PlGF mediates neutrophil elastase-induced airway epithelial cell apoptosis and emphysema

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Abstract

Background: Chronic pulmonary obstructive disease (COPD) has become the fourth leading cause of death worldwide. Cigarette smoking induces neutrophil elastase (NE) and contributes to COPD, but the detailed mechanisms involved are not fully established. In an animal model of pulmonary emphysema, there are increased expressions of placenta growth factor (PlGF) and lung epithelial (LE) cell apoptosis. This study hypothesized that excessive NE may up-regulate PlGF and that PlGF-induced LE apoptosis mediates the pathogenesis of pulmonary emphysema.

Methods: Human bronchial epithelial cells, BEAS-2B, and primary mouse type II alveolar epithelial cells were treated with NE. The PlGF promoter activity was examined by luciferase activity assay, while PlGF expression and secretion were evaluated by RT-PCR, Western blotting, and ELISA. Both cell lines were treated with PlGF to evaluate its effects and the downstream signaling pathways leading to LE cell apoptosis. PlGF knockout and wild-type mice were instilled with NE to determine the roles of PlGF and its downstream molecules in NE-promoted mice pulmonary apoptosis and emphysema phenotype.

Results: The transcriptional factor, early growth response gene-1, was involved in the NE-promoted PlGF promoter activity, and the expression and secretion of PlGF mRNA and protein in LE cells. PlGF-induced LE cell apoptosis and NE-induced mice pulmonary apoptosis and emphysema were mediated by the downstream c-Jun N-terminal kinase (JNK) and protein kinase C (PKC)δ signaling pathways.

Conclusion: The NE-PlGF-JNK/PKCδ pathway contributes to the pathogenesis of LE cell apoptosis and emphysema. PlGF and its downstream signaling molecules may be potential therapeutic targets for COPD.

Keywords: Placenta growth factor, Chronic pulmonary obstructive disease, Neutrophil elastase, Apoptosis, Emphysema

Background

Chronic pulmonary obstructive disease (COPD) is predicted to become the fourth leading cause of death worldwide by 2030 [1,2]. Due to the aging population and increasing number of smokers, the burden of medical and social resources for COPD is estimated to be US$47 trillion by 2030 [3]. Although there are many mediators (i.e., inflammatory cells, lipids, reactivate oxygen species, nitric oxide, peptides, chemokines, cytokines, growth factors, and proteases) and cellular pathways (e.g., inflammation, apoptosis, senescence and repair) involved in the pathogenesis of COPD, increasing evidence indicates that proteases provide vital contributions to all mediators and cellular pathways [4,5]. However, to date, the detailed pathogenic mechanisms of protease-mediated COPD are not fully understood [3,6].

In developed countries, the major factor for the pathogenesis and progression of COPD is cigarette smoke (CS). Exposure to CS results in chronic inflammation, elevated oxidative stress, and protease-anti-protease imbalance within the respiratory system [7]. The protease-anti-protease imbalance is triggered by the infiltration of inflammatory cells like neutrophils, macrophages, and CD8+ T lymphocytes.
[7–11]. Proteolytic enzymes of neutrophils and macrophages, neutrophil elastase (NE), and matrix metalloproteinase (MMP)-12, degrade their respective inhibitors. Thus, the interaction promotes protease-anti-protease imbalance and destroys the pulmonary parenchyma with alveolar space dilatation, i.e. emphysema, which is a major component of COPD [12].

Neutrophil elastase is a secreted serine protease that degrades extracellular matrix like elastin, which contributes to the recoil capacity of alveoli [13]. Other than proteolytic activity, NE up-regulates elafin, interleukin-8, MUC4, and MUC5AC, and promotes the secretion of mucin in LE cells [14–18]. Excessive NE also results in LE cell apoptosis through protease-activated receptor (PAR)-1, which is abrogated by treatment with retinoic acid [19,20].

Apoptosis of LE cells results in the loss of lung parenchyma and is a potential mechanistic pathway for emphysema and COPD [21]. Placenta growth factor (PIGF) induces apoptosis of type II alveolar epithelial cells (AEC II) such that PIGF transgenic mice develop a phenotype of pulmonary emphysema [22]. PIGF is a member of the vascular endothelial growth factor family that promotes angiogenesis [23,24]. PIGF expression is abundant in the placenta, heart, lungs, thyroid, brain, and skeleton muscle during fetal development, but declines in adulthood [25]. Higher levels of PIGF have been shown in serum and broncho-alveolar lavage (BAL) fluid of COPD patients and the PIGF levels is inversely proportional to lung function deterioration [26]. Porcine pancreatic elastase (PPE), a recombinant porcine elastase for the animal model of emphysema, has also been shown to increase PIGF expression in LE cells and promote LE cells apoptosis [27]. However, the role of NE in human COPD has not been established.

Under the hypothesis that NE, like PPE, up-regulates PIGF expression and leads to LE cell apoptosis and pulmonary emphysema. This study demonstrates that the NE-promoted PIGF expression and secretion in LE cells and lungs. Early growth response gene (Egr)-1 is a transcriptional factor responsible for the up-regulation of PIGF by NE in LE cells. PIGF induces apoptosis through the c-Jun N-terminal kinase (JNK) and protein kinase C (PKC)δ signaling pathways. Ablation of PIGF protects mice from NE-induced pulmonary apoptosis and emphysema. Thus, NE-induced PIGF and the downstream JNK/PKCδ signaling pathways contribute to the pathogenesis of pulmonary emphysema and COPD. Both PIGF and its downstream signaling pathways may be potential therapeutic targets for COPD.

Materials and methods
Reagents
Rabbit antibodies for phosphor-P38 MAPK (p-P38 MAPK), P38 MAPK, MTF-1, p-JNK and p-PKCδ were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies for PIGF, JNK, PKC, and Egr-1, mouse and human PIGF siRNA, mouse and human PKCδ siRNA, and the corresponding scramble siRNA were purchased from Santa Cruz (Santa Cruz, CA, USA), while NE was purchased from Abcam (Cambridge, MA, USA). Trizol reagent, SuperScript III Reverse Transcriptase and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Mouse antibody for beta-actin and rabbit antibody for HIF-1alpha were purchased from Genetex (Irvine, CA, USA). Human and mouse recombinant PIGF protein and an enzyme-linked immuno-sorbent assay (ELISA) kit were obtained from R and D Systems (Minneapolis, MN, USA).

A dual-luciferase reporter assay system was obtained from Promega (Madison, WI, USA). Hematoxylin and Eosin, Chromatin immuno-precipitation (ChIP) Assay Kit, and EZ-Zyme Chromatin Prep Kit were purchased from Merck-Millipore (Boston, MA, USA). An in situ cell Death Detection Kit and X-tremeGENE HP DNA Transfection Reagent were purchased from Roche (Mannheim, Germany). The FITC Annexin V apoptosis detection Kit I was obtained from BD Biosciences (San Jose, CA, USA). The JNK inhibitor, SP600125, was obtained from Enzo Life Science (Plymouth Meeting, PA, USA). A SuperSensitive Polymer-HRP IHC Detection System was purchased from Biogenex (Fremont, CA, USA).

Animals
This study conformed to the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85–23, revised 1996). All of the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Laboratory Animal Center, College of Medicine and Public Health of National Taiwan University. Eight-week-old male C57BL/6 wild type (WT) mice were purchased from the Laboratory Animal Center, College of Medicine and College of Public Health, National Taiwan University. The PIGF knockout (KO) mice in B6 background were provided by Dr. Po-Nien Tsao (National Taiwan University, Taiwan).

Cell culture
Human bronchial epithelial cells, BEAS-2B (ATCC number CRL-9609), were cultured in F12 nutrient mixture (Carlsbad, CA, USA) with 0.5 ng/ml recombinant epidermal growth factor, 500 ng/ml hydrocortisone, 0.005 mg/ml insulin, 0.035 mg/ml bovine pituitary extract, 500 nM ethanolamine, 500 nM phosphoethanolamine, 0.01 mg/ml transferrin, 6.5 ng/ml 3, 3′, 5-triiodothyronine, 500 ng/ml epinephrine, 0.1 ng/ml retinoic acid, 10% FCS 100 unit/ml penicillin, and 100 μg/ml streptomycin in a humidified 95%
air-5% CO₂ incubator at 37°C. Mouse primary type II alveolar epithelial cells (AEC II) and culture medium were purchased from chi scientific (Maynard, MA, USA). Primary normal human bronchial epithelial (NHBE) cells were kindly provided by Dr. Reen Wu at University of California, Davis.

**Plasmids**

Human genomic DNA was extracted from BEAS-2B by a Quick-gDNA MiniPrep kit (Zymo Research, CA, USA). The 2.0 kb human PlGF promoter region was amplified from human genomic DNA using polymerase chain reaction (PCR) performed with Hi Fi Taq DNA polymerase (Geneaid, Taipei, Taiwan) as follows: 2 minutes at 94°C, then 15 sec at 94°C, 30 sec at 59°C, and 2 min and 30 sec at 72°C for 35 cycles. The primers for 2.0 kb human PlGF promoter region were 5'-GCG GTAC CCA AAC TCA TAC ACA ATA GAC-3' (forward primer; italic, KpnI site) and 5'-TAA AGCT TCC GTA GGT AAG GCT GTG GCT-3' (reverse primer; italic, HindIII site). The amplified DNA fragments were cloned into pGL3 vector (Promega, WI, USA) and the sequences were confirmed by DNA sequence analysis. The pGL3 with mouse PlGF promoter was as previously described [27].

**Enzyme-linked immuno-sorbent assay (ELISA)**

Cellular medium from BEAS-2B and AEC II, and BAL fluid from mice were analyzed by a PlGF ELISA kit (R & D, MN, USA) according to the manufacturer’s instructions.

**Luciferase reporter assay**

The BEAS-2B and AEC II were co-transfected with the pGL3-PlGF promoter and pRenilla for 24 h via Lipofectamine 2000 and X-tremeGENE HP DNA Transfection Reagent, and then collected and analyzed on a dual-luciferase reporter assay system (Promega, WI, USA) according to the manufacturer’s instructions. The BEAS-2B and AEC II were analyzed on a Leica DM 4000B microscope (Leica, Solms, Germany).

**Protein extraction and immuno-blot analysis**

The BEAS-2B and AEC II were lysed using RIPA lysis buffer (Genestar, Taipei, Taiwan), containing 1% NP-40, 0.1% SDS, 150 mM sodium chloride, 0.5% sodium deoxycholate, and 50 mM Tris with a protease inhibitor cocktail (Bionovas, Toronto, Canada) and PhosSTOP (Roche, Basilea, Switzerland). The cell lysates were centrifuged at 12,000 rpm for 5 min and the resulting supernatant was collected.

The extracted protein was quantified by protein assay. Equal amounts of protein were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with various primary antibodies and then incubated with the corresponding secondary antibodies. The protein bands were detected using an Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) and quantified by the ImageQuant 5.2 software (Healthcare Bio-Sciences, PA, USA).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

The BEAS-2B and AEC II, and OCT-embedded lung tissue from the mice were analyzed for the apoptosis level using an in situ cell Death Detection Kit (Roche, Basilea, Switzerland) according to the manufacturer’s instructions. Fluorescence-positive cells were photographed by a Leica DM 4000B microscope (Leica, Solms, Germany).

**Flow cytometry analysis**

The BEAS-2B and AEC II were analyzed on a FITC Annexin V apoptosis detection Kit I (Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The FITC-positive cells were analyzed using a FACs Calibur flow cytometer (Becton Drive, NJ, USA).

**Immunohistochemistry (IHC) assay**

Paraffin was removed from paraffin-embedded tissue sections by xylene, dehydrated by ethanol, and rehydrated by PBS. After treatment with 3% H₂O₂, the sections were applied to a SuperSensitive Polymer-HRP IHC Detection System (Biogenex, CA, USA) and incubated with PlGF, p-JNK, and p-PKCδ antibodies as primary antibodies. The stained-sections were photographed using a Leica DM 4000B microscope (Leica, Solms, Germany).

**Hematoxylin and eosin (H and E) staining**

Paraffin was removed from paraffin-embedded tissue sections by xylene, dehydrated by ethanol, and rehydrated by PBS. Sections stained with H and E were photographed by a Leica DM 4000B microscope (Leica, Solms, Germany).

**NE-induced emphysema**

The dose of NE was four-fold higher than that of porcine pancreatic elastase according to previous report [28] and the methodology of intra-tracheal instilling NE was performed as previously described [29]. Briefly, eight-week-old mice were intra-tracheally given saline (CON), 400 μU/ml NE (NE), 400 μU/ml NE with 50 mg/kg JNK inhibitor SP600125 (NE SP), 3 mg/kg scramble siRNA (NE Si-Sc), 3 mg/kg mouse PKCδ siRNA (NE Si-PK) and 3 mg/kg PlGF siRNA (NE Si-Pl) weekly for one month. The dose of siRNA instillation was according to a previous study [27,30]. Each experimental group had five mice and the processing of lung tissues and BAL fluid were performed as previously described [27,29].
Reverse-transcriptional (RT)-PCR assay
Total RNA of BEAS-2B and AEC II were extracted by Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. Total RNA (5 μg) was used in the RT reactions using a SuperScript III Reverse Transcriptase kit (Invitrogen, CA, USA) according to the manufacturer’s instructions to synthesize the cDNA. The human PlGF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA fragments were amplified from the cDNA by PCR, performed with Dream Taq DNA polymerase (Fermentas, MA, USA) as follows: 5 min at 95°C, then 30 sec at 98°C, 30 sec at 59°C, and 1 min at 72°C for 35 cycles. The primers for 164 bp human PlGF cDNA fragment were 5′-GGC GAT GAG AAT CTG CAC TGT-3′ and 5′-GAA GAT GAA GCC GGA AAG GTG-3′. The primers for 530 bp human GAPDH cDNA fragment were 5′-GGG CGC CTG GTC ACC AGG GCT G-3′ and 5′-GGG GCC ATC CAC AGT CTT CTG-3′. The primer sets for mouse PlGF and GAPDH was as previously described [27].

Chromatin immuno-precipitation (ChIP)
Genomic DNA fragment from BEAS-2B were prepared by the EZ-Zyme Chromatin Prep Kit (Millipore, MA, USA) and analyzed using the Chromatin immuno-precipitation (ChIP) Assay Kit (Millipore, MA, USA) to evaluate the associated levels of Egr-1 and PlGF promoter regions. The antibody of Egr-1 was used for

Figure 1 NE-induced PlGF promoter activity is mediated by Egr-1. (A, E, and F) The placenta growth factor (PlGF) promoter activity was evaluated by luciferase activity. (B) Hypoxia inducible factor (HIF)-1alpha and metal-regulatory transcription factor (MTF)-1, (C) Egr-1, and β-actin were analyzed by Western blot analysis. (D) The association of Egr-1 and PlGF promoter fragment was evaluated by chromatin immuno-precipitation assay. Data are presented as mean ± SEM. *p < 0.05 vs. vehicle-treated group.
Figure 2 NE-induced PlGF expression and secretion are mediated by Egr-1. (A-C) The mRNA level of PlGF was determined by reverse-transcriptional polymerase chain reaction (RT-PCR) with primer sets for PlGF and GAPDH cDNA. (D-F) Cellular lysates were also subjected to Western blot analysis with antibodies for PlGF and β-actin. (G-I) The PlGF in the culture medium was detected by enzyme-linked immuno-sorbent assay (ELISA). Data are presented as mean ± SEM. *p < 0.05 vs. vehicle-treated group.
immuno-precipitation and the primer set (5′-CAC TTT CCA AGA ATG CCT ATG TCC ATT C-3′ and 5′-TTA AGC TTC CGT AGG TAA GGC TGT GGC T-3′) were used to amplify the human PI GF promoter fragment according to the manufacturer's instructions.

Statistical analysis
The results were presented as mean ± SEM from five independent experiments and animals. The Mann–Whitney test was used to compare two independent groups. Kruskal–Wallis with Bonferroni post hoc analysis was used for multiple testing. Statistical analyses were performed using the SPSS version 8.0 (SPSS Inc., IL, USA). Statistical significance was set at p < 0.05.

Results
NE increased PI GF promoter activity by Egr-1 in LE Cells
The results revealed that treatment with 100–300 mU/ml NE for 24 h significantly increased PI GF promoter activity dose-dependently in human bronchial epithelial cells, BEAS-2B, and primary mouse type II alveolar epithelial cell (AEC II) (Figure 1A). Previous studies indicated that several conserved metal response elements (MRE) and hypoxia response elements (HRE) reside in mouse or human PI GF promoter regions [31,32]. However, treatment with 300 mU/ml NE did not alter the expression of mental-regulatory transcription factor (MTF)-1 and hypoxia inducible factor (HIF)-1α (Figure 1B).

There was a conserved Egr-1 response element in the human and mouse PI GF promoter regions near the transcriptional start site [32,33]. Western blotting revealed that 300 mU/ml NE challenge transiently increased Egr-1 expression in BEAS-2B (Figure 1C). By ChIP, treatment of 300 mU/ml NE for 1 h triggered the binding of Egr-1 and PI GF promoter fragments in BEAS-2B (Figure 1D) and pre-treatment with Egr-1 siRNA inhibited the NE-increased PI GF promoter

Figure 3 PI GF activates JNK and PKCδ signaling pathways in LE cells. (A–C) AEC II and (D) BEAS-2B were treated with 100 ng/ml recombinant human mouse PI GF respectively for 0–24 h. Cellular lysates were subjected to Western blot analysis with antibodies for phosphorylated p38 MAPK (p-p38 MAPK) and p38 MAPK (p), phosphorylated JNK (p-JNK) and JNK (p), phosphorylated PKCδ (p-PKCδ) and PKCδ (p and d). Data are presented as mean ± SEM. *p < 0.05 vs. vehicle-treated group.
activity in BEAS-2B and AEC II (Figures 1E and F). Thus, NE increased PlGF promoter activity through the association of Egr-1 and the PlGF promoter fragment.

NE increased PlGF expression in LE Cells
NE (100 mU/ml) had been reported to up-regulate elafin expression in A549 cells [14] and PlGF was majorly secreted by AEC II [22,34]. To test whether NE could induce PlGF expression, BEAS-2B and AECII were treated with of 0–300 mU/ml NE for 24 h. PlGF mRNA and protein level were increased after NE challenge in a dose-dependent manner and Egr-1 siRNA pretreatment abrogated the NE-induced PlGF mRNA (Figure 2A-C) and protein (Figure 2D-F) expressions in BEAS-2B and AECII. Moreover, Egr-1 siRNA also blocked the NE-induced PlGF secretion in medium of BEAS-2B and AECII (Figure 2G-I).

Moreover, NE increased the PlGF expression in endothelial cell but not in fibroblast cell (Additional file 1 and Additional file 2: Figures S1A and S1B). Taken together, other than natural activity of proteolysis, NE increased the PlGF expressions and promoted PlGF secretion.

PIGF induced apoptosis in LE Cells via JNK and PKC\(\delta\) signaling pathways
A previous study indicated that 100 ng/ml PlGF induced MLE-15 cell apoptosis with an unknown mechanism [22]. It has been previously demonstrated that PlGF increased apoptosis in MLE-15 cells and BEAS-2B via JNK and p38 mitogen-activated protein kinase (MAPK) signaling pathways [27,35]. In order to confirm and explore the mechanisms underlying PIGF-induced LE cells apoptosis, BEAS-2B and AEC II were treated with 100 ng/ml recombinant PlGF for 24 h.

Although the results of Western blot analysis revealed that PlGF didn’t activate p38 MAPK significantly, PlGF induced a prolonged and enhanced phosphorylation of JNK and PKC\(\delta\) in AEC II (Figure 3A-C). PIGF also activated PKC\(\delta\) pathways in BEAS-2B (Figure 3D). Blockade of JNK or PKC\(\delta\) signaling by JNK inhibitor, SP600125, or transfection with PKC\(\delta\) siRNA had no effect on PIGF-activated PKC\(\delta\) or JNK (Additional file 3: Figure S2), suggesting no crosstalk between PIGF-activated JNK and PKC\(\delta\) signaling pathways.

Further evaluating the roles of JNK and PKC\(\delta\) in PIGF-induced apoptosis, BEAS-2B and AEC II were pretreated with JNK inhibitor or transfected with PKC\(\delta\)
siRNA to block the PI GF down-stream signaling pathways, then treated with 0–100 ng/ml PI GF for 24 h. Results of flow cytometry assay (Figure 4A) and TUNEL assay (Figure 4B) indicated that first, exogenous PI GF dose-dependently increased BEAS-2B and AEC II apoptotic levels and second, the JNK and PKCδ signaling pathways played crucial roles in PI GF-stimulated LE cell apoptosis.

The impact of NE-induced endogenous PI GF on NE-induced LE cell apoptosis was further evaluated in normal human bronchial epithelial cells (NHBE) with serum-free medium, which was the applicable condition for NE-digestion. This study also further proved that NE caused NHBE apoptosis and blocked endogenous PI GF signaling by VEGFR1 neutralized antibody, which attenuated the NE-induced NHBE apoptosis and NE-activated JNK and PKCδ signaling pathways (Additional file 1 and Additional file 4: Figure S3).

**Intra-tracheal instillation of NE increased PI GF expression and secretion and activated downstream JNK and PKCδ signaling pathways**

The role of PI GF in NE-induced LE cells apoptosis and emphysema was further confirmed in an animal model. Wild-type (C57BL/6) and PI GF KO mice were intra-tracheally treated with saline (CON) or 400 mU/ml NE (NE) weekly for one month. The pathology of the NE-treated mice showed elevated PI GF expression in alveolar epithelial cell (Figure 5A) and adjacent endothelial cells than controls (Additional file 2: Figure S1C). Moreover, NE-treated mice displayed more phosphorylated JNK and PKCδ levels than the control mice (Figure 5A).

In contrast, ablation of PI GF limited the expression of PI GF and blocked the NE instillation-induced activation of JNK and PKCδ (Figure 5B). The BAL fluid from NE-treated mice also had higher PI GF levels compared to the control mice. However, there was a lack of PI GF in
KO mice (Figure 5C). These results demonstrated that NE instillation increased the expression and secretion of PlGF, as well as the activation of JNK and PKCδ in pulmonary cells.

**PlGF and PlGF-activated JNK and PKCδ pathways were involved in NE-induced apoptosis and emphysema in mice**

To evaluate the roles of PlGF and JNK/PKCδ signaling in NE-induced apoptosis and emphysema in an animal model, 50 mg/kg of SP600125, 3 mg/kg scramble siRNA, 3 mg/kg PKCδ siRNA, or 3 mg/kg PlGF siRNA were co-treated with NE installation (NE SP, NE Si-Sc, NE Si-PK, or NE Si-Pl) on WT and PlGF KO mice weekly for one month. TUNEL assay indicated more abundant apoptotic cells in the pulmonary tissue of NE-treated mice than control mice (Figures 6A and E). In contrast, the ablation of PlGF protected mice from NE-induced pulmonary cell apoptosis (Figure 6C and E). Moreover, NE-treated mice had the emphysema phenotype with enlargement of the alveolar space (Figure 6B), as evaluated by the mean linear intercept (MLI) (Figure 6F). On the other hand, ablation of PlGF protected mice from NE-induced pulmonary destruction (Figure 6D and F). Furthermore, blocking the JNK and PKCδ signaling pathways (NE SP and NE Si-PK) and silencing of PlGF (NE Si-Pl) abrogated the levels of NE-induced pulmonary apoptosis (Figure 6A and E) and attenuated the airspace enlargement in mice (Figure 6B and F). Thus, the animal model of elastase-instillation further confirmed that the NE-increased pulmonary PlGF and the PlGF-activated JNK/PKCδ signaling pathways were involved in NE-induced pulmonary apoptosis and emphysema in vivo.

**Discussion**

There are several conserved trans-elements within the human and mouse PlGF promoter regions, including MRE and HRE [31,32]. Treatment with PlGF does not...
affect the expressions of MTF-1 and HIF-1α, which are the binding proteins for MRE and HRE. A conserved Egr-1 response element (CCCCGCCCC) [36] is observed near the transcriptional start site in both mouse and human PlGF promoter. Egr-1 is a rapid response transcription factor for UV and cigarette smoke stimuli that up-regulates several genes, including PTEN, microtubule-associated protein-1 light chain 3, and PAR-1 in LE cells [36-39]. The Egr-1-upregulated down-stream genes mediate various cellular functions like cell growth, proliferation, differentiation, and apoptosis [39]. Egr-1 also has an impact on the pathogenesis of acute lung injury [40]. A previous study has demonstrated that NE inhibitors decrease ventilator-induced Egr-1 expression [41]. In the present study, NE promotes the transient expression of Egr-1, which is involved in NE-induced PlGF expression.

The present study demonstrates that NE-induced PlGF promotes LE cell apoptosis, which corroborate the results of a previous study [22]. However, unlike previously established mechanisms of NE-induced LE cell apoptosis [19,20], this study is the first to show that NE-induced LE cell apoptosis through PlGF and PlGF-mediated downstream JNK and PKCδ signaling pathways. The results of NHBE cells further indicate that NE-promoted endogenous PlGF contributes to LE cell apoptosis. Furthermore, NE up-regulates PlGF in endothelial cells and in LE cells. The PlGF-induced LE cell apoptosis may work through both autocrine and paracrine mechanism. In addition, it is interesting to know that the up-regulation of PlGF is identified in an ovalbumin-induced asthma mice model wherein PlGF promotes neutrophilic chemotaxis [42]. Therefore, the positive feedback loop between NE and PlGF in the pathogenesis of COPD warrants further investigation.

Because of frequently ignored early symptoms and irreversible pulmonary damage, COPD remains a major cause of death worldwide [2]. As a chronic disease with insidious pathogenesis, COPD is difficult to diagnose early. Useful diagnostic markers will help in the early diagnosis, early treatment, and reduction of mortality and morbidity. A previous report indicates that the NE-digested product, Aα-Val360, may be a marker for COPD [43]. However, endogenous elastin fragments can disturb the utility of Aα-Val360 for predicting COPD.

The present study demonstrates that PlGF, which physiologically appears only in the embryonic stage, may be a suitable candidate as a diagnostic marker of early COPD. Based on the IHC results and BAL data in a previous study [26], COPD patients secrete and express more PlGF compared to non-COPD controls. Other than COPD, the up-regulation of PlGF is also associated with higher risk of several human diseases, including age-related macular degeneration, sickle cell disease, and most kinds of tumors [24]. As PlGF expression is barely detectable in healthy adults, further investigation regarding the association between PlGF and COPD may therefore support PlGF as a candidate marker for early COPD.

A previous study indicates that mouse PlGF activates p38 MAPK and JNK signaling pathway in mouse alveolar epithelial cells, and that MLE-15 and human PlGF activates the p38 MAPK and JNK signaling pathway in BEAS-2B. In the present study, PlGF promotes only JNK and PKCδ in AEC II cell. The difference in cell systems may explain why PlGF acts through different down-stream signaling pathways. However, the JNK, p38 MAPK, and PKCδ signaling pathways should all be considered as potential therapeutic targets aside from PlGF for COPD therapy [44-46].

Conclusions
Using human and mouse LE cells as well as an in vivo model, this study demonstrates that NE challenge stimulates PlGF expression and secretion, and that PlGF promotes LE cell apoptosis via the JNK and PKCδ signaling pathways. Thus, PlGF and the downstream JNK/PKCδ signaling pathways participate in the pathogenesis of CS-related COPD and should be considered potential therapeutic targets for COPD therapy.

Additional files
Additional file 1: Supplemental materials and methods.
Additional file 2: Figure S1. Neutrophil elastase (NE) increases placenta growth factor (PlGF) expression in endothelial cell. BAEC and fibroblast were treated with neutrophil elastase (NE) (0–300 μU/ml) for 24 h (A and B) and the cellular lysate were applied for Western blot analysis. (C) Wild type (WT) mice were intra-tracheally instilled with saline and 400 μU/ml NE weekly for one month. Paraffin-embedded lung tissue sections were used for immunohistochemistry (IHC) analysis and incubated with antibodies of PlGF. The arrow heads in enlarged figures indicated positive stain of PlGF only showed in endothelial cells of NE group. Data were presented as mean ± SEM. *P < 0.05 vs. vehicle-treated group.
Additional file 3: Figure S2. PlGF-activated JNK and PKCδ signaling pathways have no crosstalk in primary mouse alveolar type II epithelial cell (AEC II). (A and B) AEC II were transfected with PKCδ siRNA for 24 h (A) or pretreated with SP600125 for 2 h (B) then treated with PlGF (100 ng/ml) for 0–24 h. Cellular lysates were subjected to Western blot analysis with antibodies for phosphorylated JNK (p-JNK) and PKCδ (B). Data were presented as mean ± SEM. #P < 0.05 vs. vehicle-treated group.
Additional file 4: Figure S3. NE-upregulated endogenous PlGF promotes apoptosis and activates JNK and PKCδ signaling pathways in primary normal human bronchial epithelial (NHBE). (A and B) NHBE cells were treated with NE (300 μU/ml) for 0–60 h. Cellular lysates were subjected to caspase-3 activity (A) and trypanblue inclusion assay (B). NHBE cells were pretreated with FLT1 neutralizing antibody or IgG for 2 h then treated with NE (300 μU/ml) for 60 h. Cellular lysates were subjected to Caspase-3 activity (A) and trypanblue inclusion assay (B) and Western blot analysis with antibodies for phosphorylated JNK (p-JNK), phospho-PKCδ (p-PKCδ) and PKCδ (B). Data were presented as mean ± SEM. *P < 0.05 vs. vehicle-treated group.

Abbreviations
COPD: Chronic pulmonary obstructive disease; CS: Cigarette smoke; NE: Neutrophil elastase; MMP: Matrix metalloprotease; LE: Lung epithelial;
PIGF: Placenta growth factor; AEC II: Type II alveolar epithelial cell; MAPK: Mitogen-activated protein kinase; BAL: Broncho-alveolar lavage; WT: Wild-type; Egr-1: Early growth response gene-1; JNK: c-Jun N-terminal kinase; PKC: Protein kinase C; MTF: Mental-regulatory transcription factor; HIF: Hypoxia inducible factor; ELISA: Enzyme-linked immuno-sorbent assay; ChIP: Chromatin immuno-precipitation; NBE: Normal human bronchial epithelial cells; KO: Knockout; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; iHC: Immunohistochemistry; MRE: Metal response element; HRE: Hypoxia response element; PAR: Protease-activated receptor.

Competing interests
The authors declare that they have no competing interests.

Author contributions
HHH, SL, HCW and CJY designed research. HHH, SL, KPC and HHL conducted experiments. SCW and PNT provided PIGF KO mice. SLC, HCW and CJY provided help with data interpretation. HHFD, MP, YCC and CJY wrote the paper. All authors read and approved the final manuscript.

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References
1. Decramer M, Janssens W, Miravitlles M: Chronic obstructive pulmonary disease. Lancet 2012, 379:1341–1351.
2. Mathers CD, Loncar D: Projections of global mortality and burden of disease from 2002 to 2030 p38 MAP kinase inhibitors: a future therapy for inflammatory diseases. PLoS Med 2006, 3:e40.
3. Tudor RM, Petricek I: Pathogenesis of chronic obstructive pulmonary disease. A Clin Invest 2012, 122:2749–2755.
4. Barnes PJ: Mediators of chronic obstructive pulmonary disease. Pharmacol Rev 2004, 56:515–548.
5. Owen CA: Roles for proteinases in the pathogenesis of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 2008, 3:253–268.
6. Agusti A, Sobradillo P, Celli B: Addressing the complexity of chronic obstructive pulmonary disease: from phenotypes and biomarkers to scale-free networks, systems biology, and P4 medicine. Am J Respir Crit Care Med 2011, 183:119–137.
7. Fischer BM, Pavlikovs E, Voynow JA: Pathogenic triad in COPD: oxidative stress, protease-anti-protease imbalance, and inflammation. Int J Chron Obstruct Pulmon Dis 2011, 6:413–421.
8. Roghman A, Sallenave JM: Neutrophil elastase (NE) and NE inhibitors: canonical and non-canonical functions in lung chronic inflammatory diseases (cystic fibrosis and chronic obstructive pulmonary disease). J Aerosol Med Pulm Drug Deliv 2008, 21:15–144.
9. Lagente V, Le Quement C, Boichot E, Macrophase metalloelastase (MMP-12) as a target for inflammatory respiratory diseases. Expert Opin Ther Targets 2009, 13:287–295.
10. Shapiro SD, Goldstein AM, Kiyokawa Y, Miyake A, Isobe I, Yamamoto T, Takeda T, Ishida E, Sawada K, Morishige K, Kimura T: Metal transcription factor-1 is involved in hypoxia-dependent regulation of placenta growth factor in trophoblast-derived cells. Endocrinology 2009, 150:1801–1808.
11. Hou et al. Respiratory Research 2014, 15:106

obstruction in chronic obstructive pulmonary disease. N Engl J Med 2004, 350:2645–2653.
12. Abboud RT, Vimalanathan S: Pathogenesis of COPD. Part I. The role of protease-anti-protease imbalance in emphysema. Int J Tuberc Lung Dis 2008, 12:361–367.
13. Kimura B, Horita MS, Jeene DE, Gauthier F: Neutrophil elastase, protease 3, and cathpsins G as therapeutic targets in human diseases. Pharmacol Rev 2010, 62:76–759.
14. Reid PT, Mardsen ME, Cunningham GA, Hallett C, Sallenave JM: Human neutrophil elastase regulates the expression and secretion of elafin (elastase-specific inhibitor) in type II alveolar epithelial cells. FEBS Lett 1999, 457:33–37.
15. Devaney JM, Greene CM, Taggart CC, Carroll TP, O’Neill SJ, McEvany NG: Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. FEBS Lett 2003, 544:129–132.
16. Fischer BM, Cuellar JG, Diehl ML, de Freytas AM, Zhang J, Caraway KL, Voynow JA: Neutrophil elastase increases MUC4 expression in normal human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 2003, 284:L671–L679.
17. Shao MX, Nadel JA: Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-α-converting Enzyme. J Immunol 2005, 175:4009–4016.
18. Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB: Human neutrophil elastase induces hyper-secretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C-mediated mechanism. Am J Pathol 2005, 167:651–661.
19. Suzuki T, Moraes TJ, Vachon E, Ginzburg HH, Huang TT, Matthay MA, Hollenberg MD, Marshall J, McCulloch CA, Abreu MT, Chow CW, Downey GP: Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. Am J Respir Cell Mol Biol 2005, 33:231–247.
20. Nakajlo M, Fukushima T, Suzuki T, Yamaya M, Nakayama K, Sekizawa K, Sasaki H: Retinoic acid inhibits elastase-induced injury in human lung epithelial cell lines. Am J Respir Cell Mol Biol 2003, 28:296–304.
21. Sarafianbakhsh A, Hanania NA, Kim VP: Pathogenesis of emphysema: from the bench to the bedside. Proc Am Thorac Soc 2008, 5:475–477.
22. Tsaio PN, Yu YN, Li H, Huang PH, Chien CT, Lai YL, Lee CN, Chen CA, Cheng WF, Wei SC, Yu CJ, Hsieh FJ, Hsu SW: Over-expression of placenta growth factor contributes to the pathogenesis of pulmonary emphysema. Am J Respir Crit Care Med 2004, 169:505–511.
23. De Falco S: The discovery of placenta growth factor and its biological activity. Exp Mol Med 2012, 44:1–9.
24. Derewich M, Carmeliet P: PIGF: a multi-tasking cytokine with disease-resistant activity. Cold Spring Harb Perspect Med 2012, 2: doi:10.1101/cshperspect.a101596.
25. DiPalma T, Tucci M, Russo G, Maglione D, Lago CT, Romano A, Saccone S, Della Valle G, De Gregorio I, Drogani TA, Viglietto G, Persico MG: The placenta growth factor gene of the mouse. Mamm Genome 1996, 7:56–72.
26. Cheng SL, Wang HC, Yu CJ, Yang PC: Increased expression of placenta growth factor in COPD. Thorax 2008, 63:500–506.
27. Hou HH, Cheng SL, Liu HT, Yang FZ, Wang HC, Yu CJ: Elastase induced lung epithelial cell apoptosis and emphysema through placenta growth factor. Cell Death Dis 2013, 4:e3793.
28. Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, Kimbel P: Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. Am Rev Respir Dis 1977, 115:461–478.
29. Cheng SL, Wang HC, Yu CJ, Tsaio PN, Carmeliet P, Cheng SJ, Yang PC: Prevention of elastase-induced emphysema in placenta growth factor knock-out mice. Respi Res 2009, 10:115.
30. Lomas-Neira JL, Chung CS, Wescbe DE, Perl M, Ayala A: In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 versus KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury. J Leukoc Bio 2005, 77:846–853.
31. Nishimoto F, Sakata M, Minekawa R, Okamoto Y, Miyake A, Isobe I, Yamamoto T, Takeda T, Ishida E, Sawada K, Morishige K, Kimura T: Metal transcription factor-1 is involved in hypoxia-dependent regulation of placenta growth factor in trophoblast-derived cells. Endocrinology 2009, 150:1801–1808.
hypoxia in fibroblasts: a central role for metal transcription factor-1. Cancer Res 2001, 61:2696–2703.

33. Krämer B, Meichle A, Hensel G, Charnay P, Krönke M: Characterization of an Krox-24/Egr-1-responsive element in the human tumor necrosis factor promoter. Biochim Biophys Acta 1994, 1219:413–421.

34. Mura M, dos Santos CC, Stewart D, Liu M: Vascular endothelial growth factor and related molecules in acute lung injury. J Appl Physiol 2004, 97:1605–1617.

35. Hou HH, Cheng SL, Chung KP, Kuo YP, Yeh CC, Chang BE, Lu HH, Wang HC, Yu CJ: Elastase induces lung epithelial cells autophagy through placenta growth factor: a new insight of emphysema pathogenesis. Autophagy. In press.

36. Violle T, Adamson ED, Baron V, Birke D, Mercola D, Mustelin T, de Belle I: The Egr-1 transcription factor directly activates PTEN during irradiation-induced signaling. Nat Cell Biol 2001, 3:1124–1128.

37. Salah Z, Maoz M, Pizov G, Bar-Shavit R: Transcriptional regulation of human protease-activated receptor 1: a role for the early growth response-1 protein in prostate cancer. Cancer Res 2007, 67:9835–9843.

38. Chen ZH, Kim HP, Szurba FC, Lee SJ, Feghali-Bostwick C, Stolz DB, Dhir R, Landreneau RJ, Schuchert MJ, Yousem SA, Nakahira K, Pilewski JM, Lee JS, Zhang Y, Ryter SW, Choi AM: Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. PLoS One 2008, 3:e3316.

39. Thiel G, Cibelli G: Regulation of life and death by the zinc finger transcription factor Egr-1. J Cell Physiol 2002, 193:287–292.

40. Ngiam N, Post M, Kavanagh BP: Early growth response factor-1 in acute lung injury. Am J Physiol Lung Cell Mol Physiol 2007, 293:L1089–L1091.

41. Sakashita A, Nishimura Y, Nishiuma T, Takenaka K, Kobayashi K, Kotani Y, Yokoyama M: Neutrophil elastase inhibitor (sivelestat) attenuates subsequent ventilator-induced lung injury in mice. Eur J Pharmacol 2007, 571:62–71.

42. Bobic S, Seys S, De Vooght V, Callebaut I, Hox V, Dooms C, Vinckier S, Jonckx B, Saint-Remy JM, Stassen JM, Creupens JL, Carmellet P, Hellings PW: Placental growth factor contributes to bronchial neutrophilic inflammation and edema in allergic asthma. Am J Respir Cell Mol Biol 2012, 46:781–789.

43. Carter RI, Ungurs MJ, Mumford RA, Stockley RA: As-Val360: a marker of neutrophil elastase and COPD disease activity. Eur Respir J 2013, 41:31–38.

44. Adcock IM, Caramori G: Kinase Targets and inhibitors for the treatment of airway inflammatory diseases. BioDrugs 2004, 18:167–180.

45. Banerjee A, Kasol-White C, Panettieri RA Jr: p38 MAPK inhibitors, IKK2 inhibitors, and TNFa inhibitors in COPD. Curr Opin Pharmacol 2012, 12:287–292.

46. Dempsey EC, Cool CD, Little CM: Lung disease and PKCs. Pharmacol Res 2007, 55:545–559.

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