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Effects of the thromboxane receptor agonist U46619 and endothelin-1 on large and small airways

C. Martin*, V. Ullrich**, S. Uhlig*

Effects of the thromboxane receptor agonist U46619 and endothelin-1 on large and small airways. C. Martin, V. Ullrich, S. Uhlig. ©ERS Journals Ltd 2000.

ABSTRACT: Recently attention has been drawn to the role of small airways in asthma. However, little information exists about the responsiveness of small airways to various bronchoconstrictors in comparison to large airways. In this study, the model of precision-cut lung slices (PCLSs) was used to investigate the effects of the thromboxane receptor agonist U46619 and endothelin (ET)-1 on small (diameter <250 μm), medium (250–420 μm) and large (>420 μm) airways.

Viable PCLSs were prepared from rat lungs and the bronchoconstriction of differently sized airways inducible by U46619 and ET-1 was observed by means of a microscope and analysed by digital imaging techniques.

The median effective concentration (EC_{50}) of U46619 for inducing bronchoconstriction was 6.9 nM in small and 66 nM in large airways, respectively. This finding was corroborated by direct observations in single lung slices containing both a small and a large airway. In such slices, U46619 caused smaller airways to contract to a greater degree than larger ones. ET-1 induced bronchoconstriction was similar in small (EC_{50} 34 nM) and in medium or large (EC_{50} 22 nM) airways. This was again confirmed by direct observation of ET-1-treated PCLSs.

It is concluded that, in rat lungs, endothelin-1 affects small and large airways to the same extent, whereas thromboxane is ten times more potent in causing small airways to contract than larger ones. Precision-cut lung slices appear to be a valuable model for examining the (patho)physiology of small airways.

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*Division of Pulmonary Pharmacology, Research Centre Borstel, Borstel, and **Faculty of Biology, University of Constance, Constance, Germany.

Correspondence: C. Martin, Division of Pulmonary Pharmacology, Research Centre Borstel, Parkallee 22, D-23845 Borstel, Germany. Fax: 49 4537188478

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Thromboxane and endothelin (ET)-1 are two of the most potent bronchoconstrictors and both have been implicated in a number of airway diseases such as asthma, septic shock and acute respiratory distress syndrome [1–7]. ET-1 is a powerful spasmogen and elicits not only bronchoconstriction but also pulmonary vasoconstriction [8, 9]. Of the two known ET receptor subtypes, the ET_A receptor accounts largely for vasoconstriction and the ET_B receptor largely for bronchoconstriction [10]. However, little information is available regarding differences in receptor density or receptor subtype distribution along the airways [11, 12]. Like ET-1, thromboxane also causes both airways and vessels to constrict [2]. The thromboxane-prostanoid (TP) receptor is thought to be located mainly on smooth muscle cells of airways and vessels [13], although, unfortunately, there is no detailed information regarding its distribution in the lung.

Recently, attention has been drawn to the fact that the small airways represent an underexplored area of the lungs and more knowledge about small airways is expected to provide more insight into airway diseases such as asthma [14, 15]. With respect to both thromboxane and ET-1 almost no information about their effects on airways <2 mm exists. In perfused rat lungs treated with lipopolysaccharide [16] or the TP receptor agonist U46619 [17], the bronchoconstriction is found predominantly in terminal bronchioles. In addition, SHIOYA *et al*. [18] showed stronger responses of smaller airways to intravenously administered U46619 in dogs *in vivo*, although their experimental approach only allowed study of airway generations 0 (diameter 22 mm) to 6 (diameter 1 mm). However, these findings might be explained by the fact that both after intravenous injection and in perfused lungs compounds such as thromboxane reach the lungs through the pulmonary artery and may therefore act preferentially on smaller airways, since larger airways are supplied predominantly by the bronchial artery [19].

Although most authors define small airways in humans as those of <2 mm in diameter, the method used in the present study permits the analysis of airways considerably smaller than this. The present model is based on precision-cut tissue slices obtained from rat lungs incubated in cell culture medium, with single airways being observed directly using a microscope [20]. Compared to experiments *in vivo* or in perfused lungs, lung slice airways can be exposed to defined concentrations of an agonist independent of perfusion by the pulmonary or the bronchial artery and also largely independent of diffusion paths. Therefore, the method of precision-cut lung slices (PCLSs) can provide unique information about the sensitivity of differently sized airways to various drugs.

In the present study, PCLSs were used to examine the contractile responses of individual airways of different size to thromboxane and to ET-1.

Material and methods

Animals

Lungs were taken from 8-week-old female Wistar rats (220±20 g) obtained from Harlan Winkelman GmbH (Borchen, Germany) and kept under controlled conditions (22°C, 55% humidity, 12 h day/night rhythm) and fed a standard laboratory chow.

Design of the study

The slices were transferred from culture dishes or roller incubators to an incubation chamber and placed on the stage of an inverted microscope [20]. After pre-incubation for 10 min with 1 mL minimal essential medium (MEM), the first image was acquired. The airway area obtained from this first image served as the reference area (100%). The liquid was removed and U46619 or ET-1 diluted in 1 mL medium were transferred into the incubation cell. The airway was imaged every 30 s during the incubation time of 10 min, to determine maximal bronchoconstriction. The U46619 concentration was varied from 10^{-11} M to 10^{-4} M and increasing concentrations were added to single slices with a wash step between each concentration; the wash step always led to full relaxation of the airways. ET-1 was used in concentrations from 10^{-11} M to 10^{-4} M; for each concentration a new slice was used.

Methods

Lung slices. Lung slices were prepared essentially as described previously [20]. The lungs were perfused with Hank′s solution through the pulmonary artery until they were free of blood. The heart and lungs were removed *en bloc* and the lungs filled through the trachea with 10 mL agarose solution (0.75% in MEM, 44 mL·kg^{-1}) and a 1-mL bolus of air. For instillation and incubation, MEM supplemented with 1 mM sodium pyruvate, amino acids (MEM amino acid and glutamine supplement; Gibco BRL, Life technologies, Karlsruhe, Germany), vitamins (MEM vitamin supplement; Gibco BRL, Life technologies) and 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) (pH 7.4) was used. After the agarose had cooled to 4°C, tissue cores were prepared by advancing a rotating sharpened metal tube (diameter 8 mm). From these cores, tissue slices (220±20 µm thickness) were prepared using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA). Lung slices were floated on to Teflon mesh and cultured in glass vials containing 1 mL MEM. The vials were placed on a roller system housed in a humidified incubator. They were incubated at 37°C in a humid atmosphere and rotated at 10 revolutions per minute.

Lung slices were incubated in MEM medium lacking phenol red, since it has recently been reported that phenol red blocks the TP receptor [21]. In line with this, it was found that, in the presence of phenol red, the concentration/response curve of lung slices to U46619 was shifted ~1–3 log units to the right (data not shown).

Analysis

Image acquisition. The incubation chamber was placed on the stage of an inverted microscope (Leica DM IRB, Leica, Benzheim, Germany) and warmed to 37°C. The slices were transferred to this chamber. The airways were brought in to focus, imaged with a digital video camera (Visicam 1300, Visitron, Munich, Germany). An image of 2.3 mm^2 (on the original object) was represented by 509×639 pixels.

Image analysis. The images were analysed using an image analysis program (OPTIMAS 6.2, Optimas Corporation, Bothell, WA, USA). The luminal area was taken as the area enclosed by the epithelial luminal border and was quantified after setting the appropriate threshold value. After appropriate calibration, the airway area was measured using the image analysing program. Control airway area was defined as 100%.

Airway diameter. In order to give a more comprehensible view of airway size, airway size was expressed as the airway diameter that a perfectly circular airway of the given airway area would have. Airways of 0.05 mm in diameter roughly correspond to the terminal bronchioles in rats [22]. As before [20], small airways were defined as those having a diameter of 50–250 µm (airway generation 24 to 17 according to [22]), and medium and large airways as those with diameters of 250–420 µm (airway generation 16 to 14) and >420 µm (airway generation 13 and above), respectively.

Histology of lung slices. PCLSs were transferred to 24-well plates and fixed overnight in 10% buffered formalin (100 mM potassium phosphate buffer, pH 7.0). The tissue was dehydrated in 10, 20, and 30% sucrose solution (in 100 mM potassium phosphate buffer), each for 24 h. Frozen sections were prepared in a cryostat (Mircrom, Walldorf, Germany), transferred to a freezing block, covered by Tissue Tek (Pelco, Freiburg, Germany) and stored at -70°C. The frozen sections of 5- or 10-µm thickness were transferred to slides used for light microscopic examination and counterstained with haematoxylin and eosin.

Statistics. Data are expressed as means±SD or means±SEM, as indicated in the figure legends. Sigmoidal concentration/response curves were fitted using the four-parameter logistic equation program ALLFIT [23]. This program allows simultaneous analysis and comparison of families of sigmoidal curves. Median effective concentrations (EC_{50}) and Hill slopes for concentration/response curves were obtained from this program. Identity of EC_{50} values and slopes was tested for by comparing the sum of the squares from the constrained and the unconstrained model (F-test, $p<0.05$). Therefore, if within one group different EC_{50} are given, they were significantly different from each other ($p<0.05$).

Results

The time course of U46619- and ET-1-induced contraction of large airways in PCLSs at concentrations of 10^{-6} M is shown in figure 1. After 10 min, the contraction of large airways in response to both agonists was complete;

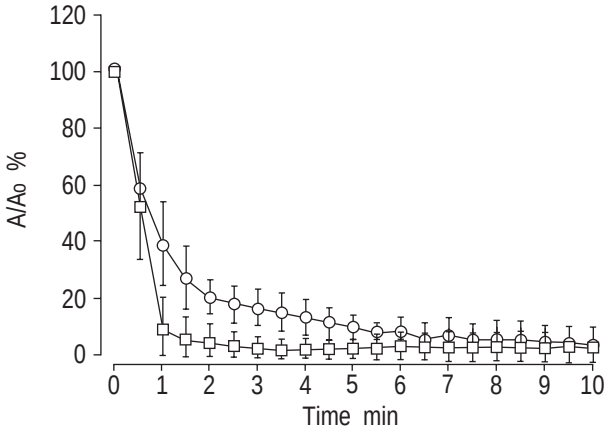

Fig. 1. – Time course of endothelin (ET)-1- and U46619-induced bronchoconstriction in individual large airways (n=6) in rat lung slices exposed to the thromboxane receptor agonist U46619 (1 μM, □) or ET-1 (1 μM, ○). Data are expressed as mean±SD. A: area; A_o: initial area.

therefore, the following data were all obtained after 10 min of exposure.

Effects of U46619

Figure 2a–f shows a lung slice with a bronchus and a vessel. After 10 min of pre-incubation, airway and vessel area were measured and defined as 100%. The slice was imaged 10 min after treatment with U46619 concentrations ranging 10^{-10}–10^{-6} M. The corresponding concentration/response curve is shown in figure 2g. A decrease in airway area was observed for U46619 concentrations of 10^{-9}–10^{-6} M. Contraction of the vessel was also measured for concentrations between 10^{-10} M and 10^{-6} M and finally reached 50% of the initial vessel area. In contrast, the bronchus was nearly closed at the latter concentration.

Bronchoconstriction was determined for U46619 concentrations of 10^{-11}–10^{-4} M in airways of different size (fig. 3). As previously [20], airways were divided into three groups: small, medium, and large. The following EC_{50} were determined: small airways 6.9 nM, medium airways 26 nM and large airways 66 nM (table 1). These three EC_{50} were significantly different ($p<0.05$) from each other. The three concentration/response curves for small, medium and large airways shared a common maximum, minimum and Hill slope (data not shown).

The preferential contraction of small airways by U46619 is illustrated in a single slice containing both a large and a small airway (fig. 4). fig. 4a–c shows one viable slice containing a bronchus, a bronchiole, a large vessel and several small vessels as seen using a video microscope. While figure 4a shows the untreated slice, figures 4b and c show the slice in the presence of 10^{-7} and 10^{-6} M U46619, respectively. In the presence of 10^{-7} M U46619, the area of the large airway was reduced to only 80% of its initial area, whereas the bronchiole was caused to contract to 10% of its initial area. Increasing the U46619 concentration to 10^{-6} M caused contraction of the large airway to 30% of its initial airway area, whereas the bronchiole was completely closed. The vessel closed to 60 and 50% of the initial luminal area in the presence of 10^{-7} and 10^{-6} M U46619, respectively.

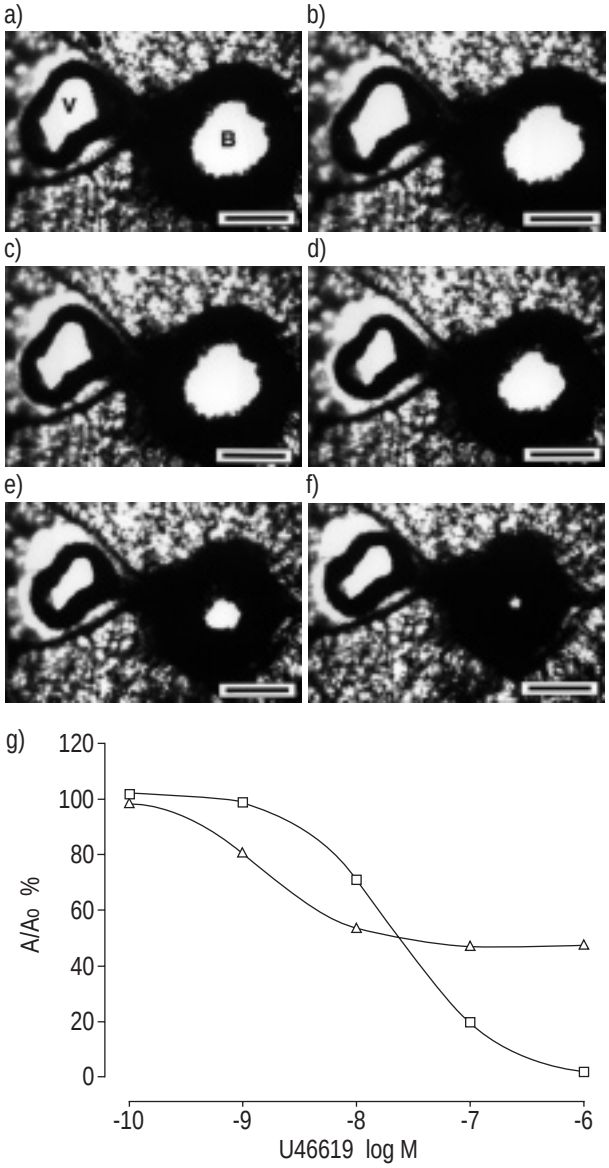

Fig. 2. – Response of a single airway (B) and vessel (V) to U46619. a–f) Images of a lung slice showing a single airway and a single vessel treated with increasing concentrations of U46619 (a) 0 M; b) 10^{-10} M; c) 10^{-9} M; d) 10^{-8} M; e) 10^{-7} M; and f) 10^{-6} M. The concentration/response curves obtained from calculation of the relative airway (□) and vessel (△) area (A) in a–f. A_o: initial area. (Internal scale bar=300 μm.)

After incubation with U46619 (10^{-6} M) the viable lung slice shown in figure 4c was fixed and frozen sections were prepared (fig. 4d and e). Histological analysis confirmed that the large airway and bronchiole were contracted, although to different extents. The bronchiole was nearly closed, whereas the large airway remained partially open. The perivascular oedema around the large vessel apparent in figure 4d is a feature that is typical of perfused lungs [16, 24].

Effects of endothelin-1

The effect of ET-1 on airways of different size is shown in figure 5. The EC_{50} for small airways was 34 nM and

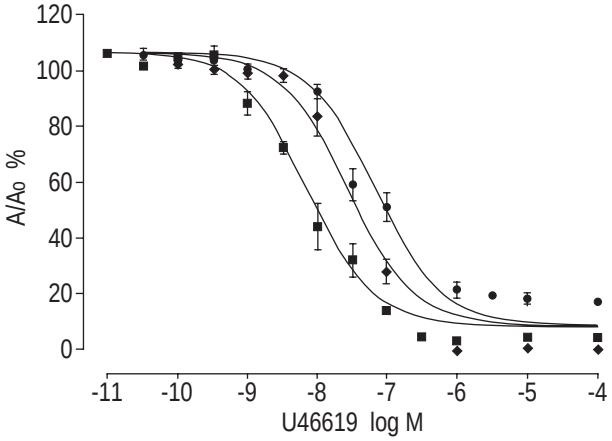

Fig. 3. – U46619-induced bronchoconstriction in rat lung slices depending on airway size. Concentration/response curves for U46619-induced bronchoconstriction in small (■), medium (◆) and large (●) airways. The corresponding EC_{50} were 6.9, 26 and 66 nM, respectively. Each curve was calculated from 83, 88 and 96 data points obtained from 25, 25 and 28 different lung slices, representing 14, nine and nine different rat lungs, respectively. Data are expressed as mean±SD. A: area; A_o: initial area.

significantly different from medium and large airways (22 nM, $p<0.05$). There was no difference between large and medium airways with ET-1 ($p=0.56$) Thus, in contrast to U46619, ET-1 caused small and large airways to contract with almost equal potency.

This is again illustrated in a single slice containing both a large and small airway (fig. 6). At an ET-1 concentration of 10^{-8} M, the area of the small and large airway did not change. At a concentration of 10^{-7} M, the small airway contracted to 45% of its initial area and the large airway to 30%. At the final ET-1 concentration of 10^{-6} M, the large and small airway were both almost completely closed; the area for large and small airways was 1% and 5%, respectively. In figure 6, in addition to the large and small airway, there is also a pulmonary vein visible. In contrast to its effects on the airways, ET-1 caused the pulmonary vein to contract only to a small extent (69% of initial area).

Discussion

Recently, the small airways have been identified as a possibly important area in the lung that needs investigation [14]. Such study could also help in the identification of sites within the lung that should be targeted by drugs. The current lack of knowledge about the small airways is reflected by them having been dubbed the "quiet zone" (see references in [15]) or the "silent zone" [25]. The method of PCLSs will be of great help in exploring this allegedly silent zone, since it allows the investigation of dynamic responses of individual airways down to the size of the terminal bronchioles. Using this method, it is shown here that small airways are approximately 10 times more sensitive to the TP receptor agonist U46619 than larger ones, whereas large and small airways respond approximately equally strongly to ET-1. The fact that the

Table 1. – Median effective concentrations (EC_{50}) of various agonists for their ability to cause differently sized airways to contract

Mediator	EC_{50} nM		
	Large airway	Medium airway	Small airway
U46619	66	26	6.9
Endothelin-1	22	22	34
Methacholine*	870	560	100

*: from [20].

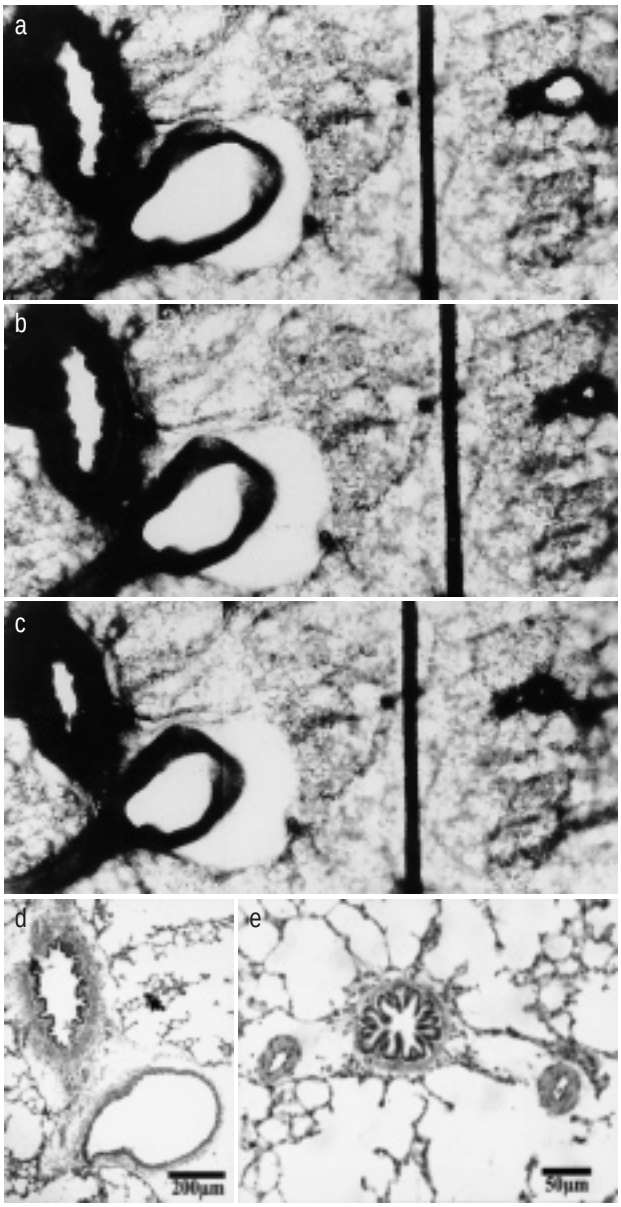

Fig. 4. – Simultaneous analysis of a large and a small airway in the same slice. a–c) Video images of a viable slice showing a pulmonary artery, a large airway (334 µm in diameter, on left) and a small airway (75 µm in diameter, on right). It was necessary to take two photographs of the same slice at almost the same time as the two airways were not very close to each other; these two photographs are separated by the thick black lines. The same slice is shown before (a; control) and after exposure to 10^{-7} M U46619 (b) or 10^{-6} M U46619 (c). d, e) Micrographs of frozen sections of the slice shown in C. (Haematoxylin and eosin stain.) (Internal scale bars a–c=300 µm; d=200 µm; e=50 µm.)

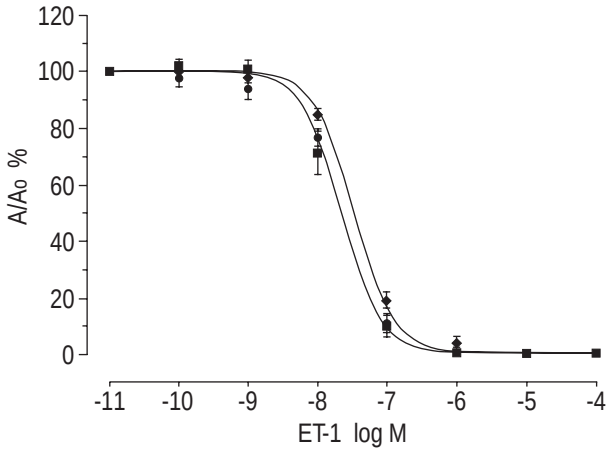

Fig. 5. – Endothelin (ET)-1-induced bronchoconstriction in rat lung slices according to airway size. Concentration/response curves for ET-1-induced bronchoconstriction in small (◆), medium (■) and large (●) airways. The corresponding median effective concentrations were 34 (small) and 22 nM (medium and large airways). Each curve was calculated from 28, 69 and 74 data points obtained from six, 25 and 27 different lung slices, representing five, nine and eight different rat lungs, respectively. Data are expressed as mean±SD. A: area; A_o: initial area.

potency of ET-1 was independent of airway size suggests that the greater responsiveness of small airways to U46619 is not due to inherent (for instance geometric) properties of the slices.

The model of precision-cut lung slices

A prerequisite for measuring responses in airways as small as terminal bronchioles is that the lungs be cut into PCLSs [20]. Only if the airways are as thin as 250 μm is it possible to study airways with a diameter of as small as 0.05 mm. These airways roughly correspond to the terminal bronchioles in rat lungs [22]. In addition, in such well-defined slices, measurements of airway contraction are more reproducible [20] than in slices cut by razor blades, as in the study of DANDURAND *et al*. [26]. Nevertheless, these authors were the first to demonstrate that lung slices can be used for assessment of lung function. The shortcomings of their technique were the relatively thick slices (>500 μm), the variability in the thickness of slices cut by razor blades and the routine use of steroids. The use of PCLSs, with a thickness of 250±20 μm compared to 500–1,000 μm, has only twice been reported previously [20, 27]. The major advantages of thinner slices are that smaller airways can be studied and experiments are more reproducible, as illustrated by the EC_{50} for methacholine-induced bronchoconstriction which varied 5.5×10^5-fold in razor-cut lung slices [26] but only 5×10^3-fold in PCLSs [20]. In addition, in precision-cut lung, it was shown that much of the variation was due to the different sizes of the airways, *i.e.* the variation was only 20-fold in smaller airways (diameter 50–250 μm) and 200-fold in medium (250–420 μm) and large (>420 μm) airways [20]. The same grouping into small, medium and large airways was used to analyse the concentration/response curves in the present study. In addition, in PCLSs, it is possible to examine airways of different size at the same time and within the same slice (figs. 4 and 6). To date, this is the only system in which a direct comparison of physiological responses of airways of different size is possible simultaneously in the same tissue. Another advantage of this model is that it is easily adapted to the lungs from other species. For example, the authors have also successfully applied this method to the preparation of mouse lung slices. In the mouse, the EC_{50} obtained with PCLS for U46619 and ET-1 were very similar to those obtained in perfused lungs [27], providing further proof of the validity of the PCLS method.

A possible concern when studying bronchoconstriction in lung slices is that the motility of the vessels might affect that of the airways, *i.e.* constriction of vessels might interact with constriction of airways. However, two observations suggest that, in the slices, changes in vessel tone do not have any influence on airway tone, or at least none that is stronger than that in the whole organ. First, in a previous study, the effects of various smooth muscle constricting agents were compared in the slice model and in the perfused mouse lung model [27]. In that study, it was found that regardless of the agonist used, *i.e.* regardless of whether only airways or airways and vessels were contracted, the EC_{50} for the airways were similar in both models. Secondly, the effects of ET-1 and the ET_B receptor agonist IRL1620 were studied recently. It was observed that even though ET-1 causes both airways and vessels to contact, and IRL1620 causes only the airways to contact, the EC_{50} for airway contraction was not significantly different for both agonists (data not shown).

Responses to U46619

In the present study, it was observed that U46619 preferentially caused smaller airways to contract. In lung slices, it was found that smaller airways were four times more sensitive to U46619 than medium ones and 10 times more sensitive than larger ones. In addition, the direct comparison of a small and a large airway in the same lung slice showed a strong effect of U46619 in small and only a minor effect in large airways (fig. 4). These findings are in line with data obtained in isolated perfused lungs, in which the major site of bronchoconstriction by U46619 was found in the terminal bronchioles [17].

One possible explanation for the preferential contraction of smaller airways in perfused lungs was the perfusion of the lungs *via* the pulmonary artery [17]. Since the pulmonary artery largely supplies the smaller airways, this route of perfusion could have been responsible for the observed results. However, the fact that a similar site specificity also occurred in lung slices, shows that smaller airways are inherently more sensitive to U46619 than larger ones. There are several possible explanations for this observation: 1) different receptor density; unfortunately, almost nothing is known about TP receptor distribution in lungs; 2) different types of TP receptor [28]; 3) larger airways may release larger quantities of an unknown bronchodilator [29]; and 4) differences in the ratio of smooth muscle and airway area [30] or in the thickness of the epithelium or adventitia [31] can largely be excluded, because, in this case, smaller airways should be more sensitive than larger ones regardless of the stimulus. However, in view of the small differences in EC_{50} for

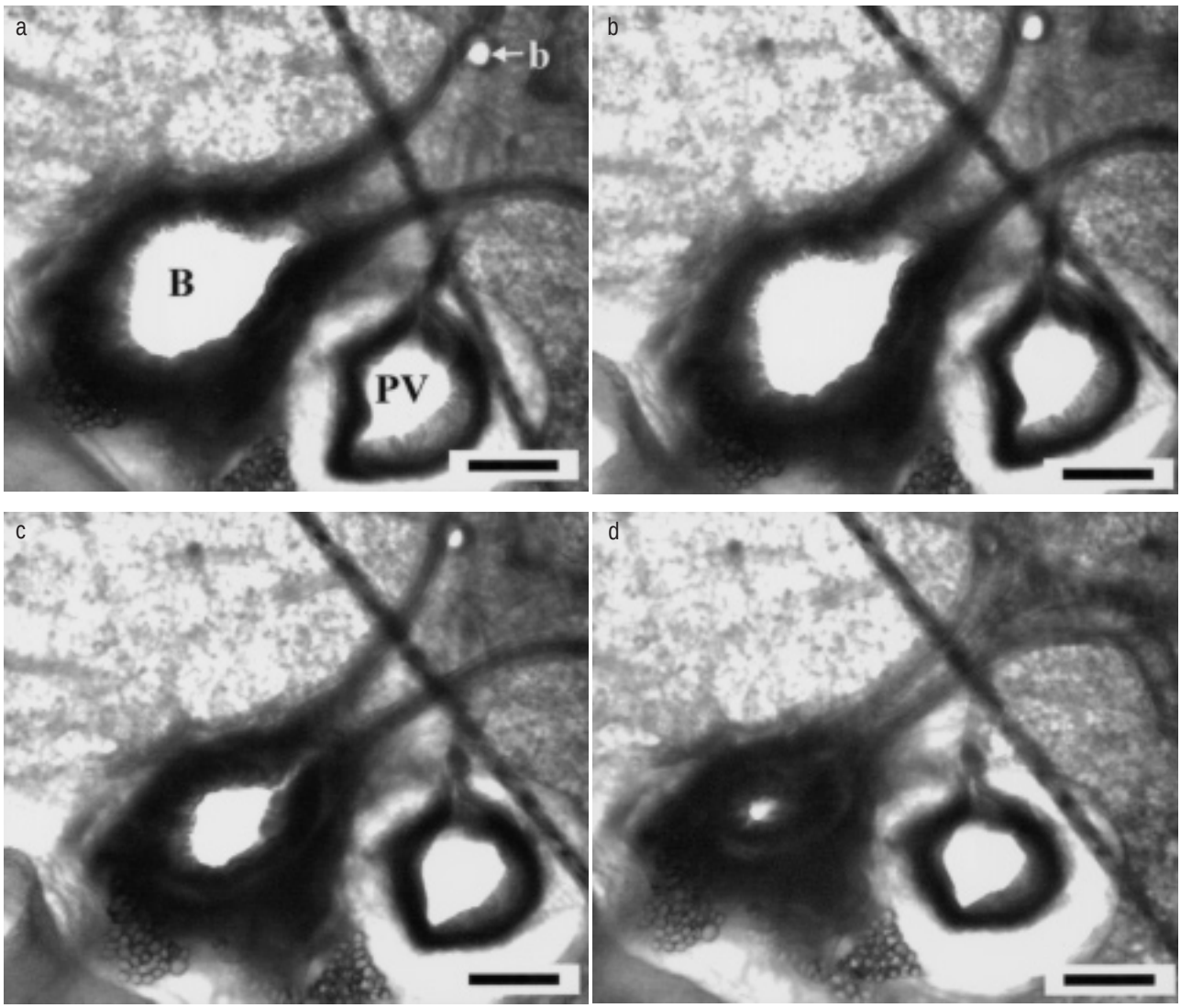

Fig. 6. – Video images of a viable slice treated with endothelin (ET). The image shows a pulmonary vein, a large airway (360 μm in diameter) and a small airway (53 μm in diameter). Image of a viable slice after: a) control; and b) 10^{-8} M; c) 10^{-7} M; and 10^{-6} M ET-1 treatment. B: bronchus; b: bronchi; PV: pulmonary vein. (Internal scale bar=200 μm.)

ET-1 in airways of different size, anatomical or geometric factors can largely be excluded.

Thus, at present, the mechanism underlying the increased sensitivity of smaller airways to stimulation of the TP receptor is unknown. Interestingly, the present authors [20] and others [31] have previously made a similar observation with methacholine. Since both U46619 and methacholine preferentially cause smaller airways to contract in PCLSs, it might be concluded that this is an inherent property of this model rather than an effect specific to the compounds themselves. Therefore, it was important to find a compound such as ET-1 that showed similar activity on differently sized airways (table 1). With respect to methacholine, studies comparing the bronchoconstrictor responses in isolated lungs perfused either *via* the pulmonary or the bronchial artery are of interest, because larger airways are predominantly supplied by the bronchial and smaller airways largely by the pulmonary artery [19]. In such studies, perfusion with both methacholine and serotonin caused a greater increase in airway resistance if perfused through the pulmonary artery [32]. Another piece of evidence suggesting that contraction of small airways may be important under physiological conditions are studies comparing the airway-relaxing effects of bronchodilatory aerosols of different particle size. The diameter of aerosol particles determines where in the lung particles are deposited; the smaller the particles, the smaller the airways they can reach [33]. In such studies, it was noted that aerosols made of small particles are more efficient in relaxing airways than those containing larger particles, suggesting that the smaller airways were the major determinant of airway resistance [34].

So far, the responses of differently sized airways to thromboxane have been studied only in dogs [18] and guinea-pigs [35]. However, compared to the present

study, in both investigations only relatively large airways were studied. WONG *et al*. [35] found that bronchial rings were less responsive to U46619 than lung strips. SHIOYA *et al*. [18] reported a stronger response of airways of the 6th generation of the dog compared to larger airways. As these experiments were performed by means of tantalum bronchography, it was impossible to detect airways smaller than those of generation six.

In addition to airway responses, in the present study, vascular responses to U46619 were also observed. In PCLSs, vessels were caused to contract by U46619 concentrations in the nanomolar range. Comparison of the pulmonary artery in fig. 4a–c with the histological section in figure 4d and e shows a good agreement between viable and fixed slices. The differences in the lumen of the pulmonary artery in figure 4c (viable lung slice) and 4d and e (fixed lung slice) may be explained by different cutting planes of the viable and the fixed lung slice or by relaxation during the fixation process.

Responses to endothelin-1

ET-1 acts on two receptor subtypes, ET_A and ET_B [12]. There is little available information about receptor density and receptor distribution in airways of different size. For the rat trachea, GOLDIE and coworkers [11, 12] reported the density of binding of ET-1; in the lung parenchyma only the relative distribution of ET_A and ET_B receptors is known, but not the density of the receptors. Therefore, the present data cannot be discussed in terms of ET receptor distribution. The ET_B receptor is predominantly responsible for the contraction of airways in several species. In parenchymal strips from guinea-pig and isolated human bronchi, contraction was inhomogeneous, which might have been due to contraction of small airways [36, 37]. In the present authors′ hands, the EC_{50} of large, medium and small airways were in the same range (22–34 nM). The ET receptor subtype distribution that is responsible for these responses requires further investigation.

In conclusion, precision-cut lung slices are a valuable tool for investigating the physiology and pathophysiology of small airways. The present findings show that smaller airways are more sensitive to the TP receptor agonist U46619 and methacholine, but similarly sensitive to endothelin-1. Such differential responsiveness suggests that small airways are subject to complex control mechanisms, indicating that this zone of the airways may not be as quiet as previously thought.

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