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Research paper

The role of C/EBP β phosphorylation in modulating membrane phospholipids repairing in LPS-induced human lung/bronchial epithelial cells



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ABSTRACT

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is a common critical emergency with high mortality in clinical practice. The key mechanism of ALI/ARDS is that the excessive inflammatory response damages the integrity of alveolar and bronchial cell membrane and thus affects their basic function. Phospholipids are the main component of cell membranes. Phospholipase A2 (PLA2), which catalyzes the cleavage of membrane phospholipids, is the most important inflammatory mediator of ALI. However, clara cell secretory protein 1 (CCSP1), an endogenous PLA2 inhibitor can increase the self-defense of membrane phospholipids. Thus, CCSP1 up-regulation and PLA2 inhibition constitutes an effective method for ensuring the stability of membrane phospholipids and for the treatment of ALI/ARDS. In the present study, we developed an in vitro model of ALI via lipopolysaccharide (LPS) stimulation of a human bronchial epithelial cell line, BEAS-2B, and assessed the mRNA and protein levels of CCSP1 and PLA2 in the model cells. The results demonstrated LPS induction inhibited the transcription and protein expression of CCSP1, but only the protein level of membrane associated PLA2 was increased, suggesting that in the in vitro ALI model, abnormally regulated CCSP1 transcription plays a crucial role in the damage of cell membrane. To find out the reason that CCSP1 expression was decreased in the ALI model, we predicted, by means of bioinformatics, putative transcription factors which would bind to CCSP1 promoter, examined their background and expression, and found that a transcription factor, CCAAT/enhancer binding protein β (C/EBP β), was correlated with the transcription of CCSP1 in the in vitro ALI model, and its phosphorylation in the model was decreased. CHIP-PCR and luciferase reporter assay revealed that C/EBP β bound to CCSP1 promoter and facilitated its transcription. Therefore, we conclude that there is a C/EBP β /CCSP1/PLA2 pathway in the in vitro ALI model. The study of underlying mechanism show that the activity of C/EBP β depends on its phosphorylation: LPS stimulation reduced C/EBP β phosphorylation and suppressed the transcription of CCSP1 in BEAS-2B cells, which resulted in enhanced PLA2 and the consequent membrane damage. And further study shows that overexpression of CDK2 (Cyclindependent kinase 2), promoted the phosphorylation of C/EBP β and inhibited PLA2 through the C/EBP β /CCSP1/PLA2 pathway, so as to attenuate membrane damage. The significance of this study lies in that artificial C/EBP β phosphorylation regulation may ease the membrane damage in ALI and improve membrane repair.

1. Introduction

ALI/ARDS is a common critical emergency in clinical practice (Xiong et al., 2016) and has become a major issue in public health due to outbreaks of severe acute respiratory syndrome (SARS) and bird flu.

Despite considerable advances in respiratory support and medications for ALI/ARDS patients, the mortality rate is greater than 40% due to the absence of an effective treatment. ALI refers to widespread damage to the pulmonary alveoli, capillaries, and bronchiole barrier as a result of excessive inflammation stimulated by a variety of factors and to

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; PLA2, Phospholipase A2; CCSP, clara cell secretory protein; LPS, lipopolysaccharide; C/EBP β , CCAAT/enhancer binding protein β ; SARS, severe acute respiratory syndrome; MOI, multiplicity of infection; TNF, tumor necrosis factor; IL, interleukin; PAF, platelet activating factor; AA, arachidonic acid; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; CDS, coding DNA sequence; PCR, polymerase chain reaction; BCA, bicinchoninic acid; ECL, enhanced chemiluminescence; GFP, green fluorescent protein; CDK2, Cyclindependent kinase 2; ELISA, enzyme linked immunosorbent assay; LSD, Least Significant Difference

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vascular leakage syndrome induced by increased microvascular permeability. The integrity of the cellular membrane is essential for maintaining the vital activities of cells (Lucas et al., 2009). The physiological function of damaged membranes can be recovered by repair, regeneration or reshaping. Phospholipids are the main component of cell membranes, and their degradation marks the start of cellular damage (De Luca et al., 2012; Elder et al., 2012). PLA2, a phospholipase highly expressed on cellular membranes, catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to release lysolecithin, platelet activating factor (PAF) and arachidonic acid (AA), which process is a key link to induce lung injury (Kitsiouli et al., 2009; Shiyu et al., 2011). CCSP is a major secretion of pulmonary clara cells, the non-ciliated epithelial cells covering distal bronchiole (Jorens et al., 1995). As an endogenous PLA2 inhibitor, CCSP1 has potent immune inhibition and anti-inflammation activity (Rastogi and McHowat, 2009) and can increase self-defense of the membrane phospholipids (Magrioti and Kokotos, 2010). CCSP1 reduces the production of lysolecithin, blocks the abnormal deposition of integrins in fibroblasts, and inhibits the release of AA derivatives as inflammatory mediators, the chemotactic response of fibroblasts to PAF, and the production and action of multiple inflammatory mediators (Wong et al., 2009). In our initial study stage, we found that the mRNA and protein of CCSP1 were reduced in the in vitro ALI model, BEAS-2B cells exposed to LPS (Shiyu et al., 2011). Up-regulation of CCSP1 can therefore stabilize membrane phospholipids and maintain normal cellular function in response to external factors, particularly endotoxin, by inhibiting PLA2 activity and thus preventing lung injury.

C/EBPs, a type of transcriptional regulation factor, were identified by Graves in 1987 (Zahnow, 2009). The C/EBP family of transcriptional regulation factors includes at least six members: C/EBP α , - β , - γ , - δ , - ϵ and - ζ . C/EBP proves to be closely associated with ALI (Okuma et al., 2015). Sugahara et al. demonstrated that C/EBP promotes the proliferation of alveolar endothelial cells in ALI (Sugahara et al., 1999). Therefore, we think that the phosphorylation of C/EBP β plays a crucial role in the regulation of LPS-induced cellular membrane damage in BEAS-2B cells. The major purposes of the study is to verify that (1) phosphorylation of C/EBP β increases the activity of the transcription factor; (2) C/EBP β can bind to the promoter of CCSP1 gene and positively regulates its transcription; (3) C/EBP β regulates the expression of PLA2 protein through CCSP1 transcription and thus affects LPS-induced membrane damage; i.e., there is a C/EBP β /CCSP1/PLA2 pathway which regulates the cellular membrane damage in the ALI model.

2. 2. Materials and methods

2.1. Cell culture

BEAS-2B cells (a gift from Professor Tianbao Zhang of Physiology Department, Second Military Medical University of China) are human normal lung/bronchial epithelia cells transformed by the SV40 T-antigen. BEAS-2B cells were cultured in LHC-8 medium (Invitrogen, CA, USA). Virus producing cell line 293 T (Systembio, CA, USA) maintained in DMEM supplemented with 10% FBS were used for lentiviral packaging, in the luciferase reporter experiments, 293 T were used as a tool cell line. All these adherent cells were passaged by 0.25% trypsin digestion (Invitrogen) and incubated in an atmosphere of 5% CO₂ at 37 °C.

2.2. Construction of vectors

2.2.1. Construction of gene expression vector

The CDS of human C/EBP β (NM_005194.3) was amplified using the primers 5'-GCTCTAGAGCCACCATGCAACGCCTGG-3' and 5'-CGGGATCCCTAGCAGT GGCCGGAGGA-3', which contain an XbaI restriction site(5'-TCTAGA-3') and Kozaksequence(5'-CCACC-3') and a

BamHI restriction site(5'-GATCC-3'), respectively, and cDNA was prepared by reverse transcription of RNA isolated from BEAS-2B cells. The PCR product was digested and cloned into the pcDH1-CMV expression vector (Systembio); the recombinant vector was named pcDH1-C/EBP β . The expression vector for human CDK2 (NM_001798.4) pcDH1-CDK2 and the expression vector for the CCSP1 gene (NM_003357.4) pcDH1-CCSP1 were similarly constructed using the primers 5'-GCTCTAGAGCCACCATGGAGAAGTCCAA-3' and 5'-CGGGATCC TCAGAGTCGAAG ATGGGGTA-3' for CDK2 and 5'-GCTCTAGAGCCACCATGGGAGCTGCTG GGA-3' and 5'-ATAAGAATG CGGCCGCTCACAACCTTCTTCTC-3' for CCSP1.

2.2.2. Construction of shRNA vector

An siRNA sequence targeting the CDS of C/EBP β was designed, and corresponding DNA hairpin structure (5'-GATCCGAG CAAGGCCAAGAAGACCCCTTCTGTGACAGGCTTCTTGGCCTTGCTC TTTTGG-3') sequences were synthesized, annealed and cloned into the pshRNA-H1 expression vector (Systembio); the resulting vector was named pshRNA-C/EBP β . The shRNA expression vectors targeting CCSP1 and CDK2, pshRNA-CCSP1 and pshRNA-CDK2, which contained the sequences 5'-GGAGCTTAACCATCCTAAT-3' and 5'-GGAGCTTAACCATCCTAAT-3', respectively, and the control vector pshRNA-NC (Negative Control), which contained the sequence 5'-GTGACGAGCGAGTCAAGAT-3', were constructed in a similar manner.

2.2.3. Construction of CCSP1 gene expression vector with specific promoter

Next, we extracted human genomic DNA from BEAS-2B cells by QIAamp DNA Mini Kit(QIAGEN, Germany) and amplified the promoter of CCSP1 using the primers 5'-GACTAGTAGGCCAGGTT AGGTGCTATGA-3' and 5'-GACTAGTGTGAGGGTGACAGCGA GTTTCAT-3', which each contain a SpeI restriction site(5'-GACTAGT-3'). The PCR product was digested and cloned into the pcDH1-CCSP1 expression vector to replace the CMV promoter; the recombinant vector was named pcDH1-wPro-CCSP1 (wPro means vector with wild type CCSP1 promoter). Bioinformatics analysis (TRANSFAC) revealed a potential C/EBP β binding site, 5'-CTTGAGAAAA-3', in the CCSP1 promoter. We mutated the sequence to 5'-CTTGCGGAAG-3' to generate a CCSP1 expression vector carrying a mutant promoter, pcDH1-mPro-CCSP1 (mPro means vector with wild mutant CCSP1 promoter).

2.2.4. Construction of luciferase reporter gene expression vector

The promoters were amplified by using pcDH1-wPro-CCSP1 and pcDH1-mPro-CCSP1 plasmids as template, and cloned to a luciferase reporter vector, pGL3-Enkancer (Promega, WI, USA), to construct the wild-type and mutant-type reporter vectors, pGL3-wPro-CCSP1 and pGL3- mPro-CCSP1. The PCR primers used are: forward, 5'-GGGTACCAGGCCAGGTTAGGTGCTATGA-3' and backward, 5'-CCAAGCTTGTGAGGGTGACAGCGAGTTTCAT-3', including KpnI (5'-GGGTACC-3') and HindIII (5'-CCCAAGCTT-3') digestion sites and protective bases.

2.3. Packaging of recombinant lentivirus and infection of BEAS-2B cells with lentivirus

The sequence-validated recombinant vectors were amplified in bacteria, and endotoxin-free DNA was prepared by EndoFree Plasmid Mini Kit (Qiagen). The expression vector or shRNA vector and Lentivirus Package plasmids mix(Systembio) were co-transfected into 293 T producer cells using Lipofectamine™ 2000 (Invitrogen) as instructed by the manufacturer. After 48 h, the supernatants were collected, centrifuged and filtered using 0.45- μ m polyvinylidene fluoride membranes (Millipore, MI, USA). The viral titer was evaluated by gradient dilution. The packaged lentiviruses were named Lv-CDK2, Lv-C/EBP β , Lv-CCSP1, Lv-wPro-CCSP1, Lv-mPro-CCSP1, Lv-shRNA-C/EBP β , Lv-shRNA-CCSP1, Lv-shRNA-CDK2 and Lv-NC.

To examine the gene delivery efficiency and gene intervention effect

of the recombinant viruses, we infected BEAS-2B cells with the over-expression and silencing viruses of CDK2, C/EBP β and CCSP1. The cells were divided into five groups: BEAS-2B, BEAS-2B + Lv-Control, BEAS-2B + Lv-CDK2, BEAS-2B + Lv-NC and BEAS-2B + Lv-shRNA-CDK2; the grouping for C/EBP β and CCSP1 gene intervention was similar. One day before viral infection, BEAS-2B cells in logarithmic phase were made into suspension by trypsin digestion, and the number of viable cells was counted by using trypan blue staining. Cells were collected by $1000 \times g$ centrifugation and re-suspended in LHC-8 medium to a concentration of 5×10^5 cells/ml. Cells were seeded on 6-well plates, 2 ml/well, and cultured overnight under normal conditions. One day after seeding, BEAS-2B cells were infected with lentiviruses diluted by LHC-8 at a MOI of 10, and the medium was refreshed after 24 h. The infection efficiency was assessed by fluorescence microscopy 72 h after infection. Total protein were extracted from the cells by a M-PER Mammalian Protein Extraction Reagent Kit (Thermo, USA) and subjected to western blotting to measure the levels of CDK2, C/EBP β and CCSP1.

2.4. Development of in vitro ALI model

We developed an in vitro ALI model using LPS to induce BEAS-2B cells. Suspension of BEAS-2B cells in logarithmic phase was prepared by trypsin digestion, and the number of viable cells was counted with a hemocytometer by trypan blue staining. Cells were suspended in LHC-8 medium and seeded to 6-well plates at 2×10^5 /well and cultured for 16 h and then the medium was replaced with the LHC-8 medium. 8 h after the medium replacement, LPS was added to a final concentration of 10 mg/ml, and the cells were cultured for 12, 24 or 48 h more. Cells were collected and total protein was extracted and quantified by the method of BCA; intracellular inflammatory factors such as TNF- α , IL-1 β and IL-10, as well as PAF and AA, were measured by ELISA, which were used to determine whether the model was established. We then assessed the expression of CDK2, CCSP1 and PLA2, and phosphorylation of C/EBP β in the cells by western blotting.

2.5. Overexpression and silencing of CDK2 and CCSP1 and their effects on phosphorylation of C/EBP β

Since it is reported that overexpression of CDK2 can regulate the phosphorylation of C/EBP β (Li et al., 2007), we overexpressed and knocked down CDK2 via a lentiviral approach in BEAS-2B cells and examined the phosphorylation of C/EBP β . At the same time, we predicted C/EBP β as a regulator of CCSP1 transcription, so we also overexpressed and silenced CCSP1 in BEAS-2B cells, and observed the effect on the phosphorylation of C/EBP β . BEAS-2B cells in logarithmic phase were seeded to 6-well plates and recombinant viral solution was added at an MOI of 10. 72 h later, cells were collected and total protein was isolated and subjected to western blotting for measuring the phosphorylation of C/EBP β . The cells were divided into five groups: cell control, the cells infected with Lv-NC, Lv-CDK2, Lv-shRNA-CDK2, Lv-CCSP1, or Lv-shRNA-CCSP1.

2.6. Effect of C/EBP β phosphorylation on CCSP1 transcription

According to bioinformatics, we found a possible binding site of C/EBP β in CCSP1 promoter, 5'-CTTGAGAAAA-3'. We overexpressed Lv-wPro-CCSP1 or Lv-mPro-CCSP1 in BEAS-2B cells and then overexpressed or silenced CDK2 to find out the effects of overexpression and depletion of CDK2 and C/EBP β phosphorylation on the mRNA level of CCSP1. By measuring CCSP1 transcription, we wanted to figure out the effect of C/EBP β phosphorylation on CCSP1 transcription and whether C/EBP β binds to CCSP1 promoter through the predicted site. The cells were divided into nine groups: cell control, cells infected with Lv-wPro-CCSP1, Lv-mPro-CCSP1, Lv-wPro-CCSP1 + Lv-NC, Lv-mPro-CCSP1 + Lv-NC, Lv-wPro-CCSP1 + Lv-CDK2, Lv-mPro-CCSP1 + Lv-

CDK2, Lv-wPro-CCSP1 + Lv-shRNA-CDK2, and Lv-mPro-CCSP1 + Lv-shRNA-CDK2. The infection condition was the same as described above, and mRNA quantification was performed 72 h after infection.

2.7. Verification of binding site of transcription factor in CCSP1 promoter by CHIP and luciferase assay

To verify the binding site of C/EBP β in CCSP1 promoter, we conducted CHIP-PCR experiment. BEAS-2B cells in logarithmic phase were infected with Lv-wPro-CCSP1 or Lv-mPro-CCSP1 and then infected with Lv-C/EBP β . 72 h later, cells were harvested and subjected to CHIP-PCR in accordance with the instruction of EZ CHIP KIT (Millipore, MI, USA). The experiment were carried out with reference (Yang et al., 2013) and in accordance with the manufacturer's protocol. The primers for real-time quantitative PCR were: 5'-TTGTGTGAGCTCAGTTTCAG-3' and 5'-GCTTGGTGGGATAGGTAA-3', producing a product of 72 bp, carrying the predicted C/EBP β binding site "5'-CTTGAGAAAA-3'".

To eliminate the interference of endogenous CCSP1 promoter on CHIP-PCR, we also performed luciferase reporter assays. 293 cells were transfected with pCDH1-C/EBP β and pGL3-wPro-CCSP1 or pGL3-mPro-CCSP1, and harvested for luciferase activity assay 48 h later. The transfection experiment was carried out in 24-well plates, following the instructions for Lipofectmaine2000. PGL-TK (100 ng) was transfected for each well as the internal reference for luciferase assay.

2.8. Effect of C/EBP β phosphorylation on C/EBP β /CCSP1/PLA2 pathway

To verify the specificity of the C/EBP β /CCSP1/PLA2 pathway, we knocked down C/EBP β and CCSP1 respectively, and overexpressed CDK2 in the in vitro ALI model established by LPS induction to observe the effect on the inhibition of PLA2 by CDK2 and analyze the specificity of the pathway. The infection condition and LPS induction condition were the same as described above; 48 h after infection, LPS induction was carried out. 24 h after LPS treatment, cells were harvested and total protein was isolated for western blotting for measuring PLA2.

2.9. Examination of effect of C/EBP β phosphorylation on damage of phospholipid membrane in in vitro ALI model

The cells were treated as in the experiment for verifying pathway specificity: LPS induction started 48 h after infection, and 24 h after induction, cells were collected and subjected to PAF and AA assessment. The inter-group difference of PAF and AA releases was used to determine the damage degree of membrane phospholipid.

2.10. Measurement of relative mRNA by real-time PCR

After removal of the culture medium, the cells were washed with pre-cooled dPBS, supplemented with pre-cooled trizol reagent, and pipetted until the lysate became viscous and clear. The lysate was transferred to a 1.5-ml centrifuge tube and extracted using the phenol-chloroform method (Han et al., 2002). The purity of the total RNA was determined by ultraviolet spectrophotometry, and the mRNA levels of CCSP1 were detected by real-time PCR. Total RNA (2 μ g) was used as the template to prepare cDNA by reverse transcription. The product of reverse transcription (2 μ l) was used as the template for real-time PCR. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method with β -actin (NM_001101.3) as the internal reference (Livak and Schmittgen, 2001). The PCR primers were as follows: β actin-forward, 5'-CCTGTACGCCA-ACACAGTGC-3', β actin-reverse, 5'-ATACTCTGCTT GCTGATCC-3'; CCSP1-forward, 5'-GCCGGCGCCGAGCTCTGGCTC-3'; CCSP1-reverse, 5'-TGTCAGTTTGCAAATACCCCA-3. Each PCR reaction contained 10 μ l of SYBR Premix Ex Taq, 0.2 μ l each of forward and reverse primers (20 μ M), 2 μ l of cDNA, and dH₂O to a final volume of 20 μ l. The reaction parameters were 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s, and elongation at 72 °C for 10 s. The

experiment was carried out on Takara fluorescent quantitative TP800.

2.11. Evaluation of protein levels by western blotting

Total protein was extracted, and proteins were detected by western blotting. Cells were rinsed with 1 ml of cooled dPBS, and 1 ml of cell lysis buffer (50 mM pH 8.0 Tris, 1 mg/ml leupeptin, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 1 M dithiothreitol, and 1 mg/ml aprotinin) was added for protein extraction. Protein concentrations were determined using the bicinchoninic acid (BCA) assay. Protein samples (10 μ l/lane) were separated by 10% SDS-PAGE and transferred to PVDF membranes by wet transfer. Blots were blocked in Tris-buffered saline containing Tween-20 (TBST) containing 5% nonfat milk at room temperature for 2 h and incubated with primary antibodies (anti-CDK2(sc-6248, santacruz), 1:300; anti-CCSP1(07–623, millipore), 1:500; anti-PLA2(sc-58,363, santacruz), 1:500; anti- β actin(sc-130,301, santacruz), 1:800; anti-C/EBP β (ab32358, Abcam), 1:400; anti-phosphorylation-C/EBP β (ab52194, Abcam), 1:400) at 4 °C overnight. The membranes were then rinsed with TBST and incubated with secondary antibodies for 2 h. Enhanced chemiluminescence (ECL) substrates (Pierce, CA, USA) and X-ray film were used to detect the bands, and the relative optical densities were analyzed using image processing software (Daniels et al., 2017).

2.12. Evaluation of protein levels by ELISA

Total protein was extracted and quantified using the BCA method, and intracellular TNF α , IL-1 β and IL-10 were measured using Human TNF α -ELISA kit, Human IL-1 β -ELISA kit and Human IL-10 ELISA kit (Invitrogen). Experiments for the PAF and AA level were performed using the Human arachidonic acid (AA) ELISA Kit (MBS703581, Cayman) and Human Platelet Activating Factor (PAF) ELISA Kit (MBS701223) according to the manufacturer's instructions. After the experiments, the value data of PAF and AA were calculated according to the A450.

2.13. Statistical analyses

All data is expressed as mean \pm SD, and analyzed by one way ANOVA. Least Significant Difference (LSD) was used for multiple comparisons between any two means. *P*-values < 0.05 were considered statistically significant. All statistical analysis was performed using SPSS 13.0 software.

3. Results

3.1. Lentiviral infection of BEAS-2B cells and determination of gene intervention efficiency

Highly efficient gene delivery is essential for effective interference. We resolved the difficulty of transfecting BEAS-2B cells by using routine approaches with a lentiviral system (Fig. 1A), resulting in GFP expression in more than 90% of cells. The lentiviral approach produced good results for overexpression and knockdown of CDK2, C/EBP β and CCSP1 in BEAS-2B cells: western blotting results show that the expression levels of CDK2, C/EBP β and CCSP1 in the overexpression groups were 3.69 ± 0.06 , 4.18 ± 0.04 and 3.06 ± 0.07 times as high as that of the control groups respectively (Fig. 1B), and CDK2, C/EBP β and CCSP1 in the knockdown groups were 0.12 ± 0.01 , 0.28 ± 0.02 and 0.20 ± 0.01 times as high as that of the control groups respectively (Fig. 1B), with significant differences between the genetic intervention groups and the cell groups (*P* < 0.05); no significant differences were observed in CDK2, C/EBP β and CCSP1 expression levels between the Lv-NC or Lv-control groups and the control groups (*P* > 0.05). These data suggest that the lentiviral approach we used effectively expressed or knocked down CDK2, C/EBP β and CCSP1

genes, and the viral system and hairpin structure themselves had little effect on these genes.

3.2. Establishment of in vitro ALI model and measurement of relevant proteins

We employed LPS to induce BEAS-2B cells to develop an in vitro ALI model, and examined TNF α , IL-1 β , and IL-10, as well as PAF and AA, in the cells to determine whether the model was established. ELISA results (Fig. 2A) show that TNF α , IL-1 β , and IL-10 were increased in BEAS-2B cells induced with LPS for 12 h, 24 h or 48 h, with significant difference in the three inflammatory factors between the LPS group and the cell group at 24 and 48 h (*P* < 0.05). Measurements of PAF and AA demonstrate that induction of 10 mg/ml LPS for 12, 24, or 48 h increased PAF and AA, both of which peaked at 48 h. The inflammatory indicators and membrane metabolic indices suggested that BEAS-2B cells displayed an obvious inflammatory response after being induced by 10 mg/ml LPS and released PAF and AA, indicating the successful establishment of the in-vitro ALI model. Based on the model, we assessed CDK2, CCSP1 and PLA2 levels and C/EBP β phosphorylation in the cells (Fig. 2C), and found the expression of CDK2 and CCSP1 and C/EBP β phosphorylation displayed similar changes—a decrease after treatment of LPS, with significant differences between the LPS treated groups and the cell groups (*P* < 0.05); PLA2 was increased by LPS treatment, significant difference found at 24 and 48 h (*P* < 0.05). Analyzing the change of four proteins in different groups, we found that in the ALI model, CDK2, CCSP1 and phosphorylation of C/EBP β were decreased, with obvious correlation between each other. In addition, the expression of PLA2 was significantly increased in the ALI model, which was negatively correlated with the expression of CDK2 and CCSP1, and the phosphorylation of C/EBP β . These findings hinted that the increase in the expression of CDK2, and CCSP1, and the phosphorylation of C/EBP β , and the decrease in PLA2 may have a positive impact on ALI.

3.3. Overexpression and knockdown of CDK2 and CCSP1 and their effects on C/EBP β phosphorylation

Xi Le et al. reported that CDK2 can phosphorylate C/EBP β through Thr188 (Xi Li et al., 2007), so in the study, we used CDK2 to regulate the phosphorylation and dephosphorylation of C/EBP β by overexpressing and knocking down CDK2 in BEAS-2B cells. At the same time, we also overexpressed and depleted CCSP1 to observe the effect on C/EBP β phosphorylation, which would help us to ascertain the relation between C/EBP β and CCSP1. The results (Fig. 3) indicate that CDK2 overexpression increased both C/EBP β phosphorylation and CCSP1 expression (*P* < 0.05 vs cell control) while CDK2 knockdown suppressed both C/EBP β phosphorylation and CCSP1 expression (*P* < 0.05 vs cell control). For C/EBP β phosphorylation or CCSP1 expression, there was no significant difference between the Lv-NC group and the control group (*P* > 0.05). In addition, the overexpression and silencing of CCSP1 had no obvious effect on the expression of CDK2 or the phosphorylation of C/EBP β (*P* > 0.05 vs cell control). Based on the analysis of these data, we speculated that CDK2 is an upstream mediator of C/EBP β and CCSP1 is a downstream element of C/EBP β . The regulation of C/EBP β by CDK2 has been reported, but is it possible for C/EBP β to regulate the expression of CCSP1? We next investigated the problem by using subsequent experiments.

3.4. Verification of C/EBP β binding site on CCSP1 promoter

According to the prediction of bioinformatics, we constructed a recombinant lentivirus with the wild CCSP1 promoter, Lv-wPro-CCSP1, and a lentivirus carrying a mutant CCSP1 promoter, Lv-mPro-CCSP1. We infected BEAS-2B cells with these lentiviruses and overexpressed CDK2, and then qualified CCSP1 mRNA to verify the predicted binding

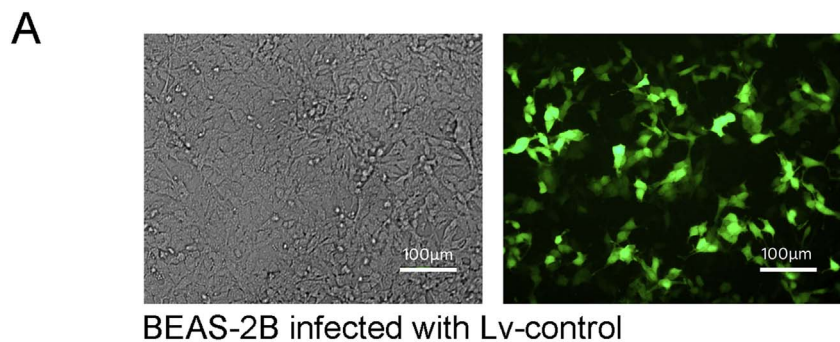
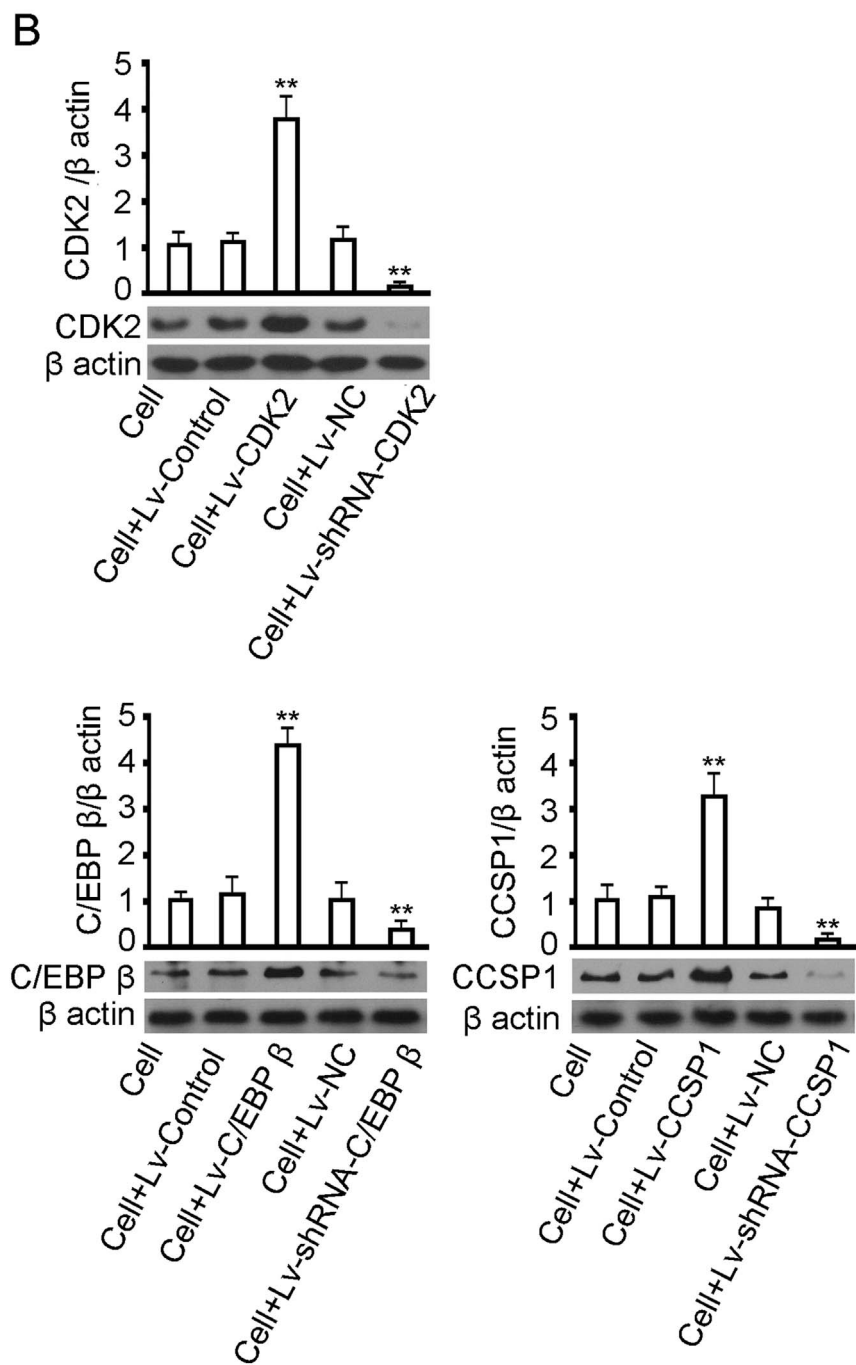


Fig. 1. Infection efficiency of BEAS-2B cells and gene intervention efficiency of CDK2, C/EBP β and CCSP1.
A. Left, cells viewed by bright-field microscopy; right, cells viewed by fluorescent microscopy. The infection efficiency was estimated by dividing the number of cells expressing the fluorescent marker with the total number of cells in the same view, five fields randomly selected for the estimation. BEAS-2B infected with Lv-Control for 72 h, MOI = 10. **B.** Expression of CDK2, C/EBP β and CCSP1 measured by western blotting. Lower: image of target blots, upper: analysis on the optical density of three proteins, β actin serving as the reference. Data are means \pm SD from at least three separate replicates. **, $p < 0.01$, vs cell group or cell infected with Lv-Control group.



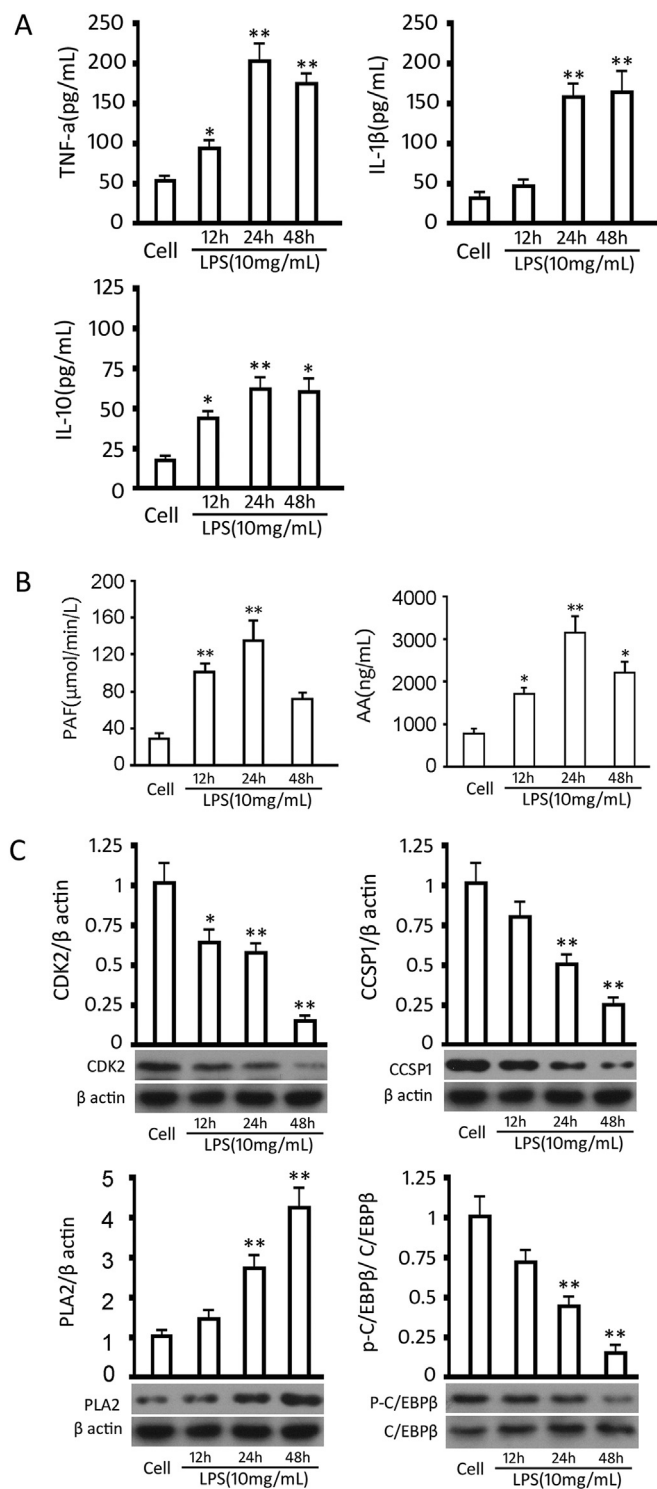


Fig. 2. Verification of ALI model and measurement of relevant proteins.

A. TNF α , IL-1 β , and IL-10 in BEAS-2B cells were examined using ELISA 12 h, 24 h or 48 h after treatment of 10 mg/ml LPS. The vertical coordinate indicates the concentration of target protein, and the horizontal coordinate indicates the experiment group. B. PAF and AA contents in BEAS-2B cells were measured using ELISA 12 h, 24 h or 48 h after treatment of 10 mg/ml LPS. The vertical coordinate indicates the concentration of target protein, and the horizontal coordinate indicates the experiment group. C. CDK2, CCSP1 and PLA2 expression levels, as well as C/EBP β phosphorylation, were assessed by western blotting 12 h, 24 h or 48 h after treatment of 10 mg/ml LPS. Lower: image of target blots, upper: analysis on the optical density of three proteins, β actin serving as the reference, and for the phosphorylated protein, the total protein of itself serving as the reference. Data are means \pm SD from at least three separate replicates. *, $p < 0.05$; **, $p < 0.01$. vs cell group.

site of C/EBP β . The results (Fig. 4A) show that infection of Lv-wPro-CCSP1 or Lv-mPro-CCSP1 increased CCSP1 mRNA ($P < 0.05$ vs control). In comparison with infection of Lv-wPro-CCSP1 alone, co-infection with Lv-CDK2 increased CCSP1 mRNA, and co-infection with Lv-shRNA-CDK2 impaired the increase ($P < 0.05$). The co-infection with Lv-CDK2 or Lv-shRNA-CDK2 did not change CCSP1 mRNA in the cells infected with Lv-mPro-CCSP1 ($P > 0.05$). All these data suggest that C/EBP β regulates the transcription of CCSP1 through the predicted binding site. We further verified the binding by using CHIP-PCR (Fig. 4B). In the BEAS-2B cells infected with Lv-wPro-CCSP1, C/EBP β bound to more DNA fragments around the binding site in CCSP1 promoter ($P < 0.05$ vs Lv-control), and in the cells infected with Lv-mPro-CCSP1, no difference was observed ($P > 0.05$). The luciferase activity assay data (Fig. 4C) demonstrated that the luciferase activity in the groups transfected with pCDH1-C/EBP β alone or pshRNA-C/EBP β and pGL3-wPro-CCSP1 was significantly higher or lower than in the group transfected with pGL3-wPro-CCSP1 alone ($P < 0.05$), but there was no significant difference in luciferase activity between the group transfected with pCDH1-C/EBP β , or pshRNA-C/EBP β and pGL3-mPro-CCSP1, and the group transfected with pGL3-mPro-CCSP1 alone ($P > 0.05$). No difference was observed in the groups transfected with pshRNA-NC with pGL3-wPro-CCSP1 or pGL3-mPro-CCSP1, compared with the groups transfected with pGL3-wPro-CCSP1 or pGL3-mPro-CCSP1 alone ($P > 0.05$).

3.5. Effect of CDK2 on C/EBP β /CCSP1/PLA2 pathway and effect of C/EBP β phosphorylation on membrane phospholipid damage in the in vitro ALI model

To verify the critical role of C/EBP β phosphorylation in the C/EBP β /CCSP1/PLA2 pathway and the specificity of the pathway, we over-expressed CDK2 in the in vitro ALI model to phosphorylate C/EBP β and then observed the change in PLA2; In addition, we also knocked down C/EBP β or CCSP1 in the ALI model with CDK2 overexpression and examined PLA2 protein levels in these groups. The results (Fig. 5A) show that CDK2 overexpression attenuated LPS-induced increase of PLA2 protein ($P < 0.05$ vs LPS group), indicating that overexpression of CDK2, or C/EBP β phosphorylation, could significantly inhibit PLA2 expression in the in vitro ALI model. Knockdown of C/EBP β or CCSP1 impaired the inhibition of PLA2 expression by CDK2 overexpression ($P < 0.05$ vs LPS + Lv-CDK2), indicating in the in vitro ALI model, there was a specific C/EBP β /CCSP1/PLA2 pathway. To illustrate the effect of C/EBP β phosphorylation on membrane phospholipid damage in the in vitro ALI model, we treated BEAS-2B cells which over-expressed or were depleted with CDK2 with LPS for 48 h and assessed PAF and AA by ELISA. The data (Fig. 5B) show that CDK2 overexpression inhibited the release of PAF and AA induced by LPS ($P < 0.05$), while the contents of PAF and AA in the group of CDK2 silencing and LPS induction were slightly higher than that in the Lv-NC group ($P > 0.05$ vs LPS group). No change in PAF or AA was found in the Lv-NC group ($P > 0.05$ vs LPS group). In conclusion, we believe that the phosphorylation of C/EBP β can promote the transcription of CCSP1 and subsequently inhibit the expression of PLA2 and then resist the disruption of phospholipids caused by ALI.

4. Discussion

C/EBP family members have a basic DNA-binding domain and a leucine zipper domain, which bind to the enhancer 5'-RTTGCGYAAY-3' (R = A or G, Y=C or T) or variants to positively or negatively regulate transcription, thus playing important roles in energy metabolism, cell growth, differentiation and tumorigenesis, hematogenesis and the immune response (Sayeed et al., 2015; Shimizu et al., 2015). C/EBP β expression varies in porcine liver during prenatal and postnatal development and sharply increases after birth, according to Tang et al. (Tang et al., 2015). C/EBP β can activate the transcription of the IGF1 gene by

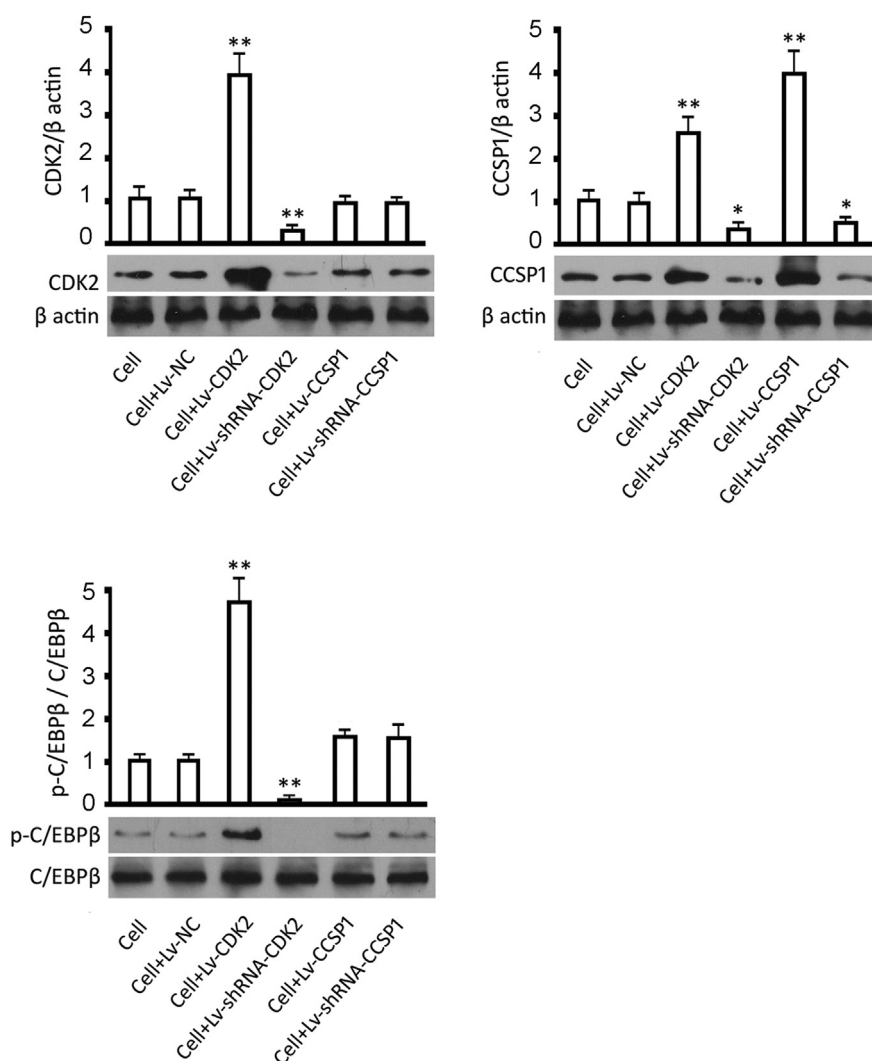


Fig. 3. Overexpression and knockdown of CDK2 and CCSP1 and their effects on C/EBP β phosphorylation.

BEAS-2B cells were infected with the indicated lentivirus, and CDK2, CCSP1 and phosphorylated C/EBP β and total C/EBP β were measured by western blotting 72 h later. Upper: representative blots of CDK2, CCSP1 and phosphorylated C/EBP β and total C/EBP β and β-actin are shown. Lower: the optical density of the target band divided by the optical density of the β-actin band. Data are means ± SD from at least three separate replicates. *, $p < 0.05$; **, $p < 0.01$ vs cell group or cell + Lv-NC group.

binding to two sites of the IGF1 gene promoter. ATP-dependent P2X7 receptor activation is also dependent on the transcriptional regulation of C/EBP β (Bilodeau et al., 2015). The above studies clearly indicate that C/EBP β mainly plays its transcriptional regulatory role as a nuclear transcription factor.

Cassel et al. identified two binding sites for C/EBPα and C/EBPδ in the sequence between –100 bp and –62 bp of the CCSP promoter through which C/EBPδ and C/EBPα enhance CCSP expression via a complex response element in A549 cells which are adenocarcinomic human alveolar basal epithelial cells (Cassel et al., 2000). Massaad et al. (Massaad et al., 2000) suggested that IL-1β can enhance the transcription of PLA2 through the promoter (–1614; +20 nt) by 6–7-fold, and the region (–210; –176 nt) is crucial to the process. C/EBP β and C/EBPδ can bind to this region, indicating that C/EBP may block IL-1β-induced PLA2 transcription. These studies may suggest that there are some potential relationships between C/EBP and CCSP/PLA2; however, the specific pathway remains unclear.

The transcriptional activity of C/EBP β depends on its phosphorylation: phosphorylation of specific amino acids allows the protein to be translocated into the nucleus and bind to the promoter of genes regulated by it, so as to regulate the transcription of downstream genes (Zhou et al., 2015). Therefore, our research objectives are to prevent the damage of membrane by effective intervention in the in vitro ALI model through the C/EBP β/CCSP1/PLA2 pathway based on C/EBP β phosphorylation. If C/EBP β inhibits PLA2 by upregulating CCSP1, we may seek an approach to repair membrane phospholipids damaged by acute

inflammation based on the C/EBP β/CCSP1/PLA2 pathway. Having confirmed the existence of the C/EBP β/CCSP1/PLA2 pathway, we attempted to suppress the phosphorylation of C/EBP β to inhibit the pathway and reduce the LPS-induced breakdown of membrane phospholipids.

Our data show that in an in vitro ALI model established by LPS induction, phosphorylation of C/EBP β was decreased and bioinformatics analysis discovered a putative binding site of C/EBP β in CCSP1 promoter, so we wondered whether C/EBP β phosphorylation regulates the transcription of CCSP1. We, using CHIP-PCR and luciferase reporter assay, demonstrated that C/EBP β binds to CCSP1 promoter and positively regulates CCSP1 transcription, and its phosphorylation increases its transcriptional activity. Based on the validation of the specificity of C/EBP β/CCSP1/PLA2 pathway, we overexpressed CDK2 to enhance the phosphorylation of C/EBP β on Thr188 (Li et al., 2007), so as to inhibit the expression of PLA2 via the C/EBP β/CCSP1/PLA2 pathway, and to control LPS-induced cellular membrane injury. The findings corroborate that overexpression of CDK2 inhibited the production of PAF and AA in the ALI model, i.e., overexpression of CDK2 attenuated the damage of membrane phospholipids. The study sheds light on the underlying signal transduction for repair of membrane phospholipids and provides a novel target for treatment of ALI.

We employed LPS to induce BEAS-2B cells, and found out the optimal condition for building the in vitro ALI model, 10 mg/ml LPS induction for 24 h by examining TNFα, IL-1β and IL-10 expression levels and the release of PAF and AA. Based on the model, we assessed CDK2,

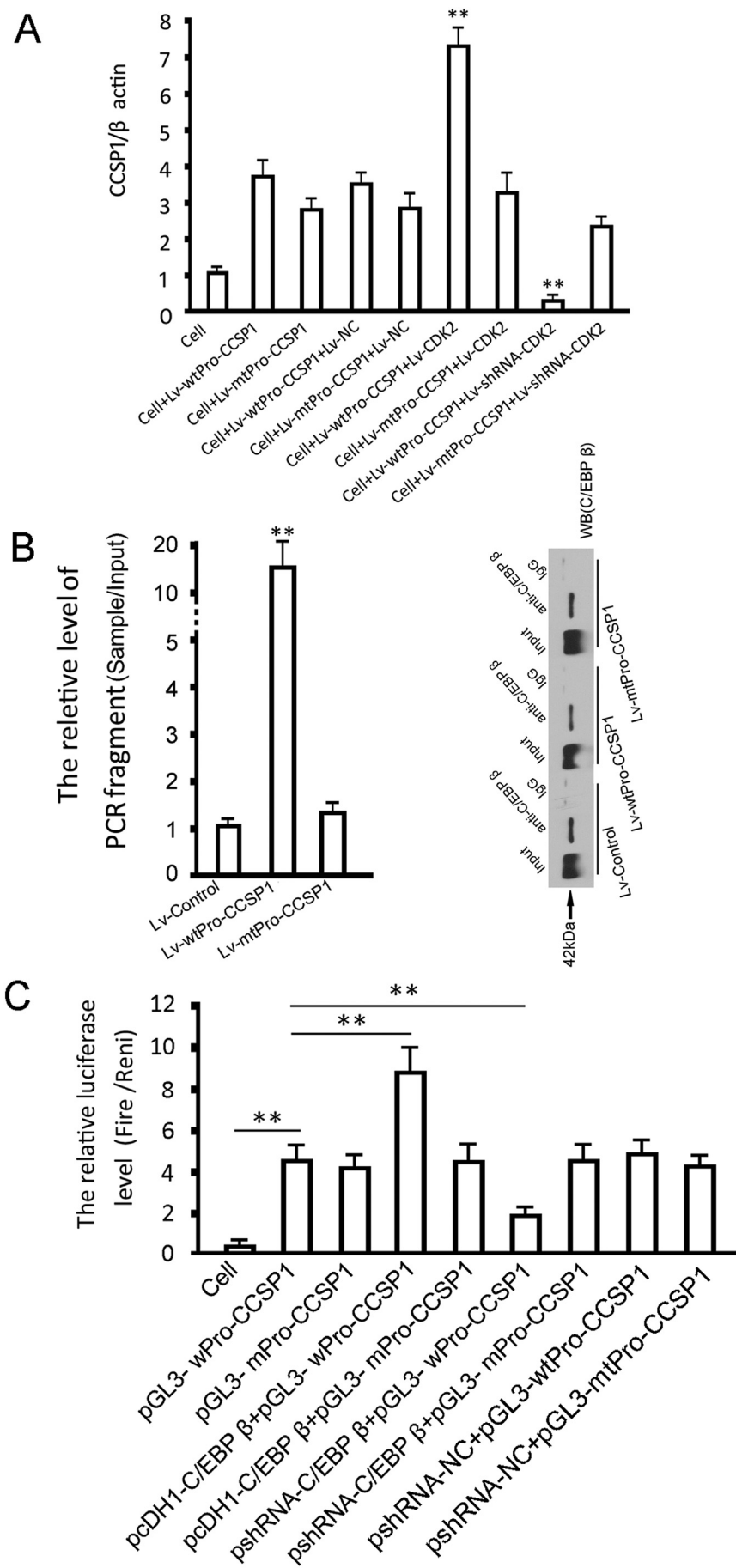


Fig. 4. Verification of C/EBP β binding site on CCSPI promoter. **A.** BEAS-2B cells were infected with the indicated lentivirus, and CCSPI mRNA was measured by real time quantitative PCR 72 h later. The vertical coordinate indicates the relative amount of CCSPI mRNA and the horizontal coordinate indicates the experiment group, β actin serving as the reference. **B.** Verification of C/EBP β binding site on CCSPI promoter by CHIP-PCR. Upper: relatively quantitative analysis on DNA segments, the vertical coordinate being the ratio of gene copy number of the sample to the control gene copy number of Input, and the horizontal coordinate being experiment group. Lower: western blotting results of C/EBP β protein, Input is the negative control without antibody, and IgG is the control group with a primary antibody against GAPDH. **C.** Measurements of luciferase activity. The vertical coordinate indicates the luciferase activity expressed as the firefly luciferase/Renilla luciferase, and the horizontal coordinate indicates the experiment group. The determination of the luciferase activity was made 48 h after transfection of 293 cells. Data are means ± SD from at least three separate replicates. *, $p < 0.05$; **, $p < 0.01$.

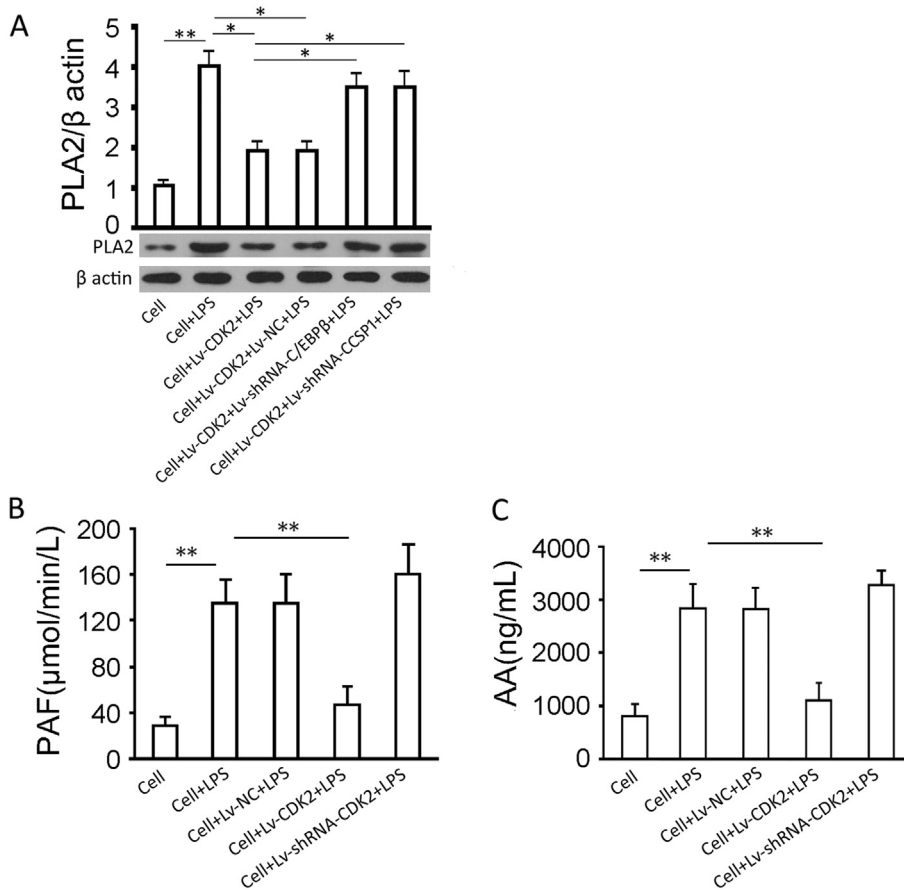


Fig. 5. Verifying the specificity of the pathway and examining membrane damage

A. BEAS-2B cells were infected with the indicated lentivirus, and PLA2 expression was measured by western blotting. Upper: representative blots and lower: the optical density of the target band divided by the optical density of the β -actin band. B. BEAS-2B cells were infected with the indicated lentivirus, and treated with LPS 48 h later, and PAF and AA were measured using ELISA. Data are means \pm SD from at least three separate replicates. *, $p < 0.05$; **, $p < 0.01$.

CCSP1 and PLA2 protein levels, and the results revealed that in the model, CDK2 and CCSP1 were decreased, and PLA2 was increased. In addition, measurement of protein phosphorylation showed that LPS induction significantly suppressed C/EBP β phosphorylation. To verify the existence of C/EBP β /CCSP1/PLA2 pathway, we analyzed the relation between C/EBP β and CCSP1 gene. The experiment revealed that C/EBP β phosphorylation or dephosphorylation could enhance or suppress CCSP1 transcription but overexpression or knockdown of CCSP1 had no effect on C/EBP β phosphorylation, hinting at CCSP1 being downstream C/EBP β . We next employed co-infection and CHIP-PCR to verify the binding site of C/EBP β in CCSP1 promoter predicted by bioinformatics. Quantitative determination of CCSP1 mRNA shows that C/EBP β phosphorylation enhanced CCSP1 transcription controlled by the wild promoter but not that controlled by the promoter with binding site mutation, and dephosphorylation of C/EBP β only inhibited CCSP1 transcription controlled by the wild promoter. CHIP-PCR results show that C/EBP β was capable of binding to the promoter DNA carrying the wild binding site but not the one carrying mutant binding site. However, though endogenous CCSP1 promoter is at a low level in CHIP-PCR experiments, it may have a certain inference on the experimental data. Therefore, we employed the luciferase reporter assay to verify the results of CHIP-PCR. The results indicated that overexpression of C/EBP β significantly increased the luciferase controlled by wild type CCSP1 promoter, but had no obvious effect on that controlled by the CCSP1 promoter with a mutant binding site. Eliminating the background impact, these data demonstrated that the transcription factor can bind to CCSP1 promoter through the predicted site and activate the transcription.

These results clarified the regulatory sequence of the proteins in the C/EBP β /CCSP1/PLA2 pathway. In addition, we also investigated the specificity of the C/EBP β /CCSP1/PLA2 pathway by knocking down C/EBP β and CCSP1, and the results indicate that the inhibition of PLA2

by CDK2 was mediated by C/EBP β phosphorylation and transcription of CCSP1. LPS stimulation inhibits the phosphorylation of C/EBP β in BEAS-2B cells, suppresses the C/EBP β /CCSP1/PLA2 pathway, and increases the expression of PLA2 protein, leading to membrane injury. Since CCSP1 is an endogenous inhibitor of PLA2, we tried to inhibit the damage of membrane in the in vitro ALI model through regulating PLA2 via C/EBP β phosphorylation. To regulate phosphorylation and dephosphorylation of C/EBP β , we overexpressed or silenced its upstream kinase CDK2, which is proved to be an effective means to phosphorylate or dephosphorylate C/EBP β by the experimental data. The findings suggest that overexpression of CDK2 suppressed LPS-induced increase of PLA2 in the cells via the C/EBP β /CCSP1/PLA2 pathway, and inhibited the release of PAF and AA, a clear inhibition of membrane phospholipid damage in the in vitro ALI model. These findings are promising, since it is of significance to the treatment of ALI that increase of C/EBP β phosphorylation could inhibit PLA2 expression and membrane injury in ALI. The results indicate that C/EBP β is a potential target for ALI treatment. However, we also have some urgent work for the future. For example, what is the mechanism by which LPS stimulation inhibits the phosphorylation of C/EBP β , and is there some approach that regulates the phosphorylation of C/EBP β in a more effective, more direct and specific way? The experimental strategy of increasing C/EBP β phosphorylation by CDK2 overexpression is obviously not suitable for clinical application. For this reason, our future research will focus on the development of specific agonist for C/EBP β phosphorylation, which may be used in the ALI treatment.

In conclusion, our study shows that C/EBP β phosphorylation could impair the increase in PLA2 in the in vitro ALI model through the C/EBP β /CCSP1/PLA2 pathway and thus restrain the membrane injury caused by PLA2, which is of significance to maintaining normal function of epithelial cells in ALI disease. It is also suggested that C/EBP β be a potential target for targeted therapy of ALI and the development of

C/EBP β phosphorylation agonist may be a direction for future research for targeted treatment of ALI.

Conflict of interest

The authors declare there are no conflict of interest.

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