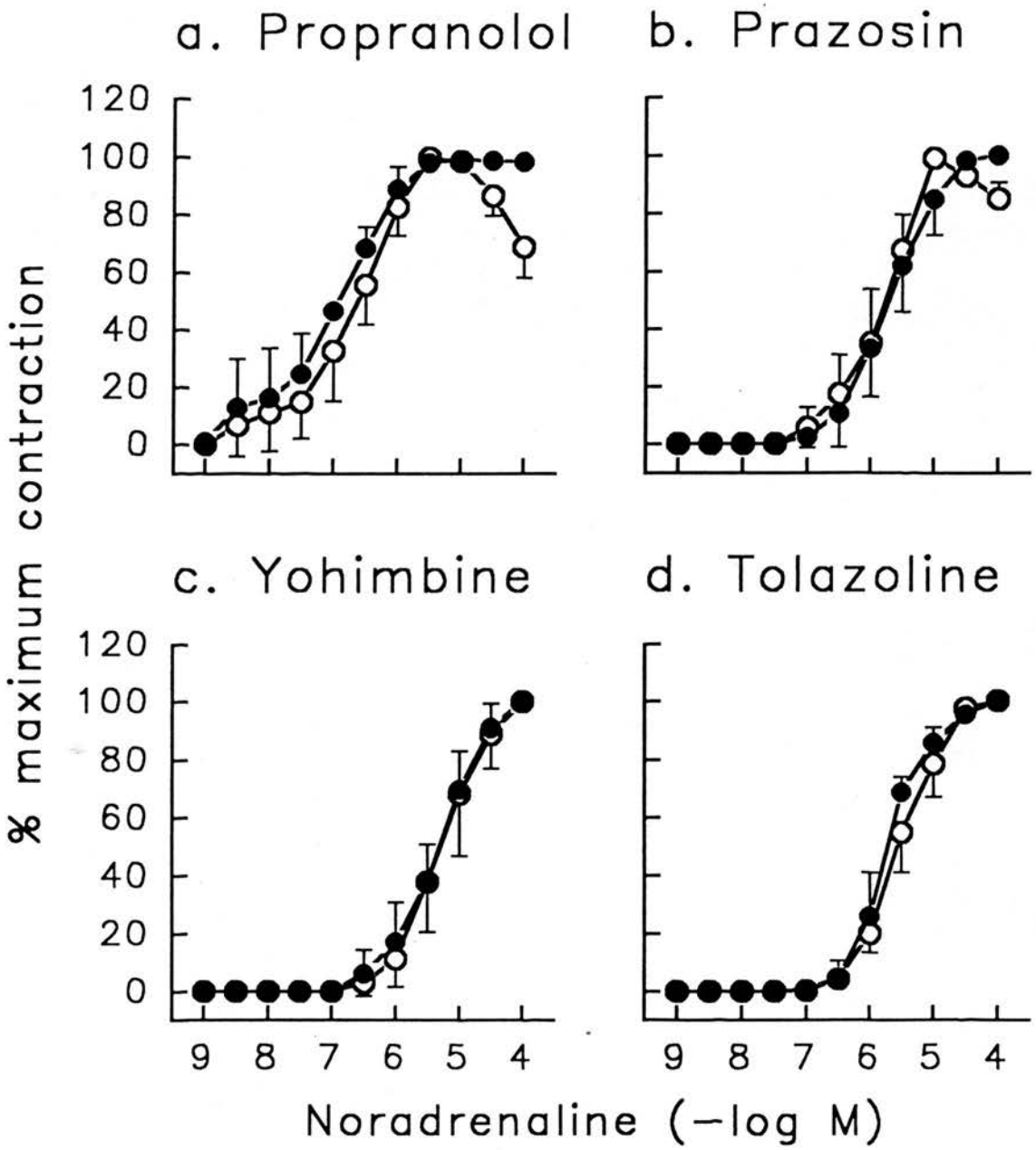
Figure 3: Effect of adrenoreceptor antagonists on the concentration-response curve to noradrenaline in pulmonary arteries from adult pigs

Figure 3a: Concentration-response curve to noradrenaline in the presence of 10^{-6}M propranolol, in pulmonary arteries with (o, n=5) and without (●, n=5) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 3b: Concentration-response curve to noradrenaline in the presence of 10^{-7}M prazosin, in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 3c: Concentration-response curve to noradrenaline in the presence of 10^{-6}M yohimbine, in pulmonary arteries with (o, n=7) and without (●, n=6) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 3d: Concentration-response curve to noradrenaline in the presence of 10^{-5}M tolazoline, in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).


a. Propranolol
b. Prazosin
c. Yohimbine
d. Tolazoline
% maximum contraction
120
100
80
60
40
20
0
9 8 7 6 5 4
Noradrenaline (−log M)

The alpha-1 adrenoreceptor antagonist prazosin (10^{-7}M) produced a significant shift in the EC50 in rings with and without endothelium ($p<0.01$), but did not alter the magnitude of the endothelium-dependent relaxation (Fig. 3b). By contrast, the alpha-2 adrenoreceptor antagonists yohimbine (Fig. 3c) and tolazoline (Fig. 3d) completely abolished the endothelium-dependent relaxation. They also produced a significant rightward shift in the concentration-response curves for noradrenaline ($p<0.01$ for yohimbine and tolazoline).

Concentration-response curves to alpha-1 and alpha-2 adrenoreceptor agonists in adult pig pulmonary artery (Tables 3, 5)

The alpha-1 adrenoreceptor agonist phenylephrine caused concentration-dependent increases in tone in pulmonary arteries with and without endothelium. There was no endothelium-dependent relaxation seen with this agonist. The EC50 values for arteries with and without endothelium were similar to those seen for noradrenaline. The maximum contractile response was not significantly different from that seen with noradrenaline.

The alpha-2 adrenoreceptor agonist clonidine caused no increase in tone in pulmonary arteries with endothelium and a small increase in tone in arteries without endothelium in the concentration range 10^{-6} to 10^{-4}M (Table 3). The alpha-2 adrenoreceptor agonist UK 14304 caused a slight increase in tone in arteries with and without endothelium (Table 3). There was no endothelium-dependent relaxation.

Noradrenaline responses in immature pig pulmonary artery

At all ages there was no significant difference in resting force in pulmonary arteries with endothelium compared with arteries denuded of endothelium. There was no significant difference in the weight of the pulmonary artery rings at different ages (Table 6).

Table 6: Weight of pulmonary artery rings from pigs of different ages

age	n	weight	endothelium
adult	8	9.75±2.2	with
	8	10.6±3.2	without
10w	7	7.75±3.5	with
	7	8.9±4.4	without
10d	7	5.9±1.8	with
	7	4.5±2.4	without
3d	10	5.7±2.3	with
	5	5.6±1.8	without
NB	8	7.8±3.0	with
	7	8.4±3.2	without

Effect of inhibitors of endothelium-derived relaxing factor and adrenoreceptor antagonists on the response to noradrenaline in immature pig pulmonary artery

1. Fifteen week old pigs

In all fifteen week old pigs, noradrenaline produced a concentration-dependent increase in tone in arteries with and without endothelium (Fig. 4a)(Table 7). However, at high concentrations ($10^{-5.5}$ to 10^{-4}M) all arteries with endothelium showed relaxation (Fig. 4a).

In the presence of the alpha-1 adrenoreceptor antagonist prazosin (10^{-7}M) endothelium-dependent relaxations to noradrenaline were still present at high concentrations (Fig. 4b)(Table 7).

Figure 4: Effect of various antagonists on the concentration-response curve to noradrenaline in pulmonary arteries from fifteen week old pigs

Figure 4a: Concentration-response curve to noradrenaline in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from fifteen week old pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 4b: Concentration-response curve to noradrenaline in the presence of 10^{-7}M prazosin, in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from fifteen week old pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 4c: Concentration-response curve to noradrenaline in the presence of 10^{-6}M yohimbine, in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from fifteen week old pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

Figure 4d: Concentration-response curve to noradrenaline in the presence of $3x10^{-6}$M LNAME, in pulmonary arteries with (o, n=6) and without (●, n=6) endothelium, from fifteen week old pigs. Responses are expressed as a % of the maximum response to noradrenaline. (mean ± SD).

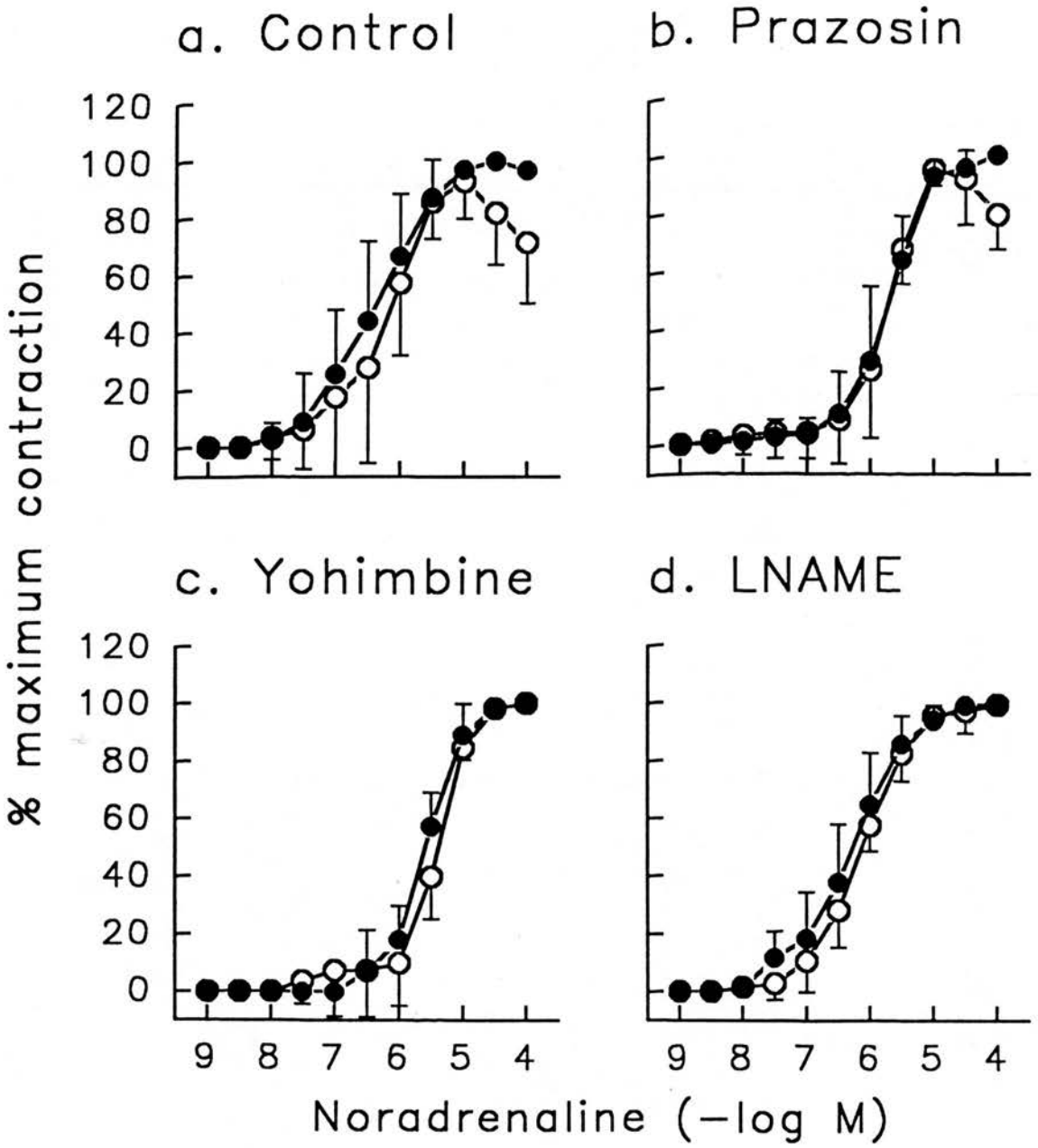

a. Control
b. Prazosin
c. Yohimbine
d. LNAME
% maximum contraction
120
100
80
60
40
20
0
9 8 7 6 5 4
Noradrenaline (−log M)

TABLE 7: Response to 10^{-4}M noradrenaline as a percentage of the maximum response to noradrenaline, and EC50 for 15 week old pigs

Endothelium	+ % max	+ EC50	- % max	- EC50
control	71.5±21.3	6.2±0.6	96.7±3.0	6.5±0.6
prazosin	79.5±12.0	5.7±0.2	100	5.7±0.3*
yohimbine	100*	5.4±0.2*	98.3±3.7	5.6±0.2*
LNAME	98.8±1.8*	6.1±0.2	99.4±1.3	6.3±0.3

In comparison with control values: * $p<0.05$, all n=6

Arteries without endothelium showed only a concentration-dependent increase in tone. In arteries both with and without endothelium, the noradrenaline concentration-response curve was significantly shifted to the right in the presence of the antagonist prazosin ($p<0.05$).

In the presence of the alpha-2 antagonist yohimbine (10^{-6} M) arteries both with and without endothelium showed only a concentration-dependent increase in tone with noradrenaline, and the concentration-response curve was significantly shifted to the right ($p<0.05$) (Table 7)(Fig. 4c). In the presence of the inhibitor of nitric oxide release LNAME ($3x\ 10^{-6}$M), noradrenaline produced an increase in tone in both arteries with and without endothelium, but no endothelium-dependent relaxations were seen (Table 7)(Fig 4d).

2. Ten week old pigs

In pulmonary arteries from ten week old pigs, noradrenaline produced a concentration-dependent increase in tone in arteries with and without endothelium (Tables 8, 9). Only arteries with endothelium showed relaxations

to noradrenaline in the concentration range 10^{-5} to 10^{-4}M. There was no difference in the maximum contraction to noradrenaline or in the EC50 between the ten week and fifteen week old animals.

3. Ten day old pigs

At ten days of age, noradrenaline increased tone in all pulmonary arteries, both with and without endothelium (Tables 8, 9). The maximum contractile response was significantly less than in the fifteen week old animals ($p<0.05$). Only four of eight arteries with endothelium showed relaxations to noradrenaline in the concentration range $10^{-4.5}$ to 10^{-4}M and no artery without endothelium relaxed.

4. Three day old pigs

At three days of age, six out of nine pulmonary arteries with endothelium, and seven out of nine arteries without endothelium responded to noradrenaline with increased tone (Tables 8, 9). The maximum contractile response to noradrenaline was significantly reduced compared with the fifteen week old animals ($p<0.05$). The EC50's for arteries with or without endothelium were significantly shifted to the right compared with the EC50's from ten day, ten week and fifteen week old animals ($p<0.05$). Noradrenaline did not produce relaxation in any of the arteries.

5. Newborn pigs

In the newborn, less than two hours of age, only one of the four pulmonary arteries with endothelium contracted to noradrenaline and then only at the maximal concentration of 10^{-4} M (Tables 8, 9). None of the pulmonary arteries without endothelium reacted to noradrenaline.

6. Fetal pigs

In the fetal studies, two out of the four arteries without endothelium responded to noradrenaline (Tables 8, 9). The maximal contraction and EC50 to noradrenaline was similar to those seen in the three day old animals. No relaxations to noradrenaline were seen.

TABLE 8: Maximum contractile response to noradrenaline and EC50 in pulmonary arteries of pigs of varying ages

Age	E	No. animals	No. animals responding	Maximum contraction (g)	EC50
15w	+	5	5	2.03±0.7	6.2±0.6
	-	5	5	2.23±0.8	6.5±0.6
10w	+	4	4	1.92±0.9	5.92±0.2
	-	4	4	1.90±0.45	6.09±0.3
10d	+	8	8	0.32±0.39*	5.7±0.4
	-	8	8	0.43±0.13*	5.9±0.5
3d	+	9	6	0.17±0.16*	5.1±0.3*
	-	9	7	0.25±0.25*	5.1±0.4*
NB	+	4	1	0.1	4.5
	-	4	0	0	-
fetal	+	4	2	0.25±0.07	4.9±0.5
	-	4	4	0.38±0.15	6.3±1.4

Compared with vessels from 15w old animals *p<0.05

TABLE 9: Response to 10^{-4}M noradrenaline as a percentage of maximum response to noradrenaline

Age	Endothelium	Response	No rings studied	No rings relaxing
15w	+	71.5±21.3	5	5
	-	96.7±3.0#	5	0
10w	+	83.3±11.2	4	4
	-	99.3±1.5#	4	0
10d	+	69.3±44.2	8	4
	-	100#	8	0
3d	+	100*	9	0
	-	100	9	0
NB	+	100	4	0
	-	0	4	0
fetal	+	100	4	0
	-	100	4	0

* $p<0.05$ compared with vessels from 15w old animals

# $p<0.05$ difference in response at 15w, 10w and 10d between rings with and without endothelium

3.3 Histamine receptor mediated responses

Effect of histamine on adult and immature pig pulmonary arteries

In pulmonary arteries from the adult pig, histamine in the presence or absence of indomethacin (10^{-5}M) produced a concentration-dependent increase in force (Fig. 5). There was no significant difference in the maximum force, or the EC50 between arteries with and without endothelium or between arteries in the presence or absence of indomethacin (Table 10).

Figure 5: Concentration-response curve to histamine in pulmonary arteries from adult pigs

Concentration-response curve to histamine in pulmonary arteries with (o) and without (●) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (n=6, mean ± SD)

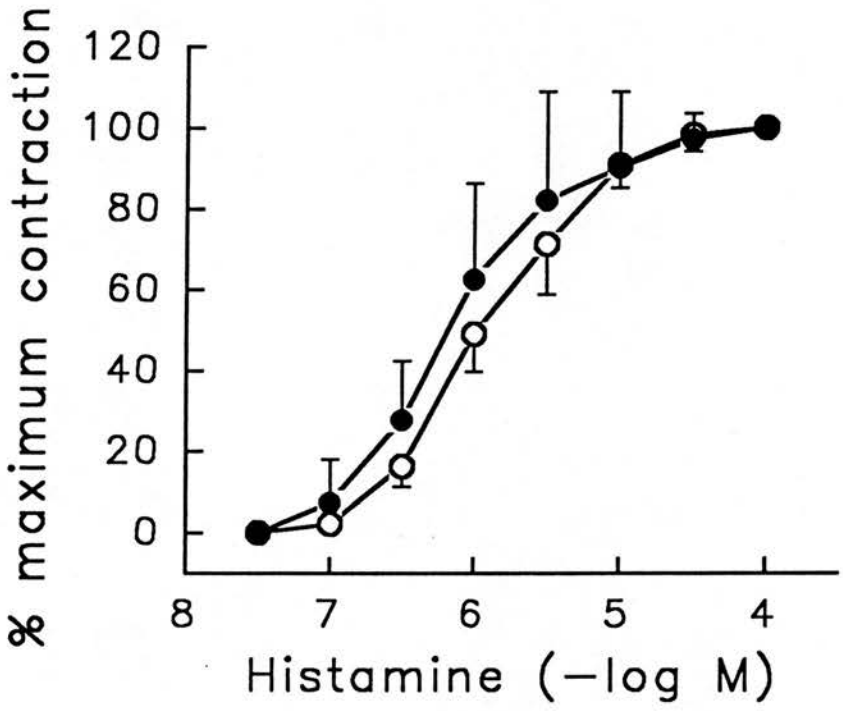

% maximum contraction
120
100
80
60
40
20
0
8
7
6
5
4
Histamine (−log M)

TABLE 10: Effect of histamine, in the presence and absence of 10^{-5}M indomethacin on pulmonary arteries with and without endothelium from adult pigs

	n	force (g) +E	force (g) -E	EC50 +E	EC50 -E
control	5	1.95±0.7	1.24±0.3	5.8±0.4	6.2±0.3
Indo	5	2.06±1.0	1.86±1.4	6.1±0.3	6.3±0.4

Indo= indomethacin

In pulmonary arteries from ten, three day old and newborn pigs, in the presence of indomethacin, contractions to histamine were readily initiated but poorly maintained in rings both with and without endothelium. This made construction of a cumulative concentration-response curve difficult, and the response to histamine was subsequently compared in young pigs using a single concentration of histamine.

TABLE 11: Response in grams to 10-4M histamine and 10^{-5}M $PGF2_{alpha}$ in arteries with and without endothelium from neonatal pigs

Age	Histamine +E	n	Histamine -E	n	PGF +E	n	PGF -E	n
10d	0.51±0.14	5	0.54±0.14	5	0.59±0.2	4	0.63±0.3	4
3d	0.45±0.17	7	0.39±0.14	7	0.4±0.4	5	0.38±0.2	5
NB	0.47±0.2	4	0.35±0.1	4	0.36±0.3	4	0.35±0.2	3

Abbreviation: E = endothelium

There was no difference in the response to histamine (10^{-4}M) in arteries from ten day old, three day old and

newborn pigs, with or without endothelium (Table 11). All arteries responded to histamine, even those which had shown no response to noradrenaline. The response to histamine was similar in all age groups.

3.4 Response to potassium

In the adult pig, KCl produced a concentration-dependent increase in force in arteries with or without endothelium (Fig. 6a). There was no significant difference in the maximum response to KCl or the EC50 in the presence or absence of endothelium (Table 12). In pulmonary arteries from three day old pigs, KCl also produced a concentration-dependent increase in force, and there was no significant difference in the maximal force or EC50 in rings with or without endothelium (Fig. 6b)(Table 12). There was a significant difference in the EC 50 between pulmonary arteries from adult and three day old pigs ($p<0.05$).

TABLE 12: Response to KCl in pulmonary arteries from adult and three day old pigs

Age	force (g) +E	force (g) -E	EC50 +E	EC50 -E
adult n=3	2.45±0.8	2.63±0.67	27.3±3.2	29.6±2.8
3d n=5	0.52 ±0.3	0.35±0.2	46.6±12.9*	53.7±11.0#

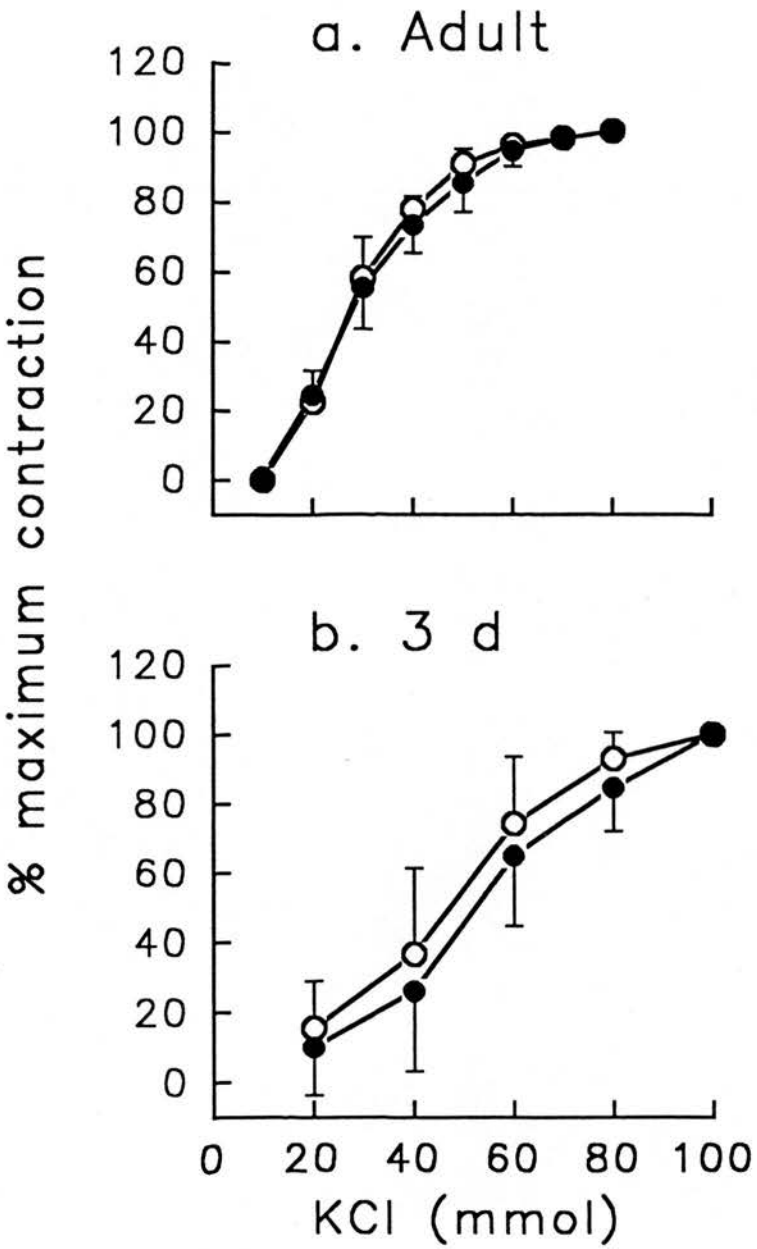
* $p<0.05$ compared with adult

\# $p<0.001$ compared with adult

Figure 6: **Concentration-response curve to potassium chloride in pulmonary arteries from adult and three day old pigs**

Figure 6a: Concentration-response curve to potassium chloride in pulmonary arteries with (o) and without (●) endothelium, from adult pigs. Responses are expressed as a % of the maximum response to noradrenaline. (n=3, mean ± SD).

Figure 6b: Concentration-response curve to potassium chloride in pulmonary arteries with (o) and without (●) endothelium, from three day old pigs. Responses are expressed as a % of the maximum response to noradrenaline. (n=3, mean ± SD).


a. Adult
b. 3 d
% maximum contraction
120
100
80
60
40
20
0
0 20 40 60 80 100
KCl (mmol)

3.5 Response to nitric oxide and sodium nitroprusside

Response to nitric oxide in adult and immature pig pulmonary arteries

Nitric oxide caused a concentration-dependent relaxation in arteries both with and without endothelium, precontracted with 10^{-4}M phenylephrine (Fig. 7a). There was no significant difference in the EC50 for arteries with (6.8±0.11) or without (6.7±0.17) endothelium. Nitric oxide ($10^{-5.5}$M) was equally effective in causing relaxation of arteries contracted with 10^{-4} phenylephrine or 80mmol KCl (Table 13).

Table 13: Effect of nitric oxide (10^{-5}M) on pulmonary arteries from adult pigs contracted with KCl or phenylephrine

Agonist	Relaxation +E	Relaxation -E
KCl	75.2±20.0	91.0±12.7
PE	94.2±11.5	96.5±7.01

Relaxation expressed as a % of precontraction

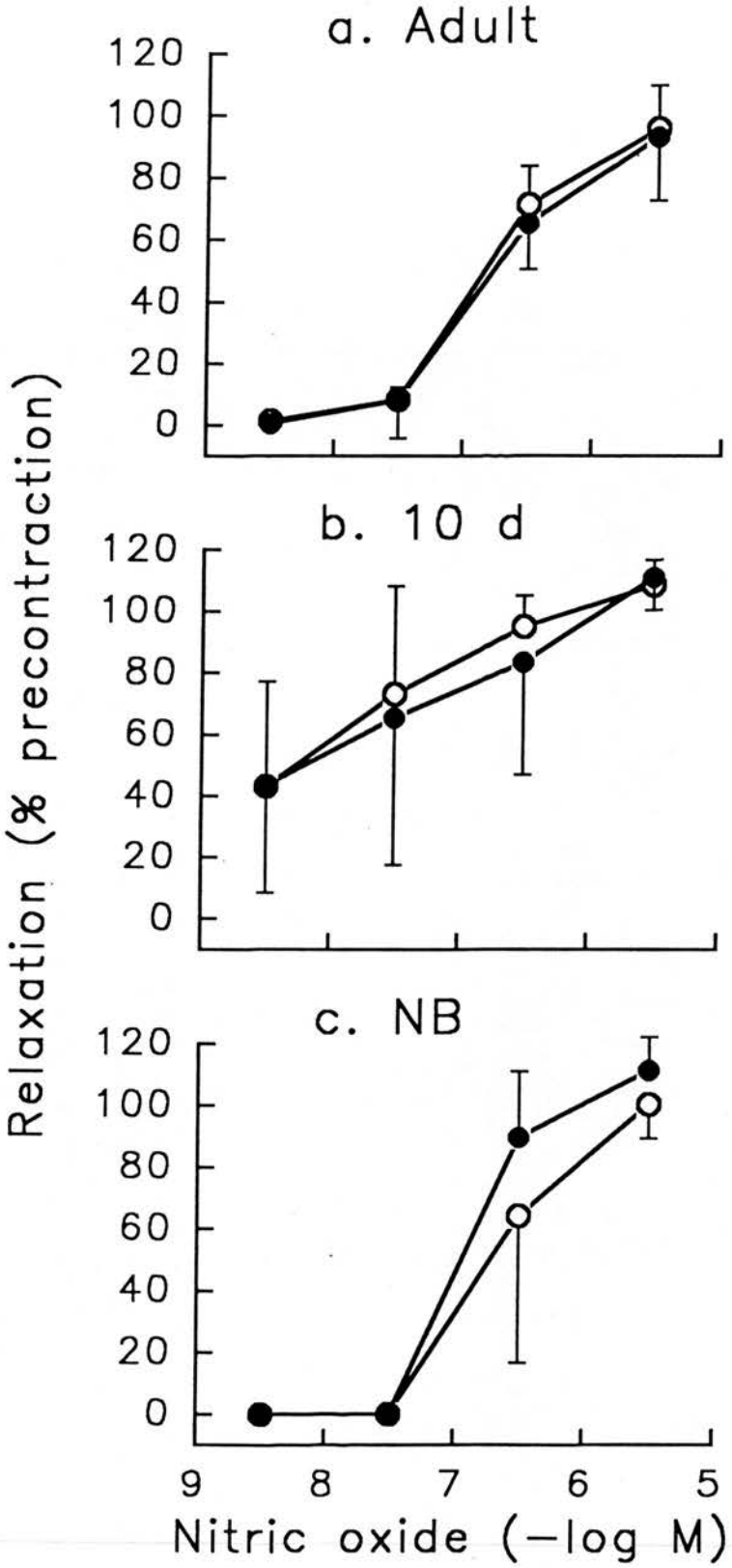
Arteries from newborn, three day and ten day old pigs contracted to 10^{-5}M $PGF2_{alpha}$ (Table 11). There was no significant difference in tone generated between arteries with and without endothelium, or between these neonatal age groups. At all ages arteries with or without endothelium precontracted with $PGF2_{alpha}$ showed a concentration-dependent relaxation to nitric oxide. However, pulmonary arteries from ten day old animals showed a marked response to nitric oxide at $10^{-7.5}$M, whilst arteries from all pigs less than two hours of age and arteries from 2 of 5 animals aged 3 days showed no relaxation at all at this concentration, but did relax at $10^{-6.5}$M.

Figure 7: **Concentration-response curve to nitric oxide in pulmonary arteries from pigs of varying ages**

Figure 7a: Concentration-response curve to nitric oxide (NO) in pulmonary arteries with (o) and without (●) endothelium. Relaxation is expressed as a % of the phenylephrine precontraction. (n=6, mean ± SD).

Figure 7b: Concentration-response curve to nitric oxide (NO) in pulmonary arteries with (o) and without (●) endothelium, from ten day old pigs. Relaxation is expressed as a % of the $PGF2_{alpha}$ precontraction. (n=6, mean ± SD).

Figure 7c: Concentration-response curve to nitric oxide (NO) in pulmonary arteries with (o) and without (●) endothelium, from newborn pigs. Relaxation is expressed as a % of the $PGF2_{alpha}$ precontraction. (n=4, mean ± SD).


a. Adult
b. 10 d
c. NB
120
100
80
60
40
20
0
9
8
7
6
5
Relaxation (% precontraction)
Nitric oxide (−log M)

Response to sodium nitroprusside in adult and immature pig pulmonary arteries

Sodium nitroprusside caused a concentration-dependent relaxation in arteries with and without endothelium contracted to 68.4±21.2% and 69.7±15.6% respectively of their maximal response to phenylephrine (Fig. 8). There was no significant difference in the EC50 for arteries with (7.4±0.5) and without (7.8 ±0.5) endothelium. Sodium nitroprusside (10^{-4}M) also caused relaxation of pulmonary arteries from adult pigs precontracted with 80mmol KCl or 10^{-4}M histamine (Table 14).

TABLE 14: Effect of 10^{-4}M sodium nitroprusside on pulmonary arteries from adult pigs contracted with phenylephrine, KCl or histamine

Contractile agonist	Relaxation +E	n	Relaxation -E	n
PE	149.0±30.4	5	126.0±12.2	5
KCl	81.3±4.1*	4	98.7±11.1*	4
Histamine	68.0±13.8*	3	69.0±8.5*	3

Relaxation expressed as a % of precontraction

* $p<0.02$ compared with vessels contracted with phenylephrine (PE)

Sodium nitroprusside (10^{-4}M) relaxed pulmonary arteries from pigs of varying ages contracted with noradrenaline (Table 15). There was no significant difference in the response to sodium nitroprusside between arteries with and without endothelium.

Figure 8: **Concentration-response curve to sodium nitroprusside in pulmonary arteries from adult pigs**

Concentration-response curve to sodium nitroprusside in pulmonary arteries with (o) and without (●) endothelium, from adult pigs. Relaxation is expressed as a % of the phenylephrine precontraction. (n=5, mean ± SD).

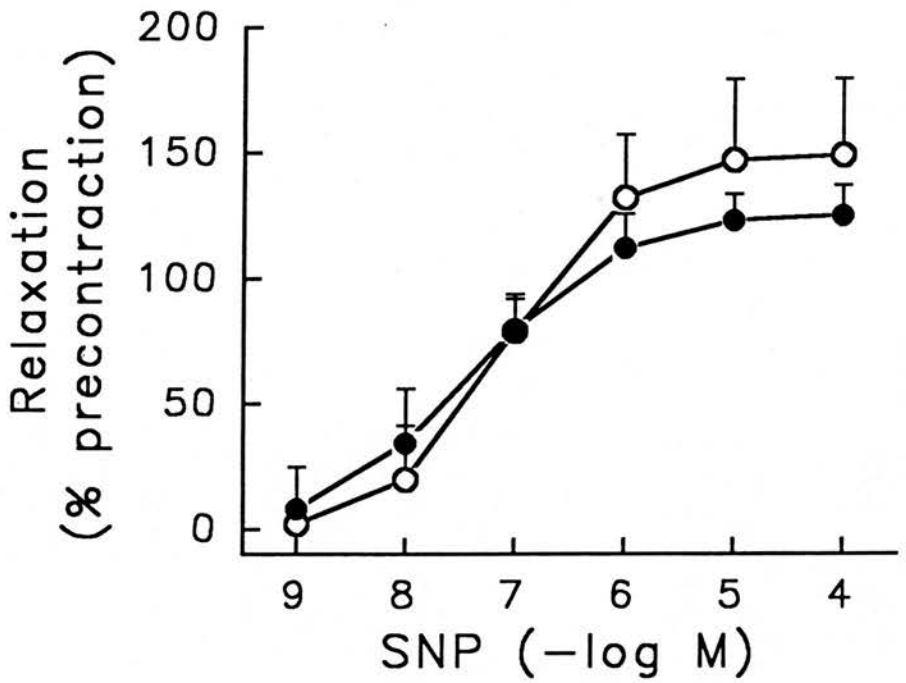

Relaxation
(% precontraction)
200
150
100
50
0
9
8
7
6
5
4
SNP (−log M)

TABLE 15: Effect of 10^{-4}M sodium nitroprusside on pulmonary arteries contracted with noradrenaline, from pigs of varying ages

Age	Relaxation +E	n	Relaxation -E	n
15w	99.7±7.3	4	94.9±11.6	4
10w	105.0±7.0	3	100	3
10d	102.3±4.0	3	114.0±19.8	3
3d	100	3	100	3

Relaxation expressed as a % of precontraction

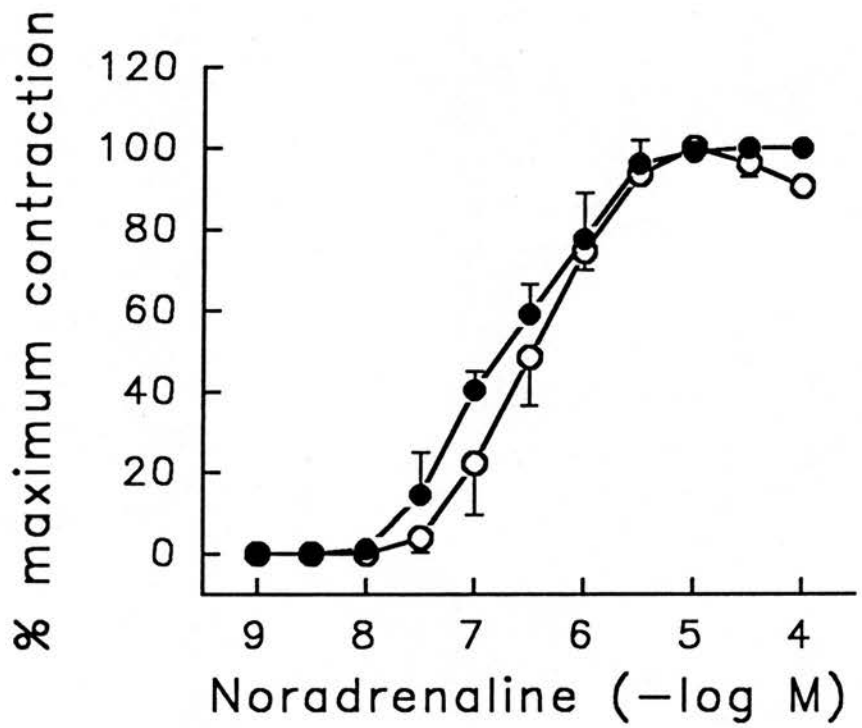
3.6 Response to oxygen

Effect of hypoxia on the response to noradrenaline in adult pig pulmonary arteries

The maximum force developed to noradrenaline was not different in arteries with (1.55±0.8g) or without (1.4±0.95) endothelium, and was not significantly different from that seen in arteries in physiological salt solution bubbled with 95%O_2 5%CO_2 ("normoxia"). Noradrenaline produced a concentration-dependent contraction at low concentrations in arteries with endothelium, and relaxation at higher concentrations (Fig. 9). No relaxations were seen in arteries without endothelium. There was no significant difference in the EC50 in arteries with (6.5±0.17) and without (6.72±0.15) endothelium, and these EC50's were not significantly different from those seen in arteries bubbled in the "normoxic" gas mix. The maximum relaxation to noradrenaline was also not significantly different from that seen in arteries bubbled in the "normoxic" gas mix.

Figure 9: **Concentration-response curve to noradrenaline in pulmonary arteries from adult pigs, maintained in physiological salt solution bubbled with 8%O_2 5%CO_2**

Concentration-response curve to noradrenaline in pulmonary arteries with (o) and without (●) endothelium, from adult pigs. The arteries are maintained in physiological salt solution bubbled with 8%O_2 5%CO_2. Responses are expressed as a % of the maximum response to noradrenaline. (n=6, mean ± SD).


% maximum contraction
120
100
80
60
40
20
0
9
8
7
6
5
4
Noradrenaline (−log M)

Effect of hypoxia on the contractile response in adult and immature pig pulmonary arteries

The "normoxic" gas mix bubbled in physiological salt solution resulted in a pH of 7.4, pO_2 57-59kPa and pCO_2 4.6-4.7 kPa. In the presence of the "hypoxic" gas mix the pO_2 was reduced to 12-15 kPa with no change in pH or pCO_2. The hypoxic gas mix caused a reversible decrease in force in pulmonary arteries with and without endothelium (Table 16).

TABLE 16: Effect of altering oxygen concentration on phenylephrine, $PGF2_{alpha}$ and KCl induced contraction (expressed as a % of the initial contraction in 95%O_2) in pulmonary arteries

Age and agonist		+E 8%O_2	+E return to 95%O_2	-E 8%O_2	-E return to 95%O_2
adult PE	3	57.6±39.0	104±24.7	60.5±25.0	96.0±39.9
6w PE	6	29.7±14.1**	77.3±22.2	60.5±31.1*	86.1±15.1
10d PGF2	4	36.5±30.7**	100±24.0	76.5±17.8*	128.0±26.7
NB PGF2	4	57.2±41.7	100	63.0±43.6	100

* $p<0.05$ compared with initial contraction in normoxia

** $p<0.01$ compared with initial contraction in normoxia

3.7 Response to electric field stimulation

In tracheal smooth muscle increasing frequency (at constant voltage and duration) produced an increase in force. (EF50 5.9±3.9 Hz) The maximal response to electric field stimulation was 78.6±14% of the maximal response to acetylcholine. By 25Hz a plateau in force had been reached (Fig 10a). An increase in voltage (25Hz, 0.5ms) also produced an increase in force with a plateau at 15V (Fig. 10b). Increasing the duration of stimulation (25Hz, 15V) caused an increase in force with a peak at 1ms (Fig. 10c). At longer pulse durations a response was found even in the presence of tetrodotoxin.

Similar responses, of increasing force with increasing frequency were found in third and fifth order adult bronchi and in the immature bronchi (Fig. 11a, b). For third order bronchi the frequency producing 20% of the maximal response was significantly greater in the immature than mature animals (Table 17). Rings from immature animals were less responsive at low voltages than bronchial rings from adult pigs (Table 18). Increasing the duration of stimulus (25Hz, 15V) caused an increase in force in bronchial rings from adult and immature pigs (Figs. 11c, 11d).

TABLE 17: Frequency (Hz) required to produce 20% of maximum force (EF20) in bronchi from pigs of varying ages

	Adult	10w	10d	3d	NB
n	5	6	5	6	4
EF20 (Hz)	1.4±0.5	6.9±1.8*	5.3±2.3*	6.0±3.0*	8.1±1.6#

* $p<0.05$ compared with adult
# $p<0.01$ compared with adult

Figure 10: Response to electric field stimulation in adult porcine tracheal smooth muscle

Figure 10a: Response to electric field stimulation (0.5ms, 15V), at varying frequencies in adult porcine tracheal smooth muscle. Contraction is expressed as a % of the maximum response to acetylcholine. (n=6, mean ± SD).

Figure 10b: Response to electric field stimulation (0.5ms, 25Hz), at varying voltages in adult porcine tracheal smooth muscle. Contraction is expressed as a % of the maximum response to acetylcholine. (n=6, mean ± SD).

Figure 10c: Response to electric field stimulation (25Hz,15V), at varying durations in adult porcine tracheal smooth muscle, in the absence (o) and presence (●) of 10^{-5}M tetrodotoxin. Contraction is expressed as a % of the maximum response to acetylcholine. (n=6, mean ± SD).

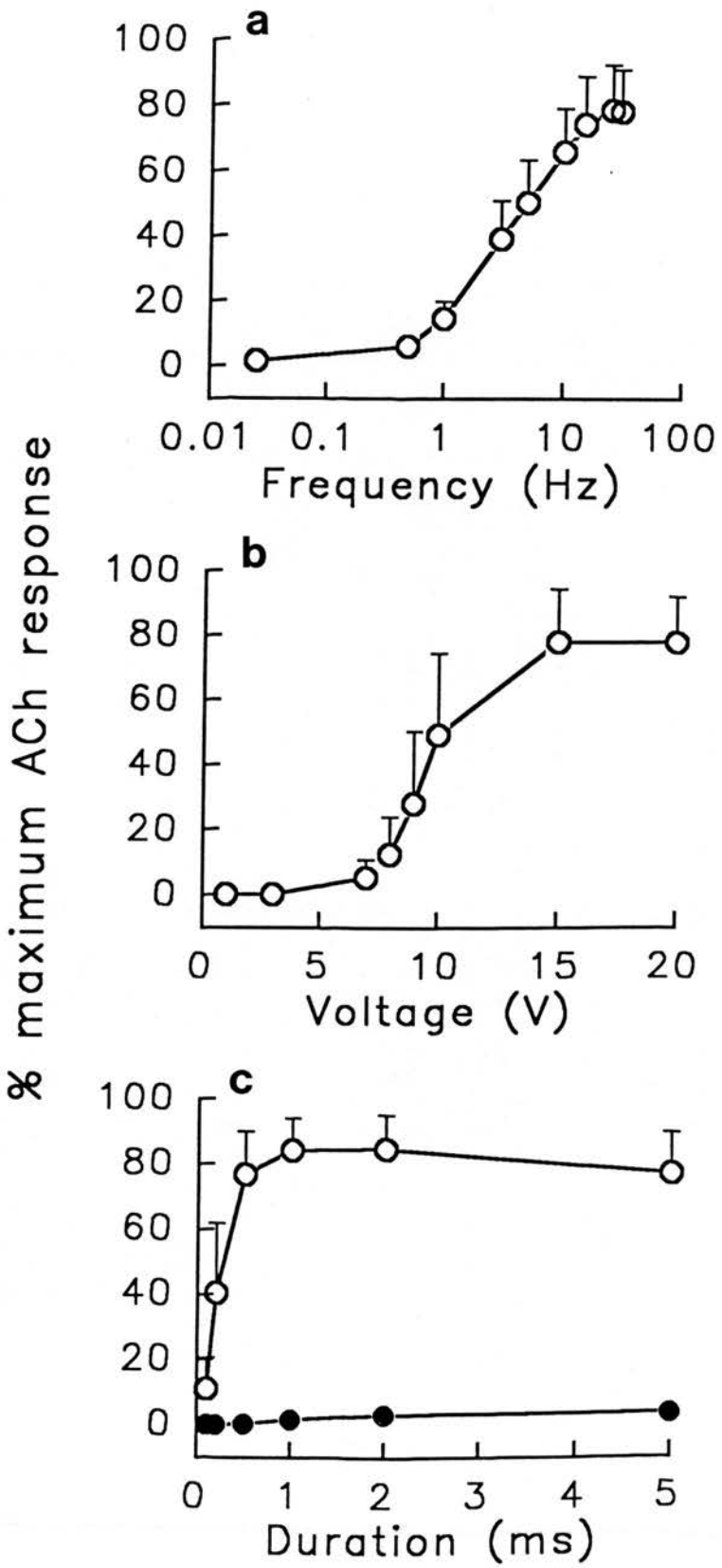

a
100
80
60
40
20
0
0.01
0.1
1
10
100
Frequency (Hz)
b
100
80
60
40
20
0
0
5
10
15
20
Voltage (V)
% maximum ACh response
c
100
80
60
40
20
0
0
1
2
3
4
5
Duration (ms)

Figure 11: **Response to electric field stimulation in third order bronchial rings from pigs of varying ages**

Figure 11a: Response to electric field stimulation (0.5ms, 15V), at varying frequencies in third order bronchial rings from adult pigs. Contraction is expressed as a % of the maximum response to acetylcholine. (n=5, mean ± SD).

Figure 11b: Response to electric field stimulation (0.5ms, 15V), at varying frequencies in third order bronchial rings from newborn pigs. Contraction is expressed as a % of the maximum response to acetylcholine. (n=4, mean ± SD).

Figure 11c: Response to electric field stimulation (25Hz,15V), at varying durations in third order bronchial rings from adult pigs in the absence (□) and presence (■) of 10^{-5}M tetrodotoxin. Contraction is expressed as a % of the maximum response to acetylcholine. (n=5, mean ± SD).

Figure 11d: Response to electric field stimulation (25Hz,15V), at varying durations in third order bronchial rings from newborn pigs in the absence (□) and presence (■) of 10^{-5}M tetrodotoxin. Contraction is expressed as a % of the maximum response to acetylcholine. (n=4, mean ± SD).

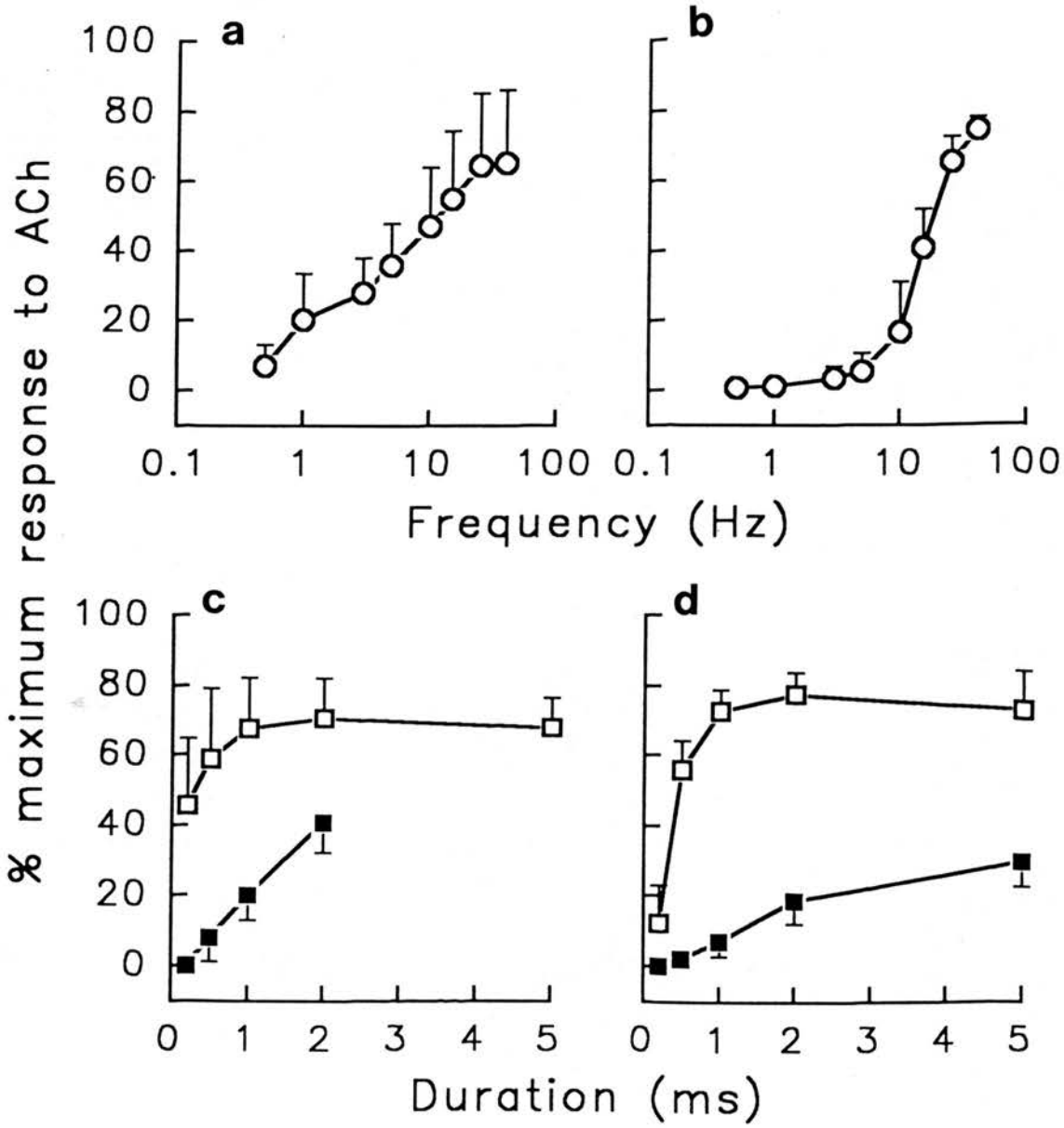

a
b
c
d
100
80
60
40
20
0
0.1
1
10
100
Frequency (Hz)
% maximum response to ACh
0
1
2
3
4
5
Duration (ms)

TABLE 18: Response (% acetylcholine maximum) to 8V, 0.5ms, 25Hz in bronchial rings from pigs of varying ages

	Adult	10d	3d	NB
n	5	5	6	4
Response	28.3±14.7	3.0±2.0*	4.6±5.5*	0.5±1.0*

* $p<0.05$ compared with adult

3.8 Response to histamine, 5HT, acetylcholine and potassium

Histamine produced a concentration-dependent increase in force in adult tracheal smooth muscle (Fig. 12). The maximum response to histamine was 73.8±23% of the maximum response to acetylcholine (n=6). No contraction was obtained with 5HT (n=6). Acetylcholine produced a concentration-dependent increase in force in tracheal smooth muscle with an EC50 of 6.06±0.17 and a maximum force of 15.9±6.2g (n=6)(Fig 13a).

Contractile responses to acetylcholine were also seen in the bronchi (Fig 13b). For third order bronchi from adult animals the maximum force was 3.25±1.8g and the EC50 5.2±0.6, was significantly less than that seen in the tracheal smooth muscle ($p<0.01$). There was no significant difference in the EC50 between third and fifth order rings from adult pigs. Bronchial rings from immature animals showed no difference in sensitivity to acetylcholine (Table 19). Tetrodotoxin did not cause a significant difference in the acetylcholine EC50.

The force developed to acetylcholine (g force per mg wet weight) was significantly greater ($p<0.01$) in the bronchial rings from newborn, three day and ten day old

pigs compared with the rings from the ten week old and adult animals (Table 20). This was also true for the agonist KCl, with a greater contraction per mg of tissue in rings from three and ten day old animals compared with the adult ($p<0.01$).

TABLE 19: EC50 for acetylcholine in the absence and presence of tetrodotoxin (TTX)

Age	Adult 3rd order	Adult 5th order	10w 3rd order	10d 3rd order	3d 3rd order	NB 3rd order
n	5	3	6	6	6	4
EC50	5.2±0.6	5.6±0.7	4.6±0.3	5.1±0.2	4.9±0.5	4.9±0.4
EC50 with TTX	-	-	-	5.0±0.2	4.8±0.3	5.4±0.4

TABLE 20: Resting force, weight and response to acetylcholine and KCl at different ages

Age	n	Resting force (g)	Weight (mg)	g Force per mg tissue: ACh	g Force per mg tissue: KCl
Adult (3rd order)	5	1.3±0.3	46.0±9.1	0.08±0.01	0.04±0.01
Adult	3	1.0±0.1	14.3±5.7	0.09±0.03	
10w	6	0.9±0.2	33.8±18.2	0.07±0.01	
10d	6	0.7±0.2	17.9±2.0	0.17±0.02*	0.15±0.03*
3d	6	0.7±0.2	20.8±10.9	0.2±0.03*	0.10±0.01*
NB	4	0.5±0.2	14.0±3.9	0.23±0.07*	

* $p<0.01$ compared with bronchial rings from adult pigs
ACh acetylcholine

Figure 12: Concentration-response curve to histamine in adult porcine tracheal smooth muscle

Concentration-response curve to histamine in adult porcine tracheal smooth muscle. Contraction is expressed as a % of the maximum response to acetylcholine. (n=6, mean ± SD).

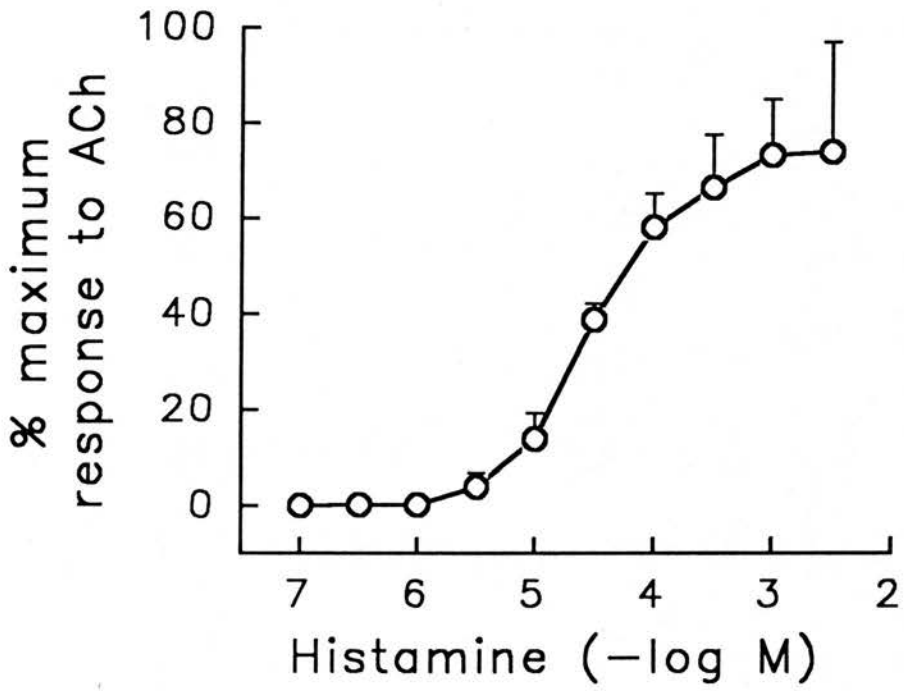
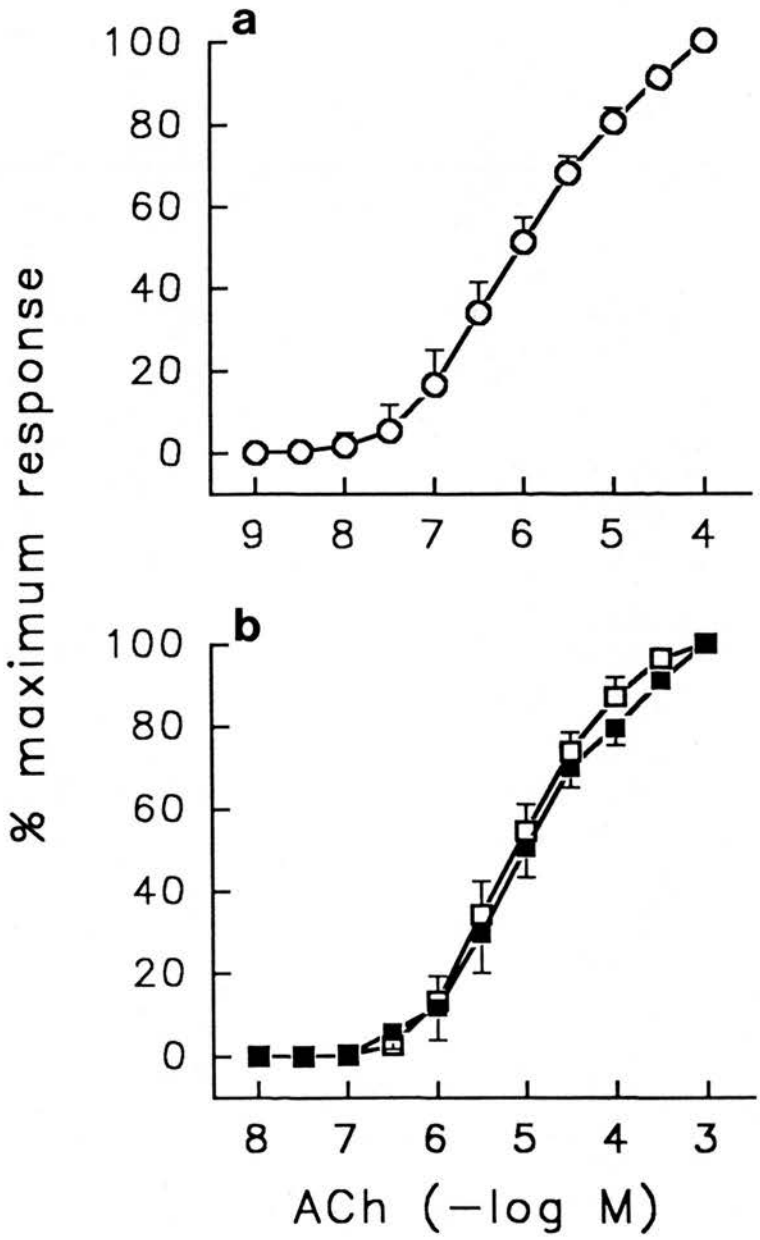

% maximum response to ACh
100
80
60
40
20
0
7
6
5
4
3
2
Histamine (−log M)

Figure 13: Concentration-response curves to acetylcholine in airway smooth muscle

Figure 13a: Concentration-response curve to acetylcholine in adult porcine tracheal smooth muscle. (n=6, mean ± SD).

Figure 13b: Concentration-response curve to acetylcholine in third order bronchial rings from ten day old pigs, in the absence (□) and presence (■) of 10^{-5}M tetrodotoxin. (n=6, mean ± SD).


a
b
100
80
60
40
20
0
9
8
7
6
5
4
3
% maximum response
ACh (−log M)

3.9 Response to ketamine: Tracheal smooth muscle

There was no significant difference in weight or resting force of the muscles exposed to acetylcholine, electric field stimulation, DMPP and potassium chloride (Table 21).

Effect of ketamine (10^{-4}M) on concentration response curves

Ketamine had no effect on resting force. It caused a rightward shift in the acetylcholine concentration-response curve ($p<0.02$)(Fig. 14a). Similarly the frequency-response curve to electric field stimulation was significantly shifted to the right by ketamine ($p<0.001$)(Fig. 14b). No response to DMPP was obtained in the presence of 10^{-4}M ketamine ($p<0.02$)(Fig. 14c).

To localise the effect of ketamine on the peripheral vagal system, the effect of ketamine was compared on muscles equally contracted with acetylcholine, electric field stimulation, and DMPP. In the absence of ketamine, $0.9 \pm 0.8 \times 10^{-6}$M acetylcholine, 2.6 ± 2.0 Hz electric field stimulation and $6 \pm 5 \times 10^{-5}$M DMPP produced contraction equal to 20% of acetylcholine maximum. In the presence of 10^{-4}M ketamine the force of contraction produced by these stimuli was reduced by 68.3 ± 24.2% for the muscles stimulated with acetylcholine, reduced by 71.6 ± 16.6% for muscles stimulated with acetylcholine, and reduced by 100% (i.e., no contraction at all) for muscles stimulated with DMPP, ($p<0.05$ for acetylcholine and electric field stimulation versus DMPP, acetylcholine versus electric field stimulation not significant).

Figure 14: **Concentration-response curves to acetylcholine, electric field stimulation and DMPP in adult porcine tracheal smooth muscle**

Concentration-response curves to (a) acetylcholine (ACh), (b) electrical field stimulation (EFS) and (c) DMPP in isolated pig tracheal smooth muscle in the absence (control o) and presence of 10^{-4}M ketamine (●). Data are expressed as a percentage of the response to 10^{-4}M acetylcholine.

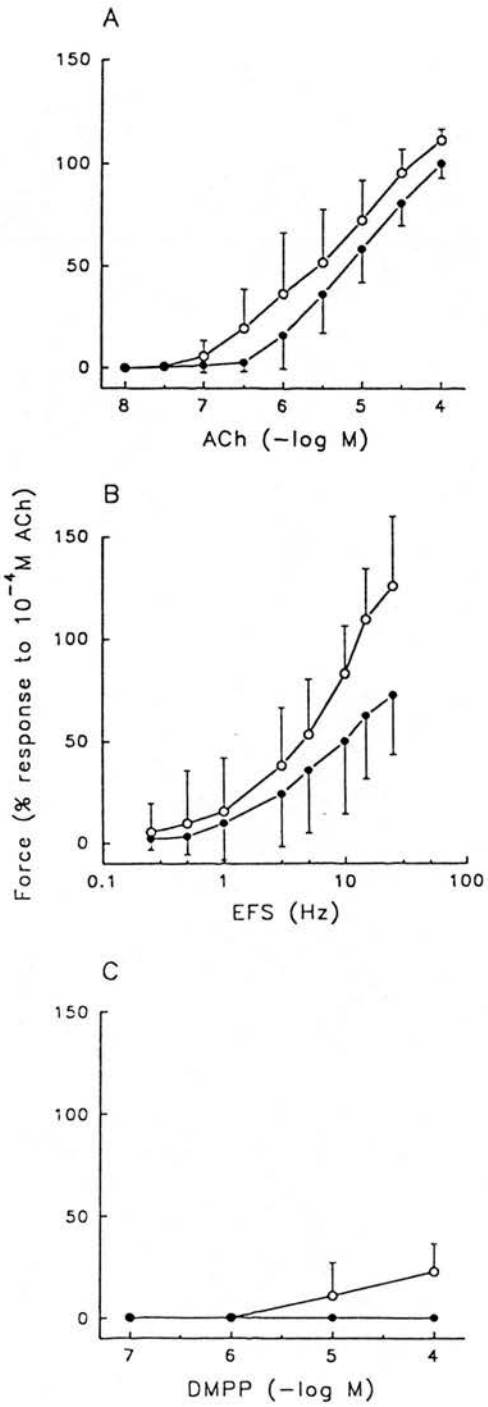

A
150
100
50
0
8
7
6
5
4
ACh (−log M)
B
Force (% response to 10^{-4}M ACh)
150
100
50
0
0.1
1
10
100
EFS (Hz)
C
150
100
50
0
7
6
5
4
DMPP (−log M)

TABLE 21: Weight, resting and maximal force in tracheal muscles exposed to various stimuli

	Weight (mg)	Resting force (g)	Maximal force (g)
ACh	23.0±5.4	0.6±0.5	10.1±5.4
EFS	20.4±8.2	0.8±0.9	9.4±4.5
DMPP	18.8±6.5	0.8±0.7	2.8±2.5*
KCl	21.0±6.4	1.0±0.9	9.4±4.4

ACh = acetylcholine
EFS = electric field stimulation
DMPP = dimethyl-phenyl-piperazinium iodide
KCl = potassium chloride

* $p<0.01$ compared with ACh, EFS and KCl.
All n=6.

Effect of ketamine on precontracted muscles

In muscles precontracted with acetylcholine to 50.4 ± 9.1% of their maximum response to acetylcholine ketamine caused a concentration dependent relaxation. This was unaffected by propranolol ($p=0.94$). Although tetrodotoxin decreased the maximal relaxation obtained with $10^{-3.5}$M ketamine from 46.7 ± 10.2% to 32 ± 13.8% ($p<0.05$), it did not significantly shift the overall concentration-response curve ($p=0.06$) (Fig. 15).

In muscles precontracted with KCl to 52.9 ± 5.1% of their maximal response to KCl, ketamine caused a concentration-dependent relaxation (Fig. 16) which was not significantly different from that seen in muscle strips precontracted with acetylcholine ($p=0.97$).

Figure 15: Concentration-response curves to ketamine in adult tracheal smooth muscle contracted with acetylcholine

Concentration-response curves to ketamine in isolated pig trachealis muscle strips contracted to EC50 with acetylcholine (control, o) and preincubated with 10^{-6} M propranolol (●) or 10^{-6}M tetrodotoxin (TTX,▽). Data are expressed as percentage relaxation from the acetylcholine induced contraction, n=6.

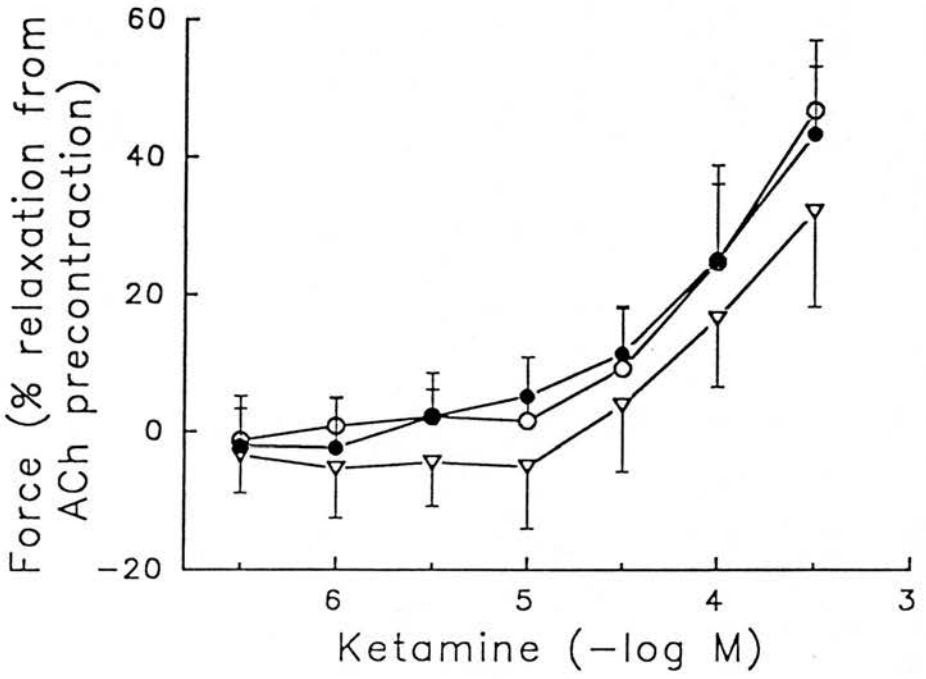
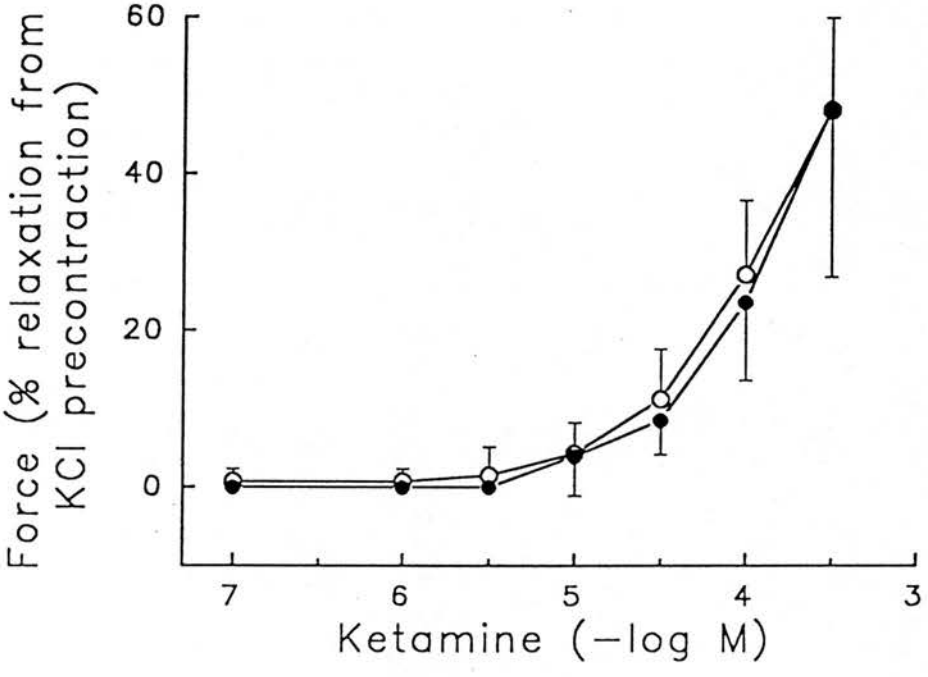

Force (% relaxation from ACh precontraction)
60
40
20
0
−20
6
5
4
3
Ketamine (−log M)

Figure 16: Concentration-response curves to ketamine in adult tracheal smooth muscle contracted with potassium chloride

Concentration-response curves to ketamine in adult tracheal smooth muscle contracted to EC50 with potassium chloride (KCl) in the absence of atropine (control, o, n=6) and in the presence of 10^{-6}M atropine (●, n=4). Data are expressed a percentage relaxation from KCl induced contraction.


Force (% relaxation from KCl precontraction)
60
40
20
0
7
6
5
4
3
Ketamine (−log M)

In muscle strips incubated with atropine and contracted with KCl to 49.5 ± 11.8% of their maximal response, ketamine caused a concentration-dependent relaxation which did not differ from that seen in the absence of atropine ($p=0.54$) or from that seen in muscles precontracted with acetylcholine ($p=0.85$).

3.10 Response to ketamine: Bronchial rings

In bronchial rings contracted with acetylcholine ketamine caused a concentration-dependent relaxation in all age groups studied (Fig. 17a). Bronchial rings from newborn animals were significantly more sensitive to ketamine than rings from adult animals ($p<0.05$)(Table 22). Ketamine had no effect on resting force.

TABLE 22: Effect of ketamine on bronchial rings from different age groups

Age	Precontraction (% ACh maximum)	n	Ketamine EC20	Maximum relaxation
Adult 3rd	48.6±7.0	6	4.15±0.3	60.1±21.9
Adult 5th	48.0±10.5	3	4.18±0.3	60.3±18.9
10w	42.9±7.9	6	4.16±0.14	40.1±14.8
10d	45.8±3.3	6	4.45±0.15	73.7±12.8
3d	41.5±4.1	6	4.45±0.4	64.1±21.9
NB	44.2±4.2	4	4.80±0.5*	69.5±14.2

Relaxation as % of precontraction.

* $p<0.05$ compared with adult

Third order bronchial rings from adult pigs, when contracted with KCl to 55.5±6.7% of their maximal contraction, also showed a concentration-dependent relaxation, with an EC20 of 3.8±0.1 and a maximum relaxation of 60±14.6% (Fig. 17b). In third order adult

bronchial rings contracted with 10^{-3}M histamine $10^{-3.5}$M ketamine caused a relaxation of 81±4.2%.

3.11 Influence of the epithelium

There was no significant difference in the weight or the resting force of the rings with and without epithelium (Table 23). Nor was there any significant difference in the maximum response to acetylcholine (Table 23). Rings with and without epithelium showed a concentration-dependent contraction to acetylcholine (Fig. 18a). There was no significant difference in the EC50 (Table 23).

Rings with and without epithelium showed a frequency-dependent increase in contraction with electric field stimulation. There was no difference in the frequency causing 20% of the maximum acetylcholine contraction (EF20) (Table 23).

Rings with and without epithelium were contracted with acetylcholine to 47.5±12.4% and 49.2±7.9% of their maximum response to acetylcholine. Ketamine caused a concentration-dependent relaxation in rings with and without epithelium (Fig 18b). There was no significant difference in the EC20 for ketamine (Table 23).

TABLE 23: Response of third order porcine bronchial rings with and without epithelium

E	Weight (mg)	Resting force (g)	Response to ACh10^{-4}M	EF20 (Hz)	ACh EC50	Ketamine EC50
+ n=6	36.1±15.8	0.7±0.4	2.6±0.6	6.5±5.3	4.6±0.5	4.1±0.4
- n=6	37.5±16.8	0.95±0.2	2.1±0.4	6.9±4.4	4.6±0.3	4.2±0.4

ACh acetylcholine

Figure 17: **Concentration-response curves to ketamine in third order bronchial rings from pigs of varying ages**

Figure 17a: Concentration-response curve to ketamine in third order bronchial rings from ten week (o), ten day (●), three day (▽) and newborn (▼) pigs. Relaxation is expressed as a % of the acetylcholine precontraction.

Figure 17b: Concentration-response curve to ketamine in third order bronchial rings from adult pigs, precontracted with acetylcholine (□) or potassium chloride (■). Relaxation is expressed as a % of the precontraction. (n=5, mean ± SD).

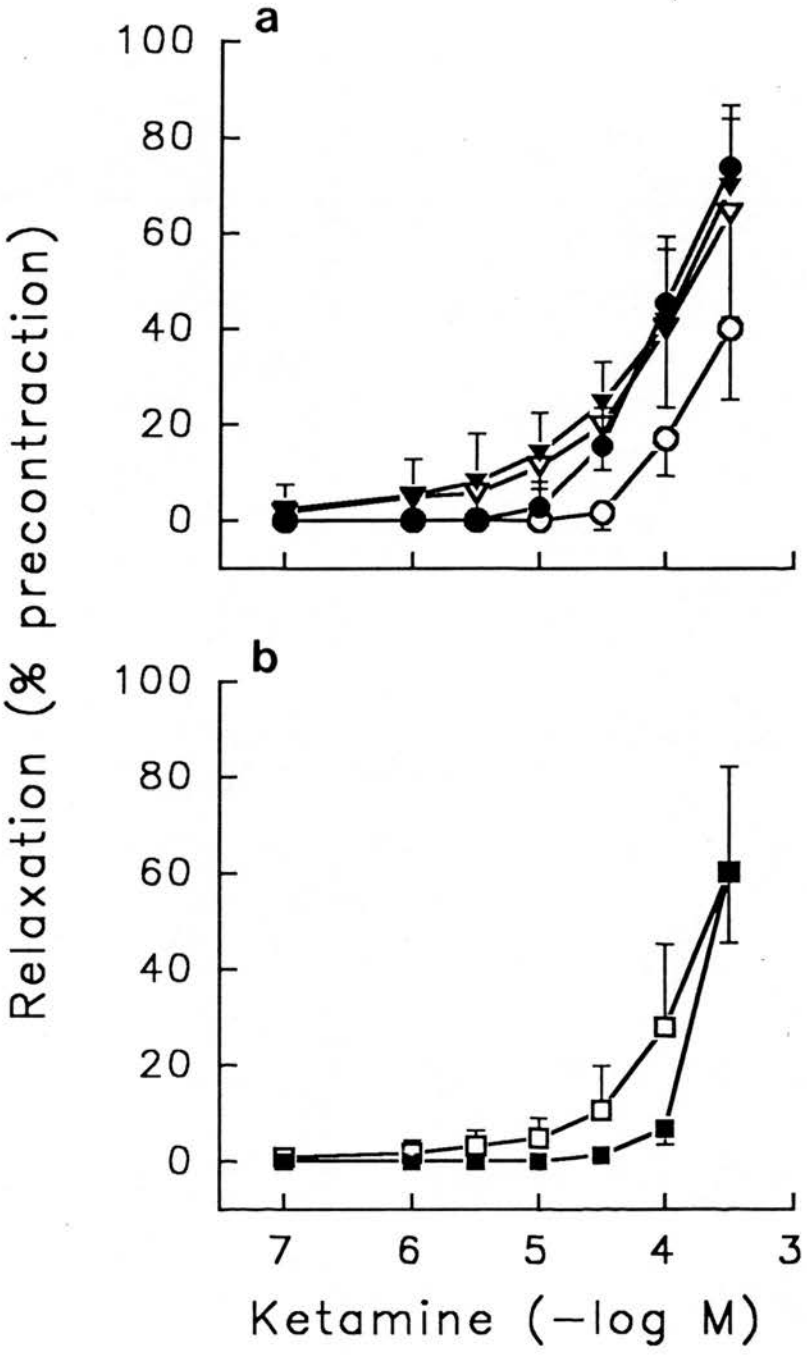
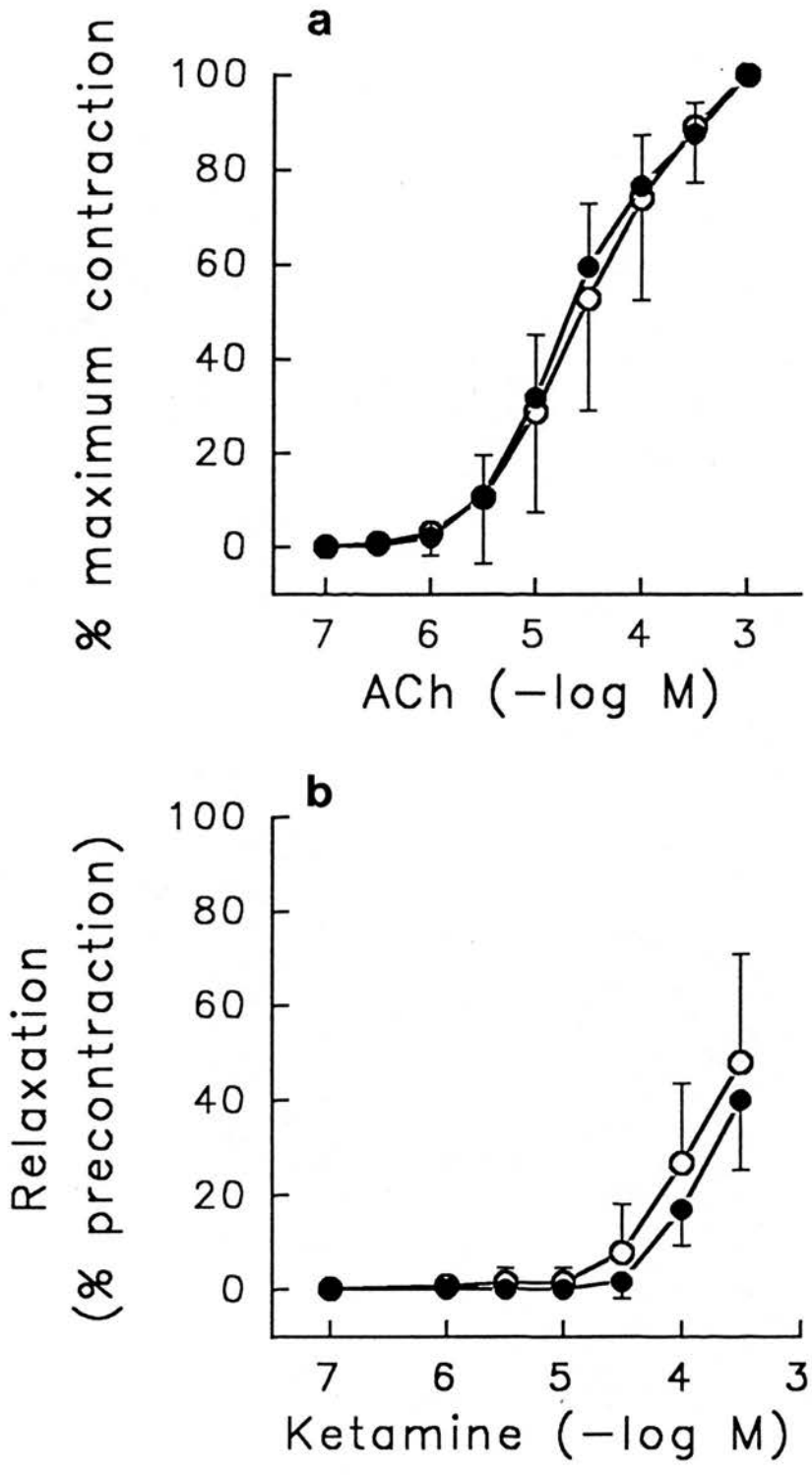

a
b
100
80
60
40
20
0
7
6
5
4
3
Relaxation (% precontraction)
Ketamine (−log M)

Figure 18: Effect of epithelium on the responses of third order bronchial rings from ten week old pigs

Figure 18a: Concentration-response curve to acetylcholine in third order bronchial rings with (o) and without (●) epithelium, from ten week old pigs. (n=6, mean ± SD).

Figure 18b: Concentration-response curve to ketamine in third order bronchial rings with (o) and without (●) epithelium, from ten week old pigs. Relaxation is expressed as a % of the acetylcholine precontraction. (n=6, mean ± SD).


a
% maximum contraction
100
80
60
40
20
0
7
6
5
4
3
ACh (−log M)
b
Relaxation
(% precontraction)
100
80
60
40
20
0
7
6
5
4
3
Ketamine (−log M)

3.12 Nonadrenergic noncholinergic responses

Electric field stimulation in tissues precontracted with acetylcholine produced a biphasic response, with an initial contraction (57.1±18% of acetylcholine maximum at 25Hz versus 14.6±8.3% at 1Hz $p<0.01$), followed by relaxation. The relaxation in tracheal smooth muscle, expressed as a percentage of the maximal contraction of the muscle to acetylcholine was greater at 25Hz (7.1±7.4%) than 1 Hz (0.08±1.1%) ($p<0.01$). The relaxation was unaffected by propranolol or phentolamine (Fig. 19). In muscles contracted with histamine, in the presence of atropine 25Hz and 1Hz electric field stimulation produced minimal contraction (3.7±4.2% and 2±2.8% respectively), with a greater relaxation at 25Hz than 1HZ (68.5±14.3% and 24±11.3% $p<0.05$).

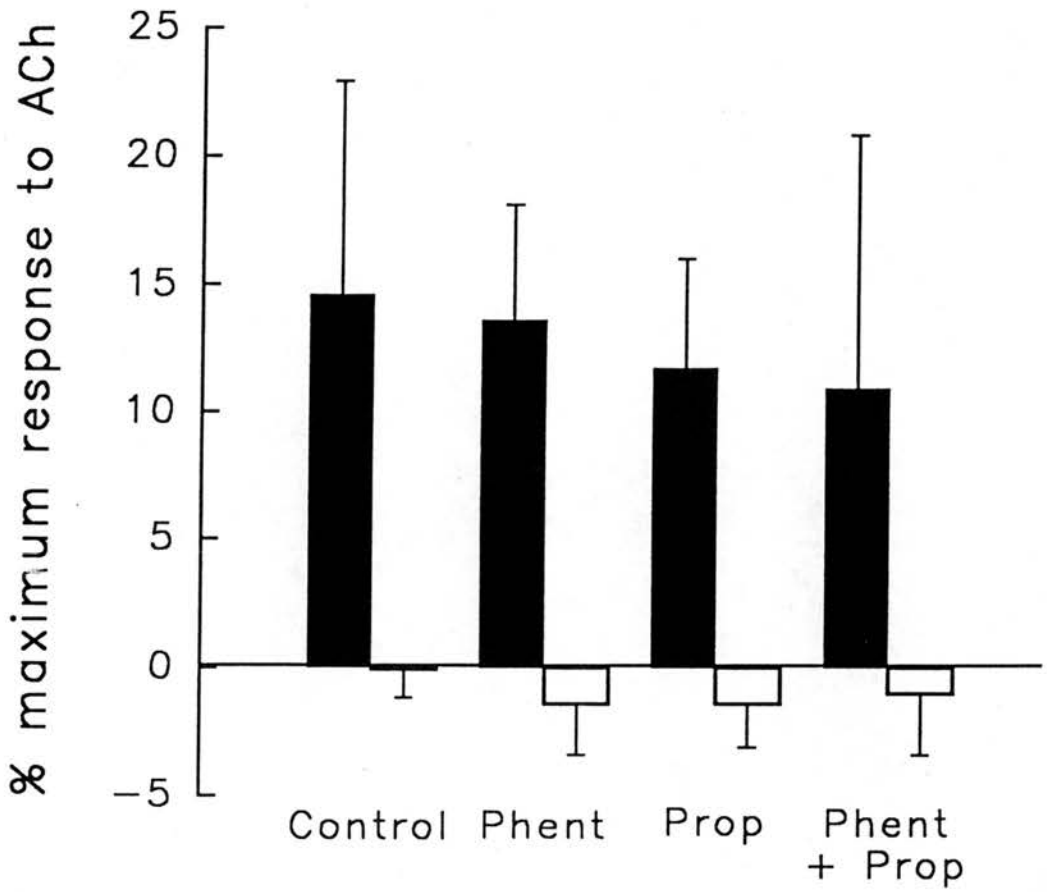
Bronchial rings from adult, ten, three day old and newborn pigs contracted with acetylcholine to an EC50 showed a similar biphasic response to electric field stimulation at 25 Hz (0.5ms, 15V) (Table 24). There was no significant difference in the degree of relaxation between the different age groups. Propranolol had no effect on the response.

TABLE 24: Response to electric field stimulation (25Hz,0.5ms,15V) in bronchial rings contracted with acetylcholine (ACh)

Age	n	ACh contraction (% Ach maximum)	Excitatory response (% ACh contraction	Inhibitory response (% ACh contraction
Adult	6	46.5±5.7	78.0±21.0	15.1±11.9
10d	6	46.2±3.8	65.5±30.0	23.7±6.8
3d	6	41.5±3.8	119.0±23.0	18.1±6.4
NB	4	45.8±9.2	53.2±50.3	26.0±16.0

Figure 19: Nonadrenergic noncholinergic responses in adult porcine tracheal smooth muscle

The excitatory (■) and inhibitory (□) response to electric field stimulation (25Hz, 15V, 0.5ms), in adult porcine tracheal smooth muscle contracted with acetylcholine. Excitatory and inhibitory responses are expressed as a % of the maximal response to acetylcholine. Responses in the absence (control) and presence of 10^{-6}M phentolamine (phent), 10^{-6}M propranolol (prop), and phentolamine with propranolol are shown. (n=6, mean ± SD).


% maximum response to ACh
25
20
15
10
5
0
−5
Control
Phent
Prop
Phent + Prop

3.13 Response to oxygen

All rings showed a significant decrease in contraction to acetylcholine when exposed to the 8%O_2 5%CO_2 87%N_2 gas mix (Table 25). This response was greater in rings from the ten day old and three day old animals compared with the adult and newborn ($p < 0.05$). After twenty minutes the pO_2 of the physiological salt solution was 12-14 kPa, compared with the normal pO_2 of 57-59kPa. There was no change in the pH. On switching the gas back to 95%O_2 5%CO_2 there was a recovery in force. The "hypoxic" gas mix had no effect on resting force.

TABLE 25: Effect of hypoxia and return to normoxia on acetylcholine contraction (expressed as % of acetylcholine maximum), in bronchial rings

	Adult	10d	3d	NB
n	4	5	3	4
Normoxia	100	100	100	100
Hypoxia	63.5±4.0*	22.8±3.6*+	37.3±8.1*+	73.5±16.6#
Normoxia	123.0±6.7	89.0±5.4	107.6±10.2	90.5±12.1

\# $p<0.02$ compared with normoxia
* $p<0.001$ compared with normoxia
\+ $P<0.05$ compared with the response to hypoxia in bronchial rings from adult or newborn pigs

3.14 Histology

Histology was used to confirm the presence or absence of endothelium in the studies involving pulmonary artery rings. In tracheal smooth muscle strips, the removal of the epithelium by mucosal stripping was confirmed. In bronchial rings the presence or absence of epithelium was confirmed.

DISCUSSION

VASCULAR SMOOTH MUSCLE

4.1 Muscarinic receptor mediated responses

Release by acetylcholine of endothelium-derived relaxing factor in adult pig pulmonary arteries

Acetylcholine caused a relaxation only in pulmonary arteries with endothelium. This effect is well known and is due to release of endothelium derived relaxing factor (Furchgott and Zawadzki, 1980). A similar relaxation to acetylcholine was seen in arteries with endothelium precontracted with histamine. In arteries with endothelium contracted with KCl no significant relaxation was found to acetylcholine. The degree of contraction was similar in arteries contracted with KCl and phenylephrine, so this does not account for the marked difference seen. The existence of endothelium-dependent relaxing factors other than nitric oxide including an endothelium-derived hyperpolarising factor (EDHF)(Nagao and Vanhoutte, 1993) has been proposed. In the femoral vein of the rat, acetylcholine causes relaxation by an endothelium-dependent mechanism and by hyperpolarisation (Nagao and Vanhoutte, 1991). In guinea-pig uterine artery both the hyperpolarisation and relaxation are reduced by Ng monomethyl L-arginine suggesting that nitric oxide itself can cause hyperpolarisation (Tare et al 1990). This was not the case in canine mesenteric artery where nitric oxide did not alter membrane potential (Komori et al, 1988).

The mechanism by which KCl produces contraction, (membrane depolarisation and activation of voltage dependent calcium channels) is markedly different from that seen with the alpha-1 agonist phenylephrine which causes contraction by increasing inositol triphosphate levels with subsequent release of calcium from intracellular stores, without the need for membrane

depolarisation. EDHF may be responsible in the pig pulmonary artery for part of the relaxation seen with acetylcholine, as endothelium-dependent hyperpolarisation and associated relaxation are impaired by elevating extracellular potassium (Mombouli et al, 1992; Eglen and Whiting, 1990; McCormack et al, 1988; O'Rourke and Vanhoutte, 1986).

Five molecular and three functional types of muscarinic receptor exist, each with different secondary messenger coupling (Minette and Barnes, 1990). The antagonists of the M1 and M2 receptors, pirenzipine and gallamine had no effect on the relaxation induced by acetylcholine. Relaxation was virtually abolished by the M3 antagonist 4-DAMP, confirming the M3 receptor as the mediator of acetylcholine induced relaxation in porcine pulmonary artery. The M3 receptor is also responsible for the endothelium-dependent relaxation seen in other species (Eglen and Whiting, 1990).

4.2 Adrenoreceptor mediated responses

Noradrenaline responses in adult pig pulmonary artery

This study demonstrates that, in the intralobar pulmonary artery of the pig, the vascular endothelium markedly alters the contractile response to noradrenaline. Noradrenaline produced concentration-dependent contractions in both rings with and without endothelium. However, at high concentrations there was an endothelium-dependent and concentration-dependent relaxation. This relaxation was not due to the release of prostaglandins from the endothelium as the inhibitor of cyclooxygenase, indomethacin, had no effect. The release of endothelium-derived relaxing factor via activation of alpha-2 adrenoreceptors appears to mediate the relaxation that occurs at high concentrations of noradrenaline.

Effect of inhibitors of endothelium-derived relaxing factor on the response to noradrenaline in adult pig pulmonary artery

The endothelium-dependent relaxation to noradrenaline tended to be less in the presence of the inhibitor of guanylate cyclase, methylene blue (Ignarro et al, 1987b), but this did not reach statistical significance. LNMMA, an analogue of L-arginine (Johns et al, 1990) is reputed to be a specific inhibitor of endothelium-derived relaxing factor formation, but it has also been shown to antagonise relaxation induced by amiloride and dibutyrl cAMP. (Thomas and Ramwell, 1992). However, LNMMA had no effect on the noradrenaline induced endothelium-dependent relaxations in this study. By contrast, in the presence of LNAME the endothelium-dependent relaxation was abolished. The difference in the response to the inhibitors of endothelium-derived relaxing factor LNMMA and LNAME (Johns et al, 1990; McMahon et al, 1991) may reflect the metabolism of LNMMA to L-citrullline and subsequently arginine by the endothelial cells (Hecker et al, 1990). The differing effects with the nitric oxide synthase antagonists may also reflect the presence of more than one enzyme metabolising L-arginine (Forstermann et al, 1991). Differences in the abilities of methylene blue and N-w-nitro-L-arginine to reduce endothelium-dependent relaxations have been noted previously (Miller and Vanhoutte, 1989). It has also been proposed that endothelium-derived relaxing factor may interact with alpha 1 and alpha 2 mediated constriction of blood vessels differently, with alpha 2 constriction being more sensitive to relaxation by nitric oxide (Ohyanagi et al, 1992).

Taken together these results indicate that noradrenaline induces endothelium-dependent relaxations of porcine pulmonary artery by stimulating the release of nitric oxide. Interestingly, there appears to be no basal release of nitric oxide as neither methylene blue nor

LNAME had any effect on basal tone. This is in marked contrast to the effect of these antagonists on pulmonary artery tone in intact lambs (Fineman et al, 1991a, Fineman et al, 1991b).

Effect of adrenoreceptor antagonists on the response to noradrenaline in adult pig pulmonary artery

The relaxation seen with noradrenaline was not due to stimulation of beta-adrenoreceptors on the endothelial cells or on the smooth muscle as the addition of propranolol had no effect. A vasodilator response to high concentrations of noradrenaline has been observed previously in canine intrapulmonary arteries (Miller and Vanhoutte, 1985) but the mechanism was not fully investigated. The presence of endothelial alpha-2 adrenoreceptors mediating the relaxant effect to noradrenaline was confirmed in these studies by demonstrating abolition of the relaxant response by yohimbine. In the presence of yohimbine the EC50 values for noradrenaline increased in intrapulmonary arteries both with and without endothelium. This may reflect an alpha-2 adrenoreceptor population on smooth muscle which contributes to the contraction (Daly et al 1988). Tolazoline, which also has alpha-2 adrenoreceptor antagonist properties also abolished endothelium-dependent relaxations to noradrenaline and produced a rightward shift in the concentration-response curve. These results confirm the role of the alpha-2 adrenoreceptor in the mediation of the noradrenaline induced relaxation. By contrast, the alpha-1 adrenoreceptor antagonist prazosin had no effect on the noradrenaline induced endothelium-dependent relaxation. However, prazosin did cause a significant rightward shift of the concentration-response curve for noradrenaline. This suggests inhibition of excitatory alpha-1 adrenoreceptors on the vascular smooth muscle.

Concentration-response curves to alpha-1 and alpha-2 adrenoreceptor agonists in adult pig pulmonary artery

The alpha-1 adrenoreceptor agonist, phenylephrine caused only concentration-dependent contractions in the pulmonary artery of the adult pig. Removal of the endothelium had no effect on the response to this drug. This suggests that in the adult porcine pulmonary artery the endothelium has no effect on the response mediated by alpha-1 adrenoreceptors. This is in contradistinction to the bovine intrapulmonary artery, where addition of the inhibitors of endothelium-derived relaxing factor methylene blue and oxyhaemoglobin cause a leftward shift of the concentration-response curve for phenylephrine implying a basal release of endothelium-derived relaxing factor (Ignarro et al, 1987c; Palmer et al, 1988). No such basal release is evident in the pulmonary artery of the pig.

The alpha-2 agonist UK 14304 produced a small increase in tone in arteries with and without endothelium, again suggesting the presence of an alpha-2 adrenoreceptor mediating contraction, located on the smooth muscle cells, or a partial agonist effect of UK 14304 on alpha-1 aadrenoreceptors. Clonidine, another alpha-2 agonist only increased tone in arteries without endothelium. Variations in the response to alpha-2 adrenoreceptor agonists may reflect opposing actions of alpha-2 adrenoreceptors on vascular smooth muscle and endothelium.

Noradrenaline responses in immature pig pulmonary artery

This study demonstrates that in the intralobar pulmonary artery of the pig the contractile response to noradrenaline alters with age as does the ability of the vascular endothelium to modulate the contractile response. Since receptor densities exhibit longitudinal variability (Kolbeck and Speir, 1987) care was taken to remove pulmonary artery rings from the same anatomical

location in pigs of all ages. The relative unresponsiveness of newborn pulmonary arteries to muscarinic (Liu et al, 1992) and alpha-2 adrenergic mediated endothelium-derived relaxing factor may mean that these mechanisms have a limited role in the adaptation of the pulmonary circulation to extrauterine life.

Effect of inhibitors of endothelium-derived relaxing factor and adrenergic antagonists on the response to noradrenaline in immature pig pulmonary artery

Pulmonary arteries with endothelium from fifteen week old animals showed increased tone to noradrenaline at low concentrations and relaxations at high concentrations. This response is similar to that seen in the adult pig. The specific blocker of nitric oxide release LNAME abolished the endothelium-dependent relaxation, implying that the relaxation was due to endothelium-derived relaxing factor. The lack of effect of LNAME on resting force implies no basal release of nitric oxide. However, in vivo studies have shown that the nitric oxide synthase inhibitor monomethyl-L-arginine can increase pulmonary artery pressure in the one to three day old guinea-pig (Davidson and Eldemerdash, 1990), and N-w-nitro-L-arginine causes a dose-dependent increase in pulmonary artery pressure in lambs (Fineman et al, 1991b). The alpha-1 adrenoreceptor antagonist prazosin did not alter the noradrenaline induced relaxations, but these were abolished by the alpha-2 adrenoreceptor antagonist yohimbine. This suggests an alpha-2 adrenoreceptor mediated endothelium-derived relaxing factor release in response to noradrenaline. The response then in the intrapulmonary artery fifteen week old pig is the same as that seen in the pulmonary artery of the adult pig. Alpha-2 adrenoreceptor mediated relaxations have been noted previously in the coronary arteries of the adult pig (Cocks and Angus, 1983) and pulmonary artery of the dog (Miller and Vanhoutte, 1985).

Endothelium-dependent relaxations to high concentrations on noradrenaline were seen in all the pulmonary arteries of ten week old pigs, and were present in the arteries of some of the ten day old animals but not in any of the younger animals. This could reflect a maturational change in receptor sensitivity, distribution, density or in the secondary messenger system leading to release of nitric oxide, or an alteration in the ability of the vascular smooth muscle to relax to nitric oxide. In an attempt to clarify this the response of neonatal pulmonary arteries to nitric oxide was studied (4.5).

The ability of the vascular smooth muscle to contract in response to noradrenaline also altered with age. In arteries from animals less than ten days of age the contractile response to noradrenaline was very variable with only some of the vessels studied responding. Rings from fetal and three day old animals showed a response to noradrenaline more frequently than did those from newborn animals, but by ten days of age and greater all arteries responded to noradrenaline. This transitory reduction in noradrenaline responsiveness after birth is similar to that seen by Dunn et al (1989) in third generation pulmonary arteries of lambs, when arteries from fetal and twenty-one day old lambs were more responsive than arteries from one and seven day old lambs. Before birth, Su et al (1977) found no change in the maximal contractile response to noradrenaline in developing lambs aged fifty-three days to term. In this study, of those arteries that did show a response to noradrenaline in the fetal, newborn, and three day old age group there was no significant difference in EC50 of arteries with or without endothelium, and no difference in EC50 between these age groups. However, the EC50 of the three day old group was significantly less than that of the ten day, ten week or fifteen week age group, which were similar to each other. These findings suggest that a maturational

change occurs in the contractile response to noradrenaline between three and ten days of age. Again this could represent changes in receptor sensitivity, distribution, density, coupling of the intracellular messenger or in the contractile apparatus itself. The effects of histamine and $PGF2_{alpha}$ on pulmonary arteries from neonatal pigs were studied to assess the maturational state of the contractile proteins (4.3). Buckley et al (1979) showed that newborn to three month old piglets studied in vivo were capable of responding to noradrenaline by an increase in systemic pressure, implying functional alpha-1 adrenoreceptors on systemic arteries. Pulmonary artery pressures were not measured. In developing lambs isolated systemic arteries were more sensitive than intrapulmonary arteries to noradrenaline. Nuwayhid et al (1975) administered noradrenaline to fetal lambs and found an increase in pulmonary artery pressure with increasing gestation, but no change in the EC50 of the response, suggesting maturation in the effector system rather that in the receptors. Mullet et al (1992) demonstrated an age-related increase in pulmonary artery pressure following intratracheal administration of noradrenaline in the isolated rabbit heart. Since birth is associated with a large increase in circulating catecholamines, a selective decrease in the ability of the pulmonary artery to vasoconstrict at birth may prove to be a protective mechanism.

4.3 Histamine receptor mediated responses

There was no endothelium-dependent component to the response to histamine. There was no indication that release of prostaglandins from tissue contributed to the response to histamine in the pulmonary artery as indomethacin was without effect. In the pulmonary arteries from immature animals, although histamine readily initiated contractions there was a failure in maintenance of contraction. This may reflect rapid desensitization to histamine, alteration in the second

messenger system or an impairment in the contractile mechanism. Changes in histamine receptor activity, as seen in the pulmonary artery of the dog may contribute (Newman et al, 1979). In smooth muscle maintenance of contraction is associated with slow-cycling crossbridges, and little is known about the age-related changes in cross-bridge cycling. The immature pigs could, however, maintain stable contractions to other agonists such as KCl and $PGF2_{alpha}$.

Vessels from newborn, three and ten day old pigs did not always show a good contractile response to noradrenaline (4.2) and therefore, following completion of the noradrenaline concentration-response curve the pulmonary artery rings were washed until resting force was regained, and then exposed to histamine (10^{-4}M). Pulmonary arteries that failed to show a response to noradrenaline contracted with histamine. Immaturity in the contractile apparatus of the vascular smooth muscle is unlikely to account for the relative lack of responsiveness to noradrenaline in the pulmonary arteries from newborn pig.

4.4 Response to potassium

KCl produced concentration-dependent increases in tone in pulmonary arteries from both adult and three day old animals. There was no effect of removal of endothelium and contractions were well maintained. The arteries from the younger animals were significantly less responsive to KCl. KCl produces contraction by depolarisation and activation of voltage-dependent calcium channels leading to an influx of calcium across the sarcolemma. Immature smooth muscle may be less sensitive to changes in intracellular calcium concentration, and this could account for the decreased response seen.

4.5 Response to nitric oxide and sodium nitroprusside

Response to nitric oxide in adult and immature pig pulmonary arteries

Nitric oxide produced a concentration-dependent relaxation in arteries from six week old pigs, both with and without endothelium. A similar effect has been seen in the pulmonary artery by other workers (Zellers and Vanhoutte, 1991). Nitric oxide activates cyclic GMP and causes relaxation, so an influence of the endothelium would not be expected. Nitric oxide was able to relax smooth muscle contracted with KCl or phenylephrine.

The response to nitric oxide in the pulmonary arteries of newborn, three day and ten day old pigs was studied. Arteries from pigs of ten days of age were significantly more responsive to nitric oxide than those from newborn or three day old animals. Maturational changes of increasing sensitivity to nitric oxide in pulmonary arteries of pigs aged three days and older have been demonstrated by Zellers and Vanhoutte (1991). Alterations in the vascular smooth muscle responsiveness to nitric oxide may partly explain the findings, and those of other groups showing diminished endothelium-dependent relaxations to acetylcholine in young pigs (Liu et al, 1992) and lambs (Abman et al, 1991b). Acetylcholine in vivo does increase pulmonary blood flow in fetal lambs (Tiktinsky et al, 1992) and nitro-l-arginine attenuates the rise in pulmonary artery blood flow during delivery of lambs (Cornfield et al, 1992) suggesting some responsiveness to nitric oxide at birth.

Response to sodium nitroprusside in adult and immature pig pulmonary arteries

Sodium nitroprusside caused a concentration-dependent relaxation of arteries precontracted with phenylephrine. The relaxant effect of sodium nitroprusside was less in arteries from adult pigs with endothelium when contracted with KCl or histamine than with phenylephrine.

The ability of an agonist to cause relaxation depends in part upon the degree of tone induced in the preparation. The contraction in grams was lesser in the phenylephrine precontracted arteries, and this may in part account for the differences seen. Sodium nitroprusside (10^{-4}M) caused a similar degree of relaxation in arteries precontracted with noradrenaline, from all ages studied. This is in agreement with the work of Zellers and Vanhoutte (1991) who found that relaxations of piglet pulmonary artery to sodium nitroprusside were unaffected by age, and in contrast with Liu et al (1992) who found that the relaxant response to 1 micromolar sodium nitroprusside increased with age. Getman et al (1991) also found a greater effect of sodium nitroprusside on pulmonary artery pressure in 10-14 day old lambs compared with 0-3 day old lambs. Pulmonary vascular tone was induced in these animals by rendering them hypoxic.

Sodium nitropusside acts as a donator of nitric oxide, with s-nitrosothiols as active intermediates (Ignarro et al, 1981). In isolated canine blood perfused lungs, vasodilation produced by sodium nitroprusside is enhanced by methylene blue, a response inconsistent with antagonism of guanylate cyclase by methylene blue (Hofman et al 1992). Sodium nitroprusside most likely causes relaxation by a cyclic GMP dependent and independent mechanism.

4.6 Response to oxygen

<u>Effect of hypoxia on the response to noradrenaline in adult pig pulmonary arteries</u>

The maximum force to noradrenaline was not altered in arteries in physiological salt solution bubbled with the "hypoxic" gas mix. There was no difference in the degree of alpha-2 adrenoreceptor mediated endothelium-derived relaxing factor release. Lowered concentrations of oxygen did not seem to impair the release or action of endothelium-derived relaxing factor. This is in contrast

How low was the P_{O_2}?

to Ogata et al (1992) who found a reduction in acetylcholine induced relaxation in small (<2mm) porcine pulmonary artery with a change in gas mix from 15% O_2 to 0%. The response to sodium nitroprusside was unaffected suggesting inhibition ocurred at a site proximal to guanylate cyclase. Shaul et al (1992) also showed in 4th generation ovine fetal pulmonary arteries that decreasing oxygen tension decreased the acetylcholine induced relaxation and cGMP production. In bovine cerebellum hypoxia markedly inhibits nitric oxide synthase (Rengasamy and Johns, 1991). Differences in the size of pulmonary artery studied,the degree of hypoxia, and in coupling of alpha-2 adrenoreceptors to endothelium-derived relaxing factor release may account for the difference. In chronically hypoxic rats responses to endothelium-dependent vasodilators are abolished, but can be restored by L-arginine (Eddahibi et al, 1992).

Effect of hypoxia on the contractile response in adult and immature pig pulmonary arteries

A decrease in oxygen tension tended to cause a decrease in phenylephrine induced tone in pulmonary arteries from adult pigs, and caused a significant decrease in tension in pulmonary arteries from six week and ten day old animals. The decrease in force was reversed by raising the oxygen tension and occurred in rings with and without endothelium, and in rings contracted with phenylephrine and $PGF2_{alpha}$.

Varying responses have been shown to occur in isolated arterial rings with changes in oxygen tension. Jin et al (1992) in isolated rat pulmonary arteries demonstrated a brief endothelium-dependent contraction, followed by relaxation not mediated by endothelium-derived relaxing factor. After approximately 20 minutes a slow contraction dependent upon extracellular calcium occurred. In the same preparation Mathew et al (1992) showed an endothelium-dependent relaxation followed by

endothelium independent vasoconstriction. A decrease in oxygen tension also produced relaxation in endothelium denuded rat aorta contracted with phenyelphrine (Spina et al, 1992).

These isolated responses differ from either the isolated perfused lamb heart where hypoxia increases pulmonary artery pressure (Tod et al, 1992) and in anaesthetised dogs (Perrella et al, 1992). Factors such as limited regeneration of ATP, or altered intracellular pH may play a role in the in vitro response.

4.7 Response to electric field stimulation

All airway preparations demonstrated a contractile repsonse to electric field stimulation (25Hz, 0.5ms, 15V). Electric field stimulation produces contraction of smooth muscle by depolarisation of nerves and release of acetylcholine. The contractile response to electric field stimulation was seen at all ages, and implies the presence of functional muscarinic receptors in the airway smooth muscle of the newborn animal. At high pulse duration the smooth muscle was activated directly, as shown by contraction in the presence of tetrodotoxin. Tetrodotoxin selectively blocks action potential generation in autonomic nerves. The tetrodotoxin resistant contractions seen at pulse durations greater than 1ms are a direct effect of electric field stimulation on the smooth muscle cells. Electric field stimulation was therefore carried out at 25Hz, 15V and 0.5ms in tracheal smooth muscle, as these parameters produced the greatest response without direct muscle activation. These results are similar to those found in canine tracheal smooth muscle (Russell, 1978).

Changes seen with age in the frequency-response curves in the third order bronchi are similar to those seen by Mitchell et al (1990a) in tracheal smooth muscle from young adult and fetal pigs, namely an increase with age in the frequency required to initiate a contraction. This may reflect changes in innervation or maturational changes in response to the acetylcholine released. By normalising force produced to the maximal acetylcholine response effects of the latter could be corrected. Electric field stimulation can activate all the nerves present in the tissue, including any nonadrenergic noncholinergic nerves, and alterations in nonadrenergic noncholinergic innervation or response to nonadrenergic

noncholinergic transmitters could account for changes in frequency-response curves.

The current output of the electrical stimulator is in part determined by the voltage output. The decreased sensitivity of the preparations from immature animals may be a reflection of size since the response at 25V (as a percentage of the acetylcholine maximum) was not significantly different between groups.

4.8 Response to histamine, 5HT, acetylcholine and potassium

The effect of a variety of agonists was first studied in adult tracheal smooth muscle, and then maturational changes in the responsiveness to two of these agonists acetylcholine, and KCl determined.

Histamine caused a concentration-dependent increase in force in adult porcine tracheal smooth muscle. This was most likely due to activation of the H1 receptor, which is the predominant histamine receptor in porcine trachea and bronchus (Mitchell and Nayler, 1986). Histamine proved to be a less potent agonist than acetylcholine. No response to 5HT was obtained at any of the concentrations studied. This lack of response to 5HT contrasts with the response seen in other species, and emphasises the importance of variation in receptor type between species.

Acetylcholine caused a concentration-dependent increase in force in all airway generations and ages studied. Tracheal smooth muscle was significantly more sensitive to acetylcholine than third and fifth order bronchial rings from adult pigs. No difference was detected in sensitivity to acetylcholine between third and fifth order bronchi. No difference in sensitivity between third and fifth generation airways has also been noted in canine airway smooth muscle (Russell, 1978). No

difference was seen in the force of contraction per mg of tissue with acetylcholine induced contractions in third and fifth order bronchi from adult pigs. This is in contrast to Shioya et al (1987) who found, in dogs, greater force normalised to smooth muscle in fifth than second order bronchi stimulated with KCl or methacholine. The lack of effect of tetrodotoxin on acetylcholine induced contraction suggests little contribution to the contraction from intramural ganglia.

With regard to maturational changes in airway reactivity in response to acetylcholine, the sensitivity of the tissue to acetylcholine as determined by the EC50, and the maximum force developed to acetylcholine was compared in different age groups. There was no significant difference in the acetylcholine EC50 in the different age groups studied. Booth et al (1992) have also shown no change in EC50 to acetylcholine in the developing fetal pig. The maximum force developed to acetylcholine was normalised to the weight of the tissue. A significantly greater force per mg of tissue was developed in bronchial rings from ten day, three day old and newborn pigs compared with adult and ten week old pigs.

Changes in acetylcholinesterase activity have been proposed to acount for the greater responsiveness to acetylcholine seen in tracheal smooth muscle from two week old compared with ten week old pigs (Murphy et al, 1989; Murphy et al, 1991b). If this were the case, then the increased responsiveness of the immature bronchi would be an agonist specific phenomenon. However, bronchi from three and ten day old pigs also showed a significantly greater force generating capacity than bronchi from adult pigs when challenged with potassium chloride. Maturation of acetylcholinesterase activity cannot by itself therefore account for the changes seen in reactivity.

The mechanism by which acetylcholine and potassium chloride produce contraction are markedly different. Activation of muscarinic receptors leads to hydrolysis of phosphoinositols and an increase in inositol triphosphate, and subsequent release of calcium from the sarcolasmic reticulum. Concommitant activation of protein kinase C occurs, which may play a role in altering the sensitivity of the contractile proteins to the intracellular calcium concentration (Rasmussen et al, 1987). Potassium chloride causes depolarisation of the muscle, and activation of voltage dependent calcium channels (Murlas et al, 1986). The increased reactivity to both acetylcholine and potassium suggests that differences may occur in the adult and immature animal at the level of the response of the contractile protein to calcium. This may be due to altered calcium-calmodulin, interaction, differences in myosin light chain kinase or phosphatase activity, or fundamental difference in the myosin heavy chain and the crossbridges formed.

Immature porcine airway smooth muscle is known to contain a greater proportion of myosin heavy chain Type I compared with the adult (Mohammad and Sparrow, 1988). Changes in acetylcholinesterase activity, muscarinic receptor density or coupling, or sensitivity to inositol triphosphate (Schramm et al, 1992) may, of course, also play a role, in the specific response to acetylcholine. Maturational changes in cartilage and other connective tissue components may also influence the response, particularly in vivo by altering the load on the muscle fibres.

In summary, a decrease in reactivity with age was manifested by a decrease in force generating capacity of third order porcine bronchial rings, and may reflect fundamental maturational changes at the level of the calcium-contractile protein interaction.

4.9 Response to ketamine: Tracheal smooth muscle

Ketamine, a phencyclidine derivative, is an anaesthetic agent whose bronchodilating action has been apparent since early studies (Huber et al, 1972). It has been used as a treatment in status asthmaticus and for the relief of bronchospasm at the induction of anaesthesia (Corssen et al, 1972; Strube and Hallam,, 1986; Rock et al, 1986; Sarma, 1992).

In vitro studies of trachealis and bronchial smooth muscle in various species have shown that ketamine produces concentration dependent relaxations (Lundy et al, 1974; Gateau et al, 1989). The exact mechanism of the bronchodilating action of ketamine remains unclear. One proposed mode of action is that ketamine may have a direct effect on the contractile properties of the smooth muscle (Lundy et al, 1974). A beta-adrenergic sympathomimetic effect has also been proposed since in antigen-sensitised dogs the prevention of bronchospasm by ketamine is abolished by propranolol (Hirshman et al, 1979). No beta-adrenoreceptor mediated role in the action of ketamine has been detected in vitro (Gateau et al, 1989), nor is the relaxation of human airway smooth muscle in vitro dependent on products of the cyclooxygenase pathway (Gateau et al, 1989). Ketamine has been shown to have an inhibitory effect exerted centrally on vagal pathways (McGrath et al, 1975), but no studies have investigated effects on the peripheral vagal motor pathway.

This study was designed to determine the site of action of ketamine on the peripheral vagal motor pathway in isolated porcine trachealis muscle strips. From the central nervous system preganglionic fibres pass down the vagus nerve to ganglia located in the airway walls, where they release acetylcholine. This acetylcholine activates nicotinic receptors in the postsynaptic membrane of the nerve fibres innervating the airway smooth muscle. 1,1-

dimethyl-4-phenyl-piperazinium iodide (DMPP) stimulates these nicotinic receptors of the intramural ganglion (Leff and Munoz, 1981). Electric field stimulation can be used to activate nerve fibres in the muscle strip, including the postganglionic cholinergic nerves. Acetylcholine can cause contraction of smooth muscle by activation of muscarinic receptors on the smooth muscle. The effect of ketamine on contractions induced by these different stimuli, which can activate the peripheral vagal motor pathway at different sites, was studied.

The results show that ketamine can attenuate contraction of isolated pig trachealis muscle at two sites along the peripheral vagal pathway, depressing the contractile response of the smooth muscle to acetylcholine and the excitability of the postsynaptic nicotinic cholinergic receptors of the intramural parasympathetic ganglion. The ability of ketamine to relax porcine trachealis smooth muscle occurred in muscles contracted with acetylcholine and KCl suggesting that the relaxant action of ketamine is not dependent on the muscarinic receptors.

Action of ketamine on concentration-response curves

The effect of ketamine on the contractile response to DMPP, electric field stimulation and acetylcholine was studied in an attempt to determine the site of action of ketamine on the peripheral vagal nervous system. In this study DMPP initiates contraction by stimulating postsynaptic nicotinic receptors of the intramural ganglia; electric field stimulation in the presence of hexamethonium, stimulates the postganglionic cholinergic nerve fibres; and acetylcholine in the presence of tetrodotoxin stimulates the muscarinic receptors on the smooth muscle.

Ketamine had no effect on the resting force of the muscle. In the presence of 10^{-4}M ketamine the concentration-response curve to acetylcholine and the

frequency-response curve to electric field stimulation were significantly shifted to the right and the response to DMPP was completely abolished. In an attempt to identify the sites of action of ketamine on the peripheral vagal nervous system the relaxant effect of ketamine was compared with muscles contracted to the same degree (20% of the maximum force obtained with acetylcholine). If ketamine affected all three sites (muscle, postganglionic cholinergic nerve fibre and intramural ganglion) equally, one would expect the least depression of force to occur with acetylcholine induced contraction (ketamine interacting with muscle-receptor complex alone), greater depression of force with electric field stimulation induced contraction (ketamine interacting with nerve and muscle-receptor complex) and the greatest reduction in force to occur with DMPP induced contraction (ketamine interacting with the intramural ganglion, nerve and muscle-receptor complex). In fact 10^{-4}M ketamine caused 68.3 ± 24.2% depression of the acetylcholine response representing the direct muscarinic receptor-muscle interaction effect. With electric field stimulation the depression of force was similar (71.6 ± 16.6%) suggesting no additional effect of 10^{-4}M ketamine on neural transmission in the postganglionic cholinergic nerves. 10^{-4}M ketamine abolished contractions due to DMPP (100% reduction), indicating an additional effect on the postganglionic nicotinic cholinergic receptors in the intramural ganglion. Similar effects have been shown with halothane, enflurane and isoflurane in canine trachealis smooth muscle, namely a decrease in the excitability of the postsynaptic nicotinic receptors of the intramural parasympathetic ganglion and an effect on the smooth muscle receptor complex (Brichant et al, 1991).

Effect of ketamine on precontracted tissue

Ketamine caused a concentration-dependent relaxation in muscles contracted with acetylcholine. Muscarinic receptor activation causes contraction via an increase in inositol 1,4,5-triphosphate and diacylglycerol (Chilvers et al, 1990). The former causes release of intracellular calcium and the latter may play a role in the maintenance of contraction by modulating the sensitivity of the contractile and structural proteins to intracellular calcium. As well as this pharmacomechanical coupling, high concentrations of acetylcholine may also cause an influx of extracellular calcium through voltage-sensitive calcium channels. The rise in intracellular calcium ultimately results in activation of the contractile proteins of the smooth muscle cell. Ketamine could exert an inhibitory effect on any of the above resulting in relaxation. Beta-adrenoreceptor activation is well known to cause relaxation of airway smooth muscle (Koenig et al, 1989). In this in vitro study propranolol had no effect on ketamine induced relaxations. By contrast, in vivo propranolol blocks the protective effect of ketamine against bronchospasm in ascaris sensitized dogs (Hirshman et al, 1979). Species differences and the absence of neural and hormonal effects in the in vitro situation may account for this difference. In the presence of tetrodotoxin, the ketamine concentration-response curve was shifted to the right in five out of six muscles and unchanged in one. The reduction in maximal relaxation obtained with ketamine in the presence of tetrodotoxin is consistent with an action of ketamine at the intramural ganglion.

The relaxant effect of ketamine in muscle strips contracted with KCl was not significantly different from that in muscle strips contracted with acetylcholine. Potassium causes contraction by membrane depolarisation and activation of voltage-dependent calcium channels resulting in an increased transsarcolemmal flux of

calcium (Murlas et al, 1986). It may also cause acetylcholine release by depolarisation of postganglionic parasympathetic nerve terminals (Mitchell et al, 1991). To eliminate any contribution of released acetylcholine to the contraction muscle strips were incubated with atropine. This did not alter the concentration-response curve to ketamine. This suggests that ketamine may affect an intracellular pathway common to KCl and acetylcholine.

The ability of ketamine to relax smooth muscle independent of the agonists used to induce tone, parallels the success of ketamine clinically as a bronchodilating agent in asthmatic patients. The lack of effect of ketamine on baseline force emphasises the need to consider intrinsic airway smooth muscle tone when clinically assessing the effect of bronchodilating agents. Although comparison between in vitro and in vivo drug concentrations across species is of limited value, it is of interest to note that an in vitro effect in this study was found at concentrations seen in clinical practice (2.5×10^{-6} to 6×10^{-5}M) (Idvall et al, 1979).

In summary, ketamine depresses the excitability of the postsynaptic nicotinic receptors of the intramural parasympathetic ganglion and affects the acetylcholine receptor-smooth muscle receptor complex. The ability of ketamine to equally relax muscle precontracted with KCl and acetylcholine suggests an effect on the smooth muscle independent of muscarinic receptor activation.

4.10 Response to ketamine: Bronchial rings

Ketamine caused a concentration-dependent relaxation in bronchial rings from adult pigs precontracted with acetylcholine, KCl or histamine. As discussed above, this suggests that the major action of ketamine is at a point distal to the mechanism of signal transduction of

acetylcholine and histamine, and may reflect alterations in the calcium-contractile protein interaction.

Ketamine caused a relaxation in bronchial rings from all age groups studied. The maximum relaxation in bronchial rings to ketamine, (as a percentage of the precontraction with acetylcholine) was not significantly different in immature animals compared with adult. Age-related differences were seen in the sensitivity of the tissue to ketamine (as manifest by change in EC50), with the airways from newborn pigs significantly more sensitive compared with the adult. As previously discussed, ketamine causes relaxation by depressing the excitability of the postsynaptic nicotinic cholinergic receptors of the intramural parasympathetic ganglion and by depressing the contractile response of the smooth muscle to acetylcholine. No attempt was made to separate these two mechanisms in the immature bronchi, thus the maturational changes seem may reflect both changes in the smooth muscle and the intramural ganlion. However, in bronchi the number of ganglia are decreased, and so it is more likely that the age-related changes are occuring in the muscle itself. Again maturational changes cartilage, extracellular matrix, muscle fibre or myofibrillar density, or changes in the contractile proteins themselves may also all play a role in these maturational changes.

4.11 Influence of epithelium

An epithelium-derived relaxing factor has been postulated to exist (Flavahan et al, 1985; Ilhan and Sahin, 1986; Gao and Vanhoutte, 1988; Munakata et al, 1990; Morrison et al, 1990), although the nature of the epithelium-derived relaxing factor has not been determined. Marked species differences have been noted to occur in the influence of the epithelium. In the dog, changes in sensitivity and maximum response occur with removal of epithelium from second but not third order bronchi (Hay

et al, 1987). No evidence for the existence of an epithelium derived relaxing factor was found in this study on third order bronchi from ten week old pigs. The action of ketamine was not influenced by, or dependent upon the presence of epithelium. There was no significant difference in the response to electric field stimulation or acetylcholine. This is in contrast with other workers (Stuart-Smith and Vanhoutte, 1988) who showed an effect of epithelium removal on the acetylcholine response in third order bronchi from adult pigs. This may be a maturational related change. The production of epithelium derived relaxing factor, like endothelium-derived relaxing factor, may alter with age, or changes may occur in the responsiveness of the airway smooth muscle to the epithelium-derived relaxing factor.

4.12 Nonadrenergic noncholinergic responses

A contractile response only was seen when electric field stimulation was applied to tracheal smooth muscle at resting force. When the tone of the muscle was raised by either acetylcholine or histamine, a frequency-dependent relaxation of tracheal smooth muscle strips to electric field stimulation could be demonstrated. Since neither phentolamine nor propranolol affected this relaxation it is not mediated by either alpha or beta-adrenergic mechanisms. With histamine induced contractions, relaxation was present in the presence of atropine indicating a noncholinergic mechanism. Recent studies have shown a nonadrenergic noncholinergic relaxation in porcine tissue blocked by LNMMA, and nitric oxide has now been proposed as a putative mediator of nonadrenergic noncholinergic relaxation (Kannan and Johnson, 1991). In muscle strips contracted with histamine and in the presence of atropine a small excitatory component to the electric field stimulation was still present. This may represent a nonadrenergic noncholinergic excitatory response, since at 10^{-6}M atropine the response to 10^{-4}M acetylcholine was abolished.

There was no significant difference in the relaxation induced by 25Hz electric field stimulation in bronchial rings from adult, ten day, three day old and newborn pigs. This is in contrast to Mitchell et al (1990a) who found a smaller nonadrenergic noncholinergic response in fetal pig airways compared with the adult. Interpretation of the force generation produced by varying electric field stimulation parameters should be made in the light of the fact that inhibitory responses are also being generated, and that the force produced is the net result of conflicting excitatory and inhibitory responses. Differences in neural maturation, or responsiveness of the airway smooth muscle to the released neurotransmitter may also play a role in the age-related changes seen in frequency-response curves generated by electric field stimulation.

4.13 Response to oxygen

Hypoxic bronchodilation is a well recognised phenomenon, although the mechanism behind it is unknown. In vivo, high resolution computed tomographic imaging reveals acute reversible bronchodilation in minipigs (Wetzel et al, 1992). In this in vitro study, all rings at all ages showed a decrease in contractile force to acetylcholine in the hypoxic gas mix. Hypoxia also decreases the development of active tension in isolated canine bronchus in rings both with and without epithelium (Gao and Vanhoutte, 1989). The decrease in contractile force on the presence of the hypoxic gas mix was not caused by damage to the muscle, as the effect was readily reversible on exposure of the muscle to the normoxic gas mix. Oxygen reaches the muscle by diffusion, and lowering the pO_2 could potentially limit the capacity of the muscle to generate ATP and maintain contraction. However, the contraction to acetylcholine was well maintained in the muscles exposed to the hypoxic gas mix, although the magnitude of the contraction was diminished

compared with the contraction in the normoxic gas mix. Bronchial rings from three and ten day old animals were the most susceptible to the decreased pO_2 with the newborn being relatively resistant to the effect. Since rings from newborn and three day old animals are very similar in size it is unlikely that this differential effect is due to alteration in the ability of oxygen to diffuse to the centre of the muscle. Lowering the pO_2 may affect the secondary messenger system or the response of the contractile mechanism itself.

4.14 Limitations of the study

The study was performed in vitro, and extrapolation to the in vivo situation is limited. In the in vitro situation, central neural control is absent as is the influence of circulating mediators. Only isometric properties of the muscle were studied, and auxotonic contractions may be more relevant to the in vivo situation.

In the vascular studies, histological studies were performed to determine the presence or absence of endothelium, but this does not indicate functional integrity of the endothelium. In the pulmonary arteries from adult pigs, the functional state of the endothelium was determined wherever possible by assessing the response to acetylcholine.

With regard to the airway smooth muscle studies, tracheal smooth muscle strips from the adult animal were studied wherever possible, to determine the effect of agonists on the airway smooth muscle. In the immature animals it was not technically feasible to mount tracheal smooth muscle strips, and so only bronchial rings were studied. Maximum force generated by agonists was normalised to the weight of the tissue as facilities were not available for analysis of the myosin content of the preparations. This may not be optimum, because of changes in the proportions

of cartilage and smooth muscle with age. Despite these limitations, maturational changes in the responsiveness of the airway smooth muscle could be demonstrated. Porcine airway smooth muscle, with the increased reactivity demonstrated in the immature animal, may be a particularly appropriate model for the human infant airway.

SUMMARY

These studies have shown that, in vitro, age-related changes occur in the responsiveness of porcine pulmonary vascular and airway smooth muscle to a range of pharmacological agents. A variety of mechanisms may contribute to the alterations in smooth muscle reactivity. Changes in receptor number, distribution or coupling may occur contributing to agonist specific maturational changes. Alterations with age in the responsiveness of the contractile proteins to calcium, or in the myosin heavy chain composition may produce more global changes in reactivity.

5.1 Response of pulmonary vascular smooth muscle to agonists causing contraction

Maturational changes occurred in the responses of pulmonary arteries to contractile agents. Arteries from pigs aged less than three days old were significantly less sensitive to noradrenaline, with a rightward shift in the EC50. In some arteries there was no response to noradrenaline, although contractions could be elicited to histamine or prostaglandin $F2_{alpha}$. Maturational changes in the distribution or coupling of adrenoreceptors may account for this. The EC50 for potassium chloride was also shifted to the right in the pulmonary arteries from three day old pigs compared with arteries from adult pigs. This may reflect a general increase in the sensitivity of the contractile apparatus to intracellular calcium with age.

5.2 Response of pulmonary vascular smooth muscle to agonists causing relaxation

Nitric oxide and sodium nitroprusside caused a concentration dependent relaxation in arteries with and without endothelium. Pulmonary arteries from newborn pigs were more resistant than those from adult pigs to the relaxant effects of nitric oxide and hypoxia, although sodium nitroprusside was equally effective in relaxing pulmonary artery rings from all ages studied. Clinically, the effectiveness of nitric oxide in decreasing pulmonary artery pressure in children of different ages has not yet been fully evaluated.

5.3 Release of endothelium-derived relaxing factor

Acetylcholine was shown to release endothelium-derived relaxing factor by interaction with type three muscarinic receptors. An alpha-2 adrenoreceptor mediated release of endothelium-derived relaxing factor was identified in pulmonary arteries from adult pigs, and was still present in the hypoxic setting. The alpha-2 adrenoreceptor mediated endothelium-derived relaxing factor release was not present in pulmonary arteries from pigs aged three days or younger. Alpha-2 adrenoreceptor stimulation and release of endothelium-derived relaxing factor may not play a major role in the decrease in pulmonary vascular resistance seen in the immediate newborn period.

5.4 Response of airway smooth muscle to agonists causing contraction

Studies performed in adult trachealis muscle enabled basic properties of the airway smooth muscle to be determined. The response to electric field stimulation and the response to acetylcholine, potassium chloride, histamine and 5HT delineated. Age-related changes in responsiveness were demonstrated in third order bronchi. A greater frequency of electric field stimulation was required to generate contractile response in immature airways. Although no change in sensitivity to

acetylcholine as measured by EC50 was detected, a greater reactivity of the immature airway was shown by the increased force normalised to tissue weight. This increased reactivity was also seen with potassium induced contractions. This suggests that differences in the calcium-force coupling mechanism in the immature animal may be present, although maturational changes in acetylcholinesterase activity may contribute to changes in the response to acetylcholine.

5.5 Response of airway smooth muscle to agonists causing relaxation

By studying adult tracheal smooth muscle, insight in to the mode of action of the bronchodilator ketamine was gained. Ketamine causes relaxation by inhibiting intramural post-ganglionic parasympathetic nicotinic receptors, and altering calcium-contractile protein interaction. Maturational differences in the response to ketamine were also found. Airways from newborn pigs showed a greater sensitivity to ketamine than airways from adult pigs. Airway smooth muscle from newborn and adult pigs was more resistant to the reduction in force caused by hypoxia compared with muscle from three and ten day old animals. Nonadrenergic inhibitory innervation was shown to be present from birth in the pig. The importance of nonadrenergic noncholinergic innervation in developing human airways is as yet unknown.

Further investigation, at a cellular level, is needed to provide the key to understanding the mechanisms behind the age-related changes in reactivity.

APPENDIX

6.1 Physiological salt solution

The composition of physiological salt solution was as follows (concentration in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 11.1 glucose.

6.2 List of chemicals

The following drugs were obtained from Sigma Chemical Company, Poole U.K.

+/- noradrenaline hydrochloride
1,1-dimethyl-4-phenyl-piperazinium iodide
5-hydroxytryptamine
acetylcholine hydrochloride
atropine sulphate
clonidine hydrochloride
dl-propranolol hydrochloride
gallamine triethiodide
hexamethonium chloride
histamine dihydrochloride
indomethacin
ketamine hydrochloride
methylene blue
N-g-methyl-D-arginine
N-g-methyl-L-arginine
n-w-nitro-L-arginine methyl ester
phenylephrine hydrochloride
phentolamine hydrochloride
pirenzipine dihydrochloride
prazosin hydrochloride
prostaglandin $F2_{alpha}$
sodium nitroprusside
tetrodotoxin
tolazoline hydrochloride
yohimbine hydrochloride

4-diphenylacetoxy-N-methyl-piperadine methiodide was obtained from R.B.I., UK.

UK 14304 was obtained from Semat Technical (UK) Ltd, St Albans, UK

Nitric oxide was obtained from Merck, Poole , UK.

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