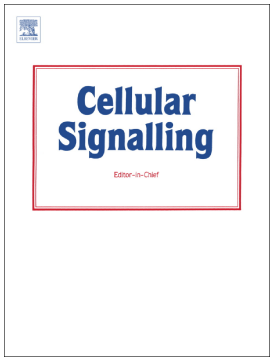

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Jian Wang, Mengchan Zhu, Linlin Wang, Cuicui Chen, Yuanlin Song

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Amphiregulin potentiates airway inflammation and mucus hypersecretion induced by urban particulate matter via the EGFR-PI3Kα-AKT/ERK pathway

Jian Wang[1,#], Mengchan Zhu[1,#], Linlin Wang[1], Cuicui Chen[1], Yuanlin Song[1,*]

1. Department of Pulmonary Medicine, Zhongshan Hospital, Fudan University, N0.180 Fenglin Road, Shanghai, 200030, China.

# Jian Wang and Mengchan Zhu contributed equally to this work.

* Yuanlin Song is the corresponding author.

E-mail: ylsong70@163.com

Abstract

Ambient particulate matter (PM) promotes the development and exacerbation of chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD) and asthma, by increasing inflammation and mucus hypersecretion. However, the biological mechanisms underlying PM-induced airway inflammation and mucus hypersecretion remain unclear. Amphiregulin (AREG) is an important ligand for epidermal growth factor receptor (EGFR) and participates in the regulation of several biological functions. Here, the PM-exposed human bronchial epithelial cell (HBEC) model was used to define the role of AREG in PM-induced inflammation and mucus hypersecretion and its related signaling pathways. The expression of AREG was significantly increased in a dose-dependent manner in HBECs subjected to PM exposure. Moreover, PM could induce inflammation and mucus hypersecretion by upregulating the expression of IL-1α, IL-1β, and Muc-5ac in HBECs. The EGFR, AKT, and ERK signaling pathways were also activated in a time- and dose-dependent manner. The AREG siRNA markedly attenuated PM-induced inflammation and mucus hypersecretion, and activation of the EGFR-AKT/ERK pathway. Exogenous AREG significantly increased the expression of IL-1α, IL-1β, and Muc-5ac, and induced activation of the EGFR-AKT/ERK pathway in HBECs. Further, under PM exposure, exogenous AREG significantly potentiated PM-induced inflammation and

mucus hypersecretion, and activation of the EGFR-AKT/ERK pathway. Tumor-necrosis factor-alpha converting enzyme (TACE) and EGFR specific inhibitor pretreatment showed that AREG was secreted by TACE-mediated cleavage to regulate PM-induced inflammation and mucus hypersecretion by binding to the EGFR. Moreover, according to the inhibitory effect of specific inhibitors of the class I PI3K isoforms, AKT and ERK, PM-induced inflammation and mucus hypersecretion was regulated by PI3Kα activation and its downstream AKT and ERK pathways. This study strongly suggests the adverse effect of AREG in PM-induced inflammation and mucus hypersecretion via the EGFR-PI3Kα-AKT/ERK pathway. These findings contribute to a better understanding of the biological mechanisms underlying exacerbation of chronic respiratory diseases induced by PM exposure.

Keywords: Particulate matter; amphiregulin; inflammation; mucus hypersecretion; epidermal growth factor receptor

1. Introduction

Air pollution has been the biggest environmental risk to public health worldwide according to the World Health Organization (WHO). It has been reported that more than 90% of the global population is suffering from the effects of air pollution[1]. Particulate matter (PM) is the major air-pollutant and exposure to PM reportedly promotes the development and exacerbation of several respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma[2, 3]. A number of epidemiological studies have demonstrated that PM is an important risk factor for COPD, and PM exposure could reduce lung function and increase the number of hospitalizations and mortality rates among COPD patients[4, 5, 6]. Moreover, the effects of PM on the exacerbation of asthma, including emergency department visits and hospitalizations, have been well-defined in both children and adults[7, 8, 9].

The airways are continuously exposed to PM from the environment and several biological processes can be triggered as a result of airway damage. Inflammation and mucus hypersecretion are the two most common biological responses to PM-induced airway damage. Our previous study showed that urban PM exposure increased the expression of pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) in human bronchial epithelial cells (HBECs) and in acutely PM-exposed mouse models[10]. Similarly, PM exposure could promote inflammatory responses in macrophages and alveolar epithelial cells[11, 12]. Mucus hypersecretion can obstruct the airway to limit airflow, reduce lung function, and cause recurrent airway infection, and thereby exacerbate COPD and asthma[13]. Muc-5ac is a key component of mucus and is considered to play an important role in mucus hypersecretion[14]. However, the molecular mechanism underlying PM-induced inflammation and mucus hypersecretion has not been clarified.

Amphiregulin (AREG) is a member of the epidermal growth factor (EGF) family. Its precursor, pro-AREG, is expressed and binds to the membrane, and could be released by tumor-necrosis factor-alpha converting enzyme (TACE)-mediated proteolytic processing as the soluble AREG[15]. Previous research shows that AREG participates in the regulation of cell proliferation, apoptosis and migration, inflammation, tissue repair and carcinogenesis by binding to the EGF receptor (EGFR)[16]. Several recent studies have shown that PM could promote the expression of AREG in bronchial epithelial cells[17]. The upregulated expression of AREG could promote the mucus hypersecretion[18]. Deacon et al.[19] found that AREG could induce the expression of CXCL8, COX2, and VEGF in bronchial epithelial cells. However, Fukumoto et al.[20] showed that AREG has a protective effect against bleomycin-induced inflammatory responses and fibrosis in the lungs. Thus, the role of AREG in PM-induced inflammation and mucus hypersecretion should be further identified, and the related signaling pathways need to be elucidated.

In the current study, the reference urban dust material SRM 1649b (which is usually abbreviated to “1649b”) was used to stimulate HBECs. The expression of AREG was upregulated by 1649b exposure. The function and related pathway

activation of AREG in PM-induced inflammation and mucus hypersecretion were identified to demonstrate whether AREG plays an important role in the regulation of PM-induced airway injury.

2. Materials and methods

2.1 Reagents

The 1649b material was purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). Recombinant human AREG (rhAREG) was obtained from Peprotech (Rocky Hill, NJ, USA). The EGFR inhibitor AG1478, TACE inhibitor TAPI-1, broad-spectrum PI3K inhibitor LY294002, PI3Kα inhibitor PIK-75, PI3Kβ inhibitor GSK2636771, PI3Kγ inhibitor AS-252424, PI3Kδ inhibitor IC-87114, AKT inhibitor MK-2206, and ERK inhibitor U0126 were purchased from Selleck (Houston, TX, USA). Antibodies against phospho-EGFR, phospho-AKT, phospho-ERK, EGFR, AKT, and ERK were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against AREG and GAPDH were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and Beyotime (Shanghai, China), respectively. The TRIzol™ reagent was purchased from Invitrogen (Carlsbad, NM, USA). The mRNA primers were synthesized by Synbio Technologies (Suzhou, China). The reagents for cDNA synthesis and real-time PCR were purchased from Takara Bio (Shiga, Japan). The reagents for western blot and immunofluorescence staining were purchased from Beyotime.

2.2 Cell culture

HBECs were obtained from the Chinese Academy of Sciences (Shanghai, China). The HBECs were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 50 U/mL penicillin and streptomycin (Gibco) at 37 °C in a culture chamber containing 5% CO_2.

2.3 Research design

The PM was prepared as previously reported[10]. The HBECs were treated with different doses of PM (50, 100, and 300 μg/cm^3) for 24 h to detect the expression of AREG, IL-1α, IL-1β, and Muc-5ac. Activation of the signaling pathway was detected by western blot analysis following exposure to different doses of PM (50, 100, and 300 μg/cm^3) over 1 h or 300 μg/cm^3 PM for different durations (0.25, 0.5, 1, 3, and 6 h). The AREG siRNA was used to inhibit the expression of AREG, whereas rhAREG was added to clarify the regulatory role of AREG in PM-induced inflammation, mucus hypersecretion, and activation of the related signaling pathways. Further, the pathway inhibitors AG1478 (10 μM), TAPI-1 (10 μM), LY294002 (10 μM), PIK-75 (500 nM), GSK2636771 (10 μM), AS-252424 (100 nM), IC-87114 (10 μM), MK-2206 (10 μM), and U0126 (10 μM) were used to treat HBECs prior to PM exposure, to address the AREG potentiated PM-induced inflammation and mucus hypersecretion the via the EGFR-PI3Kα-AKT/ERK pathway.

2.4 Real-time PCR

Total RNA was extracted using the Trizol reagent. The concentration of RNA was quantified by a NanoVue Plus spectrophotometer (GE Healthcare, Chicago, IN, USA) and 1 μg of total RNA was reverse transcribed into cDNA using Reverse Transcription Reagents (TaKaRa Bio, Shiga, Japan), according to the manufacturer's protocol. The cDNA was diluted 1:10 and 10 μg of the PCR reaction system was then prepared using the SYBR® Premix Ex Taq^{TM} reagent (TaKaRa Bio). Real-time PCR amplification was performed on a real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The primers of AREG, IL-1α, IL-1β, Muc-5ac, and GAPDH are shown in Table 1. The relative mRNA expression is shown as a $2^{-\Delta\Delta Ct}$ value, and GAPDH was used as an internal control.

2.5 Western blot

The total protein of HBECs from different groups were extracted using RIPA buffer containing phenylmethanesulfonyl fluoride (PMSF, Beyotime) and phosphatase inhibitors (Biotool, Houston, TX, USA). The concentration of proteins was measured

using a BCA Protein Assay kit (Beyotime). Protein (30μg) of each group was loaded onto a 10% sodium dodecyl sulphatepolyacrylamide gel. Subsequently, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After being blocked with western blocking buffer for 1 h at room temperature, the membrane was incubated with primary antibodies against phospho-EGFR, phospho-AKT, phospho-ERK, EGFR, AKT, ERK and GAPDH (1:1000 dilution) overnight at 4 ℃, respectively. On the following day, after being washed three times in TBST, the membrane was incubated with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature. After being washed three times in TBST, the membrane was exposed using ECL reagents (Thermo Scientific, Waltham, MA, USA) on A Bio-Rad Laboratories system. The optical densities of protein bands were quantified using the AlphaEaseFC v4.0 software, and the relative expression of p-EGFR, p-AKT, and p-ERK was normalized by comparison with total EGFR, AKT, and ERK, respectively.

2.6 AREG siRNA transfection

Three AREG siRNA were designed and synthesized by GenePharma (Shanghai, China). The sequences were shown in Supplemental Table 1. The delivery of AREG siRNA was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The inhibition efficiency of the three AREG siRNAs was measured by real-time PCR, and the most efficient AREG siRNA was selected for further study. After 24 h of transfection, HBECs were stimulated with PM to determine the role of AREG in PM-induced inflammation and mucus hypersecretion.

2.7 Immunofluorescence staining

The HBECs were seeded onto a coverslip in a 6-well plate to reach ～50% confluence. After being stimulated with PM with different doses of PM over 24 h, HBECs were washed three times and fixed using the Immunol Staining Fix Solution (Beyotime) for 10 min at room temperature. After being washed three times with PBS,

HBECs were permeabilized with 0.5% Triton X-100 for 20 min at room temperature. Subsequently, Immunol Staining Blocking Buffer (Beyotime) was used to block nonspecific protein binding sites for 1 h at room temperature, and then HBECs were incubated with primary antibody against AREG (1:100 dilution) overnight at 4 ℃. On the following day, after being washed three times with PBS, HBECs were incubated with Alexa Fluor® 488 Conjugate anti-mouse secondary antibody (1:1000 dilution) for 1 h at room temperature. The unbound secondary antibody was washed with PBS and the nuclei of the HBECs were then stained with DAPI (Beyotime) for 10 min at room temperature. The HBECs were then observed and imaged under a fluorescence microscope (Olympus, Tokyo, Japan). The relative fluorescence intensity was quantified using the Image J v1.4.3.67 software.

2.8 Statistical analysis

The data were presented as mean ± SEM. The SPSS v19.0 software (IBM, Armonk, NY, USA) was used to analyze the statistical differences between different groups using one-way analysis of variance. P value of less than 0.05 was considered significant.

3. Results

3.1 PM increases AREG expression, inflammatory responses and mucus hypersecretion in HBECs

To investigate changes in the expression of AREG, HBECs were stimulated with different doses of PM (50, 100, and 300 μg/cm^3) over 24 h. The mRNA level of AREG was increased in HBECs in a dose-dependent manner (Fig.1A). Moreover, immunofluorescence staining also confirmed that PM promoted the expression of AREG in a dose-dependent manner (Fig. 1B and C). Further, expression of the pro-inflammatory cytokines, IL-1α and IL-1β, was significantly increased in a dose-dependent manner in HBECs subjected to PM exposure (Fig. 1D). The principal mucus protein associated with mucus hypersecretion is Muc-5ac. The significantly upregulated expression of Muc-5ac was also detected in HBECs exposed to PM (Fig.

1D). These results suggested that PM could increase the expression of AREG. Moreover, inflammation and mucus hypersecretion were induced in HBECs following PM exposure.

3.2 PM activates the EGFR-AKT/ERK pathway in HBECs

Previous research has demonstrated that AREG exerts its biological functions via activation of EGFR related pathways[16]. Western blot analysis showed that PM could induce the phosphorylation of EGFR, AKT, and ERK in a time-dependent manner (Fig. 2A and B). Activation of the EGFR-AKT/ERK pathway occurred at a very early stage of PM exposure, which suggested that this pathway might play an important role in promoting PM-induced inflammation and mucus hypersecretion. Moreover, PM could increase the phosphorylation of EGFR, AKT, and ERK in a dose-dependent manner (Fig. 2C and D). These results verified that the EGFR-AKT/ERK pathway was activated in HBECs subjected to PM exposure.

3.3 AREG silencing alleviates PM-induced inflammation and mucus hypersecretion in HBECs

To explore the regulatory role of AREG in PM-induced inflammation and mucus hypersecretion, AREG siRNA was used to inhibit the expression of AREG prior to PM exposure in HBECs. Three AREG siRNAs were designed and delivered to the cells. The optimum AREG siRNA was selected according to the inhibition efficiency by comparing AREG mRNA levels in each group with the control siRNA group using RT-PCR (Fig. 3A). AREG silencing significantly attenuated the increase of the pro-inflammatory cytokines, IL-1α and IL-1β, in HBECs treated with 300 μg/cm^3 PM for 24 h. A similar inhibitory effect of AREG silencing was detected on the expression of Muc-5ac (Fig. 3B). Furthermore, under PM exposure, the phosphorylation of EGFR, AKT, and ERK was inhibited in the AREG siRNA group, compared with that in the control siRNA group (Fig. 3C and D). These results suggested that AREG potentiated PM-induced inflammation and mucus hypersecretion in HBECs via activation of the EGFR-AKT/ERK pathway.

3.4 Exogenous AREG promotes PM-induced inflammation and mucus hypersecretion in HBECs

To further confirm the pro-inflammatory role of AREG in PM-induced inflammation and mucus hypersecretion, rhAREG was added to augment the biological effect of AREG on HBECs. As expected, rhAREG significantly increased the expression of IL-1α, IL-1β, and Muc-5ac in HBECs in a dose-dependent manner (Fig. 4A). Moreover, exogenous AREG supplementation further potentiated the adverse effects of PM in HBECs by upregulating the expression of IL-1α, IL-1β, and Muc-5ac (Fig. 4A). Western blot analysis showed that exogenous AREG activated the EGFR-AKT/ERK pathway in a time-dependent manner (Fig. 4B and C). Interestingly, these signaling pathways were activated by rhAREG at a very early stage, which suggests that AREG might play an important role in PM-induced inflammation and mucus hypersecretion. Further, activation of the EGFR-AKT/ERK pathway was more significantly affected in HBECs treated with both PM and rhAREG than those treated with either PM or rhAREG alone (Fig. 4D and E). These data further confirmed that AREG was an important mediator associated with PM-induced inflammation and mucus hypersecretion.

3.5 TACE-dependent secretion of AREG regulates PM-induced inflammation and mucus hypersecretion by binding to the EGFR

As a key enzyme that facilitates the cleaving and release of AREG from the cell surface, TACE enables AREG to exert its biological functions in autocrine manner[16]. To demonstrate whether AREG is secreted by TACE-mediated cleavage to regulate PM-induced inflammation and mucus hypersecretion, HBECs were pretreated with TAPI-1, a TACE-specific inhibitor, and then stimulated with PM for 24 h. The expression of IL-1α, IL-1β, and Muc-5ac was significantly blocked by TAPI-1 treatment in PM-stimulated HBECs (Fig. 5A). Moreover, the phosphorylation of EGFR and its downstream AKT and ERK was also inhibited by TAPI-1 in HBECs treated with PM (Fig. 5B and C). Further, activation of the receptor for secreted

AREG was assessed using the EGFR specific inhibitor, AG1478. As expected, following pretreatment with AG1478, the increased expression of IL-1α, IL-1β, and Muc-5ac was attenuated in PM-stimulated HBECs (Fig. 5D). Similarly, activation of the EGFR-AKT/ERK pathway was significantly inhibited by AG1478 in PM-stimulated HBECs (Fig. 5E and F). These results suggested that AREG was shed by TACE and bound to the EGFR to promote PM-induced inflammation and mucus hypersecretion.

3.6 EGFR-PI3Kα activates AKT and ERK pathways to regulate PM-induced inflammation and mucus hypersecretion

To address the downstream pathway of EGFR in PM-induced inflammation and mucus hypersecretion, pathway-related inhibitors were used to treat cells prior to PM exposure. The expression of IL-1α, IL-1β, and Muc-5ac was significantly inhibited by pretreatment with the broad-spectrum PI3K inhibitor, LY294002, in PM-stimulated HBECs (Fig. 6A). The phosphorylation of downstream AKT and ERK was also blocked by LY294002 in PM-stimulated HBECs (Fig. 6B and C). To further explore the role of the EGFR-activated PI3K pathway in PM-induced inflammation and mucus hypersecretion, specific inhibitors of four class I PI3K isoforms (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) were used. Interestingly, PM-induced inflammation and mucus hypersecretion in HBECs were only significantly suppressed by the PI3Kα inhibitor (PIK-75), but remained unchanged by PI3Kβ, PI3Kγ, and PI3Kδ inhibition (Fig. 6D-G).

Further, pretreatment with the AKT inhibitor, MK-2206, could inhibit the expression of IL-1α, IL-1β, and Muc-5ac, as well as the phosphorylation of AKT in PM-stimulated HBECs (Fig. 7A-C). However, MK-2206 did not affect PM-induced activation of the ERK pathway. Interestingly, pretreatment with the ERK inhibitor, U0126, also blocked PM-induced expression of IL-1α, IL-1β, and Muc-5ac, as well as the phosphorylation of ERK in HBECs (Fig. 7D-F). The PM-induced activation of the AKT pathway was not inhibited by U0126 pretreatment. These data suggested that EGFR-PI3Kα could separately activate its downstream AKT and ERK pathways to

regulate PM-induced inflammation and mucus hypersecretion.

4. Discussion

The present study showed that the expression of AREG was significantly increased by urban PM 1649b exposure in HBECs. Moreover, PM could promote inflammation and mucus hypersecretion by upregulating the expression of IL-1α, IL-1β, and Muc-5ac in HBECs. The EGFR, AKT, and ERK signaling pathways were also activated. Further, increased levels of AREG could potentiate PM-induced inflammation and mucus hypersecretion by increasing the activation of the EGFR-PI3Kα-AKT/ERK pathway. The TACE-dependent cleavage and secretion of AREG is a key biological process to induce the positive effects of AREG on PM-induced inflammation and mucus hypersecretion (Fig. 7).

Several epidemiological studies have shown that PM exposure is associated with the development and exacerbation of COPD and asthma[6, 21]. Some of the underlying biological mechanisms of these processes include inflammation and mucus hypersecretion as a result of PM exposure. Recently, studies have demonstrated that PM exposure could induce the expression of specific molecules associated with inflammation and the destruction of the extracellular matrix in HBECs, alveolar epithelial cells, and alveolar macrophages, and in mouse models[10, 12]. In the present study, the expression of inflammatory cytokines (IL-1α and IL-1β) was upregulated by urban PM 1649b in a dose-dependent manner. As IL-1α and IL-1β are members of the IL-1 superfamily of inflammatory cytokines, they play a central role as mediators of innate immunity and inflammation[22]. Furthermore, Muc-5ac plays a key role in mucus hypersecretion, and is reportedly upregulated by several stimulators. In addition, PM reportedly increased the secretion of Muc-5ac in several previous studies. Chen et al.[23] showed that environmental ultrafine PM could induce the expression of inflammatory cytokines and Muc-5ac in HBECs, which were regulated by autophagy. In the present study, we also found that urban PM 1649b exposure could promote the expression of Muc-5ac in HBECs in a dose-dependent manner.

As one member of the EGF family, AREG binds to the EGFR to exert its biological functions. Several studies have shown that the expression of AREG could be promoted by different stimuli. Stolarczyk et al.[24] found that cigarette smoke increased the release of AREG in the primary bronchial epithelial cells of patients suffering from COPD. Moreover, PM exposure induces the expression of AREG in HBECs [17, 25]. In the present study, we found that urban PM 1649b could also promote the expression of AREG in HBECs in a dose-dependent manner. However, the regulatory role of AREG in airway epithelial injury is affected by different stimuli. Fukumoto et al.[20] found that AREG could protect against bleomycin-induced lung injury by suppressing the inflammatory responses and fibrosis and inhibiting apoptosis in the lung. Nordgren et al.[26] showed that docosahexaenoic acid could enhance AREG production, and upregulated AREG could promote tissue repair after inflammatory injury caused by organic dust exposure. Conversely, AREG could induce mucus cell metaplasia in naphthalene-induced lung injury[27]. Moreover, previous studies have shown that PM from various sources could promote AREG release to increase the expression of Muc-5ac in vivo and in vitro[18, 28]. In the present study, we also found that AREG could potentiate urban PM 1649b-induced mucus hypersecretion in HBECs. Moreover, the pro-inflammatory role of AREG in PM-induced airway injury was defined in the present study. Val et al.[18] showed that exogenous AREG supplementation could increase the release of the inflammatory cytokine, IL-8, in HBECs in a dose-dependent manner. To our knowledge, the present study is the first to demonstrate that AREG could regulate PM-induced IL-1α and IL-1β expression in HBECs using AREG siRNA and exogenous AREG.

The TACE-dependent release of AREG is an important step in the transactivation of EGFR under various stimuli. Chokki et al.[29] showed that TNF-α could induce AREG shedding to activate the AREG-EGFR pathway, to promote the release of IL-8 in airway epithelial cells. Similarly, cigarette smoke could induce the TACE-mediated release of AREG to activate the EGFR pathway, and thereby stimulate the proliferation of lung epithelial cells[30]. In the present study, the TACE specific inhibitor, TAPI-1, significantly inhibited PM-induced inflammation and mucus

hypersecretion, as well as activation of the EGFR-PI3Kα-AKT/ERK pathway in HBECs. These data suggested that urban PM 1649b-stimulated AREG release was mediated by TACE activity. Similar results have been observed with particles from other sources in HBECs[18].

The EGFR is considered the key receptor for AREG, and it could activate PI3K and its downstream AKT and ERK signaling pathways to regulate different biological processes. The PI3Ks are members of the lipid kinase family, and class I PI3Ks are the most extensively studied group of PI3Ks associated with chronic respiratory diseases. Four class I PI3Ks isoforms have been identified that display differential expression and functions in respiratory diseases. The PI3Kα and PI3Kβ isoforms are widely distributed, whereas PI3Kγ and PI3Kδ are highly expressed within leucocytes[31, 32]. Previous studies have shown that PI3Kα is the dominant isoform expressed in HBECs, and it plays a key role in different pathologic processes[33]. In the present study, the broad-spectrum PI3K inhibitor, LY294002, significantly inhibited PM-induced inflammation and mucus hypersecretion, indicating that PI3Ks are involved in PM-induced inflammation and mucus hypersecretion. To further identify the specific PI3Ks isoforms, four inhibitors of PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ were used. Interestingly, inhibition of PI3Kα, but not PI3Kβ, PI3Kγ, or PI3Kδ, led to a significant reduction in PM-induced inflammation and mucus hypersecretion.

The AKT signaling pathway is a central regulator in COPD pathogenesis that is associated with inflammation[34]. Moreover, the ERK signaling pathway is an important mediator that is reportedly involved in PM-induced inflammation, as evidenced by our previous study[10]. Zhao et al.[35] showed that AREG bound to the EGFR to promote the activation of AKT, ERK, and Smad signaling pathways in TGF-β-induced pulmonary fibrosis. Huang et al.[28] also demonstrated that wood smoke could activate the AREG-EGFR-ERK pathway to promote the expression of Muc-5ac in airway epithelial cells. In the present study, using western blot analysis, urban PM 1649b-induced phosphorylation of EGFR, AKT, and ERK was increased in a dose- and time-dependent manner. Pretreatment with specific pathway inhibitors of EGFR, AKT, and ERK is evidently involve in the regulation of PM-induced

inflammation and mucus hypersecretion. Interestingly, no interaction between the AKT and ERK pathways was evident. This made sense, because no direct crosstalk between AKT and ERK was addressed yet[36, 37]. Furthermore, both AKT and ERK pathways were activated by EGFR-mediated PI3Kα, and had synergistic effects on the PM-induced inflammation and mucus hypersecretion.

In conclusion, urban PM 1649b increased the expression of AREG in HBECs. Furthermore, AREG could potentiate PM-induced inflammation and mucus hypersecretion by increasing the activation of the EGFR-PI3Kα-AKT/ERK signaling pathway. Moreover, the TACE-mediated release of AREG is the key step that promotes PM-induced inflammation and mucus hypersecretion. The present study contributes to a better understanding of the biological mechanisms underlying the exacerbation of chronic respiratory diseases induced by PM exposure. It could help us to find efficient methods to prevent PM-induced adverse health effects.

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Conflict of interest

The authors have no conflicts of interest to declare.

Legends

Figure1. PM induces the expression of AREG, inflammatory responses, and mucus hypersecretion in HBECs. The HBECs were treated with PM in a dose-dependent manner (50, 100, and 300 μg/cm^3) for 24 h. (A) The mRNA level of AREG was increased in a dose-dependent manner according to the results of real-time PCR. (B, C) Immunofluorescence analysis of HBECs treated with different doses of

PM over 24 h. (D) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was increased in a dose-dependent manner according to the results of real-time PCR, which indicated that PM exposure promoted inflammatory responses and mucus hypersecretion in HBECs. Values represent the mean ± SEM; *, P<0.05 or **, P<0.01, compared with the control group; n=3. HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure 2. PM induces activation of the EGFR-AKT/ERK pathway in HBECs. The HBECs were first treated with 300 μg/cm^3 PM for different durations (0.25, 0.5, 1, 3, and 6 h), and then treated with different doses of PM (50, 100, and 300 μg/cm^3) for 1 h. (A) Phosphorylation of EGFR, AKT, and ERK in HBECs treated with PM in a time-dependent manner were detected by western blot. The optical densities of protein bands are shown in (B). Values represent the mean ± SEM; *, P<0.05 or **, P<0.01, compared with the control group; n=3. (C) Phosphorylation of EGFR, AKT, and ERK in HBECs treated with PM in a dose-dependent manner were detected by western blot. The optical densities of protein bands are shown in (D). Values represent the mean ± SEM; *, P<0.05 or **, P<0.01, compared with the control group; n=3. HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure 3. AREG silencing attenuates PM-induced inflammatory responses, mucus hypersecretion, and activation of the EGFR-AKT/ERK pathway in HBECs. The AREG siRNA was transferred to HBECs 24h prior to PM exposure. (A) The most efficient AREG siRNA was selected using real-time PCR. (B) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was inhibited by AREG siRNA in PM-stimulated HBECs according to the results of real-time PCR. (C) Phosphorylation of EGFR, AKT, and ERK in HBECs was inhibited by AREG siRNA in PM-stimulated HBECs based on western blot analysis. The optical densities of protein bands are shown in (D). Values represent the mean ± SEM; **, P<0.01, compared with the PM + NC siRNA group; n=3. AREG, amphiregulin; HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure 4. Exogenous AREG promotes PM-induced inflammatory responses, mucus hypersecretion, and activation of the EGFR-AKT/ERK pathway in HBECs. The HBECs were treated with recombinant human AREG and PM for 24 h. (A) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was increased by exogenous AREG in HBECs that had been, or had not been exposed to PM according to the results of real-time PCR. Values represent the mean ± SEM; *, P<0.05 or **, P<0.01, compared with the control group; #, P<0.05 or ##, P<0.01, compared with the PM group; n=3. (B) Exogenous AREG induces the phosphorylation of EGFR, AKT, and ERK in a time-dependent manner in HBECs. The optical densities of protein bands are shown in (C). Values represent the mean ± SEM; *, P<0.05 or **, P<0.01, compared with the control group; n=3. (D) Phosphorylation of EGFR, AKT, and ERK in HBECs was enhanced by exogenous AREG in HBECs following PM exposure according to the results of western blot analysis. The optical densities of protein bands are shown in (E). Values represent the mean ± SEM; **, P<0.01, compared with the control group; #, P<0.05 or ##, P<0.01, compared with the PM group; n=3. AREG, amphiregulin; HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure 5. TACE-dependent release of AREG regulates PM-induced inflammation and mucus hypersecretion via binding to the EGFR. The HBECs were pretreated with TAPI-1 or AG1478 before PM exposure. (A) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was inhibited by TAPI-1 pretreatment in PM-exposed HBECs according to the results of real-time PCR. (B) Phosphorylation of EGFR, AKT, and ERK was inhibited by TAPI-1 pretreatment in PM-exposed HBECs according to the results of western blot analysis. The optical densities of protein bands are shown in (C). (D) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was inhibited by AG1478 pretreatment in PM-exposed HBECs according to the results of real-time PCR. (E) Phosphorylation of EGFR, AKT, and ERK was inhibited by AG1478 pretreatment in PM-exposed HBECs based on western blot analysis. The optical densities of protein bands are shown in (F). Values represent the mean ± SEM; **,

P<0.01, compared with the PM group; n=3. AREG, amphiregulin; TACE, tumor-necrosis factor-alpha converting enzyme; HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure 6. Differential effects of PI3K isoforms on PM-induced inflammation and mucus hypersecretion. The HBECs were pretreated with LY294002, PIK-75, GSK2636771, AS-252424, or IC-87114 prior to PM exposure. (A) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was significantly inhibited by LY294002 pretreatment in PM-exposed HBECs according to the results of real-time PCR. (B) Phosphorylation of AKT and ERK was inhibited by LY294002 pretreatment in PM-exposed HBECs according to western blot analysis. The optical densities of protein bands are shown in (C). (D) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was significantly inhibited by PIK-75, but not PI3Kβ, PI3Kγ, or PI3Kδ in PM-exposed HBECs according to the results of real-time PCR. Values represent the mean ± SEM; **, P<0.01, compared with the PM group; n=3. HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure7. EGFR-dependent AKT and ERK pathways regulate PM-induced inflammation and mucus hypersecretion. The HBECs were pretreated with MK-2206 or U0126 prior to PM exposure. (A) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was inhibited by MK-2206 pretreatment in PM-exposed HBECs according to the results of real-time PCR. (B) Phosphorylation of AKT and ERK was detected using western blot in HBECs with MK-2206 pretreatment. The optical densities of protein bands are shown in (C). (D) The mRNA expression of IL-1α, IL-1β, and Muc-5ac was inhibited by U0126 pretreatment in PM-exposed HBECs according to the results of real-time PCR. (E) Phosphorylation of AKT and ERK was detected using western blot in HBECs with U0126 pretreatment. The optical densities

of protein bands are shown in (F). Values represent the mean ± SEM; **, P<0.01, compared with the PM group; n=3. HBEC, human bronchial epithelial cell; PM, particulate matter.

Figure 8. Schematic diagram of the mechanism for the pro-inflammatory role of AREG in PM-induced inflammation and mucus hypersecretion in HBECs. AREG, amphiregulin; TACE, tumor-necrosis factor-alpha converting enzyme; HBEC, human bronchial epithelial cell; PM, particulate matter.

Table1. Primers used in the study.

Genes	Forward	Reverse
AREG	5'-TGGATTGGACCTCAATGACA-3'	5'-AGCCAGGTATTTGTGGTTCG-3'
IL-1α	5'-AGGCTGCATGGATCAATCTGTGTC-3'	5'-AUGGUUCACGCUUCCCAGATT-3'
IL-1β	5'-TGGCAATGAGGATGACTTGT-3'	5'-TGGTGGTCGGAGATTCGTA-3'
Muc-5ac	5'-GAGTACCAGGAGCAGAGCGG-3'	5'-CTGGTGGGTCACACAGTGGT-3'
GAPDH	5'-CCACCCATGGCAAATTCCATGGCA-3'	5'-TCTACACGGCAGGTCAGGTCCACC-3'

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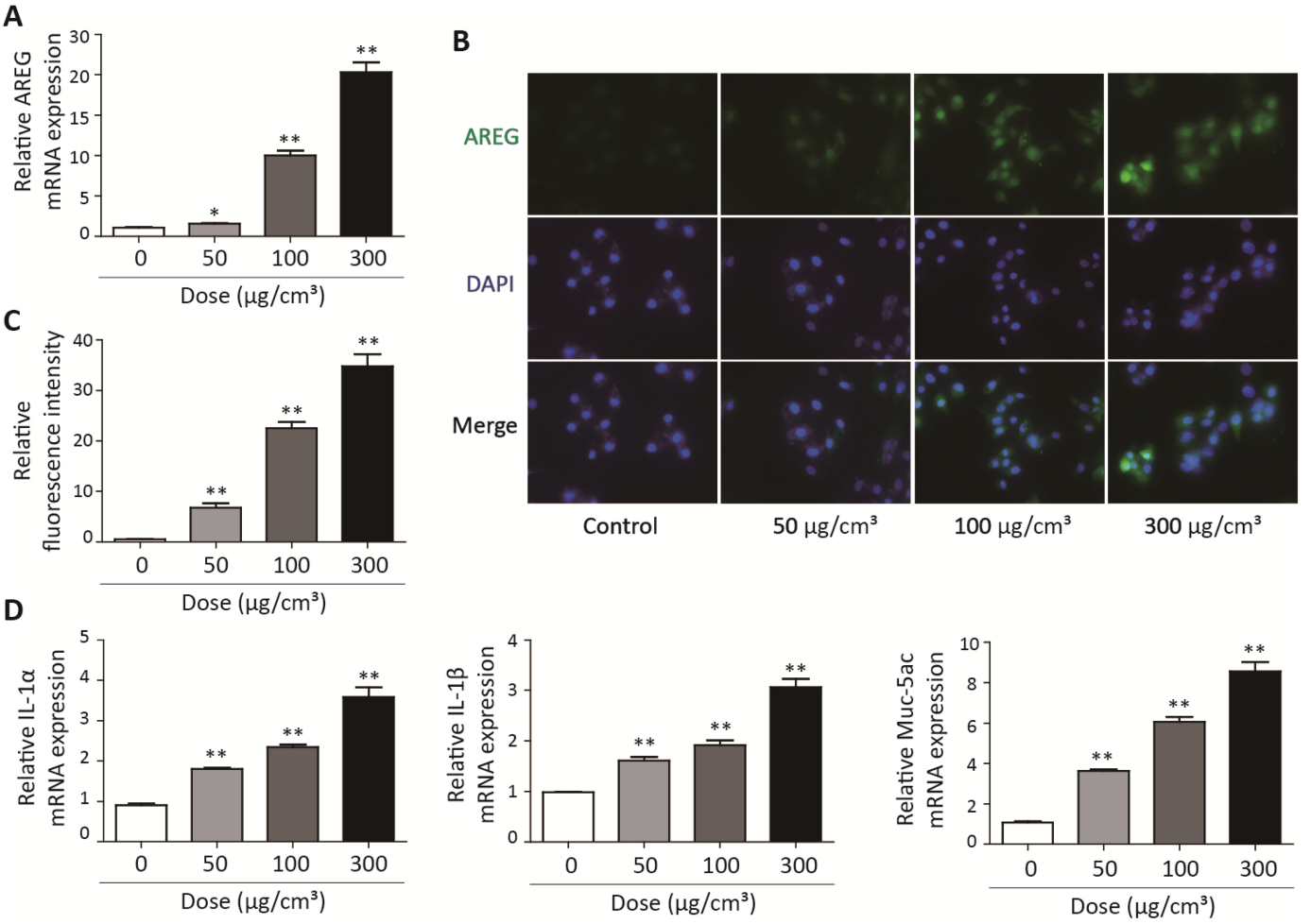
Amphiregulin potentiates airway inflammation and mucus hypersecretion induced by urban particulate matter via the EGFR-PI3Kα-AKT/ERK pathway

Jian Wang[1,#], Mengchan Zhu[1,#], Linlin Wang[1], Cuicui Chen[1], Yuanlin Song[1,*]

Hightlights

1. Particulate matter increased the expression of AREG, and induced inflammation and mucus hypersecretion by upregulating the expression of IL-1α, IL-1β, and Muc-5ac in HBECs.
2. AREG potentiated particulate matter-induced inflammation and mucus hypersecretion by increasing the activation of the EGFR-PI3Kα-AKT/ERK signaling pathway in HBECs.
3. TACE-dependent cleavage and secretion of AREG was a key biological process to induce the positive effects of AREG on particulate matter-induced inflammation and mucus hypersecretion in HBECs.
4. PI3Kα is the dominant class I PI3Ks isoform to regulate particulate matter-induced inflammation and mucus hypersecretion in HBECs.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Relative AREG mRNA expression
30
20
15
10
5
0
*
**
**
0
50
100
300
Dose (μg/cm³)
B
AREG
DAPI
Merge
Control
50 μg/cm³
100 μg/cm³
300 μg/cm³
C
Relative fluorescence intensity
40
30
20
10
0
**
**
**
0
50
100
300
Dose (μg/cm³)
D
Relative IL-1α mRNA expression
5
4
3
2
1
0
**
**
**
0
50
100
300
Dose (μg/cm³)
Relative IL-1β mRNA expression
4
3
2
1
0
**
**
**
0
50
100
300
Dose (μg/cm³)
Relative Muc-5ac mRNA expression
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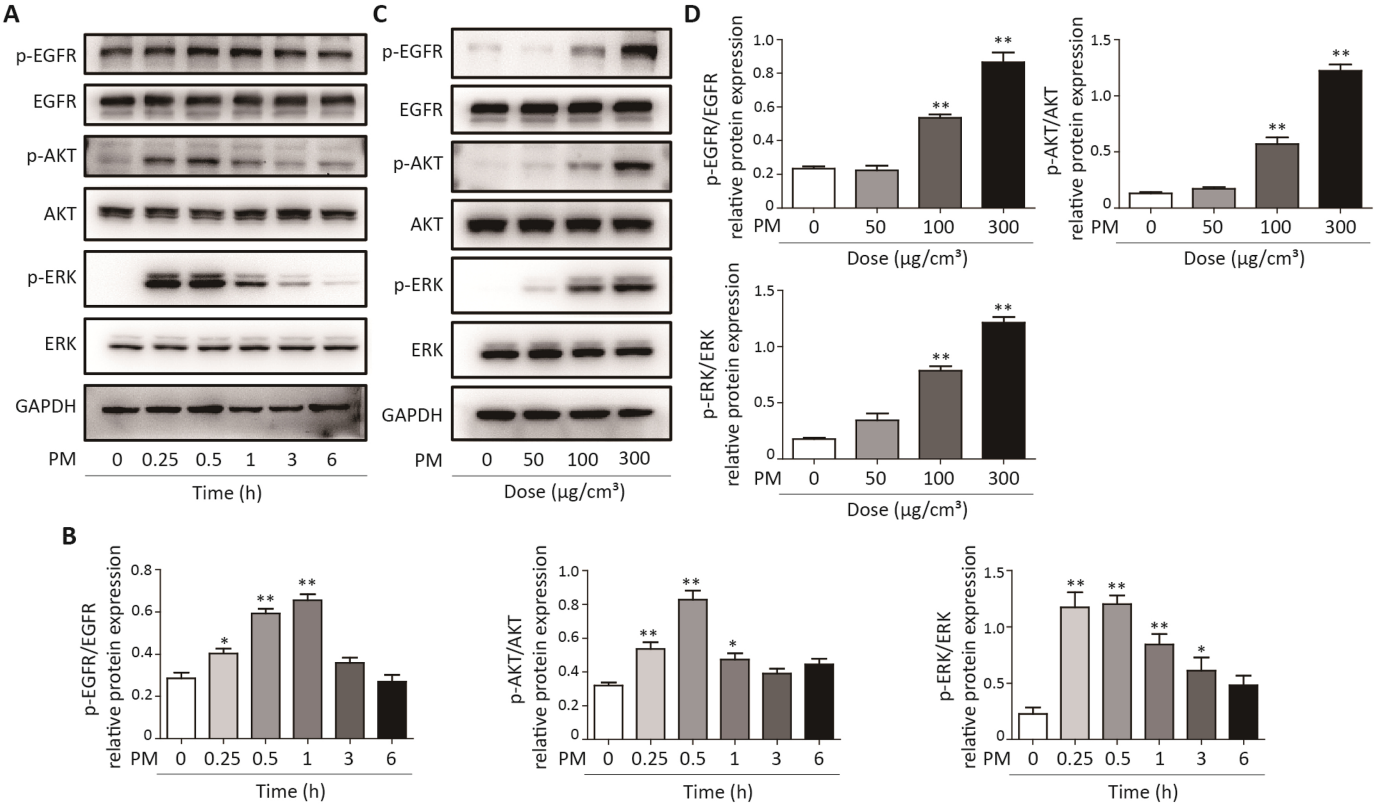
8
6
4
2
0
**
**
**
0
50
100
300
Dose (μg/cm³)

Figure 1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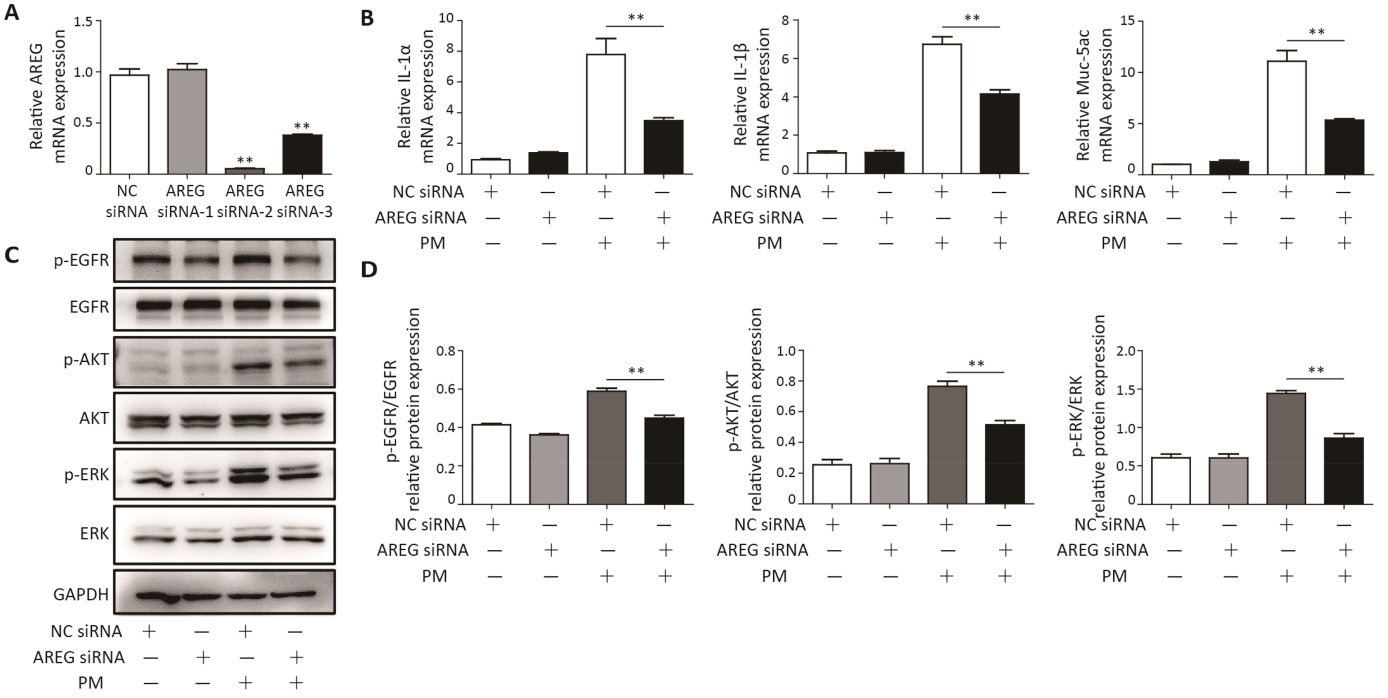
p-EGFR
EGFR
p-AKT
AKT
p-ERK
ERK
GAPDH
PM 0 0.25 0.5 1 3 6
Time (h)
C
p-EGFR
EGFR
p-AKT
AKT
p-ERK
ERK
GAPDH
PM 0 50 100 300
Dose (μg/cm³)
D
p-EGFR/EGFR relative protein expression
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
PM 0 50 100 300
Dose (μg/cm³)
B
p-EGFR/EGFR relative protein expression
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
PM 0 0.25 0.5 1 3 6
Time (h)

Figure 2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1.0
0.5
0
**
**
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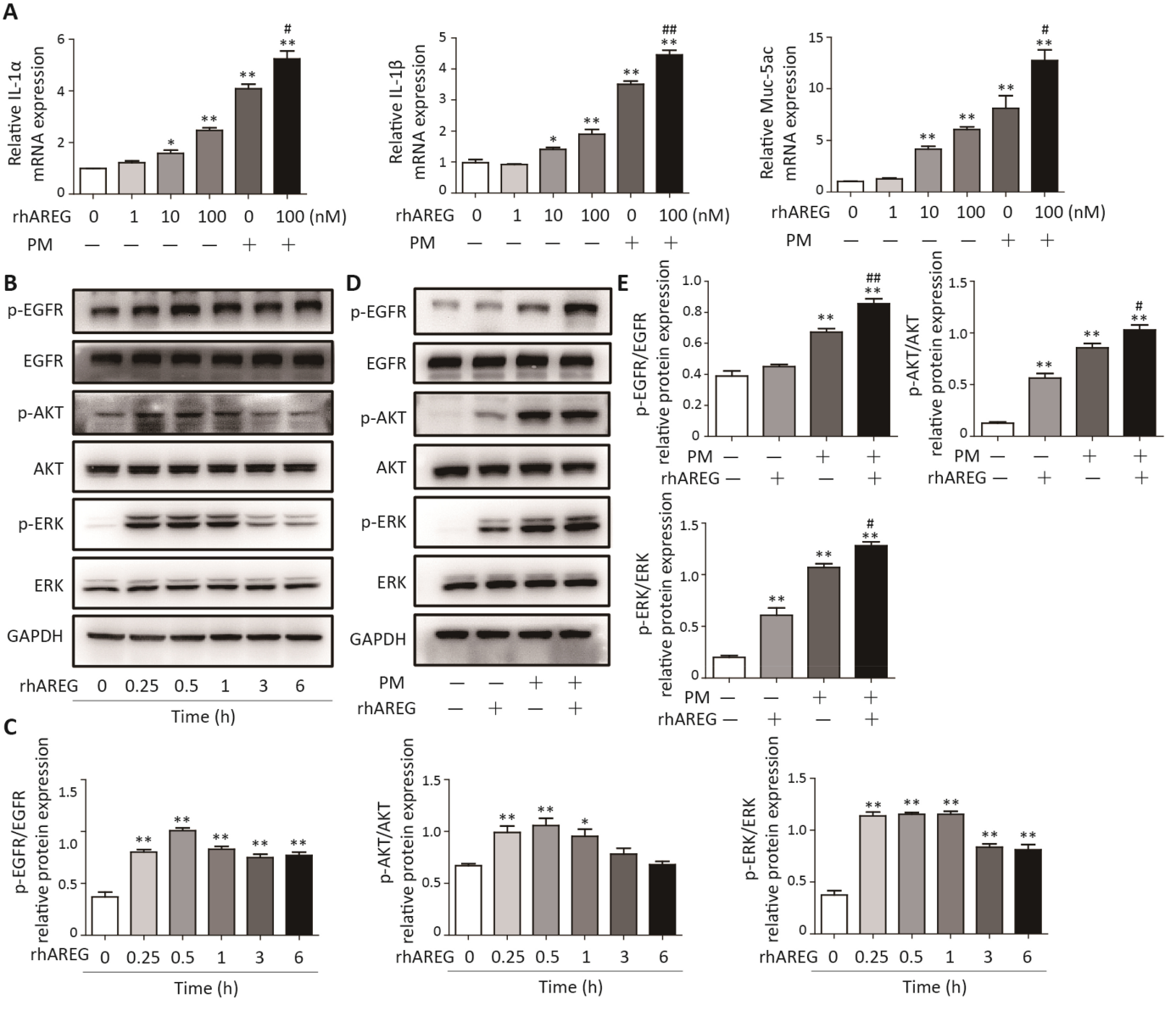
AREG siRNA-1
AREG siRNA-2
AREG siRNA-3
B
Relative IL-1α mRNA expression
Relative IL-1β mRNA expression
Relative Muc-5ac mRNA expression
**
NC siRNA + − + −
AREG siRNA − + − +
PM − − + +
C
p-EGFR
EGFR
p-AKT
AKT
p-ERK
ERK
GAPDH
NC siRNA + − + −
AREG siRNA − + − +
PM − − + +
D
p-EGFR/EGFR relative protein expression
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
**
NC siRNA + − + −
AREG siRNA − + − +
PM − − + +

Figure 3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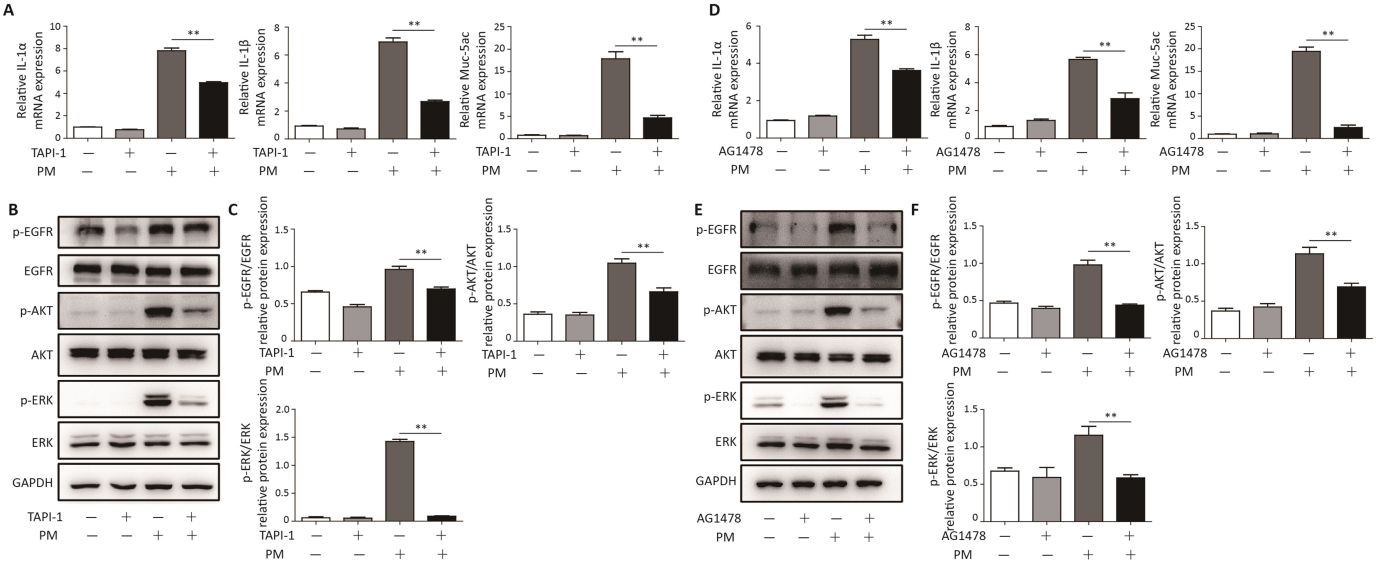
Relative IL-1β mRNA expression
Relative Muc-5ac mRNA expression
rhAREG 0 1 10 100 0 100 (nM)
PM — — — — + +
B
p-EGFR
EGFR
p-AKT
AKT
p-ERK
ERK
GAPDH
rhAREG 0 0.25 0.5 1 3 6
Time (h)
D
PM — — + +
rhAREG — + — +
E
p-EGFR/EGFR relative protein expression
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
C
rhAREG 0 0.25 0.5 1 3 6
Time (h)

Figure 4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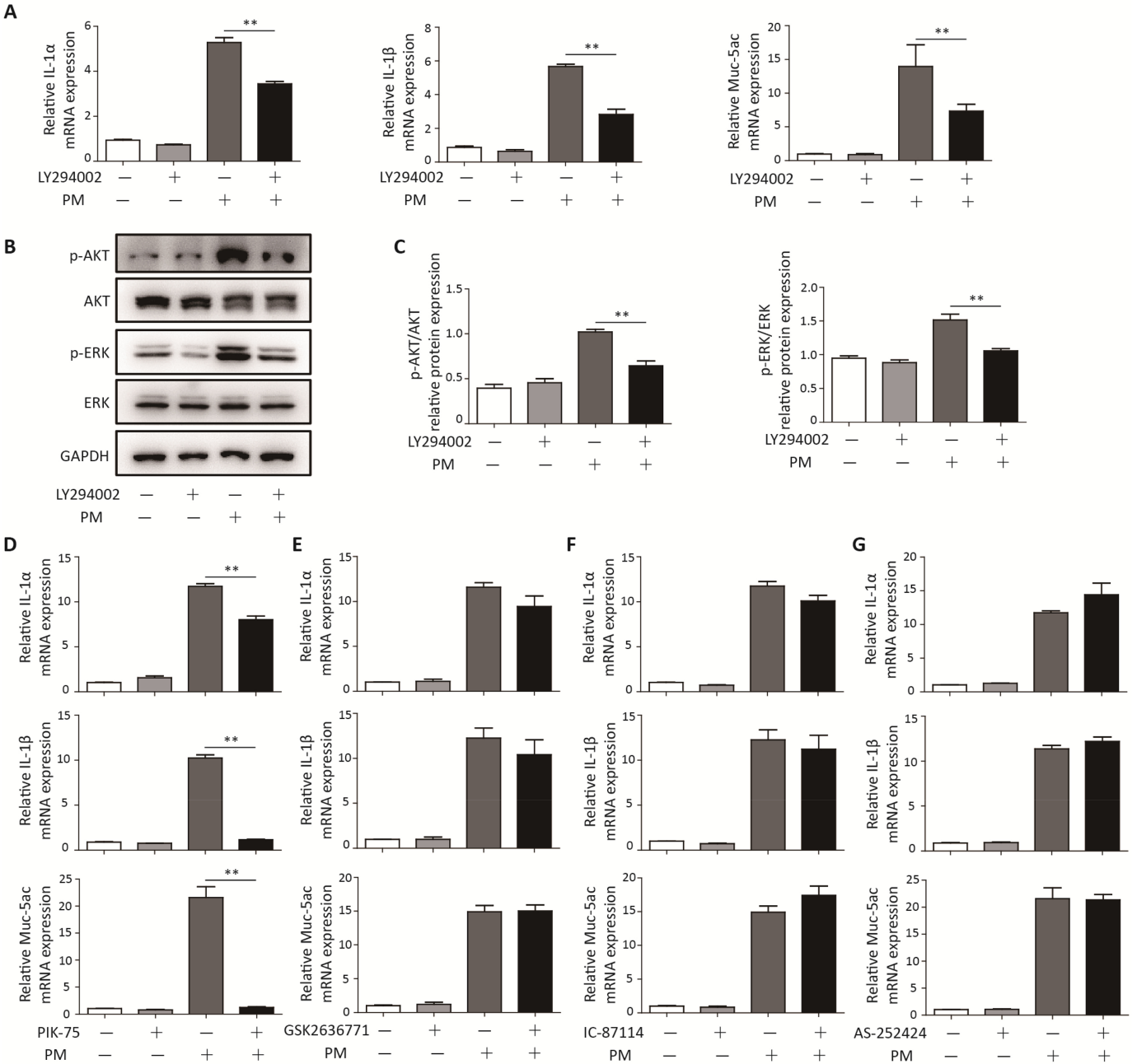
Relative IL-1α mRNA expression
Relative IL-1β mRNA expression
Relative Muc-5ac mRNA expression
TAPI-1
PM
B
p-EGFR
EGFR
p-AKT
AKT
p-ERK
ERK
GAPDH
C
p-EGFR/EGFR relative protein expression
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
D
AG1478
E
F

Figure 5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Relative IL-1α mRNA expression
Relative IL-1β mRNA expression
Relative Muc-5ac mRNA expression
LY294002
PM
B
p-AKT
AKT
p-ERK
ERK
GAPDH
C
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
D
E
F
G
PIK-75
GSK2636771
IC-87114
AS-252424

Figure 6

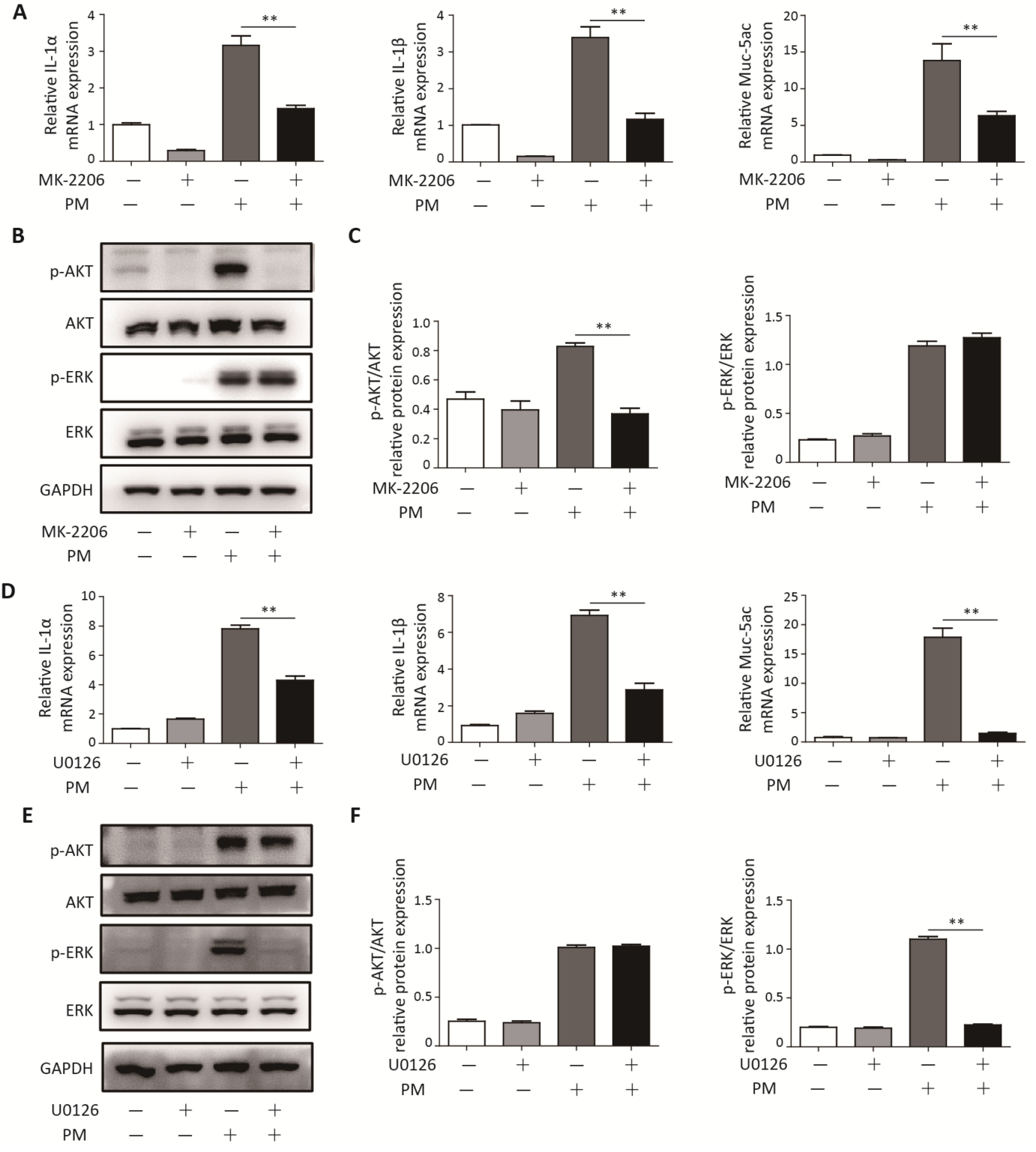
A
Relative IL-1α mRNA expression
Relative IL-1β mRNA expression
Relative Muc-5ac mRNA expression
**
MK-2206
PM
B
p-AKT
AKT
p-ERK
ERK
GAPDH
C
p-AKT/AKT relative protein expression
p-ERK/ERK relative protein expression
D
U0126
E
F

Figure 7

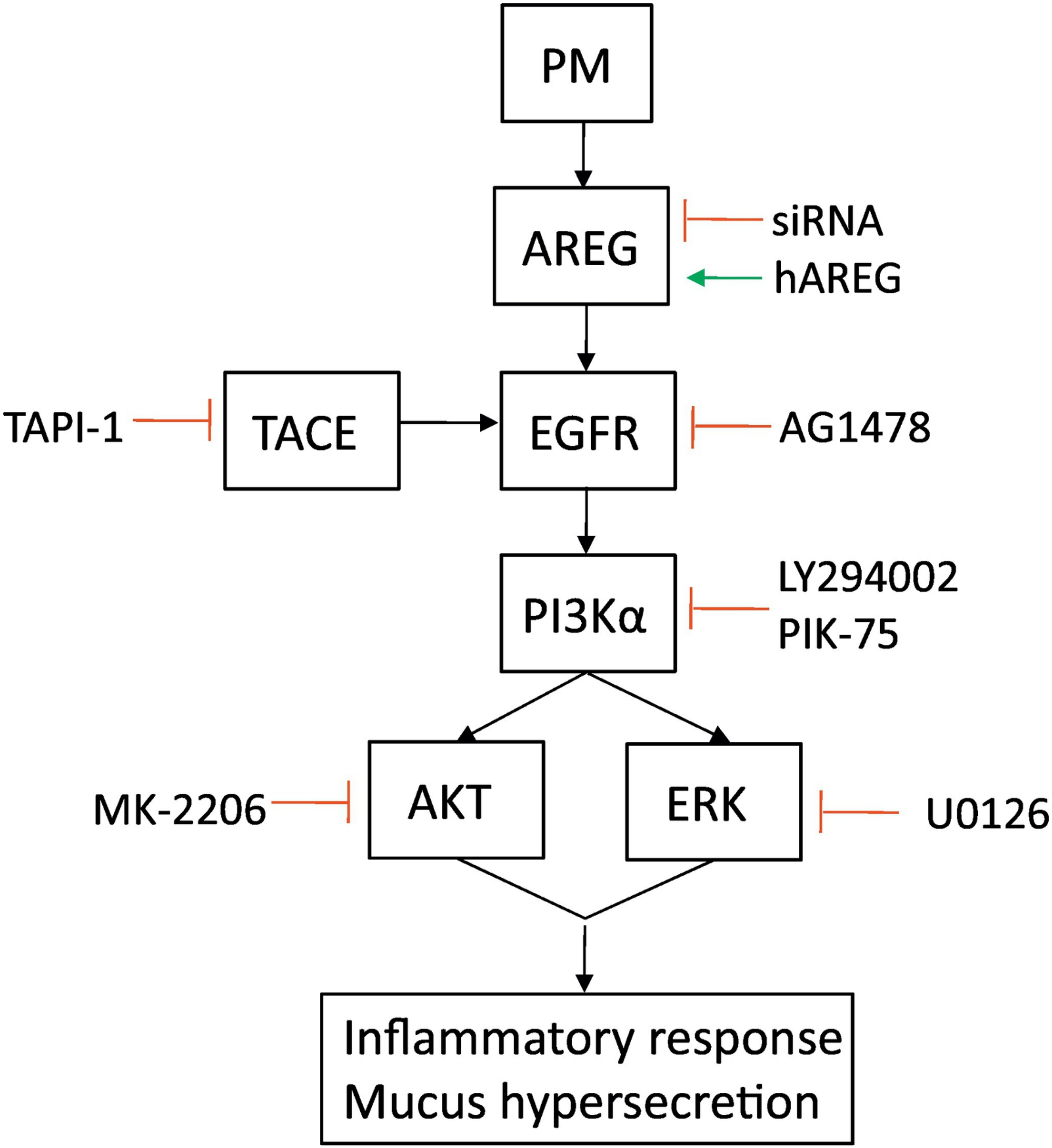
PM
AREG
siRNA
hAREG
TAPI-1
TACE
EGFR
AG1478
PI3Kα
LY294002
PIK-75
MK-2206
AKT
ERK
U0126
Inflammatory response
Mucus hypersecretion

Figure 8