

Effect of platelet-activating factor on cell proliferation & NF- κ B activation in airway smooth muscle cells in rats

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Background & objectives: Despite the established pro-inflammatory actions of platelet activating factor (PAF) observed on chronic obstructive pulmonary disease (COPD), its action on airway remodeling has been still unclear. It has been reported that nuclear factor-kappa B (NF- κ B) activity is necessary for ASMC proliferation. Further, PAF has been identified as the proximal inducer of NF- κ B. The present study was thus aimed to investigate the effect of PAF on airway smooth muscle cells (ASMC) proliferation and to evaluate the potential role of NF- κ B in this regulation.

Methods: Healthy male Sprague-Dowley rats of 6-8 wk age were used for obtaining ASMCs. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay was to investigate the effects of PAF on ASMC proliferation and to confirm its optimum concentration for action. Additionally, cell proliferation was also examined using cell cycle assay by flow cytometry and immunocytochemical staining for proliferating cell nuclear antigen (PCNA). And NF- κ B DNA-binding activity was assayed by electrophoretic mobility shift assay (EMSA).

Results: PAF stimulated ASMC proliferation with its peak at 100nM. At this optimum concentration, PAF significantly increased the cell proliferation index (PI) and the PCNA-positive rate in the ASMCs, as well as NF- κ B DNA-binding activity. Whereas, 20mM N-acetylcysteine (NAC) pre-treatment effectively blocked all of these events.

Interpretation & conclusion: The present findings demonstrated that PAF could promote ASMC proliferation, suggesting its potential involvement in airway remodeling. Our study also suggested the promising action of 20mM NAC on the alleviation of airway remodeling due to direct inhibition of ASMC proliferation. The involved mechanism would be relevant to the change of NF- κ B activation in ASMCs.

Key words Nuclear factor- κ B - N-acetylcysteine - platelet-activating factor - proliferation - smooth muscle cell

Platelet activating factor (PAF), a phospholipid mediator with many biological activities, has been implicated in pathophysiological processes ranging from shock to cell differentiation¹. The majority of its actions are due to its interaction with PAF receptor (PAF-R), a specific G-protein-coupled transmembrane receptor². In lungs, PAF extensively participates in inflammatory processes, including the activation of many leukocytes, such as monocytes, macrophages, eosinophils, neutrophils and platelets, and the induction of vascular permeability, vasoconstriction, broncho-constriction and airway hyper-reactivity³. All these responses are of pathophysiological relevance in chronic inflammation of chronic obstructive pulmonary disease (COPD). Despite its established pro-inflammatory actions on COPD, the action of PAF on airway remodeling has been still unclear.

In COPD airway wall remodeling is known to be responsible for chronic airflow limitation, for the decline in lung function and for the relatively poor responses to available therapies⁴. Airway smooth muscle cells (ASMCs) are regarded as the major constituent of smooth muscle layer and profoundly implicated in remodeling processes of the small airways⁴. Hence, a better understanding of the mechanisms by which ASMC growth is regulated is crucial to the development of potential and effective therapies for COPD.

PAF has also been identified as the proximal inducer of nuclear factor- κ B (NF- κ B)^{5,6}, which is a major regulator of gene transcription involved in inflammation and the processes of cell growth. NF- κ B activation is shown to be involved in the proliferation of ASMCs⁷. N-acetylcysteine (NAC) has been extensively used in clinical practice of COPD because of its mucolytic action. It possesses both antioxidant and SH-donating properties^{9,10} and can also modulate NF- κ B activity. However, its regulation appears to be cell-type specific¹⁰. Taking account of the crucial role of NF- κ B in ASMC, there is a need to further investigate how PAF is involved in the regulation of NF- κ B in ASMCs and how NAC influences these events. We undertook this study to investigate the effect of PAF on ASMC proliferation and to study the role of NF- κ B in the regulation of these events.

Material & Methods

Reagents: MTT (3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium-bromide), PAF (1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine), TPS (trypsin), NAC, PDTTC (pyrrolidine dithiocarbamate) and monoclonal antibody against PCNA (proliferating cell nuclear antigen) were purchased from Sigma Corporation,

St.Louis, USA. Collagenase IV and DMEM (Dulbecco's modified Eagle's medium) were from Gibco Corporation, California, USA. SABC kit was purchased from Boster Biotechnology Company, Wuhan, China. Foetal calf serum (FCS) was from Hangzhou Sijiqing Biological Engineering Materials Corporation, Hangzhou, China. NE-PER™ kit was purchased from Pierce Biotechnology Incorporation, Rockford, USA. Gel Shift Assay System kit was from Promega Corporation, Madison, USA.

Culture and identification of ASMCs : The rat airways were drawn from healthy male Sprague-Dawley (SD) rats at 6-8 wk of age, each weighing 100-120g. Airway smooth muscle cells (ASMCs) were obtained as previously described¹¹. Primary culture of ASMCs was maintained in DMEM containing 10 per cent FCS and 1 per cent penicillin/streptomycin at 37°C in a humidified 5 per cent CO₂-95 per cent air atmosphere and their SMC identity was verified by standard immuno-cytochemistry with anti-smooth muscle α -actin polyclonal antibody (1:100 dilution). Cells at passages 5-10 were used for further experiments. The study protocol was approved by the medical ethics committee at Fourth Military Medical University, Xi'an, China.

MTT assay : ASMCs were seeded at 7×10^3 cells/well into 96-well cell culture plates and randomly divided into two groups - control and PAF group. Following synchronization for 24 h, the medium in the control group was replaced with DMEM only containing 5 per cent FCS, while the medium in the PAF group was replaced with DMEM containing 5 per cent FCS and PAF at different final concentrations of 1, 10, 100 nM and 1 μ M. According to these final concentration, cells in the PAF group were subdivided into four small subgroups. Then ASMCs were incubated for 12, 24, 36, 48, 60 and 72 h, respectively. At the end of each cultural time point, 20 μ l/well of MTT was added. Following further incubation at 37°C, 5 per cent CO₂ for 4 h, the cultural medium was aspirated and replaced with 150 μ l/well of DMSO. Plates were then agitated for 10 min and the optical density (OD) was measured at a test wavelength of 492 nm in an ELISA plate reader (Bio-Rad Labs, California, USA). Based on these values, the optimum concentration of PAF was determined for the subsequent experiments.

Additionally, another two MTT assays were performed. One was performed to identify the specific binding of PAF on ASMCs with the pretreatment of 1 μ M WEB 2086 (a specific antagonist of PAF), and another was conducted with the pretreatment of 100 μ M

PDTC (a special inhibitor of NF- κ B activation) to confirm the role of NF- κ B in PAF-promoted ASMC proliferation.

Flow cytometry analysis : After adherence, ASMCs were divided randomly into three groups: the control, PAF and NAC groups. ASMCs in the control group were growth-arrested with serum-free DMEM for 24 h and then maintained in DMEM containing 5 per cent FCS. Following the same growth-arrest, cells in the PAF group were maintained in DMEM containing 5 per cent FCS and 100 nM PAF. In the NAC group, after the same synchronization for 2.5 h, NAC (20 mM) was added into the serum-free arrest medium for further synchronization for 21.5 h and then treated in the same way as the PAF group. Cells were incubated for 12, 24 and 36 h, respectively. At the end of each time point, ASMCs were harvested and fixed with ice-cold 75 per cent ethanol overnight at -20°C . Then cells were washed with PBS and stained with propidium iodide (50 $\mu\text{g}/\text{ml}$) in $1\times$ PBS containing RNase (10 mg/ml) for 20 min at room temperature. The percentage of the cells in phase G_0/G_1 , S and G_2/M in the cell cycle was determined by flow cytometry. And the cell proliferation index (PI) was calculated according to the following equation: $PI = (S + G_2/M) / (S + G_0/G_1 + G_2/M) \times 100$ per cent.

Immunocytochemistry detection for PCNA expression: ASMCs were seeded in coverslips and grouped in the same way as previously described. After the stimulation period for 48 h, cells were fixed in 4 per cent paraformaldehyde and then stained by standard immunocytochemical techniques with SABC kit. A mouse monoclonal antibody against PCNA protein was used at a dilution of 1:100 as the primary antibody. The substitution of PBS for the specific primary antibody was performed as the negative control. Then 5 high-power (HP) sights were randomly chosen and 500 cells were randomly counted. Accordingly, the per cent of cells with positive stained nuclei was calculated.

Electrophoretic mobility shift assay for NF- κ B DNA-binding activity: After adherence, the culture medium was replaced with DMEM containing 5 per cent FCS. When the cells reached 60 per cent confluence, ASMCs were randomly divided into three groups: the control, PAF and NAC groups. The medium in the NAC group was changed to DMEM containing 20 mM NAC and 5 per cent FCS. After further treatment for 21.5 h, the medium in the PAF and NAC groups was replaced with DMEM containing 5% FCS and 100 nM PAF, but the medium of the control group was not changed. ASMCs were further maintained for 0.5, 1, 2 and 4 h, respectively. At the end of each

culture period, cells were harvested and nuclear extracts were prepared using NE-PER™ kit. Then NF- κ B DNA-binding activity was examined by EMSA using Gel Shift Assay System kit. The double-stranded oligonucleotide containing the NF- κ B binding site: 5'-AGTTGAGGGGACTTCCAGGC-3' was radioactively end-labeled with ($\gamma^{32}\text{P}$) ATP and used as a probe. Specific binding was confirmed by competition experiments with an excess of unlabeled but identical oligonucleotide or SP1-containing oligonucleotide. The protein-DNA complexes were resolved by electrophoresis on 5 per cent nondenaturing polyacrylamide gels in $0.5\times$ Tris-borate EDTA at 4°C for 1.5 h and visualized by autoradiography. Band intensities were quantitatively analyzed using a gel analysis system and normalized to the intensity of standard reaction using HeLa nuclear extract and $\gamma^{32}\text{P}$ -labeled SP-1 oligonucleotide.

Statistical analysis : All experiments were carried out in triplicate with consistent results. Inter-group comparisons were established using one-way analysis of variance (one-way ANOVA). Student-Newman-Keuls tests were used for multiple comparisons. Statistical significance was considered at $P < 0.05$.

Results

ASMC identification: When examined with phase-contrast microscopy, the cells displayed the typical "hill-and-valley" appearance of SMC cultures. In addition, nearly 100 per cent of the cells at 5-10th passage showed a positive staining for smooth muscle α -actin. Thus the cultured cells were identified as smooth muscle cells.

Effect of PAF on cell proliferation: PAF significantly promoted cell proliferation in a time-dependent fashion. With PAF concentrations ranging from 1 nM to 1 μM , ASMC proliferation was noted not to be a concentration-dependent-response to PAF with a peak at 100 nM but a reduced effect at 1 μM , (Fig.1A) which was similar to previous data showing a PAF bell-shaped response in other cell types^{12,13}. Accordingly, 100 nM was regarded as the optimum concentration of PAF for the successive experiments.

Pre-treatment with 1 μM WEB2086 counteracted, the growth-promoting effect of 100nM PAF (Fig.1B), indicating that the action of PAF in ASMCs was receptor-mediated and not due to nonspecific actions. On the other hand, 100 μM PDTC significantly attenuated the PAF-mediator ASMC proliferation, which suggested that PAF had its effect in the NF- κ B-dependent manner (Fig. 1C).

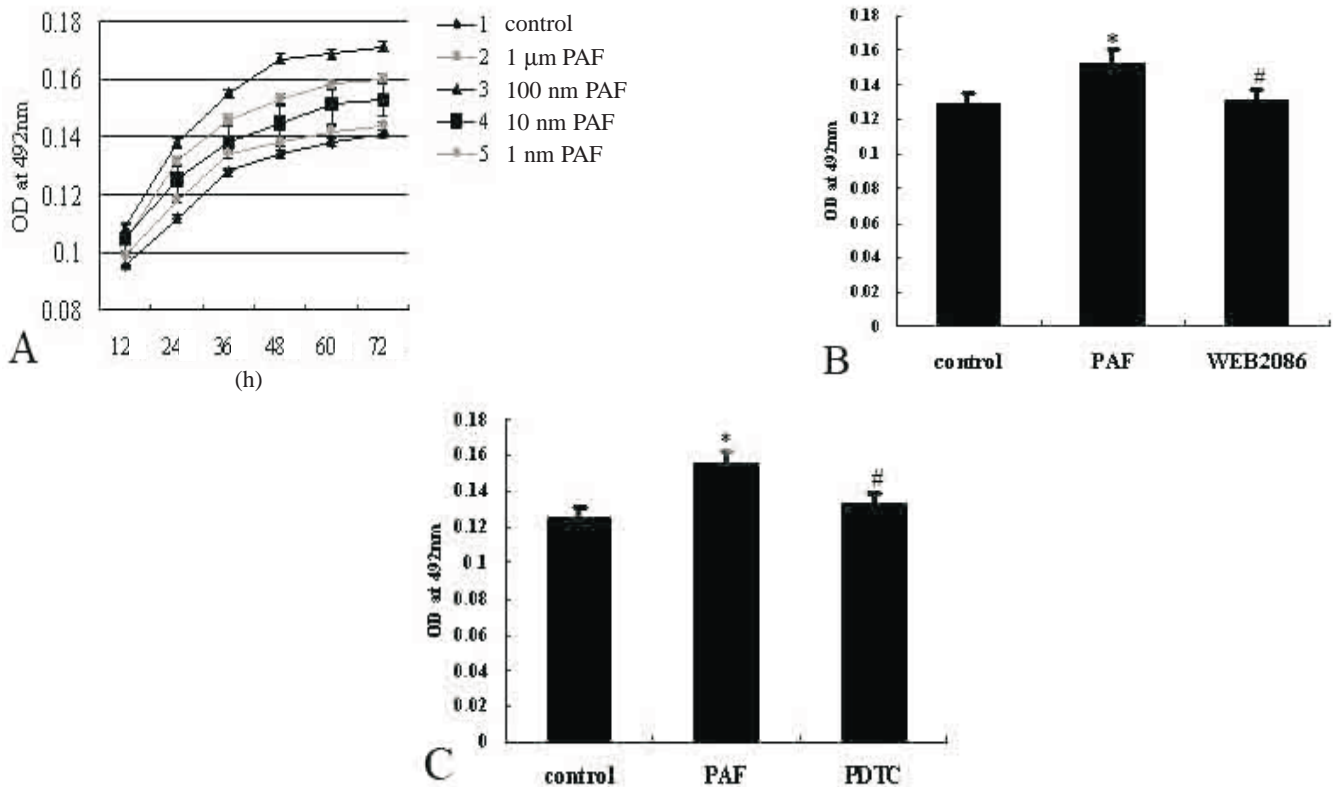


Fig. 1. Effect of PAF on ASMC proliferation using MTT assay. (A) Growth-arrested ASMCs (7×10^3 cells/well) were incubated with or without PAF at different final concentrations for indicated periods. Values represent mean \pm SE from three independent experiments. (B) Growth-arrested ASMCs (5×10^3 cells/well) were pretreated with or without $1 \mu\text{M}$ WEB 2086 for 30 min prior to PAF treatment (100 nM) for 48 h. (C) Growth-arrested ASMCs (5×10^3 cells/well) were pretreated with or without $100 \mu\text{M}$ PDTC for 1h prior to PAF treatment (100 nM) for 48 h. Each bar represents mean \pm SE from three independent experiments. (* $P < 0.05$ compared to the control group, # $P < 0.05$ compared to the PAF group).

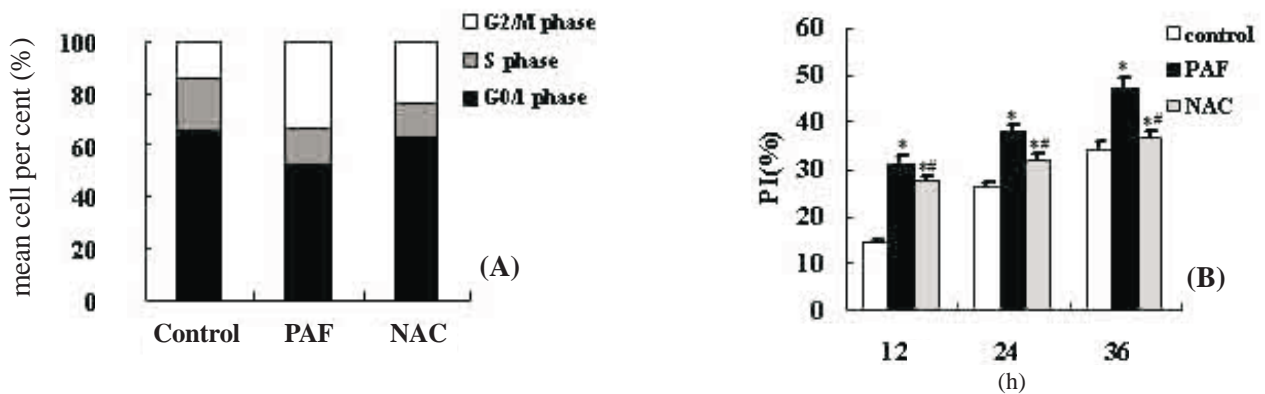


Fig. 2. Cell cycle analysis of ASMCs. Results shown are representative of three independent experiments. (B) depicts the calculated PI in the control, PAF and NAC groups. (A) shows the distribution of the cell cycle up to 36 h. Each bar is presented as the mean \pm SE from three independent experiments. (* $P < 0.05$ compared to the control group, # $P < 0.05$ compared to the PAF group).

Cell cycle analysis of ASMCs: As shown by flow cytometry, treatment with PAF (100 nM) markedly decreased the per cent of ASMCs at G₀/G₁ phase and correspondingly increased their per cents at S and G₂/M

phase. NAC blocked this event (Fig. 2A). PAF treatment resulted in a significant increase ($P < 0.005$) in cell proliferation index (PI) of ASMCs and NAC caused a significant ($P < 0.05$) inhibitory effect (Fig. 2B).

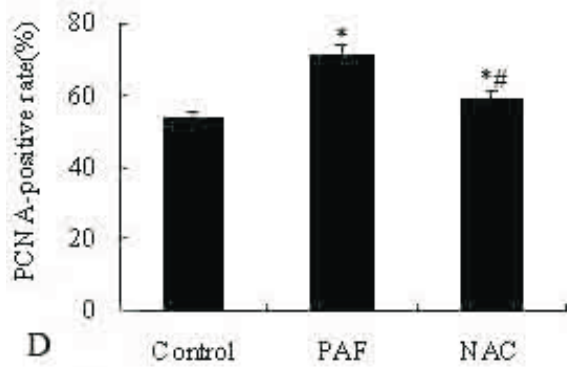


Fig. 3. Calculated PCNA -positive rates in ASMCs. Each bar represents mean ± SE from three independent experiments (* $P < 0.05$ compared to control group, # $P < 0.05$ compared to PAF group).

Expression of PCNA in ASMCs: The results revealed that PAF increased the intensity and number of PCNA-stained ASMCs, but pre-treatment with NAC reduced these stimulative effects. (Fig. 3). A significant augment ($P < 0.05$) in the PCNA-positive rate of cultured ASMCs was obtained in the PAF group compared with that in the control group, whereas NAC pre-incubation markedly decreased ($P < 0.05$) the increased PCNA-positive rate.

NF-κB DNA-binding activity in ASMCs: To demonstrate the influence of PAF on the NF-κB signal pathway in ASMCs, nuclear extracts of ASMCs were analyzed by EMSA. As shown in Fig. 4(A), following the stimulation with PAF for indicated periods, the

nuclear extracts of ASMCs yielded larger bands in comparison to the control cells, exhibiting relatively higher levels of NF-κB DNA-binding activity. However, NAC pre-treatment caused a marked reduction in PAF-mediated DNA-protein complex binding activity, as evidenced by a decrease in the band intensities (Fig. 4B). Additionally, the binding was specific since it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, non-specific oligonucleotide (data not shown).

Discussion

Because of little progress in drug therapy of COPD, more specific targets underlying the pathogenesis need to be aimed at¹⁴. ASMCs have been regarded as a new therapeutic target for this disease. In recent years, there has been increasing interest in the identification of the mechanisms involved in ASMC proliferation. So far, many cytokines have been demonstrated to participate in ASMC proliferation process, including transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and epidermal growth factor (EGF)^{15,16}. Overexpression of some proto-oncogenes, such as *c-fos* and *c-jun*, also leads to ASMC overproliferation¹⁷.

Besides its well-known pro-inflammatory action, PAF has shown its growth-promoting activity in a broad range of cell types such as epidermal cells, NRK cells and human myeloma cells^{12,18,19}. Conversely, PAF-R antagonists have inhibitory effects on the cell

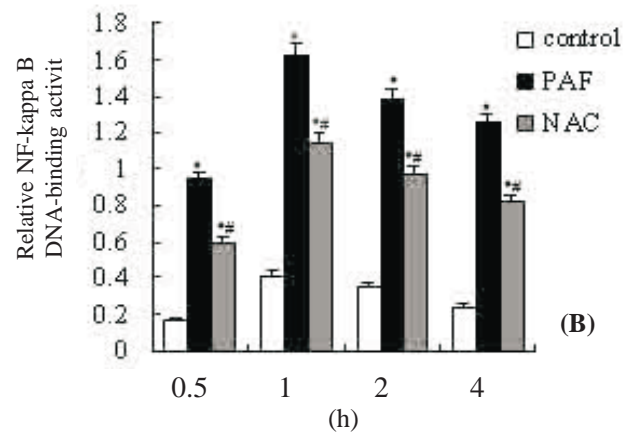
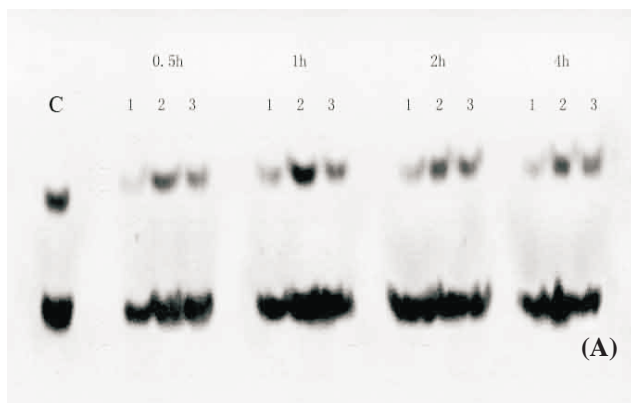


Fig. 4. Changes of NF-κB binding activity in ASMCs at different time points. (A) shows EMSA gel using γ^{32} P-labeled oligonucleotide probes for NF-κB. Results shown are representative of three independent experiments. Lane C: standard reaction; Lane 1: control group; Lane 2: PAF group; Lane 3: NAC group. (B) depicts the densitometric evaluation of NF-κB DNA-binding activity, normalized to the level of standard reaction. Each bar represents mean ± SE from three independent experiments (* $P < 0.05$ compared to the control group, # $P < 0.05$ compared to the PAF group).

growth²⁰⁻²². PAF can induce proliferation of vascular SMCs²³, and CV-3988, a specific PAF-R antagonist, has been shown to inhibit (s)-albutetol-induced rat bronchial SMC proliferation²⁴. These observations have raised the possibility that PAF could stimulate ASMC proliferation, and our findings also showed the same.

The major finding of this study was that PAF markedly promoted ASMC proliferation. Flow cytometry analysis further revealed the PAF-mediated reduction of ASMCs in G₀/G₁ phase and the accumulation of ASMCs at S and G₂/M phases, consequently resulting in its growth-promoting effect. Accordingly, the present study suggests a potential involvement of PAF in airway remodeling due to its direct stimulation of ASMC proliferation, which broadens the role of PAF in the pathogenesis of COPD and provides a new theoretical basis for the special treatment of COPD targeting against PAF.

The mechanism of the stimulation on ASMC proliferation by PAF is still unclear. As a proximal inducer of NF- κ B, PAF is directly involved in its biphasic activation⁶⁻²⁵. In this way, PAF could affect many biological functions of cells. For example, in RBL-2H3 cells, the NF- κ B activation by PAF was associated with the CC chemokine receptor ligand 2(CCL2) production²⁶, in ECV304 and HEC-1A cells with matrix metalloproteinase-9 expression^{27,28}, in mouse peritoneal macrophages with the production of macrophage-derived angiogenic factors²⁹ and in epidermal cells with the decreased apoptosis³⁰. Interestingly, the present study found that PAF also upregulated NF- κ B activation in ASMCs. It was noted that the changes in NF- κ B activation in response to PAF and NAC paralleled the alterations in ASMC proliferation; the upregulation of NF- κ B activity by PAF coincided with its stimulation on ASMC proliferation. While pre-treatment with NAC attenuated the PAF-induced ASMC proliferation, which coincided with its suppression on NF- κ B activation enhanced by PAF. Further, PDTC pre-treatment was found to markedly attenuate the PAF-promoted ASMC proliferation. Hence, the increased activity of NF- κ B may be regarded as the cause of the stimulative effect of PAF on ASMCs.

NAC has been proposed as an agent that could influence both oxidative stress and inflammatory processes present in COPD, aiding in improving the clinical status of these patients³¹⁻³³. The administered dose of NAC has not been optimized according to many COPD-related clinical trials¹⁰. Though 0.1 mM NAC could reduce ASMC proliferation³⁴, rather high NAC concentrations should be necessary for the inhibition of

NAC on NF- κ B activation, as well as its anti-inflammatory effects *in vitro*¹⁰. NAC is known to exert toxic effects only at concentrations >30 mM in vascular SMCs³⁵. The present study focused on the influence of NAC at a relatively high dose of 20 mM on airway remodeling. Similar to the previous studies^{36,37}, our study demonstrated that without decreasing cell viability as judged by trypan blue staining (data not shown), and showing any cytotoxic effect, NAC (20 mM) could significantly inhibit ASMC proliferation. Thus our findings not only suggest the promising action of NAC on the alleviation of airway remodeling in COPD but also provide a potentially theoretical basis for clinical administration of NAC at higher doses.

To conclude, our study demonstrated that PAF could directly promote ASMC proliferation and NAC could attenuate this effect. The underlying mechanism would be relevant to the change of NF- κ B activation in ASMCs.

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