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Calcitonin gene-related peptide down-regulates bleomycin-induced pulmonary fibrosis

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Abstract

We have found that eIF3a plays an important role in bleomycin-induced pulmonary fibrosis, and up-regulation of eIF3a induced by TGF- β 1 is mediated via the ERK1/2 pathway. Whether ERK1/2/ eIF3a signal pathway is involved in CGRP-mediated pathogenesis of bleomycin-induced pulmonary fibrosis remains unknown. Pulmonary fibrosis was induced by intratracheal instillation of bleomycin (5 mg/kg) in rats. Primary pulmonary fibroblasts were cultured to investigate the proliferation by BrdU incorporation method and flow cytometry. Sensory CGRP depletion by capsaicin exacerbated bleomycin-induced pulmonary fibrosis in rats, as shown by a significant disturbed alveolar structure, marked thickening of the interalveolar septa and dense interstitial infiltration by inflammatory cells and fibroblasts, accompanied with increased expression of TGF- β 1, eIF3a, phosphorylated ERK1/2, α -SMA, collagen I and collagen III. Exogenous application of CGRP significantly inhibited TGF- β 1-induced proliferation and differentiation of pulmonary fibroblasts concomitantly with decreased expression of eIF3a, phosphorylated ERK1/2, α -SMA, collagen I and collagen III. These effects of CGRP were abolished in the presence of CGRP8-37. These results suggest that endogenous CGRP is related to the development of pulmonary fibrosis induced by bleomycin, and the inhibitory effect of CGRP on proliferation of lung fibroblasts involves the ERK1/2/ eIF3a signaling pathway.

Keywords

calcitonin gene-related peptide; pulmonary fibrosis; pulmonary fibroblasts; eukaryotic translation initiation factor 3a; TGF- β 1

Introduction

Pulmonary fibrosis (PF) is characterized by fibroblast proliferation and extracellular matrix remodeling, which result in irreversible distortion of the lung architecture [Selman et al. 2001; Cook et al. 2002]. However, the underlying molecular mechanisms responsible for fibroblast proliferation and excessive deposition of collagen in fibrotic lesions are not fully understood.

Pulmonary fibroblasts have been implicated as a major participant in pulmonary fibrosis and are currently being studied as targets for therapy [Wynn 2011]. Histologic sections of diseased lung from patients with PF show clusters of proliferating fibroblasts termed “fibroblastic foci” [Scotton and Chambers 2007]. These clusters of fibroblasts are composed primarily of myofibroblasts, contractile cells that express both fibroblast and smooth muscle cell markers such as α -smooth muscle actin (α -SMA) [Hinz et al. 2007]. Myofibroblasts are one of the main effectors cells in fibrosis, as they are responsible for the excess production of extracellular matrix components, including collagen and fibronectin [Phan 2002]. In vitro differentiation of fibroblasts to myofibroblasts is driven by the cytokine transforming growth factor- β_1 (TGF- β_1), which is greatly increased in patients with PF [Gu et al. 2007; Hashimoto et al. 2001].

TGF- β_1 has been shown to play a key role in pulmonary fibrosis through its functions to attract fibroblasts and to stimulate their proliferation by activating Smad or extracellular signal-regulated protein kinase 1/2 (ERK1/2) signaling pathways [Border and Noble 1994; Yoshida et al. 2002]. TGF- β_1 signaling through the Smad pathway has been well described. In addition to the Smad pathway, there is increasing evidence that other pathways including ERK1/2 signal pathway are also important in TGF- β_1 signaling [Bou-Gharios et al. 2004]. It has been reported that ERK1/2 was involved in the regulation of lung inflammation and injury [Bou-Gharios et al. 2009; Li et al. 2008]. Recent studies also demonstrated that inhibition of the ERK1/2 pathway by PD98059 markedly reduced bleomycin induced lung fibrosis and TGF- β_1 induced proliferation of lung fibroblasts [Galuppo et al. 2011; Jiang and Guan. 2009].

Translational control plays a major role in regulating gene expression and occurs primarily at the initiation step involving multiple eukaryotic translation initiation factors (eIFs) [Maitra et al. 1982]. eIF3a (also known as p170), the largest subunit of eIF3 complex, has been suggested to play roles in regulating translation of a subset of mRNAs and in regulating cell cycle progression and cell proliferation [Dong et al. 2009; Dong and Zhang 2006]. Suppressing endogenous eIF3a expression has been shown to reverse malignant phenotype of human cancer cells while over-expression of ectopic eIF3a has been shown to promote malignant transformation of mammalian cells [Dong et al. 2004; Zhang et al. 2007]. These observations suggest that eIF3a plays an important role in tumorigenesis and in cancer cell response to chemotherapeutics, possibly by regulating gene expression. Our previous study has found that eIF3a plays an important role in bleomycin-induced pulmonary fibrosis and TGF- β_1 -induced proliferation and differentiation of pulmonary fibroblasts is mediated via ERK1/2/ eIF3a pathway [Li et al.2015]. This means that eIF3a is involved in the regulation of fibroblasts proliferation in pulmonary fibrosis.

Calcitonin gene-related peptide (CGRP), a 37-amino acid peptide, is a predominant neurotransmitter in sensory nerves [Bell and McDermott 1996] and it has been found among neuroendocrine cells, sensory C fibers, blood vessels and lymphoid tissues in the normal lung [Komatsu et al.1991].It has been reported that the expression of CGRP in lung was decreased in pulmonary fibrosis patients evoked by crystalline silica and crocidolite [Morimoto et al.2007].In recent study has found that CGRP mediated bleomycin induce pulmonary fibrosis in endothelin-converting enzyme-1 (ECE-1) heterozygous knock-out mice via CGRP-cAMP/EPAC1 pathway [Hartopo et al.2013]. Capsaicin, the active substance in chilli peppers, activates the vanilloid type 1 receptor (VR1) in the trigeminal ganglion and nucleus of small and medium C- and Ad-fibres and induces CGRP release when VR1 receptors are activated. It has been reported that rats pretreated with high-dose capsaicin to desensitize sensory nerves, the cardioprotection by pacing-induced preconditioning was abolished [Atzori et al.1998]. And our previous study has found that sensory CGRP depletion by capsaicin exacerbated hypoxia-induced pulmonary hypertension in rats [Li et al.2012]. And we

have also found that CGRP inhibited proliferation of PASMCs induced hypoxia via inactivation of ERK1/2 signal pathway [Li et al.2012]. It has been shown that ERK1/2 signal pathway is necessary for up-regulation of eIF3a, and is also crucial in mediating TGF- β_1 -induced proliferation and differentiation of pulmonary fibroblasts [Li et al.2015]. Whether ERK1/2/p27 signal pathway is involved in CGRP-mediated pathogenesis of pulmonary fibrosis and pulmonary fibroblasts proliferation and differentiation remains unknown. So in the present study we used capsaicin to deplete endogenous CGRP to explore whether capsaicin exacerbated bleomycin-induced pulmonary fibrosis in rats and the effect of CGRP on proliferation of lung fibroblasts and its mechanism in pulmonary fibrosis.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 180–220 g were obtained from Laboratory Animal Center, Xiangya School of Medicine, Central South University (Changsha, China). All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Medicine Animal Welfare Committee of Xiangya School of Medicine, Central South University.

Animal experiments

Rats were randomly divided into three groups: (i) Control group; (ii) Bleomycin group, SD rats were anesthetized with sodium pentobarbital (P3761, 30 mg/kg; Sigma) by intraperitoneal injection followed by intratracheal instillation of 5 mg/kg bleomycin (Nippon Kayaku, Tokyo, Japan) in 1 mL of saline; (iii) Bleomycin plus Capsaicin group, rats was given capsaicin (M2028, 50 mg/kg, s.c.; Sigma, St Louis, MO, USA) 4 days before intratracheal instillation of bleomycin to deplete endogenous CGRP. Control animals received the same volume of intratracheal saline instead of bleomycin. Pulmonary fibrosis was assessed by lung histology as described in the following section [Ashcroft et al.1998].The rats were sacrificed 28 days after bleomycin injection.

Histology and Immunohistochemistry

For light microscopic investigation, lung tissues were fixed by inflation with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 24 h and embedded in paraffin. Tissue sections (5 μ m) were stained with hematoxylin and eosin (HE) and Masson's trichrome stain to enable histological evaluation of lung fibrosis. Masson's trichrome stain was used to demonstrate collagen deposition, and collagen fiber is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink. The procedure is according to the manufacturer's instructions (KeyGEN Biotech, Nanjing, China). For Lung CGRP immunohistochemistry staining, sections were stained with anti-CGRP antibody (C8198, 1:4000; Sigma), developed with DAB, and counterstained with hematoxylin.

Measurement of plasma concentration of CGRP and TGF- β_1

Plasma concentration of CGRP was measured by radioimmunoassay and TGF- β_1 was measured by ELISA kits (RD Systems Inc, Hong Kong, China) as previously described and the procedure according to the manufacturer's instructions [Li et al.2012; Kang et al.2007].

Cell experiments

Primary rat pulmonary fibroblasts were prepared from the lung tissue of male 10-week-old healthy SD rats using trypsin digestion method as described previously [Phan et al.1985]. The cells were cultured at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. Fibroblasts were identified by immunohistochemistry staining with the antibody of Vimentin (ab8978, 1:50; Abcam, Hong Kong, China). The cells between passages 3 and 8 were used for the experiments. The cell experiments were to explore the effect of CGRP on TGF- β_1 -induced proliferation of pulmonary fibroblasts and the correlation with ERK1/2/ eIF3a pathway. The cells were divided into 8 groups as follows: i) Control, cells were incubated with double distilled water (TGF- β_1 solvent) for 24 h; ii) TGF- β_1 , cells were incubated with TGF- β_1 (5ng/mL) for 24 h; iii) +DMSO: cells were pre-treated with DMSO (PD98059 solvent) for 1 h, and then subjected to TGF- β_1 (5ng/mL) for 24 h; iv)+PD98059 1 μ M: cells were pre-treated with PD98059 1 μ M for 1 h, and then subjected

to TGF- β_1 (5ng/mL) for 24 h. v) +CGRP 1 nM: cells were pre-treated with CGRP 1 nM for 1 h, and then subjected to TGF- β_1 (5ng/mL) for 24 h. vi) +CGRP 10 nM: cells were pre-treated with CGRP 10 nM for 1 h, and then subjected to TGF- β_1 (5ng/mL) for 24 h. vii) +CGRP 100 nM: cells were pre-treated with CGRP 100 nM for 1 h, and then subjected to TGF- β_1 (5ng/mL) for 24 h. viii) +CGRP 100 nM and CGRP₈₋₃₇ 1 μ M: cells were pre-treated with CGRP 100 nM and CGRP₈₋₃₇ 1 μ M for 1 h, and then subjected to TGF- β_1 (5ng/mL) for 24 h. Cell proliferation assays were performed. The expressions of collagen I, collagen III, α -SMA, ERK1/2 and eIF3a were analyzed. The 24 h duration of TGF- β_1 was based on our pilot study.

Cell Proliferation Assays

As described previously [Li et al.2015; Shi et al.2011], cell proliferation was measured by two methods; the DNA synthesis and cell cycle were analysed by BrdU marking and flow cytometry, respectively. For the BrdU incorporation assay, cells were counted and seeded into 96-well culture plates (6×10^3 cells per well). BrdU (10 μ L/ per well; Roche, Mannheim, Germany) was added. Cells were fixed and stained after 4 h according to the manufacturer's instructions. Colorimetric analysis was performed with an ELISA plate reader (DTX880; Beckman, Miami, FL) at 450 nm. For cell cycle analysis using flow cytometry, cells were counted and seeded into six-well culture plates (1×10^5 cells per well). After 24 h, the medium was changed to DMEM containing 1% FBS to make them quiescent for 24 h. The cells were fixed gently with 70% cold alcohol at 4°C for over night. The cells were then treated with 0.25% Triton X-100 for 5 min in an ice bath and resuspended in 300 ml of phosphate-buffered saline containing 50 μ g/ mL propidium iodide and 0.1mg/mL RNase. Cells were incubated in a dark room for 1 h at 37°C and then subjected to cell cycle analysis using a FACScan flow cytometer (BD Biosciences, Bedford, MA) and FACS Di Va software. For each measurement, at least 10000 cells were counted. Data are presented as the percentage of cells in a given sub-population.

Real-time PCR analysis

Total RNA was extracted by using TRIzol reagent (Invitrogen). RNA (0.2-0.5 μ g) was subjected to reverse transcription reaction using the PrimeScript reverse

transcription reagent Kit (DRR037S; TaKaRa, Dalian, China). Quantitative analysis of the change in expression levels was performed using SYBR® Premix Ex Taq™ (DRR420A; TaKaRa) by the ABI 7300 system. PCR cycling conditions were an initial incubation at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 31 s. Primers for α -CGRP were: forward 5'-GTGTCAGTCCCA GAAGAGATC-3', reverse 5'-CAAAGTTGTCCTTCACCA CACC-3'. Primers for β -CGRP were: forward 5'-CCAGTGTCATTT CCCAGAAGA GA-3', reverse 5'-CAGCCGATGGGTCACACA-3'. Primers for TGF- β_1 were: forward 5'-TGGCGTTACCTTGGTAACC-3', reverse 5'-GGTGTGAGCCCTTTCC AG-3'. Primers for eIF3a were: forward 5'-TCAAGTCGCCGGGACGATA-3', reverse 5'-CCTGTCATCAGCACGTCTCCA-3'. Primers for α -SMA were: forward 5'-CTATTCCTTCGTGACTACT-3', reverse 5'-ATGCTGTTATAGGTGGTT-3'. Primers for collagen I were: forward 5'-CCAACTGAACGTGACCAAAAACCA-3', reverse 5'-GAAGGTGCTGGGTAGGGAAGTAGGC-3'. Primers for collagen III were: forward 5'-ATTCTGCCACCCTGAACTCAAGAGC-3', reverse 5'-TCCATGTAGGCAATGCTGTTTTTGC -3'. Primers for GAPDH were: forward 5'-TGGCCTCCAAGGAGTAAGAAAC-3', reverse 5'-GGCCTCTCTCTTGCTCTCA GTATC-3'. Data analysis was performed by comparative Ct method using the ABI software. GAPDH was used to normalize the expression of mRNA.

Western blot analysis

Protein was extracted from lung tissues and fibroblasts with RIPA buffer (containing 0.1% PMSF), and equal amounts of protein from each sample (50 μ g) were separated by 10% SDS/PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with primary antibodies overnight at 4°C, and horseradish peroxidase (HRP)-coupled goat anti-mouse or goat anti-rabbit secondary antibody (sc-2005, 1:2,000, sc-2030, 1:5,000; Santa Cruz, CA, USA). The chemiluminescence signals were detected with the EasySee Western Blot Kit (Beijing TransGen Biotech, Beijing, China). The densitometric analysis was conducted with Image J 1.43 (National Institutes of Health). Primary antibodies against eIF3a (#3411, 1:1,000), phospho-ERK1/2 (#9106, 1:2,000), ERK1/2(#9107, 1:1,000) were

purchased from Cell Signaling (Boston, MA, USA). Primary antibodies against α -SMA (ab5694, 1:2000), TGF- β_1 (ab27969, 1:500), collagen I (ab34710, 1:1000), and collagen III (ab7778, 1:1000) was purchased from Abcam (Hong Kong, China), and GAPDH (sc-137179, 1:2000) were obtained from Santa Cruz (CA, USA).

Statistical analysis

The results were presented as means $\pm$ SEM (standard errors). Statistical analysis was performed by ANOVA followed by Newman-Student-Keuls test for multiple comparisons. Results were considered statistically significant when $P < 0.05$.

Results

Effect of capsaicin on Lung Histological and α -SMA expression in bleomycin-induced pulmonary fibrosis rats

In keeping with previous study [Ashcroft et al.1998], after 4 four weeks of exposure to bleomycin induced pulmonary fibrosis rats, as shown by a significant disturbed alveolar structure, marked thickening of the interalveolar septa and dense interstitial infiltration by inflammatory cells and fibroblasts (Fig. 1A). Myofibroblasts are generally considered to be key effect or cell in the development of pulmonary fibrosis, the hallmark of which is the expression of α -smooth muscle actin (α -SMA). In line with previous studies [Hinz et al. 2007], bleomycin dramatically increased the expression of α -SMA (both mRNA and protein) in lung tissues of rats (Fig. 1B and C). All these effects of bleomycin were further aggravated by pre-treatment of rats with capsaicin

Effect of capsaicin on lung collagen accumulation in bleomycin-induced pulmonary fibrosis rats

Masson staining of lung specimens demonstrated that bleomycin instillation induced severe distortion of lung structure and accumulation of collagen fiber (blue) in rat lungs, whereas a well-alveolized normal histology was seen in rats treated with saline (Fig.2A). And bleomycin also markedly up-regulated the expression of collagen I and collagen III (both mRNA and protein) in lung tissues of rats (Fig. 2B, C, D and E). All these effects of bleomycin were further aggravated by pre-treatment of rats with capsaicin

Effect of capsaicin on plasma CGRP level and CGRP expression in lung tissue of bleomycin-induced pulmonary fibrosis rats

It has been reported that CGRP mediated bleomycin-induced pulmonary fibrosis [Hartopo et al.2013]. In the present study, we found that bleomycin dramatically decreased the plasma CGRP level and expression of CGRP (both mRNA and protein) in lung tissues of rats (Fig. 3), an effect that was further enhanced by pre-treatment of rats with capsaicin.

Effect of capsaicin on the expression of TGF- β_1 , ERK1/2 and eIF3a in lungs from bleomycin-induced pulmonary fibrosis rats

TGF- β_1 seems to play a major profibrotic role, inducing fibroblast to myofibroblast differentiation and increasing collagen expression. In line with previous studies [Hashimoto et al. 2001], bleomycin dramatically increased the plasma TGF- β_1 level and the expression of TGF- β_1 (both mRNA and protein) in lungs of rats (Fig. 4A, B and C). On this basis, we further found that the expression of ERK1/2 and eIF3a were obviously increased in lungs from bleomycin-induced pulmonary fibrosis rats (Fig. 4D, E and F), an effect that was further enhanced by pre-treatment of rats with capsaicin.

Effect of CGRP on TGF- β_1 -induced cell proliferation and expression of α -SMA, type I collagen and III collagen in cultured pulmonary fibroblasts

Recent studies demonstrated that inhibition of the ERK1/2 pathway by PD98059 markedly reduced bleomycin induced lung fibrosis and TGF- β_1 induced proliferation of lung fibroblasts [Galuppo et al. 2011; Jiang and Guan. 2009]. In consistent with previous study, exogenous TGF- β_1 stimulated proliferation of pulmonary fibroblasts as shown by an increase in BrdU incorporation and the percentage of cells in S+G₂ phase (Fig. 5A and B). To further conform whether TGF- β_1 -induced pulmonary fibroblasts proliferation via ERK1/2 signal pathway, phosphorylation ERK1/2 inhibitor PD98059 was used. The results showed that PD98059 markedly inhibited TGF- β_1 -induced fibroblasts proliferation (Fig. 5A and B). We also found that PD98059 obviously inhibited TGF- β_1 -induced the up-regulated expression of α -SMA, collagen I, and collagen III (both mRNA and (or) protein) (Fig. 5C, D and 6)

To further confirm the role of CGRP in mediating bleomycin-induced pulmonary fibrosis in rats, the effect of exogenous CGRP application on TGF- β_1 -induced proliferation of pulmonary fibroblasts was measured. In the present study we found that CGRP (1, 10, 100 nM) concentration-dependently inhibited TGF- β_1 -induced proliferation of pulmonary fibroblasts, an effect that was abolished in the presence of CGRP₈₋₃₇ (Fig. 5). We also found that CGRP concentration-dependently inhibited TGF- β_1 -induced the up-regulated expression of α -SMA, collagen I, and collagen III (both mRNA and (or) protein). The inhibitory effects of CGRP were also abolished in the presence of CGRP₈₋₃₇ (Fig. 6).

Effect of CGRP on TGF- β_1 -induced the expression of ERK1/2 and eIF3a in cultured pulmonary fibroblasts

As mentioned above, TGF- β_1 -induced pulmonary fibroblasts proliferation via ERK1/2 signal pathway. Our previous study has found that TGF- β_1 -induced proliferation and differentiation of pulmonary fibroblasts is mediated via ERK1/2/eIF3a pathway [Li et al.2015]. And we have also found that CGRP inhibits hypoxia-induced proliferation of pulmonary artery smooth muscle cells (PASMCs) via ERK1/2 signal pathway [Li et al.2012]. We therefore evaluated the effect of CGRP on expression of ERK1/2 and eIF3a in pulmonary fibroblasts. CGRP (1, 10, 100 nM) was found to concentration-dependently blunt TGF- β_1 -induced phosphorylation of ERK1/2 and down-regulation of eIF3a, an effect that was abolished in the presence of CGRP₈₋₃₇ (Fig. 7).

Discussion

Calcitonin gene-related peptide (CGRP) is 37-amino acid neuropeptide found among neuroendocrine cells, sensory C fibers, blood vessels and lymphoid tissues in the normal lung [Komatsu et al.1991]. It has been reported that in the models of lung injury induced by crystalline silica, crocidolite asbestos and bleomycin, pathological findings of lung inflammation increased progressively in a time dependent manner, while low levels of CGRP concentration in lungs were observed [Morimoto et al.2007]. In recent study has also found that the activity of ECE-1 in vivo, under pathological conditions, is particularly associated with the degradation of CGRP, and

further confirms that reduced ECE-1 activity exerts beneficial effects on bleomycin lung injury, which in turn preserves lung CGRP concentrations and activates CGRP signaling, thereby preventing the transition from inflammation to fibrosis [Hartopo et al.2013]. In the present study, we found that bleomycin dramatically decreased the plasma CGRP level and expression of CGRP in lung tissues of rats, and CGRP depletion by capsaicin exacerbates bleomycin-induced pulmonary fibrosis in adult rats. These findings suggest that CGRP plays a crucial role in pathogenesis of pulmonary fibrosis, and may be a potential therapeutic target in pulmonary fibrosis.

Fibroblasts have been implicated as a major participant in pulmonary fibrosis and are currently being studied as new therapeutical targets [Wynn 2011]. Histologic sections of diseased lung from patients with PF show clusters of proliferating fibroblasts termed “fibroblastic foci” [Scotton and Chambers 2007]. These clusters of fibroblasts are composed primarily of myofibroblasts, contractile cells that express both fibroblast and smooth muscle cell markers such as α -smooth muscle actin (α -SMA) [Hinz et al. 2007]. Myofibroblasts are largely responsible for the excess production of extracellular matrix components, such as collagen and fibronectin [Phan 2002]. While, intervention in fibroblast proliferation and differentiation may be a key to treatment. In the present study, direct application of exogenous CGRP (1, 10, 100 nM) dose-dependently inhibited TGF- β_1 -induced proliferation and differentiation of fibroblasts, and the effect of CGRP was abolished in the presence of CGRP₈₋₃₇, a selective CGRP receptor antagonist. These finding suggest that inhibition of proliferation of fibroblasts by CGRP, at least in part, mediates the role of CGRP in pathogenesis of pulmonary fibrosis.

eIF3a is the largest subunit of eIF3 complex, which has been suggested to play roles in regulating translation of a subset of mRNAs and hence in regulating cell cycle progression and cell proliferation [Dong et al. 2009; Dong and Zhang 2006]. Suppressing endogenous eIF3a expression has been shown to reverse malignant phenotype of human cancer cells while over-expression of ectopic eIF3a has been shown to promote malignant transformation of mammalian cells [Dong et al. 2004; Zhang et al. 2007]. eIF3a expression has also been correlated with better prognosis of

human cancer patients and eIF3a up-regulation in lung cancer patients correlates with their response to platinum-based chemotherapy and contributes to increased cisplatin sensitivity [Shen et al. 2014; Yin et al. 2011]. These observations suggest that eIF3a plays an important role in cancer cell responding to chemotherapeutics, possibly by regulating gene expression. Our previous study has found that eIF3a is involved in TGF- β_1 -induced proliferation and differentiation of pulmonary fibroblasts [Li et al. 2015]. In the present study, we found that pulmonary fibrosis induced by bleomycin was accompanied by up-regulation of TGF- β_1 and eIF3a expression. Sensory CGRP depletion by capsaicin exacerbates pulmonary fibrosis and up-regulation of TGF- β_1 and eIF3a expression induced by bleomycin. Furthermore, application of exogenous CGRP inhibited TGF- β_1 -induced proliferation of fibroblasts concomitantly with inhibition of eIF3a expression, an effect that was reversed in the presence of CGRP₈₋₃₇. These results demonstrate the importance of eIF3a in mediating the role of CGRP in pathogenesis of pulmonary fibrosis.

It has been reported that ERK1/2 was involved in the regulation of lung inflammation and injury [Bou-Gharios et al. 2009; Ponticos et al. 2008]. Recent studies have also demonstrated that inhibition of the ERK1/2 pathway by PD98059 markedly reduced bleomycin induced lung fibrosis and TGF- β_1 induced proliferation of lung fibroblasts [Galuppo et al. 2011; Jiang and Guan. 2009]. Our previous findings have found that TGF- β_1 -induced proliferation and differentiation of pulmonary fibroblasts is mediated via ERK1/2-eIF3a pathway. Our present study confirmed the previous observation that the expression of ERK1/2 was obviously increased in lungs from bleomycin-induced pulmonary fibrosis rats, an effect that was further enhanced by pre-treatment of rats with capsaicin. In addition, application of exogenous CGRP inhibited TGF- β_1 -induced proliferation of pulmonary fibroblasts concomitantly with down-regulation of ERK1/2 phosphorylation and expression of α -SMA, collagen I and collagen III, an effect that was reversed in the presence of CGRP₈₋₃₇. Reasonably, ERK1/2 inhibitor PD98059 markedly inhibited up-regulated α -SMA, collagen I and collagen III expression induced by TGF- β_1 . These findings suggest that inhibitory effect of CGRP on TGF- β_1 -induced proliferation and

differentiation of pulmonary fibroblasts is mediated via ERK1/2-eIF3a pathway.

In conclusion, the present study suggests that endogenous CGRP is related to the development of pulmonary fibrosis induced by bleomycin, and the inhibitory effect of CGRP on proliferation of lung fibroblasts involves the ERK1/2/ eIF3a signaling pathway.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Fig.1. Lung histology and α -SMA expression in bleomycin-induced pulmonary fibrosis of rats. (A) Hematoxylin-eosin staining of lung tissue. (B and C) The expression of α -SMA mRNA and protein was determined by real-time PCR and Western blot. Data are means $\pm$ SEM ($n=8$ in each group). ** $P<0.01$ vs. Control; # $P<0.05$ vs. Bleomycin.

Fig.2. Collagen expression of lung tissue in bleomycin-induced pulmonary fibrosis rats. (A) Masson's trichrome staining of lung tissue. (B and C) The expression of collagen I and collagen III mRNA was determined by real-time PCR. (D and E) The expression of collagen I and collagen III protein was determined by Western blot. Data are means $\pm$ SEM ($n=8$ in each group). ** $P<0.01$ vs. Control; # $P<0.05$ vs. Bleomycin.

Fig.3. Effect of capsaicin on plasma CGRP level and CGRP expression in lung tissue of bleomycin-induced pulmonary fibrosis rats. (A) Plasma CGRP level was measured by radioimmunoassay. (B and C) The expression of α -CGRP and β -CGRP mRNA was determined by real-time PCR. (D) The expression of CGRP protein was determined by immunohistochemistry staining (arrows indicated CGRP positive staining). Data are means $\pm$ S.E.M ($n=8$ in each group). ** $P<0.01$ vs. Control; # $P<0.05$ vs. Bleomycin.

Fig.4. Effect of capsaicin on the expression of TGF- β_1 , ERK1/2 and eIF3a in lungs from bleomycin-induced pulmonary fibrosis rats. (A) Plasma TGF- β_1 level was measured by enzyme linked immunosorbent assay (ELISA). (B and C) The expression of TGF- β_1 in lung tissue was determined by real-time PCR and Western blot. (D) The expression of p-ERK1/2 in lung tissue was determined by Western blot. (E and F) The expression of eIF3a in lung tissue was determined by real-time PCR and Western blot. Data are means $\pm$ SEM ($n=8$ in each group). ** $P<0.01$ vs. Control; # $P<0.05$ vs. Bleomycin.

Fig.5. Effect of CGRP on TGF- β_1 -induced cell proliferation and expression of α -SMA in cultured pulmonary fibroblasts. (A) Cell proliferation was measured by BrdU incorporation assay. (B) Cell cycle analysis by flow cytometry. (C and D) The expression of α -SMA mRNA and protein was determined by real-time PCR and Western blot. The values are means $\pm$ SEM from three independent experiments in vitro. ** P <0.01 vs. Control; # P <0.05, ## P <0.01 vs. TGF- β_1 ; \$\$ P <0.01 vs. CGRP (100 nM).PD98059: phosphorylation ERK1/2 inhibitor.

Fig.6. Effect of CGRP on TGF- β_1 -induced expression of type I collagen and III collagen in cultured pulmonary fibroblasts. (A and B) The expression of collagen I and collagen III mRNA was determined by real-time PCR. (C and D) The expression of collagen I and collagen III protein was determined by Western blot. The values are means $\pm$ SEM from three independent experiments in vitro. ** P <0.01 vs. Control; # P <0.05, ## P <0.01 vs. TGF- β_1 ; \$\$ P <0.01 vs. CGRP (100 nM).PD98059: phosphorylation ERK1/2 inhibitor.

Fig.7. Effect of CGRP on TGF- β_1 -induced the expression of ERK1/2 and eIF3a in cultured pulmonary fibroblasts. (A)The expression of p-ERK1/2 in lung tissue was determined by Western blot. (B and C)The expression of eIF3a was determined by real-time PCR and Western blot. The values are means $\pm$ SEM from three independent experiments in vitro. ** P <0.01 vs. Control; # P <0.05, ## P <0.01 vs. TGF- β_1 ; \$\$ P <0.01 vs. CGRP (100 nM).PD98059: phosphorylation ERK1/2 inhibitor.

Control

Bleomycin

Bleomycin + Capsaicin

B

C

Control

Bleomycin

Bleomycin + Capsaicin

A **B** **C** **D**

A **B** **C** **D**

A **C** **B**