

Effects of Cigarette Smoke and Hypoxia on the Pulmonary Circulation in the Guinea Pig

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ABSTRACT

Rationale: Cigarette smoke (CS) and chronic hypoxia (CH) can produce pulmonary hypertension. Similarities and differences between both exposures and their interaction have not been explored.

Objective: Investigate the effects of CS and CH, as single factors or in combination, on the pulmonary circulation in the guinea pig.

Methods: Fifty-one guinea pigs were exposed 12 weeks to CS, and 32 sham-exposed. 50% of the animals in each group were additionally exposed to hypoxia the last 2 weeks. We measured pulmonary artery pressure (PAP) and the weight ratio between right ventricle (RV) and left ventricle+septum. Pulmonary artery contractility in response to norepinephrine (NE), endothelium-dependent vasodilatation and distensibility were evaluated in organ bath chambers. The number of small intrapulmonary vessels showing immunoreactivity to smooth muscle (SM)- α -actin and double elastic laminae was assessed microscopically.

Results: CS and CH induced similar increases of PAP and RV hypertrophy ($p < 0.05$, each), effects that were further enhanced when both factors were combined. CH increased the contractility to NE ($p < 0.01$) and reduced the distensibility ($p < 0.05$) of pulmonary arteries. Animals exposed to CS showed an increased number of small vessels with positive immunoreactivity to SM- α -actin ($p < 0.01$) and those exposed to CH a greater proportion of vessels with double elastic laminae ($p < 0.05$).

Conclusions: We conclude that chronic hypoxia amplifies the detrimental effects of CS on the pulmonary circulation by altering the mechanical properties of pulmonary arteries and enhancing the remodeling of pulmonary arterioles.

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Key words: cigarette smoke, COPD, hypoxia, pulmonary hypertension, vascular remodeling.

INTRODUCTION

Pulmonary hypertension is a common and serious complication of chronic obstructive pulmonary disease (COPD). It is considered to result from the effects of chronic hypoxemia on pulmonary vessels. Indeed, acute and chronic exposure to hypoxia results in smooth muscle cell (SMC) and adventitial fibroblast proliferation[1]. Nevertheless, structural changes in the pulmonary arteries of COPD patients differ from those observed in subjects exposed to a hypoxic environment. Whereas highlanders show medial hypertrophy, COPD patients show prominent changes in the intima[2]. Intimal hypertrophy is present in non-hypoxemic COPD patients and in smokers with normal lung function, suggesting that vascular changes may be triggered by cigarette smoke (CS) before hypoxemia develops.

Cigarette smoking is associated with endothelial dysfunction[3], increased expression of growth factors[4] and inflammatory cell infiltrate in pulmonary arteries[5]. These factors may induce SMC proliferation and increase pulmonary vascular resistance. The molecular mechanisms by which CS induces vascular changes remain unknown, but they might be related to oxidative damage[6].

The effects of hypoxia on human lungs are difficult to characterize because it is usually related to the presence of primary lung diseases that may be associated to vascular remodeling by additional mechanisms (i.e. inflammation). Nevertheless, there is evidence that in humans chronic hypoxia (CH) *per se* may induce endothelial dysfunction[7], increased expression of growth factors[8] and inflammation[9].

Some of the observations in pulmonary vessels of COPD patients have been replicated in a guinea pig model chronically exposed either to CS or hypoxia. Exposure to CS induces muscularization of precapillary vessels and increases pulmonary artery pressure (PAP)[10;11], which runs in parallel to endothelial dysfunction[12;13], and develops

before pulmonary emphysema is apparent. CH on the other hand, induces pulmonary hypertension, right ventricle (RV) hypertrophy, vascular remodeling[14] and increased plasma levels of endothelin-1 (ET-1) in the guinea pig[15].

We have hypothesized that in COPD changes in pulmonary vessels are initiated at early disease stages as a result of a direct effect of CS products on the pulmonary endothelium. Subsequently, with the progression of the disease hypoxemia may add on, producing further vascular damage potentially resulting in pulmonary hypertension. The combined effects of CS and CH on the pulmonary circulation have not yet been assessed in experimental models.

Accordingly, the present study aimed to evaluate the effects of CS exposure and CH, alone and as combined stimuli, on pulmonary hemodynamics, RV structure, pulmonary vascular reactivity and vessel remodeling in the guinea pig.

METHODS

Animals and management

Eighty-three male Hartley guinea pigs weighing 350g were divided into four groups. One group was exposed to CS for 12 weeks (n=33); a second group was kept in a normal atmosphere for 10 weeks and subsequently exposed to FiO₂ 0.12 for 2 weeks in a hypoxic chamber (CH) (n=16); a third group was exposed to CS for 12 weeks and to CH for the last two weeks (CSCH) (n=18); and a Control group was sham-exposed to CS and kept in a normal atmosphere (n=16).

Cigarette smoke exposure

Animals were exposed to the smoke of four research cigarettes (2R4F; Kentucky University Research; Lexington, KY, USA) per day, 5 days a week for 12 weeks using a nose-only system[13;16].

Exposure to chronic hypoxia

Animals were placed in hypoxic glass chambers continuously fluxed with gas mixture (12% O₂ in N₂; PO₂≈85 mmHg; equivalent to ≈4300m).

Pulmonary hemodynamics

In half of the animals in each group a catheter was placed in the pulmonary artery through the right ventricle. The right carotid artery was cannulated to simultaneously measure systemic arterial pressure. Measurements were performed in normoxic and hypoxic conditions (4 min at a 0.10 fraction of inspired oxygen (FiO₂)). The recovery profile was evaluated by pumping again air with a FiO₂ of 0.21.

Vessel distensibility and endothelial function

Half of the animals in each group underwent assessment of vessel distensibility and reactivity. Vessel distensibility (Dis) was evaluated in rings of pulmonary artery and aorta (3mm length) according to the equation $Dis = \frac{\Delta D}{\Delta P} \cdot D$ (where ΔD is the difference of diameter before and after 1mm of stretching, ΔP is the difference in pressure at the same points and D is the final diameter of the ring) [17;18]. Endothelial function was assessed as the change in wall tension in response to cumulative doses of adenosine-5'-diphosphate (ADP), as previously described[13].

Macroscopic and microscopic morphologic studies

Right ventricular hypertrophy was measured as the ratio between the RV weight and the weight of the left ventricle plus septum.

Wall thickness of the aorta and main pulmonary artery were measured [13]. To further assess the characteristics of extracellular matrix in the vessel wall, the area occupied by mucopolysaccharides, collagen and elastin was evaluated in adjacent sections of main pulmonary artery stained with Alcian blue, Masson trichrome and Orcein, respectively, and expressed as a percent of total vessel area.

The number of intrapulmonary vessels with a diameter $<50\mu\text{m}$ showing positive immunoreactivity to smooth muscle (SM)- α -actin was counted and expressed as percent of total number of small vessels. These vessels were further classified into non-muscularized, partially muscularized and fully muscularized, according to the proportion of vessel wall positive for SM- α -actin [19]. Additionally, the number of intrapulmonary vessels $<50\mu\text{m}$ with double elastic laminae, assessed in Orcein stained sections, were counted and expressed as percent of total number of small intrapulmonary vessels.

Assessment of cell proliferation

Lung tissue sections were immunostained with a monoclonal antibody against proliferating cell nuclear antigen (PCNA) to assess cell proliferation. The number of cells with nuclei showing immunoreactivity to PCNA was counted and expressed as the ratio between positive and negative cells in each vessel wall.

Real-Time PCR

Total RNA was extracted from lung tissue using an RNeasy Microkit. Real-time PCR for endothelial nitric oxide synthase (eNOS) and β -actin was performed as previously described[13]. Results were normalized to β -actin expression levels and relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method[20].

Western blot analysis

Endothelial NOS activity was determined in lung homogenates by the ratio between the phosphorylated form (PeNOS) and the total eNOS protein expression, analyzed by western blot.

Plasma chemistry of vasoactive agents

A method based on the Griess reaction[21] was used to measure nitrites and nitrates in the plasma. Endothelin-1 (ET-1) levels were determined by ELISA assay. Plasma levels of norepinephrine (NE), epinephrine and serotonin were measured by HPLC.

Statistics

Data in tables are expressed as mean \pm SD and in graphs as mean \pm SEM. A two-way ANOVA was used to evaluate the main effects of CS and hypoxia, as well as their interaction. *Post-hoc* pairwise comparisons were performed using the Student t-test.

Additional details on Methods are provided in the on-line supplementary material.

RESULTS

Survival and body weight

During the first 10 weeks, the mortality rate of animals exposed to CS was 45% whereas no animal died in the Control group. During the last 2 weeks, when hypoxia was added, one animal of each experimental group died, resulting in an additional mortality rate of 6.3% in CS, 6.3% in CH and 9.1% in CSCH groups. All the non-exposed animals completed the study. The main cause of death (88% of cases) was due to bronchoconstriction during the exposure to CS. Animals that died during the study were excluded from the final analysis.

From week 4 the weight gain in the CS group decreased markedly. Hypoxia induced weight loss from the first week of exposure (week 11 of the study). As a result, at the end of the study, body weight in the three experimental groups was lower than in the Control group, showing the CSCH group the lowest value (supplemental Figure 1).

Pulmonary hemodynamics

Exposure to CS or to hypoxia raised the PAP, both under normoxic and hypoxic conditions (Table 1, Figure 1A). The increase of PAP in the CS and CH groups was of similar magnitude, and was much more pronounced in animals subjected to both stimuli (CSCH group) (Figure 1A). Interestingly, PAP did not change during acute hypoxic challenge in any of the experimental groups, reflecting a lack of hypoxic pulmonary vasoconstriction in this experimental model (Figure 1B). Exposure to hypoxia (CH and CSCH groups) was associated with an increase in systemic arterial pressure (Table 1, Figure 1C).

Right ventricular weight increased in animals exposed to hypoxia (Table 1) and increased further in animals exposed to hypoxia and CS (CSCH group) (Figure 1D).

At the end of the study, compared with the Control group, the hematocrit was 7% higher in the CS group, 5% in the CH group and 12% in the CSCH group (Supplemental Figure 2).

Mechanical properties and reactivity of pulmonary artery and aorta

Vascular distensibility. The distensibility of pulmonary arteries was significantly reduced in animals exposed to hypoxia, the effect being more pronounced in the CSCH group (Table 2 and supplemental Figure 3). Overall, the distensibility of the aorta was lower than that of pulmonary arteries and was similar in all groups.

Contractile responses. Table 2 shows the vascular responses to KCl and NE in the pulmonary artery. Contraction induced by KCl was greater in pulmonary arteries from animals exposed to CS or CH, compared with controls ($p < 0.05$ each), being further increased when both stimuli were combined (Table 2 and supplemental Figure 4). Contraction induced by NE was greater in pulmonary arteries of animals exposed to hypoxia (CH and CSCH groups), whereas in the CS group it was similar to that in the controls (Table 2 and supplemental Figure 5). No differences were observed in the contractility of the aorta.

Endothelium-dependent vasorelaxation. Figure 2 shows the changes in wall tension of pulmonary artery and aorta induced by cumulative doses of ADP, after pre-contraction with NE. The initial wall tension, after NE pre-contraction, was higher in pulmonary arteries of animals exposed to hypoxia (CH and CSCH groups). As a result, when evaluating absolute values of wall tension, the area under the relaxation curve (AUC) in these two groups was larger than in the other two groups (Control and CS) (Table 2). To account for differences in initial wall tension we evaluated change in tension as percent of initial value. After performing this correction, no differences between groups in the

AUC of endothelium-dependent responses were observed (Table 2).

The AUC in the aorta was similar in all groups (Figure 2B).

Pulmonary vascular remodeling

Wall thickness of pulmonary arteries was greater in the three experimental groups (CS, CH, CSCH), as compared with controls. No additive effects were observed when CS and hypoxia were combined (Figure 3A). In contrast, wall thickness in the aorta was not affected by any of the experimental conditions (Supplemental Table 1), although it was thinner in the CH group compared with the CS group (Figure 3B).

In animals exposed to hypoxia (CH and CSCH groups) the content of mucopolisaccharydes in main pulmonary artery was increased, whereas collagen was diminished, as compared with Control and CS groups. Elastin content was greater in the CSCH group compared with the Control group (Figure 4).

The proportion of small vessels showing positive immunoreactivity to SM- α -actin was higher in the 3 experimental groups, with a highly significant effect for CS exposure (Table 3). When arterioles were scored according to the degree of muscularization, it was apparent that in the CS and CSCH groups there was a decrease in the proportion of non-muscularized vessels and a concomitant increase in the proportion of fully-muscularized intrapulmonary vessels (Figure 5).

Animals exposed to hypoxia (CH and CSCH) showed a significantly greater proportion of small intrapulmonary vessels with double elastic laminae as compared with control animals (Table 3 and supplemental Figure 6)

Assessment of proliferating cells (PCNA⁺/PCNA⁻ nucleus ratio) in small intrapulmonary vessel walls revealed no differences between groups (Supplemental Figure 7). The total number of nuclei per vessel remained unchanged irrespective the

type exposure.

Gene expression and activity of eNOS

The gene expression of eNOS was evaluated by real-time PCR in lung homogenates and normalized by the expression of β -actin. Lung expression of eNOS was reduced in animals exposed to CS ($p=0.028$ for CS effect in ANOVA) (Supplemental Figure 8A). No additive effect was observed when both stimuli were combined. Changes in PeNOS and eNOS protein expression were evaluated by western blot in lung homogenates. The ratio PeNOS/eNOS was calculated as a measure of the enzyme activity. No significant changes were observed in the eNOS activity in none of the 3 experimental groups, although a tendency to decrease was present in CS and CSCH groups (Supplemental Figure 8B).

Plasma chemistry of nitrites and nitrates, endothelin-1 and catecholamines

Nitrites and nitrates. Plasma levels of nitrites and nitrates decreased in animals exposed to CS, and tended to increase in those exposed to hypoxia (Table 4).

Endothelin-1. There was strong interaction between the effects of CS and hypoxia on plasma levels of ET-1. Whereas in normoxic animals ET-1 increased in those exposed to CS, CS reduced ET-1 levels in animals subjected to CH (Table 4).

Catecholamines. There were no significant differences between groups in the plasma levels of NE, epinephrine and serotonin (Table 4).

Correlations

In pulmonary arteries, the contraction to KCl correlated with the vessel wall thickness ($r=0.45$, $p=0.02$).

The PAP was related to the percentage of SM- α -actin positive intrapulmonary vessels. As shown in Figure 6A, PAP increased to similar extent in animals exposed either to CS or CH, which showed a similar degree of muscularization, and increased further in those subjected to both exposures (CSCH group), which also showed the greatest number of muscularized vessels.

The number of small intrapulmonary vessels positive for smooth muscle SM- α -actin was inversely correlated with the RNA expression of eNOS in lung homogenates (Figure 6B).

Moreover, distensibility correlated positively with the content of mucopolysaccharide ($p=0.013$, $R=0.470$) and inversely with the content of collagen ($p=0.013$, $R=-0.469$).

DISCUSSION

Results of the present study show that both CS and CH produced a similar increase in PAP and RV hypertrophy, and that the combination of both agents had a synergistic effect on these alterations. Furthermore, CS and CH exerted different effects on the reactivity and mechanical properties of large pulmonary arteries and on the morphological characteristics of small intrapulmonary vessels.

We have previously hypothesized that pulmonary vascular changes that take place in COPD start at early disease stages, since they are apparent in patients with moderate disease severity and also in smokers with normal lung function[2;3]. Nevertheless, we acknowledge the pivotal role that hypoxia has in the development of pulmonary hypertension in this condition [22]. Given that COPD patients develop hypoxemia when airflow obstruction becomes severe, we hypothesized that the effects of hypoxemia on pulmonary vessels may add to the pre-existing effects of CS. In an attempt to mimic this sequence, in the present study we exposed guinea pigs to a hypoxic environment 10

weeks after initiating CS exposure. In addition, we evaluated the individual effects of CS and hypoxia on pulmonary and systemic vessels, as they have never been compared in the same animal model.

Interestingly, CS and hypoxia exerted similar effects on PAP, right ventricular hypertrophy and pulmonary vessel remodeling, the effects on PAP and RV hypertrophy being further enhanced when both types of exposure were combined. On average, compared with the values observed in the Control group, CS exposure induced a 25% increase in PAP, exposure to hypoxia a 34% increase, and exposure to both factors a 64% increase. It is of note that the effect of 12 weeks of CS on PAP was of a similar magnitude as that induced by 2 weeks of hypoxia, thus emphasizing the prominent effect of CS exposure on pulmonary vessels, which is in agreement with the previous observation made by Yamamoto et al [10]. Yet, the current investigation demonstrates that the hemodynamic effects of CS exposure are of a similar magnitude to those produced by hypoxia and that the effects of both factors are further enhanced when they are combined.

The increase in PAP induced by CS or hypoxic exposures might be due to the effects of these agents on pulmonary vessel remodeling, distensibility or tone. Exposure to CS and/or to hypoxia resulted in an increased proportion of muscularized intrapulmonary vessels. Interestingly the morphological characteristics of muscularized arterioles were non-uniform and appeared to be related with the type of stimulus. Whereas CS exposure was strongly associated with an increased proportion of small vessels showing positive immunostaining to SM- α -actin, exposure to hypoxia was associated with a greater proportion showing double elastic laminae. These changes were not due to cell proliferation since the number of cells present in the vessel wall and the proportion of those showing positive immunoreactivity to the proliferating cell nuclear antigen

(PCNA) did not differ between groups. Our findings are in agreement with those reported by King *et al* [23] who showed ultrastructural differences in smooth muscle cells in vessels of rats treated with monocrotaline and rats submitted to hypoxia. While arterioles of monocrotaline-treated rats contained immature smooth muscle cells with coarse peripheral myofilaments, bounded by thin indistinct elastic laminas, arteriolar smooth muscle cells of hypoxic rats were mature with fine myofilaments and bounded by electron dense laminas. It can be hypothesized, therefore, that hypoxia may have a stimulating effect on the production of elastin by mature smooth muscle cells, whereas CS may induce changes in cell phenotype with increased cytoplasmatic content of SM- α -actin.

Exposure to hypoxia had greater effect on vessel distensibility than CS (Table 2), although guinea pigs subjected to both exposures showed the lowest distensibility (Table 2 and supplemental Figure 3). The cellular and molecular mechanisms responsible for pulmonary artery stiffening as a result of hypoxia are not well understood. Deposition of extracellular matrix in pulmonary arteries has been shown in animal models of hypoxia-induced pulmonary hypertension[24;25]. Interestingly, we observed a significant increase in the content of mucopolysaccharides in the wall of main pulmonary arteries, with a concomitant decrease of collagen, both in the CH and CSCH groups, as compared with the Control group. The content of both proteins correlated with the distensibility of pulmonary arteries, suggesting that an imbalance in extracellular matrix protein content may modulate the mechanical properties of the vessels wall.

The increase in PAP in the experimental groups could be also explained, at least in part, by an increase in vessel tone. In humans, exposure to hypoxia induces pulmonary vasoconstriction. However, in the present investigation we did not observe any change

in PAP when animals were exposed to acute hypoxia, which is consistent with the lack of hypoxic pulmonary vasoconstriction in the guinea pig [26]. Accordingly, the increased PAP in the CH group might be explained by adjustments in the contractile/synthetic phenotype of SMC in response to reduced oxygen concentrations[27;28]. In this respect, it should be noted that *in vitro*, pulmonary arteries from animals exposed to hypoxia showed greater contraction to NE than controls (Table 2), likely reflecting changes in SMC adrenergic receptors that regulate vessel tone. Regretfully, we were unable to show a clear increase in the plasmatic levels of NE and epinephrine in animals exposed to hypoxia (Table 4), presumably due to the variability in these measurements and the reduced number of animals in each group. Overall, these results suggest that the effects of hypoxia on the pulmonary circulation exceed those produced by the stimulation of a sympathetic response mediated by peripheral chemoreceptors and baroreceptors in the systemic circulation.

The assessment of endothelium-dependent vasodilatation of pulmonary arteries was largely influenced by the pre-contraction induced by NE, since in arteries from animals exposed to CH the contraction to NE doubled that shown in arteries of the Control or CS-exposed groups (Figure 2). When ADP-induced relaxation was evaluated as the AUC of the absolute change in tension, values in animals exposed to hypoxia were greater than in the other two groups. However, we do not interpret such a difference as demonstrative of the impairment of endothelium-dependent relaxation, but as the result of different baseline tension after NE precontraction. To account for this difference we evaluated the change in tension as a percentage of the baseline value. In this case, we did not observe significant differences in the AUC of the relaxation curve among the groups (Table 2). Accordingly, we conclude that in the current investigation neither CS nor exposure to hypoxia produced endothelial dysfunction in pulmonary arteries. In a

previous study we showed that exposure to CS for 3 and 6 months induced endothelial dysfunction in the guinea pig[13]. Differences between the two studies can be explained by the fact that CS exposure in the present study (4 cigarettes/day) was lower than that used in the previous study (7 cigarettes/day). We used a lower dose of CS because we anticipated greater mortality in animals exposed to a hypoxic environment for two weeks after being exposed to CS for 10 weeks, as indeed it occurred. The lack of impairment of endothelial function in the main pulmonary arteries contrasts with the observation of decreased expression of eNOS mRNA and protein activity in lung tissue and reduced plasma levels of nitrites/nitrates in the CS group. We speculate that this situation may represent an initial step that antecedes endothelial dysfunction as assessed *in vitro* in organ bath chambers. Interestingly, the lung expression of eNOS was inversely related to the SM- α -actin content in small intrapulmonary vessels, suggesting that vascular cells of these vessels may be a first target of CS, whereas large vessels may require more intense exposure to CS.

We evaluated ET-1 and serotonin levels in plasma because of its actions on SMC physiology and vascular tone. Levels of ET-1 were increased in CS and CH groups, in keeping with the increased levels observed in smokers and COPD patients[29;30]. However, no differences in ET-1 levels were observed in CSCH animals, suggesting that the synergistic effect on PAP does not appear to be mediated by ET-1 in plasma.

The greatest effects on PAP and RV hypertrophy were observed in guinea pigs subjected to combined CS and CH exposure. Since animals subjected to both factors showed similar vessel remodeling and contractile response to NE, we hypothesize that the greater hemodynamic effect observed in this group can be explained by the combined effects of CS on vessel remodeling and the greater reactivity induced by CH.

This observation in the guinea pig is in keeping with the observations made in COPD in which patients with moderate disease severity show a similar degree of pulmonary vessel remodeling as patients with severe disease, whereas they differ markedly in PaO₂ and PAP[31]. Furthermore, in patients with COPD and chronic respiratory failure the degree of pulmonary vessel remodeling is not related to the presence of pulmonary hypertension or its severity[32]. Accordingly, our findings suggest that the presence of pulmonary hypertension in COPD, which is commonly associated with chronic respiratory failure, may be due to factors related to hypoxia that add to an underlying process of vessel remodeling produced by cigarette smoking. The different intensity of vessel remodeling and reactivity might explain the great variability in the relationship between PAP and PaO₂ observed in COPD[33].

Animals exposed to CS and CH showed greater hematocrit values, likely due to the additive effects of hypoxia and carboxyhemoglobin on red cell production. Eventually, the increase in blood viscosity resulting from increased red cell concentration could contribute to some degree to the increase in both PAP and SAP. The CSCH group was also the group with less weight gain. Presumably, the mechanisms responsible for lower weight gain could be related to the systemic effects induced either by CS or chronic hypoxia, akin to those observed in COPD. Body weight was unrelated to mortality probably because the principal cause of death was bronchoconstriction induced by CS.

Our study has limitations. Cardiac output was not measured due to technical difficulties for its assessment in our experimental setting. Accordingly we cannot disregard that sympathetic changes might underline some of the differences noticed in SAP. Further, the use of anesthesia during the acute hypoxic challenge might have attenuated hypoxic pulmonary vasoconstriction (HPV)[34]. Accordingly, we cannot completely exclude

that the guinea pig may exhibit some degree of HPV, as it has been suggested by Thompson *et al* [35].

In summary, results of the present investigation show that in the guinea pig exposure to CS or to CH has similar effects on pulmonary hypertension and RV hypertrophy, and when the two factors are combined the hemodynamic effects are magnified. In animals exposed to CS, mechanisms underlying these hemodynamic changes appear to be related to the remodeling of small intrapulmonary vessels. Exposure to hypoxia modifies the mechanical properties of large pulmonary arteries, presumably by altering extracellular matrix deposition in the vessel wall, enhances their sensitivity to adrenergic agonists, and induces small vessel remodeling. Altogether, this indicates that hypoxemia represents a critical step in the progression of pulmonary vascular impairment that accelerates and, in some aspects amplifies, the initial effects of CS. These findings contribute to unravel the mechanisms underlying the development of pulmonary hypertension in COPD and to clarify the variability in the relationship between arterial oxygenation and pulmonary hypertension.

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Reference List

1. Tozzi, C. A., D. L. Christiansen, G. J. Poiani, and D. J. Riley. Excess collagen in hypertensive pulmonary arteries decreases vascular distensibility. *Am J Respir Crit Care Med* 1994; 149:1317-1326.
2. Santos, S., V. I. Peinado, J. Ramirez, T. Melgosa, J. Roca, R. Rodriguez-Roisin, and J. A. Barbera. Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD. *Eur Respir J* 2002; 19:632-638.
3. Peinado, V. I., J. A. Barbera, J. Ramirez, F. P. Gomez, J. Roca, L. Jover, J. M. Gimferrer, and R. Rodriguez-Roisin. Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *Am J Physiol* 1998; 274:L908-L913.
4. Santos, S., V. I. Peinado, J. Ramirez, J. Morales-Blanhir, R. Bastos, J. Roca, R. Rodriguez-Roisin, and J. A. Barbera. Enhanced expression of vascular endothelial growth factor in pulmonary arteries of smokers and patients with moderate chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003; 167:1250-1256.
5. Peinado, V. I., J. A. Barbera, P. Abate, J. Ramirez, J. Roca, S. Santos, and R. Rodriguez-Roisin. Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999; 159:1605-1611.
6. Rahman, I. and I. M. Adcock. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur Respir J* 2006; 28:219-242.

7. Le Cras, T. D. and I. F. McMurtry. Nitric oxide production in the hypoxic lung. *Am J Physiol Lung Cell Mol Physiol* 2001; 280:L575-L582.
8. Nicolls, M. R. and N. F. Voelkel. Hypoxia and the lung: beyond hypoxic vasoconstriction. *Antioxid Redox Signal* 2007; 9:741-743.
9. Stenmark, K. R., N. J. Davie, J. T. Reeves, and M. G. Frid. Hypoxia, leukocytes, and the pulmonary circulation. *J Appl Physiol* 2005; 98:715-721.
10. Yamato, H., A. Churg, and J. L. Wright. Guinea pig pulmonary hypertension caused by cigarette smoke cannot be explained by capillary bed destruction. *J Appl Physiol* 1997; 82:1644-1653.
11. Wright, J. L., H. Tai, and A. Churg. Vasoactive mediators and pulmonary hypertension after cigarette smoke exposure in the guinea pig. *J Appl Physiol* 2006; 100:672-678.
12. Wright, J. L. and A. Churg. Short-term exposure to cigarette smoke induces endothelial dysfunction in small intrapulmonary arteries: analysis using guinea pig precision cut lung slices. *J Appl Physiol* 2008; 104:1462-1469.
13. Ferrer, E., V. I. Peinado, M. Diez, J. L. Carrasco, M. M. Musri, A. Martinez, R. Rodriguez-Roisin, and J. A. Barbera. Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig. *Respir Res* 2009; 10:76.
14. Thompson, B. T., D. M. Steigman, C. L. Spence, S. P. Janssens, and C. A. Hales. Chronic hypoxic pulmonary hypertension in the guinea pig: effect of three levels of hypoxia. *J Appl Physiol* 1993; 74:916-921.

15. Underwood, D. C., S. Bochnowicz, R. R. Osborn, M. A. Luttmann, and D. W. Hay. Nonpeptide endothelin receptor antagonists. X. Inhibition of endothelin-1- and hypoxia-induced pulmonary pressor responses in the guinea pig by the endothelin receptor antagonist, SB 217242. *J Pharmacol Exp Ther* 1997; 283:1130-1137.
16. Ardite, E., V. I. Peinado, R. A. Rabinovich, J. C. Fernandez-Checa, J. Roca, and J. A. Barbera. Systemic effects of cigarette smoke exposure in the guinea pig. *Respir Med* 2006; 100:1186-1194.
17. Fischer, E. C., D. B. Santana, Y. Zocalo, J. Camus, E. D. Forteza, and R. Armentano. Effects of Removing the Adventitia on the Mechanical Properties of Ovine Femoral Arteries In Vivo and In Vitro. *Circ J* 2010.
18. Brouwers-Ceiler, D. L., H. J. Nelissen-Vrancken, J. F. Smits, and J. G. De Mey.. The influence of angiotensin II-induced increase in aortic wall mass on compliance in rats in vivo. *Cardiovasc Res* 1997; 33:478-484.
19. Crosby, A., F. M. Jones, M. Southwood, S. Stewart, R. Schermuly, G. Butrous, D. W. Dunne, and N. W. Morrell. Pulmonary vascular remodeling correlates with lung eggs and cytokines in murine schistosomiasis. *Am J Respir Crit Care Med* 2010; 181:279-288.
20. Livak, K. J. and T. D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25:402-408.

21. Granger, D. L., R. R. Taintor, K. S. Boockvar, and J. B. Hibbs, Jr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol* . 1996; 268:142-151.
22. Weitzenblum, E., C. Hirth, A. Ducolone, R. Mirhom, J. Rasaholinjanahary, and M. Ehrhart. Prognostic value of pulmonary artery pressure in chronic obstructive pulmonary disease. *Thorax* 1981; 36:752-758.
23. King, A., P. Smith, and D. Heath. Ultrastructural differences between pulmonary arteriolar muscularization induced by hypoxia and monocrotaline. *Exp Mol Pathol* 1994; 61:24-35.
24. Kobs, R. W., N. E. Muvarak, J. C. Eickhoff, and N. C. Chesler. Linked mechanical and biological aspects of remodeling in mouse pulmonary arteries with hypoxia-induced hypertension. *Am J Physiol Heart Circ Physiol* 2005; 288:H1209-H1217.
25. Bank, A. J., H. Wang, J. E. Holte, K. Mullen, R. Shammass, and S. H. Kubo. Contribution of collagen, elastin, and smooth muscle to in vivo human brachial artery wall stress and elastic modulus. *Circulation* 1996; 94:3263-3270.
26. Schwenke, D. O., C. P. Bolter, and P. A. Cragg. Are the carotid bodies of the guinea-pig functional? *Comp Biochem Physiol A Mol Integr Physiol* 2007; 146:180-188.
27. Rensen, S. S., P. A. Doevendans, and G. J. van Eys. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Neth Heart J* 2007; 15:100-108.

28. Owens, G. K., M. S. Kumar, and B. R. Wamhoff. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004.; 84:767-801.
29. Goerre, S., C. Staehli, S. Shaw, and T. F. Luscher. Effect of cigarette smoking and nicotine on plasma endothelin-1 levels. *J Cardiovasc Pharmacol* 1995; 26 Suppl 3:S236-S238.
30. Spiropoulos, K., G. Trakada, E. Nikolaou, E. Prodromakis, G. Efremidis, A. Pouli, and A. Koniavitou. Endothelin-1 levels in the pathophysiology of chronic obstructive pulmonary disease and bronchial asthma. *Respir Med* 2003; 97:983-989.
31. Magee, F., J. L. Wright, B. R. Wiggs, P. D. Pare, and J. C. Hogg. Pulmonary vascular structure and function in chronic obstructive pulmonary disease. *Thorax* 1988; 43:183-189.
32. Wright, J. L., T. Petty, and W. M. Thurlbeck. Analysis of the structure of the muscular pulmonary arteries in patients with pulmonary hypertension and COPD: National Institutes of Health nocturnal oxygen therapy trial. *Lung* 1992; 170:109-124.
33. Scharf, S. M., M. Iqbal, C. Keller, G. Criner, S. Lee, and H. E. Fessler. Hemodynamic characterization of patients with severe emphysema. *Am J Respir Crit Care Med* 2002; 166:314-322.
34. Busch, C. J., F. A. Spohr, J. Motsch, M. M. Gebhard, E. O. Martin, and J. Weimann. Effects of ketamine on hypoxic pulmonary vasoconstriction in the

isolated perfused lungs of endotoxaemic mice. *Eur J Anaesthesiol* 2010; 27:61-66.

35. Thompson, B. T., P. M. Hassoun, R. L. Kradin, and C. A. Hales. Acute and chronic hypoxic pulmonary hypertension in guinea pigs. *J Appl Physiol* 1989; 66:920-928.

TABLE 1. PULMONARY HEMODYNAMICS AND RIGHT VENTRICLE HYPERTROPHY

	Control	CS	CH	CSCH	Two-way ANOVA main effects		
					CS Exposure	Hypoxia	Interaction
PAP at FiO₂ 0.21, mmHg	6.4±1.7	8.0±0.7	8.6±2.6	10.5±1.8	0.028	0.005	0.843
PAP at FiO₂ 0.12, mmHg	6.2±2.0	8.2±1.4	8.2±2.2	10.2±1.9	0.016	0.016	0.994
PAP at FiO₂ 0.21, mmHg (recovery)	6.9±1.7	8.0±1.8	8.4±1.9	10.1±1.9	0.092	0.033	0.677
Mean systemic arterial pressure, mmHg	51.9±12.5	50.9±2.2	59.1±5.7	55.8±4.1	0.474	0.048	0.682
Fulton Index	0.30±0.01	0.32±0.03	0.34±0.05	0.37±0.05	0.063	<0.001	0.713

Values are mean±SD.

Definition of abbreviation: PAP: Mean pulmonary artery pressure.

Fulton Index = Right ventricle weight / (left ventricle weight + septum weight).

TABLE 2. MECHANICAL PROPERTIES AND REACTIVITY OF PULMONARY ARTERY AND AORTA

Pulmonary Artery	Control (n=7)	CS (n=6)	CH (n=7)	CSCH (n=8)	Two-way ANOVA main effects		
					CS Exposure	Hypoxia	Interaction
Distensibility, mmHg⁻¹*10⁻³	14.9±1.9	14.2±2.9	13.1±1.7	11.2±2.8	0.152	0.013	0.534
Contraction, mN							
KCl (60 mM)	20.3±3.2	31.9±4.1	30.5±5.5	33.8±8.5	0.002	0.008	0.059
NE (0.2·10⁻⁶M)	5.5±2.9	6.6±3.9	10.8±4.3	9.0±3.0	0.815	0.007	0.284
Relaxation in response to, AUC:							
ADP % of change^a	207.8±46.7	171.7±47.8	176.8±19.2	205.2±43.6	0.808	0.936	0.043
 wall tension^b	22.6±8.5	25.7±16.2	52.7±13.2	46.5±16.2	0.775	<0.001	0.406
ADP+L-NAME^b	44.4±22.5	66.8±33.9	107.7±29.7	69.6±28.3	0.903	0.016	0.230
SNP^b	18.4±8.1	23.6±12.3	35.4±8.5	32.1±13.1	0.820	0.005	0.316

Values are mean±SD.

Definition of abbreviations: KCl: potassium chloride, NE: Norepinephrine, AUC: area under the curve, ADP: Adenosine diphosphate; L-NAME: N^G-monomethyl-L-arginine; SNP: Sodium nitroprusside

Vascular response is shown as ^a percentage of change in tension from pre-contraction to NE and ^b absolute change in tension. The AUC values of ADP+L-NAME and SNP are shown as the delta change in the wall tension.

TABLE 3. MUSCULARIZATION OF INTRAPULMONARY ARTERIES

					Two-way ANOVA main effects		
	Control (n=8)	CS (n=8)	CH (n=15)	CSCH (n=13)	CS Exposure	Hypoxia	Interaction
Wall thickness, μm							
Aorta	105 \pm 28	126 \pm 19	98 \pm 26	114 \pm 49	0.148	0.486	0.838
Pulmonary artery	137 \pm 22	171 \pm 2	170 \pm 33	176 \pm 26	0.062	0.084	0.183
Small intrapulmonary vessels							
% of arteries α-actin⁺	48.9 \pm 13.0	63.59 \pm 11.0	54.79 \pm 12.5	63.09 \pm 8.7	0.004	0.469	0.392
% of arteries with double elastic lamina	6.8 \pm 5.1	9.6 \pm 11.8	17.4 \pm 14.5	14.3 \pm 8.6	0.883	0.039	0.472

Values are mean \pm SD

TABLE 4. BLOOD CHEMISTRY

	Control (n=7)	CS (n=6)	CH (n=8)	CSCH (n=8)	Two-way ANOVA main effects		
					CS Exposure	Hypoxia	Interaction
Levels in plasma:							
Nitrites and nitrates, mM	4.15±0.93	3.46±0.44	5.15±1.16	3.71±0.85	0.005	0.082	0.297
ET-1, pg/mL	1.02±0.10	1.12±0.23	1.56±0.64	0.75±0.32	0.022	0.580	0.005
Catecholamines, pmoles/mL of plasma:							
Norepinephrine	6.17±1.14	7.35±6.5	8.20±2.47	7.29±3.54	0.922	0.488	0.462
Epinephrine	4.02±1.16	5.09±2.66	6.44±5.59	8.05±5.71	0.464	0.149	0.83
Serotonin	2.66±0.67	2.31±2.1	7.40±8.16	2.94±2.84	0.229	0.183	0.303

Values are mean±SD

Definition of abbreviation: ET-1: endothelin-1. *P*-values < 0.05 were considered significant.

Figure legends

Figure 1. Pulmonary and systemic hemodynamics. (A) Mean pulmonary artery pressure (PAP) at baseline. Bars indicate mean \pm SEM value in each experimental group. (B) Individual values of mean PAP in: normoxia (FiO_2 0.21), acute hypoxia (FiO_2 0.10) and recovery to normoxia (FiO_2 0.21). (C) Mean systemic arterial pressure at baseline. (D) Right ventricle (RV) hypertrophy, assessed as the ratio between the RV weight and the left ventricle weight plus the septum weight. CS: exposed to cigarette smoke; CH: exposed to chronic hypoxia; CSCH: exposed to cigarette smoke and chronic hypoxia.

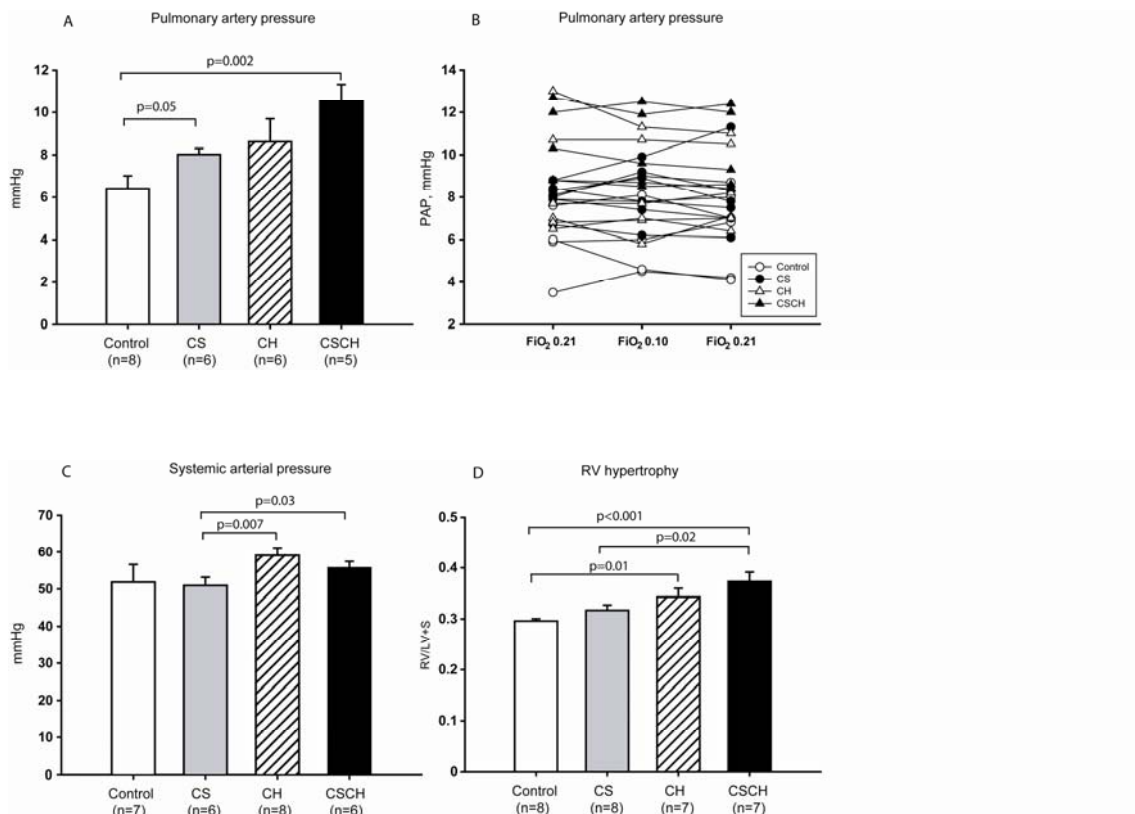


Figure 2. Vascular reactivity of main pulmonary artery and aorta. (A) Changes in wall tension of pre-contracted pulmonary artery rings, expressed in mN/mm of circumference, in response to cumulative doses of ADP. (B) Changes in wall tension of pre-contracted aorta rings, expressed in mN/mm of circumference, in response to cumulative doses of ADP. Values are mean \pm SEM at each condition of ADP.

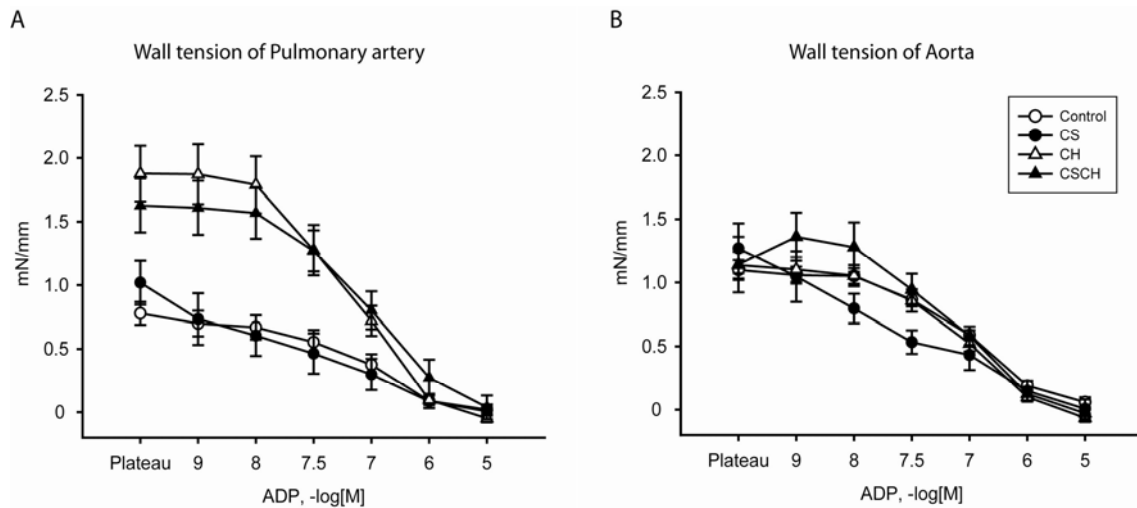


Figure 3. Morphometry of main pulmonary artery and aorta. Bars indicate mean \pm SEM. Wall thickness of pulmonary artery (A) and aorta (B) in each experimental group.

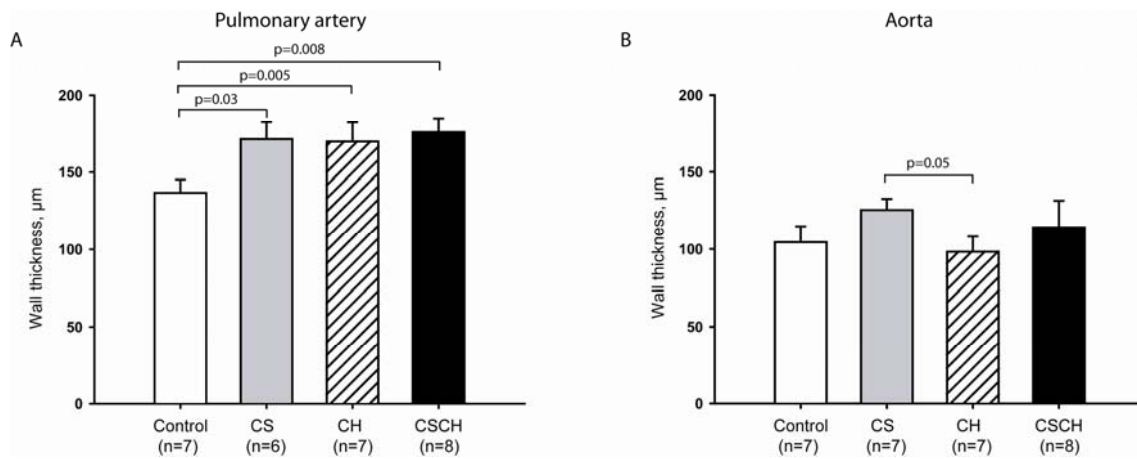


Figure 4. Extracellular matrix protein content in the main pulmonary artery. (A) Bar charts indicate the percentage of the area occupied by mucopolysaccharide in the total vessel area. (B) Representative microphotograph of Alcian blue staining in the 4 experimental groups. (C) Graph representation of the percentage of collagen in the pulmonary artery wall. Panel (D) shows representative images of Mason trichrome staining in each group of animals. (E) Elastin content shown as the percentage of the total vessel area. Panel (F) shows representative images of orcein staining in each experimental group.

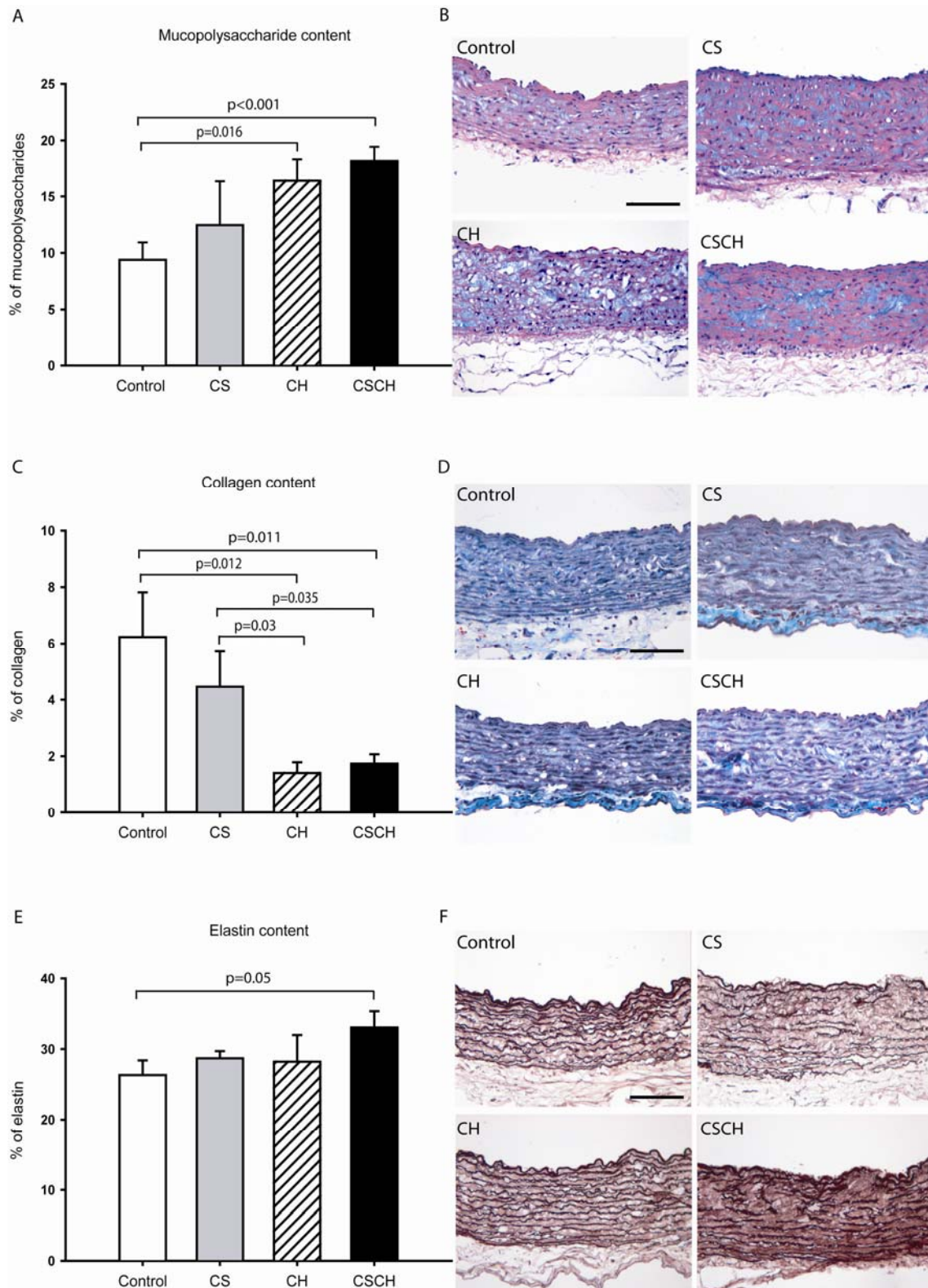


Figure 5. Classification of the intrapulmonary arteries according to their degree of muscularization. Bar charts show the % of non-, partially- and fully-muscularized vessels evaluated in lung sections by immunohistochemistry against SM- α -actin.

Animals exposed to both cigarette smoke and chronic hypoxia (CSCH group) developed a greater number of fully-muscularized arteries. Values are mean \pm SEM.

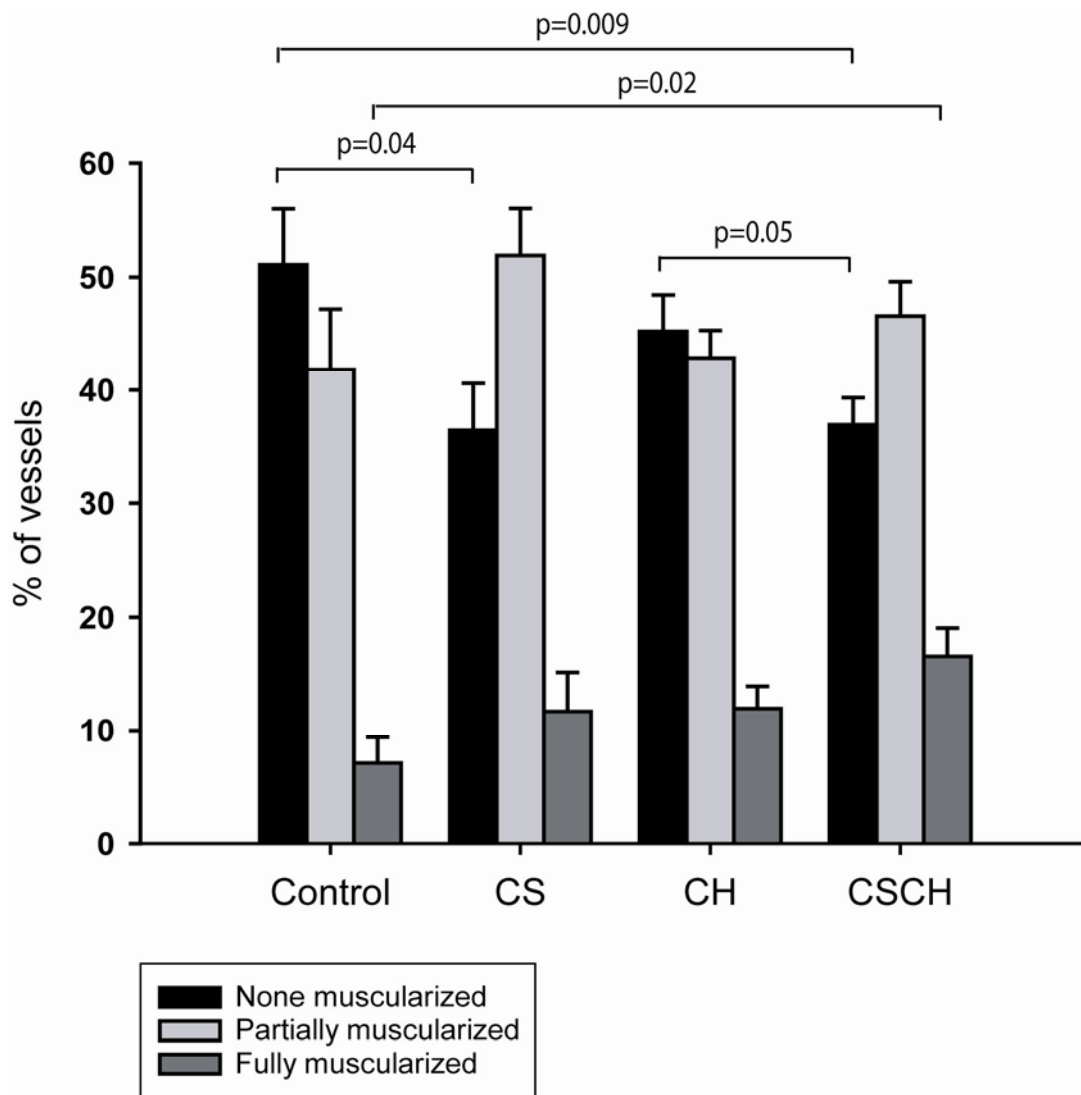
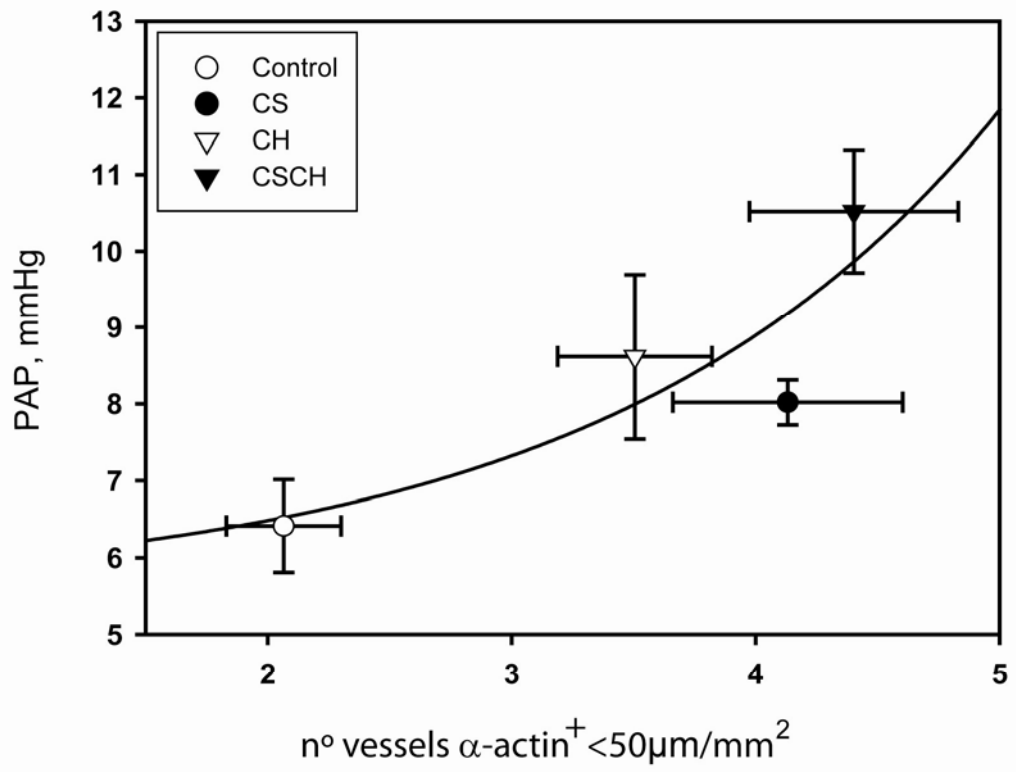


Figure 6. (A) Plot of pulmonary artery pressure (PAP) against the degree of vascular remodeling, expressed as the number of smooth muscle α -actin⁺ vessels per mm² of area. The equation was fitted to a modified simple exponential curve. Each data point corresponds to the mean value \pm SEM of each group. (B) Relationship between the RNA expression of eNOS in lung homogenates and the degree of muscularization of small intrapulmonary arteries. The lower expression of eNOS is related to a higher content of smooth muscle α -actin in arteries $<50\mu\text{m}$.

A



B

