

Original Article

Effects of S100A12 reduction on H₂O₂-induced injury of human vascular smooth muscle cells (HVSMCs)

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Abstract: Thoracic aortic dissection is a catastrophic acute aortic disease with a high postoperative mortality. Although TAD results from various risk factors, the final common pathway for its development is tunica media dysfunction with vascular inflammation. The aim of the present study was to investigate the protective effects of S100A12 reduction on hydrogen peroxide (H₂O₂)-induced human vascular smooth muscle cells (HVSMCs) injury and evaluate the relevance of S100A12 and aortic disease. In this study, HVSMCs were exposed to the H₂O₂ in the presence or absence of S100A12, then cell viability was detected by MTT assay, cell apoptosis was performed with the flow cytometry kit, IL-6 and TNF α production evaluated by ELISA and apoptotic proteins were investigated by western blot. The results showed that H₂O₂ inhibited cell proliferation, induced cell apoptosis, IL-6 and TNF α release, the increase of caspase-3 protein and the decrease of Bcl-2, while transfection with S100A12 shRNA significantly repaired the situation above. Our findings suggested that reduction of S100A12 protects HVSMCs against H₂O₂-induced injury, and may be useful as a treatment for aortic disease.

Keywords: Thoracic aortic dissection, S100A12, HVSMCs, H₂O₂

Introduction

Thoracic aortic dissection (TAD) is a highly lethal vascular with a high postoperative mortality. The abnormal aortic middle structure, pathology and blood pressure increase transverse shear stress of vessel wall, and then lead to the spread of intramural hematoma and avulsion of aortic intima, ultimately aortic dissection is forming [1, 2]. The disease was extremely dangerous, if untreated, 33% of patients died within 24 h, 50% died within 48 h and 80% died within a week [3]. TAD is most common in men and the elderly and is estimated to occur at a rate of 3-4 cases per 100,000 persons every year [4]. However, reported rates are probably underestimates of the actual occurrence of TAD because of difficulties in diagnosis. Up to date, few biochemical factors and biomarkers are reported to predict the outcomes of TAD.

S100 is a multigenic family of calcium-modulated proteins and most of S100 members exist in the form of binding target proteins in a cal-

cium-dependent manner to regulate specific steps of signaling pathways and cellular functions [5]. S100 calcium binding protein A12 (S100A12) is one of ligands of receptor for advanced glycation end products (RAGE), and is extensively involved in a series of pathological and physiological processes associated with inflammatory, metabolic and neoplastic diseases [6]. S100A12 has been proven to be useful as diagnostic markers of inflammation especially in non-infectious inflammatory [7]. S100A12 interacts with RAGE to activate downstream of signaling pathways and have been reported to play a vital role in occurrence and development of atherosclerosis and coronary artery diseases [8, 9]. In our previous study, elevated S100A12 level could play a crucial role in systemic inflammation and may be a promising biomarker for predicting cardiovascular events and perioperative complications in patients with TAD [10].

Oxidative stress (OS) such as hydrogen peroxide (H₂O₂) stimulation induces vascular smooth

Reduction of S100A12 protects HVSMCs

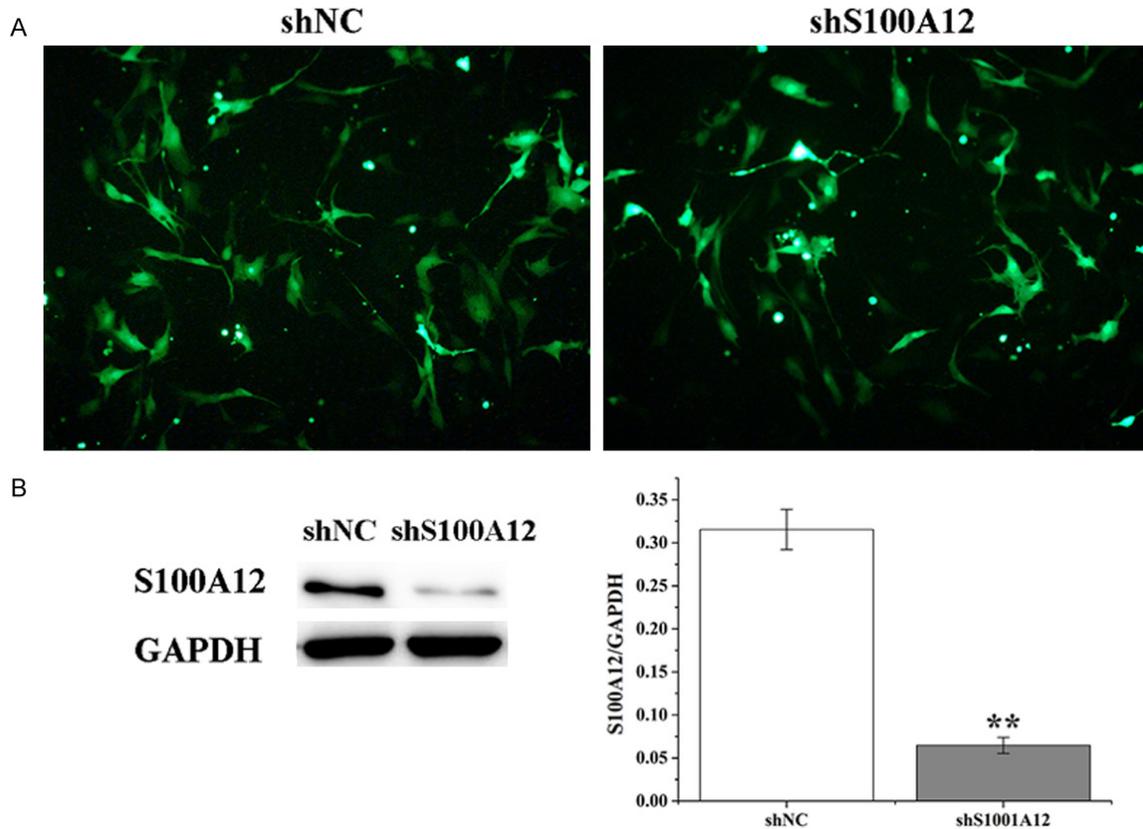


Figure 1. The expression of S100A12 was inhibited by S100A12 interference vector. A: S100A12 interference vector and shNC vector were inserts into HVSMCs and detected by fluorescence microscope. B: The protein levels of S100A12 in HVSMCs. All values are expressed as the means \pm S.E.M (n=3). **P<0.01 as compared with shCN.

muscle cell (VSMC) apoptosis in the atherosclerotic plaque, leading to plaque instability and rupture associated with atherosclerosis and restenosis [11, 12]. Thus, the development of protective strategies for prevention of VSMC apoptosis is a therapeutic target. Our present study aims to investigate the protective effects of S100A12 on H₂O₂-induced human vascular smooth muscle cell (HVSMC) injury and evaluate the association between S100A12 and aortic disease.

Materials and methods

Cell culture

Human vascular smooth muscle cells (HVSMCs) were maintained in F12K medium containing 0.05 mg/ml ascorbic acid, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement (ECGS), 10% fetal bovine serum (FBS), 10 mM HEPES, 10 mM TES, 100 μ g/ml penicillin/streptomycin and 0.25 μ g/ml

fungizone. Cells were kept at 37°C and 5% CO₂ humidified environment.

Construction of interference vector and cell transfection

Negative control pGPU6/GFP/Neo-shNC (target sequence: GTTCTCCGAACGTGTACAGT), and S100A12 interference vector pGPU6/GFP/Neo-shS100A12 (target sequence: CTAAGGGTGAGCTGAAGCAG) were purchased from GenePharma (China). Cells were seeded in 24-well plates and cultured until the cells were grown to 70-80% confluence, and then were transfected with 1 μ g/well of interference plasmid using Lipofectamine 2000 (Invitrogen) as specified by the manufacturer. 100 μ M H₂O₂ was added into cells before transfection of sh-S100A12.

MTT assay

Cell proliferation was measured by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoli-

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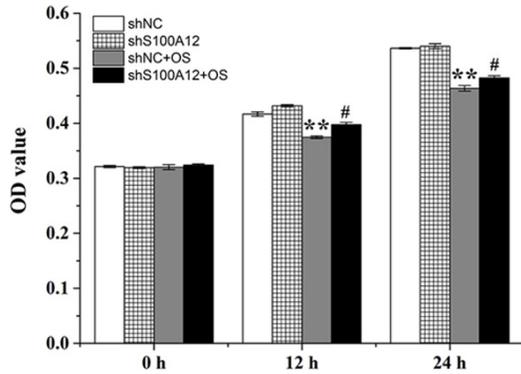


Figure 2. S100A12 reduction increased H_2O_2 -reduced cell proliferation. At 0, 12, 24 h after transfection, the proliferation of cells stimulated with H_2O_2 was lower than that of the shNC group, while the proliferation is higher in shS100A12+OS groups than shNC+OS groups. ** $P < 0.01$ as compared with shNC. # $P < 0.05$ as compared with the shNC+OS group.

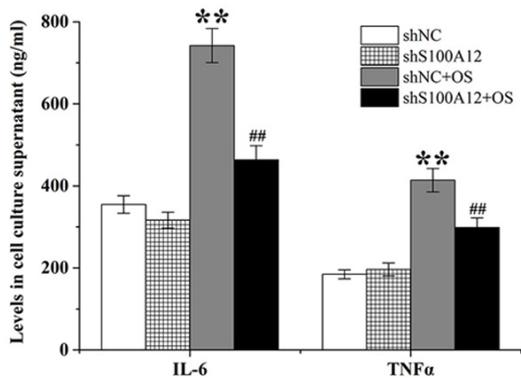


Figure 3. S100A12 inhibited H_2O_2 -induced IL-6 and TNF α production. The release of IL-6 and TNF α was assessed by ELISA. Statistically significant differences (** $P < 0.01$) compared to the shNC group. ## $P < 0.01$ as compared with the shNC+OS group.

um bromide) (Sigma-Aldrich, USA) colorimetric method. Cells were seeded in 96-well plates with 1×10^4 cells in each well. MTT (20 μ l) was added into each well after treatment for 0 h, 12 h and 24 h, respectively, and incubated for 2-4 h at 37°C. After the purple precipitate was visible, the medium were removed and 150 μ l DMSO was added to each well. After shaking at low speed for 10 min, the absorbance was recorded at 570 nm. For each detect, the total procedure was repeated 3 times.

ELISA

The levels of IL-6 and TNF α in cell culture supernatant were quantified by ELISA assay and the

enzyme-linked immunosorbent assay was purchased from Bioswamp (China). The assay was carried out in accordance with the manufacturer's instructions. The optical densities were measured at 450 nm by a microplate reader. The cytokine concentrations were determined using a standard curve established with the appropriate recombinant cytokine, and are expressed in ng/ml.

Assessment of apoptosis

Apoptosis analysis was performed by Annexin-V-FITC/PI (propidium iodide) flow cytometry kit (BD) according to the manufacturer's instructions. Cells were washed twice with ice-cold PBS and resuspend 200 μ l of binding buffer at a concentration of 1×10^6 cells/ml. 10 μ l Annexin V-FITC and 10 μ l PI were added and cells were incubated for 30 min at 4°C in the dark. Finally, 300 μ l binding buffer was added and analyzed by flow cytometry (Beckman Coulter, Cytomics FC 500, CA) within 1 h.

Western blot analysis

Cells were harvested and washed twice with PBS, and the total protein was obtained after cell lysis using lysis buffer [components: 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and sodium orthovanadate sodium fluoride, EDTA and leupeptin (Beyotime Institute of Biotechnology)]. Western blot analysis was performed as previously described [13]. Antibodies against caspase-3, Bcl-2 and β -actin were purchased from Abcam.

Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed using one-way analysis of variance without interaction terms, followed by Dunnett's or Duncan's test for multiple comparisons. Differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

Results

The expression of S100A12 was blocked by S100A12 interference vector

As showing in **Figure 1A**, the S100A12 interference vector and shNC vector was successfully transfected in HVSMCs. The expression level of S100A12 in the shS100A12 group was lower than that of shNC group (**Figure 1B**).

Reduction of S100A12 protects HVSMCs

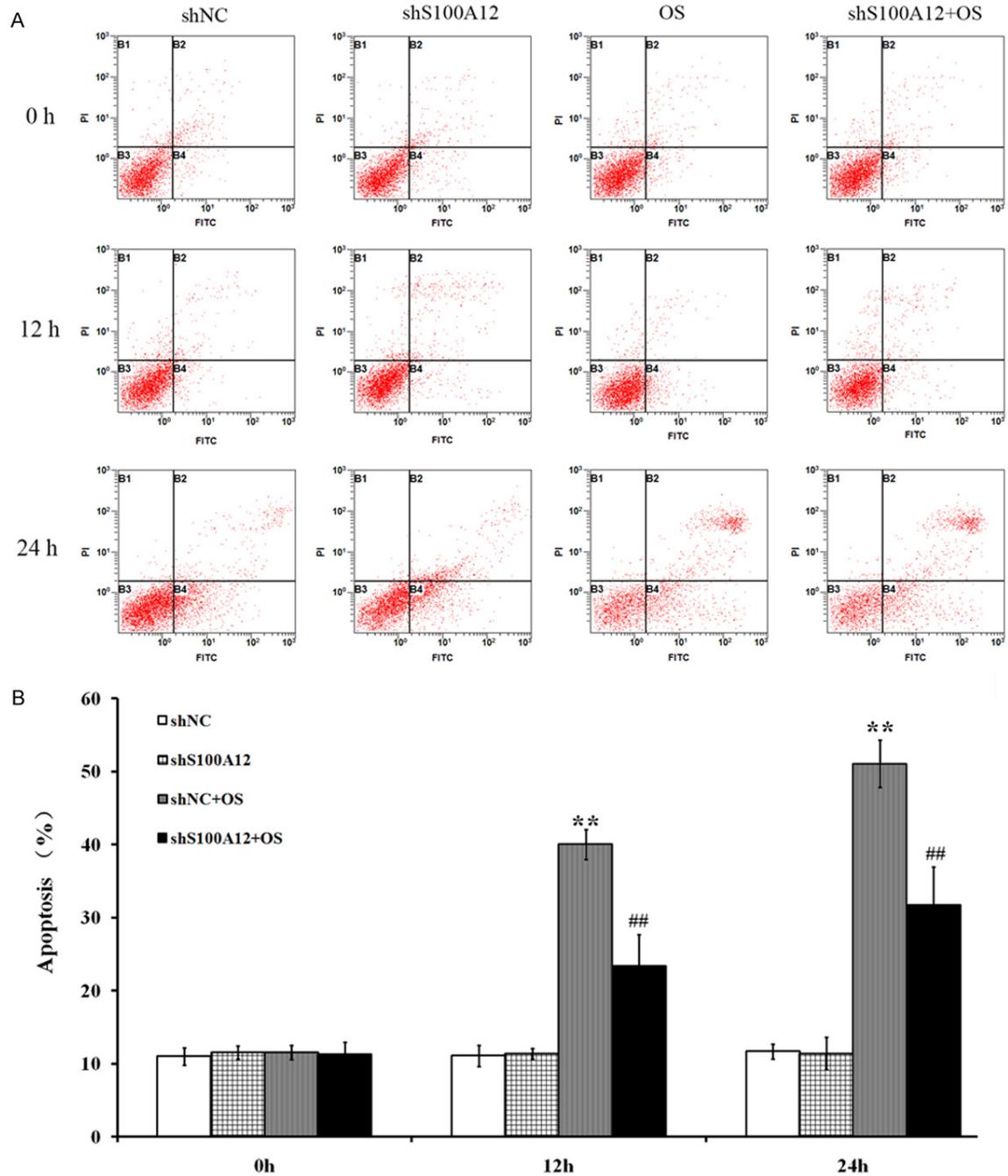


Figure 4. S100A12 inhibited H_2O_2 -induced cell apoptosis. The percentage of apoptotic cells in OS group was higher than shNC group at 12 h and 24 h. The percentage of apoptotic cells in shS100A12+OS group was decreased significantly compared with OS group at 12 h and 24 h. ** $P < 0.01$ as compared with shNC. ## $P < 0.01$ as compared with the shNC+OS group.

The effect of S100A12 on H_2O_2 -induced cell proliferation

To investigate the effect of S100A12 on cell proliferation, MTT assay was performed. Four groups were divided: shNC (transfection of ne-

gative control vector), shS100A12 (transfection of S100A12 interference vector), shNC+OS (transfection of shNC and H_2O_2 stimulation) and shS100A12+OS (transfection of shS100A12 and H_2O_2 stimulation). As shown in **Figure 2**, cells stimulated with H_2O_2 significant-

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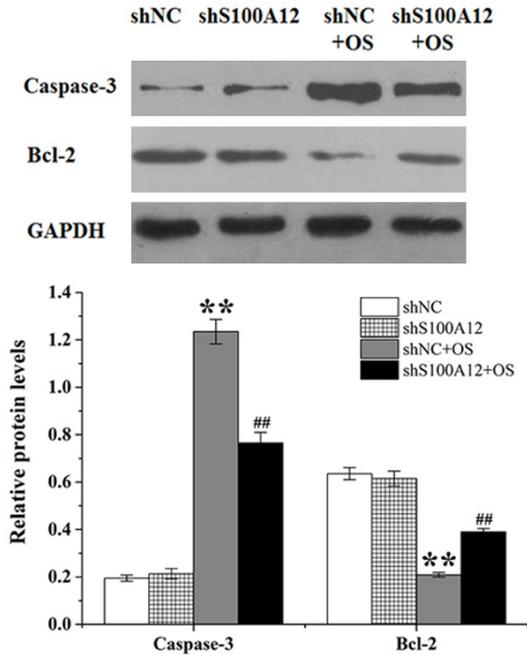


Figure 5. Effect of S100A12 on the expression of apoptotic genes. The expression of caspase-3 and Bcl-2 in HVSMCs was detected by Western blot.

tly decreased the cell growth compared with shNC group ($P<0.01$). Compared to shNC+OS group, the shS100A12+OS group significantly elevated the cell proliferation ($P<0.05$). These results indicated that H_2O_2 inhibit cell growth but reduction of S100A12 could repair H_2O_2 -induced reduction of cell viability.

The effect of S100A12 on H_2O_2 -induced IL-6 and TNF α release

High level of IL-6 and TNF α induced inflammatory response apoptosis in cells. ELISA was performed to examine the concentration of IL-6 and TNF α in HVSMC culture supernatant. As shown in **Figure 3**, compared with the shNC group, the IL-6 and TNF α levels significantly increased in shNC+OS group ($P<0.01$). S100A12+OS group significantly reduced IL-6 and TNF α production compared as that of shNC+OS group ($P<0.01$). These results indicated that H_2O_2 induced cell inflammation and toxicity but S100A12 reduction protect the H_2O_2 -induced injury of HVSMCs.

The effect of S100A12 on H_2O_2 -induced cell apoptosis

H_2O_2 stimulation could induce VSMC apoptosis, but the effect of S100A12 on H_2O_2 -induced

cell apoptosis is still unknown. Cell apoptosis was measured by Annexin V-FITC/PI flow cytometry. As shown in **Figure 4**, cells stimulated with H_2O_2 markedly induced cell apoptosis ($P<0.01$). Compared with shNC+OS group, S100A12+OS group significantly decreased the OS-induced HVSMCs apoptosis. These results indicated that S100A12 reduction could protect from H_2O_2 -induced cell death.

The effect of S100A12 on H_2O_2 -induced apoptosis genes

Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family and it is the most important nucleic acid-cleaving enzyme of the apoptosis pathways. Bcl-2 is specifically considered an important anti-apoptotic protein. So, the expression of caspase-3 and Bcl-2 was used to evaluate H_2O_2 -induced apoptosis in HVSMCs in this study. As shown in **Figure 5**, the protein level of cleaved caspase-3 was increased by H_2O_2 stimulation but Bcl-2 decreased. The S100A12+OS group exhibits lower level of caspase-3 but higher level of Bcl-2 than that of shNC+OS group. These results indicated that H_2O_2 induced apoptosis of HVSMCs while S100A12 reduction repaired it.

Discussion

TAD is a fatal vascular disease which remains a serious challenge, and includes a broad range of degenerative, genetic, structural, and acquired disease states [2]. Various risk factors are associated with TAD occurrence, but tunica media dysfunction with vascular inflammation is the final common pathway for dissection formation [14]. In our previous study, we found that the level of S100A12 in patients with aortic dissection before aortic arch replacement was associated with the incidence of postoperative complications and hospital stay [10]. S100A12 is an acalcium-, zinc- and copper-binding protein which plays a prominent role in the regulation of inflammatory processes and immune responses [15]. S100A12 is thought to activate pathogenic pathways through the modulation of oxidative stress, inflammation and vascular remodeling, whereas anti-inflammatory soluble RAGE may protect against vascular disease [16]. The focus of this study was to verify the influence of S100A12 on aortic disease, and find an effective

way to reduce S100A12-mediated accelerated inflammation.

There is evidence showing that S100A12 is highly expressed in the medial layer in acute type A aortic dissection and S100A12 expression in aortic smooth muscle may be a common response to multifactorial injury [17]. To better understand the specific effects of S100A12 on inflammatory responses, H₂O₂ was used in this study to produce oxygen stress in cells. In this study, oxidative stress was enhanced by H₂O₂, and reduction of S100A12 repaired the cell injury. The findings showed that S100A12 was an important factor of oxidative stress, and effectively controlling S100A12 level could reduce oxidative stress responses. Our study showed that reduction of S100A12 increased cell growth and decreased H₂O₂-induced cell apoptosis and apoptotic proteins, indicating the excess of S100A12 could induce apoptosis and cell death. The results are consistent with the previous studies also support the hypothesis that S100A12 is an important regulator of cell survival and cell death [17].

S100A12 was reported to regulate target genes related inflammation, apoptosis and adhesion molecule through cell signaling pathway [18]. However, the precise mechanisms underlying effect of S100A12 on signaling transduction in TAD still need further research. In summary, our data demonstrate that S100A12 reduction protect HVSMCs against H₂O₂-induced injury and identify S100A12 may be a potential therapeutic target for aortic disease.

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Disclosure of conflict of interest

None.

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