

Full Length Research Paper

Proteomic analysis of oxymatrine-induced changes in lung fibroblasts

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Oxymatrine, one kind of alkaloids extracted from *Sophora flavescens* Ait, has been demonstrated to attenuate bleomycin-induced lung fibrosis. The mechanisms of this action are poorly known. This study aimed to use proteomics to provide new insights into the mechanisms of the pharmacological effects. Proteins from murine lung fibroblasts exposed to oxymatrine were separated by two-dimensional electrophoresis. Differentially expressed proteins were identified using mass spectrometry. A two-dimensional cell migration assay was used to study the effects of oxymatrine on fibroblast migration activity. Among these detectable protein spots on the 2-DE gel 9 protein, spots were significantly altered in oxymatrine treated cells. 5 proteins were successfully identified. All were down-regulated by oxymatrine. Mass spectrometer analysis indicated that one of these proteins is a metabolic enzyme, while others are related to cytoskeleton or involved in cellular transcription and translation. The attenuated expressions of mRNA and protein of the cytoskeleton-related protein, HSP27, were confirmed by Western blotting and RT-PCR analysis. Besides these, the migration activity of fibroblast was reduced by oxymatrine. It is suggested that regulation of HSP27 expression by oxymatrine is associated with its inhibitory effects on motility of lung fibroblasts, which contributes to the protective action of this drug against lung fibrosis.

Key words: Oxymatrine, fibroblasts, proteomics, heat shock protein 27, motility

INTRODUCTION

Pulmonary fibrosis is characterized by an absolute increase of collagen-rich extracellular matrix. The accumulation of extracellular matrix, damage of alveolar wall and loss of functional capillary units can lead to respiratory failure within a few years after diagnostic confirmation. Specific treatments for fibrotic lung diseases are not yet available. Therefore, novel therapeutic agents with improved efficacy are needed (Datta et al., 2011; Gross and Hunninghake, 2001).

Up to now, our understanding of the pathogenesis of this condition is incomplete. However, it is likely that

multiple abnormalities in a myriad of biological pathways affect inflammation and wound repair, including matrix regulation, epithelial reconstitution, the coagulation cascade, neovascularization and antioxidant pathways. Current research results indicated that fibroblast activation lead to progressive fibrosis. Therefore, regulation of fibroblasts should be an important therapeutic strategy (Datta et al., 2011).

Oxymatrine (OM) is an alkaloid extracted from *Sophora flavescens* Ait (a traditional Chinese medicine belonging to the pulse family) (Wu et al., 2006). In China, OM as a clinical drug has been used to treat viral hepatitis, cardiac arrhythmia and skin inflammations (Dong et al., 2002; Liu et al., 1994, 2003). It has been proven to be an effective inhibitor of tissues fibrosis, including hepatic fibrosis, myocardial fibrosis, pulmonary fibrosis (Shi and Li, 2005;

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Shen et al., 2011; Liu et al., 2012). The protective effects of OM on pulmonary fibrosis are related with the inhibition of inducible nitric oxide synthase expression and the TGF- β /Smad signaling pathway (Shen et al., 2011; Liu et al., 2012). However, the mechanisms of the effects of OM are largely unknown, especially the effects of OM on lung fibroblasts.

Proteomic methodologies, including two-dimensional electrophoresis (2-DE) and mass spectrometry (MS), provide a general route to analyze the global protein expression of cells at different status. Proteomic analysis has been successfully applied to investigate the molecular effects of drugs and to obtain information regarding their mode of action (Elrick et al., 2006). In this paper, fibroblasts derived from lung of mice were treated with OM, and the protein profiling of fibroblasts was analyzed and compared.

The functional implications of the differentially expressed proteins, which may play important roles in mediating OM's effects on fibroblasts, were discussed. To confirm the expression changes of proteins by proteomics analysis, Western blot and Reverse transcription polymerase chain reaction (RT-PCR) were performed. We believe that such global proteins expression study could allow improved understanding of the mechanisms of OM's protective effects on mice with pulmonary fibrosis.

MATERIALS AND METHODS

Reagents

OM was purchased from Yixin pharmaceuticals (Zhejiang, China). It was dissolved in distilled Dulbecco's modified Eagle's medium (DMEM) and stored at 4°C. IPG strip (pH 3 to 10), pharmalyte (pH 3 to 10), high-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) were purchased from Bio-Rad (Hercules, CA, USA). α -Cyano-4-hydroxycinnamic acid (CHCA), ammonium persulphate (APS), sodium dodecyl sulphate (SDS), glycine, glycerol, ultra pure urea, acrylamide, dithiothreitol (DTT), 3-[3(cholamidopropyl) dimethylammonio]-1-propane-sulphonate (CHAPS), ultra pure Coomassie R-250 were purchased from Sigma-Aldrich Corp. (St. Louis MO, USA). Rabbit polyclonal IgG antibody to rodent heat shock protein 27 (HSP27) was provided by Cell Signaling Technology, Inc. (Danvers, USA). Chemiluminescence Western blotting detection reagent (ECL Plus) was purchased from Pierce Biotechnology (Rockford, USA). The Prime ScriptTM 1st Strand cDNA Synthesis Kit and PCR Amplification Kit were purchased from TaKaRa Biotechnology (Otsu, Japan). All other chemicals were of analytical grade.

Cell culture

Lung fibroblasts from health adult mice were isolated according to the method by Phan et al. (1985) as before. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) maintained at 37°C in a humidified incubator in an atmosphere of 5% CO₂ in air. After reaching subconfluence, cells were cultured in serum-free DMEM for 24 h, then challenged with or without OM 1.0 mg/mL over 72 h. All procedures were approved by Ethics Committee of The Guangzhou Medical College.

Two-dimensional gel electrophoresis

OM treated or the control cells were collected and lysed in lysis buffer (7 mmol/mL urea, 2 mmol/mL thiourea, 4% CHAPS, 65 μ mol/mL DTT, 0.15 μ mol/mL EDTA, 2 μ mol/mL PMSF), sonicated in six cycles of 15 s. After centrifugation at 12000 \times g for 1 h at 4°C, the protein concentration was estimated with the method of Bradford (Bio-Rad, Richmond, CA, USA) (Tsai et al., 2008; Ruiz-Romero et al., 2009). The first dimension (IEF) was performed on a Protean IEF Cell apparatus (Bio-Rad) as follows: 800 μ g protein samples were applied to an IPG strip, pH 3 to 10 (Bio-Rad). The strips were soaked in the sample solution for 1 h to allow uptake of the proteins. The strip was then actively rehydrated in a Protean IEF cell (Bio-Rad) for 16 h at 50 V. The isoelectric focusing was at 300 V for 2 h linearly, 500 V for 2 h linearly, 1000 V for 2 h linearly, 8000 V for 8 h linearly, and 8000 V for 10 h rapidly. All the processes above were carried out at 22°C. After equilibration, SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a Protean II XL system (Bio-Rad). The gels were then stained overnight using R-250 Coomassie blue and destained in 1% (v/v) acetic acid solution on a gently continuous rocker.

Image analysis

The two-dimensional gels were scanned using an Image Scanner (GE HealthCare). The peptide spots were analyzed with ImageMaster 2D Elite 5.0 software (Amersham Biosciences). All well-separated and clearly focused spots that were 2-fold differentially expressed between the control and OM treated fibroblasts were classified as up- or down- regulated spots, and then selected for mass spectrometric analysis (Tsai et al., 2008; Ruiz-Romero et al., 2009).

Mass spectrometry analysis and protein identification

Selected protein spots were excised from the 2-DE gel and destained and digested in-gel with trypsin, as previously described (D'Hertog et al., 2007). Then, the collected samples were analyzed with a MALDI-TOF/TOF (ABI 4700 Proteomics Analyzer, Applied Biosystems). For protein identification, the obtained mass information was searched against the National Center for Biotechnology Information (NCBI) protein databases using the MASCOT search engine (www.matrixscience.com) (Qian et al., 2008).

Western blotting

Cells were scraped off the plates after treated with 0, 1.0, 0.5, 0.25 or 0.125 mg/mL of OM and incubated for 30 min in lysis buffer. After centrifugation, a portion of supernatant was used for protein concentration determination using the Bio-Rad protein assay. Equal amounts of protein (20 μ g) were loaded onto a 10% polyacrylamide gel, electrophoresed and transferred to polyvi polyvinylidene difluoride (PVDF) membrane. The blots were blocked for 1 h with 5% non-fat milk reagent dissolved in TBST (1 \times Tris-buffered saline with 0.05% Tween-20). Membranes were incubated with an anti-HSP27 monoclonal antibody (1 : 1000). Anti-GAPDH polyclonal antibody was used as an internal standard. Detection was done using an enhanced chemiluminescence (ECL) system.

Reverse-transcription polymerase chain reaction

The mRNA expressions of HSP27 were determined by reverse-transcription polymerase chain reaction (RT-PCR) analyses. After

being treated with or without OM, cells were harvested and total RNA was extracted using standard Trizol RNA isolation method. Reverse transcription of 5 µg RNA was carried out according to the instruction of TaKaRa RT-PCR kit (TaKaRa Biotechnology, Otsu, Japan). The PCR amplification was conducted by a Biometra T-Gradient thermal cycler (Biometra, Tampa, FL, USA) according to the following protocol: solutions were denatured at 94°C for 30 s, annealed at 58°C for 30 s, extended at 72°C for 60 s, and amplified by 35 cycles. The primers used were listed in Table 1. PCR products were separated by electrophoresis on 2.0% agarose gels and stained with ethidium bromide. The density of bands was analyzed using a Bio-Rad GS-700 densitometer (Foster City, CA, USA). Ratios of target genes to house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated.

Two-dimensional cell migration assay

A two-dimensional cell migration assay using a wound-healing model was performed as previously described (Santos et al., 1997). Briefly, confluent cells seeded on 24-well culture plates were treated with OM at dose of 0, 1.0, 0.5, 0.25 or 0.125 mg/mL for 24 h. The monolayer cells were then scraped with a sterile 200 µl pipette tip to create a double-sided wound. After being washed with PBS, media were replaced with fresh media containing DMEM supplemented with 0, 1.0, 0.5, 0.25 or 0.125 mg/mL of OM. After this, time-lapse images were captured using an inverted phase contrast microscope at 100x magnification for 24 h. Cell migration was evaluated by calculating the average cell migration velocity using the following formula:

The average cell migration velocity = migration distance/migration time

Cell proliferation

MTT assay was used to measure cell proliferation of OM on fibroblasts. Cells were seeded on 96-well plates (1×10^4 cells/well). After fibroblasts were treated with different concentrations of OM for 48 h, media were carefully aspirated. 200 µl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added into wells and incubated for 4 h. Then, 150 µl of dimethyl sulphoxide was added and the absorbance at 490 nm was determined. Percentage of cell proliferation was calculated as (OD of treated sample/OD of untreated control) \times 100.

Statistical analysis

Data were expressed as mean \pm standard deviation. Data analysis was performed using SPSS 12.0 software package. After homogeneity of variances was confirmed by Levene's test ($P > 0.05$), homogeneous data were analyzed with the least significant difference (LSD) test for multiple comparisons following one-way ANOVA. To determine inter-group differences, heterogeneous data were analyzed with Dunnett T3 test. A value of $P < 0.05$ was considered significant.

RESULTS

Two-dimensional protein maps of the fibroblasts

The protein expression profile of the control reagent or

OM treated fibroblasts was examined by 2-DE. The experiments were repeated at least three times with reproducible results. Representative high resolution 2-DE was able to resolve hundreds of proteins as shown in Figure 1. A total of 1136 protein spots were detected in the control fibroblasts, while 1020 spots were detected in OM treated fibroblasts when analyzed with ImageMaster 5.0.

Changes in protein expression

Furthermore, we used ImageMaster 5.0 to identify the quantitatively differentially expressed proteins on the 2-DE gel between the control and OM treated cells. Among these detectable protein spots, we were able to pinpoint a total of 9 spots expressed differentially in response to OM treatment (-fold changes in protein expression ≥ 3). Of these protein spots, 2 were of higher intensities, 7 were of lower intensities (Figure 2). Using MALDI-TOF/TOF analysis and NCBI protein databases, 5 proteins were successfully recognized. They were heat shock protein 27 (HSP27), myosin regulatory light chain 1 (MRLC1), eukaryotic translation initiation factor 3 (EIF3), mitochondrial phosphoenolpyruvate carboxykinase 2 (PEPCK), mus musculus transcription factor S-II-related proteins (TFSII). All expressions were markedly decreased in OM exposed cells. The identification of these proteins along with their accession numbers, MW, PI and protein score was shown in Table 2.

Changes of HSP27 induced by OM

To verify the effects of OM on HSP27 expression in primary lung fibroblasts, the RT-PCR and western-blot studies were conducted. By RT-PCR analysis, it was found that the expression of HSP27 mRNA of fibroblasts derived from lung of mice was significantly lower than that of control cells ($P < 0.01$) (Figure 3). In addition, western-blot results presented in Figure 4 showed that OM can also lower the protein expression of HSP27 at the dose from 0.25 to 1.0 mg/mL. The feature was the drug concentration-effect dependence. There is a significant difference compared with the control group ($P < 0.05$). The result was in accordance with that of two-dimensional gel electrophoresis.

Effects of OM on migration of fibroblasts

HSP27 has been proven to behave as an actin-capping protein to regulate many cell activities, including motility, proliferation, contraction, and so on. In this study, we studied the effect of OM on fibroblast migration using an *in vitro* scrape-injury model. As shown in Figure 5, the average cell migration velocity was significantly reduced

Table 1. Primer pairs for reverse-transcription polymerase chain reaction in the study.

	Forward	Reverse	Product (base pair)
Hsp27	CAAGGAAGGCGTGGTGGGA	GCCTCGAAAAGTAACCGGAATG	222
GAPDH	ATGACCTTGCCCACAGCC	CCCATCACCATCTTCCAG	445

Table 2. Identified proteins by MALDI-TOF/TOF.

Spot No.	protein name	Accession No.	M.W. Da	PI	Score
1	Heat shock protein 27	IPI00468068	21947.2	2.45	364
2	Myosin light chain	IPI00109044	19882.5	4.67	431
3	Eukaryotic translation initiation factor 3	IPI00115580	42489.9	5.5	331
4	Mitochondrial phosphoenolpyruvate carboxykinase 2	IPI00223060	73371	7.88	312
5	Mus musculus transcription factor S-II-related proteins	IPI00323639	9342	11.35	50

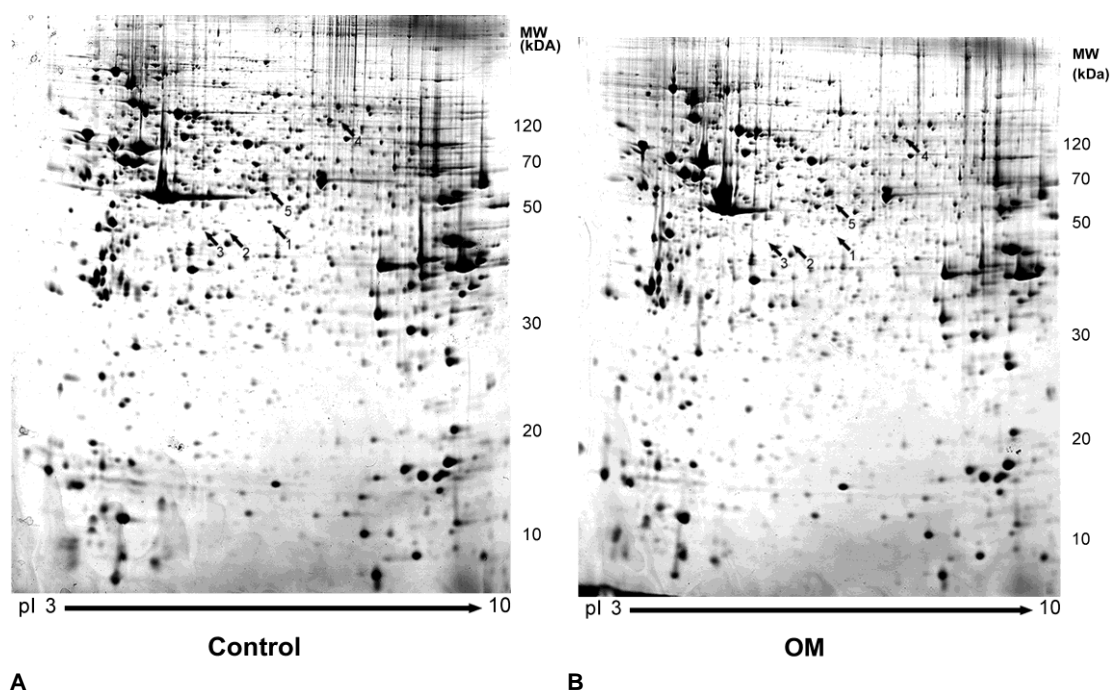


Figure 1. Differential expression of proteins from mouse lung fibroblasts visualized by two-dimensional polyacrylamide gel electrophoresis (2-DE) gel. Protein extracted from the lung fibroblasts of mouse untreated (a) or treated (b) with OM was separated by 2-DE (first dimension, pH 3 to 10, non-linear gradient of IPG strips; second dimension, 12% SDS-PAGE) and visualized by coomassie blue staining. The orientation of the IEF strips is indicated at the top of gels. Molecular mass standards are shown on the right of the 2-DE gel.

by OM with a dose dependent manner ($P < 0.05$). The results indicated that OM suppresses fibroblast migration, possibly through HSP27.

Effects of OM on proliferation of fibroblasts

Fibroblast proliferation was evaluated using MTT analysis. Lung fibroblasts from mice were cultured in the

presence of OM. As shown in Figure 6, no statistical differences were obtained between the control group and the OM-treated groups at doses of 1.0, 0.5, 0.25 or 0.125 mg/mL at 48 h.

DISCUSSION

Two-dimensional electrophoresis offers an efficient tool to

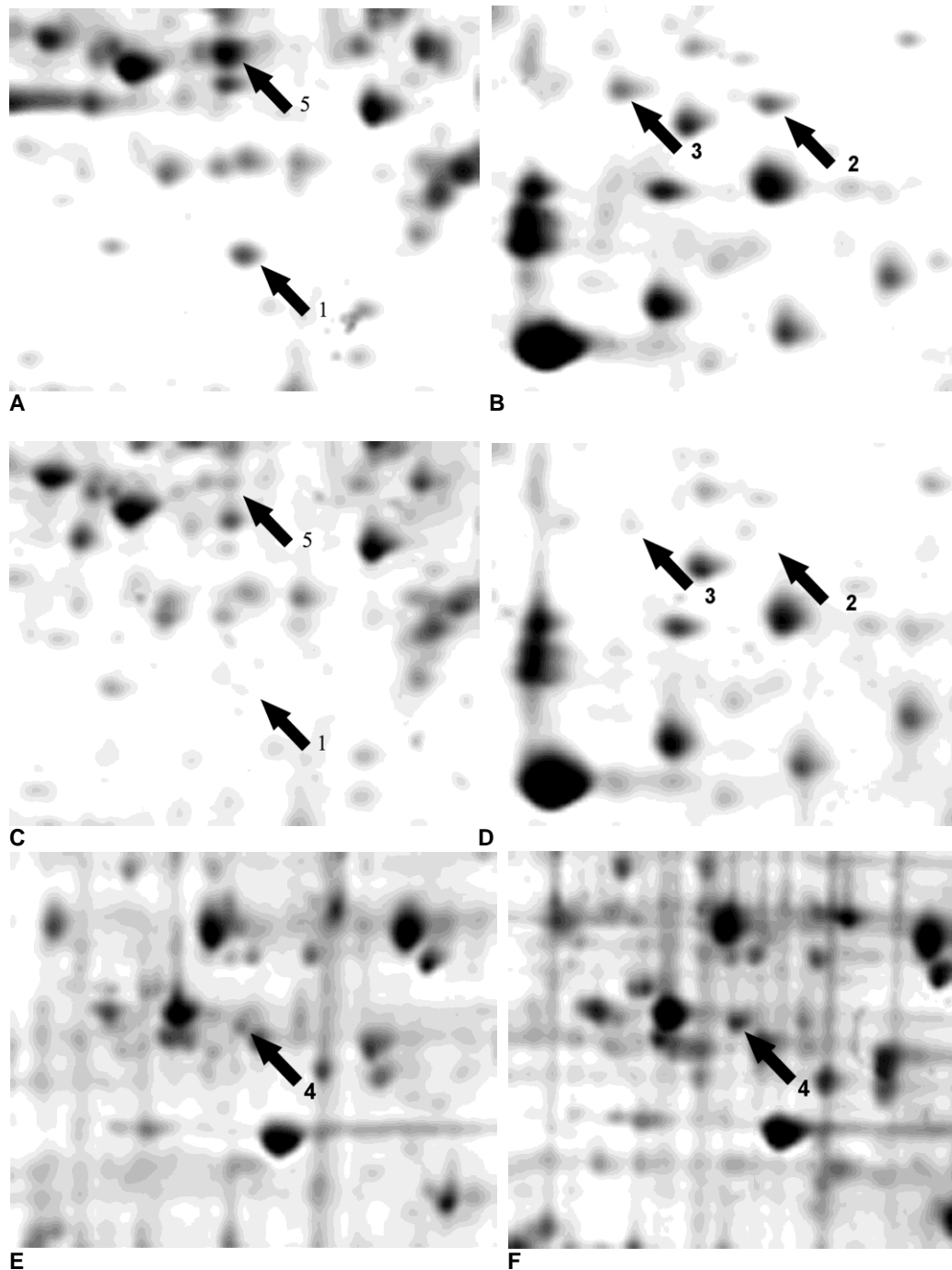


Figure 2. Enlargement of selected portions of the two-dimensional electrophoresis (2-DE) gel images, shown of control (A, B and C) and OM treated fibroblasts (D, E and F). The locations of spots that significantly altered after oxymatrine use were indicated by arrows.

screen for abundant protein changes in cells in different states. In the present study, a full characterization of the fibroblast proteome and the global changes in protein expression caused by OM at the dose of 1 mg/mL were demonstrated with 2-DE and MOLDT-TOF/TOF MS analysis.

Our results showed that a total of 9 spots differentially

expressed in fibroblasts treated with OM compared with the control cells. 5 proteins were successfully identified. All were down-regulated in cells exposed to OM. Mass spectrometer analysis indicated that one of these proteins is a metabolic enzyme, while others are related to cytoskeleton or involved in cellular transcription and translation.

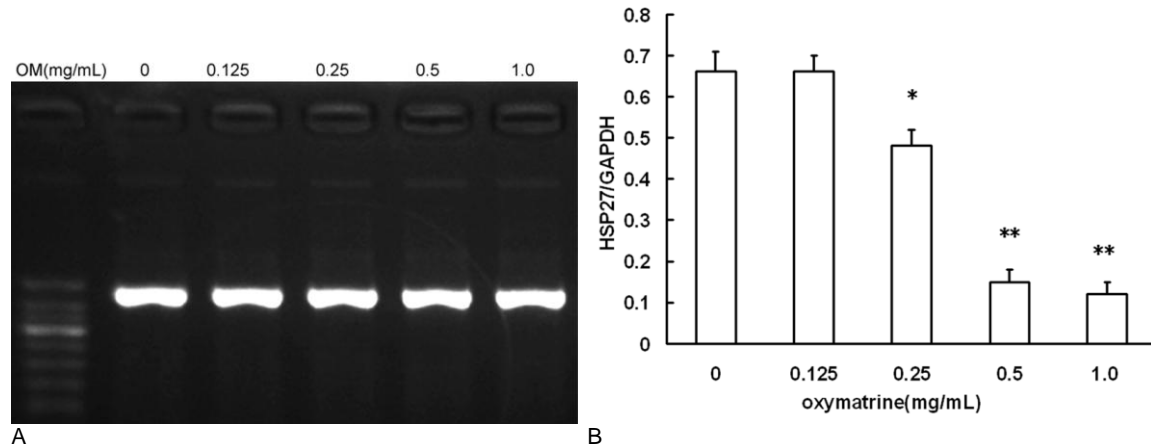


Figure 3. Effects of oxymatrine treatment on the mRNA levels of heat shock protein 27 (HSP27) in lung fibroblasts. The mRNA expression was determined by semiquantitative reverse-transcription PCR. (A) Expression of HSP27 mRNA in cultured lung fibroblasts detected 48 h after oxymatrine administration, (B) relative ratio of densitometry of HSP27 to GAPDH ($n = 3$ in each group). Data are mean \pm S.E.M. ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

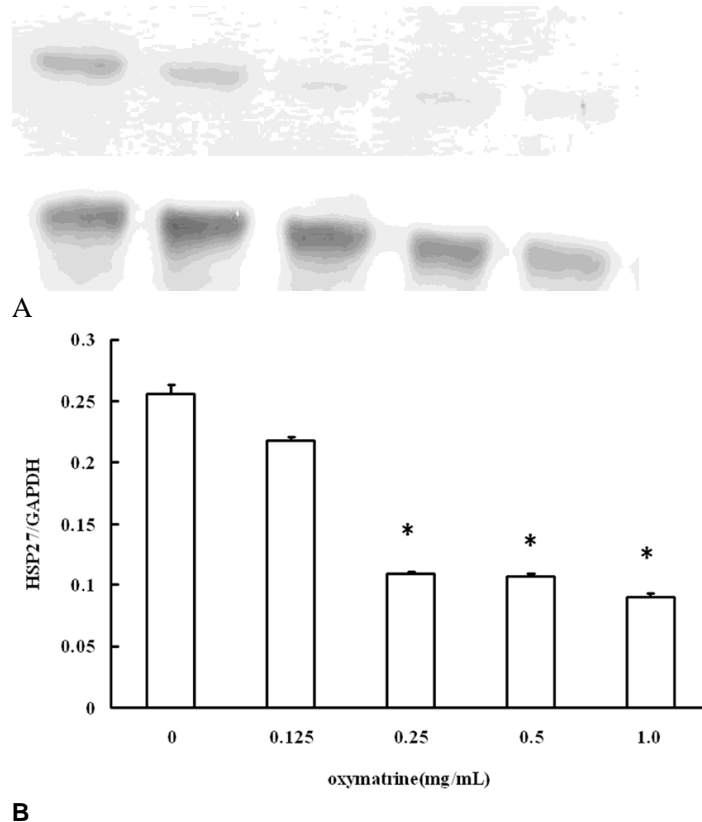


Figure 4. The effects of oxymatrine on the protein levels of heat shock protein 27 (HSP27) in lung fibroblasts. The effects of oxymatrine on the protein levels of heat shock protein 27 (HSP27) were detected by Western blot analysis. (A) Expression of HSP27 protein in lung fibroblasts incubated with or without oxymatrine for 48 h, (B) relative ratio of densitometry of HSP27 to GAPDH ($n = 3$ in each group). Data are mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$, compared with the control group.

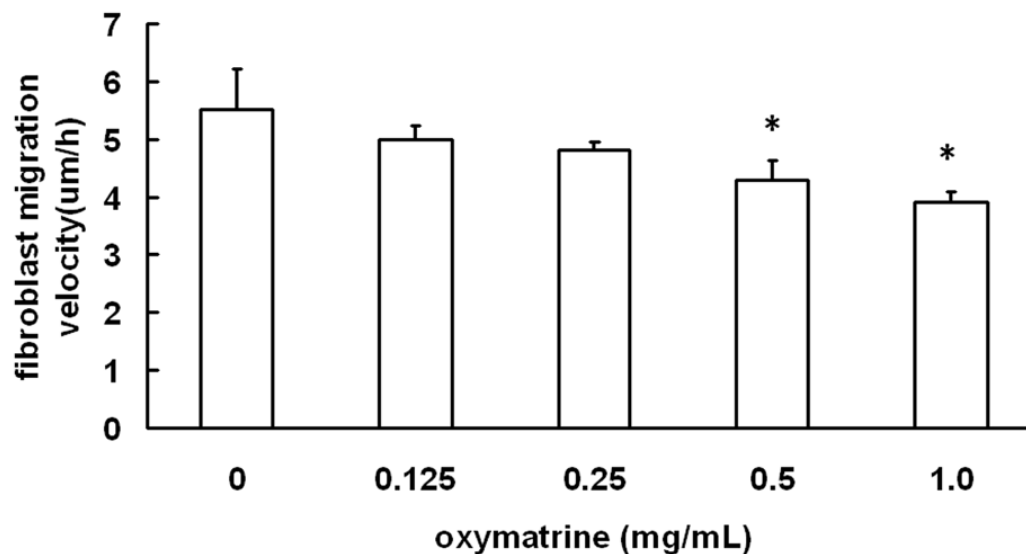


Figure 5. Effect of oxymatrine on the migration of lung fibroblasts in a two-dimensional cell migration assay. Confluent fibroblasts were damaged with a pipette tip. After wound healing for 24 h in the present or not present of oxymatrine, the cell migration velocity was computed ($n=3$ in each group). Data are mean \pm S.E.M. * $P < 0.05$ compared with the control group.

Myosin regulatory light chain 1 (MRLC1) and HSP27, both found to be reduced by OM, are cytoskeleton-related proteins and can influence cellular processes, such as proliferation, contraction, cell adhesion, cytokinesis, cell motility, and migration. MRLC1 is a regulatory protein and belongs to the myosin light chains family (Swant et al., 2005) while HSP27 is a member of the stress-inducible small heat shock protein family, heat shock proteins (HSPs). It behaves as an actin-capping protein, interfering with its polymerization by modulating RhoA-ROCKII pathway, then regulating phosphorylation of MRLC (Hiragami et al., 2009). In this study, changes of HSP27 induced by OM have been confirmed by RT-PCR and western-blot analysis at mRNA and protein levels, the latter was in accordance with the result of two-dimensional gel electrophoresis.

Recently, HSP27 was found expressed at very high levels in fibroblast foci, a relevant morphologic marker of idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP). Usually, fibroblast foci are recognized as sites where fibrotic responses are initiated and/or perpetuated in this severe disease. Thus, HSP27 was considered partly responsible for the abnormal proliferation and migration of mesenchymal fibroblasts and has a major role in the remodeling process characterizing IPF/UIP (Chilosi et al., 2006). In this present study, MTT assay indicated that proliferation of fibroblasts exposed to OM for 48 h showed no significant changes compared with that of the control group. However, the results of cell motility showed that the average cell migration velocity was significantly reduced by OM at 48 h, with a dose dependent manner ($P < 0.05$). Based on those

observations, we suggest the mechanisms by which OM attenuates pulmonary fibrosis involve inhibition of fibroblast migration, and it might be partly related to regulation of HSP27 expression.

Phosphoenolpyruvate carboxykinase (PEPCK) is a rate controlling enzyme in gluconeogenesis which catalyzes the synthesis of phosphoenolpyruvate from oxaloacetate. Recent studies have suggested that PEPCK plays important role in the maintenance of blood glucose concentration (Huang et al., 2010; Kim et al., 2009). In this paper, PEPCK decreased in cells exposed to OM, which indicates that OM may disrupt glucose homeostasis by inhibiting gluconeogenesis. The results suggested that the inhibition effect of OM on PEPCK at least partly contributed to its attenuation of proliferation and mobility of fibroblast. Moreover, transcription factor S-II (TFSII) and eukaryotic translation initiation factor 3 (EIF3) were found down regulated in the OM treated cells. TFSII was a transcription elongation factor which directly binds the transcription motor, RNA PolymeraseII and allows it to be read by various transcription arrest sites.

Recent studies revealed that knockdown of TFSII reduced cancer cell proliferation and induced apoptosis in cancer cells (Hubbard et al., 2008; Kanai et al., 1991). The results of the study indicated that OM may interfere with the proliferation of fibroblast by the transcription machinery. EIF3 is a large multiprotein complex. Its major effect on initiation complex formation of eukaryotic translation is to loosen the interactions between the ribosomal subunits, making the small subunit available for fMet-tRNA binding. Recently, some evidences

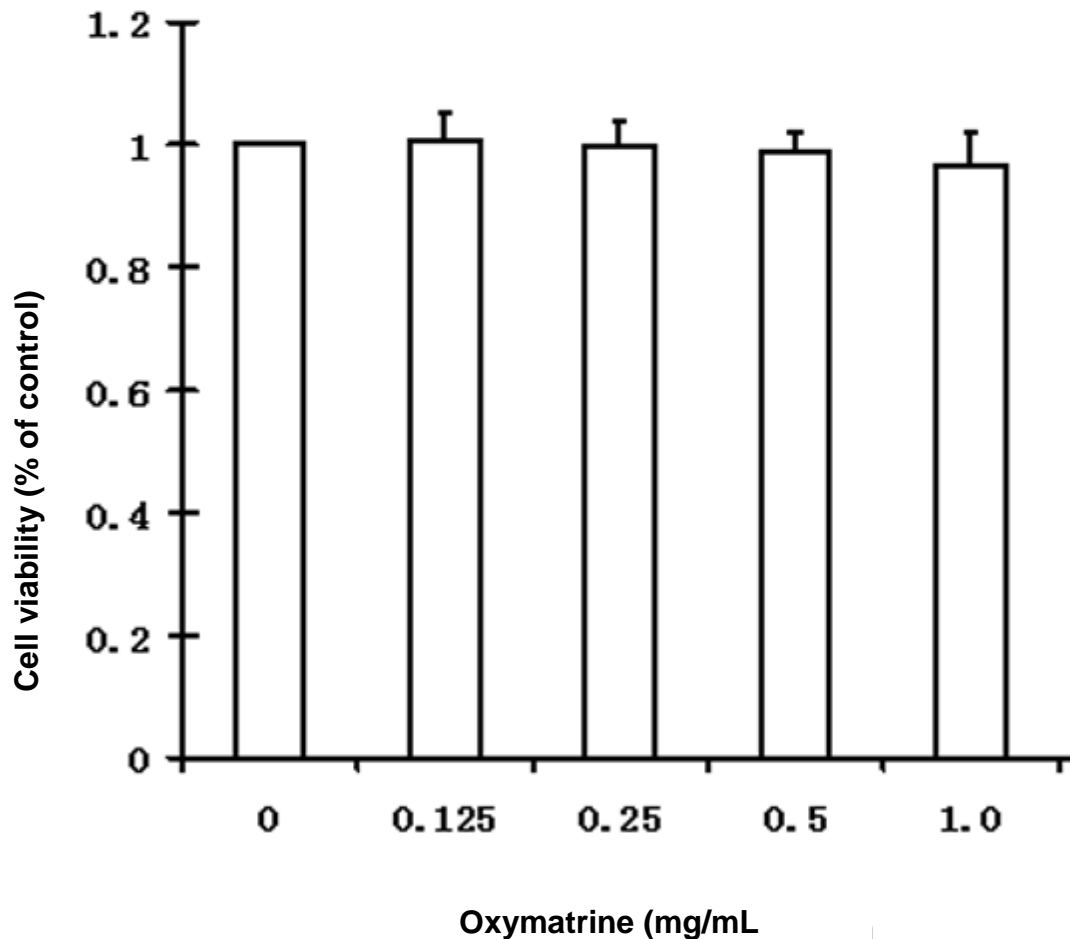


Figure 6. Effect of oxymatrine on the proliferation of lung fibroblasts. MTT assay was used to measure cell proliferation after fibroblasts were treated with different concentrations of OM for 48 h. Percentage of cell proliferation was calculated as (OD of treated sample/OD of untreated control) \times 100. Data are mean \pm S.E.M.

indicated that EIF3 are involved in cell cycle, tumorigenesis and DNA repair. In humans, over-expression of EIF3 has been found in prostate, breast, and liver cancer. At the cellular level, EIF3 over-expression increases proliferation, growth and survival (Cappuzzo et al., 2009; Wang et al., 2008).

In this study, EIF3 was significantly down-regulated by OM, which indicates the important roles of this protein in mediating the inhibiting effects of OM on fibroblasts. In the present study, using proteomic approach, we identified 5 protein spots that have altered expression in fibroblasts exposed to OM, compared with the control cells. The changed proteins were MRLC1, HSP27, PEPCK, TFSII and EIF3. Among them, the reduction of HSP27 induced by OM has been confirmed by RT-PCR and western-blot analysis at mRNA and protein levels. Moreover, since HSP27 is a cytoskeleton regulatory protein, the inhibitory effect of OM on motility of fibroblast was proven in the experiment. To verify the role of HSP27 and other changed proteins in mediating effects

of OM on lung fibroblasts, further research needs to be carried out.

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