Original Paper

Somatostatin Reduces the Acute Lung Injury of Mice via Increasing the Affinity of Glucocorticoid Receptor

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Key Words

Acute Lung Injury • Somatostatin • Heat Shock Protein 90 • Glucocorticoid Receptor

Abstract

Background/Aims: Although it has been reported that somatostatin (SOM) upregulated the level of 90-kD heat shock protein (Hsp90), which participates in the inflammatory regulation by its client proteins, such as glucocorticoid receptor (GR), it remains unclear if it has a protective role against acute lung injury (ALI). **Methods:** ALI model was established by the injection of oleic acid (OA) into the tail vein of mice. Lung injury was assessed by histological analysis, lung water content and arterial blood gases. The levels of Hsp90 and GR, the binding capacity and the affinity of GR were examined. **Results:** It was showed that pretreatment with SOM significantly increased Hsp90 levels and alleviated lung injuries in OA-injected mice. Furthermore, SOM increased the GR expression and improved the affinity of the GR in animals with lung injury. However, little alteration was found in the maximum binding capacity of the GR in mice with or without SOM. **Conclusion:** The data indicate SOM exerts a protective effect by increasing Hsp90 abundant and further enhancing the affinity of the GR. The beneficial effects of SOM treatment provide a new strategy for modulation of GR efficiency and alleviation of acute lung injury.

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Introduction

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are critical illnesses with high morbidity and mortality. Despite the advances in

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the strategies targeting cell growth factors, complements, antioxidants, signal transduction inhibitor [1, 2] and stem cell [3, 4], no specific treatment exists for these diseases. Recently, several lines of evidence showed that somatostatin (SOM) has protective effects against central nervous system, pancreatic and gastrointestinal tract diseases [5-7], but the role of SOM in ALI remains to be clarified.

Interestingly, SOM also plays an inhibitory role in the effect of calpain, an enzyme that degrades Hsp90, in rat brain [8], which indicates the possibility that SOM could upregulate the Hsp90 level. Heat shock proteins play an important role under various stresses (trauma, operation, radiation, tumor, etc.) and maintain the internal homeostasis. Recent studies have found that Hsp90 participates in innate immunity and in the signaling pathways of many inflammatory factors, in addition to aiding damaged proteins in achieving their proper folding as a chaperone molecular. Moreover, pre-elevated levels of heat shock proteins help to attenuate organic injuries and reduce mortalities in various kinds of animal models and cellular experiments [9-11]. Among many Hsp90 clients, the glucocorticoid receptor (GR) which is a key effector in the hypothalamic-pituitary-adrenal axis (HPA axis) shows an important regulatory function under stresses. The participation of Hsp90 is indispensable for the activity of the GR. Treatment with specific Hsp90 inhibitors, such as geldanamycin, or mutant constructs of Hsp90 can lead to decrease of nuclear translocation of the GR and further significantly decrease its effects [12]. Glucocorticoids (GCs) are extensively used as anti-inflammatory agents in clinical treatments and experimental researches of animal in various inflammatory lung diseases [13-16], and they are closely associated with the duration of intensive care unit stay and ALI survivors' physical outcomes [17-21].

Therefore, SOM might have a protective effect on ALI in mice via elevating the Hsp90 level and then enhancing the activity of GR, which is a main protective factor against the development of ALI. To confirm this hypothesis, the present study explored the effect of SOM on ALI, levels of Hsp90 and GR, the affinity and the binding capacity of GR using an oleic acid-induced ALI model. This work will identify the protective role of SOM in ALI and provide an alternative strategy to our knowledge for the improving of the outcomes of ALI and ARDS with reduced side effects of GCs.

Materials and Methods

Animals and drugs treatment

A total of 120 male Kunming mice weighing 18-25g (purchased from Shanghai Laboratorial Animal Center, China) were housed in a 12-h light-dark cycle facility and allowed free access to food and water. Animals were randomized into three groups: OA group, SOM group and SOM+OA group. Animals were administered intraperitoneally with SOM (Sigma-Aldrich Co., USA) at the doses of 0.1, 1 and 10 µg/g body weight in the dose dependent experiment and at a dose of 1 µg/g in the rest of the experiments. Mice in OA group were injected with pure OA (Sigma-Aldrich Co., USA) slowly through the tail veins at a volume of 0.6ml/kg by a Micro Sample instrument to induce acute lung injury, and saline was administered 30min before OA injection as vehicle treatment. In SOM+OA group, animals received SOM (1µg/g) 30min before OA (0.6ml/kg) injection [22, 23]. All procedures were approved by Experimental Animal Ethics Committee at the Third Military Medical University (TMMU) and performed in accordance with the TMMU guidelines for the care and use of experimental animal which are consistent with the NIH guidelines.

Histological analysis

Animals were anesthetized with 1.5% sodium pentobarbital. The lungs were isolated and fixed in 4% paraformaldehyde. Paraffin-embedded tissues were sectioned, mounted, stained with hematoxylin and eosin. Images were obtained using a Leica light microscope.

Lung/body weight ratio and wet/dry lung weight ratio

Lung/body weight ratio and wet/dry lung weight ratio were measured as indicators of pulmonary edema. Mice were euthanized with an overdose of sodium pentobarbital at the designated time points and

the lungs were excised immediately after animals died. Lung/body weight ratio was calculated by the body and the wet lung weights. The lungs were weighted before and after they were dried to a constant weight at 80°C for 72 h to determine wet/dry lung weight ratio.

Arterial blood gas analysis

Arterial blood gas analysis is important to evaluate the condition of animals with acute lung injury. Mice were anesthetized with sodium pentobarbital (50 mg/kg) and a 0.3-ml arterial blood sample was collected with a heparinized syringe from the abdominal aorta of each animal. The pH value, partial pressures of oxygen (PaO₂) and carbon dioxide (PaCO₂) were measured with an IL1302-type blood gas analysis instrument (Instrumentation Laboratory Inc.) immediately after blood samples were collected.

Western blot analysis

Cytoplasmic protein was isolated from lung tissues as previously described with modifications [24]. Samples were separated on a 10% SDS-polyacrylamide gel, transferred to PVDF membrane and probed with the rabbit anti-Hsp90 and anti-GR (Santa Cruz, USA) primary antibodies. The samples were then incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Santa Cruz, USA) and detected with an ECL substrate kit (Thermo Scientific, USA). In these experiments, β-actin (Santa Cruz, USA) served as an endogenous control. The intensity of the bands was analyzed by a UVP Bio-imaging acquisition and image analysis system (Ultra-Violet Products Ltd, UK).

The mRNA expression of Hsp84, Hsp86 and IL-6

The mRNA level of Hsp84 and Hsp86, two subtypes of Hsp90 in mice, and IL-6 were examined. Total RNA was isolated from the lung tissues at the indicated time points using Trizol (Invitrogen, USA) and then reverse-transcribed into cDNA using the GoScript™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. Real-time PCR was performed in a Stratagene Mx3000P system (Stratagene, USA). The following primers were used, forward: 5' AAT GGA GAG CAA GGC AAA G 3', reverse: 5' GCC ATC ATG TAG CCC ATT GTA G 3' for Hsp84, forward: 5' GAG GCT GAC AAG AAT GAC 3', reverse: 5' TCT TCC ATG CGT GAT GTG 3' for Hsp86, forward: 5' CCA GAA ACC GCT ATG AAG 3', reverse: 5' TTC CAC GAT TTC CCA GAG 3' for IL-6 and forward: 5'AGG TTG TCT CCT GCG ACT TCA3', reverse: 5'TGG TCC AGG GTT TCT TAC TCC 3' for GAPDH. Samples were normalized to GAPDH.

Glucocorticoid receptor binding assays

Saturation binding assays were performed to test the affinity (indicated by K_d value) and the binding capacity (indicated by maximum binding capacity) of GR to [³H]-dexamethasone, a synthesized glucocorticoid. The lung tissues from different treatment groups were homogenized in the lysis buffer (25mM Sucrose, 25mM KCl, 1mM MgCl₂, 1.5mM CaCl₂, 10mM Sodium Molybdate, 50mM Tris, 0.5M 1,10-Phenanthroline, 0.5M Iodoacetamide and 2M Pepstatin A) followed by centrifugation at 170,000g for 20 minutes at 4°C. Homogenates used for saturation binding assay were incubated with [³H]-dexamethasone (D6310, Sigma) alone and in the presence of 1000-fold excess unlabeled dexamethasone for 20min at 23°C with a 400-μL total volume. Following incubation, homogenates were resuspended in 5-mL scintillation cocktail and then counted using a LS 6500 Liquid Scintillation Counter (Beckman Coulter, USA). The data from each assay were analyzed according to the method of Scatchard.

Statistical analysis

One-way analysis of variance and a Bonferroni post hoc test were used for multiple comparisons. Data are indicated as means ± S.D. and the differences are considered to be statistically significant at *p* < 0.05.

Results

Effects of SOM on lung histology

In the OA-induced ALI model, obvious pulmonary edema and hemorrhage were found at 3 and 12 h post injury by histological analysis. A marked infiltration of inflammatory cells (a number of neutrophils) and thickening of alveolar walls were observed at 1, 3 and

Fig. 1. Effect of SOM on lung histology. A significant edema (marked with Arrow a) appeared at 1, 3 and 12 h after OA injection. A thickening alveolar septum (marked with Arrow b) and marked infiltration of inflammatory cells (marked with Arrow c) were also observed after administration of OA. Lung sections were stained with hematoxylin and eosin. Magnification, $\times 200$. A indicated control, B-E indicated OA group and F-I indicated OA+SOM group from 1 to 24 h respectively.

Fig. 2. Effects of SOM on the lung injuries. Lung/body weight ratio (A), wet/dry lung weight ratio (B) and IL-6 mRNA expressions (C) of mice were measured at 1, 3, 12 and 24 h post injury as described in the Methods section. $^*P < 0.05$, SOM+OA versus OA, $^{\#}P < 0.05$, versus 0 h. Data are expressed as the means $\pm$ S.D. ($n = 5$ per group, one-way ANOVA followed by Bonferroni post hoc test).

12 h and a disorganized alveolar structure were found at 24 h after injury. SOM treatment prior to OA injection significantly alleviated pulmonary edema, hemorrhage and neutrophil accumulation compared to saline-treated animals in OA group (Fig. 1).

Effects of SOM on the lung injury

To investigate the effects of SOM on the pulmonary edema in mice, the lung/body ratio and wet/dry lung weight ratio were examined. Results showed both the lung/body and wet/dry lung weight ratio were gradually increased from 1 to 12 h and slightly dropped at 24 h in OA-administered animals with or without SOM pretreatment. However, data revealed that the lung/body ratio in animals pre-treated with SOM prior to OA injection was significantly decreased at 3, 12 and 24 h compared to those of SOM-untreated OA group ($p < 0.05$) (Fig. 2 A). Similarly, SOM treatment decreased the wet/dry lung weight ratio significantly at 1, 3 and 12 h ($p < 0.05$) (Fig. 2B). Moreover, SOM significantly decreased the IL-6 mRNA level at 3 and 12 h post injury in OA-induced ALI animals ($p < 0.05$) (Fig. 2C). Thus, these results revealed the SOM pretreatment mitigated the lung injury induced by OA injection.

Fig. 3. Effects of SOM on the arterial blood gas and biochemical test. The experimental animals were anesthetized with sodium pentobarbital and a 0.3-ml arterial blood sample collected from abdominal aorta with a heparinized syringe. pH value (A), PaO₂ (B), PaCO₂ (C), Na⁺ (D), K⁺ (E), HCO₃⁻ (F) and anion gap (G) at designed time points were tested. *P<0.05, SOM+OA versus OA, #P<0.05, versus 0 h. Data are expressed as the means ± S.D. (n=5 per group, one-way ANOVA followed by Bonferroni post hoc test).

Arterial blood gas analysis and biochemical test

Arterial blood gas analysis which is used to characterize the type of disorder and quantify the magnitude of lung injury was performed to evaluate the influences of SOM on OA-induced lung injury. When mice were administered with OA, pH value and PaO₂ were decreased significantly from 1 to 24 h after injection. Animals with SOM treatment prior to OA injection had an increased pH value compared with those without SOM pretreatment at 1 h ($p=0.04$), 3 h ($p=0.027$) and 24 h ($p=0.032$) (Fig. 3A). The PaO₂ value was also increased after SOM treatment in ALI mice at 1 h ($p=0.036$), 3 h ($p=0.022$) and 24 h ($p=0.044$) post injury (Fig. 3B). In contrast, animals had an increased PaCO₂ in OA group, and there is no significant difference between OA and SOM+OA group from 1 to 24 h (Fig. 3C). Above data indicated that mice presented obvious dysfunction of gas exchange in OA-induced lung injury model and SOM pretreatment alleviated the disorder in pH value and PaO₂ at 1, 3 and 24 h after injury. In addition, the results showed the concentrations of Na⁺ and K⁺ kept unchanged in all three groups (Fig. 3D and 3E). But HCO₃⁻ concentration decreased and anion gap increased significantly 1 h post injury in mice with ALI (Fig. 3F and 3G). Our data indicated acidosis presented at 1 h post injury in ALI animals and SOM obviously alleviated this pathological change.

Effects of SOM on Hsp90 expression

We used three SOM dosages (0.1, 1 and 10 µg/g body weight) and found it dose-dependently increased Hsp90 level at 12 h in mice with lung injury induced by OA ($P<0.05$) (Fig. 4A and 4B). Thus, based on published method for dose conversion from human to mice [25] and our results, we chose to use the dose of 1 µg/g in the present experiments. Hsp90 level was increased at 1, 3 and 12 h after injury in response to OA ($P<0.05$). Similarly, it was increased from 1 to 24 h in animals with SOM pretreatment before OA injection ($P<0.05$). Moreover, SOM alone increased Hsp90 from 1 to 24 h after injury (Fig. 4C and 4D). These data demonstrated that SOM produced a significant increase in Hsp90 level in both normal and injured animals.

Fig. 4. The expression of heat shock proteins. Representative results of western blot (A) and relative Hsp90 expression (B) in mice treated with SOM (0.1, 1 and 10 μg/g body weight) at 12 h post injury were shown (n=3). Representative results of western blot and the drugs administration (C) and relative Hsp90 expression (D) at 1, 3, 12 and 24 h were shown (n=5). The mRNA levels of Hsp84 (E) and Hsp86 (F) (two subtypes of Hsp90 in mice) were measured by q-PCR at designed time points (n=5). Data were normalized to GAPDH. *P<0.05, SOM+OA versus OA, #P<0.05, versus 0 h, N.S. indicates no significant difference between indicated groups. Data are expressed as the mean ± S.D. (one-way ANOVA followed by Bonferroni post hoc test).

In order to confirm whether the changes of Hsp90 derived from the alteration of its mRNA levels, we examined the mRNA levels of Hsp84 and Hsp86, two subtypes of Hsp90 in mice. However, the data showed that there was no significant difference between the OA and OA+SOM groups or SOM and OA+SOM groups (Fig. 4E and 4F). It suggests that the up-regulation of Hsp90 protein is not due to the changes at the translational level.

Effects of SOM on GR expression

Due to the importance of GR for inflammation and our recent findings that the nuclear translocation of GR could be regulated by Hsp90, western blot analyses of GR were performed.

We found that the GR level increased temporarily at 1 h, then decreased gradually from 3 to 12 h after OA administration. SOM-treated animals with OA injection had a higher GR

Fig. 5. The expression of glucocorticoid receptor. Representative results of western blot (A) and the statistical data (B) at 1, 3, 12 and 24 h were shown. * $P < 0.05$, SOM+OA versus OA, # $P < 0.05$, versus 0 h. Data are expressed as the mean $\pm$ S.D. (n=5 per group, one-way ANOVA followed by Bonferroni post hoc test).

Fig. 6. The binding capacity and the affinity of GR. Kd value (A) and max binding capacity (B) of GR were tested at 1, 3, 12 and 24 h post injury by [3 H]-dexamethasone radioligand binding assay. * $P < 0.05$, SOM+OA versus OA; # $P < 0.05$, versus 0 h. Data are expressed as the mean $\pm$ S.D. (n=4 per group, one-way ANOVA followed by Bonferroni post hoc test).

expression at 1, 12 and 24 h compared to the SOM-untreated mice in OA group. Unlike the expression of Hsp90 protein, SOM had no effect on GR protein under normal condition (Fig. 5A and 5B).

Effects of SOM on ligand-binding ability of GR

In order to determine if SOM affects ligand binding to the GR, saturation binding assays were performed using [3 H]-dexamethasone, a ligand of glucocorticoid receptor, and the dissociation constant (Kd) and the maximum number of binding sites (Bmax) were calculated. As shown in Fig. 6, the Kd values of the GR increased at 1 and 3 h post-injury and were resolving subsequently at 12 and 24 h. The Kd values of SOM+OA group were profoundly lower than OA group (Fig. 6A). Moreover, the maximal numbers of binding sites (Bmax) of the GR were decreased from 3 to 24 h within the OA group and there was no obvious difference for Bmax of the GR between the SOM+OA group and OA group across all time points in an acute lung injury model (Fig. 6B).

Discussion

The OA model is one of the three most widely used animal models (broncho-alveolar lavage, OA injection and LPS injection) in research concerning acute respiratory diseases, such as acute lung injury and acute respiratory distress syndrome [26, 27]. This model causes

the increasing in the permeability in endothelial cells and impairs gas exchange [28-30].

In the present study, the results showed that OA treatment resulted in a strong lung inflammation which was characterized by vascular leakage. The changes were determined by the presence of lung edema and strong leukocyte infiltration into the lung and also occur in patients with ALI. The IL-6 level was increased significantly in OA-induced ALI animals. This is in agreement with previous studies, which found SOM inhibits secretion of IL-6 in ALI mice [31] and IL-8 and IL-1 β from the intestinal epithelial cells [32]. SOM intervention significantly alleviated the OA-induced lung injuries, indicating it could be an effective method for ALI treatment. Although published documents reported that the effects of anesthesia on murine models of lung injury should be considered, the dosage (50 mg/kg) we used is a relatively low dose in mice [33] and no pentobarbital-induced death was observed in the present study. The O₂ saturation (SPO₂) at this dose is approximately 90% at 35–40 min after anesthesia [34], which would not have a significant effect on ALI.

In addition, our data demonstrated that the levels of Hsp90 were markedly increased from 1 to 12 h with OA injection alone due to the intrinsic protection of organs when they suffered insults and SOM treatment dramatically increased Hsp90 level in animals with lung injury. It also showed that Hsp90 level was increased in animals with SOM treatment alone. The heat shock response has been classically defined as a highly conserved cellular defense mechanism. It has been well documented that pre-condition of heat shock restored alveolar fluid clearance in cultured alveolar type II cells and alveolar macrophages [35, 36]. Heat shock proteins also had the similar effect on the animals with lung injury induced by sepsis [37] and ischemia-reperfusion [38].

In this study, though the level of Hsp90 was increased in OA-induced lung injury, it was insufficient to cope with the severe injury. In addition, increases in Hsp90 protein appeared as early as 1 h after injury, and the mRNA level of Hsp84 and Hsp86 kept unchanged in all three groups, which implied that the upregulation of Hsp90 was not caused by *de novo* synthesis. Interestingly, recent data showed that calpain could cause Hsp90 cleavage in several cell systems and its activity is inhibited by SOM via blocking of Ca²⁺ channels [39-41]. Therefore, SOM-induced increase in Hsp90 level might due to the inhibition of its degradation, which has a distinct mechanism from the elevation of Hsp90 level triggered by heat stress.

In addition to functioning as a chaperone, increased Hsp90 would affect the activity of its clients. Hsp90 is also required for GR to bind their ligands and exert anti-inflammatory action [42]. Thus, it is possible that the increased Hsp90 protect mice from acute lung injury via improving the activity of GR.

Here, we showed that SOM had no effect on the GR expression in mice without lung injury. Nevertheless, SOM pretreated animals had a significantly higher GR level at 1, 3 and 24 h compared to OA group. Moreover, the K_d value of GR was increased at 12 and 24 h, and almost reached the normal level at 24 h, which implied that the protective action of GR in lung injury was associated closely with its affinity at 12 and 24 h. It might because the level of GR at these time points was insufficient to meet the needs for the body to maintain the balances of internal environment, and the enhanced affinity of GR was beneficial to the supplement of GR effects.

In addition, during OA-induced acute lung injury the binding capacity of GR was reduced strongly because Hsp90 is largely consumed during the recovery of misfolded proteins. In this case, it was undoubtedly important that the Hsp90 level was increased rapidly by blocking its degradation and further promoting the function of the GR. The present study demonstrated that the binding capacity was not a key factor accounting for the altered activity of the GR. Thus, the affinity of the GR was separately enhanced without alteration of the binding capacity at 12 and 24 h in this case. However, SOM exerted positive effects at an early stage via increasing the expression of GR, especially at 1 and 3 h after injury.

So far, several lines of evidence have showed that SOM exerts a protective action in inflammatory response. Exogenous SOM has been demonstrated to inhibit vasodilatation [43] and plasma protein extravasation [44]. Another study revealed that SOM inhibited the function of several types of cells which are able to produce pro-inflammatory mediators

[32]. In addition, it's well known that GR as a nuclear factor play an important role in inflammatory response via regulating the target genes related to inflammation. Taken above factors into account, we think it is possible that the inhibition effect of SOM on inflammatory mediators is modulated by GR activity, at least partly.

Besides the mechanism we found in the present experiments, the differences in genetic background could also alter the function of GR. Our previous study has found the mutation in Hsp84 gene affect the nuclear translocation of GR [12]. Another study found the key structure of GR domain mutations would also change its function. For example, the mutations located at hormone combined domain can reduce affinity of GR to its ligand [45]. In addition, hypothalamo-pituitary-adrenocortical (HPA) axis dysregulation [46] and an excessively high concentration of endogenous cortisol and administered steroid drugs [47] could alter the expression and activity of GR. These factors could not be excluded in the current experiments.

In summary, we demonstrate that SOM exerts a protective action against OA-induced ALI in mice. It is a positive regulatory drug for GR efficiency because it rapidly promotes increases in Hsp90 level and further in the activity of GR. Though glucocorticoid is an extensively used and effective drug, the overdose of it causes several severe side effects, such as osteoporosis and inhibition of the HPA axis by negative feedback. SOM could improve the affinity of GR to its ligand, which provides a better way for experimental research on improving the effect of corticosteroids without increasing the dosage of the hormone.

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Disclosure Statement

The authors have declared that no conflict of interest exists.

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