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Cigarette smoke exposure causes constriction of rat lung explant airways

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Cigarette smoke exposure causes constriction of rat lung explant airways. JL. Wright, J-P. Sun, A. Churg. © ERS Journals Ltd 1999.

ABSTRACT: Cigarette smoke is known to cause acute increases in airway resistance, but the mechanisms behind this effect are unknown.

Lung explants were utilized to examine the constrictive effects of acute cigarette smoke exposure on bronchioles from rats *in vitro* that had or had not been previously exposed to cigarette smoke *in vivo*.

It was found that smoke induced a small but consistent degree of contraction of the airways *in vitro*, which could be reduced by an endothelin receptor antagonist in the animals which had had no previous smoke exposure *in vivo*, and reduced by the oxidant scavengers superoxide dismutase or catalase in the animals with previous smoke exposure.

In conclusion, cigarette smoke induces acute small airways constriction through both endothelin release and direct oxidant effects; which mechanisms are operative depends on the prior smoking status.

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In humans, chronic cigarette smoking is associated with abnormalities of airway structure and function such that the airways become narrowed and there is an increase in airflow resistance (reviewed in [1]). However, acute exposure to cigarette smoke appears to produce an acute bronchospastic response [2–7], although the mechanism of this smooth muscle constriction is not clear. NAKAMURA *et al.* [8] found that acute inhalation of smoke produced increases in both central and peripheral resistance; only the central resistance was abolished by vagotomy. In a previous study in the authors' laboratory [7], it was suggested that constriction of the peripheral airways was due to the inflammatory response induced by the smoke, data which supported the studies of O'BYRNE *et al.* [9] and HOLTZMAN *et al.* [10]. However, cigarette smoke contains a large number of oxidants and it is quite possible that these act directly on the smooth muscle to produce constriction [11]. Likewise, it is possible that the smoke stimulates the release of bronchoconstrictive mediators. For example, it has been shown that endothelin receptor antagonists block smoke-induced airway and vascular cell proliferation [12]; since endothelin is also a powerful bronchoconstrictor, this finding raises the possibility that endothelin is also involved in the very acute effects of smoke on the airways.

Partitioning bronchoconstrictive effects in whole animals is often complicated because it is difficult to separate central from peripheral resistance, and, in determining peripheral resistance, it is difficult to examine other than small segments. Lung explants have proven to be an excellent vehicle by which to study directly the effects of constrictor agents on the peripheral airways [13, 14] in an environment which is free of the complicating effects of inflammatory cells and circulating mediators. In this study, the explant technique has been utilized to examine the possible direct smooth muscle constrictor effects of smoke on the noncartilaginous (peripheral) airways.

Methods

The experimental protocol was approved by the Committee on Animal Care at the University of British Columbia, Vancouver, Canada.

Sources of materials

Male Sprague-Dawley rats were obtained from Charles River (Quebec, Canada). Dulbecco's modified Eagle's medium (DMEM) and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) were obtained from Gibco (Grand Island, NY, USA), endothelin receptor antagonist BQ-610 from Peptides International (Louisville, KY, USA), catalase (CAT) from Boehringer-Mannheim (Laval, Canada), and superoxide dismutase (SOD), agarose and atropine from Sigma (St Louis, MO, USA). The CAT was inactivated by boiling for 30 min.

Culture media

Bicarbonate-buffered culture medium (BCM) was prepared from DMEM powder by adding salts and supplements as previously detailed [14]. The supplemented culture medium was adjusted to pH 7.2 and sterilized using a 0.22 μm filter (Nalgene Company, Rochester, NY, USA), resulting in a BCM with a final pH of 7.3. HEPES-buffered culture medium (HCM) was prepared in a manner identical to the BCM except that 5.96 $g \cdot L^{-1}$ HEPES was substituted for the sodium bicarbonate. Agarose type

VII solution (2%) was prepared in BCM without supplements. Equal volumes of the liquid 2% agarose solution at 37°C and BCM containing twice the normal level of supplements were mixed to produce the 1% agarose/BCM solution.

Explant preparation and exposure

Groups of explants taken from animals in two experimental groups, each consisting of five Sprague-Dawley rats weighing 300–325 g were examined. The first group of rats was exposed to the smoke of 10 cigarettes using a nose-only exposure apparatus [15]; each rat is enclosed in a chamber and wears a flattened Elizabethan collar so that it cannot turn its head away from the smoke. The cigarettes have been characterized as containing 16 mg tar, 1.1 mg nicotine and 11 mg carbon monoxide when smoked to a 23-mm butt length. Twenty-four hours after smoke exposure, the rats were sacrificed by exsanguination under anaesthesia, and lung explants prepared. Explants from this group are referred to as "*in vivo*/*in vitro*" (VV). The second group was exposed to room air as a sham smoke, and sacrificed as above 24 h later. Explants from this group are referred to as "*in vitro* only" (VO).

Lung explants were prepared according to a modification [14] of the method of DANDURAND *et al.* [13] which is presented here in brief. Using sterile technique, the lungs were removed from the chest cavity, the heart dissected free, and a 14-gauge catheter inserted into the trachea. The lungs were then inflated to 90% of the calculated total lung capacity (5.5 mL·100 g) using 1% agarose/BCM, with a final 1.0-mL bolus of air to clear the solution from the larger conducting airways. After instillation, the tracheal tube was clamped and the lungs cooled to 4°C for 30 min in order to allow the agarose to gel. The lungs were then placed in a small chamber and sectioned to give 0.5–1.0-mm transverse slices using a hand-held microtome blade. The lung explant slices were placed into a 60×15 mm culture plate containing 3 mL BCM and incubated overnight at 37°C in an incubator supplemented with 5% CO_2.

As DANDURAND *et al.* [13] have previously shown that the degree of inter-animal variability in airway responsiveness is less than intra-animal variability, and it has been demonstrated that intra-animal variability depends largely on airway size [14], all of the explants in each group were pooled. Explants from smoke-exposed and control rats were then randomly divided into six groups; the explants in each of the tests were incubated in medium plus supplement, as indicated below, for 30 min. The treatment groups shown in table 1 were created.

Measurement of airway constriction

In order to examine the airways, the explants were transferred immediately after exposure to one of the protocols listed above into Lab-Tec chamber slides (Nunc, Naperville, IL, USA) with 300 µl HCM, and placed on the stage of an inverted microscope equipped with a video camera. The image of each identified airway was captured using a computerized frame grabber (ScreenMachine™, Munich, Germany), and stored on the computer for printing later. The technique is illustrated in figure 1, with figure 1a illustrating an airway at baseline, and figure 1b illustrating the same airway after constriction. *In vitro* smoke exposure of the explants was then performed using the method of LANNAN *et al.* [16], slowly injecting 35 mL of fresh whole cigartte smoke over a period of 1 min on to the explant surface. The smoke was obtained by immediately drawing 35 mL of room air through a lighted nonfiltered cigarette; a new cigarette was freshly lighted for each explant. The same cigarette type as used for the *in vivo*

Table 1. – Treatment groups

	Airways n	
	VO	VV
Unsupplemented medium	29	38
Media+atropine 500 mg·mL^{-1}	32	29
Media+SOD 1200 IU·mL^{-1}	32	26
Media+CAT 1600 IU·mL^{-1}	38	27
Media+BQ-610 3 µM	33	30
Media+Boiled CAT 1600 IU·mL^{-1}	30	31

VO: *in vitro* only; VV: *in vivo*/*in vitro*; SOD: superoxide dismutase; CAT: catalase.

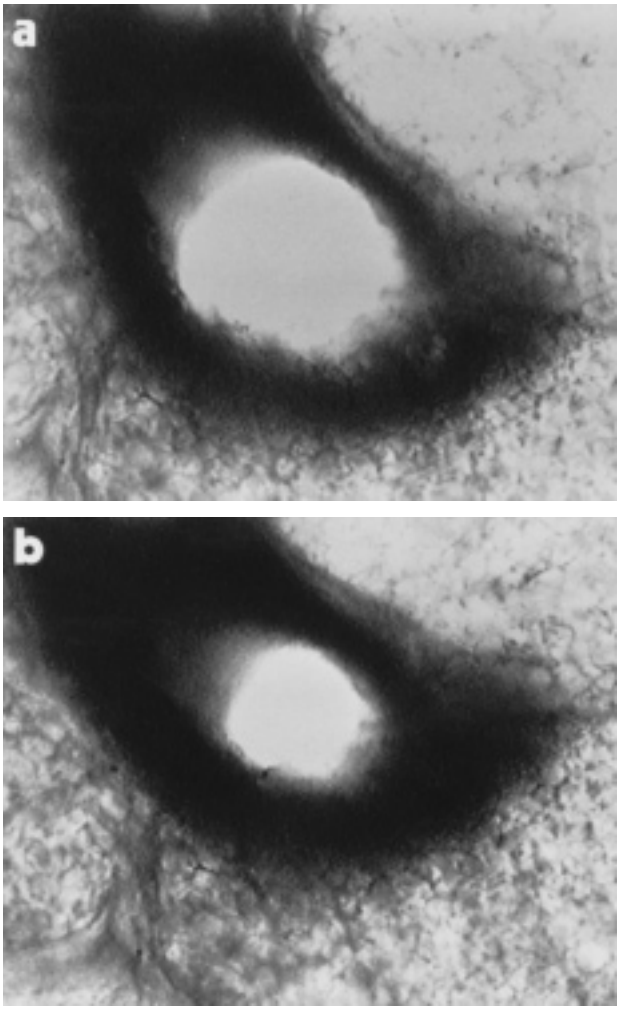

Fig. 1. – Explant airways illustrating the technique: a) an airway in an explant at baseline; and b) the same airway in a constricted state.

smoke exposure was used, although, under the protocol described above, only the distal 2.5 cm of the cigarette were consumed to provide the 35 mL smoke. Following smoke delivery, the same airways were reimaged, and stored on the computer for later analysis.

All images were calibrated at each magnification; it has previously been shown, using this methodology, that a single pixel at 4 × magnification represents 60 μg^2 on the printed image [14]. The lumen area and perimeter were then measured using a computer-linked digitizer, with the airway lumen designated as the space enclosed by the border of the epithelial image. It has previously been shown that 10 repeated measurements using this methodology are highly reproducible, with a coefficient of variation of 3.4% [14].

Statistical analysis

All analyses were performed using the SYSTAT statistical package (Evanston, IL, USA) [17]. The Kolmogorov-Smirnov test was used on the airway area data to ascertain whether the distribution of airway sizes were similar in all groups. To test for the effect of smoke on the airways, a paired t-test was used on the lumenal areas. Because the data were skewed, the nonparametric Kruskal-Wallis analysis of variance was used on the percentage of baseline area data in order to compare the acute effects of smoke exposure among treatment groups for VO or VV protocols, or between VO and VV treatments. Appropriate corrections of significance values by means of the Bonferroni technique were performed to adjust for multiple comparisons.

Results

The test groups did not differ statistically as regards the distribution of airway sizes (data not shown). This check is important because, in a previous study from this laboratory [14], the explant technique was used to partition airway contractility according to the size of the airways, and it was found that methacholine-induced airway contraction occurred primarily in the airways of >0.32 min in diameter (>1 mm internal perimeter). It is, therefore, possible to be confident that any alteration in contractility in the present experiments cannot be explained by a predominance of the larger or smaller sizes of airway examined.

Administration of cigarette smoke to the surface of the explant produced limited but, within each treatment protocol, consistent contraction of the airways in both the VO and VV groups (areas of all post-smoke airways compared to all presmoke airways for each treatment protocol, $p<0.01$). The mean±SD degree of contraction is shown in table 2. The unsupplemented explants (medium alone) were statistically identical in the VO and VV groups. In the VO animals, the explants which had been incubated with BQ610 showed a significantly lesser degree of contraction than did the untreated (medium alone) explants ($p<0.05$). Figure 2 illustrates this data in graphical form and shows that the effect is consistent from airway to airway. Atropine, CAT, SOD and boiled CAT had no effect. In the VV animals, the explants which had been incubated with either SOD or catalase contracted to a significantly lesser degree than did the unsupplemented explants (both $p<0.05$). Figures 3 and 4 again show that this effect is consistent from airway to airway. Boiling abolished the effects of CAT. There was no effect of BQ610 or atropine on contraction.

Table 2. – Effect of cigarette smoke exposure

	Airway contraction % baseline airway human area	
Treatment group	VO	VV
Medium alone	93.4±5.8	93.6±5.5
Atropine	95.7±4.9	91.8±13.6
SOD	94.9±6.5	96.0±5.3*
Catalase	94.6±6.8	96.4±5.7*
BQ-610	95.7±6.5*	93.8±5.4
Boiled catalase	92.0±7.3	92.6±7.8

Data are presented as mean±SD. VO: *in vitro* only; VV: *in vivo*/*in vitro*; SOD: superoxide dismutase; *: $p<0.05$ *versus* medium alone.

Discussion

It has been shown that, in this *sub vivo* system, cigarette smoke directly causes small airway contraction. Although the amount of constriction appears small, with luminal areas reduced to 90–95% of baseline, it should be remembered that resistance is inversely proportional to the 4th power of the airway radius. Since small peripheral airways are being considered, a small degree of contraction will result in a proportionally larger increase in resistance than that which would be produced in the larger airways. Thus the changes seen here may be quite significant in terms of function.

The mechanism by which cigarette smoke induces airway constriction is not known, but bronchoconstriction has most commonly been attributed to the effects of the smoke-evoked inflammatory response, since such a response can be detected in lavage fluid in experimental conditions [7, 18], and a similar association has been found in bronchoconstriction induced by ozone exposure [9, 10] which is also accompanied by an inflammatory response. However, the present study suggests that this hypothesis

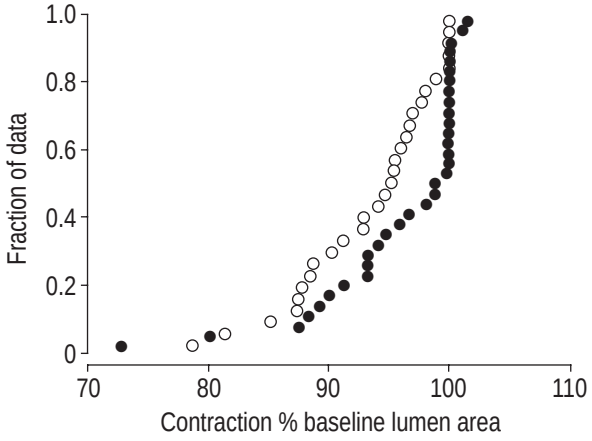

Fig. 2. – Explants from the *in vitro* smoke exposure only group treated with medium alone (○) and BQ610 (●). The Q-plots demonstrate that those airways in explants pretreated with BQ610 have a lesser degree of contraction after *in vitro* smoke exposure, and this is true of almost all of the airways in this treatment protocol.

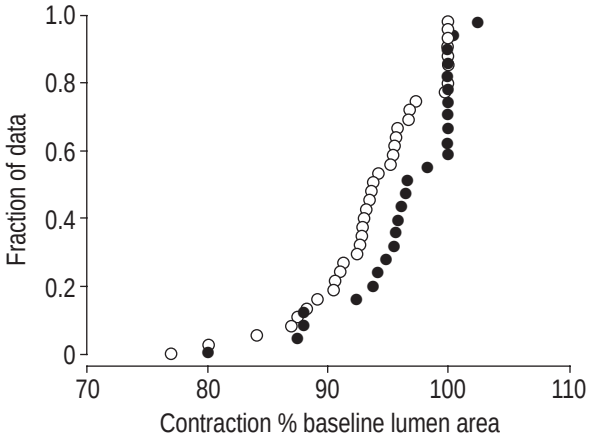

Fig. 3. – Explants from the *in vivo*/smoke exposure group treated with medium alone (○) and superoxide disumutase (SOD); (●). The Q-plots demonstrate that those airways in explants pretreated with SOD have a lesser degree of contraction after *in vitro* smoke exposure, and this is true of almost all of the airways in this treatment protocol.

cannot completely explain immediate bronchoconstriction, since there is little opportunity for inflammatory cell migration or recruitment in the lung explants. Furthermore, contraction after smoke exposure is almost immediate, suggesting direct bronchial muscle stimulation by smoke components.

STERLING [19] suggested that bronchoconstriction could be caused by a vagal cholinergic reflex mechanism. Obviously, vagal mechanisms are not operative in explants, and pretreatment with atropine failed to prevent constriction. Although the role of nicotine was not examined in this study, other workers have found that nicotine produces bronchial smooth muscle relaxation [20, 21], and therefore this component of smoke would be an unlikely explanation for the present results.

Endothelin is a known constrictor of bronchial smooth muscle, and it has previously been shown that cigarette smoke induces an endothelin$_A$ receptor-mediated associated increase in cell proliferation [12]. The present study found that pretreatment of the explants with the endothelin$_A$ inhibitor BQ610 diminished contractility in group

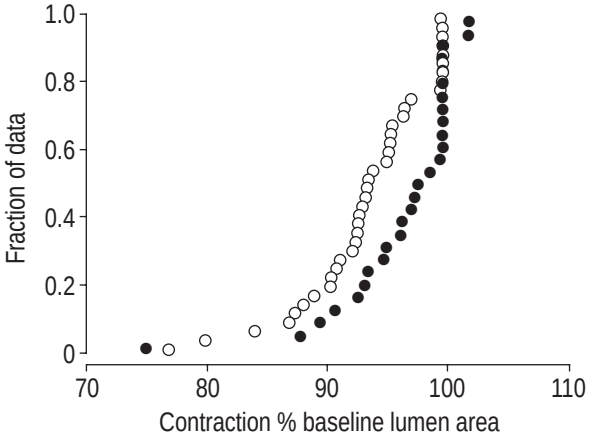

Fig. 4. – Explants from the *in vivo*/*in vitro* smoke exposure group treated with medium alone (○) and catalase (CAT); (●). The Q-plots demonstrate that those airways in explants pretreated with CAT have a lesser degree of contraction after *in vitro* smoke exposure, and this is true of almost all of the airways in this treatment protocol.

VO, suggesting that, at least in airways that had not previously encountered cigarette smoke, endothelin was one of the mediators of constriction. This effect was not, however, present in explants from animals which had undergone *in vivo* smoke exposure 24 h previously. Since smoke is known to cause an immediate increase in plasma endothelin in humans [22, 23], and since a similar effect has been observed in animals (J.L. Wright, unpublished data), it is possible that in the VV group the endothelin receptors have been completely saturated and thus no further response can be obtained.

In the animals which had undergone *in vivo* smoke administration, both SOD and catalase reduced the degree of constriction produced by acute smoke exposure of the explants. Although cigarette smoke contains high concentrations of reactive oxygen species [24], there appears to be a time-dependent and complex interaction of the lung antioxidant system with cigarette smoke. RAHMAN *et al.* [25] have shown that the plasma antioxidant capacity of smokers is reduced, data supported by a recent study in cigarette smokers [26], which found reduced antioxidant capacity in both plasma and lung lavage fluid. In their experimental model, LI *et al.* [27] found that instillation of cigarette smoke condensate into lungs immediately reduced both lung lavage and lung tissue glutathione levels. By contrast, CANTIN *et al.* [28] demonstrated an increased amount of glutathione in the lung lavage of chronic smokers, data which were supported by the authors′ previous experimental work, in which lung explants exposed to smoke had an increased glutathione concentration 24 h later [29]. In that study, it was found that the balance between oxidants and antioxidants is important in cigarette smoke-induced cell proliferation in the airways [29]; the present data would suggest that this balance is also important in determining the degree of airway constriction. Depending on the conditions of administration, oxidants appear to have both vasoconstrictor and vasodilator effects on vascular smooth muscle [11]. There appear to be several potential mechanisms for smooth muscle constriction including arachidonate mediator stimulation, guanylate cyclase activation or potentiation of the cholinergic response [11, 30]. The present data do not support the latter mechanism since no effect of atropine on the constrictor response to smoke could be found.

In summary, the present study demonstrated that direct administration of cigarette smoke to lung explants will produce constriction of the small airways. The constriction appears to be related to endothelin in animals without previous cigarette smoke exposure, and to the balance of oxidants and antioxidants in animals which have undergone smoke exposure *in vivo*.

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