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Selenoprotein S Is Highly Expressed in the Blood Vessels and Prevents Vascular Smooth Muscle Cells From Apoptosis

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ABSTRACT

Atherosclerosis and related cardiovascular diseases (CVD) represent one of the greatest threats to human health worldwide. The protection of vascular smooth muscle cells (VSMCs) from apoptosis in the plaque has become an important therapeutic target for atherosclerotic plaque stabilization. A significant association of selenoprotein S (SelS) gene polymorphism with atherosclerotic CVD has been reported in epidemiologic studies, but the underlying mechanism remains unknown. In this paper, SelS expression in the thoracic aorta and its role in the protection of VSMCs from apoptosis have been studied. Western blot analysis showed that SelS was highly expressed in rat thoracic aorta. SelS gene silence by small interference RNA (siRNA) rendered VSMCs more sensitive to hydrogen peroxide- or tunicamycin- induced injury and apoptosis, as determined by MTT assay, Hoechst staining, and annexin V/propidium iodide staining. SelS silence aggravated hydrogen peroxide-induced oxidative stress and phosphorylation of p38 MAPK and c-Jun N-terminal kinase (JNK) in VSMCs. Furthermore, SelS silence enhanced endoplasmic reticulum (ER) stress induced by hydrogen peroxide or tunicamycin, as showed by the increased protein levels of ER chaperone 78 kDa glucose-regulated protein (GRP78), ER stress transducer phosphorylated protein kinase RNA like ER kinase (PERK), and the proapoptotic transcription factor C/EBP homologous protein (CHOP). In conclusion, the present study suggested that SelS highly expressed in the blood vessel might protect VSMCs from apoptosis by inhibiting oxidative stress and ER stress. Our finding provided mechanistic insights for the potential preventive role of SelS in atherosclerotic CVD. J. Cell. Biochem. 117: 106–117, 2016.

KEY WORDS: SELENOPROTEIN S; VASCULAR SMOOTH MUSCLE CELLS; APOPTOSIS; OXIDATIVE STRESS; ENDOPLASMIC RETICULUM STRESS

Atherosclerosis and related cardiovascular diseases (CVD) represent one of the greatest threats to human health worldwide. As they constitute the vessel wall structure and are the major cellular components in the maintenance of vascular tone, vascular smooth muscle cells (VSMCs) are important in the progression of atherosclerosis. Particularly in the advanced atherosclerotic lesions, VSMC apoptosis induces destabilization and rupture of atherosclerotic plaques [Clarke et al., 2006; Tabas, 2010]. Plaque rupture often leads to thrombosis with clinical manifestations of myocardial infarction or stroke. Oxidative stress and endoplasmic reticulum (ER) stress, two intracellular stresses that have been implicated in the pathogenesis of atherosclerosis [Hulsmans and Holvoet, 2010; Tabas, 2010], can induce VSMC apoptosis [Li et al., 1997; Okura et al., 2000; Stocker and Keaney, 2004; Brunt et al., 2006; Duan et al., 2013; Liu et al., 2014; Spitler and Webb, 2014]. Among several intracellular signaling pathways involved in oxidative stress responses, the mitogen-activated protein kinase (MAPK) cascade is one of the most important signaling pathways. MAPKs are a family of serine/threonine kinases, composed of extracellular-regulated kinase (ERK), p38 MAPK, and JNK. Among them, p38 MAPK and JNK are typically activated by oxidative stress, and play pivotal roles in regulating oxidative stress-induced cell death [Runchel et al., 2011].

The physiological roles of selenium (Se) as an essential micronutrient occur mainly through the function of selenoproteins, which incorporate selenium in the form of one or more selenocysteine residues [Hatfield et al., 2014]. In humans, 25 selenoproteins have been identified, many of which have unknown functions [Kryukov et al., 2003]. Known functions of selenoproteins include antioxidant protection and cellular redox balance (attributed to glutathione

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peroxidases (GPx), thioredoxin reductases, and methionine sulfoxide reductases), thyroid hormone metabolism (catalyzed by iodothyronine deiodinases), and selenium transport to peripheral tissues (through plasma selenoprotein P).

Selenoprotein S (SelS, also known as SEPS1, VIMP, Tanis, and SELENOS) is localized to the endoplasmic reticulum (ER) membrane and also enriched at perinuclear speckles [Bubenik et al., 2013]. Human SelS is a single-spanning membrane protein (21 kDa, 189 amino acids) with a short N-terminal ER-luminal tail and a longer cytosolic Sec-containing tail from amino acids 49–189. The Sec residue is the 188th amino acid [Christensen et al., 2012]. Expression of SelS protein has so far been detected in astrocytes and neurons as well as macrophage, pancreatic β-cell, hepatocarcinoma cell lines, and intestinal epithelial cells [Gao et al., 2004; Kim et al., 2007; Fradejas et al., 2008, 2011; Du et al., 2010; Speckmann et al., 2014]. SelS was identified as an essential component of ER-associated protein degradation (ERAD) complex, together with selenoprotein K, p97 ATPase, and Derlins [Shchedrina et al., 2011; Lee et al., 2014; Turanov et al., 2014]. This complex mediates the retrotranslocation of unfolded and misfolded proteins from the ER lumen to cytosol for degradation. The function of SelS in ERAD plays a critical role in neutralizing ER stress and cell survival. Up-regulation of SelS was observed in many cells under ER stress conditions: glucose deprivation and treatments with N-glycosylation inhibitor tunicamycin, Ca^{2+}-ATPase blocker thapsigargin, and reducing agent β-mercaptoethanol [Gao et al., 2004; Kim et al., 2007; Fradejas et al., 2008, 2011; Du et al., 2010; Lee et al., 2014; Speckmann et al., 2014]. SelS protected these cells from apoptosis induced by ER stress [Kim et al., 2007; Fradejas et al., 2008; Du et al., 2010; Lee et al., 2014]. Furthermore, SelS participates in intracellular membrane transport and maintenance of protein complexes by anchoring them to the ER membrane [Turanov et al., 2014]. In addition, SelS was proposed to function as an efficient reductase as well as a moderate peroxidase in vitro [Christensen et al., 2012; Liu and Rozovsky, 2013; Liu et al., 2013]. SelS overexpression protected Min6 pancreatic β-cells from H_2O_2-induced cell death [Gao et al., 2004]. However, very recently, SelS has been reported to be a marker but not a regulator of ER stress in intestinal epithelial cells. Depletion of SelS in intestinal cells line did not cause or modulate ER stress and had no effect on H_2O_2-induced cell death [Speckmann et al., 2014].

The effect of genetic polymorphisms in SelS on the risk of CVD was first investigated in two independent prospective Finnish cohorts by Alanne et al. [2007]. They found a significant association of SelS gene polymorphism with coronary heart disease and ischemic stroke. Recently, Cox et al. [2013] reported the association of SelS polymorphism with subclinical atherosclerotic CVD in individuals with type 2 diabetes mellitus. These results suggest a potential role for SelS in the prevention of CVD. However, the mechanisms underpinning these relationships remain unknown. To our best knowledge, SelS expression and its functions in the vessel wall have not been studied before. In this paper, we found that SelS was highly expressed in rat thoracic aorta. Considering that SelS has been reported to protect many other types of cells from apoptosis induced by ER stress [Kim et al., 2007; Fradejas et al., 2008; Du et al., 2010; Lee et al., 2014] or oxidative stress [Gao et al., 2004], we examined the effect of SelS on VSMC apoptosis induced by oxidative stress or ER stress. Our results indicated that SelS protected VSMCs from apoptosis by inhibiting oxidative stress, p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways, and ER stress. Our findings provide important mechanistic insights into the potential role of SelS in the prevention of CVD.

MATERIALS AND METHODS

MATERIALS

Dulbecco modified Eagle's medium (DMEM), newborn calf serum, Lipofectamine 2000 and Trizol were purchased from Invitrogen (Carlsbad, CA). Tunicamycin, Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, and 2,7-dichlorofluorescein diacetate (2,7-DCFH-DA) were obtained from Sigma–Aldrich (St. Louis, MO). M-MLV reverse transcriptase and SYBR Green PCR Master Mix kit were purchased from Toyobo (Osaka, Japan). SelS-specific polyclonal antibody was purchased from Abcam (Hong Kong) Ltd. Antibodies against C/EBP homologous protein (CHOP) and β-actin were obtained from Santa Cruz Technology (Santa Cruz, CA). Antibodies against phosphorylated p38 MAPK and JNK, p38 MAPK, JNK, 78 kDa glucose-regulated protein (GRP78), and phosphorylated protein kinase RNA (PKR)-like endoplasmic reticulum kinase (PERK) were obtained from Cell Signaling Technology (Beverly, MA). All secondary antibodies were purchased from Thermo Scientific (Rockfora, IL). The enhanced chemiluminescence (ECL) kit and PVDF membrane were purchased from Millipore (Billerica, MA). Total superoxide dismutase (SOD) activity assay kit, GPx activity assay kit, and lipid peroxidation assay kit were obtained from Jiancheng BioEngineering, (Nanjing, China). An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Beijing Biosea Biotechnology Co., LTD, China. All other reagents used were of analytical grade.

DETECTION OF SelS PROTEIN LEVELS IN VARIOUS RAT TISSUES

Fourteen tissues were quickly harvested from male SD rats. The procedure was approved by the Animal Ethics Committee of Huazhong University of Science and Technology. Each tissue was immediately washed in 0.9% NaCl and frozen at −80°C. Protein was extracted from each tissue by homogenizing 0.5 g of tissue on ice in lysis buffer (50 mM HEPES, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.4% SDS, protease inhibitor cocktail, 1 mM Na_3VO_4, and 1 mM NaF). Homogenate was centrifuged at 12,000 rpm for 30 min at 4°C and supernatant were removed and stored at −80°C. The total protein concentration of the supernatants was determined with the Lowry method [Lowry et al., 1951] and 20 μg total protein in each tissue was used to detect SelS protein level by Western blot analysis.

CELL CULTURE

Primary VSMCs used in the present studies were isolated from the thoracic aortas of male SD rats and identified as described by Smith and Brock [1983]. The procedure was approved by the Animal Ethics Committee of Huazhong University of Science and Technology. We cultured VSMCs in DMEM with 20% newborn calf serum and antibiotic solution at 37°C in 5% CO_2. Cells between passages three and seven were used for all experiments.

SMALL INTERFERENCE RNA (siRNA) TREATMENT

siRNAs to target rat SelS (GeneBank number NM_173120.2) were designed and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The effective sequence was sense 5′-GCGUC-CUUCUCUACAUUGUTT-3′, and antisense 5′-ACAAUGUAGA-GAAGGACGC TT-3′. A scramble siRNA served as a negative control (NC) was from RiboBio (Guangzhou, China). VSMCs were transfected with 50 nM SelS siRNA or control siRNA using Lipofectamine 2000. To evaluate the efficiency of SelS gene silence, real-time polymerase chain reaction (PCR) was used to quantify SelS mRNA level after transfection for 24 h and Western blot was used to analyze SelS protein level after transfection for 48 h, respectively. For cell treatment, after transfection for 24 h, the cells were treated with or without indicated concentrations of H_2O_2 or tunicamycin for indicated time and then harvested for analysis.

QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from VSMCs using Trizol and reverse-transcribed into cDNA. SYBR Green-based real-time PCR was performed on a DNA Engine Opticon 2 (MJ Research, Boston, MA) using the SYBR Green PCR Master Mix kit, according to the vendor's protocol. Quantification was performed using the $\Delta\Delta C_T$ method [Livak and Schmittgen, 2001] with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as internal control. The forward and reverse PCR primers (rat) were for SelS, 5′-CATCATCTGGTGGAT-GAAGC-3′ and 5′-GTGAAAGTGTGCGTAAGGCAATT-3′; and for GAPDH, 5′-CCATCCACAGTCTTCT GA-3′ and 5′-CACCAC CATG-GAGAAGGC-3′.

ANALYSIS OF CELL VIABILITY

Cell viability was measured quantitatively with the MTT reduction assay, showed the activity of living cells [Denizot and Lang, 1986]. Briefly, VSMCs after treatment were incubated with 0.5 mg/ml MTT for 4 h at 37°C. After that, the medium was carefully removed, and the formed formazan products were dissolved with dimethyl sulfoxide. The absorbance of the resulting solution was measured at 570 nm using a UV-1200 spectrophotometer (Mapada, Shanghai, China). The cell viability data from treated cells are expressed as percentages of the control cells.

APOPTOSIS DETECTION

For detection of apoptosis, Hoechst 33342 staining and FITC-labeled annexin V/propidium iodide (PI) double-staining were employed to visualize the nuclear morphology and quantify the percentage of apoptotic cells, respectively. After they were washed three times with PBS, VSMCs were stained with 5 μg/ml Hoechst 33342 in PBS at 37°C for 30 min. The nuclear morphology was visualized using a fluorescence microscope (Olympus IX71, Olympus Optical Co., Ltd, Tokyo, Japan). Cells with condensed or fragmented nuclei were defined as apoptotic cells. At least 200 cells were counted for each treatment. Next, FITC-labeled annexin V/PI double-staining was performed using a commercially available annexin V-FITC apoptosis detection kit. The percentage of apoptotic cells was then analyzed with bivariate flow cytometry (FC500, Beckman Coulter).

WESTERN BLOT ANALYSIS

Whole cell extracts from VSMCs were prepared in lysis buffer (50 mM HEPES, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.4% SDS, protease inhibitor cocktail, 1 mM Na_3VO_4, and 1 mM NaF). After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatants were collected for Western blot analysis. The total protein concentration of the supernatants was determined with the Lowry method [Lowry et al., 1951]. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrically transferred to a PVDF membrane. Non-specific binding of the membrane was blocked by using 5% bovine serum albumin for 1 h at room temperature. The membranes were incubated with the indicated primary antibody overnight at 4°C, and then horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The blots were visualized using an ECL detection system. Relative levels of target proteins were quantified using Quantity One software (Bio-Rad Laboratories).

MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

The level of intracellular ROS in VSMCs was assayed using the fluorescence probe 2,7-DCFH-DA as previously described [Liu et al., 2011]. Briefly, VSMCs cultured in Petri dish after treatment were loaded with 5 μg/ml 2,7-DCFHDA for 20 min at room temperature in modified Kreb's Ringer buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 6 mM glucose, 1.2 mM $MgSO_4$, and 1.0 mM $CaCl_2$). After that, excess 2,7-DCFH-DA was carefully removed, and the cells were washed three times with modified Kreb's Ringer buffer. The cells were photographed with a fluorescence microscope (Olympus IX71, Olympus Optical Co., Ltd, Tokyo, Japan). The level of intracellular ROS in VSMCs was quantified by measurement of fluorescence intensity of at least eight fields per dish using NIH ImagingJ software. The data were from a representative of three experiments shown as the mean ± SD of fluorescence intensity.

DETERMINATION OF CELLULAR LIPID PEROXIDATION, SOD ACTIVITY, AND GPx ACTIVITY

Malondialdehyde (MDA) was measured in VSMCs using a commercial assay kit and is expressed as nanomoles per milligram protein. Cellular total SOD activity and GPx activity were measured using commercial assay kits. One unit of GPx activity was defined as a decrease of 1 μmol/min in the glutathione (GSH) concentration after the decrease in the GSH concentration of the non-enzymatic reaction was subtracted. One unit of SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of reduction of nitroblue tetrazolium. SOD activity and GPx activity are expressed in units per milligram protein.

STATISTICAL ANALYSIS

Results from a representative of at least three independent experiments are shown as the mean ± SD. Data analysis was performed by one-way ANOVA. For comparison of two groups, Student's *t*-test was used. A value of $P < 0.05$ was considered statistically significant.

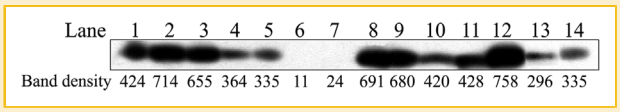

Fig. 1. The protein levels of SelS in different tissues of rat. Western blot analysis was performed using 20 μg total protein of each tissue. The band density was quantified with Quantity one software. One blot representative of three experiments was present. Lane 1, skeletal muscle; lane 2, lung; lane 3, liver; lane 4, cerebellum; lane 5, cerebrum; lane 6, kidney; lane 7, eye; lane 8, adipose tissue; lane 9, thoracic aorta; lane 10, spleen; lane 11, stomach; lane 12, thymus gland; lane 13, pancreas; lane 14, heart.

RESULTS

SelS PROTEIN EXPRESSION IN VARIOUS RAT TISSUES

Western blot analysis of 14 different rat tissues (skeletal muscle, lung, liver, cerebellum, cerebrum, kidney, eye, adipose tissue, thoracic aorta, spleen, stomach, thymus gland, pancreas, and heart) displayed ubiquitous SelS protein distribution, with particularly high levels in thymus gland, lung, adipose tissue, thoracic aorta, and liver (Fig. 1). The high expression of SelS in the thoracic aorta means that SelS must play an important role in maintaining the normal function of blood vessels. Since the total anatomical volume taken up by VSMCs in the vessel wall is much greater than that of endothelial cells, SelS function in VSMCs was investigated in the present paper. We studied whether SelS influences the cells' survival when challenged with oxidative stress or ER stress, two intracellular stresses that have been implicated in the pathogenesis of atherosclerosis [Hulsmans and Holvoet, 2010; Tabas, 2010].

SelS SILENCE EXACERBATED H_2O_2-INDUCED APOPTOSIS IN VSMCs

To assess SelS function in rat VSMCs, one specific siRNA was used to silence SelS gene. After SelS siRNA transfection into VSMCs for 24 h, the mRNA level of SelS was suppressed approximately by 77%, compared with negative control (Fig. 2A). Also, the protein level of SelS was efficiently reduced by SelS siRNA in VSMCs after 48 h (Fig. 2B). The result showed that SelS gene silence was successful. Then, these cells were challenged with H_2O_2 (range 0–0.5 mM) for 24 h or 48 h to assess SelS function. H_2O_2 at relatively lower pathophysiological concentrations (nano- to micromolar range) plays important signaling roles in cells. However, relatively high concentrations of H_2O_2 was apoptotic for VSMCs [Li et al., 1997; Okura et al., 2000; Stocker and Keaney, 2004; Brunt et al., 2006; Duan et al., 2013; Liu et al., 2014; Spitler and Webb, 2014]. Therefore, 0.2 and 0.5 mM H_2O_2 were used in this work.

Cell viability was first measured using the MTT assay. As shown in Fig. 2C and D, H_2O_2 caused a concentration- and time-dependent reduction in VSMC viability. SelS silence had no significant effect on cell viability, however, rendered cells more susceptible to H_2O_2-induced injury (Fig. 2C,D). After treatment with 0.5 mM H_2O_2 for 24 h, the cell viability in control cells was 84.3%, while the cell viability in SelS silence cells was 52.2% (Fig. 2C). Furthermore, the viabilities of control cells treated with 0.2 and 0.5 mM H_2O_2 for 48 h

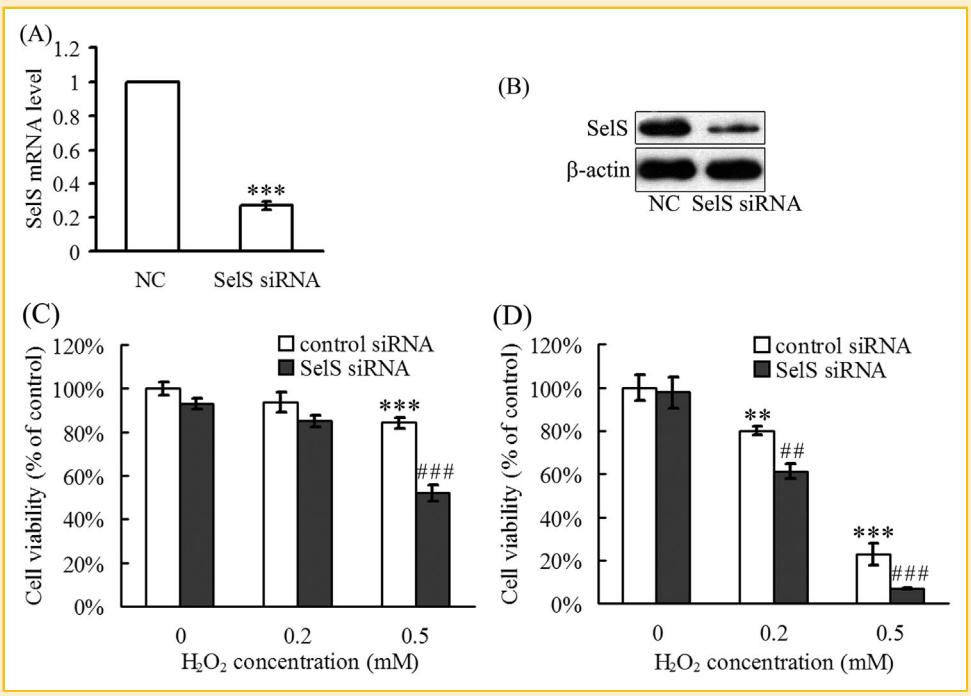

Fig. 2. SelS silence enhanced H_2O_2-induced cell death in VSMCs. (A) VSMCs were transfected with 50 nM SelS siRNA or control siRNA, then SelS mRNA expression was quantified with real-time PCR after 24 h (means ± SD, n = 3) and (B) SelS protein level was determined by Western blot analysis after 48 h (blot representative of four experiments). NC: negative control. (C,D) VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h, and then treated with indicated concentrations of H_2O_2 for another 24 h (C) or 48 h (D). The cell viability was determined by MTT assay (means ± SD, n = 4). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control cells; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the control cells treated with H_2O_2.

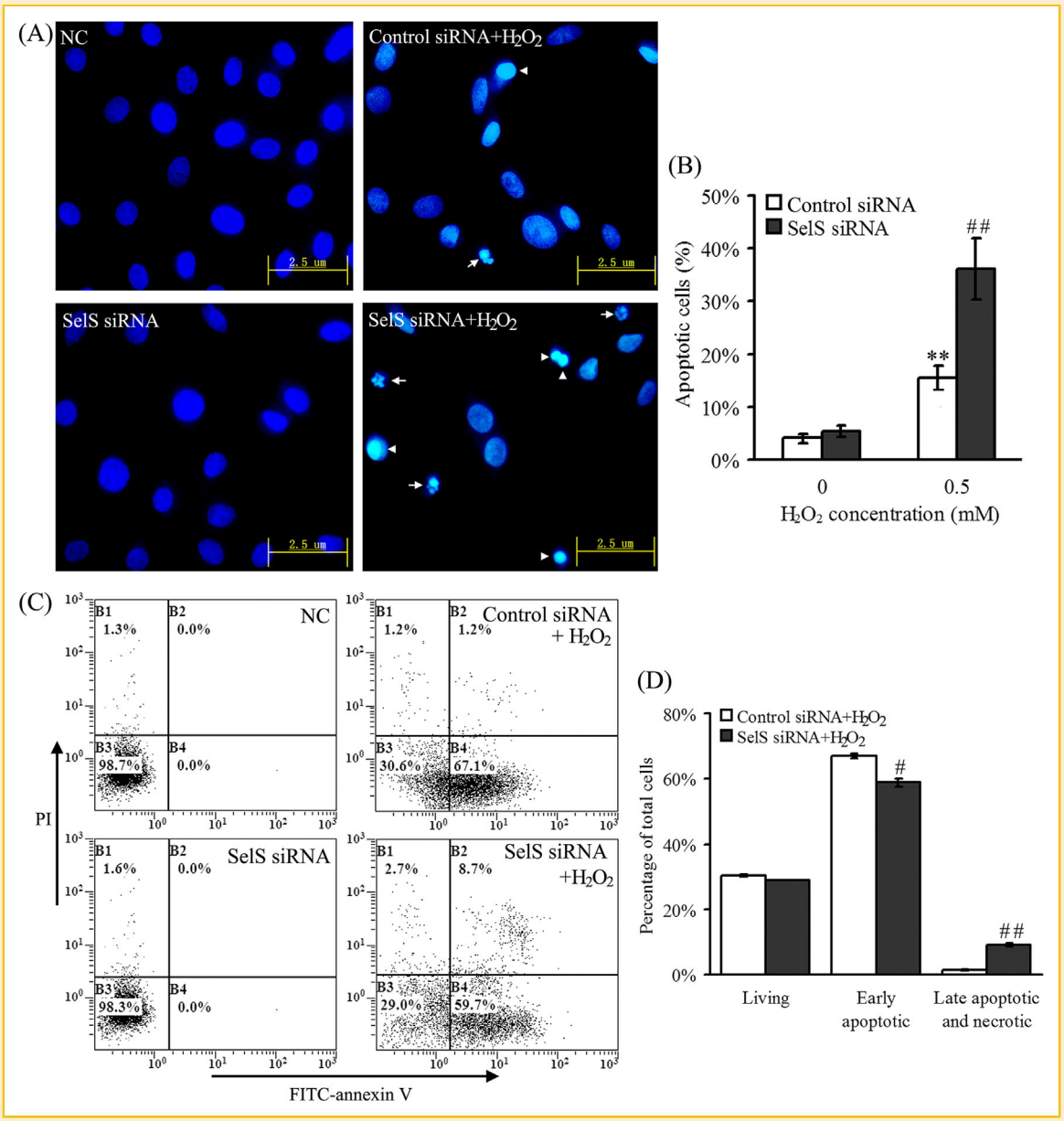

Fig. 3. SelS silence promoted H_2O_2-induced apoptosis in VSMCs. (A) VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then incubated in fresh media with or without 0.5 mM H_2O_2 for 24 h. Apoptotic cells with condensed (▶) or fragmented (➔) nuclei were visualized by Hoechst 33342 staining, and (B) the percentage of apoptotic cells was counted independently for each treatment (mean ± SD, n = 3). NC: negative control. (C) VSMCs transfected with SelS or control siRNA were treated with 0.5 mM H_2O_2 for 48 h. The percentages of living, early and late apoptotic cells were measured by flow cytometry after annexin V/PI double-staining. Early apoptotic cells appear in the lower right quadrant, while living cells are in the lower left quadrant, and late apoptotic and necrotic cells are in the upper right region. (D) Quantitative analysis of flow cytometry results (mean ± SD, n = 2). A representative experiment is shown in C. $^{**}P < 0.01$, compared with the control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the control cells treated with H_2O_2.

were 80.1% and 22.8%, respectively, while the viabilities of SelS silence cells treated with 0.2 and 0.5 mM H_2O_2 for 48 h were 61.3% and 6.9%, respectively (Fig. 2D).

Our following experiment indicated that SelS protective function on VSMCs' survival implied a prominent antiapoptotic effect, as determined by Hoechst 33342 staining and FITC-labeled annexin V/PI double staining. Hoechst 33342 staining revealed many apoptotic nuclei (characteristically condensed or fragmented nuclei) in control cells treated with 0.5 mM H_2O_2 for 24 h, and this response was exacerbated in SelS silence cells treated with H_2O_2 (Fig. 3A,B). Few apoptotic nuclei were visualized in SelS silence cells without H_2O_2, compared with the control cells (Fig. 3A,B). Furthermore, the percentage of apoptotic cells was quantified by flow cytometric analysis after FITC-labeled annexin V/PI double staining. SelS silence without H_2O_2 had no significant effect on VSMCs apoptosis. The proportions of early apoptotic cells and late apoptotic cells were

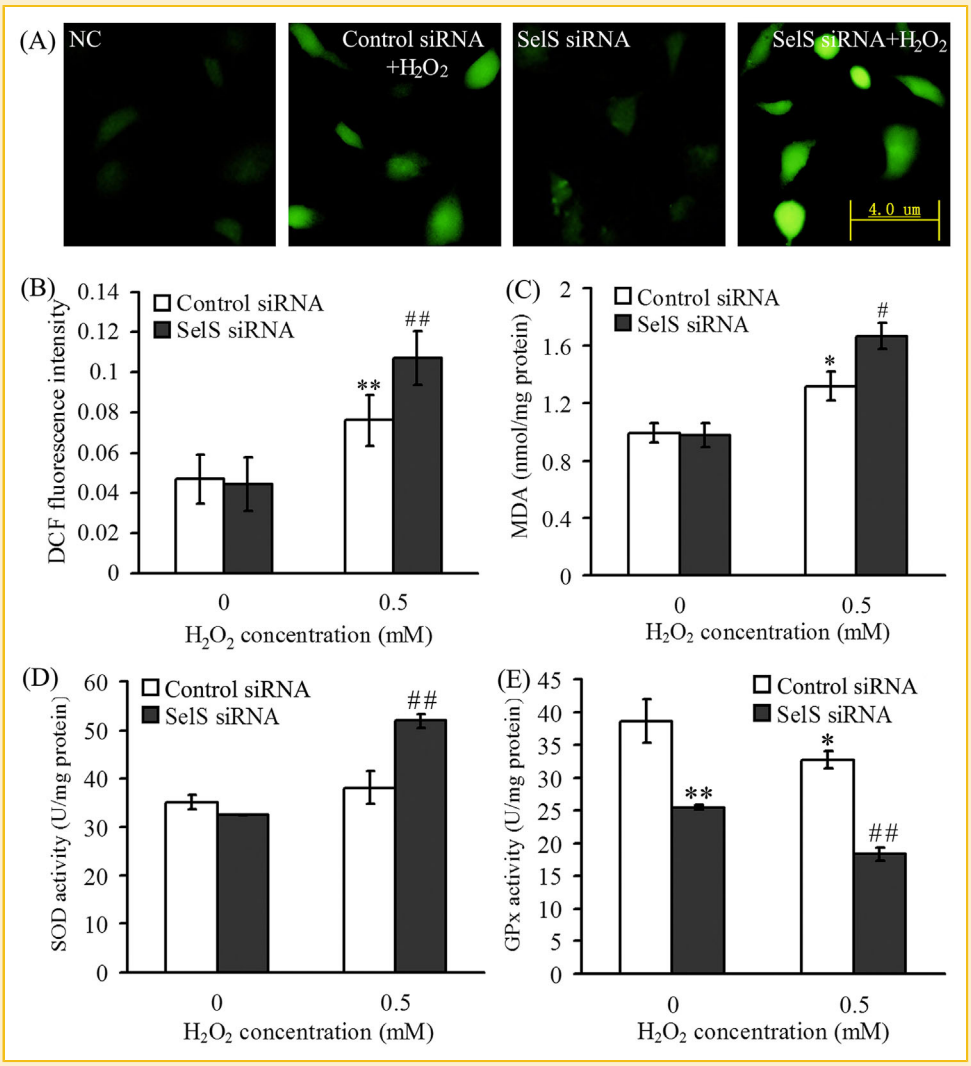

Fig. 4. Effects of SelS silence on H_2O_2-induced oxidative stress in VSMCs. VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then incubated in fresh media with or without 0.5 mM H_2O_2 for 24 h. (A) The level of intracellular ROS was visualized by DCF fluorescence and (B) quantified with NIH ImagingJ software (mean ± SD, n = 6). NC: negative control. (C–E) MDA content (C), SOD activity (D), and GPx activity (E) were measured with commercial kits, respectively (mean ± SD, n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the control cells treated with H_2O_2.

67.1% and 1.3%, respectively, in control cells treated with 0.5 mM H_2O_2 for 48 h. However, in SelS silence cells treated with H_2O_2, the proportions of early apoptotic cells and late apoptotic cells were 58.8% and 9.1%, respectively (Fig. 3C,D). Taken together, the above data indicate that SelS plays an important role in protecting VSMCs from H_2O_2-induced apoptosis.

SelS SILENCE AUGMENTED H_2O_2-INDUCED OXIDATIVE STRESS IN VSMCs

Oxidative stress was first assessed by examining the contents of intracellular ROS and MDA. MDA is an end product of lipid peroxidation and was often used to estimate the level of lipid peroxidation in cells. The levels of intracellular ROS (Fig. 4A,B) and MDA (Fig. 4C) were both significantly increased in control cells treated with 0.5 mM H_2O_2 for 24 h, compared with untreated control cells. These responses were further exacerbated in SelS silence cells treated with H_2O_2 (Fig. 4A–C), suggesting that SelS could inhibit H_2O_2-induced oxidative stress in VSMCs. We further measured the activities of SOD and GPx, two antioxidant enzymes which can regulate the metabolism of H_2O_2. The activity of SOD was unaffected by H_2O_2 (0.5 mM, 24 h), but markedly enhanced in SelS silence cells treated with H_2O_2 (Fig. 4D). In contrast, the activity of GPx was significantly inhibited by H_2O_2 (0.5 mM, 24 h) or SelS silence, and this response was aggravated in SelS silence cells treated with H_2O_2 (Fig. 4E). In addition, the GPx activity was significantly decreased in SelS siRNA-treated cells without H_2O_2 (Fig. 4E), compared with the control cells. However, real-time PCR data showed that the mRNA level of cellular GPx was not changed in SelS siRNA-treated cells without H_2O_2 (data not shown).

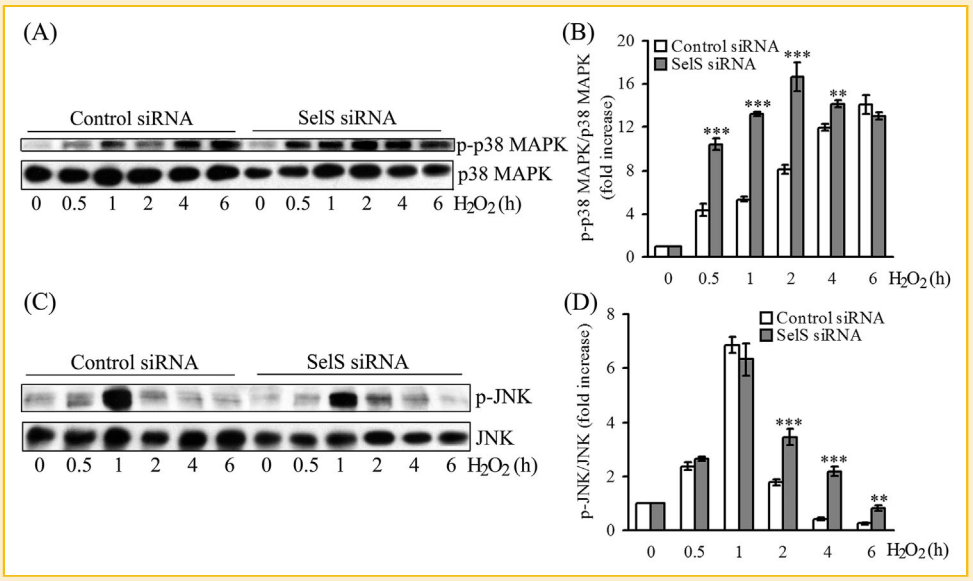

Fig. 5. Effects of SelS silence on H_2O_2-induced phosphorylation of p38 MAPK and JNK in VSMCs. VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then were either left untreated or treated with 0.5 mM H_2O_2 for indicated hours. The phosphorylated p38 MAPK (p-p38 MAPK) (A) or JNK (p-JNK) (C) was determined by Western blot with either anti-p-p38 MAPK or anti-p-JNK antibodies. Semi-quantification analysis (mean ± SD, n = 2) of p-p38 MAPK (B) and p-JNK (D) normalized to p38 MAPK and JNK, respectively. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the respective control cells treated with H_2O_2.

EFFECTS OF SelS SILENCE ON H_2O_2-INDUCED PHOSPHORYLATION OF p38 MAPK AND JNK IN VSMCs

Because the activation of p38 MAPK and JNK have been implicated in oxidative stress-induced apoptosis of VSMCs [Tsujimoto et al., 2006; Li et al., 2008], we examined the activation of these kinases in control and SelS silence VSMCs treated with H_2O_2 (Fig. 5). Western blot analysis revealed that the level of phosphorylated p38 MAPK was increased after exposing control cells to 0.5 mM H_2O_2 for 0.5 h, and continued to be elevated after exposure to H_2O_2 for 6 h. By contrast, in SelS silence VSMCs, the level of phosphorylated p38 MAPK was markedly increased after exposure to H_2O_2 for 0.5 h, and maximized after 2 h (Fig. 5A). The level of phosphorylated p38 MAPK was significantly higher in SelS silence VSMCs than in control cells after exposure to H_2O_2 for 0.5, 1, 2, 4 h (Fig. 5B).

The level of phosphorylated JNK was significantly elevated in both control and SelS silence VSMCs after exposure to H_2O_2 for 0.5 h, and maximized after exposure to H_2O_2 for 1 h (Fig. 5C). However, the level of phosphorylated JNK was significantly higher in SelS silence VSMCs than in control cells after exposure to H_2O_2 for 2, 4, 6 h (Fig. 5D).

SelS SILENCE ENHANCED H_2O_2-ACTIVATED ER STRESS IN VSMCs

GRP78 is a major ER chaperone protein. PERK is a major transducer of the ER stress response, and activation of PERK leads to the phosphorylation of the α subunit of eukaryotic initiation factor 2, resulting in translational attenuation. CHOP is a transcriptional factor that mediates ER-initiated apoptotic cell death [Tabas, 2010]. Because ER stress is involved in H_2O_2-induced apoptosis in many other types of cells [Ariyama et al., 2008; Min et al., 2008; Katsoulieris et al., 2010; Wei et al., 2010; Weber et al., 2013], Western blot of selected ER stress markers (GRP78, phosphorylated PERK, and CHOP) was performed to examine whether their protein expression levels were affected by SelS silence with or without H_2O_2. Analysis of SelS expression confirmed its suppression in SelS silence cells. Whether in control cells or in SelS silence cells, an up-regulation of SelS was observed in response to 0.2 mM H_2O_2, while a down-regulation of SelS was observed in response to 0.5 mM H_2O_2 (Fig. 6A, B). GRP78, phosphorylated PERK, and CHOP were increased by H_2O_2 at all concentrations used and their levels were further increased in SelS silence cells treated with H_2O_2 (Fig. 6A,C–E). In addition, significant increases in the protein levels of phosphorylated PERK and CHOP were seen in SelS silence cells without H_2O_2 treatment, compared with the control cells (Fig. 6A,D,E). These results suggested that SelS could attenuate H_2O_2-activated ER stress in VSMCs.

SelS SILENCE AGGRAVATED ER STRESS-INDUCED APOPTOSIS IN VSMCs

To further explore the role of SelS in regulating ER stress, Western blot of GRP78, phosphorylated PERK, and CHOP in VSMCs was performed in control or SelS silence cells treated with tunicamycin, a well known ER stress inducer. Tunicamycin (1–5 μg/ml) concentration-dependently induced SelS expression in control cells, but decreased SelS expression in SelS silence cells (Fig. 7A,B). ER stress was induced by tunicamycin in a concentration-dependent manner, as demonstrated by the significant increase in the protein levels of GRP78, phosphorylated PERK, and CHOP (Fig. 7A,C–E). Compared with the control cells treated with tunicamycin, the protein levels of phosphorylated PERK and CHOP were further enhanced in SelS silence cells treated with tunicamycin (Fig. 7A,D,E). The above results suggested that SelS is not only a marker, but also a regulator of ER stress in VSMCs.

Next, MTT assay was performed to determine whether SelS influences VSMC survival when challenged with ER stress. As shown

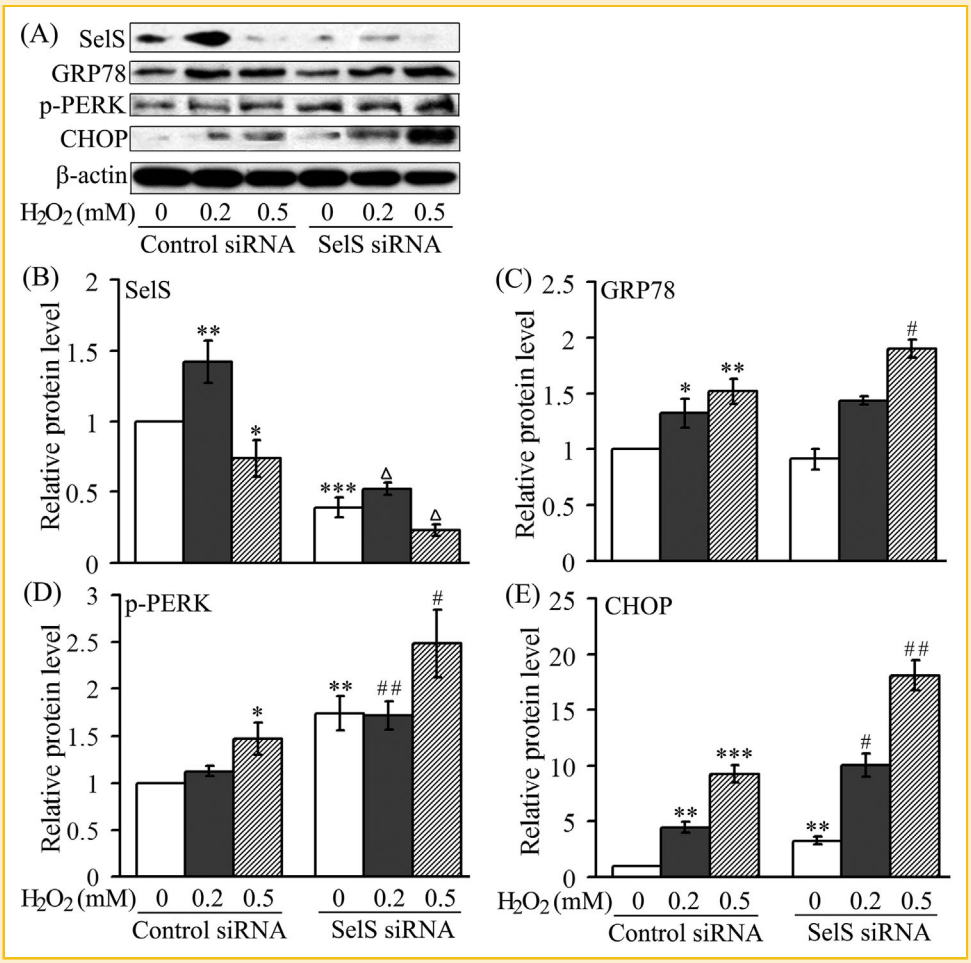

Fig. 6. SelS silence aggravated H_2O_2-induced ER stress in VSMCs. (A) Western blot analysis of protein levels of SelS, GRP78, phosphorylated PERK (p-PERK) and CHOP and (B–E) semi-quantification analysis of protein levels (mean ± SD, n = 3) using β-actin as a control. VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then incubated in fresh media with or without indicated concentrations of H_2O_2 for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the control cells treated with H_2O_2; $^{\Delta}P < 0.05$, compared with SelS silence cells.

in Figure 8A, tunicamycin significantly decreased cell viability in a concentration-dependent manner (1–5 μg/ml). The cell viability was further decreased in SelS silence cells treated with tunicamycin. For example, after treatment with 5 μg/ml Tm for 48 h, the cell viability in control cells was 77.4%, while the cell viability in SelS silence cells was 63.3% (Fig. 2C).

Furthermore, Hoechst staining and annexin V/PI double staining were used to evaluate VSMC apoptosis under SelS silence condition. As determined by Hoechst staining, severe VSMC apoptosis was induced by tunicamycin (5 μg/ml, 48 h) treatment after SelS silence, revealing many condensed or fragmented nuclei (Fig. 8B,C). Flow cytometric analysis after FITC-labeled annexin V/PI double staining showed that the percentage of apoptotic cells was increased 12.8% in SelS silence cells treated with tunicamycin (5 μg/ml, 48 h), compared with that in control cells treated with tunicamycin (Fig. 8D,E).

Taken together, the above results confirmed that SelS silence rendered VSMCs more susceptible to tunicamycin-induced apoptosis, suggesting that SelS could protect VSMCs from ER stress-induced apoptotic death by down-regulating ER stress.

DISCUSSION

A significant association of SelS gene polymorphism with CVD has been reported in three independent epidemiologic studies, suggesting a potential role for SelS in the prevention of atherosclerotic CVD [Alanne et al., 2007; Cox et al., 2013]. However, the underlying mechanisms underpinning this relationship remain unknown. In this paper, we found that SelS was highly expressed in rat thoracic aorta. Furthermore, SelS could protect VSMCs from apoptosis induced by oxidative stress and ER stress. These results provided further support and partial mechanistic explanation for the potential role of SelS in the prevention of atherosclerotic CVD.

Some previous works reported SelS gene expression in the different tissues. Walder et al. [2002] detected for the first time SelS gene expression by Taqman PCR and Northern blots in Israeli sand rat tissues. They found that SelS gene was expressed in a range of tissues, including liver, adipose tissue, hypothalamus, and skeletal muscle. Hoffmann et al. [2007] evaluated the mRNA levels for SelS by real-time PCR in eight different mouse tissues: brain, testes, liver,

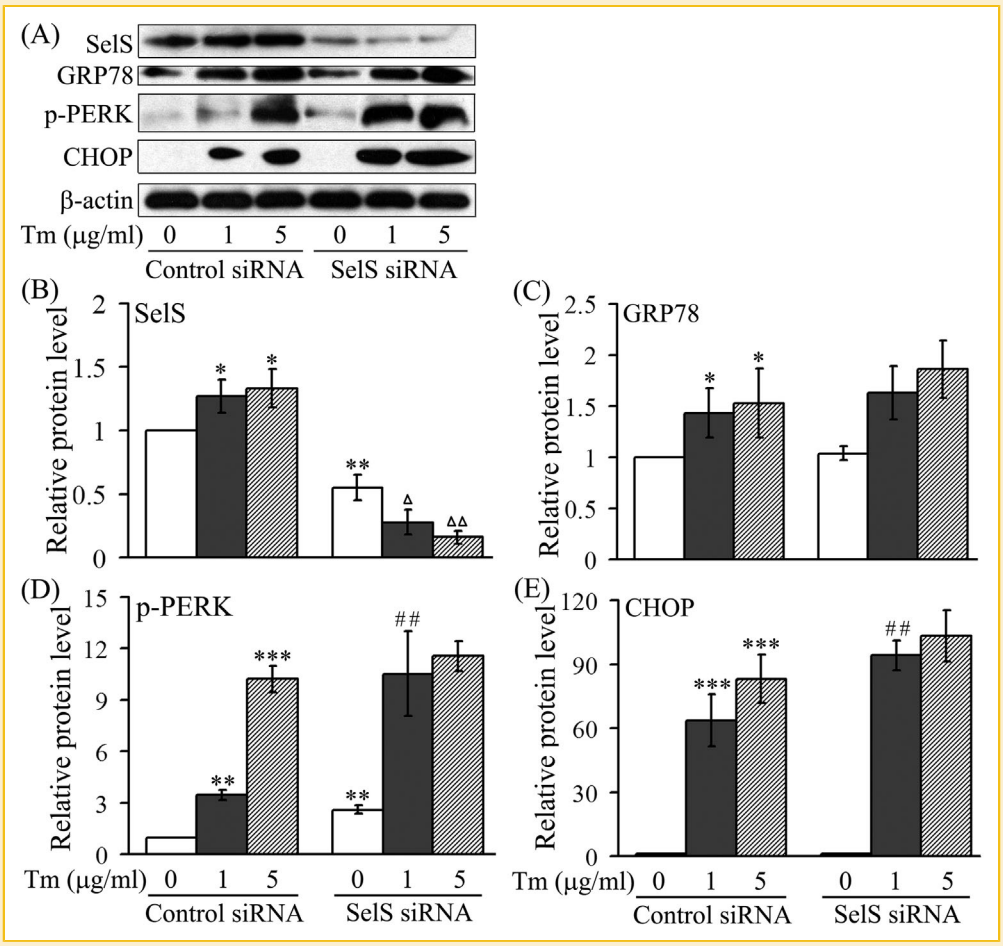

Fig. 7. SelS silence aggravated tunicamycin (Tm)-induced ER stress in VSMCs. (A) Western blot analysis of protein levels of SelS, GRP78, phosphorylated PERK (p-PERK), and CHOP and (B–E) semi-quantification analysis of protein levels (mean ± SD, n = 3) using β-actin as a control. VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then incubated in fresh media with or without indicated concentrations of tunicamycin for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the control cells treated with tunicamycin; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, compared with SelS silence cells.

kidney, lung, heart, intestine, and spleen. In the present work, the protein levels of SelS were determined in 14 different rat tissues, including skeletal muscle, lung, liver, cerebellum, cerebrum, kidney, eye, adipose tissue, thoracic aorta, spleen, stomach, thymus gland, pancreas, and heart. We found that SelS was highly expressed in rat thoracic aorta, behind thymus gland, lung, adipose tissue. This is the first time to evaluate the abundance of SelS protein in the vessel wall. Our result suggests that SelS must play an important role in the blood vessels, giving a support for the association of SelS gene polymorphism with atherosclerotic CVD.

We next found that SelS silence by siRNA rendered VSMCs more sensitive to H_2O_2-induced injury and apoptosis, as determined by MTT assay, Hoechst staining, and annexin V/PI double staining (Figs. 2 and 3). This finding indicated that SelS could prevent VSMCs from H_2O_2-induced apoptosis, which was in agreement with previous study showing that SelS protected Min6 pancreatic β-cells from H_2O_2-induced cell death [Gao et al., 2004]. Furthermore, SelS silence exacerbated oxidative stress triggered by H_2O_2, as evidenced by the increase of intracellular ROS and MDA (Fig. 4A–C). Additionally, a significant increase of SOD activity and a sharp decrease of GPx activity were observed in SelS silence cells treated with H_2O_2 (Fig. 4D,E). SOD catalyses the dismutation of superoxide anion radicals to H_2O_2, and GPx catalyses the reduction of H_2O_2. The increase in SOD activity and the decrease in GPx activity may account for the highest level of intracellular ROS observed in SelS silence cells treated with H_2O_2 (Fig. 4A,B). SelS was proposed to function as a peroxidase in vitro by using selenocysteine [Christensen et al., 2012; Liu and Rozovsky, 2013; Liu et al., 2013] and might play a role in the antioxidant defense in VSMCs. The lowest level of SelS protein in SelS silence cells treated with H_2O_2 (Fig. 6A,B) means the weakest antioxidant capability. Taken together, our results implied that SelS might protect VSMCs from H_2O_2-induced apoptosis partly by inhibiting oxidative stress. However, further studies are needed to clarify the underlying molecular cause by which SelS silence increased SOD activity and decreased GPx activity under H_2O_2 condition. The increase in SOD activity in SelS silence cells treated with H_2O_2 might be a compensatory up-regulation when cells response to excessive ROS. The decreased GPx activity may be explained by the fact that in vitro the Sec active site in GPx1 can be oxidatively

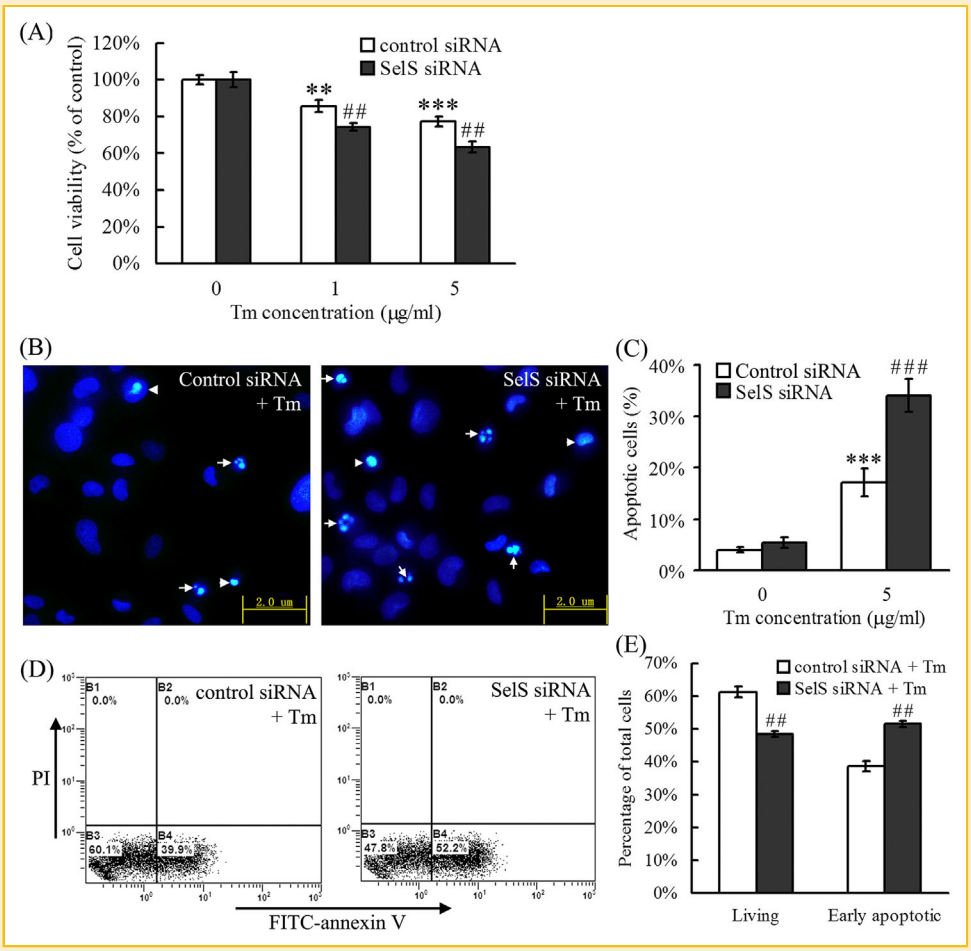

Fig. 8. SelS silence enhanced tunicamycin (Tm)-induced apoptotic death and apoptosis in VSMCs. (A) VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then incubated in fresh media with or without indicated concentrations of tunicamycin for 48 h. The cell viability was determined by MTT assay (means ± SD, n = 4). (B–E) VSMCs were transfected with 50 nM SelS siRNA or control siRNA for 24 h and then incubated in fresh normal media with or without 5 μg/ml tunicamycin for 48 h. (B) Apoptotic cells with condensed (▶) or fragmented (➔) nuclei were visualized by Hoechst 33342 staining, and (C) the percentage of apoptotic cells was counted independently for each treatment (mean ± SD, n = 3). (D) The percentages of living, early and late apoptotic cells were measured by flow cytometry after annexin V/PI double-staining. Early apoptotic cells appear in the lower right quadrant, while living cells are in the lower left quadrant, and late apoptotic and necrotic cells are in the upper right region. (E) Quantitative analysis of flow cytometry results (mean ± SD, n = 2). A representative experiment is shown in D. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control cells; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the control cells treated with tunicamycin.

inactivated by H_2O_2 [Cho et al., 2010]. In addition, the unchanged GPx mRNA level (data not shown), but the lowered GPx activity (Fig. 4E) were observed in SelS siRNA-treated cells without H_2O_2, compared with the control cells. This result suggested that SelS siRNA might regulate GPx1 expression by post-transcriptional, translational, or post-translational mechanism. Further studies are needed to illustrate this point.

JNK and p38 MAPK are both strongly activated by H_2O_2, and are critical mediators of oxidative stress-induced apoptosis [Runchel et al., 2011]. In the present study, p38 MAPK and JNK were also strongly activated by H_2O_2 in VSMCs, and these responses were significantly enhanced under SelS silence condition (Fig. 5). Because previous study has provided evidence for an important role of p38 MAPK and JNK pathways in the process of VSMC apoptosis induced by H_2O_2 [Li et al., 2008; Tsujimoto et al., 2006], we suggested that the inhibitory effect of SelS on VSMC apoptosis might be partly due to its regulation on p38 MAPK and JNK pathways involved in oxidative stress-induced apoptosis.

ER stress is involved in H_2O_2-induced apoptosis in many other types of cells, such as pancreatic AR42J cells [Weber et al., 2013], pancreatic β-cells [Ariyama et al., 2008], mesenchymal stem cells [Wei et al., 2010], renal proximal tubular cells [Katsoulieris et al., 2010], human oral keratinocytes [Min et al., 2008]. In this paper, ER stress was activated by H_2O_2 in VSMCs, as shown by the increased protein levels of three ER stress markers, GRP78, phosphorylated PERK, and CHOP (Fig. 6). Additionally, ER stress and apoptotic death were simultaneously induced in VSMCs by an ER inducer, tunicamycin (Figs. 7 and 8). These results indicated that H_2O_2 might trigger apoptosis partly by activating ER stress pathway in VSMCs. Compared with the control cells, more severe ER stress was activated in SelS silence cells when exposure to H_2O_2 or tunicamycin (Figs. 6 and 7). Moreover, SelS silence sensitized VSMCs to tunicamycin-

induced cell death and apoptosis (Fig. 8). All these results suggested that SelS is an ER stress regulator in VSMCs and might protect VSMCs from apoptosis induced directly by ER stress or indirectly by H_2O_2-activated ER stress. Similarly, previous studies reported the inhibitory effect of SelS on ER stress-induced apoptosis in astrocyte, RAW264.7 cells, HepG2 cells, and N2a cells [Kim et al., 2007; Fradejas et al., 2008; Du et al., 2010; Lee et al., 2014]. The inhibitory effect of SelS on ER stress may be attributed to its binding to the ERAD components selenoprotein K, p97 ATPase, and Derlins, which participate the retrotranslocation of unfolded and misfolded proteins from the ER lumen to cytosol for degradation [Shchedrina et al., 2011; Lee et al., 2014; Turanov et al., 2014]. However, SelS did not influence ER stress and H_2O_2-induced cell death in intestinal cell lines [Speckmann et al., 2014]. Thus, the involvement of SelS in the regulation of ER stress seems to be cell-type-dependent.

In the present paper, SelS expression in VSMCs was shown to be regulated not only by ER stress, but also by oxidative stress. We found that SelS expression was induced by tunicamycin in normal VSMCs (Fig. 7), which was consistent with previous works showing up-regulation of SelS in many other types of cells under ER stress conditions [Gao et al., 2004; Kim et al., 2007; Fradejas et al., 2008, 2011; Du et al., 2010; Lee et al., 2014; Speckmann et al., 2014]. In addition, an up-regulation of SelS in response to lower concentration of H_2O_2 (0.2 mM) was observed surprisingly, whether in control cells or SelS silence cells. This phenomenon could be interpreted with a novel translational control mechanism, which was been proposed very recently by Touat-Hamici et al. [2014] who also observed an up-regulation of SelS in response to oxidative stress. Therefore, SelS might be not only an ER stress marker, but also a potential oxidative stress maker. However, the decrease of SelS protein level induced by higher concentration of H_2O_2 (0.5 mM) both in control cells or SelS silence cells can not be explained clearly here. Further studies are needed to clarify this phenomenon.

In summary, our study demonstrated that SelS was highly expressed in rat thoracic aorta and could protect VSMCs from apoptosis by inhibiting oxidative stress and ER stress. This is the first time to study the expression of SelS in the vessel wall and its function in VSMCs. VSMC apoptosis induces destabilization and rupture of atherosclerotic plaques [Clarke et al., 2006; Tabas, 2010], leading to thrombosis with clinical manifestations of myocardial infarction or stroke. Thus, the protection of VSMCs from apoptosis in the plaque has become an important therapeutic target for plaque stabilization. Our findings provide important mechanistic insights into the potential role of SelS in the prevention of atherosclerotic CVD. Further investigations of the role of SelS in the pathogenesis of CVD should be done, especially in vivo in a model of CVD.

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