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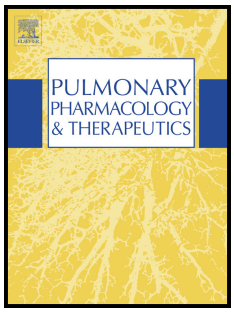

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Direct effect of cigarette smoke on human pulmonary artery tension

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Running head: Cigarette smoke and human pulmonary artery tension

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Abstract

The effect of chronic cigarette smoke on pulmonary artery (PA) tension has been studied extensively; nevertheless, the direct effect of cigarette smoke is poorly understood. We investigated the direct effect of cigarette smoke extract (CSE) on PA tension in non-smokers, smokers, and COPD patients *in vitro*. PA samples from 35 patients who underwent lung resection were examined by measuring isometric tension in response to increasing serotonin concentrations. CSE dose-dependently inhibited the response to serotonin in smokers and COPD patients, and to a lesser extent in non-smokers. CSE-induced relaxation was similarly inhibited by the nonspecific nitric oxide synthase (NOS) inhibitor L-NOARG and the specific inducible NOS (iNOS) inhibitor L-NIL, mainly in non-smokers and smokers, and to a lesser extent in COPD patients. Immunostaining of iNOS in PA samples was greater for smokers and COPD patients compared with non-smokers, which explains the lesser effect of CSE on PA tension in non-smokers. Moreover, CSE induced the release of nitrite via iNOS in human PA smooth muscle cells. In conclusion, CSE inhibition of serotonin-induced PA contraction was mediated mainly by iNOS in non-smokers, smokers, and COPD patients, but in different ways, which may be explained by differential iNOS expression in the PA of these patients.

Key words: Cigarette smoke extract, Inducible nitric oxide synthase, Pulmonary artery.

Abbreviations: CSE, cigarette smoke extract; COPD, chronic obstructive pulmonary disease; DMSO, dimethyl sulfoxide; eNOS, endothelial nitric oxide synthase; FEV1, forced expiratory volume in 1 second; FCS, foetal calf serum; FVC, forced vital

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capacity; HPAECs, human pulmonary artery endothelial cells; HPASMCs, human pulmonary artery smooth muscle cells; iNOS, inducible nitric oxide synthase; NO, nitric oxide; L-NOARG, N^G-Nitro-L-arginine; L-NIL, L-N^6-(1-Iminoethyl)-lysine HCl; $PaCO_2$, carbon dioxide tension in arterial blood; PaO_2, oxygen tension in arterial blood; 5-HT, serotonin; TLC, total lung capacity.

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1. Introduction

The chronic effects of cigarette smoke on pulmonary arterial tension have been studied widely [1, 2], but the direct effects of cigarette smoke are poorly understood. Acute exposure to cigarette smoke extract (CSE) has been reported to have contradictory effects. Acute exposure to cigarette smoke has been shown to increase pulmonary arterial pressure in dogs [3], whereas CSE has been shown to induce pulmonary arterial relaxation in isolated pig lungs [4, 5].

CSE has been reported to decrease endothelial NO production through the inhibition of endothelial nitric oxide synthase (eNOS) expression and activity in smokers and patients with chronic obstructive pulmonary disease (COPD), thus impairing endothelium-derived vasodilation [6-8]. In smokers and COPD patients, CSE causes intimal thickening in small pulmonary muscular arteries, which increases pulmonary resistance [8]. In contrast to its effect on eNOS, cigarette smoke leads to the induction of inducible nitric oxide synthase (iNOS) expression, especially in the media of the pulmonary vessels; this has been explained as compensatory vasodilator and antiproliferative effects of NO to limit the extent of pulmonary vascular resistance [9-11]. In contrast, NO may contribute to oxidative injury to the walls of the pulmonary vessels, which appears to initiate their morphological remodelling [12].

Several studies have analysed the relationship between cigarette smoke, NO, and the impairment of endothelium-dependent relaxation. However, less is known about the role of iNOS-derived NO in cigarette smoke-induced pulmonary artery tension.

We hypothesized that the direct action of cigarette smoke could modulate NO production and the consequent vascular tension in human pulmonary arterial vessels in different ways, depending of the stage and severity of the arterial pulmonary

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dysfunction, and that different patterns of iNOS expression could explain these differences. Therefore, the objectives of the present work were to investigate whether CSE directly modulates human pulmonary artery tension induced by serotonin (5-HT) in pulmonary artery rings from non-smokers, smokers, and COPD patients *in vitro,* and to elucidate the role of iNOS expression and NO release in this process.

2. Materials and methods

Unless stated otherwise, all reagents used were obtained from Sigma Chemical Co. (Madrid, Spain). N^G-Nitro-L-arginine (L-NOARG) and L-N^6-(1-iminoethyl)-lysine HCl (L-NIL) were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions. Several dilutions of the stocks were prepared, using cell culture medium. The final concentration of DMSO in the culture medium did not exceed 0.01% and had no significant pharmacological activity.

2.1. Patients

Peripheral human lung tissue was obtained from 35 (5 women, 30 men) patients who were undergoing surgery for lung carcinoma. With the approval of the local ethics committee, informed consent was obtained. None of the patients exhibited clinical evidence of pulmonary hypertension. Pulmonary function tests (forced spirometry) and arterial blood gas measurements were performed during the days prior to surgery. According to their spirometry results and smoking habits, patients were classified into three groups: 1) non-smokers, patients with normal lung function and who did not smoke; 2) Smokers, patients with normal lung function and who had smoked for more than 10 pack-years; and 3) Patients with COPD, who had smoked more than 10 pack-years and with airflow obstruction evidenced by a forced expiratory volume in one

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second (FEV1) of <80% predicted and a FEV1/forced vital capacity (FVC) ratio of <70%. The patients' characteristics are summarized in Table 1.

2.2. Preparation of pulmonary artery rings

Lung tissue was obtained as described previously [13]. Pulmonary arteries were carefully dissected free of adjoining connective tissue and lung parenchyma. Although the lung resection and pulmonary artery dissection were performed at the most distant point from and completely free of tumour tissue, we cannot rule out a possible influence of the inflammatory neoplastic microenvironment, which may be one limitation of this study. The preparations were placed in cold Krebs-Henseleit's solution (mM): NaCl, 118; $NaCO_3H$, 24; KCl, 4.7; KH_2PO_4, 12; $MgSO_4$, 1.2; $CaCl_2$, 2.5; and glucose, 11.1; pH 7.35–7.45. Arterial segments with an external diameter of 4-5 mm were carefully dissected and cut into rings of 4-5 mm in length. The endothelium was removed from some pulmonary artery rings, as described previously [14]. Arteries were used within 1 to 5 h post-surgery. The rings were mounted in a 10-ml organ bath chamber (Pan-Lab, USA) under an initial load of 1 to 2 g, and the isometric tension was recorded with a transducer (Grass FT03 isometric force transducer; Grass Instruments, Quincy, MA, USA) connected to a PowerLab® data acquisition system (AD Instruments, Castle Hill, New South Wales, Australia), as reported previously [15]. The tissues were allowed to equilibrate in Krebs-Henseleit's solution for 90 min at 37°C, while being aerated with 5% CO_2 in O_2. Pulmonary artery rings that failed to reach a tension of 0.5 g in response to 80 mM KCl were discarded.

2.3. Isolation and culture of human pulmonary artery endothelial cells and pulmonary arterial smooth muscle cells

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Segments of pulmonary artery (4-5 mm internal diameter) were dissected free from parenchyma lung tissue, cut longitudinally, and digested with 1% collagenase (Gibco, UK) in RPMI-1640 culture medium for 30 min at 37°C. The digestion was neutralized by adding RPMI 1640 supplemented with 20% foetal calf serum (FCS), and the homogenate was separated by centrifugation at 1100 rpm. The pellet was resuspended, and the cells were cultured in EGM-2 endothelial culture medium supplemented with Single Quotes (Clonetics, UK), 10% FCS, 1% fungizone, and 2% streptomycin/penicillin.

The selection of human pulmonary arterial endothelial cells (HPAECs) was performed as described previously [16], modified to include the use of a commercially available Dynabeads CD31 endothelial cell kit (Dynal Biotech, Germany). Briefly, cells were trypsinized (0.25% trypsin), and the cell mixture was incubated with CD-31-coated Dynabeads for 30 min at 4°C with end-over-end rotation. After incubation, the HPAECs were collected using a magnetic particle concentrator (MCP-1; Dynal) and washed four times with cold phosphate-buffered saline (PBS)/bovine serum albumin (BSA). Clusters of purified HPAECs retained on the CD-31-coated Dynabeads were separately resuspended in EGM-2 full growth medium supplemented with 10% FCS, 1% fungizone, and 2% streptomycin/penicillin. The cells not retained on the CD-31-coated Dynabeads, *i.e.*, human pulmonary arterial smooth muscle cells (HPASMCs), were cultured in DMEM supplemented with 10% FCS, 1% fungizone, and 2% streptomycin/penicillin to selectively separate the HPASMCs.

For positive identification of HPASMCs, the cells were subcultured for one passage, and α-actin expression was examined using a monoclonal antibody against α-smooth muscle actin (1:100 dilution; Sigma); >95% of the cells were positively stained. The

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cells were incubated for 16 h in 1% FCS culture medium before each experiment and were returned to 10% FCS culture medium at the start of each experimental condition.

2.4. Preparation of CSE solutions

Based on previous reports, we prepared CSE, which has been used to study the effects of cigarette smoke on isolated vessels and various cultured cells [17, 18]. Briefly, the smoke of a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, KY, USA) was generated by a respiratory pump (Apparatus Rodent Respirator 680; Harvard, Germany) through a puffing mechanism related to the human smoking pattern (3 puff/min; 1 puff 35 ml; each puff of 2 s duration with 0.5 cm above the filter) and was bubbled into a flask containing 25 ml of pre-warmed (37°C) DMEM or EGM-2 medium. The CSE solution was sterilized by filtration through a 0.22-µm cellulose acetate sterilizing system (Corning, NY). The resultant CSE solution was considered to be 100% CSE and was used for experiments within 30 min of preparation. CSE 10% corresponds approximately to the exposure associated with smoking two packs per day [18].

The quality of the prepared CSE solution was assessed based on the absorbance at 320 nm, which is the specific absorption wavelength of peroxynitrite. Stock solutions with an absorbance value of 3.0 ± 0.1 were used. To test for cytotoxicity from CSE, HPAECs, HPASMCs, and isolated arteriolar pulmonary vessels were treated with CSE concentrations of up to 20% for 24 and 48 h. No significant difference in the lactate dehydrogenase supernatant level (lactate dehydrogenase cytotoxicity assay; Cayman, Spain) was observed, compared with the control group (data not shown).

2.5. Experimental protocol

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After an equilibration period of 1 h at 1.5 g of basal tension, preparations were contracted with 1 μM noradrenaline (NA) and relaxed with 1 μM acetylcholine (Ach), to confirm endothelium function. The tissues were washed until resting tone was re-established, and then the artery rings were contracted maximally using 80 mM KCl, to establish the maximal contractile response. After rinsing and equilibration, increasing 5-HT concentrations (10 nM to 100 μM) were added, and the tension was expressed as a percentage of the maximal contraction with KCl. In other experiments, rings were incubated for 30 min with CSE 2.5%, 5%, and 10%, and the changes in tension were measured. To determine the effect of CSE on contractility, the responses to cumulative doses of 5-HT were measured while maintaining CSE 2.5%, 5%, or 10% in the bath medium.

To determine the potential roles of the endothelium, NO, and prostaglandins in the effect of CSE on 5-HT-induced arterial contraction, artery rings in the presence or absence of endothelium were incubated for 30 min with or without CSE 10% and in the presence of a cyclooxygenase inhibitor (5 μM indomethacin), a nonspecific NOS inhibitor (100 μM L-NOARG), or a specific iNOS inhibitor (20 μM L-NIL). Indomethacin, L-NOARG, and L-NIL were maintained during the whole experiment. All of these experiments were conducted in all three study groups.

2.6. NO production

NO was measured as nitrates and nitrites in HPAEC and HPASMC culture supernatant samples, using a commercially available nitric oxide assay kit (Calbiochem-Novabiochem, San Diego, CA) according to the manufacturer's protocol.

2.7. Immunohistochemistry and immunofluorescence

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For eNOS and iNOS immunohistochemical analysis of human pulmonary arteries, specimens were fixed, embedded in paraffin, cut into sections (4-6 μm), and stained with haematoxylin, as reported previously [19]. The sections were incubated with rabbit monoclonal antibody to iNOS (1:50; NeoMarkers, Fremont, CA) for 24 h at 4°C. A secondary anti-rabbit antibody (1:100; Vector Laboratories, Burlingame, CA) with avidin-biotin complex/horseradish peroxidase was used for immunohistochemistry, and a secondary anti-rabbit antibody labelled with FITC (1:100) was used for immunofluorescence. The slices were observed under an epifluorescence microscope (×200; Nikon-XB0-100, Tokyo, Japan).

2.8. Statistical analysis

All data are expressed as the mean ± SEM. Comparisons between groups were performed using analysis of variance (ANOVA) on the maximal effect (E_{max}) of 5-HT. The Bonferroni test was applied to compare points in cumulative dose-response curves (GraphPad Software Inc., San Diego, CA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Direct effect of CSE on 5-HT-induced contraction curve

Experiments were performed in three different groups, which were based on the clinical characteristics defined in Table 1. After 1 μM NA-induced contraction of the artery rings, the relaxing effects of 1 μM Ach were 40 ± 8%, 36 ± 7%, and 20 ± 9% for non-smokers, smokers, and COPD patients, respectively. The Ach relaxing effect in

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COPD patients was significantly lower than that in non-smokers ($P < 0.05$, data not shown), suggesting an endothelial dysfunction in COPD patients.

The addition of 80 mM KCl produced a plateau contraction of 1.01 ± 0.06 g in artery rings from non-smokers; 1.10 ± 0.07 g, in smokers; and 1.12 ± 0.09 g, in COPD patients. After the washout of KCl, the new basal tone did not differ statistically from the initial tension. The stimulation of pulmonary arteries with 5-HT resulted in a concentration-dependent contraction that was significantly lower in non-smokers (E_{max} 102.7 ± 3.7%; Fig. 1A) and smokers (E_{max} 96.6 ± 2.8%; Fig. 1B) compared with the COPD group (E_{max} 145.9 ± 16.5; $P < 0.05$ vs. E_{max} from non-smokers and smokers; Fig. 1C). In contrast, the EC_{50} under different experimental conditions was not statistically different among the groups.

The direct application of CSE (2.5% to 10%) for 30 min did not produce any change in pulmonary artery tension in non-smokers, smokers, or COPD patients (data not shown). In contrast, 5-HT-induced contraction was inhibited significantly by CSE in a dose-dependent fashion in non-smokers (56% inhibition by CSE 10%; $P < 0.05$; Fig. 1A), smokers (79% inhibition by CSE 10%; $P < 0.05$; Fig. 1B), and COPD patients (88% inhibition by CSE 10%; $P < 0.05$; Fig. 1C). Importantly, the inhibitory effect of CSE on 5-HT-induced contraction was significantly higher in smokers and COPD patients compared with non smokers ($P < 0.05$).

3.2. Effect of CSE on endothelium-dependent relaxation of human pulmonary arteries

The removal of the endothelium significantly increased the concentration-response curve for 5-HT in pulmonary artery rings from the non-smokers and smokers ($P < 0.05$;

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Figs. 2A and 2B), whereas the COPD patients showed the same concentration-response curves with and without intact endothelium (Fig. 2C).

On the other hand, pre-treatment with CSE 10% attenuated the 5-HT-induced contraction of pulmonary artery rings without endothelium in non-smokers (34.3% inhibition; $P < 0.05$; Fig. 1A), smokers (71.9% inhibition; $P < 0.05$; Fig. 1B), and COPD patients (72.3% inhibition; $P < 0.05$; Fig. 1C). The relaxing effect of CSE on pulmonary arteries without endothelium was significantly higher in smokers and COPD patients compared with the non-smokers ($P < 0.05$).

3.3. Role of cyclooxygenase and nitric oxide pathways in the effect of CSE on 5-HT-induced contraction

The direct action of CSE has been shown to induce a relaxing effect in pig pulmonary arteries, and this was reversed by indomethacin [5]. To explore the possible role of the cyclooxygenase pathway, we incubated the human arterial pulmonary rings with indomethacin (5 μM) for 30 min before the addition of CSE 10%. Indomethacin did not significantly change the relaxing effect of CSE in any of the three study groups (Fig. 3A-C). In contrast, L-NOARG (100 μM) and L-NIL (20 μM) completely reversed the effect of CSE on 5-HT-induced pulmonary arterial ring contraction in non-smokers and smokers, and to a lesser extent in COPD patients (Fig. 3A-C, $P < 0.05$).

3.4. Expression of iNOS in human pulmonary arteries from non-smokers, smokers, and COPD patients

Cigarette smoke leads to the induction of iNOS expression in pulmonary arteries [9-11]. To understand the relaxing effect of CSE on 5-HT-induced pulmonary arterial tension, we examined the pattern of iNOS expression in the pulmonary arteries of the

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three study groups. iNOS was expressed markedly in the pulmonary artery smooth muscle of smokers and COPD patients (Fig. 4, red arrows) and to a lesser extent in the endothelium (Fig. 4, black arrows). In contrast, non-smokers did not exhibit detectable iNOS immunostaining.

3.5. Effect of CSE on NO release in HPAECs and HPASMCs

Both HPAECs and HPASMCs were isolated from non-smoking controls, as it has been shown that after normal culture conditions for 1-4 passages in the absence of an inflammatory environment, the expression of iNOS returns to basal levels [20]. Cultured cells from the different groups did not express basal iNOS (data not shown). To study the direct action of CSE on NO release, we incubated HPAECs with CSE for 30 min. Compared with basal conditions, the application of CSE significantly reduced nitrite release (basal, 9.4 ± 0.8 μM; CSE 10%, 2.3 ± 0.2 μM; $P < 0.05$; Fig. 5A). In contrast, CSE augmented nitrite release into HPASMC culture medium (basal, 6 ± 0.7 μM; CSE 10%, 15.43 ± 2.1 μM; $P < 0.05$; Fig. 5B). Pre-incubation with L-NOARG (100 μM) or L-NIL (20 μM) completely suppressed the CSE-induced nitrite release ($P < 0.05$; Fig. 5B). The addition of CSE 10% to the HPASMC culture increased iNOS protein expression, which reached appreciable levels at 3-5 h (Fig. 5C).

4. Discussion

The present study demonstrates for the first time that direct cigarette smoke modifies the *in vitro* human pulmonary artery tension in a different fashion in non-smokers, smokers, and COPD patients, through iNOS activation. CSE inhibited the 5-HT-induced pulmonary artery contraction in smokers and COPD patients, and to a lesser extent in non-smokers. Furthermore, the endothelium mediated a relaxing effect against

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5-HT contraction in non-smokers and smokers, but not in COPD patients, suggesting a dysfunctional endothelium in the latter group. The high basal iNOS expression found in pulmonary arteries from smokers and COPD patients, as well as the activation of iNOS by CSE in HPASMCs, may explain the main role of iNOS in CSE-induced pulmonary artery relaxation and why CSE causes a lesser effect in non-smokers.

In this work, we chose 5-HT as a contractile stimulus because it has been demonstrated to mediate the functional and remodelling consequences of pulmonary arterial hypertension [21]. In a previous *in vitro* study, CSE produced pulmonary arterial relaxation in pig pulmonary artery rings, and this was fully reversed by indomethacin [5]. In the same sense, an *in vivo* study performed in pigs suggested that CSE induces direct pulmonary vasodilatation through two main mechanisms. First, by the actions of the NO and CO present in the cigarette smoke, and second, by the release of endogenous NO, as demonstrated the inhibitory effect of L-NOARG [4]. In contrast to these results, we could not detect a significant direct action of CSE on the artery rings, probably due to the absence of physiological basal tension in the *in vitro* preparations. However, the physiological tension induced by 5-HT in the human pulmonary arteries was inhibited by CSE, primarily through endogenous NO release, as demonstrated by the inhibitory effects of L-NOARG and L-NIL (Fig. 3A-C); however, we cannot discard a direct action of the NO content of CSE in this process. It has been shown previously that cigarette smoke challenge in the lower airways of pigs caused a marked vasodilator response in the bronchial circulation [22]. Both cigarette smoke and NO rapidly increased the level of guanosine 3′-5′-cyclic monophosphate (cGMP), the second messenger for NO, in lung tissue, inducing relaxation in the pulmonary circulation of the pig lung. Furthermore, the changes in blood pressure induced by intermittent cigarette smoke challenge were correlated with the NO levels in the smoke,

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and only cigarette smoke free from particles (vapour phase) relaxed the pulmonary circulation [23]. In fact, our results showed that in COPD patients, neither L-NOARG nor L-NIL could completely suppress the inhibitory effect of CSE on 5-HT-induced contraction, implicating another mechanism in the CSE relaxing effect. As we used CSE immediately after its extraction, it is likely that the NO and CO concentrations in the aqueous solution were sufficient to directly produce relaxing effects, as described previously [24-26]. On the other hand, we observed only a slight change in tension when indomethacin was used, suggesting a lesser role of prostacyclin in this mechanism.

The effect of cigarette smoke on *in vivo* NO formation is poorly understood and sometimes appears to be contradictory. Some studies have shown an increased NO level in response to cigarette smoke in pigs and rats [4, 27], whereas others have indicated that exposure to cigarette smoke decreases the endothelium-dependent relaxation in pigs and rabbits [1, 28, 29]. By comparison, the *in vitro* studies are more uniform, demonstrating that cultured endothelial cells exposed to CSE show decreased endothelial NO production owing to the inhibition of eNOS activity [7], as we have shown in cultured HPAECs. In the present study, the inhibition of 5-HT-induced contractility of pulmonary artery rings by CSE was probably the result of NO release, as evidenced by the effect of L-NOARG. Furthermore, the relaxing effect of CSE was mediated mainly by iNOS activation; as the specific iNOS inhibitor L-NIL had the same inhibitory effect as the nonspecific L-NOARG, a direct activation of iNOS by CSE is likely. Although NOS inhibition suppressed the relaxing effect of CSE in non-smokers and smokers, we only observed partial inhibition in COPD patients. It is known that pulmonary arteries from COPD patients exhibit elevated levels reactive oxygen species such as O_2^- and H_2O_2, which modulate vascular tone associated with

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hypertension. Vasoconstriction induced by O_2^- occurs through several different mechanisms; in one well-established mechanism, an extremely fast reaction between NO and O_2^- yields the highly cytotoxic and contraction-inducing peroxynitrite anion, $ONOO^-$ [30]. Furthermore, elevated levels of $ONOO^-$ have been found in human patients with COPD [31]. Therefore, we hypothesized that the high iNOS expression that we observed in COPD patients may be associated with $ONOO^-$ production and with inflammatory and remodelling changes of the intima, which may in turn impair the ability of iNOS in the vasodilation process. The activation of iNOS by CSE in COPD artery rings may share both pathways, NO and $ONOO^-$ production, reducing the action of NOS inhibitors on the CSE relaxing effect (Fig. 3C). Hence, the relaxing effect of CSE in COPD patients should be due largely to the NO and CO content of the CSE, as described previously [24-26].

Smokers and COPD patients exhibit an endothelial dysfunction characterized by impaired NO-dependent relaxation of the pulmonary arteries [8, 32]. In the present study, we found that the relaxing effect of Ach after NA contraction was significantly lower in COPD patients than in non-smokers, suggesting an endothelial dysfunction. In addition, removal of the endothelium appreciably increased the 5-HT-induced contraction in non-smokers and smokers, but not in COPD patients, further suggesting the presence of a damaged endothelium in COPD patients. The endothelium plays an important role in regulating vascular tone and controlling cell growth. In COPD patients, where the endothelium is damaged [33], there is abundant smooth muscle cell (SMC) proliferation and intense deposition of both elastin and collagen fibres in the intimal layer, contributing to vascular remodelling and tension of the pulmonary arteries [33]. Furthermore, in patients with mild to severe COPD, increased inflammatory infiltrate in the pulmonary arteries with an overexpression of $5\text{-}HT_{1B\text{-}2A\text{-}2B}$

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receptors has been reported [21]. This may account for the increase in 5-HT-induced contraction observed in COPD patients in the current study.

Recently, it has been shown that smokers and patients with mild to severe COPD exhibit increased iNOS expression in skeletal muscle and peripheral lung tissue [34, 35]. In these cases, iNOS expression is augmented by inflammatory factors, including cigarette smoke [36], resulting in 1,000-fold quantities of NO, which mediates defence and pathological processes [35]. In the present study, iNOS was expressed mainly in the smooth muscle of arteries and to a lesser extent in the endothelium from smokers and COPD patients. In contrast, iNOS immunostaining was not detectable in non-smokers. Moreover, CSE reduced the nitrite release by cultured HPAECs and increased the nitrite release by HPASMCs via iNOS activation and iNOS expression, as described previously [6-11]. This may help to explain why CSE induced a major relaxing effect in smokers and COPD patients, compared with non-smokers.

Despite limitations of the *in vitro* characteristics of this study, our results demonstrate that CSE directly reduces the 5-HT-induced pulmonary arterial tension in non-smokers, smokers, and COPD patients by activating iNOS, and the high basal expression of iNOS in the pulmonary arteries of smokers and COPD patients may enhance this effect. Furthermore, the increased 5-HT-induced tension in COPD patients may be explained by the endothelial dysfunction observed previously in this population. These results help to elucidate how pulmonary artery tension responds to the direct action of cigarette smoke and how pulmonary arteries are modified in the course of pulmonary artery diseases related to cigarette smoking.

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Figure Legends

Figure 1

Effect of CSE on 5-HT-induced human pulmonary arterial ring contraction. Tension was measured in human pulmonary arteries in response to cumulative concentrations of 5-HT alone or in combination with different concentrations of CSE in non-smokers (panel A), smokers (panel B), and chronic obstructive pulmonary disease (COPD) patients (panel C). Changes in tension are expressed as percentages of the maximal pre-contraction with KCl (80 mM). Data represent means ± SEM of 7-11 preparations per condition from five different patients. *P <0.05 compared to the control (●).

Figure 2

Effect of CSE on endothelium-dependent relaxation of human pulmonary arteries. Tension was measured in human pulmonary arteries with (black symbol) or without (white symbol) endothelium, in response to cumulative concentrations of 5-HT alone or in combination with CSE in non-smokers (panel A), smokers (panel B), and chronic obstructive pulmonary disease (COPD) patients (panel C). Changes in tension are expressed as percentages of the maximal pre-contraction with KCl (80 mM). Data are

means ± SEM of 6-8 preparations per condition from five different patients. *$P < 0.05$ compared with control (●). ♦$P < 0.05$ compared with the control without endothelium (○).

Figure 3

Modulation of CSE-induced relaxation of human pulmonary arteries by indomethacin, L-NOARG, and L-NIL.

Tension was measured in human pulmonary arteries in response to cumulative concentrations of 5-HT in the absence and presence of CSE, and with CSE in the presence of indomethacin, L-NOARG, or L-NIL in non-smokers (panel A), smokers (panel B), and chronic obstructive pulmonary disease (COPD) patients (panel C). Changes in tension of are expressed as percentages of the maximal pre-contraction with KCl (80 mM). Data are means ± SEM of 6-11 preparations per condition from five different patients. *$P < 0.05$ compared with control (●). #$P < 0.05$ compared with CSE 10% (■).

Figure 4

Immunohistochemical detection of iNOS expression in lung sections from smokers and chronic obstructive pulmonary disease (COPD) patients.

iNOS staining (brown) was intense in the smooth muscle (red arrows) and was less strong in the endothelium (black arrows) from smokers and COPD patients (original magnification, ×200). The different panels represent the three study groups.

Figure 5

Effect of CSE on nitrite release in isolated HPAECs and HPASMCs.

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HPAECs and HPASMCs were cultured in 6-well flasks until □ 90% confluent. Then, CSE 2.5%, 5%, or 10% was added. **A)** CSE decreased nitrite release in a dose-dependent manner in HPAECs. **B)** CSE increased nitrite release in a dose-dependent manner in HPASMCs, and this was suppressed by pre-treatment with L-NOARG (100 μM) or L-NIL (20 μM). Data are the means ± SEM of four experiments per condition. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with CSE 10%. **C)** HPASMCs were incubated with CSE 10% for different periods of time, and the cells were immunostained for iNOS (original magnification, ×200).

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	Non smokers (n=6)	Smokers (n= 14)	COPD (n= 15)
Age, yr	65±4	67±7	65±9
Tobacco consumption, pack-yr	0	37.5±6	40.6±8
FEV1, % pred	94±2	90.1± 6	68.1±8
FVC, % pred	98±3	93.3±7	85±7
FEV1/FVC %	89±6	87.7±4	64.1±4
TLC %pred	94±5	89±6	97.5±2
PaO_2, mmHg	94±7	86±6	83±7
$PaCO_2$ mmHg	38±4	39±4	41±2

Table 1. Clinical features. COPD: chronic obstructive pulmonary disease; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TLC: total lung capacity; PaO_2: oxygen tension in arterial blood; $PaCO_2$: carbon dioxide tension in arterial blood; Pack-yr = 1 year smoking 20 cigarettes-day.

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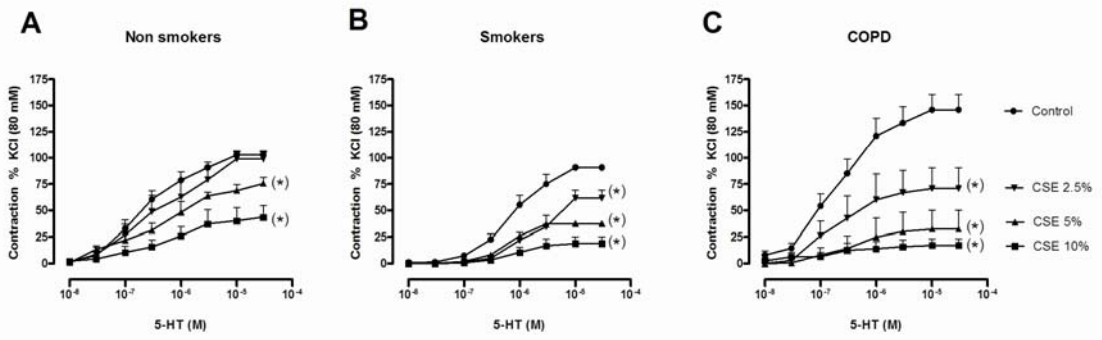

Figure 1

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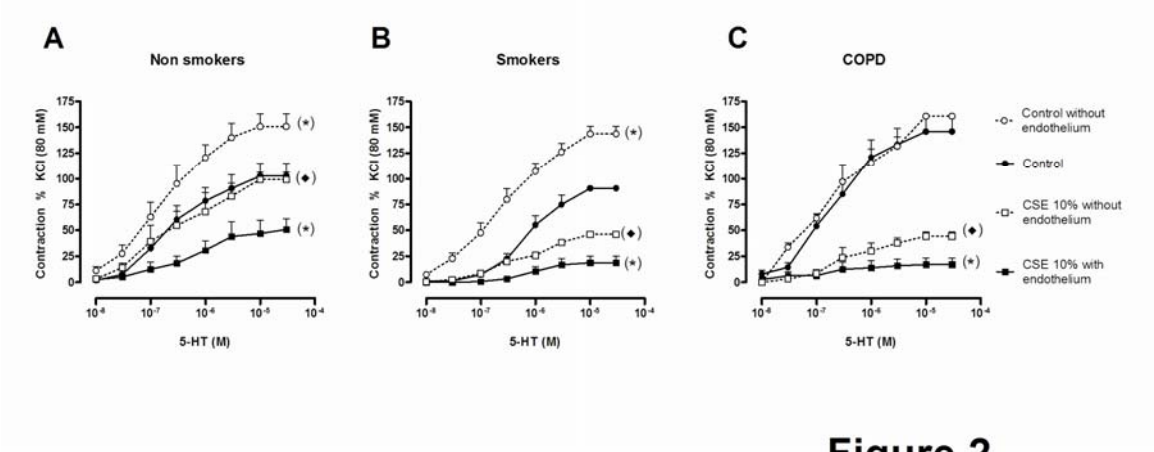

Figure 2

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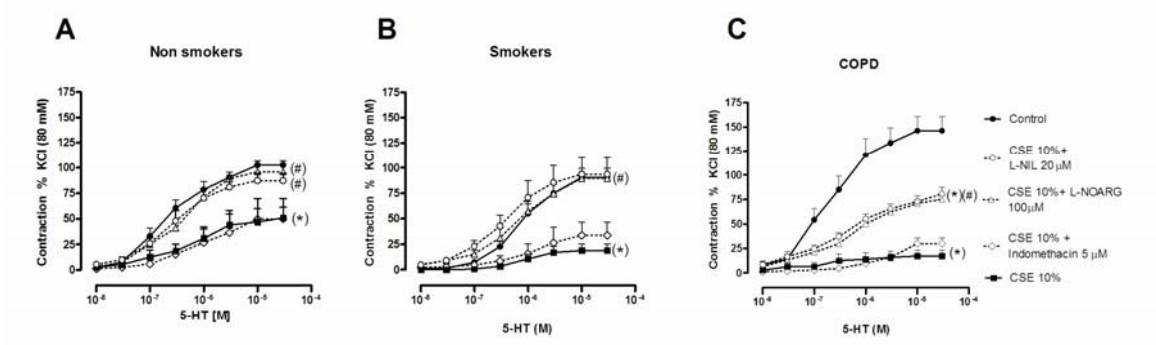

Figure 3

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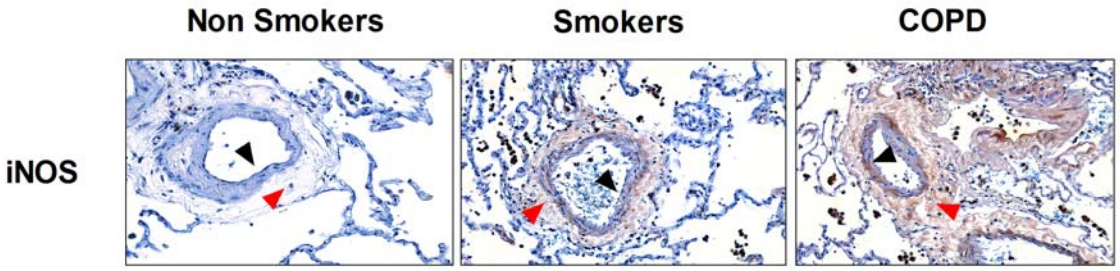

Figure 4

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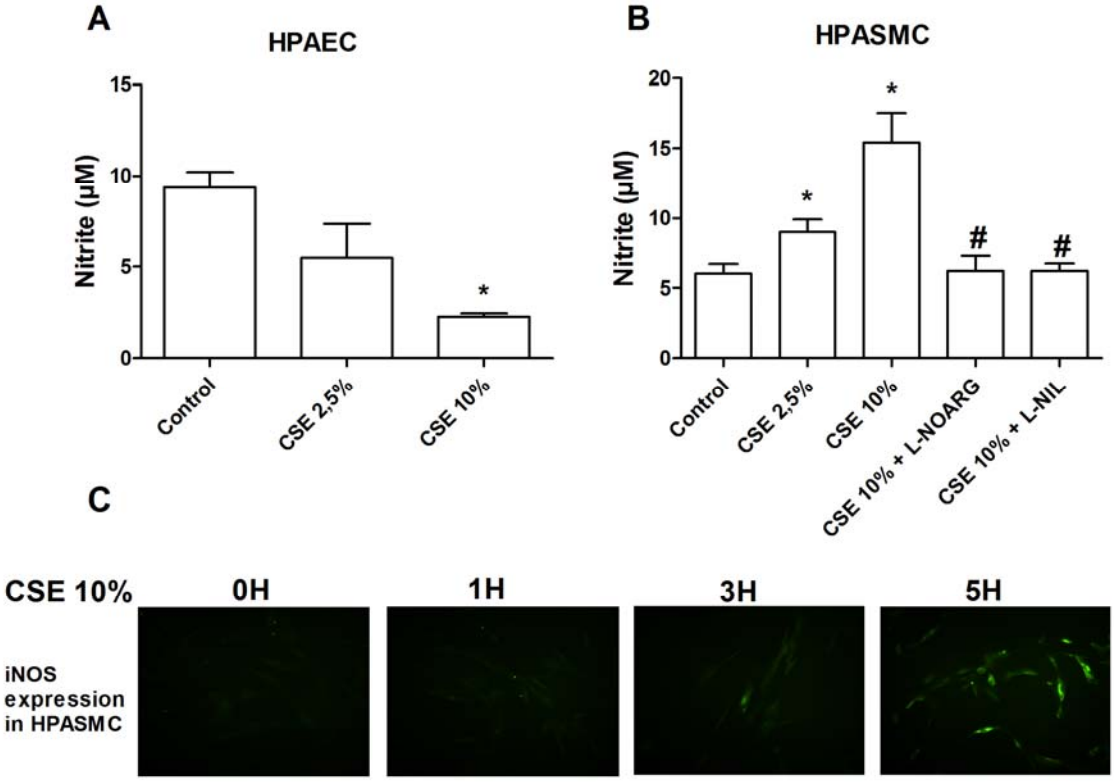

Figure 5

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