

Original article

Increased RhoGDI₂ and peroxiredoxin 5 levels in asthmatic murine model of β_2 -adrenoceptor desensitization: A proteomics approach

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Keywords: β_2 -adrenoceptor desensitization; two-dimensional gel electrophoresis; mass spectrometry; peroxiredoxin 5; RhoGDP-dissociation inhibitor 2

Background β_2 -adrenoceptor (β_2 AR) desensitization is a common problem in clinical practice. β_2 AR desensitization proceeds by at least such three mechanisms as heterologous desensitization, homologous desensitization and a kind of agonist-induced rapid phosphorylation by a variety of serine/threonine kinases. It is not clear whether there are other mechanisms. This study aimed to investigate potential mechanisms of β_2 AR desensitization.

Methods Twenty-four BALB/c (6–8 weeks old) mice were divided into three groups, which is, group A, phosphate buffered saline (PBS)-treated; group B, ovalbumin (OVA)-induced; and group C, salbutamol-treated. Inflammatory cell counts, cytokine concentrations of bronchoalveolar lavage fluid (BALF), pathological sections, total serum IgE, airway responsiveness, membrane receptor numbers and total amount of β_2 AR were observed. Asthmatic mouse model and β_2 AR desensitization asthmatic mouse model were established. Groups B and C were selected for two-dimensional gel electrophoresis (2DE) analysis so as to find key protein spots related to β_2 AR desensitization.

Results Asthmatic mouse model and β_2 AR desensitization asthmatic mouse model were verified by inflammatory cell count, cytokine concentration of BALF, serum IgE level, airway hyperreactivity measurement, radioligand receptor binding assay, Western blot analysis, and pathologic examination. Then the two groups (groups B and C) were subjected to 2DE. Two key protein spots associated with β_2 AR desensitization, Rho GDP-dissociation inhibitor 2 (RhoGDI₂) and peroxiredoxin 5, were found by comparative proteomics (2DE and mass spectrum analysis).

Conclusion Oxidative stress and small G protein regulators may play an important role in the process of β_2 AR desensitization.

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Asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion and airway hyperresponsiveness.^{1,2} The prevalence and mortality rate of asthma have been increasing worldwide in the past two decades. Treatments currently used for asthma are aiming at bronchodilation by short-/long-acting β_2 -agonists and other bronchodilators or inhibition of inflammation by corticosteroids.³ But β_2 -agonists are limitedly used clinically because of β_2 -adrenoceptor (β_2 AR) desensitization and side-effects. The mechanisms underlying the pathogenesis of β_2 AR desensitization are still not fully understood.

For β_2 AR, desensitization proceeds by at least three mechanisms. Upon exposure to agonist, the receptors are rapidly phosphorylated by a variety of serine/threonine kinases. Heterologous desensitization, mediated by protein kinase A (PKA) and protein kinase C (PKC), can target both agonist-occupied and unoccupied receptors. Homologous desensitization, mediated by G protein-coupled receptor kinases (GRKs), specifically targets agonist-occupied receptors. These phosphorylation events lead to a functional uncoupling of β_2 ARs from their transductional heterotrimeric G proteins (Gs). Within minutes of agonist exposure, most receptors will

translocate from the cell surface into endosomes. Within these endocytic vesicles, receptors are dephosphorylated and then predominantly recycle back to the cell surface in a fully sensitized state. After chronic agonist treatment, some β_2 ARs are lost from the cell by a process termed as downregulation. Downregulation likely results from both changes in receptor synthesis owing to the inhibition of gene transcription and mRNA instability and increases receptor degradation attributable to the sorting of some receptors to lysosomes.^{4,5} Besides the above mechanisms, however, are there other mechanisms associated with β_2 AR desensitization and more detailed molecular processes related to ubiquitination which is essential to β_2 AR degradation⁶ and cellular trafficking of β_2 ARs

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involved in β_2 AR signaling pathways? They are still unclear, especially those related to the β_2 AR desensitization of asthmatic patients and the signaling pathway of β_2 AR desensitization.

Proteomics is an effective high-throughput technology for the determination of global proteins of related disease.^{7,8} In order to study the potential mechanism of β_2 AR desensitization, BALB/c mice sensitized and challenged to ovalbumin (OVA) served as a murine model of acute asthma, meanwhile the model of β_2 AR desensitization of asthmatic mice was also established by the OVA-induced and salbutamol-treated method. Complex protein mixtures extracted from the lung tissues of the two groups of mice were separated by two-dimensional gel electrophoresis (2DE). Subsequently, isolated protein spots were compared and analyzed by ImageMaster software and mass spectrometry, and thus proteins were identified accurately. Bioinformatic tools were used to analyze these protein spots and to find related protein spots associated with the mechanism of β_2 AR desensitization. In the end, these proteins were verified by Western blot.

METHODS

Animals

The study and all procedures concerning animals were approved by the Institutional Animal Care and Use Committee and Conformed to the International Guidelines on the Ethical Use of Animals. Every effort was made to minimize the number of animals used and their suffering. Female BALB/c mice (6–8 weeks old) purchased from Shanghai Laboratory Animal Inc., China prior to experiment were fed in air-conditioned cages with free access to food and water and maintained on a 12-hour dark/light cycle for a week to acclimatize to their new surroundings.

Antigen sensitization, challenge and treatment

All BALB/c mice were divided into three groups (groups A, B, C). Group A or control group ($n=8$) was sensitized and challenged with phosphate-buffered saline (PBS). Group B ($n=8$) was sensitized on days 0, 14 and 21 by intraperitoneal injection of 200 μ g chicken egg OVA (grade V, Sigma, St. Louis, MO, USA) together with 20 mg aluminum hydroxide in a total volume of 200 μ l. One week after the last sensitization, the mice were challenged with an aerosol of 1% (w/v) OVA in saline using an ultrasonic nebulizer 30 min/d for 1 week. Group C ($n=8$), salbutamol-treated asthmatic mice (salbutamol, provided by Glaxo Operations UK Ltd., Hamire Road, Barnard Castle, Durham, UK) underwent the same procedure as group B besides daily intraperitoneal injection of 60 μ g salbutamol and inhaling an aerosol of 0.01% salbutamol 30 min/d for a week half an hour before they were challenged with OVA.⁹

Measurement of airway hyperreactivity

The mice were anesthetized by intraperitoneal

administration of 1% pentobarbital sodium 0.15 ml 24 hours after the last challenge, and then airway hyperresponsiveness to acetylcholine chloride (ACh) was measured. Each mouse was placed in a plethysmograph chamber and then mechanically ventilated with an animal ventilator (AniRes2005, Beijing SYNOL High-Tech Co. Ltd., China) with a tidal volume of 150 μ l and a frequency of 110 breaths per minute. Airway expiration resistance (R_e) was continuously measured and recorded for later analysis. After measurement of baseline R_e lasting for one minute, the animals were injected with acetylcholine chloride in progressively doses (0.5 mg/ml, 1.5 mg/ml, 5.0 mg/ml, 15 mg/ml, 45 mg/ml) by caudalis vein (35 μ l each time) at one minute interval.

Sample preparation

After measurement of airway hyperreactivity, the mice were made bled by retroorbital puncture using heparinized capillary tubes, and blood samples were centrifuged and sera were stored at -70°C until used. After removal of blood, the mice were used either for collecting bronchoalveolar lavage fluid (BALF) ($n=4$) or tissue ($n=4$). The BALF was used for measurement of cytokines. The tracheas of mice were cannulated and lavaged with two 0.8 ml aliquots of PBS. The BALF was centrifuged and supernatants were collected and stored at -70°C until use. At the same time, the number of mononuclear cells in BALF was counted. After BALF was acquired, the four mice lungs of each group were used to identify the number of β_2 AR in lung member using a radioligand receptor binding assay (see below). For tissue collection and protein extraction, right lungs of the other four mice of each group were isolated and frozen in liquid nitrogen and stored at -70°C . When used, one hundred milligram lung tissues of each mouse were taken out and were cut into slices respectively. These slices were transferred to 5 ml sterilized centrifuge tubes containing 700 μ l of sample buffer (7 mol/L urea, 2 mol/L thiourea, 4% (w/v) Chaps, 40 mmol/L DTT, 2% (v/v) IPG buffer, 1% (v/v) protease inhibitor cocktail). The mixtures were homogenized using dispersing driver (6500 r/min, 3–5 times, 3–5 seconds every time, T18 basic ULTRA-TURRAX[®], China). Homogenized tissues were incubated for 1 hour at 4°C , and then sonicated 10 times at an interval setting 15 seconds and working setting 10 seconds. Next, samples were put in ice for 30 minutes and then centrifuged for 30 minutes at 14 000 r/min at 4°C . Supernatants were transferred to clean vials. Subsequently the protein concentrations were quantified using Bradford assay and stored at -70°C until used (for 2DE and Western blot analysis). Left lungs were immersed in 10% formaldehyde overnight. Then they were embedded in paraffin, sectioned into 5 mm thickness, and stained with HE. Histologically, the sections were observed under a microscopy by specialists.

Measurement of total IgE, IL-4, and IFN- γ

Total serum IgE, BALF cytokines, interleukin 4 (IL-4) and interferon-gamma (IFN- γ) from each group were measured by standardized sandwich enzyme linked

immunosorbent assay (ELISA) according to the manufacturer's instructions.

Protein separation and visualization

Proteins (120 µg) from groups B ($n=3$) and C ($n=3$) (randomly selected 3 samples from 4 samples) were applied to a 24 cm Immobiline Dry-Strip, pH 3–10 (Amersham Biosciences, Roosendaal, Netherlands), respectively. Strips were rehydrated for 12 hours at 30 V, followed by focusing for 1 hour at 100 V, 1 hour at 500 V, 1 hour at 1000 V, 1 hour at 5000 V and subsequently at 8000 V to 140 kV·h on an IPGPhor (Amersham Biosciences) and stored at -70°C . Focused IPG strips were equilibrated twice for 15 minutes with gentle shaking in equilibration buffer containing 50 mmol/L Tris-HCl buffer, pH 8.8, 6 mol/L urea, 20% (v/v) glycerol, 0.1% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) DTT. In the second equilibration buffer, DTT was replaced with 2.5% (w/v) iodoacetamide in order to remove excess DTT that causes point streaking in silver-stained patterns.¹⁰ The equilibrated IPG strips were gently rinsed with distilled water, blotted to remove excess equilibration buffer and then applied onto a 12.5% polyacrylamide gradient gel (26 cm $\times$ 20 cm). The Ettan DALT II system (Amersham Biosciences) was used to make the second dimension separation sequentially with a constant voltage of 70 V for 1 hour, 150 V for 2 hours, and 400 V for 6 hours. After SDS-PAGE, the separated gels were visualized by silver staining according to the kit procedures (PlusOne silver staining kit, Amersham Biosciences).

Image analysis

Digitized images of the stained gels were analyzed using the 2-DE gel analysis software ImageMaster 2D ver. 4.0 (Amersham Pharmacia Biotech, Uppsala, Sweden). A comparison report was generated of the qualitative and quantitative differences between the samples for the data.

In-gel digestion and mass spectrometric analysis

Differentially expressed protein spots were taken from the gels, cut into smaller pieces, and digested with trypsin (Promega, Madison, WI, USA), as described by Gharahdaghi et al.¹¹ and Joo et al.¹² For matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-MS) analysis, tryptic peptides were concentrated on POROS R2 columns (Applied Biosystems, Foster City, CA, USA). After successive column washings with 40% methanol, 100% acetonitrile, and 50 mmol/L ammonium bicarbonate, the samples were placed onto the R2 column and eluted in 2 ml of α -cyano-4-hydroxycinnamic acid before MALDI-TOF analysis. The spectra for the protein samples were obtained using a Voyager DE PROMALDI-TOF spectrometer (Applied Biosystems, USA). Protein databases were searched with the MSFit program (<http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm>) using monoisotopic peaks. A mass tolerance within 50 per million was allowed for the first analysis; the system was subsequently recalibrated at 20 per million using the list

of proteins obtained from the initial analysis. The spectra were also internally calibrated using trypsin autolysis products. The resulting peptide masses were used to search the databases managed by SWISSPROT (<http://kr.expasy.org>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

Radioligand receptor binding assay

The four mice lung tissues which had been lavaged were respectively minced coarsely with scissors and suspended in 10 $\times$ volume of 25 mmol/L Tris-HCl buffer (pH 7.4) containing 0.32 mol/L sucrose at 4°C , and then homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland) at setting 6 in 30-second bursts. The homogenate was centrifuged at $1000 \times g$ for 10 minutes at 4°C to remove unhomogenized debris, the supernatant was then centrifuged at $40\,000 \times g$ for 20 minutes at 4°C , and the resulting pellet was washed and recentrifuged at the same speed. The final homogenate was frozen in liquid nitrogen and stored at -80°C without loss of binding characteristics. Portions of lung membrane at a protein concentration of 10 µg/tube were incubated with [^{125}I] iodocyanopindolol (ICYP) (sp act: 7400×10^{10} Bq/mmol; 3–100 pmol/L) in the presence or absence of excess Iso (200 µmol/L) in 25 mmol/L Tris-HCl buffer (pH 7.4) containing 154 mmol/L NaCl and 1.1 mmol/L ascorbic acid (to prevent oxidation of Iso) in a final volume of 250 µl. The density of β_1 -receptors was analyzed by ICYP saturation binding in the presence of 0.1 µmol/L ICI 118551, a β_2 -selective antagonist, a concentration at which practically all β_2 -receptors were occupied. The density of β_2 -receptors was analyzed by ICYP saturation binding in the presence of 0.1 µmol/L CGP 20712 A, a β_1 -selective antagonist, a concentration at which practically all β_1 -receptors were occupied. Incubation was carried out at 37°C for 120 minutes, which was found to be optimal for specific binding. Incubations were performed in triplicate. The incubation was terminated by rapid filtration through GF/C glass-fiber filters (Whatman Inc., Clifton, NJ, USA). Each filter was rapidly washed with 3×5 ml ice-cold 25 mmol/L Tris-HCl buffer (pH 7.4). The filters were counted in the Auto Gamma Counting System at an efficiency of 80%. Specific binding was calculated by subtracting nonspecific binding from total binding. Protein concentration was determined by the method of Lowry with bovine serum albumin as a standard.

Immunoblotting

Samples of denatured protein (40 µg) ($n=3$, the same 3 samples as those of 2-DE, samples from groups A, B and C) were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked in 5% milk in $1 \times$ TBST (Tris-buffered saline, 0.5% Tween 20) at 4°C overnight, and probed with anti- $\beta_2\text{AR}$ antibody (1:500 dilution, SC-9042 Santa Cruz Biotechnology, USA) and appropriate secondary antibody conjugated to horseradish peroxidase (1:2000 dilution, Abcam, UK) at room temperature for 2 hours,

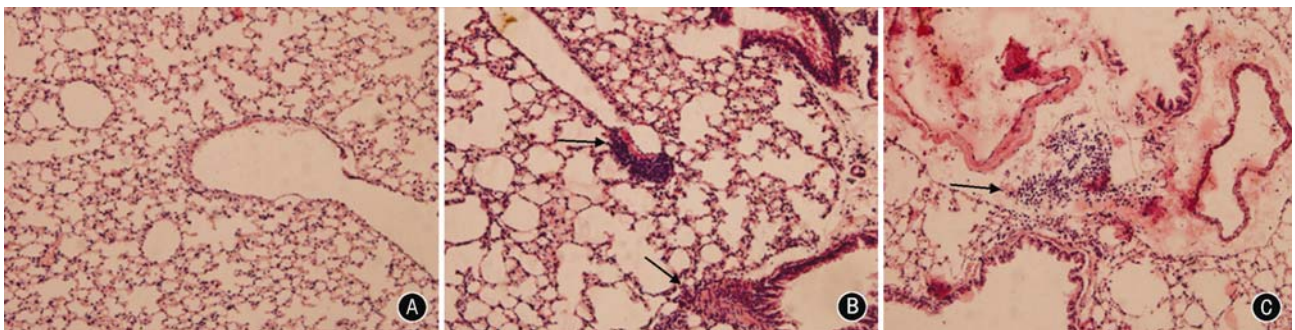
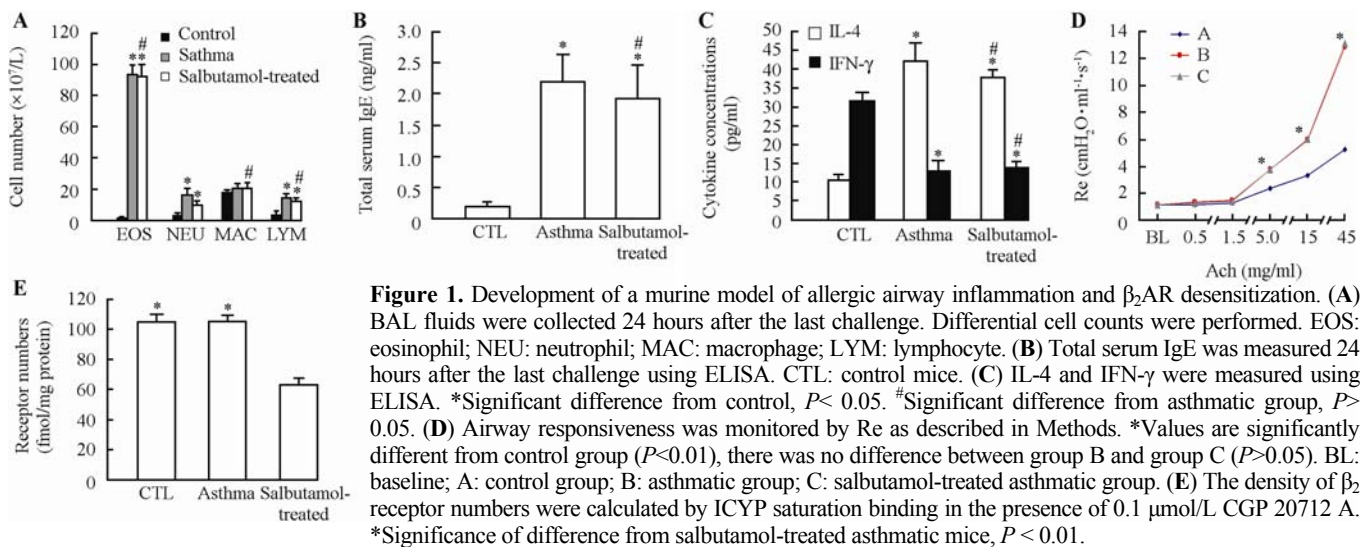


Figure 2. Histological changes. Representative photomicrographs of lung sections from control mice (A), asthmatic mice (B), and salbutamol-treated asthmatic mice (C). Lung sections were stained with HE, original magnification $\times 200$. Arrows indicate areas of peribronchial and perivascular inflammation.

respectively. Meanwhile, the membranes were stripped and reprobed with anti- α -tubulin antibody to confirm equal amounts of loaded samples. Immunoblots of protein bands were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, USA). At the same time, in order to confirm the up-regulation of peroxiredoxin 5 and Rho GDP-dissociation inhibitor 2 (RhoGDI₂) identified by 2-DE, the expression of these lung tissue proteins was also examined using Western blotting referred to the above method.

Statistical analysis

Values are expressed as the mean $\pm$ standard error (SE). Differences between the control, asthmatic, and salbutamol-treated asthmatic groups were determined by analysis of variance (ANOVA). The different protein expression levels of asthmatic and salbutamol-treated asthmatic mice were analyzed using Student's t test. The critical level for significance was set at $P < 0.05$.

RESULTS

Construction of asthmatic mouse model and β_2 AR desensitization asthmatic mouse model

BAL fluid and blood were collected 24 hours after the last OVA aerosol challenge. Eosinophil, neutrophil and lymphocyte counts in the BAL fluid and total serum IgE

level were increased ($P < 0.05$) in the asthmatic and salbutamol-treated asthmatic groups compared with the PBS-challenged group (Figures 1A and 1B). The IL-4 concentration in BALF of the asthmatic and salbutamol-treated asthmatic groups was higher ($P < 0.05$) than that of the PBS-challenged group, but the IFN- γ concentration was lower ($P < 0.05$) than that of the PBS-challenged group (Figure 1C). Airway responsiveness, determined by an animal ventilator,¹³ to inhaled acetylcholine chloride was markedly increased ($P < 0.05$) in the asthmatic and salbutamol-treated asthmatic groups compared with the PBS-challenged group (Figure 1D). In addition, the asthmatic and salbutamol-treated asthmatic groups induced marked infiltration of inflammatory cells, especially eosinophils, into the lamina propria, perivascular, and peribronchiolar areas compared to the PBS-challenged group (Figure 2). Furthermore, the number of β_2 receptors of the PBS-challenged group and asthmatic group was obviously higher than that of the salbutamol-treated asthmatic group (Figure 1E). Western blot showed the total amount of β_2 AR in the asthmatic group, PBS-challenged group and salbutamol-treated group, and α -tubulin was used as internal control (Figure 3). With inflammatory cell count, cytokine concentration of BALF, pathological result, total serum IgE, airway responsiveness, membrane receptor number and total amount of β_2 AR, asthmatic mouse model and

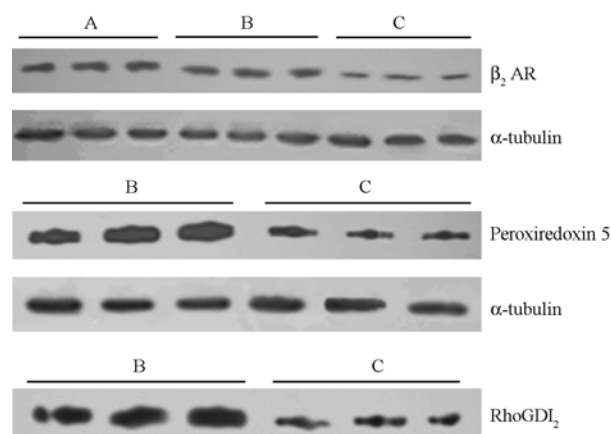


Figure 3. Western blot analysis of lung tissues using polyclonal antibodies targeted at β_2 AR, peroxiredoxin 5, and RhoGDI₂. Proteins (20 μ g/lane) were separated by SDS-PAGE and probed with anti β_2 AR, antiperoxiredoxin 5 and antiRhoGDI₂ antibodies. The blot was detected by ECL, α -tubulin was used as internal control. **A:** control group; **B:** asthmatic group; **C:** salbutamol-treated asthmatic group.

β_2 AR desensitization asthmatic mouse model were successfully established.

2-DE analysis and identification of differentially expressed protein in asthmatic mice and salbutamol-treated asthmatic mice

Lungs were isolated from each mouse 24 hours after the last challenge. Proteins from the asthmatic group and salbutamol-treated asthmatic group (three samples per group) were resolved on 2-D gels and visualized by silver staining (Figure 4). The six gels were analyzed using the 2-DE gel analysis software ImageMaster 2D ver. 4.0 (Figure 5). Then MALDI-TOF MS was used to identify all the differentially expressed protein spots between the two groups and the results were enumerated in the Table.

Immunoblotting

To verify the 2-DE findings, immunoblotting analysis was made on selected proteins. Western blot analysis revealed a significant increase in peroxiredoxin 5 and RhoGDI₂ protein levels in the salbutamol-treated asthmatic group (Figure 3), and α -tubulin was used as internal control.

DISCUSSION

We present the comparative proteome analysis of asthmatic and salbutamol-treated asthmatic lung tissues from mice. The pH ranges used for the first-dimension gel electrophoresis in this study (3–10) separated a proportion of soluble proteins in the lung tissues. To avoid individual and experimental variation, we produced a large number of replicate gels from the same sample or treatment group in order to obtain statistically significant changes in protein levels between the different groups, i.e., asthma versus salbutamol-treated asthma. Using

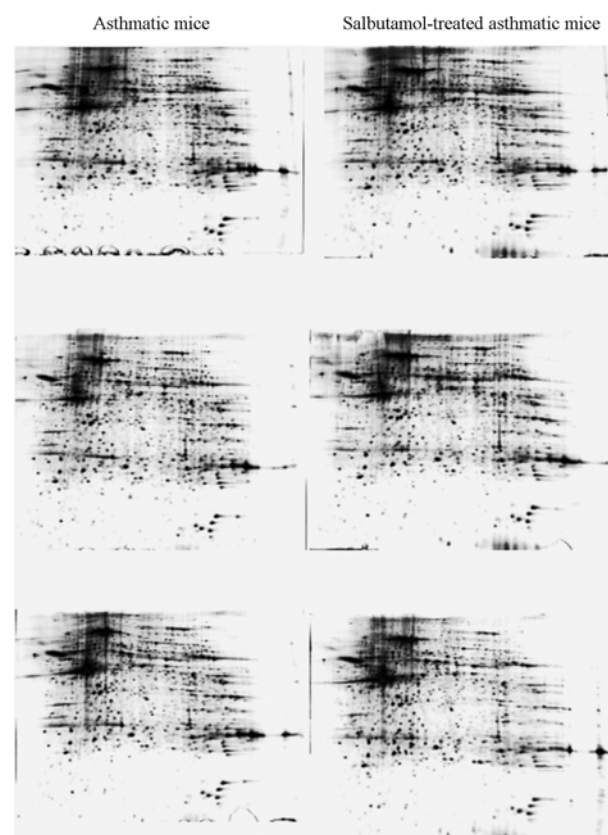


Figure 4. Representative 2-DE profiles of lung samples from asthmatic mice and salbutamol-treated asthmatic mice. Proteins on 2-DE (pH 3–10) gels were visualized by silver staining.

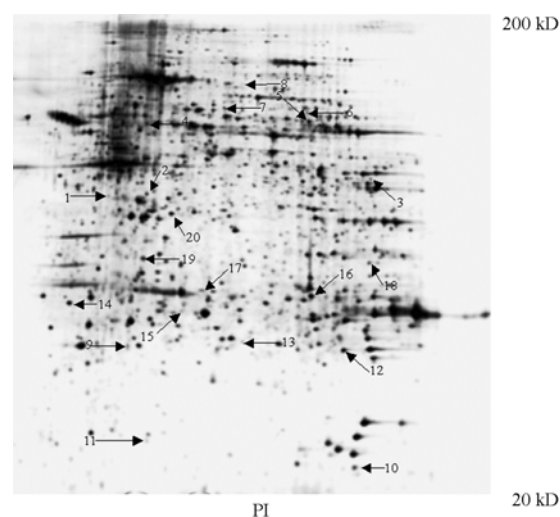


Figure 5. Effect of differential protein expression in 2-DE. Differential protein expressions from asthmatic mouse gel were compared with salbutamol-treated asthmatic mouse gel. The spots identified by MALDI-TOF MS were enumerated.

2-DE, we found 20 proteins that were differentially expressed in the lung tissues of salbutamo-treated asthmatic mice. Eight protein spots were down-regulated and 12 protein spots were up-regulated in salbutamol-treated asthmatic lungs compared with asthmatic lungs. These protein spots were identified by in-gel digestion and MALDI-TOF MS.

In our study three groups were designed: group A, PBS-treated; group B, OVA-induced; and group C, salbutamol-treated. We presumed that the number of β_2 AR was not altered in the OVA-induced group but decreased significantly in the salbutamol-treated group compared with the PBS-treated group. This finding was proved by the results of our experiments. Meanwhile, groups B and C were selected for 2-DE analysis, attempting to find key protein spots related to β_2 AR desensitization.

Salbutamol is reportedly an effective bronchodilator used to treat asthma, but it can induce β_2 AR desensitization as other β_2 AR agonists.¹⁴ In this study we found that salbutamol can lead to β_2 AR desensitization in a murine model of acute asthma by measuring the number of membrane β_2 AR (radioligand receptor binding assay) and checking total amount of β_2 AR receptor (Western blot analysis). To investigate the mechanism, we used a proteomic approach to identify protein expressions between asthmatic mice and salbutamol-treated asthmatic mice and further to detect key proteins of β_2 AR desensitization. Most of the identified spots were found to be proteins involved in epithelial cytoskeleton, oxidative stress, apoptosis, and other metabolic functions. Some of these proteins were responded to β_2 AR desensitization.

Oxidative stress associated with β_2 AR desensitization

The mechanism of β_2 AR desensitization is mainly due to the above mentioned three aspects. Besides these, some interesting spots may be related to β_2 AR desensitization. Peroxiredoxin 5, a member of the peroxiredoxins superfamily, is an important antioxidant enzyme in oxidative stress, which plays a vital role in reducing active oxygen from intracellular oxygen metabolism. But its role in β_2 AR desensitization is not elucidated. Our 2-DE data showed that the expression of peroxiredoxin 5 and superoxide dismutase (SOD), a kind of antioxidant enzyme, was statistically increased compared with asthmatic mice, indicating that oxidative stress may play an important role in β_2 AR desensitization. Oxidative stress is reported to mediate downregulation of β -adrenergic receptors and SR Ca^{2+} ATPase in cultured myocytes, ischemic-reperfused hearts, and the failing hearts.¹⁵⁻¹⁷ Similarly, β_2 AR of the airway smooth muscle may also be induced into desensitization by oxidative stress. But the mechanism is obscure.¹⁸ Because oxidative stress at first leads to protein ubiquitination¹⁹ and β_2 AR degradation is closely associated with ubiquitination,⁶ the possible mechanism of β_2 AR desensitization may be related to β_2 AR ubiquitination caused by oxidative stress. After ubiquitination, β_2 AR may be transferred to lysosome or proteasome, followed by degradation.⁵ At the same time, however, oxidative stress decreases G protein-coupled receptor kinase 2.²⁰ As G protein-coupled receptor kinase 2 plays a fundamental role in β_2 AR desensitization, its decrease in number may result in β_2 AR resensitization. This seems to be contradictory.

Hence it is very important to know which plays a predominant role: β_2 AR ubiquitination or G protein-coupled receptor kinase 2 decreases in number. In the different physiopathologic processes, the two statuses may be different. Our experimental results suggest that oxidative stress may play an important role in β_2 AR desensitization.

RhoGDI₂

The second interesting protein spot we found is RhoGDI₂. Rho, one of the Ras superfamily, is a kind of small G proteins, which have been established as key signaling nodes strategically positioned as integral components of complex signaling networks that control diverse facets of cellular biology.²¹ Rho can hydrolyze GTPases, so it is also called Rho GTPases. It has several functions in signal transduction, and aberrant Rho GTPase function has also been implicated in human cancer development. In addition to cancer, Rho GTPase deregulation is also associated with other human diseases. RhoGDI is identified as a down-regulator of the Rho family GTPases typified by its ability to prevent nucleotide exchange and membrane association. Despite the initial negative roles attributed to RhoGDI, recent evidence has suggested that it may also act as a positive regulator necessary for the correct targeting and regulation of Rho activities by conferring cues for spatial restriction, guidance and availability to effectors.²² In our experiment, the expression of RhoGDI₂ in salbutamol-treated asthmatic mice was statistically increased. β_2 AR desensitization is thought to be closely correlated with intracellular signaling pathways for the β_2 -receptor. Activation of the β_2 -receptor by β_2 -agonists causes the α -subunit of the associated Gs protein to dissociate and couple with adenylate cyclase, leading to enhanced production of cAMP and stimulation of PKA. PKA induces phosphorylation of the receptor protein and uncoupling of Gs. The phosphorylated receptor, with its residual $\beta\gamma$ -subunits, then couples to Gi. After the binding of β -arrestin, which acts as an assembly scaffold for Src, SOS, and RAS, the mitogen-activated protein kinases (MAPK) pathway is activated.²³ SOS serves as an activator of the MAPK signaling pathway in the process of β_2 AR desensitization, meanwhile, it is also a kind of guanine nucleotide exchange factor (GEF) which can regulate Rho activities. From the above signaling pathway, we can find that β_2 AR desensitization is closely related to small G protein regulators (SOS, etc). Combined with our results, RhoGDI₂ as a kind of small G protein regulators, its increasing prognosticates β_2 AR desensitization. At the same time, some research results indicated that small G proteins were closely associated with G protein-coupled receptor kinase-2 (GPRK₂),²⁴ suggesting that RhoGDI₂ termed as a kind of small G protein regulators is not only the result of β_2 AR desensitization but also the reason for salbutamol-induced β_2 AR desensitization. So, small G proteins or small G protein regulators may be a major convergence. This must be verified by further investigation

Table. Identification of differential protein

Spot protein name	Protein accession number	Change in intensity B/C	Chromosome location	TheoreticalMr (kD) /PI	MOWSE score	Percent sequence coverage (%)
Serum albumin precursor	P07724	2.0	5E2; 550 cM	70.7/5.75	54	17
Isocitrate dehydrogenase (NAD) subunit alpha	Q9D6R2	0.5	9A5.3	40.07/6.27	124	40
Hadha protein	Q8QZU4	0.3	5 B1	44.17/8.40	72	24
Cytosolic nonspecific dipeptidase	Q9D1A2	1.4	18 E3	53.19/5.43	153	43
Dihydrolipoyl dehydrogenase	O08749	0.6	12A3; 1215.1 cM	54.75/7.97	118	35
Fibrinogen, alpha polypeptide	Q99K47	2.1	3F1; 344.8 cM	61.8/7.16	129	40
Dihydropyrimidinase-related protein 2	O08553	1.2	14D1; 1428.2 cM	62.5/5.95	120	35
Annexin A2	P07356	3.0	9C; 937 cM	38.8/7.53	98	35
Melanocyte cDNA	Q3UGS0	0.6	5G2;580 cM	42.08/5.37	82	26
Peroxiredoxin 5	P99029	0.2	19A;190 cM	22.23/9.10	135	46
Actin, cytoplasmic1	P60710	1.9	5G2;580 cM	42.05/5.29	109	26
Superoxide dismutase (Mn)	P09671	0.2	17A1; 177.6 cM	24.82/8.80	80	38
Guanylate kinase	Q64520	2.1	11B1.3	21.89/6.14	67	35
Rho GDP-dissociation inhibitor 2	Q61599	0.3	6G1	22.89/4.97	101	70
Annexin A2	P07356	3.0	9C; 937 cM	38.81/7.53	104	52
Phosphoglycerate mutase 1	Q9DBJ1	0.5	19C3	28.8/6.75	130	62
Two days neonate thymus thymic cells cDNA	Q8C7C7	0.2	5E2; 550 cM	67.01/5.49	94	25
Adult male lung cDNA	Q9DC07	0.3	2A2	31.49/8.54	95	46
Chloride intracellular channel protein 5	Q8BXX9	0.3	17C	28.44/5.64	87	58
L-lactate dehydrogenase B chain	P16125	0.4	6G2; 662 cM	36.7/5.7	132	39

B: asthmatic group; C: salbutamol-treated asthmatic group.

in the future.

Some spots may be related to β_2 AR desensitization

Some cytoskeleton elements decrease in salbutamol-treated asthmatic mice, such as beta actin. As a house-keeping gene, beta actin is usually unchanged in the physiopathologic process. Our 2-DE data indicated that it might be associated with β_2 AR desensitization. Although some research results supported this view, we still cautiously deal with its function in β_2 AR desensitization.⁵ β_2 AR desensitization is closely related to GPRK₂, and GPRK₂-mediated ezrin-radixinmoesin (ERM) phosphorylation serves to link G protein coupled receptors (GPCR) activation to cytoskeletal reorganization.²⁵ The role of cytoskeleton actin has not been fully elucidated in β_2 AR desensitization, it still needs a lot of experiments to support.

Other protein spots

All protein spots are listed in the Table. Besides cytoskeleton and oxidative stress, the remaining protein spots include apoptosis, other metabolic functions, and so on. These functions are not described because they may not play a major role in β_2 AR desensitization. Noticeably, none of the known protein spots linked to β_2 AR desensitization was observed in our experiment, suggesting that these proteins may be low-abundant, low-dissolved or they may be lost in the process of extracting. Meanwhile, this may be associated with the

technology of 2-DE since it is a new method for protein separation.

In summary, we used 2-DE to identify proteins that are differentially expressed in asthmatic and salbutamol-treated asthmatic mouse lung tissues. Some of the identified proteins are related to oxidative stress, small G protein and cytoskeleton actin, which may be connected to the mechanism of β_2 AR desensitization in asthmatic mice. Recent reports have demonstrated the relationship between the expression of certain proteins and asthma. However, no comparative analysis has been reported of the different protein spots between asthmatic and salbutamol-treated asthmatic mouse lung tissues, ie, β_2 AR desensitization asthmatic mouse lung tissues.²⁶ The proteins we identified may provide tools for successful therapeutic interventions in patients with β_2 AR desensitized asthma. Although we have found the altered protein spots in response to β_2 AR desensitization, additional studies are needed to characterize the precise mechanism of action.

REFERENCES

- Robertson CF, Price D, Henry R, Mellis C, Glasgow N, Fitzgerald D, et al. Short course montelukast for intermittent asthma in children: a randomised controlled trial. *Am J Respir Crit Care Med* 2007; 175: 323-329.
- Mao H, Wen FQ, Li SY, Liang ZA, Liu CT, Yin KS, et al. A

- preliminary study towards downregulation of murine bone marrow eosinophilopoiesis mediated by small molecule inhibition of interleukin-5 receptor α gene *in vitro*. *Respiration* 2007; 74: 320-328.
3. Antoniu SA. Formoterol as a rescue medication for asthma. *Expert Opin Pharmacother* 2006; 7: 2439-2441.
 4. Hanania NA, Moore RH. Anti-inflammatory activities of β_2 -agonists. *Curr Drug Targets Inflamm Allergy* 2004; 3: 271-277.
 5. Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2 -adrenergic receptor. *Nature* 1999; 401: 286-290.
 6. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Regulation of receptor fate by ubiquitination of activated β_2 -adrenergic receptor and beta-arrestin. *Science* 2001; 294: 1307-1313.
 7. Banci L, Bertini I, Cusack S, de Jong RN, Heinemann U, Jones EY, et al. First steps towards effective methods in exploiting high-throughput technologies for the determination of human protein structures of high biomedical value. *Acta Crystallogr D Biol Crystallogr* 2006; 62: 1208-1217.
 8. Liu H, Yin KS. The status quo and perspective of proteomic study in bronchial asthma. *Int J Respir (Chin)* 2007; 27: 29-32.
 9. Finney PA, Belvisi MG, Donnelly LE, Chuang TT, Mak JCW, Scorer C, et al. Albuterol-induced downregulation of Gs α accounts for pulmonary β_2 -adrenoceptor desensitization *in vivo*. *J Clin Invest* 2000; 106: 125-135.
 10. Jang HS, Oh CK, Jo JH, Kim YS, Kwon KS. Detection of telomerase activity in psoriasis lesional skin and correlation with Ki-67 expression and suppression by retinoic acid. *J Korean Med Sci* 2001; 16: 623-629.
 11. Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* 1999; 20: 601-605.
 12. Joo WA, Kang MJ, Son WK, Lee HJ, Lee DY, Lee E, et al. Monitoring protein expression by proteomics: human plasma exposed to benzene. *Proteomics* 2003; 12: 2402-2411.
 13. Bian T, Yin KS, Jin SX, Zhang XL, Zhou JY, Ma XQ, et al. Treatment of allergic airway inflammation and hyperresponsiveness by imiquimod modulating transcription factors T-bet and GATA-3. *Chin Med J* 2006; 119: 640-648.
 14. Rovati GE, Baroffio M, Citro S, Bricchetto L, Ravasi S, Milanese M, et al. Cysteinyl-leukotrienes in the regulation of β_2 -adrenoceptor function: an *in vitro* model of asthma. *Respir Res* 2006; 7: 103-113.
 15. Shite J, Qin F, Mao W, Kawai H, Stevens SY, Liang C. Antioxidant vitamins attenuate oxidative stress and cardiac dysfunction in tachycardia-induced cardiomyopathy. *J Am Cell Cardiol* 2001; 38: 1734-1740.
 16. Siwik DA, Tzortzis JD, Pimental DR, Chang DL, Pagano PJ, Singh K, et al. Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes *in vitro*. *Circ Res* 1999; 85: 147-153.
 17. Temsah RM, Netticadan T, Chapman D, Takeda S, Mochizuki S, Dhalla NS. Alterations in sarcoplasmic reticulum function and gene expression in ischemic-reperfused rat heart. *Am J Physiol* 1999; 277: H584-H594.
 18. Qin F, Yan C, Patel R, Liu W, Dong E. Vitamins C and E attenuate apoptosis, β -adrenergic receptor desensitization, and sarcoplasmic reticular Ca²⁺ ATPase downregulation after myocardial infarction. *Free Radic Biol Med* 2006; 40: 1827-1842.
 19. Fasanaro P, Magenta A, Zaccagnini G, Cicchillitti L, Fucile S, Eusebi F, et al. Cyclin D1 degradation enhances endothelial cell survival upon oxidative stress. *FASEB J* 2006; 20: 1242-1244.
 20. Lombardi MS, Kavelaars A, Penela P, Scholtens EJ, Roccio M, Schmidt RE, et al. Oxidative stress decreases g protein-coupled receptor kinase 2 in lymphocytes via a calpain-dependent mechanism. *Mol Pharmacol* 2002; 62: 379-388.
 21. Bustelo XR, Sauzeau V, Berenjano IM. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions *in vivo*. *Bioessays* 2007; 29: 356-370.
 22. Dovas A, Couchman JR. RhoGDI: multiple functions in the regulation of Rho family GTPase activities. *Biochem J* 2005; 390: 1-9.
 23. Johnson M. Molecular mechanisms of β_2 -adrenergic receptor function, response, and regulation. *J Allergy Clin Immunol* 2005; 117: 18-24.
 24. Bagrodia S, Bailey D, Lenard Z, Hart M, Guan JL, Premont RT, et al. A tyrosine-phosphorylated protein that binds to an important regulatory region on the cool family of p21-activated kinase-binding proteins. *J Biol Chem* 1999; 274: 22393-22400.
 25. Cant SH, Pitcher JA. G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. *Mol Biol Cell* 2005; 16: 3088-3099.
 26. Murphy VE, Johnson RF, Wang YC, Akinsanya K, Gibson PG, Smith R, et al. Proteomic study of plasma proteins in pregnant women with asthma. *Respirology* 2006; 11: 41-48.

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