Cigarette smoke extract-induced p120-mediated NF-κB activation in human epithelial cells is dependent on the RhoA/ROCK pathway

Chao Zhang*, Shenghui Qin*, Lingzhi Qin, Liwei Liu, Wenjia Sun, Xiyu Li, Naping Li, Renliang Wu & Xi Wang

Cigarette smoke exposure is a major cause of chronic obstructive pulmonary disease (COPD), but the underlying molecular inflammatory mechanisms remain poorly understood. Previous studies have found that smoke disrupts cell-cell adhesion by inducing epithelial barrier damage to the adherens junction proteins, primarily E-cadherin (E-cad) and p120-catenin (p120). Recently, the anti-inflammatory role of p120 has drawn increasing attention. In this study, we demonstrate that p120 has a role in the cigarette smoke extract-induced inflammatory response, presumably by regulating NF-κB signaling activation. Mechanistically, we show that p120-mediated NF-κB signaling activation in airway epithelial inflammation is partially RhoA dependent and is independent of E-cad. These results provide novel evidence for the role of p120 in the anti-inflammatory response.
ROCK pathway might regulate NF-κB activity to up-regulate inflammatory genes and mediate the development of diabetic nephropathy. The blockade of ROCK inhibits the activation of NF-κB and the production of proinflammatory cytokines, suggesting a critical role for ROCK in the synovial inflammation of rheumatoid arthritis. Rho kinase is upstream of NF-κB in driving LPA-mediated adhesion molecule expression. As noted above, p120 and RhoA/ROCK are shown to be involved in inflammation and inflammation-related diseases; therefore, we hypothesize that p120 might also play an important role in cigarette smoke extract (CSE)-induced airway injury through the NF-κB signaling pathway via the RhoA/ROCK axis.

Results

p120 expression was down-regulated after exposure to CSE at different concentrations and time points. To investigate the effects of CSE on cell viability, a cytotoxicity assay was performed after 16HBE cells were exposed to different concentrations of CSE for 24 h. The MTT analysis revealed a dose-dependent reduction in cell viability. The cells remained approximately 80% viable at 15% CSE and had only approximately 40% viability at 20% CSE (Fig. 1A). Hence, six concentrations of CSE (0%, 1%, 5%, 10%, 15%, 20%) were chosen to study the effects of CSE on p120 expression in epithelial cells. After exposure to different concentrations of CSE for 24 h, a western blot showed that the expression of p120 gradually decreased as the concentration of CSE increased, and this effect was most obvious at 15% (Fig. 1B). Therefore, 15% CSE, which was the maximum concentration that did not cause a significant change in cell viability, was chosen for the following experiments.

After exposure to 15% CSE, a western blot revealed that p120 expression was reduced in a time-dependent manner (Fig. 1C). An RT-PCR analysis also indicated that p120 mRNA decreased in a time-dependent manner (Fig. 1D).

NF-κB signaling pathway was activated after CSE stimulation in 16HBE cells. The activation of NF-κB was assessed in 16HBE cells after exposure to 15% CSE at different time points. First, we found that the NF-κB static binding protein IκBα decreased (Fig. 2A), which was accompanied by an increase in p-IκBα (Fig. 2B) after the cells were exposed to 15% CSE for 12 h. Next, immunofluorescence and nuclear/cytoplasmic protein extraction experiments were used to directly confirm the NF-κB-p65 activation. We found obvious p65 translocation into the nucleus and a decrease in cytoplasmic p65 (Fig. 2C,D, Supplementary Fig. S1). We also found a dramatic increase in proinflammatory cytokines at both the mRNA (Fig. 2E) and the protein levels (Fig. 2F) after CSE exposure.

CSE-induced NF-κB activation was inhibited by p120 3A over-expression in 16HBE cells. To investigate the effects of p120 on the activation of NF-κB signaling by smoke, we transfected the plasmid RcCMVp120 3A into 16 HBE cells to observe the effects on NF-κB activity. The transfection efficiency was evaluated by western blot (Fig. 3A, Supplementary Fig. S2A). The over-expression of p120 3A had no obvious effect on the total NF-κB-p65 expression regardless of CSE exposure (Fig. 3A,B, Supplementary Fig. S2). However, using ELISA, we found that the phosphorylation of NF-κB-p65 (Ser536) was attenuated after 6 h of CSE exposure in the p120 3A over-expression group (Fig. 3C). Moreover, RT-PCR showed that IL-8 mRNA was down-regulated in the p120 3A over-expression group after CSE exposure for different time points (Fig. 3D). Additionally, the secretion of IL-8, IL-1β and IL-6 was decreased in the p120 3A over-expression group after CSE exposure for different time points as measured by ELISA (Fig. 3E–G).

Knockdown of p120 enhanced CSE-induced NF-κB activation in 16HBE cells. We further examined NF-κB activation after p120 knockdown in 16HBE cells. A western blot showed that p120 expression was significantly knocked down by siRNA (Fig. 4A, Supplementary Fig. S3A). The total NF-κB-p65 was not changed in both the scrambled and the p120-knockdown group after CSE exposure (Fig. 4A,B, Supplementary Fig. S3), but phospho-NF-κB-p65 (Ser536) was increased significantly in the p120-knockdown group after 6h of exposure (Fig. 4C) compared with the scrambled group. At the same time, RT-PCR showed an increased up-regulation of IL-8 in the p120-knockdown group after CSE exposure for different time points (Fig. 4D). Meanwhile, the up-regulation of IL-8, IL-1β and IL-6 was enhanced in the p120-knockdown group after CSE exposure for different time points as measured by ELISA (Fig. 4E–G).

CSE-induced NF-κB activation was regulated through the RhoA/ROCK axis in 16HBE cells. The RhoA/ROCK pathway might regulate NF-κB activity to up-regulate inflammatory genes and mediate the development of diabetic nephropathy. Therefore, we explored whether the RhoA/ROCK axis can also regulate NF-κB activation during airway inflammation in 16HBE cells. By western blot, we found the levels of total RhoA had not changed (Fig. 5A), but G-LISA showed that RhoA activity dramatically increased after 4.5 h of CSE exposure (Fig. 5B). Moreover, ROCK1, a protein downstream of RhoA, increased after 15% CSE exposure (Fig. 5C).

The ROCK inhibitor Y27632 was used to further investigate the association between RhoA and NF-κB. Before CSE exposure, the cells were pretreated with 20 μM Y27632 for 12 h to inhibit the RhoA pathway. Immunofluorescence staining revealed that NF-κB-p65 nuclear translocation decreased significantly after 6h of CSE stimulation (Fig. 5D), and the ELISA results also showed that the phosphorylation of NF-κB-p65 (Ser536) decreased in the Y27632 group (Fig. 5E).

p120-regulated NF-κB activation occurs partially through the RhoA/ROCK axis in human bronchial epithelial cells after CSE exposure. We first detected RhoA activity in p120-knockdown 16 HBE cells. Although the western blot revealed no obvious change in the total RhoA (Fig. 4A, Supplementary Fig. S3A), the G-LISA showed that RhoA activity was sharply up-regulated in the p120-knockdown group compared with
the scrambled group after 15% CSE exposure (Fig. 6A). Moreover, ROCK1 increased in the p120-knockdown group (Fig. 4B, Supplementary Fig. S3B).

On the other hand, we observed that the over-expression of p120 influenced RhoA activity. The western blot revealed that both RhoA and ROCK1 were decreased in the p120 3A over-expressed groups compared with the MT groups after 15% CSE exposure (Fig. 3B, Supplementary Fig. S2B). The G-LISA showed that RhoA activity was down-regulated in the p120 3A over-expression group after 15% CSE exposure (Fig. 6B). Furthermore, we found RhoA and p120 could combine with each other in 16HBE cells by coimmunoprecipitation (Fig. 6C).

Finally, to further assess whether p120 might regulate airway inflammation via NF-κB upstream of the RhoA pathway, we conducted rescue experiments by transfecting 16HBE cells with a p120 mutant lacking the putative Rho GTPase regulatory domain (p120ΔRRD-GFP). The western blot demonstrated that the over-expression of p120ΔRRD-GFP had no effect on the expression of RhoA and NF-κB-p65 (Fig. 6D,E, Supplementary Fig. S4). The G-LISA showed that RhoA activity was not affected by the over-expression of p120ΔRRD-GFP (Fig. 6F). ELISA showed that the level of phosphorylation of NF-κB-p65 (Ser536) did not differ from that of the MT group.
These results demonstrated that p120-mediated NF-κB signaling activation in airway epithelial inflammation is at least partially RhoA dependent.

CSE-induced p120-mediated NF-κB signaling is not E-cadherin dependent. Many studies have reported that E-cadherin participates in RhoA regulation of NF-κB; thus, E-cadherin attracted our attention. Firstly, the western blot revealed that, after 15% CSE exposure, the expression of E-cadherin gradually decreased with the increased exposure time (Fig. 7A). Next, the plasmid p120ΔEcad-GFP, which cannot combine with E-cadherin, was transfected into the 16HBE cells. The western blot showed that the expression of RhoA and NF-κB-p65 did not change when p120ΔEcad-GFP was over-expressed in the No-CSE group (Fig. 7B, Supplementary Fig. S5A). However, RhoA expression was inhibited in the CSE exposure group (Fig. 7C, Supplementary Fig. S5B), and RhoA activity was also inhibited by the over-expression of p120ΔEcad-GFP as
measured by G-LISA (Fig. 7D). The phosphorylation of NF-κB-p65 (Ser536) was attenuated after 6 h of CSE exposure in the p120ΔEcad-GFP over-expression group as measured by ELISA (Fig. 7E).

**Discussion**

In the present study, we demonstrated that p120 plays a role in the cigarette smoke-induced inflammatory response presumably by regulating the RhoA-NF-κB axis. Using a human bronchial epithelial cell line as a model for the cigarette smoking-induced airway inflammatory response, we demonstrated that p120 and E-cad were both decreased in CSE-exposed BECs. Additionally, NF-κB was activated, and the expression of IL-8, IL-6 and IL-1β was also increased. Transfection with the plasmid p120 3A or with p120 siRNA confirmed that p120 regulated NF-κB signaling in CSE-induced airway inflammation. Most importantly, we found that this regulation may be at least partially occurring in a RhoA/ROCK-dependent manner. p120 directly co-precipitates with RhoA in human bronchial epithelial cells. The over-expression of p120 3A inhibited RhoA activity but p120ΔRRD-GFP did not, and the silencing of p120 expression by siRNA activated RhoA. The RhoA/ROCK signaling inhibitor Y27632 greatly inhibited NF-κB-p65 (Ser536) phosphorylation and its subsequent nuclear translocation. The over-expression of mutant p120ΔEcad-GFP also inhibited both RhoA expression and activity and the phosphorylation of NF-κB-p65 (Ser536). These results demonstrate that p120-mediated NF-κB signaling activation in airway epithelial inflammation is partially RhoA dependent but not E-cad dependent.
Cigarette smoke is a major risk factor for COPD and other lung diseases, and it induces sustained NF-κB nuclear translocation, the production of inflammatory mediators, and mucus cell hypersecretion. Cigarette smoke also reduces epithelial integrity and repair, causing further progression of the COPD symptoms. The present study confirmed that CSE reduced the expression of the adhesion molecules p120 and E-cad in 16HBE cells. Moreover, CSE-induced NF-κB activation was accompanied by IκBα phosphorylation and IL-8, IL-6 and IL-1β production. Cigarette smoke exposure activates NF-κB signaling and leads to lung inflammation, but its underlying molecular mechanism remains poorly understood. Erk1/2 is involved in regulating NF-κB signaling in mice lungs that are exposed to cigarette smoke. In addition, the RAGE-Ras–NF-kB axis has been reported to likely be associated with several smoking-related inflammatory lung diseases. CSE disrupts the AJs by down-regulating p120, which modulates smoke-induced cell migration via the EGFR/Src-pathway. In our study, p120 may represent a potentially novel upstream regulator of the NF-κB pathway in CSE-exposed airway inflammation.

The depletion of p120 in conditional knockout mice exhibits an inflammatory response in the intestines and epidermis as evidenced by tissue immune cell infiltration and proinflammatory cytokine release. Consistent with these observations, our study provided clear evidence that p120 was down-regulated in a dose- and time-dependent manner by CSE exposure, and p120 regulated the NF-κB signaling pathway in the CSE-induced airway inflammatory response. p120 3A over-expression attenuated the CSE-stimulated phosphorylation of NF-κB-p65 (Ser536) and IL-8 mRNA expression and protein synthesis. On the contrary, p120 siRNA significantly enhanced the CSE-stimulated phosphorylation of NF-κB-p65 (Ser536) and IL-8 expression. Owing to the

Figure 4. Knockdown of p120 enhances CSE-induced NF-κB activation in 16HBE cells. The cells were transfected with a p120 siRNA or a scrambled siRNA sequence for 48 h. (A) p120, NF-κB-p65 and RhoA expression were analyzed by western blot. β-actin was the loading control. The quantified results can be found as Supplementary Fig. S3A. (B) After 6 h of CSE exposure, the cells were analyzed by western blot to detect p120, ROCK1, NF-κB-p65 and RhoA. β-actin was the loading control. The quantified results can be found as Supplementary Fig. S3B. (C) After 6 h of CSE exposure, the cells were measured to assess the phosphorylation of NF-κB-p65 (Ser536) by ELISA. The data are expressed as the means ± SD (n = 3), **P < 0.01 vs. the Scrambled group. (D) IL-8 mRNA was measured by RT-PCR. The data are expressed as the means ± SD (n = 3), ***P < 0.001 vs. the Scrambled + CSE groups. (E–G) IL-8, IL-1β and IL-6 protein was examined by ELISA. The data are expressed as the means ± SD (n = 3), ***P < 0.001 vs. the Scrambled + CSE groups, ###P < 0.001 vs. the CSE 0 h group.
alternative splicing, p120 mRNAs exhibit many isoforms. In humans, this splicing produces four different p120 isoforms. Isoform 1 and 3 are the most commonly expressed. Different isoforms display tissue- or cell-specific expression patterns. Isoform 1 is predominantly expressed in highly motile cells. In contrast, isoform 3, whose molecular weight 100 kD, is the most abundant in epithelial cells. In our study, only p120 isoform 3 was detected in 16HBE cells and was found regulate the NF-κB activity and the expression of IL-8 after CSE exposure. This finding may to some extent be agreed with Zhang et al. Our results illuminate the function of p120 3A in the inflammatory process.

In our study, GTP-RhoA increased after 3 h of CSE exposure compared with other time points by G-LISA, while the total RhoA protein in the 16HBE cells showed no difference after CSE expose at different time points. All of the p120 isoforms can bind RhoA in vitro via a central RhoA binding site. However, only the cooperative binding of RhoA to both the central p120 domain and the alternatively spliced p120 N terminus can stabilize RhoA binding and inhibit RhoA activity. Our study showed RhoA was the binding partner of p120 3A and over-expression of p120 3A inhibited RhoA expression and activity in 16HBE cells when treated with CSE. Yanagisawa reported that the existence of the N-102-234 and N-622-628 amino acid residues of p120 may be responsible for the binding to RhoA. The over-expressed p120ΔRRD-GFP that lacks its Rho GTPase-binding domain, which consists of the N-622-628 amino acid residues, did not suppress CSE-induced activation of RhoA and NF-κB. This result suggests that the interaction between p120 and RhoA is necessary to prevent RhoA from activating NF-κB.

Smoke caused a time-dependent dissociation of the junctional p120/E-cad/β-ctn complexes with rapid translocation and degradation of E-cadherin. CSE reduces barrier function and the expression of the adhesion molecule E-cadherin, specifically at the cell membrane, in 16HBE cells. Kuphal, S. et al. reported that the loss of E-cadherin leads to the up-regulation of NF-κB activity in malignant melanoma. Our results confirmed that...
Figure 6. p120-mediated NF-κB activation occurs partially through the RhoA/ROCK axis in human bronchial epithelial cells after CSE exposure. (A) RhoA activity in the scrambled and p120 siRNA groups after 15% CSE exposure was assessed by G-LISA. The data are expressed as the means ± SD (n = 3), *P < 0.05 vs. Scrambled + CSE group. (B) RhoA activity in the MT and the p120 3A over-expression groups after 15% CSE exposure was assessed by G-LISA. The data are expressed as the means ± SD (n = 3), *P < 0.05 vs. the MT + CSE group. (C) Co-immunoprecipitation was performed to observe the relationship between p120 and RhoA, normal goat serum was used as negative control. (D) The cells were transiently transfected with p120ΔRRD-GFP or MT for 48 h. Whole-cell protein was extracted and analyzed by western blot to detect p120, NF-κB-p65 and RhoA, β-actin was the loading control. The quantified results can be found as Supplementary Fig. S4A. (E) The cell lysates from the MT and the p120ΔRRD-GFP over-expression groups after 6 h of CSE exposure were analyzed by western blot to detect p120, NF-κB-p65 and RhoA 48 h after transfection, β-actin was the loading control. The quantified results can be found as Supplementary Fig. S4B. (F) The G-LISA analysis was used to detect the active RhoA after the 16HBE cells were exposed to 15% CSE in the MT and the p120ΔRRD-GFP over-expression groups. The data are expressed as the means ± SD (n = 3). (G) After CSE exposure, the cell lysates from the MT and the p120ΔRRD-GFP over-expression groups were measured to detect the phosphorylation of NF-κB-p65 (Ser 536) by ELISA. The data were expressed as the means ± SD.
E-cadherin was decreased in a time-dependent manner after CSE exposure in 16HBE cells. Using a mutant p120ΔEcad-GFP that cannot interact with E-cadherin, we found that the over-expression of this mutant p120ΔEcad-GFP decreased E-cadherin expression. The cell lysates were analyzed by western blot to detect E-cadherin. β-actin was the loading control. The quantified results can be found as Supplementary Fig. S5A. The cell lysates from the MT and the p120ΔEcad-GFP groups after 6 h of CSE exposure were analyzed by western blot to detect p120, NF-κB-p65 and RhoA. β-actin was the loading control. The quantified results can be found as Supplementary Fig. S5B. The G-LISA results showed that RhoA activity was down-regulated in the p120ΔEcad-GFP over-expression groups compared with the MT group after 15% CSE exposure. The data are expressed as the means ± SD (n = 6), *P < 0.05 vs. the MT + CSE group. After 15% CSE exposure, the cell lysates from the MT and the p120ΔEcad-GFP groups were measured to detect the phosphorylation of NF-κB-p65 (Ser 536) by ELISA. The data were expressed as the means ± SD (n = 3), *P < 0.05 vs. MT + CSE group.

Figure 7. CSE-induced p120-mediated NF-κB signaling is not E-cadherin dependent. (A) E-cad was decreased after the 16HBE cells were exposed to CSE. The cell lysates were analyzed by western blot to detect E-cad. β-actin was the loading control. (B) The cells were transiently transfected with p120ΔEcad-GFP or MT for 48 h. Whole-cell protein was extracted and analyzed by western blot to detect p120, NF-κB-p65 and RhoA. β-actin was the loading control. The quantified results can be found as Supplementary Fig. S5A. (C) The cell lysates from the MT and the p120ΔEcad-GFP groups after 6 h of CSE exposure were analyzed by western blot to detect p120, NF-κB-p65 and RhoA 48 h after transfection. β-actin was the loading control. The quantified results can be found as Supplementary Fig. S5B. (D) The G-LISA results showed that RhoA activity was down-regulated in the p120ΔEcad-GFP over-expression groups compared with the MT group after 15% CSE exposure. The data are expressed as the means ± SD (n = 6), *P < 0.05 vs. the MT + CSE group. (E) After 15% CSE exposure, the cell lysates from the MT and the p120ΔEcad-GFP groups were measured to detect the phosphorylation of NF-κB-p65 (Ser 536) by ELISA. The data were expressed as the means ± SD (n = 3), *P < 0.05 vs. MT + CSE group.
could still down-regulate CSE-induced NF-κB activation. Moreover, this over-expression also inhibited the activity of RhoA. Thus, this result suggests that the role of p120 in modulating the RhoA-NF-κB pathway in airway epithelial inflammation was independent of E-cadherin.

Perez-Moreno, M. et al. reported that p120 affects NF-κB activation and immune homeostasis is partially through regulation of RhoA in the skin inflammation. In addition, previous studies showed that p120 was involved in inflammatory responses in a RhoA dependent manner in acute pneumonia caused by LPS and mechanical scratch caused inflammatory response. Why p120-mediated NF-κB signaling activation in airway epithelial inflammation is partially RhoA dependent but not E-cad dependent? To our knowledge, RhoA is the downstream of p120 in the regulation of NF-κB signaling pathway, p120 combines with E-cadherin in the cell membrane, while p120 and RhoA are co-located in the cytoplasm. A mutant p120ΔEcad-GFP could still inhibit NF-κB activation and RhoA activity, suggesting this process may occur in the cytoplasm, not in the membrane. In this situation, p120 may function as a GDI, and the binding of p120 to RhoA prevents the GEFs from switching RhoA to the GTP state independent of the binding of p120 with E-cadherin.

In summary, our data confirmed that the down-regulation of p120 can activate the NF-κB signaling pathway in CSE-exposed human bronchial epithelial cells. This process may be partially mediated in a RhoA-dependent manner. p120 directly co-precipitates with RhoA, and its down-regulation activates RhoA, which then leads to IκBα degradation and NF-κB-p65 translocation, implying that p120 has an effect on NF-κB signaling in CSE-exposure induced airway inflammation. The study of this mechanism of airway inflammation is useful because it may lead to the development of novel therapeutic strategies for a number of airway inflammatory diseases.

Methods

Preparation of CSE and inhibitor treatment. CSE was prepared as previously described. In short, three cigarettes without filters were combusted with a modified syringe-driven apparatus. The smoke was bubbled through 25 mL of medium (100% CSE). The extract was prepared freshly and sterilized using a 0.22 mm filter and used within 30 min.

Y27632, an inhibitor of ROCK, was obtained from Sigma (St. Louis, MO, USA). The cells were starved in basal medium without growth factors for 6 h and pretreated with the above inhibitors for 12 h followed by incubation in CSE-free or CSE medium as indicated in the text.

Cell cultures, CSE treatment, and cytotoxicity assay. The 16HBE cells, which are a simian virus 40 large T antigen transformed human bronchial epithelial cell line that retains the differentiated morphology and function of normal human bronchial epithelia, were a kind gift from Dr. D. Gruenert (California Pacific Medical Center, CA, USA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, GibcoBRL, Paisley, UK) and supplemented with 10% heat-inactivated fetal bovine serum (FBS, GibcoBRL) and antibiotics of 1% streptomycin and penicillin at 37 °C in 5% CO₂. The cells were cultured in the presence and absence of CSE (1%, 5%, 10%, 15% and 20%) for different time points.

To investigate the effects of CSE on cell activity, the 16HBE cells were exposed to CSE (0%, 10%, 20%, 30%, 40% and 50%) for 24 h. The cytotoxicity assay has been described previously. When the cells reached 80–90% confluence, they were rinsed two times with PBS and incubated in serum-free culture medium with or without the different concentrations of CSE (according to the MTT assay).

Immunofluorescent analysis. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and incubated with primary antibodies at 4 °C overnight. The antibodies directed against NF-κB-p65 were obtained from Cell Signaling Technology (San Jose, CA, USA) and used according to the manufacturer’s instructions. The nuclei were stained with DAPI. The images were photographed with an Eclipse FV500 confocal laser scanning microscopy system (Olympus, Tokyo, Japan) with the appropriate filter sets.

Enzyme-linked immune sorbent assay. After stimulation, the cell lysate supernatants were collected and measured for IL-8, IL-6, IL-1β and NF-κB-p65 (Ser536) by ELISA using a commercial kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s protocol. The absorbance corresponding to the phosphorylation of NF-κB-p65 (Ser536) was measured at 450 nm, and the concentrations of IL-8, IL-6 and IL-1β were determined using an internal standard curve.

Quantitative real-time PCR. Total RNA was extracted with the TRIzol Reagent (Invitrogen Carlsbad, CA, USA), and the concentration was measured using an ultraviolet (UV) spectrophotometer (UV-1201; Shimadzu Corporation, Kyoto, Japan). Reverse transcription (RT) was performed as described previously. Real-time PCR was conducted using the SYBR-Green PCR kit (Takara, Osaka, Japan) in a Rotor-Gene 3000 machine (Corbett Life Science, Sydney, Australia). The quantitative analysis of the transcription of p120 and IL-8 was described previously. Each reaction was performed in a 25 μL volume containing 2 μL of cDNA, 0.5 μL of 10 μM per each primer, and 12.5 μL of the 2 × SYBR-Green mixture. p120: For: 5′-GGA CAC CCT CTT ACC CTC G-3’, Rev: 5′-GCT TGC TAA ACT TCC TCG CTC-3’, product of 122bp. IL-8: For: 5′- AGA CTG GCC CAA CAC AGA AAT TA-3’, Rev: 5′-TTT GCT TGA AGT TTC ACT GTC ATG-3’, product of 185bp.

Coimmunoprecipitation and Western blot analysis. Coimmunoprecipitation, cytoplasmic and nuclear extracts, and Western blot analysis were performed as described previously.

RNA interference. The human p120 small interfering RNA (p120 siRNA) oligonucleotide was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The cells were seeded onto a 6-well plate at 30%
confluence in complete medium and transfected with p120 siRNA using Lipofectamine 2000 according to the manufacturer's recommended procedure. The nonsilencing siRNA (scrambled) was used as the control.

**Plasmids and transient transfection.** The plasmid RecMV mp120-3A was generously provided by Professor Enhua Wang. The plasmids p120ΔRRD-GFP and p120ΔEcad-GFP were generously provided by Professor Mirna Perez-Moreno. To achieve a transient gene transfection, the cells were used in the exponential phase of growth, and the transfection was performed using Lipofectamine 2000 according to the manufacturer's recommendation and the method described by Tucker et al. with minor modifications.

**RhoA activation assay.** The RhoA G-LISA® kit was purchased from Cytoskeleton Inc. (Cat. #BK124; Denver, CO, USA). According to the protocol, the 16HBE cells were collected in ice-cold cell lysis buffer and immediately snap frozen in liquid nitrogen and stored at −80 °C to minimize GTP hydrolysis. An aliquot was set aside for protein concentration determination using the Precision Red™ Advanced Protein Assay Reagent supplied with the kit. Equal protein aliquots were added to the individual wells in an eight-well strip coated with an appropriate RBD, and the plates were incubated on a cold orbital microplate shaker at 400 rpm at 4 °C for exactly 30 min. The strips were washed and incubated with an anti-RhoA primary antibody followed by an HRP-conjugated secondary antibody, and the HRP detection reagent supplied with the kit was used. The signal was read by measuring the absorbance at 490 nm using a microplate spectrophotometer (SpectraMax 340 Microplate Reader; Molecular Devices). The samples from at least three independent experiments were assayed in triplicate.

**Statistical analysis.** The results are expressed as the means ± SD of experiments repeated at least three independent times. The statistical significance was determined using SPSS 19.0 software. The data were evaluated with one-way analysis of variance (ANOVA) combined with a post hoc analysis (Fisher’s PLSD). A value of p < 0.05 was considered significant.

**References**

1. Sutherland, E. R. & Martin, R. J. Airway inflammation in chronic obstructive pulmonary disease: comparisons with asthma. *J Allergy Clin Immunol* **112**, 819–827; quiz 828, doi: 10.1016/S0091-6749(03)63059-2 (2003).
2. Rusznak, C. et al. Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **23**, 530–536, doi: 10.1165/ajrcmb.23.4.3959 (2000).
3. Beadsmoor, C., Cheow, H. K., Szczepura, K., Ruparelia, P. & Peters, A. M. Healthy passive cigarette smokers have increased pulmonary alveolar permeability. *Nucl Med Commun* **28**, 75–77, doi: 10.1097/MMM.0b013e3280b1e1b6 (2007).
4. Pryor, W. A. & Stone, K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrite, and peroxynitrite. *Ann NY Acad Sci* **686**, 12–27, discussion 17–28 (1993).
5. Rahman, I. & MacNee, W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* **21**, 669–681 (1996).
6. Jeffrey, P. K. Histological features of the airways in asthma and COPD. *Respiration* **59 Suppl 1**, 13–16 (1992).
7. Reynolds, A. B. & Roczniak-Ferguson, A. Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene* **23**, 7947–7956, doi: 10.1038/sj.onc.1208161 (2004).
8. Wang, X., Wu, R., Hao, T. & Chen, F. Effects of cigarette smoke extract on E-cadherin expression in cultured airway epithelial cells. *J Tongji Med Univ* **20**, 32–35 (2000).
9. Zhang, L., Gallup, M., Zlock, L., Finkbeiner, W. & McNamara, N. A. p120-catenin modulates airway epithelial cell migration induced by cigarette smoke. *Biochem Biophys Res Commun* **417**, 49–55, doi: 10.1016/j.bbrc.2011.11.048 (2012).
10. Jiang, H., Zhu, Y., Xu, H., Sun, Y. & Li, Q. Activation of hypoxia-inducible, HIF-1 alpha via nuclear factor-kappaB p65. *Toxicol Lett* **42**, 483–488, doi: 10.1093/abbs/gmp041 (2010).
11. Perez-Moreno, M. et al. p120-catenin mediates inflammatory responses in the skin. *Cell 124*, 631–644, doi: 10.1016/j.cell.2005.11.043 (2006).
12. Perez-Moreno, M., Song, W., Pasolli, H. A., Williams, S. E. & Fuchs, E. Loss of p120 catenin and links to mitotic alterations, inflammation, and skin cancer. *Proc Natl Acad Sci USA* **105**, 15399–15404, doi: 10.1073/pnas.0807301105 (2008).
13. Smalley-Freed, W. G. et al. Adenoma formation following limited ablation of p120-catenin in the mouse intestine. *PLoS One* **6**, e19880, doi: 10.1371/journal.pone.0019880 (2011).
14. Stairs, D. B. et al. Deletion of p120-catenin results in a tumor microenvironment with inflammation and cancer that establishes it as a tumor suppressor gene. *Cancer Cell* **19**, 470–483, doi: 10.1016/j.cccr.2011.02.007 (2011).
15. Wang, Y. L. et al. Innate immune function of the adherens junction protein p120-catenin in endothelial response to endotoxin. *J Immunol* **186**, 3180–3187, doi: 10.4049/jimmunol.1001252 (2011).
16. Wang, M. et al. Involvement of p120 in LPS-induced NF-kappaB activation and IL-8 production in human bronchial epithelial cells. *Toxicol Lett* **195**, 75–81, doi: 10.1016/j.toxlet.2010.02.011 (2010).
17. Qin, L. et al. p120 modules LPS-induced NF-κB activation partially through RhoA in bronchial epithelial cells. *Biomed Res Int* **2014**, 1932340, doi: 10.1155/2014/1932340 (2014).
18. Qin, S. et al. p120-Catenin modulating nuclear factor-kappa B activation is partially RhoA/ROCK-dependent in scratch injury. *Wound Repair and Regeneration* **23**, 231–240, doi: 10.1111/wrr.12270 (2015).
19. Etienne-Manneville, S. & Hall, A. Rho GTPases in cell biology. *Nature* **420**, 629–635, doi: 10.1038/nature01148 (2002).
20. Castano, J. et al. Specific phosphorylation of p120-catenin regulatory domain differently modulates its binding to RhoA. *Mol Cell Biol* **27**, 1745–1757, doi: 10.1128/MCB.01974-06 (2007).
21. Guo, F. et al. GEF-H1-RhoA signaling pathway mediates LPS-induced NF-κB transactivation and IL-8 synthesis in endothelial cells. *Mol Immunol* **50**, 98–107, doi: 10.1016/j.molimm.2011.12.009 (2012).
22. Xie, X. et al. Activation of RhoA/ROCK regulates NF-κB signaling pathway in experimental diabetic nephropathy. *Mol Cell Endocrinol** **369**, 86–97, doi: 10.1016/j.mce.2013.01.007 (2013).
23. He, Y. et al. Antiinflammatory effect of Rho kinase blockade via inhibition of NF-kappaB activation in rheumatoid arthritis. *Arthritis Rheum* **58**, 3366–3376, doi: 10.1002/art.23986 (2008).
24. Shimada, H. & Rajagopalan, L. E. Rho kinase-2 activation in human endothelial cells drives lysophosphatidic acid-mediated expression of cell adhesion molecules via NF-kappaB p65. *J Biol Chem* **285**, 12536–12542, doi: 10.1074/jbc.M109.099630 (2010).
25. Hejink, I. H., Brandenburg, S. M., Postma, D. S. & van Oosterhout, A. J. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. *Eur Respir J* **39**, 419–428, doi: 10.1183/16000617.00193810 (2012).
Author Contributions
X.W. designed the research, analyzed the data and wrote the manuscript; C.Z. and S.H.Q. conducted the experiments, analyzed the data and wrote the manuscript; L.Z.Q., L.W.L., W.J.S., X.Y.L., N.P.L. and R.L.W. participated in scientific discussions, analyzed the data and provided detailed feedback on all areas of the project.

Additional Information
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