Effect of Oxotremorine and Epinephrine on Lung Surfactant Secretion in Neonatal Rabbits

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Summary

The phosphatidylcholine (PC) content (mg/g dry lung weight; mean $\pm$ S.E.) of lung washes of 1 to 3-day-old rabbits was significantly greater 30 min after the injection of the muscarinic agonist oxotremorine (0.2 mg/kg; 50.5 ± 2.9 mg/g; $2 P < 0.01$) or epinephrine (50 μ g/kg; 78.0 ± 14.8 mg/g; $2 P < 0.001$) than saline (25.7 ± 1.9 mg/g). Injection of atropine (2 mg/kg), dl-propranolol (1 mg/kg) but not d-propranolol (1 mg/kg) at 40 min or adrenalectomy at 45 min before killing abolished the increase produced by oxotremorine ($2 P < 0.001$). Also, dl-propranolol antagonized the increase produced by epinephrine. Palmitic and myristic acids were the major fatty acids in the lung wash PC of control and oxotremorine-treated rabbits. The residual lung tissue PC content following atropine (2 mg/kg; 104.4 ± 18.5 mg/g) was significantly greater ($2 P < 0.05$) than following saline (72.6 ± 6.0 mg/g) but not different ($2 P > 0.05$) in other treatment groups. The total lung content of PC was 55% greater ($2 P < 0.001$) than controls 30 min after epinephrine (50 μ g/kg).

The surface activity of lung washes (mg/g dry lung weight; mean $\pm$ S.E.), assayed on a surface tension balance using dipalmitoyl-phosphatidylcholine as standard, was significantly greater ($2 P < 0.05$) 30 min following injection of oxotremorine (0.2 mg/kg; 73.8 ± 9.9 mg/g) or epinephrine (50 μ g/kg; 72.2 ± 10.2 mg/g) than saline (42.8 ± 2.0 mg/g). Ten-min infusions of oxotremorine (0.34 μ g/ml) into isolated, ventilated, perfused neonatal rabbit lung preparations failed to alter the PC content of the subsequent wash, but this was significantly ($2 P < 0.05$) raised from 3.7 (± 0.8) to 4.8 (± 0.8) mg/g dry lung wt by a 10 min infusion of epinephrine (3.4 μ g/ml).

Speculation

Alteration of the rate of secretion of lung surfactant into alveoli may control the rate of biosynthesis. Disorders of lung surfactant secretion may be involved in the pathogenesis of respiratory distress syndrome.

INTRODUCTION

There is now substantial evidence that lung surfactant is mainly composed of phospholipids, particularly dipalmitoyl phosphatidylcholine (DPPC) (21), synthesized in type 2 pneumocytes and stored as osmiophilic lamellar bodies (OLB) (4, 15). There are several morphologic studies describing discharge of these OLB into alveoli (3, 5, 29). In other tissues where substances are synthesized and stored before release, there are discrete physiologic processes controlling secretion as well as synthesis (22, 32). Secretion of stored lung surfactant into alveoli may be a controlled process; possible modulators include agonists at muscarinic receptors and β -adrenoceptors and ventilation.

Goldenberg *et al.* (16) described enhanced expulsion of OLB into alveoli in adult rats following injection of the muscarinic

agonist pilocarpine. Pilocarpine injection has also been shown to decrease the number of OLB per type 2 pneumocyte, increase the phospholipid content of lung lavages in adult rats (24), and increase the air retention on deflation of fetal rabbit lungs (7, 8). Morgan and Morgan (23) demonstrated earlier appearance and higher specific activity of [¹⁴C]palmitate in disaturated phosphatidylcholines (PC's) in lavage fluid of adult rats following pilocarpine. These observations are compatible with an increase in surfactant secretion after pilocarpine. Corbet *et al.* (8) have suggested that pilocarpine acts indirectly to increase the static compliance of fetal rabbit lungs because its effect could be antagonized by large doses of phenoxybenzamine or propranolol. More direct evidence of a role for muscarinic receptors mediating surfactant secretion has come from studies showing enhanced release of radiolabeled PC from explants of fetal rat lungs (27) by prolonged incubation with pilocarpine and from a culture of cell line A549, whose cells resemble type 2 pneumocytes, by acetylcholine, and by carbachol (31).

β -adrenoceptors may also be involved in controlling lung surfactant secretion. Injection of isoproterenol into adult rats increased the phospholipid content of lung lavages and reduced the number of OLB per type 2 pneumocyte (24). These effects were antagonized by propranolol. Three to 5 hr after isoxsuprine injection into fetal rabbits, there were increases in lung static compliance (12, 34) and lecithin/sphingomyelin ratio and surface activity of pulmonary fluid and decreases in the number of OLB per type 2 pneumocyte (12). Terbutaline, a β -adrenoceptor agonist, increased the release of ¹⁴C-disaturated PC from adult rat type 2 alveolar cells *in vitro* (11), providing the most direct evidence that secretion is sensitive to β -adrenoceptor agonists.

Increased minute ventilation in lightly anesthetized adult rabbits raised the phospholipid content of lung washes, and this action could be antagonized by atropine, propranolol, sotalol, indomethacin, or sodium meclofenamate (25, 26). This suggests the involvement of muscarinic receptors, β -adrenoceptors, and prostaglandins in ventilation-induced surfactant secretion.

We have therefore examined the effect of a selective muscarinic agonist, oxotremorine (9), on lung surfactant secretion by measuring the PC content and surface activity in lung washes in neonatal rabbits and the residual lung tissue PC content at a short time interval (30 min) after injection of this drug and after various pretreatments. Furthermore, the effect of oxotremorine and epinephrine have been studied on a perfused, isolated, ventilated lung preparation. Preliminary results have been published (1, 2).

MATERIALS AND METHODS

One- to 3-day-old Dutch rabbits (Nottingham University, United Kingdom) were used. They were given injections of the drugs or saline at the doses (in terms of base) and times before killing given in Table 1. Other groups of rabbits were given injections of oxotremorine or saline 15 min after bilateral adrenalectomy under ether anesthesia.

ISOLATED LUNG PREPARATION

All rabbits, whether pretreated or not, had their isolated lungs perfused and ventilated (1). Each rabbit was tracheotomized under sodium pentobarbitone (45 mg/kg) anesthesia, its pulmonary artery was cannulated, and the weighed lungs were removed into a well-sealed, water-heated (38°C) perspex box with the tracheal cannula open to the air. The lungs were perfused with Krebs-Henseleit solution (37°C) bubbled with 95% oxygen and 5% carbon dioxide at 2.9 ml/min via the pulmonary artery. Successful perfusion was assessed by removal of blood from the lungs. The perfusate collected in the lung chamber, maintained the humidity, and was removed at 5-min intervals. The box was evacuated 140 times/min to a negative pressure of about 37 mm Hg using a small animal ventilator (Scientific Research Instruments, Ltd.). In most experiments, at 5-min intervals ventilation was stopped, 1.5 ml of saline were injected slowly into the lungs via the tracheal cannula, and after 1 min, as much saline as possible was withdrawn to produce a lung wash. A total of 6 successive washes from each rabbit were combined. These and samples of the residual lung tissues were stored at -20°C until assayed chemically, or their surface activity was measured. Other weighed samples of residual lung tissues were dried at 150°C for 2 days to determine lung dry weight.

In a further group of *in vitro* experiments, the first four lung washes were discarded. Oxotremorine (0.34 µg/ml), epinephrine (3.4 µg/ml), or saline was added to the infusing Krebs solution for 10 min after the fifth wash. Following a further 5-min period with Krebs solution only, the sixth wash was obtained. The fifth and sixth washes were assessed individually for their PC contents.

CHEMICAL ASSAYS

Aliquots (0.5 ml) of lung washes were assayed in duplicate for their PC content. Following three extractions with chloroform/methanol (2/1, v/v), the pooled chloroform layer was evaporated at 50°C under nitrogen. After separation of PC by thin-layer chromatography on silica gel (Merck F₂₅₄) using chloroform/methanol/glacial acetic acid/water (75/25/7/3, v/v/v/v) as solvent. PC was measured by phosphorus assay (6) using DPPC as standard. The minimum quantity of PC that could be readily distinguished from blanks was 10 µg. Samples of lung tissue were assayed in a similar manner after homogenisation for 2 min in chloroform/methanol (3/1, v/v) using an Ultra-Turrax type TP 18/2 homogenizer and filtration. Recovery of a 60 µg standard of DPPC was 82.2 ± 2.7% (mean ± S.E.; *n* = 20). Phosphatidylcholine content was corrected for procedural losses and standardized per g of dry lung weight.

Further samples of lung washes from two oxotremorine (0.2 mg/kg) and two saline-pretreated rabbits were subjected to two-dimensional thin-layer chromatography using chloroform/methanol/ammonia, (65/35/5; v/v/v) and chloroform/acetone/methanol/glacial acetic acid/water (50/20/10/10/5; v/v/v/v/v) as first and second solvents, respectively. Phosphomolybdic acid and stannous chloride or charring were used to identify the phospholipids present. After location with stannous chloride, the PC spots were removed and digested with methanolic sodium methoxide, and the methyl esters of the fatty acids were separated by gas-liquid chromatography (19). The relative peak areas were used to determine the proportion of each fatty acid in PC.

SURFACE ACTIVITY

Measurement of surface activity were made using an apparatus based on the Wilhelmy surface tension balance (Ref. 15; Fig 1a). This consisted of a platinum plate hung from a torsion arm and dipping in a 0.9% (w/v) saline subphase held in a perspex trough (1 × 5.2 × 12 cm). The recording system was an attached Polaroid type 87 camera. Vertical movement of the platinum plate by a change in surface tension produced a proportional deflection of a small mirror attached to the torsion arm and, consequently, move-

Fig. 1. *a*, surface tension balance. 1, Polaroid camera for continuous recording of surface tension; 2, manual reading scale; 3, shutter; 4, balance arm; 5, platinum plate dipping in saline; 6, perspex trough; 7, lamp; 8, motor; 9, perspex barrier to compress surface. *b*, continuous recording of surface tension on compression and expansion of 18 µl of lung wash from control rabbit.

ment of a spot of light reflected onto the film (Fig. 1b). Therefore, one axis of the film was surface tension and was calibrated by adding weights (50 to 300 mg) to the platinum plate. The surface was compressed by 83% by means of a perspex barrier driven by a motor at the rate of 1 cm/min and then expanded to the original dimensions. This movement produced a proportional deflection on the other axis of the film. A calibration curve was obtained by adding 6 to 11 µg of DPPC standard in chloroform/methanol (3/1; v/v) to the saline surface using a micropipet and 5 min later measuring the surface pressure at 83% compression relative to the surface tension of the surface-swept saline solution. The surface pressures produced by 6 to 18 µl of saline lung washes were obtained in a similar manner 15 min after addition to the surface (Fig 1b). The surface activity of lung washes was calculated in DPPC equivalents in mg/g of dry lung weight.

Statistical analysis was by analysis of variance (17) or Mann-Whitney *U* test (30).

The drugs and chemicals used and the suppliers (*parentheses*) were: atropine sulfate, epinephrine hydrogen tartrate (British Drug Houses); oxotremorine (R. Emmanuel Ltd.); d-propranolol, dl-propranolol hydrochloride (Imperial Chemical Industries); dl-dipalmitoyl phosphatidylcholine (Sigma Chemical Co.).

RESULTS

In control experiments (*n* = 5), it was found that 6 washes removed 95.7 ± 0.7% (mean ± S.E.) of the PC that could be removed with 12 washes. The total PC content of 6 washes was not significantly different (2 *P* > 0.05; Mann-Whitney *U* test) whether the interval was 5 or 30 min between successive washes.

Thirty min after the IP injection of oxotremorine (0.2 mg/kg), the PC content of the 6 pooled lung washes was approximately twice that of saline-injected control rabbits (Table 1). Atropine (2 mg/kg), dl-propranolol (1 mg/kg), or d-propranolol (1 mg/kg) were given IP at 40 min and oxotremorine at 30 min before killing. The antagonists had no effect themselves on the lung wash PC content. Atropine and dl-propranolol but not d-propranolol abolished the rise in lung wash PC content induced by oxotremorine.

Table 1. PC content of lung washes and residual lung tissue from neonatal rabbits¹

Treatment	Dose	Time treatment to killing (min)	PC contents (mg/g dry lung wt)		n
			Lung wash	Residual lung tissue	
Saline		30	25.7 ± 1.9 ^{a,b,2}	72.6 ± 6.0 ^a	5
Oxotremorine	0.2 mg/kg	30	50.5 ± 2.9 ^{a,c,d,e}	71.8 ± 6.8	7
Atropine	2 mg/kg	40	20.8 ± 2.0	104.4 ± 18.5 ^a	5
Atropine	2 mg/kg	40			
+			21.5 ± 1.1 ^c	83.4 ± 15.3	5
Oxotremorine	0.2 mg/kg	30			
dl-Propranolol	1 mg/kg	40	32.9 ± 8.4	70.9 ± 4.6	5
dl-Propranolol	1 mg/kg	40			
+			20.4 ± 1.7 ^d	85.4 ± 7.6	5
Oxotremorine	0.2 mg/kg	30			
d-Propranolol	1 mg/kg	40	23.9 ± 2.6	65.1 ± 5.3	5
d-Propranolol	1 mg/kg	40			
+			42.6 ± 6.2	78.3 ± 6.1	5
Oxotremorine	0.2 mg/kg	30			
Epinephrine	20 µg/kg	30	40.9 ± 6.8	55.5 ± 6.6	4
Epinephrine	50 µg/kg	30	78.0 ± 14.8 ^{b,f}	76.4 ± 13.1	5
dl-Propranolol	1 mg/kg	40			
+			20.4 ± 3.4 ^f	68.1 ± 2.7	5
Epinephrine	50 µg/kg	30			
Adrenalectomy		45	29.5 ± 5.3	63.0 ± 7.3	4
Adrenalectomy		45			
+			26.9 ± 4.5 ^e	73.9 ± 1.2	4
Oxotremorine	0.2 mg/kg	30			

¹ Values within columns with same superscripts were compared and are significantly different (analysis of variance; 2 *P* < 0.05).

² Mean ± S.E.

As expected, oxotremorine also produced tremors, salivation, and hypothermia. Only atropine produced clear antagonism of these effects.

Epinephrine (20 or 50 µg/kg) was given SC 30 min before killing. The higher dose produced a significant (2 *P* < 0.001; analysis of variance) three-fold increase in lung wash PC content (Table 1). This effect was antagonized by dl-propranolol (d-propranolol was not tested).

To further investigate the possible indirect action of oxotremorine, this drug or saline was given 15 min following bilateral adrenalectomy, and the animals were killed 30 min later. The PC content of lung washes was not altered 45 min after bilateral adrenalectomy compared to intact controls but the action of oxotremorine was abolished (Table 1).

The residual lung tissue content of PC in saline controls was significantly greater 40 min following atropine (2 mg/kg) pretreatment but not significantly different in any of the other experimental groups (Table 1). The total volume of saline washes withdrawn was not different between experimental groups. The total lung content of PC (lung wash plus lung tissue) after epinephrine (50 µg/kg) was 154.4 ± 13.8 mg/g dry lung weight which was 55% greater (2 *P* < 0.001) than for controls (98.3 ± 7.2 mg/g dry lung weight).

Further qualitative chemical analysis of the lung washes from two saline and two oxotremorine (0.2 mg/kg) pretreated rabbits showed that PC was the major phospholipid present, with only a small amount of phosphatidylserine which would cochromatograph with the PC on one-dimensional thin-layer chromatography. The main fatty acids present in the PC from the control rabbits were palmitic acid (74.6%), myristic acid (15.2%), and oleic acid (10.2%; each value mean from 2 animals). The proportions were similar from the oxotremorine pretreated rabbits.

In further groups of rabbits, the surface activity of lung washes was assayed using the surface tension balance. The surface tension of saline was 69.8 ± 1.5 dynes/cm (mean ± S.E.; *n* = 10). There was a linear relationship between the amount of DPPC standard (6 to 11 µg) added to the surface of a saline subphase and the surface pressure produced. Injection of rabbits with oxotremorine (0.2 mg/kg IP) or epinephrine (50 µg/kg SC) 30 min before killing

Table 2. Surface activity¹ of lung washes from neonatal rabbits

Drug	Dose	Time injection to killing (min)	Surface activity ¹ (mg/g dry lung wt)	n
Saline		30	42.8 ± 2.0 ^{2,3a,3b}	5
Oxotremorine	0.2 mg/kg	30	73.8 ± 9.9 ^a	5
Epinephrine	50 µg/kg	30	72.2 ± 10.2 ^b	5

¹ Surface activity measured on Wilhelmy surface tension balance in terms of equivalents of dipalmitoyl phosphatidylcholine.

² Mean ± S.E.

³ Superscripts *a* and *b*, significantly different from controls (2 *P* < 0.05; Mann Whitney *U* test).

approximately doubled the surface activity, measured as DPPC equivalents, in lung washes compared to saline controls (Table 2). There was a significant (2 *P* < 0.05) and positive correlation (*r* = 0.63) between PC content and surface activity in individual washes from these three groups.

Because oxotremorine and epinephrine could increase the PC content of lung washes *in vivo*, so their effects were studied in the isolated lung by a 10 min infusion via the pulmonary artery between the fifth and sixth washes. The PC contents of the sixth wash following saline (2.0 ± 0.3 mg/g; *n* = 5) or 0.34 µg/ml oxotremorine (0.9 mg/g; *n* = 2) were less than their respective fifth washes (2.8 ± 0.5 and 1.5 mg/g). However, following 3.4 µg/ml epinephrine, the PC content was significantly raised (2 *P* < 0.05; Mann Whitney *U* test) from 3.7 ± 0.6 mg/g (fifth wash) to 4.8 ± 0.8 mg/g (sixth wash; *n* = 6).

DISCUSSION

These experiments demonstrated that the PC content and surface activity of lung washes of neonatal rabbits was increased only 30 min after the injection of oxotremorine or epinephrine. The simplest explanation of these observations is that these drugs increased the secretion of lung surfactant into alveoli.

Lung surfactant is defined by its ability to lower surface tension

at the air-liquid interface (15). Saline washes (6 to 18 μ l) of neonatal rabbit lungs produced measurable surface pressures on compression and so the surface activity could be assayed quantitatively. Because DPPC is thought to be the principal component of lung surfactant (14, 15, 21), this was used as the standard for both chemical and surface activity assays. Increased PC content or surface activity of washes after oxotremorine or epinephrine does not necessarily imply that the DPPC content was increased. However, a combination of two-dimensional thin-layer chromatography and gas-liquid chromatography suggested that palmitic acid formed about 75% of the fatty acids in the PC spot, and this proportion was similar after oxotremorine. This proportion is slightly higher than the findings of others in neonatal rabbits (14, 28). The results suggest that the major PC present in the lung washes was DPPC. Also, there was a significant positive correlation between PC content and surface activity of lung washes from oxotremorine, epinephrine, and control treated rabbits. Therefore, we suggest that assay of PC content of lung washes is a reasonable measure of surfactant content.

A major criterion that one is studying stimulation of a secretory rather than a biosynthetic process is the measurement of an increase in the concentration of the secreted substance in an appropriate extracellular space a short time interval after application of the stimulus. The finding of increased PC content and surface activity of lung washes only 30 min after oxotremorine and epinephrine *in vivo* and increased PC content of washes only 15 min after commencement of epinephrine perfusion *in vitro* are more direct evidence of stimulation of secretion than the results of most earlier workers (7, 8, 12, 18, 25, 26, 34) who have used time intervals of more than 3 hr between stimulus and measurement.

The increase in surfactant in lung washes after oxotremorine and epinephrine *in vivo* and the abolition of these effects with atropine and dl-propranolol, respectively, provide further support for the modulation of surfactant secretion via muscarinic receptors and β -adrenoceptors (see "Introduction"). The effect of oxotremorine *in vivo* was antagonized by dl-propranolol and acute adrenalectomy. Also, oxotremorine was ineffective in increasing the lung wash PC contents in the isolated, perfused, and ventilated lung in two experiments and as published previously (1), but epinephrine could increase lung wash PC contents. These results therefore suggest that oxotremorine does not act directly on type 2 pneumocytes to cause secretion and support the hypothesis that pilocarpine, another muscarinic agonist, also acts indirectly (8). Rather, the results suggest that oxotremorine increases catecholamine secretion from the adrenal medulla which then act on β -adrenoceptors in the lung to produce surfactant secretion. Recently, oxotremorine has been shown to increase norepinephrine and epinephrine plasma concentrations in the adult rat (33). The resolved d-propranolol was also used as this isomer has the local anaesthetic properties of dl-propranolol but is less potent as a β -adrenoceptor antagonist (20). The lack of antagonism by d-propranolol of oxotremorine's effects provide further support for oxotremorine's action via β -adrenoceptors rather than by other mechanisms. In most tissues where there is autonomic control of secretory processes, this is mediated by muscarinic receptors rather than by adrenoceptors. However, there is a precedent in that renin release from the kidney is modulated by β -adrenoceptors (10).

It was anticipated that the increased lung wash content of PC after oxotremorine and epinephrine would be reflected in a proportionate decrease in the PC content of the residual lung tissue; this was not the case. In lung tissue, compared to lung washes, a greater proportion of the PC's are unsaturated, non surface active and form cell membranes (14, 28). This could have obscured any changes in the saturated, surface active PC in the residual lung tissue. Because the total lung content of PC was 55% greater than control 30 min after epinephrine (50 μ g/kg), this must reflect increased biosynthesis. Our results and those of others (8, 11, 12, 18, 24, 26, 34) provide evidence for β -adrenoceptor control of secretion. We therefore suggest that increased biosynthesis *in vivo* was secondary to increased secretion. Primary control of secretory rate with feedback control of biosynthesis rate is a common feature

where substances are stored in a granular form before secretion (22). Measurements of turnover time after drug treatment are necessary to investigate this important hypothesis further.

The absence of an alteration in lung wash PC content after atropine, dl-propranolol or adrenalectomy suggest little tonic activity via muscarinic receptors or β -adrenoceptors in causing a regular secretion of lung surfactant in neonatal rabbits. We are as yet unable to explain the increased residual lung tissue PC content after atropine alone. It is possible that a 40-min interval between injection and killing was too short to demonstrate any effects of the antagonists on a tonically active system. Oyarzún and Clements (25, 26) demonstrated a slightly decreased phospholipid content of lung washes in adult, lightly anesthetised rabbits 2 hr after dl-propranolol but no change following atropine. It is possible that muscarinic receptor and β -adrenoceptor mediated control of lung surfactant secretion is usually minimal but may be more important under certain situations such as during increased ventilation (13, 25, 26) and at birth.

CONCLUSION

The PC content of lung washes of neonatal rabbits was increased 30 min after oxotremorine. This effect was antagonized by atropine, dl-propranolol, acute adrenalectomy but not by d-propranolol. The PC content of lung washes was also increased 30 min after epinephrine, the effect being antagonized by dl-propranolol. Oxotremorine and epinephrine increased the surface activity in lung washes. Epinephrine, but not oxotremorine, increased the PC content of lung washes of isolated, perfused, and ventilated lungs. The results suggest that oxotremorine and epinephrine increased the secretion of surfactant into alveoli. Oxotremorine probably acts indirectly by increasing secretion of catecholamines from the adrenal medulla which then act on β -adrenoceptors in the lung. The total lung tissue content of PC was increased after epinephrine suggesting that biosynthesis was stimulated secondarily to increased secretion.

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