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Original Article

Hsp90 protects LPS induced pancreatic inflammation of AR42J cells by inhibition of p38MAPK/NF-κB pathways

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Abstract: Aims: The purpose of this study was to evaluate the role of Hsp90 in the AR42J treated with LPS through over-expression and knock-out Hsp90. This study has further expounded the mechanism that Hsp90 inhibited the activation of p38MAPK/NF-κB pathway, and thus decreased the LPS-induced inflammatory reaction in AR42J. Methods: Cell proliferation (MTT) assay and apoptosis assay were respectively used to explore the viability and apoptosis in AR42J cells. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to confirm the expression of Hsp90. Western blot was performed to determine the expression of p38MAPK/NF-κB pathway related protein. Results: Compared with control, over-expression Hsp90 could increase the viability and decrease the apoptosis in AR42J cells treated with LPS. Over-expression Hsp90 could significantly down-regulate the expression of the inflammatory factors such as IL-1α, IL-6, IL-8, and TNF-α in AR42J cells treated with LPS ($P<0.05$). Western blotting showed that up-regulation of Hsp90 could significantly inhibit the LPS-induced inflammatory reaction in AR42J cells ($P<0.05$). Conclusion: In the present study, we drew a conclusion that over-expression Hsp90 could protect LPS-induced pancreatic inflammation of AR42J cells by inhibition of p38MAPK/NF-κB pathways.

Keywords: Hsp90, pancreatic inflammation, AR42J, p38MAPK/NF-κB pathway

Introduction

Acute pancreatitis (AP) is a common clinical emergency, which shows an increasing trend in recent years [1]. Acute abdominal pain is the most common symptom [2]. AP is an inflammatory disease owning to the activation of pancreatic enzyme in pancreas, which leads to autodigestion, edema, hemorrhage and necrosis of pancreas [3]. Alcohol abuse and gallstone migration into the common bile duct are the most frequent causes of pancreatitis in adults [1], which together accounts for about 70% of cases [2, 3]. Other factors such as abdominal trauma, pancreatic carcinoma, elevated triglycerides, viral infections, metabolic disorders, prior abdominal surgery, side effect of drugs, and hereditary disease have been reported [4-7]. AP is divided into mid AP and severe acute pancreatitis (SAP). 80% of acute pancreatic patients is mild, who recover without complications, having an associated mortality of 1%. However, approximately 20% of patients may turn to SAP with evidence of organ dysfunction [8]. SAP with a mortality approaching 30% is an urgent onset and rapid progress clinical emergency, which has become a threat to human health.

Despite improvements in intensive care treatment during the past few decades, the death rate for AP has not declined significantly [8, 9]. Most studies believe that many inflammatory factors contribute to serious circulatory, respiratory, urinary and nervous system damage in SAP. However the majority of patients with AP can prevent excessive inflammatory response development, so that pancreatitis is mild with self-healing tendency [10, 11]. Why about 20% of patients will develop SAP. There are several hypotheses attempting to explain this phenomenon, but its mechanism still has not been clarified. At present a growing number of scholars have argued disturbed balance between pro-inflammatory and anti-inflammatory cytokines play an important role.

Heat shock protein 90 (Hsp90) plays an important role in maintaining the conformation, stability, and function of many proteins. It is a molecular chaperone required for the stability and function of a number of conditionally acti-

vated or expressed signaling proteins, as well as multiple mutated, chimeric, or overexpressed signaling proteins, which promote cancer cell growth or survival or both [12]. One Hsp90 inhibitor, 17-allylamino-17-desmethoxygeldanamycin (17-AAG), has shown antitumor activity in several human xenograft models, including prostate, breast, and colon cancer [13-15]. The majority of identified Hsp90 clients are oncogenic proteins [16], including signaling proteins, transmembrane tyrosine kinases, transcription factor, cell cycle regulators, and so on. Many of these oncoproteins directly participate in the growth and angiogenesis of pancreatic cancer. It was also reported that Hsp90 was over-expressed 2 to 10 folds in various human cancers [13-15], which could remain the activity of excessive activated or mutated onco-proteins and inhibit the degradation of these proteins, in order to accelerate the malignant phenotype and tumor progression. Targeting of Hsp90 chaperone exhibits combined antitumor effect by simultaneous destabilization and inactivation of various oncogenic proteins [17]. Hsp90 is existed in an activated, super-chaperone complex form in tumor cells, which has a much higher affinity for Hsp90 inhibitors versus normal cells [18]. Therefore, targeting Hsp90 became an appealing, selective and validated target for cancer therapy.

Recent work has indicated that the signal pathway in the interaction of various cytokines, leading to various inflammatory factors and thus lead to systemic inflammation response syndrome (SIRS), finally resulting in multiple organ failure (MOF) [19]. How can we effectively control the production of cytokines, to prevent the occurrence of SIRS or reduce their severity, thereby enhancing the therapeutic effect of SAP and reduce the mortality became a huge problem. It has been confirmed that p38 mitogen-activated protein kinase (P38MAPK) signal transduction pathway are closely related with the appearance and regulation of various cytokines [20, 21]. In the present study, we sought to evaluate the role of Hsp90 in the AR42J treated with LPS and further expounded the mechanism by inhibiting the activation of p38MAPK/NF-κB pathway.

Materials and methods

Cell culture and LPS treatment

AR42J cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 1X penstrep and 2 mM glutamine. The cells were treated in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. For Dex treatment, the cells were inoculated into 6-well plates, T-75 or T-25 flasks. The second day after Dex treatment, complete media containing 50 nMDex was added to the cells. The cells were assayed or harvested 2-3 days after the addition of Dex. AR42J cells were treated with LPS (100 ng/mL) for 24 h.

Plasmids transfection

An Hsp90 expression vector (pc-Hsp90) was constructed by sub-cloning the full-length wild-type Hsp90 coding sequence into pcDNA3.1, and confirmed by sequencing. The empty construct pcDNA3.1 was transfected as a control. The target sequence for Hsp90-specific siRNA was (5'CGCATGATCAAGCTAGGTCTA3', and 5'CCAACTCATGTCCCTCATCAT3'), and control siRNA (no silencing) was synthesized by GenePharma Co (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol. Stable Hsp90 transfection was generated under G418 (Gibco, Paisley, UK) selection as described.

MTT assay

The cell proliferative and invasive capacities were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay and a Matrigel invasion chamber assay, respectively, according to standard methods described before [25]. Each experiment was performed three times.

Apoptosis

Apoptosis analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The Schwann cells (100,000 cells/well) were seeded in 6 well-plates. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and

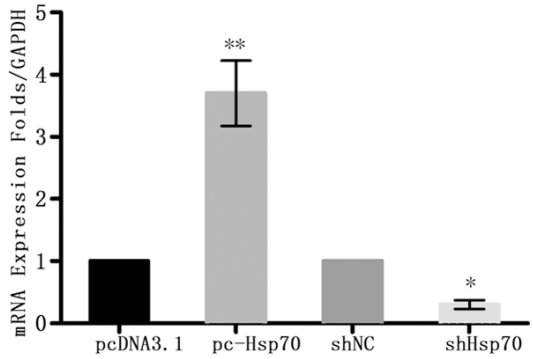

Figure 1. Confirm the expression efficiency of Hsp90 by western blot after Hsp90 over-expression and knock-out. The expression of Hsp90 was determined by western blot. Error bars indicate means ± SD and *indicates significant difference compared with blank group (P<0.05).

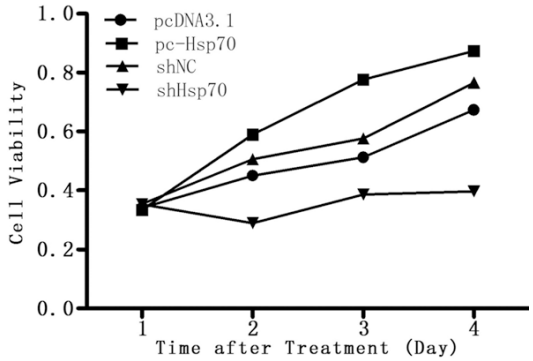

Figure 2. Detect viability of AR42J injured by LPS after Hsp90 over-expression and knock-out. At 24 h after treated with LPS, the AR42J cells transfection with pc-Hsp90 and sh-Hsp90 were collected for analysis. The cell viability was determined by the MTT assay. Error bars indicate means ± SD and *indicates significant difference compared with blank group (P<0.05).

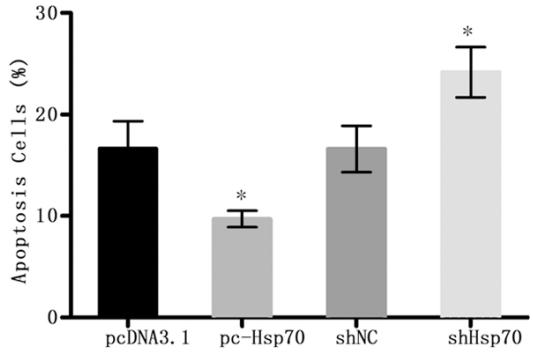

Figure 3. Detect apoptosis of AR42J injured by LPS after Hsp90 over-expression and knock-out. At 24 h after treated with LPS, the AR42J cells transfection with pc-Hsp90 and sh-Hsp90 were collected for analysis. The cell apoptosis was determined by the apoptosis assay. Error bars indicate means ± SD and *indicates significant difference compared with blank group (P<0.05).

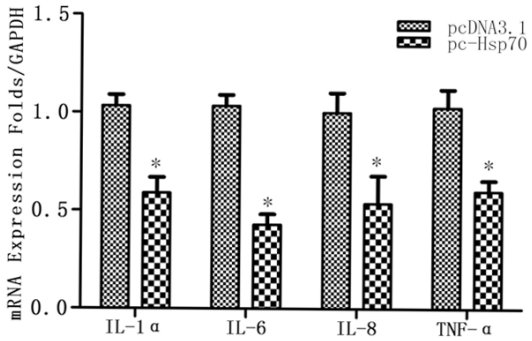

Figure 4. Detect the expression of inflammatory cytokines of AR42J treated with LPS after over-expression. The expression of inflammatory cytokines was determined by qRT-PCR. Error bars indicate means ± SD and *indicates significant difference compared with blank group (P<0.05).

PI-negative) from necrotic cells (Annexin-V and PI-positive).

qRT-PCR

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega). Reverse transcription was performed by using the MultiscribeRTkit (Applied Biosystems) and random hexamers or oligo(dT). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C. The sequences of the primers were as follows, Hsp90 forward primer: 5'TGGACAGCAAACATGGAGAG3', reverse primer: 5'AGACAGGAG CGCAGTTTCAT3'. GAPDH forward primer: 5'GCACCGTCAAGGCTGAGAAC3', reverse primer: 5'TGGTGAAGACGCCAGTGGA3'.

Western blot

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Rabbit-anti-human Hsp90 antibody for chromatin immunoprecipitation was purchased from Abcam

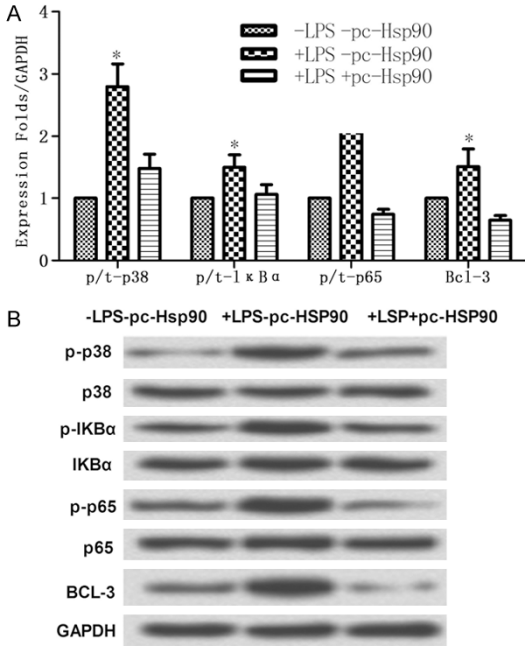

Figure 5. Detect the expression level of p38MAPK/NF-κB signal pathway related factors. The p38MAPK/NF-κB signal pathway related protein was determined by Western blot. Error bars indicate means ± SD and *indicates significant difference compared with blank group (P<0.05).

(Shanghai, China). GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the PolyvinylideneDifluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using Graphpad statistical software. The *P*-values were calculated using a one-way analysis of variance (ANOVA). A *P*-value of <0.05 was considered to indicate a statistically significant result.

Results

Confirm the expression efficiency of Hsp90 after Hsp90 over-expression and knock-out in AR42J

As shown in **Figure 1**, the Hsp90 protein expression levels of over-expression Hsp90 were significantly increased compared with that of control group (P<0.05). On the contrary, the treatment of sh-Hsp90 significantly decreased the Hsp90 expression levels compared with control group (P<0.05).

Detect viability of AR42J after Hsp90 over-expression and knock-out

We found that when the AR42J cells were treated by LPS, the cells viability of Hsp90 over-expression were significantly increased compared with that of the control group (P<0.05), while shHsp90 significantly decreased the cells viability compared with shNC (P<0.05) (**Figure 2**).

Detect apoptosis of AR42J after Hsp90 over-expression and knock-out

We found that when the AR42J cells were treated by LPS, the cells apoptosis of Hsp90 over-expression were significantly decreased compared with that of the control group (P<0.05), while shHsp90 significantly increased the cells apoptosis compared with shNC (P<0.05) (**Figure 3**).

Detect the expression of inflammatory factors in AR42J after over-expression Hsp90

As shown in **Figure 4**, over-expression Hsp90 could significantly decrease the expression of the inflammatory factors such as IL-1α, IL-6, IL-8, and TNF-α in AR42J cells treated with LPS (P<0.05). This result showed that Hsp90 could significantly inhibit the LPS-induced inflammatory reaction in AR42J.

Detect the expression of signal pathway factors

As shown in **Figure 5**, the expression of p/t-p 38 in the normal cells treated with LPS was increased compared with the pc-Hsp90 group (P<0.05). The expression of pt-p65 and Bcl-3 followed the same trend. These results showed that over-expressed Hsp90 could significantly inhibit the activation of p38MAPK/NF-κB pathway in AR42J cells induced by LPS.

Discussion

The incidence of AP is increasing per year, mainly owing to an increase in biliary pancreatitis [22, 23]. About 20% of patients will develop severe AP, which is associated with a 30% mortality rate, mostly attributed to infection of (peri) pancreatic necrotic tissue and infectious complications [24]. These infections are considered to be the sequelae of a cascade of incidents starting with small-bowel bacterial overgrowth, intestinal barrier failure (IBF), and a proinflammatory response leading to bacterial translocation of intestinal bacteria [25, 26]. In the present study, we demonstrated that Hsp90 could reduce the inflammatory reaction in AR42J.

It is believed that the severity of pancreatitis may be affected by the incident to determine the extent of acinar cell injury. Start early inflammatory response, inflammatory cytokines and chemokines produced by the acinar cells may have the original signal, leading to macrophages and neutrophils and other inflammatory cell infiltration. It has been shown that neutrophil aggregation is an important regulator of serious reactions and systemic factors [27]. Inflammatory cell recruitment and activation of various inflammatory cells cause further damage in acinar cells. Besides, the acinar cells will release a large number of inflammatory cytokines [28], such as tumor necrosis factor (TNF-α), interleukin (IL)-1, IL-2, IL-6, and so on. These inflammatory mediators play an important regulatory role in the pancreatic cell injury.

Many intracellular signal transduction pathway involved in inflammatory response in pancreatitis have been identified, including p38 mitogen-activated protein kinase (P38MAPK), nuclear factor-κB(NF-κB), phospholipids inositol 3 kinase (PI-3K), and activator protein-1 (AP-1). It has been shown that inhibition of these signals may reduce inflammation and improve the severity of pancreatitis.

P38MAPK, primary information linking, plays an important role in inflammatory reaction [29]. p38MAPK can be activated by different stress, which mediates the cell product inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8 [30-32]. AP is a clinical entity which is characterized by the abnormally activation of pancreatin in pancreatic acinar cell and autodigestion of pancreas [33]. Digestive enzymes and lysosomal enzymes were activated, which cause autodigestion of pancreas and activate p38-MAPK signal transduction pathway in pancreatic acinar cell, leading to the release of proinflammatory cytokines [34]. However, the acinar cells will release a large number of inflammatory cytokines. The release of inflammatory mediators and proinflammatory cytokines can activate cytokine cascade reactions and lead to systemic inflammatory reaction syndrome (SIRS), multiple organ dysfunction syndromes (MODS) [19], or even multiple organ failure (MOF). Therefore, effective control the release of inflammatory cytokines can reduce the severity of AP. In the present study, we found that over-expression Hsp90 could significantly down-regulate the expression of the inflammatory factors. This result indicated that Hsp90 could decrease the severity of AP.

In the heat shock protein family Hsp90 is a pleiotropic molecular chaperone and functions as a key protein in the conformational maturation and stability of client proteins [35], which play an important role in cell proliferation, cancer chemoresistance and signaling transduction. The clients of Hsp90 are various, including glucocorticoid receptor (GR) which is a major downstream molecular of the hypothalamus-pituitary-adrenal axis and toll-like receptor 4 signaling pathway related molecules which induce inflammatory reaction. In the present study, we found that over-expression Hsp90 could significantly down-regulate the expression of p38MAPK signal pathway related factors and thus induce inflammatory reaction.

This paper has preliminarily studied the mechanism of Hsp90 inhibiting the inflammatory response in AR42J cell. In the present study, we drew a conclusion that over-expression Hsp90 could protect LPS induced pancreatic inflammation of AR42J cells by inhibition of p38MAPK/NF-κB pathways. It is necessary that further study on the function and mechanism of Hsp90 in SAP. These results will provide the theory basis for the clinical treatment.

Disclosure of conflict of interest

None.

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