Hypoxia-induced Preadipocyte factor 1 expression in human lung fibroblasts through ERK/PEA3/c-Jun pathway

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Abstract

Background

Several studies have reported that hypoxia plays a pathological role in severe asthma and tissue fibrosis. Our previous study showed that hypoxia induces A disintegrin and metalloproteinase 17 (ADAM17) expression in human lung fibroblasts. Moreover, preadipocyte factor 1 (Pref-1) is cleaved by ADAM17, which participates in adipocyte differentiation. Furthermore, Pref1 overexpression is involved in liver and heart fibrosis. Studies have demonstrated that polyoma enhancer activator 3 (PEA3) and activator protein 1 (AP-1) play crucial roles in lung fibrosis, and the Pref-1 promoter region contains PEA3 and AP-1 binding sites, as predicted. However, the roles of extracellular signal-regulated kinase (ERK), PEA3, and AP-1 in hypoxia-stimulated Pref-1 expression in human lung fibroblasts remain unknown.

Methods

The protein expression in ovalbumin (OVA)-induced asthmatic mice was performed by immunohistochemistry and immunofluorescence. The protein expression or the mRNA level in human lung fibroblasts (WI-38) was detected by western blot or quantitative PCR. Small interfering (si) RNA was used to knockdown gene expression. The collaboration with PEA3 and c-Jun were determined by coimmunoprecipitation. Translocation of PEA3 from the cytosol to the nucleus was observed by immunocytochemistry. The binding ability of PEA3 and AP-1 to Pref-1 promoter was assessed by chromatin immunoprecipitation.

Results

Pref-1, PEA3, and hypoxia-inducible factor 1α (HIF-1α) were expressed in the lung sections of ovalbumin-treated mice. Hypoxia induced Pref1 protein upregulation and mRNA expression in human lung fibroblasts (WI38 cells). Moreover, PEA3 small interfering (si) RNA decreased the expression of hypoxia-induced Pref1 in WI38 cells. Hypoxia induced PEA3 phosphorylation, translocation of PEA3 from the cytosol to the nucleus, PEA3 recruitment and AP-1 binding to the Pref1 promoter region, and PEA3-luciferase activity. Furthermore, hypoxia induced c-Jun-PEA3 complex formation, and U0126 (an ERK inhibitor) or curcumin (an AP1 inhibitor) downregulated hypoxia-induced Pref-1 expression.

Conclusions

These results implied that ERK, PEA3, and AP1 participate in hypoxiainduced Pref1 expression in human lung fibroblasts.

1 Introduction

Asthma is an allergic airway disease characterized by airway inflammation, epithelial apoptosis, and airway remodeling (1, 2). A study showed that approximately 3.7% of patients with asthma developed severe asthma (3). Severe asthma is a difficult-to-control airway disease; it requires inhalation of high doses of corticosteroids to relieve symptoms (4). Airway fibrosis occurs when fibroblasts differentiate into α-smooth muscle actin myofibroblasts with massive deposition of extracellular matrix, including collagen I (5–7). Numerous studies have shown that subepithelial collagen deposition and fibrosis are correlated with asthma severity (5, 8, 9).

Hypoxia contributes to the fibrosis of several organs, including the kidney, liver, and lung (10–13). A study indicated that hypoxia plays a vital role in asthma (1). Hypoxia regulates gene expression through hypoxia-inducible factors (HIFs), and HIF-α is a key transcript factor produced in response to hypoxia (14). Airway inflammation and fibroblast proliferation cause airway remodeling, leading to hypoxia (1, 15, 16). Furthermore, hypoxia exacerbates airway remodeling and fibrosis (17–19). Thus, hypoxia plays a major role in airway fibrosis.

Preadipocyte factor 1 (Pref-1) is a transmembrane protein and is processed to the soluble protein by A disintegrin and metalloproteinase 17 (ADAM17; (20). Pref-1 was originally found to be responsible for adipogenesis inhibition in preadipocytes (21). A study reported that Pref-1 is involved in mesenchymal cell differentiation (22). Moreover, soluble Pref-1 participates in heart and liver fibrosis (23, 24). However, the role of Pref-1 in human lung fibrosis remains unknown. A study indicated that hypoxia induces ADAM17 expression in human lung fibroblasts, and ADAM17 is involved in hypoxia-induced fibroblast differentiation (25). Whether hypoxia can induce Pref-1 expression in human lung fibroblasts needs further investigation.

Polyoma enhancer activator 3 (PEA3), also called ETs translocation variant 4, is a transcription factor and plays a crucial role in cancer cell metastasis and fibrotic gene expression (26, 27). Studies have revealed that extracellular signal-regulated kinase (ERK)-induced PEA3 phosphorylation mediate gene expression in collaboration with another transcription factor (28, 29). According to the NCBI database, the PEA3 binding sequence is present in the Pref-1 promoter. However, the role of PEA3 in hypoxia-induced Pref-1 needs clarification.

The activator protein 1 (AP-1) transcript factor is a heterodimer composed of c-Jun and c-Fos. It usually regulates gene expression in cell growth, differentiation, and apoptosis (30). A study showed that AP-1 is involved in profibrotic protein expression in lung fibrosis (31). Moreover, hypoxia activates AP-1, which induces Cyr61 expression in retinal vascular endothelial cells (30). In hepatoma cells, PEA3 regulates *IL-8*/*CXCL8* gene expression in collaboration with AP-1 (32). Nevertheless, whether hypoxia induces Pref-1 expression through the ERK/PEA3/AP-1 pathway remains unknown.

In the present study, we revealed that hypoxia increased ERK phosphorylation, which in turn induced PEA3 and AP-1 activation and theirs recruitment to the Pref-1 promoter region to increase Pref-1 expression in human lung fibroblasts. Examining the mechanism may provide a potential maker of airway fibrosis in patients with severe asthma.

2 Materials And Methods

2.1 Materials

Antibodies specific for Pref-1, HIF-1α, and phospho-serine were purchased from Abcam (Cambridge, MA, USA). Secondary antibodies against IP detection reagent, Alexa Fluor-488 and Alexa Fluor-555 were purchased from Abcam (Cambridge, MA, USA). An antibody specific for c-Jun Ser63 was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for PEA3, c-Jun, horseradish peroxidase (HRP)-linked antibodies, including anti-goat immunoglobulin G (IgG), anti-rabbit IgG, and anti-mouse IgG antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A luciferase assay kit was purchased from Promega (Madison, WI, USA). The human PEA3 luciferase reporter was a obtained from Peter Hollenhorst (Watertown, MA, USA). Furthermore, α-tubulin antibody, fetal bovine serum, control small interfering RNA (siRNA) (scrambled), and PEA3 siRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 3000 reagent, minimum essential medium (MEM), penicillin, and streptomycin were acquired from Invitrogen Life Technologies (Carlsbad, CA, USA). A Novolink Max Polymer Detection System was purchased from Leica (Wetzlar, Germany)

2.2 Cell culture

Human lung fibroblast (WI-38) cells, purchased from American Type Culture Collection (Manassas, VA, USA), were grown in MEM supplemented with 10% fetal calf serum, penicillin G (100 U/mL), streptomycin (100 μg/mL), and MEM nonessential amino acids. WI-38 cells were maintained in a humidified 37°C incubator with 5% CO_2. After reaching confluence, cells were seeded onto 12-well plates for the transfection and luciferase reporter assay, onto 6-cm dishes for Western blot analysis, and onto 10-cm dishes for the immunoprecipitation assay.

2.3 Ovalbumin-induced animal model of airway fibrosis

For sensitization, on days 1, 7, and 14, C57BL/6 mice were intraperitoneally injected with 200 μL of 50 μg ovalbumin (OVA) emulsified in 2 mg of aluminum hydroxide. From day 21, 8- to 10-week-old C57BL/6 mice were challenged with aerosolized 5% OVA in phosphate-buffered saline (PBS) or PBS alone for 9 weeks. The frequency of the OVA challenge was twice weekly. After the final OVA aerosol challenge, C57BL/6 mice were sacrificed, and the lung tissue of mice was analyzed with further experiments.

2.4 Western blot analysis

Protein extraction and Western blot analysis were performed as previously described (33). In brief, cells were lysed with lysis buffer containing 20 mM Tris (pH 7.5), 1 mM $MgCl_2$, 125 mM $NaCl_2$, 1% Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM Na_3VO_4. Whole cell lysates (30 μg) were electrophoresed through SDS-PAGE, and the gels were transferred onto PVDF membranes. The whole membranes were blocked through incubation with 5% bovine serum albumin for 1 hour. Subsequently, proteins were incubated with specific primary antibodies for 20 hours at 4°C. Then, the membranes were incubated with HRP-conjugated secondary antibody for 1

hour at room temperature. Immunoreactivity was analyzed using enhanced chemiluminescence following the manufacturer's protocol.

2.5 Cell transfection

For transient transfection of siRNA into WI-38 cells, the transfection reagent/siRNA mixture was incubated at room temperature for 10 minutes and then added dropwise to MEM supplemented with 10% fetal bovine serum; it was then incubated in a humidified 37°C incubator for 24 hours.

2.6 PEA3-luciferase activity assay

In brief, WI-38 cells were transfected with PEA3-Luc (0.8 μg) and pBK-CMV-Lac Z (0.1 μg) by using transfection reagent for 24 hours. Cells were stimulated with hypoxia (1% O_2) for 18 hours. Luciferase activity was measured using the luciferase assay kit (Promega, Madison, WI, USA).

2.7 Immunohistochemistry and immunofluorescence

Lung tissues were fixed in 10% formaldehyde overnight, embedded in paraffin, and sectioned for immunohistochemistry (IHC) and immunofluorescence staining. For IHC staining, 2-μm sections were deparaffinized and then processed for antigen retrieval by using citrate buffer (pH 6.0). Endogenous peroxidase in tissue was neutralized through peroxidase blocking. The tissue was then blocked with a blocking buffer and incubated with Pref-1 and PEA3 antibodies for 40 minutes followed by polymer secondary antibody incubation for 30 minutes. Finally, the tissues were stained with hematoxylin and DAB solution. The integrated density of IHC staining was analyzed using ImageJ Fiji software. For immunofluorescence staining, 2-μm sections were deparaffinized and then processed for antigen retrieval by using EDTA buffer (pH 9.0). The tissue was blocked with a blocking buffer and incubated with Pref-1 and HIF-1α antibody for 24 hours at 4°C. Then, the tissue was incubated with Alexa Fluor 488-conjugated and Alexa Fluor 555-conjugated secondary antibodies for 1 hour at room temperature. Images were scanned using a ScanScope CS or fluorescence microscope.

2.8 Immunocytochemistry

WI-38 cells were cultured on slides. After reaching confluence, cells were subjected to hypoxia (1% O_2) for 30 minutes and then fixed through incubation in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Permeabilization with PBS containing either 0.5% Triton X-100, and washed slides in PBS three times for 5 minutes. The coverslips were blocked with 5% bovine serum albumin in PBST for 1 hour and incubated at 4°C overnight with antibodies specific to PEA3. Cells were incubated with Alexa Fluor 488-conjugated secondary antibody for an additional 1 hour. Counter staining was performed DAPI for 1 minute and the coverslip was mounted with a mounting medium. They were then observed under a fluorescence microscope.

2.9 Coimmunoprecipitation

WI-38 cells were seeded onto 10-cm dishes. After reaching confluence, cells were subjected to hypoxia (1% O_2) for the indicated time intervals. Cells were then harvested, lysed in 100 μL of IP lysis buffer

(Thermo Fisher Scientific, MA, USA), and centrifuged. The supernatant was then immunoprecipitated with a specific Ab against PEA3 (Santa Cruz, CA, USA) or c-Jun (Santa Cruz, CA, USA) in the presence of protein A/G beads at 4°C overnight. The immunoprecipitated beads were washed three times with IP lysis buffer. The immune complex was analyzed through 8% SDS-PAGE, transferred to PVDF membranes, and then subjected to immunoblot analysis with Abs specific for serine (Abcam, Cambridge, UK), PEA3 (Santa Cruz, CA, USA), or c-Jun (Santa Cruz, CA, USA).

2.10 Chromatin immunoprecipitation (ChIP)

WI-38 cells were subjected to hypoxia (1% O_2) for 30 minutes and then fixed with 10% formaldehyde for 10 minutes. Cells were collected and subjected to sonication; then, anti-PEA3, or anti-c-Jun was used for immunoprecipitation, and mouse anti-IgG antibody was used as the control. The Pref-1 promoter region was amplified through the polymerase chain reaction, and the following primers were used: AP-1, 5'-ACCACGAGTCAGCTGGGTAT-3' (sense) and 5'-TGCACACCCAAACACGCAAA-3' (antisense) and PEA3, 5'-TTGTGTTTCAGCGCGGCTA-3' (sense) and 5'-CAAGCGGACCTGCGGTTA-3' (antisense). DNA was analyzed with 1% agarose gel containing ethidium bromide.

2.11 Study approval

All animal protocols were approved by the Animal Ethics Committee of Taipei Medical University (approval no. LAC-2016-0361 and LAC-2019-0042).

2.12 Statistical analysis

All experimental data are presented as the mean value ± standard error of the mean for at least three independent experiments. The results of comparisons were conducted using one-way analysis of variance (ANOVA) followed by Dunnett's test analysis, unpaired t test, or Mann–Whitney *U* test. The results were considered statistically significant if p was <0.05.

3 Results

3.1 Colocalization of Pref-1 and HIF-1α in lung sections from OVA-treated mice

To evaluate Pref-1 expression in severe asthma, we used a mouse model sensitized with the allergen OVA. Mice received an intraperitoneal injection of 50 μg OVA in 2 mg aluminium hydroxide or PBS alone on days 1, 7, and 14 (Fig. 1A). These mice were challenged with aerosolized 5% OVA twice per week from day 21 to 81 and were then sacrificed for further analysis. In the OVA-sensitized mouse model, colocalization of Pref-1 and HIF-1α was observed through dual-label immunofluorescent staining (Fig. 1B). Moreover, we examined PEA3 expression in the lung section. Increased PEA3 expression was observed in the subepithelial layer of OVA-treated mice through IHC staining (Fig. 1C). These results suggest that Pref-1 and PEA3 are involved in hypoxia-induced airway fibrosis in OVA-sensitized mouse.

3.2 Hypoxia-induced Pref-1 mRNA and protein upregulation in WI-38 cells

Hypoxia induced Pref-1 expression in preadipocytes (34). A study showed that Pref-1 is involved in human adipose tissue fibrosis (35). However, the mechanism by which hypoxia induces Pref-1 expression in human lung fibrosis remains unknown. Our data demonstrated that the Pref-1 mRNA level increased after hypoxic stimulation of WI-38 cells for 1 hour (Fig. 2A). Furthermore, we found that Pref-1 protein expression was upregulated with a decrease in oxygen concentration (Fig. 2B). These data indicated that hypoxia induced Pref-1 expression in WI-38 cells.

3.3 PEA3 was involved in hypoxia-induced Pref-1 expression in WI-38 cells

To determine the role of PEA3 in hypoxia-induced Pref-1 expression in human lung fibroblasts, WI-38 cells were treated with PEA3 siRNA (50 ng/mL) overnight and then subjected to hypoxia (1% O_2). We found that hypoxia-induced Pref-1 expression significantly decreased after PEA3 siRNA transfection (Fig. 3A). Moreover, we exposed WI-38 cells to hypoxia for various time intervals. PEA3 serine phosphorylation was observed at 10 minutes, which declined after 60 minutes of exposure (Fig. 3B). We used the NCBI database to predict the transcription factor binding site in the promoter region of Pref-1; we found that PEA3 and AP-1 are the binding sequences in the promoter region of Pref-1. Furthermore, the ChIP assay showed PEA3 binding to the promoter region of Pref-1 in WI-38 cells during hypoxia (Fig. 3C). Hypoxia-stimulated cells showed a increase in PEA3-luciferase activity at 24 hours (Fig. 3D). PEA3 was translocated to the nucleus from the cytoplasm after hypoxia in WI-38 cells, as revealed by immunocytochemistry (Fig. 3E). Taken together, these data indicated that PEA3 activation was involved in hypoxia-induced upregulation of Pref-1 expression.

3.4 Involvement of ERK phosphorylation in PEA3 and AP-1 activation for hypoxia-induced Pref-1 expression in WI-38 cells

PEA3 plays a role in hypoxia-induced Pref-1 expression. A study indicated that ERK plays a crucial role in PEA3 activation in gastric adenocarcinoma (36, 37). We aimed to elucidate whether hypoxia induced Pref-1 expression through ERK/PEA3/AP-1 signaling in WI-38 cells. Treatment of cells with U0126 (10 μM), an ERK inhibitor, downregulated hypoxia-induced Pref-1 expression (Fig. 4A). Moreover, U0126 downregulated hypoxia-induced PEA3 and c-Jun phosphorylation in WI-38 cells (Fig. 4B, C). These results demonstrated that ERK mediated hypoxia-induced Pref-1 expression, PEA3 and c-Jun phosphorylation in WI-38 cells.

3.5 Formation of the AP-1-PEA3 complex mediated Pref-1 gene expression in WI-38 cells

A study showed that the AP-1-PEA3 complex binds to *IL-8/CXCL8* promoter in human hepatocellular carcinoma (32). Whether the AP-1-PEA3 complex is involved in hypoxia-induced Pref-1 expression remains unknown. In this study, PEA3 antibodies coprecipitated PEA3 and c-Jun after exposure to hypoxia in WI-38 cells (Fig. 5A). Moreover, curcumin (10 μM), an AP-1 inhibitor, attenuated hypoxia-induced Pref-1 expression in WI-38 cells (Fig. 5B). We observed that AP-1 bound to the promoter of Pref-1 after hypoxia exposure in the ChIP assay (Fig. 5C). Collectively, the results showed that AP-1-PEA3 complex was involved in Pref-1 expression in WI-38 cells.

4 Discussion

The present study demonstrated that hypoxia induces ERK phosphorylation, which in turn activates PEA3 and AP-1 that binds to the promoter region of Pref-1; this upregulates Pref-1 expression in human lung fibroblast cells. These results indicate that hypoxia-induced Pref-1 may play a crucial role in airway fibrosis.

In fibroblastic lung diseases, fibroblast proliferation and differentiation can rapidly decline lung function (38, 39). Studies have demonstrated that moderate to severe hypoxia (0.1–5%) can promote the proliferation of airway smooth muscle cells and lung fibroblasts (1, 39, 40). However, the correlation of hypoxia and Pref-1 with airway fibrosis remains unknown. Moreover, HIF-1α regulates hypoxia-induced lung fibroblast proliferation (39). In the present study, Pref-1 and HIF-1α colocalized in the OVA-induced allergic airway. It can be implied that Pref-1 is associated with hypoxic airway fibrosis. However, the mechanism of Pref-1-induced lung fibroblast differentiation requires further investigation.

Pref-1 suppressed adipogenesis by binding to the integrin receptor and then inhibited C/EBPβ and C/EBPδ genes in preadipocytes (22). Pref-1 showed strong correlations with various metabolic conditions, including hepatic steatosis, blood pressure, and insulin sensitivity (41). Moreover, Pref-1 impaired proinflammatory cytokine expression in human bone marrow mesenchymal stem cells (42). These findings suggest that Pref-1 is involved in chronic inflammation and metabolic diseases. A study reported that hypoxia induces Pref-1 expression in preadipocytes during adipogenesis but does not increase Pref-1 levels in adipocytes because hypoxia alters histone modification in preadipocytes (34). In this study, hypoxia upregulated Pref-1 mRNA and protein expression through the ERK/PEA3/AP-1 cascade in human lung fibroblasts.

A study showed that PEA3 is involved in many processes, including epithelial mesenchymal transition, apoptosis, cell invasion, and chemotherapy resistance (43). A study showed that mitogen-activated protein kinase (MAPK) signaling upregulated PEA3 expression (36). Moreover, PI3K/Akt signaling activated PEA3 expression in renal cell carcinoma (44). In this study, PEA3 was activated with ERK, and then, it bound to the Pref-1 promoter region after hypoxia exposure in WI-38 cells. We found that hypoxia

induced PEA3 phosphorylation and translocation to the nucleus, which in turn induced Pref-1 expression in WI-38 cells. Furthermore, PEA3 siRNA downregulated hypoxia-induced Pref-1 expression. These results demonstrated that PEA3 contributed to hypoxia-induced Pref-1 expression.

MAPK/ERK regulates lung fibrogenesis and cell growth and proliferation (45). Several studies have reported that ERK is essential for mediating profibrotic gene expression through the activation of transcriptional factors, including AP-1 (33, 46). MAPK/ERK regulates transforming growth factor β1-induced human alveolar type II cell senescence and epithelial mesenchymal transition (47). Moreover, hypoxia induces ERK Tyr204 phosphorylation, which in turn contributes to AP-1 activation and connective tissue growth factor expression in WI-38 cells (48). In the present study, ERK was involved in hypoxia-induced PEA3 phosphorylation, c-Jun phosphorylation and Pref-1 expression in human lung fibroblasts. Thus, hypoxia induced Pref-1 expression through the ERK/PEA3/AP-1 pathway.

A study demonstrated that hypoxia induced AP-1 expression and promoted downstream gene expression (30). A study showed that hypoxia induced AP-1 phosphorylation and contributed to fibrogenic protein expression in human lung fibroblasts (48). In retinal vascular endothelial cells, AP-1 activated JNK under hypoxia and then induced Cyr61 protein expression (30). Here, we demonstrated that curcumin inhibited hypoxia-induced Pref-1 expression, and the AP-1-PEA3 complex was recruited to the Pref-1 promoter after hypoxia in human lung fibroblasts.

In conclusion, these findings showed that hypoxia activates the ERK/PEA3/AP-1 signaling pathway, which in turn. Figure 6 shows a simplified diagram of the signaling pathway that demonstrates hypoxia-induced Pref-1 upregulation in human lung fibroblasts. Our findings demonstrate how hypoxia induces Pref-1 expression in human lung fibroblasts.

Declarations

Ethics approval and consent to participate

All animal protocols were approved by the Animal Ethics Committee of Taipei Medical University (approval no. LAC-2016-0361 and LAC-2019-0042).

Availability of data and materials

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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Formal analysis: WHC, BCC.

Funding acquisition: WHC, BCC. Investigation: WHC, BCC.

Methodology: CLC, JYC, BCC. Project administration: CLC BCC.

Resources: BCC. Software: WHC, BCC.

Supervision: BCC.

Validation: WHC, CLC, CHL, BCC. Visualization: WHC, CLC, CHL, BCC

Writing - original draft: WHC. Writing - review & editing: BCC.

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Figures

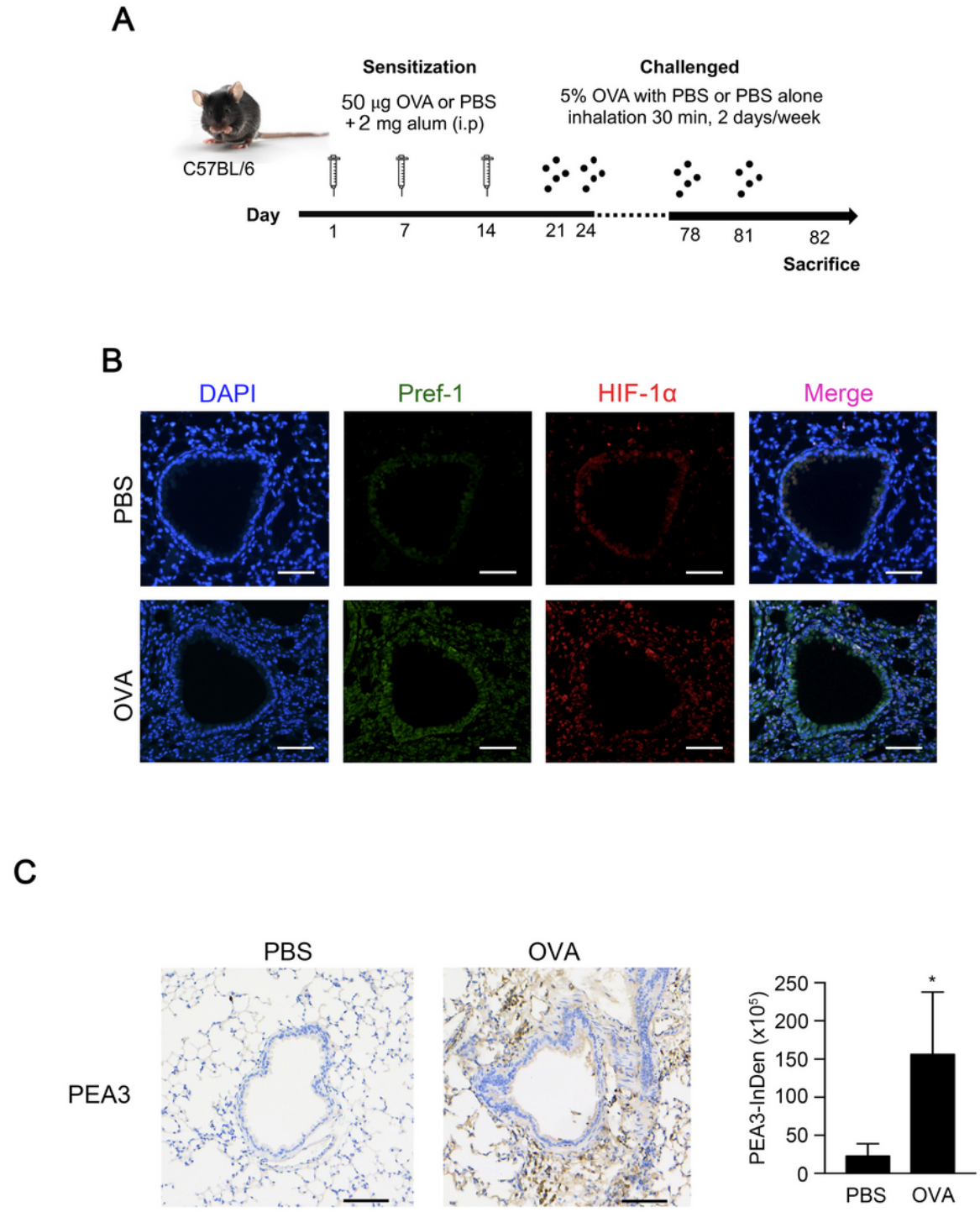

Figure 1

Pref-1 and PEA3 expression in lung tissue sections obtained from OVA-treated mice. (A) Mice were sensitized with OVA through three intraperitoneal injections on days 1, 7, and 14 with OVA absorbed to aluminium hydroxide. Mice were sensitized and challenged with aerosolized OVA twice weekly for up to 82 days. Control mice were treated with PBS. Lung tissue sections were stained through (B) immunofluorescence for Pref-1 (green) and HIF-1α (red) and (C) immunohistochemistry for PEA3

expression (original magnification, 20×; n = 5). PEA3 Integrated density are presented as the mean ± SEM. *p < 0.05, compared with PBS.

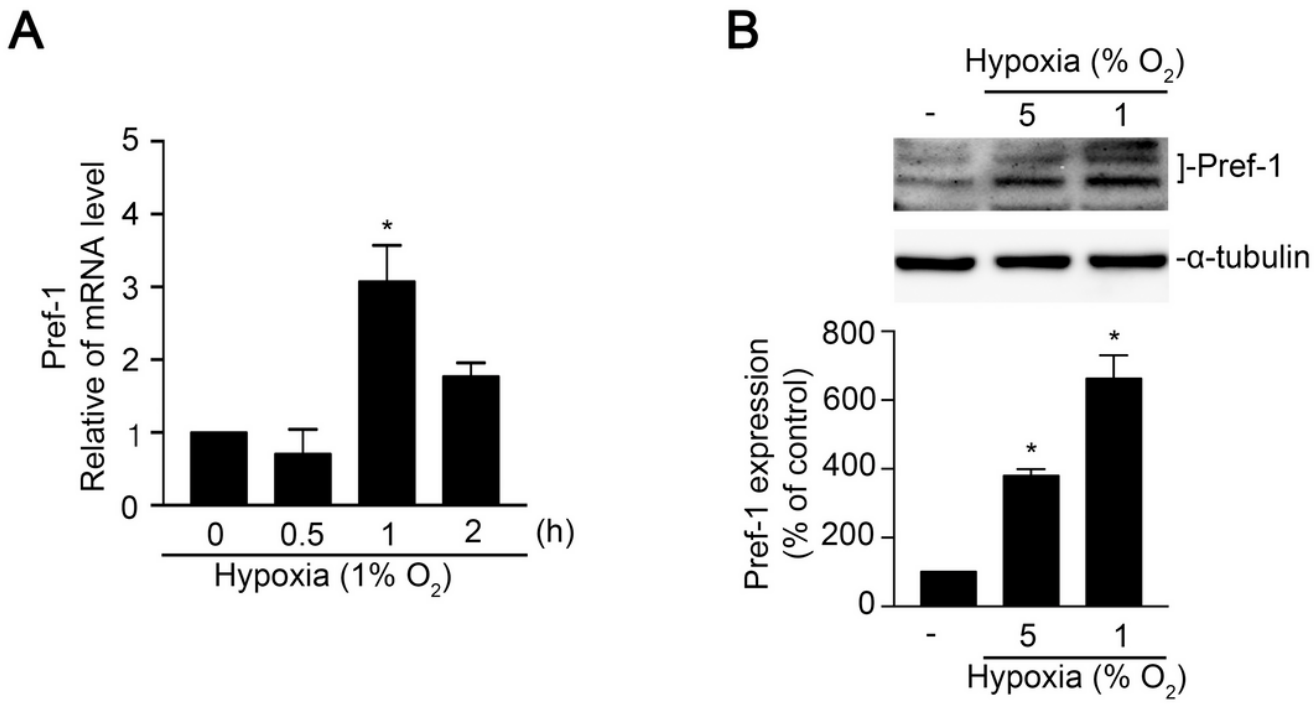

Figure 2

Hypoxia-induced Pref-1 expression in WI-38 cells. (A) WI-38 cells were incubated with 1% O2 for 0–2 hours. The levels of Pref-1 messenger RNA were detected by using qPCR . Data are presented as the mean ± SEM of three experiments. (B) WI-38 cells were incubated with hypoxia for different concentrations, and then, Pref-1 and α-tubulin were determined through immunoblots. These are presented as the mean ± SEM (n = 3). *p < 0.05, compared with control (at O2 21%).

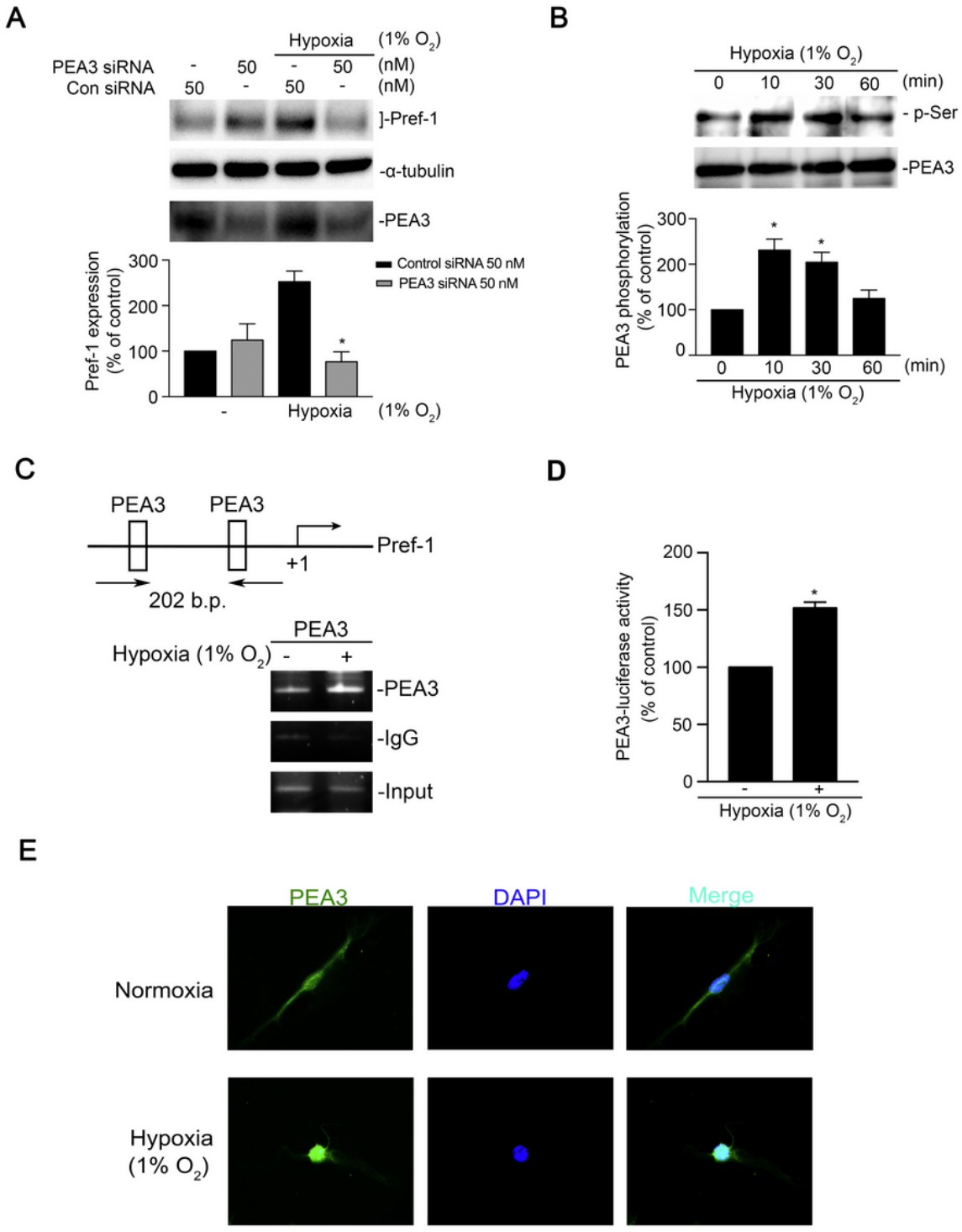

Figure 3

Involvement of PEA3 in hypoxia-induced Pref-1 expression in WI-38 cells. (A) WI-38 cells were transfected with control siRNA or PEA3 siRNA for 24 hours and then subjected to hypoxia (1% O2) for another 4 hours. Western blotting was performed to assess the levels of Pref-1, α-tubulin, and PEA3 in cell lysates. Data are presented as the mean ± SEM of three experiments. *p < 0.05, compared with the control siRNA group. (B) WI-38 cells were subjected to hypoxia for the indicated time, and cell lysates were

immunoprecipitated with an anti-PEA3 antibody; further, they were immunoblotted with antibodies for serine and PEA3. Data are presented as the mean ± SEM of three experiments. *p < 0.05, compared with the control at 0 minutes. (C) Cells were subjected to hypoxia (1% O2) for 30 minutes; the PEA3 binding site of the Pref-1 promoter region was detected through the ChIP assay. Input for for use as a positive control. Mouse polyclonal IgG for use as a negative control. Traces indicate that the three experiments produced similar results. (D) Cells were transfected with 0.8 μg of PEA3-Luc and 0.1 μg of pBK-CMV-Lac Z for 24 hours and then subjected to hypoxia (1% O2) for another 16 hours. Cells were harvested for a luciferase activity assay. Data are shown as the mean ± SEM, n = 3. *p < 0.05, relative to nonstimulated cells. (E) WI-38 cells were subjected to hypoxia (1% O2) for 30 minutes. In confocal microscopy, the cells were incubated with antibodies specific for PEA3, and immunoreactivity was performed through the incubation of the cells with an FITC-conjugated secondary antibody. All slides were counterstained with DAPI (blue) to distinguish the nucleus, which were visualized under an immunofluorescence microscopy (original magnification, 20×; n = 3).

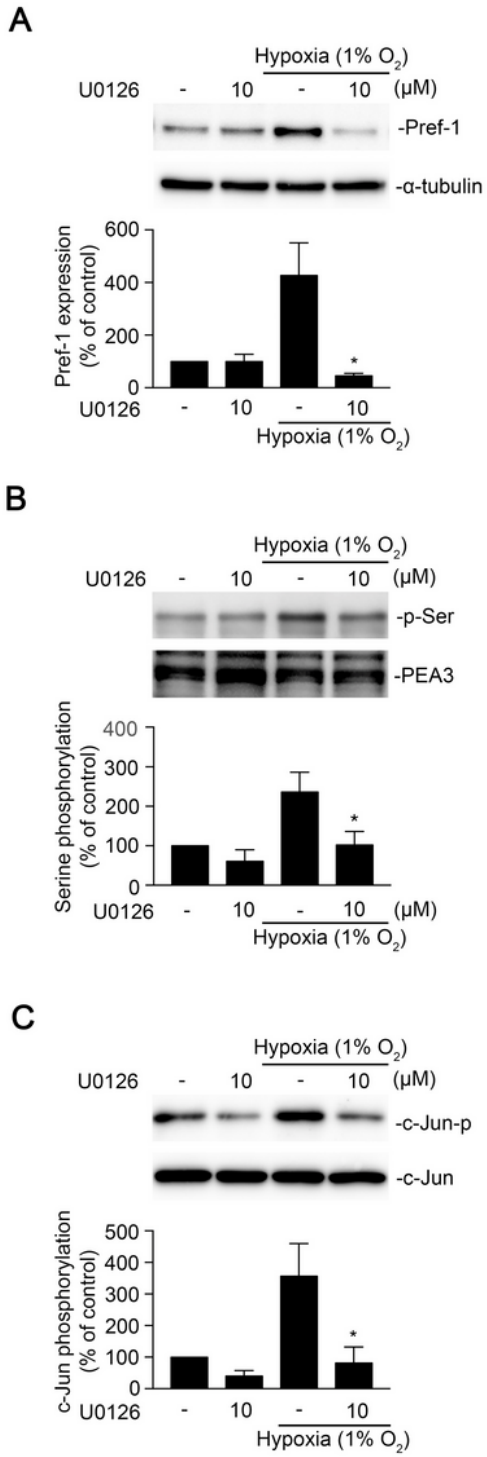

Figure 4

Involvement of ERK in hypoxia induces a Pref-1 expression in WI-38 cells. (A) WI-38 cells were pretreated with U0126 for 30 minutes and then incubated with the hypoxia (1% O2) for 4 hours. Levels of Pref-1 and α-tubulin in cell lysates were determined. Data are presented as the mean ± SEM of three experiments. *p < 0.05, compared with the hypoxia-exposed group. Cells were pretreated with U0126 for 30 minutes and then subjected to hypoxia (1% O2) for 30 minutes. (B) PEA3 was immunoprecipitated using anti-PEA3

antibody, and PEA3-p-serine was detected using anti-p-serine antibody. The quantified results were adjusted with PEA3 and expressed as a percentage of control. (C) Levels of phosphor-c-Jun Ser63 and c-Jun were detected using Western blotting in cell lysates. Values represent the means ± SEM of three experiments. *p < 0.05.

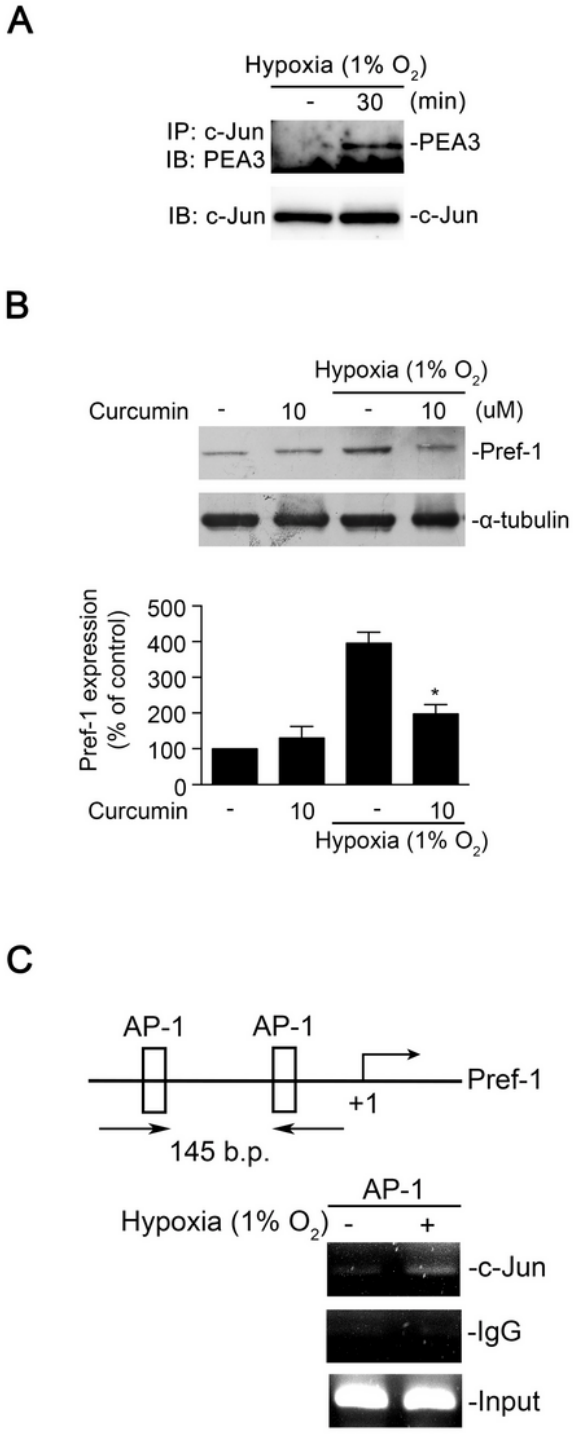

Figure 5

Involvement of AP-1 in hypoxia-induced Pref-1 expression in WI-38 cells. (A) WI-38 cells were exposed to hypoxia (1% O2) for 30 minutes. Cells were lysed with IP lysis buffer and then immunoprecipitated with the anti-c-Jun antibody. The immunoprecipitated complex was detected through immunoblotting with an anti-PEA3 antibody. Typical traces were demonstrative of three experiments. (B) Cells were pretreated with curcumin (10 μM) for 30 minutes and then stimulated with hypoxia (1% O2) for another 4 hours. Pref-1 and α-tubulin were detected in cell lysates. Data are presented as the mean ± SEM for three independent experiments. *p < 0.05, compared with the hypoxia group. (C) WI-38 cells were exposed to hypoxia (1% O2) for 30 minutes. The AP-1 binding site of the Pref-1 promoter region was detected through ChIP assay. Typical traces were presented in all two experiments.

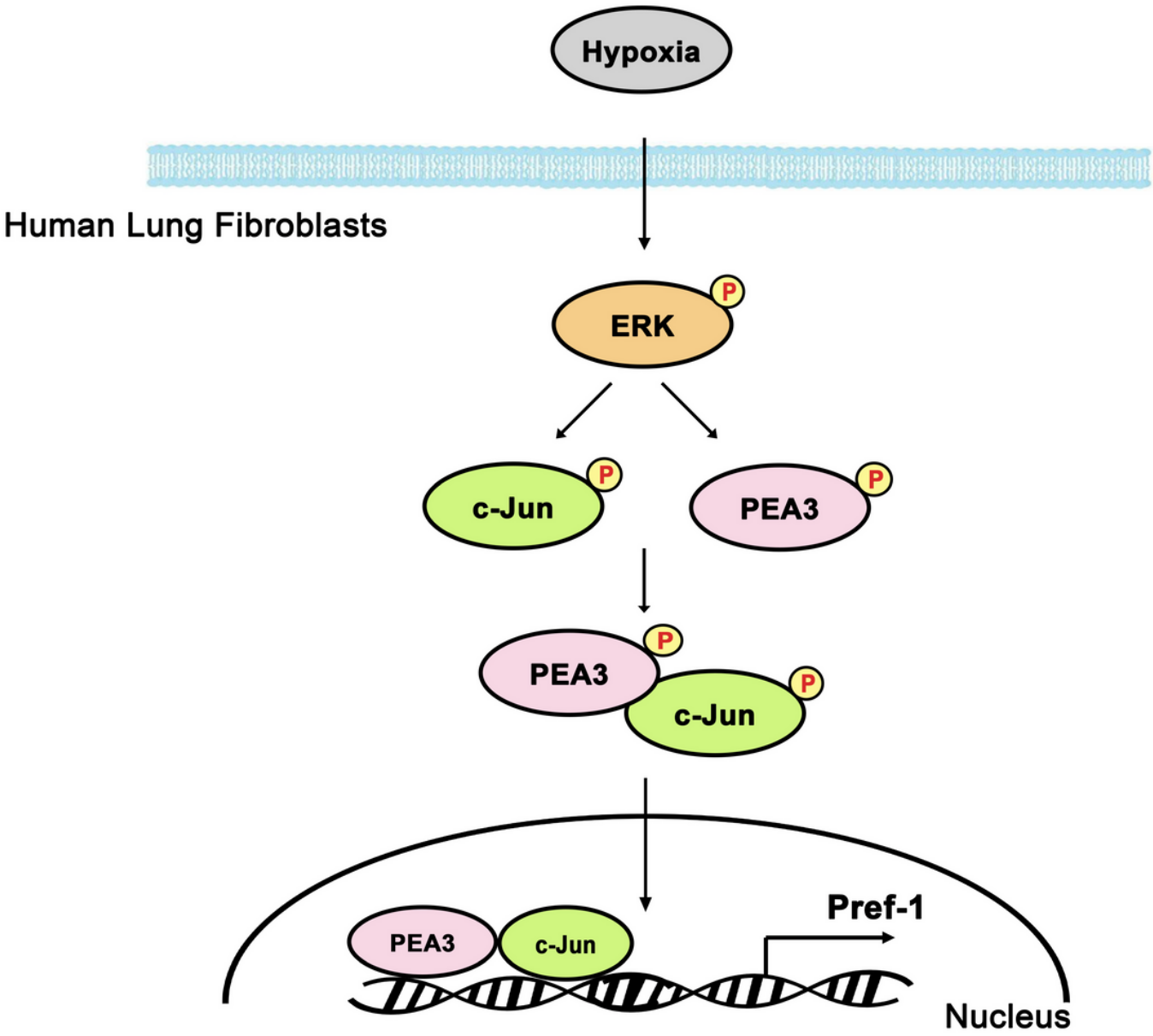

Figure 6

Simplified image displaying the results of the expression of hypoxia-induced Pref-1 through the ERK/PEA3/AP-1 pathway in human lung fibroblasts. Hypoxia induced the activation of ERK, which in turn caused PEA3 and AP-1 phosphorylation and complex formation. Moreover, PEA3 and AP-1 complex mediates Pref-1 expression through hypoxia stimulation in human lung fibroblasts.