

Increased Rho-kinases expression and activity and pulmonary endothelial dysfunction in smokers with normal lung function

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Abstract

Endothelial dysfunction is one of the main consequences of the toxic effects of cigarette smoke on the vascular system. Increasing evidence suggests that the small G-protein Rho-A and its downstream effectors, the Rho-kinases (ROCKs), are involved in systemic endothelial dysfunction induced by cigarette smoke. This study aimed to evaluate the role of the RhoA/ROCKs pathway in pulmonary artery endothelial function in current smokers with normal lung function.

Lung tissues were obtained from nonsmokers and smokers who underwent lobectomy for lung carcinoma. Arterial relaxation in response to acetylcholine (ACh) was assessed in isolated pulmonary arterial rings. Protein expressions and activities of endothelial nitric oxide synthase (eNOS), ROCKs, and the myosin phosphatase subunit-1 (MYPT-1) were sought.

Relaxation in response to ACh was significantly lower in smokers as compared with nonsmokers (n=8 in each group), consistent with reduced eNOS activity in the former as compared with the latter. eNOS protein expression remained however the same in both groups. Expression of ROCKs, GTP-RhoA, and p-MYPT-1 were significantly increased in smokers as compared with controls.

Pulmonary endothelial dysfunction is present in smokers whose lung function has not yet been impaired. Reduced activity of eNOS accounts at least in part for this endothelial dysfunction. Increased expression and activity of ROCKs accounts for another part through direct or indirect inhibition of the Rho-A/ROCKs pathway on NO synthesis and sustained pulmonary vasoconstriction through inhibition of myosin phosphatase.

Key-words: Tobacco smoke; endothelial nitric oxide synthase; cGMP; RhoA; Rho-kinases; endothelial dysfunction.

INTRODUCTION

Tobacco smoke is associated with high mortality and morbidity related to cardiovascular disease [1], and is currently the leading cause of chronic obstructive pulmonary disease (COPD) [2]. The most prominent pathological changes in COPD patients are progressive airflow limitation, peripheral airways inflammation, and pulmonary vascular remodelling [3, 4]. Structural changes of the pulmonary vasculature, however, are not exclusive to patients with severe COPD, as they have been found in patients with milder degree of airways obstruction, and even in heavy smokers without airflow limitation [5]. Together with its deleterious effects on peripheral airways, tobacco smoke also causes systemic endothelial dysfunction in active and passive smokers [6, 7] in whom endothelial dysfunction often precedes structural changes of pulmonary vessels [8, 9].

In smokers, endothelial dysfunction might directly result from the toxic effects of, or indirectly due to the release of inflammatory mediators induced by free radicals derived from, tobacco smoke [10]. The precise mechanisms of tobacco smoke-related endothelial dysfunction are not fully understood but it is hypothesized that decreased production, or reduced bio-availability, of nitric oxide (NO) in the pulmonary vasculature of smokers might be partly implicated [11].

Recent studies suggest that the small G-protein RhoA, and its downstream effectors the Rho-kinases (ROCK-1 or ROCK- β and ROCK-2 or ROCK- α) are also implicated in the pathogenesis of endothelial dysfunction [12]. In smooth muscle cells, activation of Rho-kinases by GTP-RhoA causes phosphorylation of the myosin-binding subunit of myosin phosphatase (MPT), also called myosin phosphatase subunit 1 (MYPT-1). The resulting inhibition of MPT prolongs actin-myosin interaction and sustains preexisting vasoconstriction [13]. Furthermore, RhoA/Rho-kinase downregulates endothelial NO synthase (eNOS) expression and impairs NO production, thus reducing a potent counteracting mechanism to increased vascular tone [14].

Increased RhoA/Rho-kinase activity has been implicated in systemic endothelial dysfunction but evidence is still lacking regarding its role in pulmonary endothelial dysfunction. The aims of the present study was two-fold, first to assess the presence (or the absence) of impaired endothelium-dependent relaxation of pulmonary arteries in smokers with normal lung function, and second, to determine the respective contributions of reduced eNOS/NO and increased RhoA/Rho-kinase in tobacco smoke-related endothelial dysfunction.

METHODS

Subjects and tissue preparations

Patients, who underwent lung resection for carcinoma, were enrolled in the present study after giving their informed consents, as approved by the local Ethics Committee. They were divided in two groups: nonsmokers and current smokers. All but 2 nonsmokers had never smoked. The two remaining subjects were ex-smokers (5 and 10 pack-years) but had quit smoking for 20 and 25 years, respectively. All patients had normal lung function as defined by slow and forced vital capacity (SVC and FVC), total lung capacity (TLC) and forced expiratory volume in one second (FEV_1) greater or equal to 80% of predicted values. Absence of airflow obstruction as defined a ration of FEV_1/FVC greater or equal to 0.7 [15].

Peripheral lung tissues and proximal pulmonary arteries were carefully dissected from lobectomy tissue free from macroscopic signs of lung tumour. Rings of 2-3 mm external diameter and 4-6 mm of length were cut and immediately placed in cold Krebs-Henseleit solution (4°C) for vasoreactivity study within the following half an hour. The others specimens of pulmonary arterial and lung parenchyma were stored at -80°C for Western blotting or they were fixed overnight in 4% formalin and embedded in paraffin for immunohistochemistry assessment.

Chemical products

eNOS antibody was purchased from BD Transduction Laboratories, Lexington, KY. Primary antibodies for ROCK-1, ROCK-2, and RhoA were provided by Santa Cruz Biotechnology Inc. Horseradish peroxidase-conjugated secondary antibody was purchased from Pierce Biotechnology, Inc. Rho activation kit for pull-down assay was purchased from Stressgen Bioreagent Corp. Rho-kinase Assay Kit was supplied by CycLex, Japan. Nitric Oxide Synthase Assay Kit was purchased from Cayman Chemical Europe, France. cGMP Enzyme immunoassay Biotrak (EIA) System was provided by GE Healthcare Life Sciences, UK. All other chemical products were purchased from Sigma-Aldrich (Steinheim, Germany).

Measurement of isometric tension of pulmonary arterial vascular rings

Each pulmonary arterial ring was mounted between two parallel stainless steel wires for measurement of isometric tension in organ chambers (Emka Technologies, France). Individual organ baths were filled with 20 ml of Krebs-Henseleit solution (glucose 10 mM, pyruvate 2 mM, HEPES 10 mM, EDTA 0.03 mM, NaCl 118 mM, KCl 4.7 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 15 mM), heated at 37°C and continuously aerated with 95 % O₂ and 5 % CO₂. The isometric force generated by the ring segment was measured and recorded by Mac Intosh Performa 630-Software. Pulmonary arterial rings were initially stabilized for 30 min between 1 - 1.5 g, at a resting tension that has been previously determined as corresponding to the optimal length for tension development.

The arterial rings were precontracted with increasing concentrations of phenylephrine (from 10⁻⁸ to 10⁻⁵ mol/L) as previously described [16]. For each ring, the effective concentration of phenylephrine needed to achieve optimal contraction was calculated. Indomethacin (10⁻⁵ mol/L) was added to the organ baths 30 min prior to phenylephrine to exclude the influence of cyclooxygenase pathway products [17]. After the phenylephrine contraction series, the baths were washed three times with fresh Krebs solution and the rings were allowed to stabilize over a resting tension for an hour.

Endothelium-dependent relaxation was assessed by exposing pulmonary arterial rings to increasing concentrations of acetylcholine (ACh, 10^{-9} - 10^{-4} mol/L). At least four rings from each patient were studied for obtained results.

Immunohistochemistry assessment for eNOS, ROCK-1, and ROCK-2

For eNOS immunohistochemical staining, sections were prepared as previously described to block nonspecific binding of primary and secondary antibodies and nonspecific reaction [18]. The sections were incubated with the specific eNOS antibody (1/100 titer). All bound antibody sections were incubated with horseradish peroxidase-conjugated secondary antibody. The sections were incubated with metal enhanced DAB peroxidase substrate (3, 3'-diaminobenzidine) until the desired staining was obtained. Tissues were counterstained with hematoxylin and examined using light microscopy. Negative-control sections were incubated in blocking buffer alone without primary antibody. eNOS immunostaining expression was measured in endothelial cells of pulmonary arterial rings. The staining expression was measured by using intensity score. At least ten counted images were performed for each patient.

For ROCK-1 and ROCK-2 immunostaining, sections were stained with ImmunoCruz™ Staining System. Primary antibody (goat polyclonal immunoglobulin G) was diluted to 1/50 as determined by titration in serum block. After 2 hours of incubation with the primary antibody, sections were washed in PBS and incubated with biotinylated secondary antibody and HRP-streptavidin complex during 30 minutes for each. The desired stain was obtained in 60 seconds after addition of HRP substrate mixture to the sections. The last steps with counterstained, dehydrated and mounted slides were then performed. The expression of ROCK-1 and ROCK-2 peroxidase staining were measured in SMC of pulmonary arteries. Ten magnified visual fields were performed for each subject.

Proteins semi-quantification of eNOS, ROCK-1, ROCK-2, RhoA, and p-MYPT-1 by Western blot

Equal amounts of pulmonary arterial homogenates adjusted to protein content were subjected to 7.5 % SDS-PAGE (for eNOS, ROCKs: ROCK-1 and ROCK-2, p-MYPT-1) or 12 % gels (for RhoA) for electrophoresis and then transferred to PVDF membranes (Immobilon-P). Membranes were blocked in 5 % non-fat milk, 0.1 % Tween-20 in PBS for 1 hour at room temperature. The membranes were hybridized overnight with antibodies against eNOS (dilution 1/100), ROCKs (dilution 1/500), RhoA (dilution 1/100), and p-MYPT-1 (dilution 1/200), which were then incubated with HRP-linked goat anti-mouse (eNOS, dilution 1/5.000) or donkey anti-goat IgG-HRP as secondary antibodies during 45 minutes for ROCKs (dilution 1/10.000), RhoA and p-MYPT-1 (dilution 1/5.000). The equal sample loadings were confirmed by β -actin. Protein bands were developed on the film by using enhanced chemiluminescence reagent, according to the manufacturer's instructions. The protein densities were quantified by using Image Software System (Genius 2) and normalized with β -actin densities.

eNOS activity

The activity of eNOS was measured by using Nitric Oxide Synthase Assay Kit with colorimetric method. This kit was used to evaluate the production of NO from L-Arginine by the action of eNOS in the presence of NADPH, an essential co-factor of this enzyme. The best index of total NO produced was the sum of both nitrite (NO_2^-) and nitrate (NO_3^-) concentration and it was represented indirectly the activity of total eNOS. All the steps of assay had been done according to the manufacturers' instruction. The final products of reaction were nitrate (NO_3^-). The results were read at the absorbance of 540 nm. The concentrations of nitrate from the samples were determined via the standard curve of provided nitrate standard.

cGMP concentration

cGMP concentration in pulmonary arteries was measured by Enzyme immunoassay Biotrak (EIA) System according to the manufacturers' instruction. It combined the use of a peroxidase-labelled cGMP conjugate, a specific antiserum that can be immobilized on pre-coated microplates, and a one-pot stabilized substrate solution. The assay was based on competition between unlabelled cGMP and a fixed quantity of cGMP labelled with peroxidase-, for a limited number of binding sites on a cGMP specific antibody. Results were obtained by colorimetric reading with the absorbance at 450 nm. The concentrations from the sample were quantified by interpolating absorbance readings from a standard curve generated with the calibration of provided cGMP protein standard.

Measurement of RhoA active form by pull-down assay

Rho activation was determined by Rho activation kit. It was used to measure the active form of RhoA (GTP-RhoA). This assay used a GST-fusion protein containing the Rho-binding domain (RBD) of mouse Rhotekin to affinity precipitate active Rho (GTP-Rho) from pulmonary arterial homogenates (500 µg total proteins). After pull-down assay, 25 µL of eluted samples were separated by 12 % SDS-PAGE gels, transferred to PVDF membrane and probed with antibodies against RhoA. The protein bands were detected and quantified as describe above.

Determination of the activity of Rho-kinase by Elisa

Rho-kinase activity in pulmonary arteries was assessed by Elisa, using Rho-kinase Assay Kit. Plates were pre-coated with a substrate corresponding to recombinant the C terminus of MBS (Myosin-Binding Subunit of myosin phosphatase or MYPT-1), which contains a threonine residue that was phosphorylated by Rho-kinase. The specific detector antibody was HRP (horseradish peroxidase) conjugated anti-phospho-MBS (AF 20) that detected only the phosphorylated form of threonine-696 on MBS. The amount of phosphorylated substrate was measured by binding it with a HRP of AF20 which then

catalyzed the conversion of the chromogenic substrate TMB (tetra-methylbenzidine) from a colorless solution. It was then quantified by spectrophotometry and reflected the relative amount of Rho-kinase activity on phosphorylation of MYPT in pulmonary arterial SMC.

Statistical analysis

Results are presented as mean \pm SEM. The software SPSS-version 13.0 was used for all statistical analysis. Comparisons between groups for characteristics were performed with the unpaired Student's T-test. Comparisons of dose-response curves for relaxation response were analyzed by ANOVA for repeated measure. Values of $p < 0.05$ was considered statistically significant.

RESULTS

Baseline clinical and functional characteristics

All 16 subjects (8 non smokers and 8 smokers) had normal lung function test. Their clinical and functional characteristics are shown in Table 1.

Endothelium-dependent relaxation in response to ACh

Endothelium-dependent pulmonary arterial relaxation in response to ACh (10^{-9} - 10^{-4} mol/L) was significantly reduced in smokers as compared with controls ($p < 0.001$; Figure 1).

eNOS protein expression (immunostaining and western blotting)

Semi-quantitative analysis for positive peroxidase staining and protein density of eNOS expression by western blotting in pulmonary arteries showed no significant difference between smokers and non-smoker subjects ($p > 0.05$; Figure 2a).

Concentration of nitrate and cGMP

Nitrate concentration in homogenates of pulmonary arterial in smokers was significantly lower than that in nonsmokers ($p < 0.01$). Similarly, the concentration of cGMP obtained from pulmonary arterial homogenates of smokers was significantly lower as compared with nonsmokers ($p < 0.01$; Figure 2b).

ROCK-1 and ROCK-2 immunostaining and protein expression

Peroxidase staining scores and protein densities showed that expression of ROCK-1 (Figure 3a) and ROCK-2 (Figure 3b) in pulmonary arterial of smokers were significantly higher than nonsmokers ($p < 0.01$).

GTP-RhoA and total RhoA protein expression

Western blot analysis of total RhoA protein in pulmonary arteries showed no difference between the 2 groups ($p > 0.05$, Figure 4). Results of pull-down assay showed GTP-RhoA protein expression in smokers was significantly higher than nonsmokers ($p < 0.01$; Figure 4). Activity of RhoA in pulmonary arteries, as assessed by the ratio of GTP-RhoA/total RhoA, was significantly higher in smokers as compared with non-smoker subjects ($p < 0.01$; Figure 4).

p-MYPT-1 protein expression

p-MYPT1 protein density measured by Western blot, was significantly increased in smokers in comparison with nonsmokers ($p < 0.01$; Figure 5). The concentration of p-MYPT-1, quantified by Elisa, showed it was higher in smokers as compared with nonsmokers ($p < 0.001$; Figure 5).

DISCUSSION

The deleterious effect of cigarette smoke on endothelial function has already been reported in the systemic circulation of asymptomatic smokers [6, 7, 11]. Only few reports have to date investigated the underlying mechanisms of pulmonary endothelial dysfunction in smokers with mild COPD [9, 10] or normal lung function [19]. The main results of the present study, assessing pulmonary endothelial function of smokers with normal lung function as compared with nonsmokers, can be summarized as follows. (1) Pulmonary endothelium-dependent relaxation in response to ACh was significantly impaired in smokers; (2) this

impairment was associated with reduced activity but not the expression of eNOS together with (3) increased activity and expression of the Rho kinases (ROCK-1 and ROCK-2).

If the results of the present study showing significant impairment of pulmonary arterial relaxation in response to ACh in smokers with normal lung function have not yet been reported, several studies have previously suggested the possibility of endothelial dysfunction in both the systemic [6, 7, 11] and pulmonary circulation [10] of healthy smokers. This observation is consistent with the hypothesis that pulmonary endothelial dysfunction not only occurs in patients with end-stage lung disease [20], but it can also be seen in patients with milder degree of COPD [5, 9, 10], and in supposedly healthy smokers [10, 19]. As endothelial dysfunction is not restricted to the pulmonary circulation, it is likely that the underlying mechanisms are related to systemic oxidative stress caused by tobacco smoking [21, 22].

Decreased production and/or reduced bio-availability of NO might account for endothelial dysfunction [8, 11, 19]. In the present study, protein expression of eNOS did not significantly differ (Figure 2A), but eNOS activity - as assessed by nitrates measurement - and the concentration of cGMP (Figure 2B) were significantly reduced in smokers as compared with nonsmokers. Thus, impairment of relaxation is unlikely due to reduced eNOS expression but rather to impaired eNOS activity (nitrate production) and NO bio-availability (cGMP concentration) in pulmonary arteries from smokers. Results from the present study differed from previous reports showing impaired eNOS expression in pulmonary arteries from healthy smokers [19]. The reduced activity of eNOS found in the present study, however, is consistent with all previous findings showing impaired NO mediated-pulmonary relaxation in smokers either without [19], moderate [9, 10] or severe [20] lung disease.

The role of the RhoA/Rho-kinase pathway in causing impairment of endothelium-dependent relaxation has been recently demonstrated in the systemic circulation of smokers [12]. Augmented Rho-kinases activity in peripheral leukocyte is related to impaired flow-mediated vasodilatation [23] and increased aortic stiffness [24] in cigarette smokers. There is

mounting evidence suggesting important and complex cross-talks between RhoA/Rho-kinase and the NO/cGMP pathways. Rho-kinases can downregulate eNOS expression and activity, and NO bioavailability [25], whereas eNOS mRNA stability is increased by Rho-kinase inhibitors [26]. The latter can furthermore negate the inhibition of Rho-kinases on Akt, a protein kinase that stimulates eNOS activity through a calcium-independent pathway, thus leading to increase in NO production [27, 28].

Together with protein expression of Rho-kinases, the active form of RhoA (RhoA-GTP) and the activity of Rho-kinase (as assessed by measurement of the phosphorylated form of MYPT-1) have also been evaluated in the present study. The results showed that impairment of endothelium-dependent relaxation in smokers was associated with significant increase of expression of RhoA-GTP (Figure 4) and p-MYPT-1 (Figure 5). As the phosphorylation of MYPT-1 caused by Rho-kinases results in the inhibition of MPT, thereby sustaining actin-myosin interaction and prolongs preexisting vasoconstriction [13], it is conceivable that increased RhoA/Rho-kinase activity further aggravates the imbalance between pulmonary vasoconstrictors and dilators towards increased vascular tone in the pulmonary circulation of healthy smokers. Consistent with the central role of Rho-kinases in the modulation of pulmonary vascular tone is the recent demonstration that Fasudil, a Rho-kinase inhibitor significantly improved experimental pulmonary arterial hypertension to a larger extent than endothelin receptor antagonists and inhibitors of phosphodiesterase 5 [29].

CONCLUSION

Limitations of the present study include the relative small number of patients with different male/female ratios in each study group and the lack of assessment of endothelium-dependent relaxation in the presence of the Rho-kinase inhibitors. Our study has nevertheless provided the evidence linking pulmonary endothelial dysfunction in smokers with normal lung function and to the reduction of eNOS activity and related to the upregulation of

RhoA/Rho-kinase pathway. The interaction between RhoA/Rho-kinase pathway and eNOS/NO signalization in smokers should be investigated.

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Table 1. Patients' Clinical and Functional Characteristics

Variables	Nonsmokers (n = 8)	Smokers (n = 8)
Age, years	65 ± 11	55 ± 8
Male/female	1/7	7/1
Tobacco, pack-year		37 ± 11
FEV ₁ , liters	2.47 ± 0.49	2.81 ± 0.55
FEV ₁ , % pred	110 ± 26	96 ± 5
FEV ₁ /FVC, % pred	81 ± 5	78 ± 4
PaO ₂ , mmHg	86 ± 8	83 ± 9
PaCO ₂ , mmHg	36 ± 4	39 ± 4

FEV₁: forced expiratory volume in one second; FVC: forced vital capacity.

Figure 1. Relaxation endothelium-dependent of pulmonary arterial rings.

Endothelium-dependent relaxation in response to acetylcholine (ACh) of pulmonary arterial rings from nonsmokers and smokers with normal lung function. Values are presented as means \pm SEM. n=8 for each group.

Figure 1.

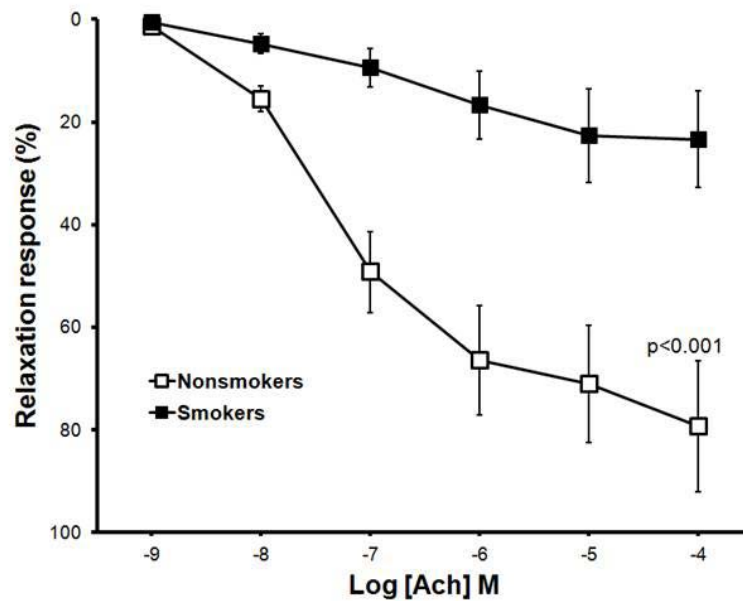


Figure 2.

Figure 2a. Expression of eNOS in nonsmokers and smokers.

eNOS protein expression measured by immunohistochemistry and Western blot in pulmonary arteries of nonsmokers and smokers. Protein density is presented as eNOS/ β -actin ratio. On the top, endothelial pulmonary arteries stained with eNOS (magnification x 10; left) and samples of eNOS protein expression measured by Western blot (right). NS: no significant difference. n=8 for each group.

Figure 2a.

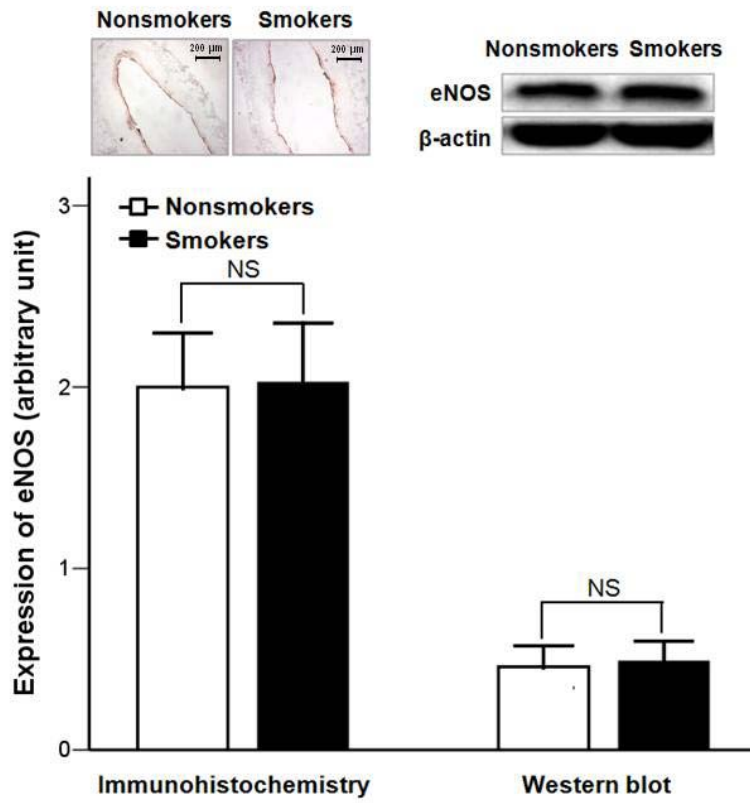


Figure 2b. Concentration of nitrate and cGMP.

Concentration of nitrate and cGMP measured by Elisa from pulmonary arterial homogenates of nonsmokers and smokers. Values are presented as means \pm SEM. n=8 for each group.

Figure 2b.

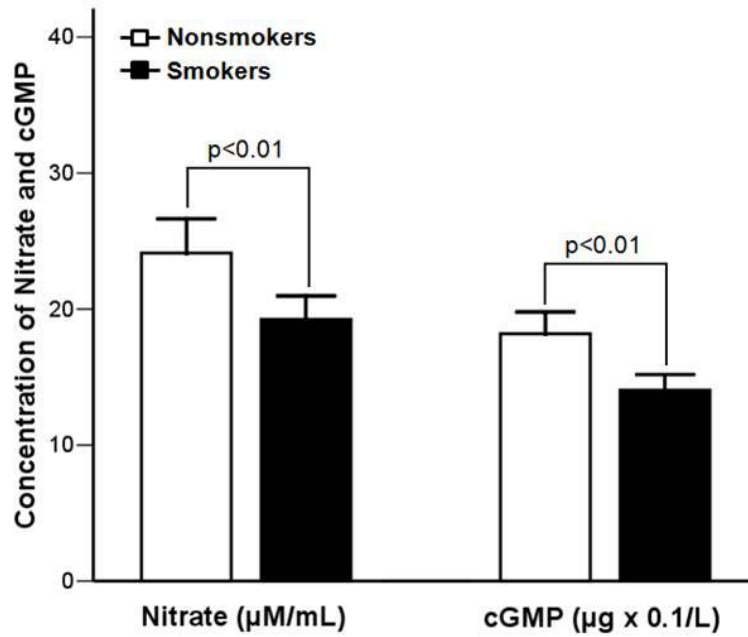


Figure 3.

Figure 3a. Expression of ROCK-1 in nonsmokers and smokers.

ROCK-1 protein expression measured by immunohistochemistry (left; magnification x 5) and Western blot from pulmonary arteries of nonsmokers and smokers (right). Protein density is presented as ROCK-1/ β -actin ratio. On the top, smooth muscle cells of pulmonary arteries stained with ROCK-1 (magnification x 5; left) and samples of ROCK-1 protein expression measured by Western blot (right). n=8 for each group.

Figure 3a.

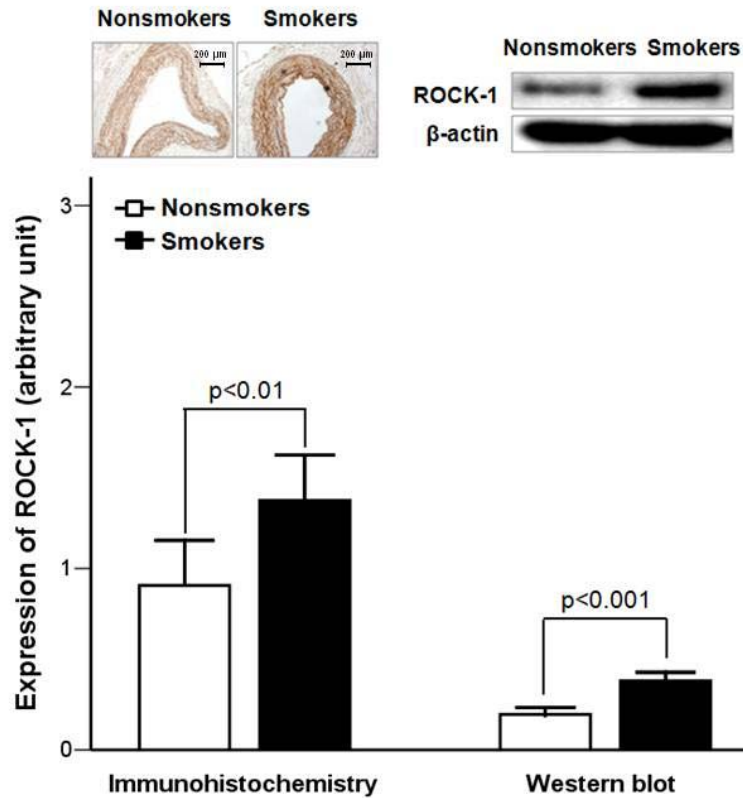


Figure 3b. Expression of ROCK-2 in nonsmokers and smokers.

ROCK-2 protein expression measured by immunohistochemistry (left; magnification x 5) and Western blot from pulmonary arteries of nonsmokers and smokers (right). Protein density is presented as ROCK-2/β-actin ratio. On the top, smooth muscle cells of pulmonary arteries stained with ROCK-2 (magnification x 5; left) and samples of ROCK-2 protein expression measured by Western blot (right). n=8 for each group.

Figure 3b.

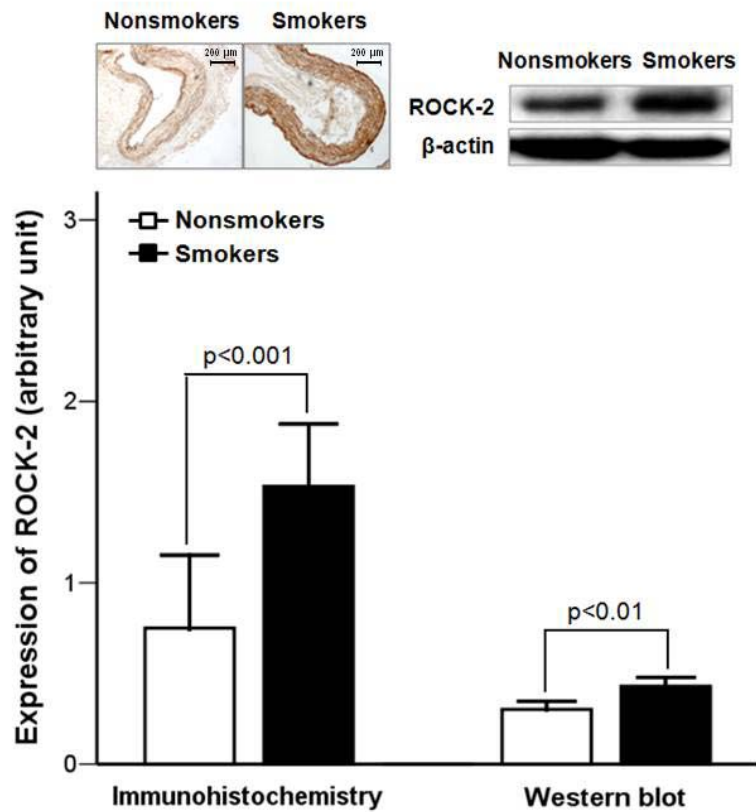


Figure 4. Expression of GTP-RhoA and RhoA in nonsmokers and smokers.

Protein expression of GTP-RhoA measured by pull-down assay and RhoA measured by Western blot from pulmonary arterial homogenates of nonsmokers and smokers. Protein density is presented as GTP-RhoA/β-actin and RhoA/β-actin ratio. GTP-RhoA/total RhoA presented as RhoA activity. On the top, samples of GTP-RhoA and RhoA protein expression. NS: no significant difference. n=8 for each group.

Figure 4.

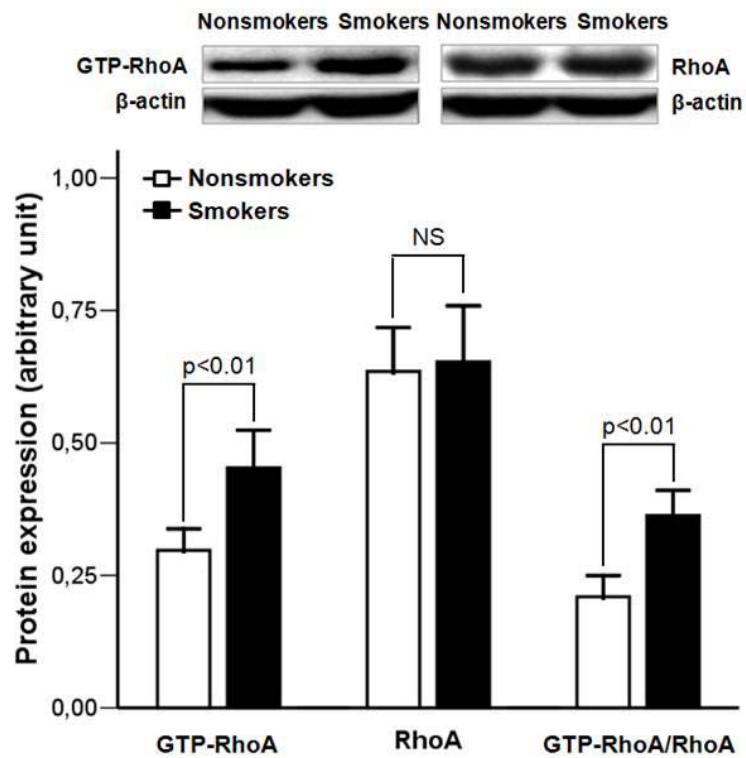


Figure 5. Expression of p-MYPT-1 in nonsmokers and smokers.

p-MYPT-1 protein expression measured by Western blot and Elisa from pulmonary arterial homogenates of nonsmokers and smokers. Protein density measured by Western blot is presented as p-MYPT-1/ β -actin. On the top, samples of p-MYPT-1 protein expression measured by Western blot. n=8 for each group.

Figure 5.

