Sphingomyelin synthase 2 promotes H_2O_2-induced endothelial dysfunction by activating the Wnt/β-catenin signaling pathway

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Abstract. Atherosclerosis (AS) is the primary cause of various cardiovascular and cerebrovascular diseases and has high morbidity and mortality rates. Oxidative stress-induced endothelial cells (ECs) dysfunction is the pathological basis of AS. In addition, sphingomyelin (SM) and the Wnt/β-catenin signaling pathway are considered to be closely associated with AS; however, the specific mechanism is not clear. Therefore, the present study investigated whether SM may induce ECs dysfunction through the Wnt/β-catenin signaling pathway. Firstly, a sphingomyelin synthase 2 (SMS2) overexpression cell model was constructed. It was identified that the expression of SMS2 was increased when ECs were treated with H_2O_2. In addition, these results demonstrated that SMS2 overexpression promoted apoptosis and macrophage adhesion of H_2O_2-induced ECs, thereby increasing the expression of β-catenin. Furthermore, SMS activity was inhibited with Dy105, combined with simultaneous treatment with LiCl or H_2O_2. This additionally confirmed that Dy105 significantly inhibited SMS activity and decreased the level of ECs dysfunction and β-catenin content; however, LiCl served a key role in activating the Wnt/β-catenin signaling pathway to promote ECs dysfunction. Collectively, these results suggested that SMS2 overexpression may promote ECs dysfunction by activating the Wnt/β-catenin signaling pathway, while Dy105 may inhibit the evolution of oxidative stress-induced dysfunction.

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Introduction

Atherosclerosis (AS) is the primary cause of various cardiovascular and cerebrovascular diseases and has high morbidity and mortality rates (1). The dysfunction of endothelial cells (ECs), the infiltration of inflammatory cells and the formation of vascular smooth muscle cells and foam cells are considered to be the key mechanisms involved in the development of AS; however, ECs dysfunction is also hypothesized to serve a pivotal role in the initiation of AS (2). In addition, oxidative stress and mitochondrial dysfunction are responsible for the development of endothelial dysfunction (3,4). Oxidative stress leads to a disruption of the capabilities of intracellular antioxidants, resulting in the presence of a large number of reactive oxygen species (ROS), thereby causing lipid peroxidation and biological macromolecular degeneration, which are considered to be the primary factors leading to vascular endothelial dysfunction (5,6). In addition, H_2O_2 is a critical type of ROS that has good stability and diffusibility (5,6). Therefore, in the present study, H_2O_2 was used to simulate oxidative stress to construct an EC dysfunction model.

Sphingomyelin (SM) is a type of sphingolipid, which are key modulators of a number of physiological and pathophysiological processes that include the cell cycle, apoptosis, angiogenesis, and stress and inflammatory responses (7,8). Sphingomyelin synthase (SMS) is involved in the biosynthesis of SM. SMS has two isoforms, sphingomyelin synthase 1 (SMS1) and sphingomyelin synthase 2 (SMS2), which are the key enzymes in the last step of a series of enzymatic processes involved in SM synthesis (9). In previous years, a number of studies have demonstrated that SM is involved in AS occurrence and development (10-12). For example, epidemiological studies and clinical trial data identified that serum SM content and AS were positively correlated (11,12). Animal and cellular experiments have also indicated that SMS may cause AS by inhibiting reverse cholesterol transport (RCT), leading to lipid deposition (13-15). However, these studies were focused on the effects of SM on the RCT monocyte-macrophage infiltration and lipid deposition (13-15). Although Zhou *et al* (16) hypothesized that lovastatin may decrease the levels of sphingomyelin increased by 27-hydroxycholesterol on cultured vascular ECs and may attenuate cellular calcification, the role of SM in endothelial dysfunction remains unclear.

The canonical Wnt/β-catenin signaling pathway has been established as being involved in a number of cellular processes, including cell proliferation, differentiation and tissue patterning, and is also associated with multiple pathological processes, including metabolic disorders and cancer (17,18). However, several recent studies have suggested that the Wnt/β-catenin signaling pathway may cause AS (19,20). Animal experiments and human endarterectomy samples have demonstrated that the expression of protein Wnt-5a is increased in AS and is associated with the severity of atherosclerotic lesions (21,22). In addition, ECs dysfunction is also associated with the Wnt/β-catenin pathway. For example, activation of the Wnt/β-catenin pathway may cause endothelial dysfunction and augment monocyte adhesion to ECs (23,24). Ma *et al* (25) also suggested that the Wnt/β-catenin pathway may be activated in the process of ECs dysfunction induced by oxidized low-density lipoprotein (ox-LDL). Concomitantly, the Wnt/β-catenin pathway is hypothesized to induce vascular smooth muscle cell proliferation and migration, which is one of the hallmarks of the development of atherosclerotic plaques (26,27).

As aforementioned, the SM and Wnt/β-catenin pathways are involved in AS. However, the correlation between SM and Wnt/β-catenin in the development of AS induced by oxidative stress remains unclear. Therefore, the present study focused on identifying their association in the process of H_2O_2-induced ECs dysfunction. Firstly, an SMS2 overexpression and SMS-silenced human umbilical vein endothelial cells (HUVECs) model was constructed. This model was subsequently treated with H_2O_2, and the dysfunction of HUVECs, the expression of SMS2 and the activation of the Wnt/β-catenin pathway were examined.

Materials and methods

Cell culture. HUVECs were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; cat.no. 12100-500; Beijing Solarbio Bioscience & Technology Co., Ltd., Beijing, China) containing penicillin and streptomycin (100 U/ml and 0.1 mg/ml, respectively) and 10% certified fetal bovine serum (FBS; Biological Industries Israel Beit Haemek, KibbutzBeit Haemek, Israel) at 37˚C containing 5% CO_2. In addition, THP-1 cells (Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were grown in RPMI-1640 (cat. no. 31800; Beijing Solarbio Bioscience & Technology Co., Ltd.) containing 10% FBS and incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. Cell proliferation was detected by using an MTT assay. The untreated HUVECs were cultured in a 96-well plate (Beaver Nano-Technologies Co., Ltd., Suzhou, China) at a density of $5x10^4$/well for 24 h, and then were treated with different concentrations of H_2O_2 (0, 200, 400, 600, 800 and 1,000 μmol/l) for 24 h. Then, 40 μl MTT (5 mg/ml; cat. no. M8180; Beijing Solarbio Bioscience & Technology Co., Ltd.) was added to each well and the plates were incubated for 4 h at 37˚C. After 4 h, the medium was removed, and 150 μl dimethyl sulfoxide (cat. no. D8370; Beijing Solarbio Bioscience & Technology Co., Ltd.) was added to the wells, which were then agitated on a shaking table for 10 min. Finally, the absorbance was measured at a wavelength of 490 nm. A total of 6 repeat wells were measured at each time point, and the experiment was performed in triplicate.

Transfection and grouping. To establish the SMS2 overexpression cell model, a SMS2-overexpression plasmid [pcDNA3.1(+)], provided by Dr Tingbo Ding (School of Pharmacy, Fudan University, Shanghai, China) was used to transfect the HUVECs. The transfection protocol was performed as described by Luo *et al* (28). In brief, cells were seeded into 6-well plates (Beaver Nano-Technologies Co., Ltd., Suzhou, China) containing antibiotic-free RPMI-1640 medium (Biological Industries Israel Beit Haemek Ltd.). At the time of transfection, the cells were grown to 60-70% confluency as described previously (27). The plasmid SMS2 (4 μg; SMS2 group) or a control empty plasmid (4 μg; Control group) was diluted with 50 μl DMEM (FBS-free and antibiotic-free medium) or 5 μl Entranster™-D-4000 (Engreen Biosystem Ltd., Auckland, New Zealand) was also diluted with 50 μl DMEM. After 5 min, the dilutions were mixed together gently and incubated at 37˚C for 20 min, and the mixture and 2 ml DMEM (10% FBS) were subsequently added to each well. After 6 h, the medium was replaced by fresh DMEM (containing 10% FBS and antibiotics) as described previously (14). The control and SMS2 group were divided into two groups after 24 h, resulting in four groups; the control, SMS2, CH (Control+H_2O_2) and SH (SMS2+H_2O_2) groups, and both the CH and SH groups were treated with H_2O_2 (500 μmol/l). After 24 h, to collect all of these cells, HUVECs were centrifuged at 1,520 x g for 5 min (room temperature) as appropriate according to the needs of the experiments.

Measurement of lactate dehydrogenase (LDH) level in medium, intracellular superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. HUVECs were cultured at a density of $1x10^5$/well in 6-well plates and incubated overnight at 37˚C. Following transfection and treatment with H_2O_2 (500 μmol/l) for 24 h, the supernatant were collected, HUVECs were centrifuged (1,520 x g, 5 min, room temperature) and harvested following being digested by trypsin according to the protocol of the manufacturer. The levels of LDH (cat. no. A020-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), intracellular MDA content (cat. no. A003-1; Nanjing Jiancheng Bioengineering Institute) and SOD activity (cat. no. A001-1-1; Nanjing Jiancheng Bioengineering Institute) levels were measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). LDH was measured at the wavelength of 450 nm, however, SOD and MDA were measured at the wavelength of 560 nm.

Cell adhesion assay. Following transfection of the HUVECs and treatment with H_2O_2 (500 μmol/l), THP-1 cells at a density of $5x10^3$/well were added and incubated for 30 min at 37˚C. Next, the medium was discarded, and the cells were washed gently with PBS twice to remove non-adherent THP-1 cells. As described previously (29), the adherent THP-1 cells were counted in a single field under a phase contrast inverted

microscope (Magnification, x20; Olympus IX71; Olympus Corporation, Tokyo, Japan). Experiments were repeated three times to obtain the average number of adhesive monocytes/well.

Flow cytometry analysis. HUVECs were collected by trypsin digestion without EDTA (trypsin 1:250 from porcine pancreas; cat. no., 9002-07-7; Sangon Biotech Co., Ltd., Shanghai, China) digestion and washed twice with PBS. Thereafter, V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were added into the HUVECs according to the protocol of the ANNEXIN V-FITC/PI apoptosis assay kits (cat. no., 17210J; Beijing Zoman Biotechnology Co., Ltd., Beijing, China). The apoptosis results were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA), and FlowJo Software (v.10; Tree Star, Inc., Ashland, OR, USA) was used to analyze the data as previously described (28,30).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. After HUVECs were treated by different concentrations of H_2O_2 for 24 h, the RNA was isolated using TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA (2 μg) was used for RT transcription in a total volume of 20 μl (RevertAid First Strand cDNA; cat. no. K1622; Thermo Fisher Scientific, Inc.). The primers for β-actin and SMS2 were synthesized by Shanghai Sangon Biotech Co., Ltd., (Shanghai, China) and their sequences were as follows: β-actin forward, 5'-GATCATTGCTCCTCCTGAGC-3'; β-actin reverse, 5'-ACT CCTGCTTGCTGATCCAC-3'; SMS2 forward, 5'-ATAGCC CTCAGTCATGATT-3'; and SMS2 reverse 5'-CAGCACATG ACA ACGGTTCA-3' (30). Gene expression was measured using qPCR with SYBR-Green (cat. no. PR820A; Takara Biotechnology Co., Ltd., Dalian, China). The samples were examined using a StepOnePlus™ Real-Time PCR System, with the following thermocycler conditions: 95˚C for 3 min, then 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Fluorescence data and melting curves were subsequently obtained. Experimental data are presented as the mean ± standard deviation of three biological replicates. The gene expression was normalized to the expression of β-actin. The quantification cycle (Cq) value was defined as the number of cycles required for the fluorescent signal to reach the threshold. Using the comparative Cq method, the relative expression levels were calculated using the formula for $2^{-\Delta\Delta Cq}$ (31).

Western blot analysis. Following treatment with 500 μmol/l H_2O_2, only HUVECs that were not used for measuring the cell adhesion ability to THP-1 were used for western blot analysis. Total proteins of all groups were extracted by radioimmunoprecipitation assay buffer (cat. no. ROO20; Beijing Solarbio Bioscience & Technology Co., Ltd.), and protein content was measured using a BCA assay kit (cat. no. CW0014; Beijing Kangwei Century Biotechnology Co., Ltd., Beijing, China). Equal amounts of protein (~60 μg) were separated by electrophoresis on 10-12% SDS-PAGE gels and then transferred onto polyvinylidene fluoride membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA). Equal transfer was examined by staining with Ponceau red (cat. no. CW0057S; Beijing Kangwei Century Biotechnology Co., Ltd.). The membranes were blocked with 10% skimmed milk or 10% BSA in TBS for 1 h at room temperature and incubated with primary antibodies in TBS, which included 0.05% Tween 20, 2% bovine serum albumin (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) and 0.05% sodium azide overnight at 4˚C (28,32). The antibodies were used to detect the expression levels of SMS2 [cat. no AP9740b; dilution, 1:1,000; Abgent Biotech (Suzhou) Co., Ltd., Suzhou, China], apoptosis-associated proteins B-cell lymphoma 2 (Bcl-2; cat. no. BS-0032R; dilution, 1:500; BIOSS, Beijing, China), Bcl-2-associated X protein (Bax; cat. no. 60267-1-Ig; dilution, 1:2,000; ProteinTech Group, Inc., Chicago, IL, USA) and adhesion-associated proteins intracellular adhesion molecule-1 (ICAM-1; cat. no. BS-0608R; dilution, 1:1,000; BIOSS), vascular cell adhesion molecule-1 (VCAM-1; cat. no. BS-0920R; dilution, 1:500; BIOSS), monocyte chemoattractant protein-1 (MCP-1; cat. no. BS-1101R; dilution, 1:500; BIOSS), and the Wnt/β-catenin signal pathway-associated proteins β-catenin (cat. no. 66379-1-Ig; dilution, 1:2,000; ProteinTech Group, Inc.) and phosphorylated β-catenin (cat. no. DF2989; dilution, 1:1,000; Affinity Biosciences, Cincinnati, OH, USA). GAPDH (cat. no. HRP-60004; dilution, 1:8,000; ProteinTech Group, Inc.) was used as the internal control protein to ensure the equal loading of the proteins in each lane. Following washing of the membranes three times, horseradish peroxidase-conjugated anti-rabbit (cat. no. BA1054; dilution, 1:8,000; Boster Biological Technology, Inc., Wuhan, China) and anti-mouse (cat. no. SA0000I-I; dilution, 1:8,000; ProteinTech Group, Inc.) antibodies were used as the secondary antibodies at 1:8,000 in 10% skimmed milk in TBS. Finally, subsequent to washing the membranes three times, the blots were visualized using an enhanced chemiluminescence reagent (cat. no. CW0049M; Beijing Kangwei Century Biotech Co., Ltd.) and an autoradiography system (Chemiluminescence Imaging System; version 5.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each assay was repeated three times. The results were analyzed by Image Lab (version 5.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Sphingomyelin synthase enzyme activity assay. HUVECs were treated with H_2O_2 for 24 h as aforementioned. HUVECs were treated with different concentrations of Dy105 (provided by Dr Deyong Ye, School of Pharmacy, Fudan University; 0, 15, 20 and 25 μmol/l) (33) for 24 h. NBD-ceramide (0.1 μg/μl, cat. no. 62527; Cayman chemical company, Ann Arbor, MI, USA) was incubated with treated cells at 37˚C to synthesize sphingomyelin *in vitro*. After 3 h of incubation, the cells were harvested by 1,520 x g for 5 min, and the medium was also collected at room temperature. According to the protein content in each group, protein levels were adjusted to the volume of the reaction system (700 μl) to ensure the consistency of the total protein and medium added. Lipids were extracted in chloroform: Methanol (2:1), dried under N_2 gas, and separated by thin layer chromatography using chloroform: MeOH: NH_4OH (14:6:1) at room temperature for 10 min. The chromatography film was scanned after 10 min with an autoradiography system (Chemiluminescence Imaging System, Clinx Science Instruments Co., Ltd., Shanghai, China), and the intensity of each band was measured using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) (15).

Treatment with SMS inhibitor and LiCl on HUVECs. The cells were cultured as aforementioned. The HUVECs were seeded at a density of $1x10^6$/well into a 6-well plate prior to the addition of 20 μmol/l Dyl05 or lithium chloride (LiCl, 40 μmol/; Shanghai Qingfang Chemical Technology Co., Ltd., Shanghai, China) treatment for 24 h. Next, the Dyl05 and the LiCl groups were treated with H_2O_2 for 24 h, resulting in the following five groups: Control, CH (Control+H_2O_2), DH (Dyl05+H_2O_2), LH (LiCl+H_2O_2) and DLH (Dyl05+LiCl+H_2O_2). The medium and cells were harvested and analyzed by LDH, MDA and SOD kits and western blot analysis as aforementioned.

Statistical analysis. Statistical analysis was performed with statistical software SPSS 22.0 (IBM Corp., Armonk, NY, USA), and the significant differences between groups were evaluated using an independent samples (un-paired) t-test. For multiple comparisons, a one-way analysis of variance with Tukey's post-hoc analysis was used. All experiments were repeated at least three times, and P<0.05 was considered to indicate a statistically significant difference.

Results

H_2O_2 inhibits HUVECs viability in a dose-dependent manner. To investigate whether SMS2 was associated with the oxidative stress-induced dysfunction, the HUVECs were treated with H_2O_2 for 24 h. Firstly, the results (Fig. 1A) indicated that H_2O_2 inhibited HUVECs proliferation, and the proliferation rate of HUVECs gradually decreased to 25.49% when the H_2O_2 dose was increased from 0 to 1,000 μmol/l. Concurrently, the LDH leakage rate into the media of the HUVECs was also markedly increased in response to H_2O_2 (Fig. 1B), which suggested that H_2O_2 inhibited the viability of the HUVECs in a dose-dependent manner. Furthermore, the present study measured the levels of Bax and Bcl-2 by western blot analysis, and as demonstrated in Fig. 1C, the protein expression of Bax was increased, while the expression of Bcl-2 was decreased. Notably, as indicated in Fig. 1C, it was also observed that the protein expression level of SMS2 was increased concomitant with the dose of H_2O_2 (P<0.05; n=3), and similar results were also identified for the mRNA levels (Fig. 1D; P<0.05, n=3). The expression of SMS1 was also measured, and no significant differences in its expression levels were observed (data not shown). Therefore, SMS2 was selected as the target gene. These data suggest that SMS2 may be involved in H_2O_2-induced cellular dysfunction.

SMS2 overexpression inhibits proliferation and increases apoptosis of HUVECs. To additionally analyze the role of SMS2 in the process of HUVECs dysfunction, an SMS2 overexpression cell model was constructed. Fig. 2A indicated that the expression of SMS2 was significantly upregulated by ~130.7, 231.4 and 320.6% in the SMS2, CH and SH groups, respectively, compared with the control group (P<0.05; n=3), and the expression of SMS2 in the SH group was increased compared with the CH group (P<0.05; n=3). These results demonstrated the successful establishment of a SMS2 overexpression cell model, and again, verified that the oxidative stress-induced dysfunction may upregulate SMS2 in HUVECs. In addition, the results indicated that the expression of the pro-apoptotic protein Bax was markedly increased (Fig. 2B; P<0.05; n=3), while the level of apoptosis inhibitory protein Bcl-2 was decreased significantly (Fig. 2B; P<0.05; n=3) in the SMS2, CH and SH groups compared with the control group; additionally, the expression of Bcl-2 in the SH group was decreased compared with the CH group (P<0.05; n=3).

The overexpression of SMS2 may inhibit the proliferation of HUVECs and induce apoptosis. Therefore, to determine the apoptosis rate of HUVECs, the present study analyzed the apoptosis levels of the HUVECs by flow cytometry. As demonstrated by Fig. 2C and D, it was observed that H_2O_2 led to significantly increased levels of HUVECs apoptosis compared with the control group (P<0.001; n=3), and the apoptosis rate was 16.86, 32.43 and 55.40% in the SMS2, CH and SH groups, respectively. The results also suggested that the apoptosis rates of the SH group were increased compared with the CH group (P<0.001; n=3), and the rate in the SH group was increased by ~6.11% compared with the total increased rate in the CH and SMS2 groups (Fig. 2C and D). Collectively, these data indicate that H_2O_2 may induce HUVECs apoptosis and that SMS2 may increase the level of apoptosis induced by oxidative stress in HUVECs, and that it may serve an important role in endothelial dysfunction.

SMS2 overexpression increases the LDH content of medium, and intracellular MDA content and SOD activity. H_2O_2 is an important type of ROS that may lead to ECs dysfunction through oxidative stress, and we hypothesized that SMS2 may also aggravate the development of oxidative stress. As it is well-known, the content of MDA is a marker that reflects the level of antioxidants in cells; the content of LDH is considered to be an index for measuring cell viability and membrane integrity, and SOD activity is hypothesized to prevent cell dysfunction by eliminating oxygen free radicals (34). Therefore, the activity of LDH, and the intracellular MDA and SOD activity levels were detected. The results indicated that MDA and LDH production in HUVECs following exposure to H_2O_2 or SMS2 overexpression was significantly increased compared with the control group (P<0.05; n=3; Fig. 3A and B), and the levels in the SH group were increased compared with the CH group (P<0.05; n=3). Conversely, the level of SOD activity was decreased (Fig. 3C; P<0.05; n=3). Treatment with H_2O_2 or SMS2 may augment the dysfunction of HUVECs induced by oxidative stress.

SMS2 overexpression increases monocyte adhesion in HUVECs. To identify whether SMS2 may affect the adhesion rate of HUVECs, the present study measured the adhesion ability of HUVECs to THP-1 cells. As indicated in Fig. 4A, the adhesion ability of the SMS2, CH and SH groups were significantly increased compared with the control group (Fig. 4A; P<0.05; n=3), and the adhesion ability was improved by 29.94, 441.24 and 586.56% in the SMS2, CH and SH groups, respectively. Therefore, SMS2 may promote the adhesion ability of HUVECs. In addition, MCP-1, VCAM-1 and ICAM-1 are markers of HUVECs adhesion ability (35). Compared with the control group, the SMS2, CH and SH groups exhibited significantly upregulated levels of the adhesion molecules MCP-1, VCAM-1 and ICAM-1 (Fig. 4B; P<0.05; n=3). In addition, the increased levels of these proteins in the SH group was increased by ~75.99, 22.85 and 54.2% compared with the CH group. These

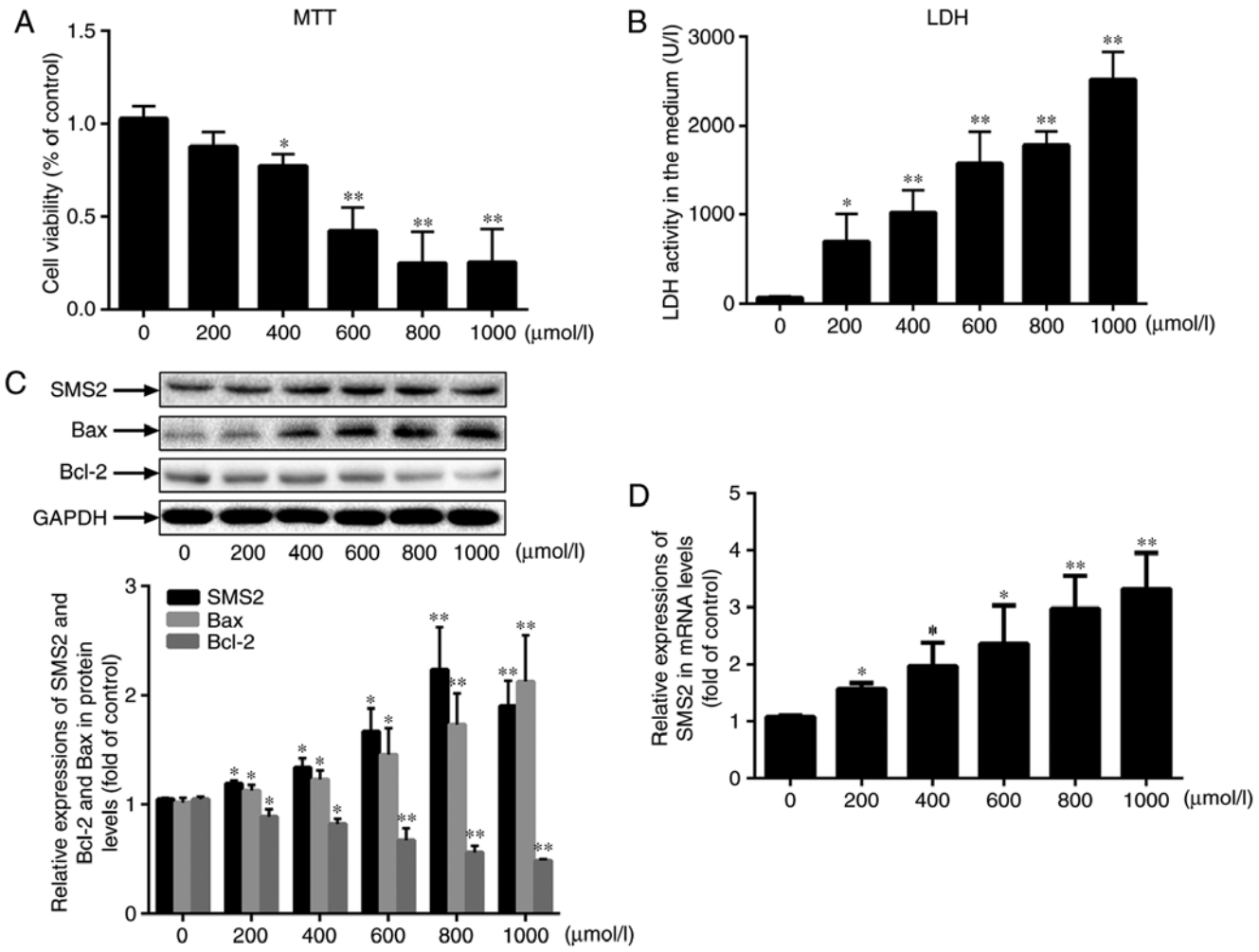

Figure 1. H_2O_2 inhibits HUVECs viability in a dose-dependent manner. HUVECs were treated with H_2O_2 at different doses (0, 200, 400, 600, 800 and 1,000 μmol/l) for 24 h. (A) Cell viability was measured by an MTT assay. (B) The activity of LDH in the cellular supernatant was also analyzed. (C) The protein levels of Bax, Bcl-2 and SMS2 were measured by western blot analysis. (D) The mRNA expression of SMS2 was measured by reverse transcription quantitative polymerase chain reaction. Values are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.001 vs. control group. SMS2, sphingomyelin synthase 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; LDH, lactate dehydrogenase; HUVECs, human umbilical vein endothelial cells.

results suggested that the overexpression of SMS2 may increase MCP-1, VCAM-1 and ICAM-1 expression and adhesion ability in the HUVECs dysfunction model induced by H_2O_2.

SMS2 overexpression activates the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway regulates a number of biological and pathological processes. However, the association between the Wnt/β-catenin signaling pathway and EC dysfunction remains unclear. To determine whether SMS2 overexpression may increase H_2O_2-induced HUVECs dysfunction by the Wnt/β-catenin signaling pathway, the present study analyzed the expression of β-catenin by western blot analysis. Fig. 5A indicated that the protein expression of β-catenin was significantly upregulated in the CH, SMS2 and SH groups compared with the control group (Fig. 5A; P<0.05; n=3); in addition, the SH group exhibited higher expression levels compared with the CH group (Fig. 5A; P<0.001; n=3). The levels of phosphorylated β-catenin were also investigated, and the results suggested that the relative content of phosphorylated β-catenin in the SMS2, CH and SH groups was decreased compared with the control group (Fig. 5A; P<0.05; n=3). Additionally, it was also observed that its expression level in the SH group was decreased compared with the CH group (Fig. 5A; P<0.05; n=3). As indicated in Fig. 5B, the ratio of phosphorylated-β-catenin/β-catenin was investigated, and the results demonstrated that this ratio was decreased in the SMS2, CH and SH groups compared with the control group (P<0.05; n=3). The ratio in the SH group was decreased compared with the CH group (Fig. 5B; P<0.05; n=3). All of these results indicated that H_2O_2-induced and SMS2 overexpression may increase the expression of β-catenin and prevent β-catenin phosphorylation.

Inhibition of SMS2 activity may decrease EC dysfunction and inactivate the Wnt/β-catenin signaling pathway. To confirm that SMS2 may regulate the Wnt/β-catenin signaling pathway, the present study simultaneously inhibited SMS activity and activated the Wnt/β-catenin signaling pathway by using an SMS inhibitor (Dy105) and LiCl, respectively. Firstly, HUVECs were treated with different concentrations of Dy105 (0, 15, 20 and 25 μmol/l) for 24 h. Then, the expression levels of SMS2 and β-catenin were analyzed by western blot analysis; Fig. 6A indicated that the levels of SMS2 and β-catenin were

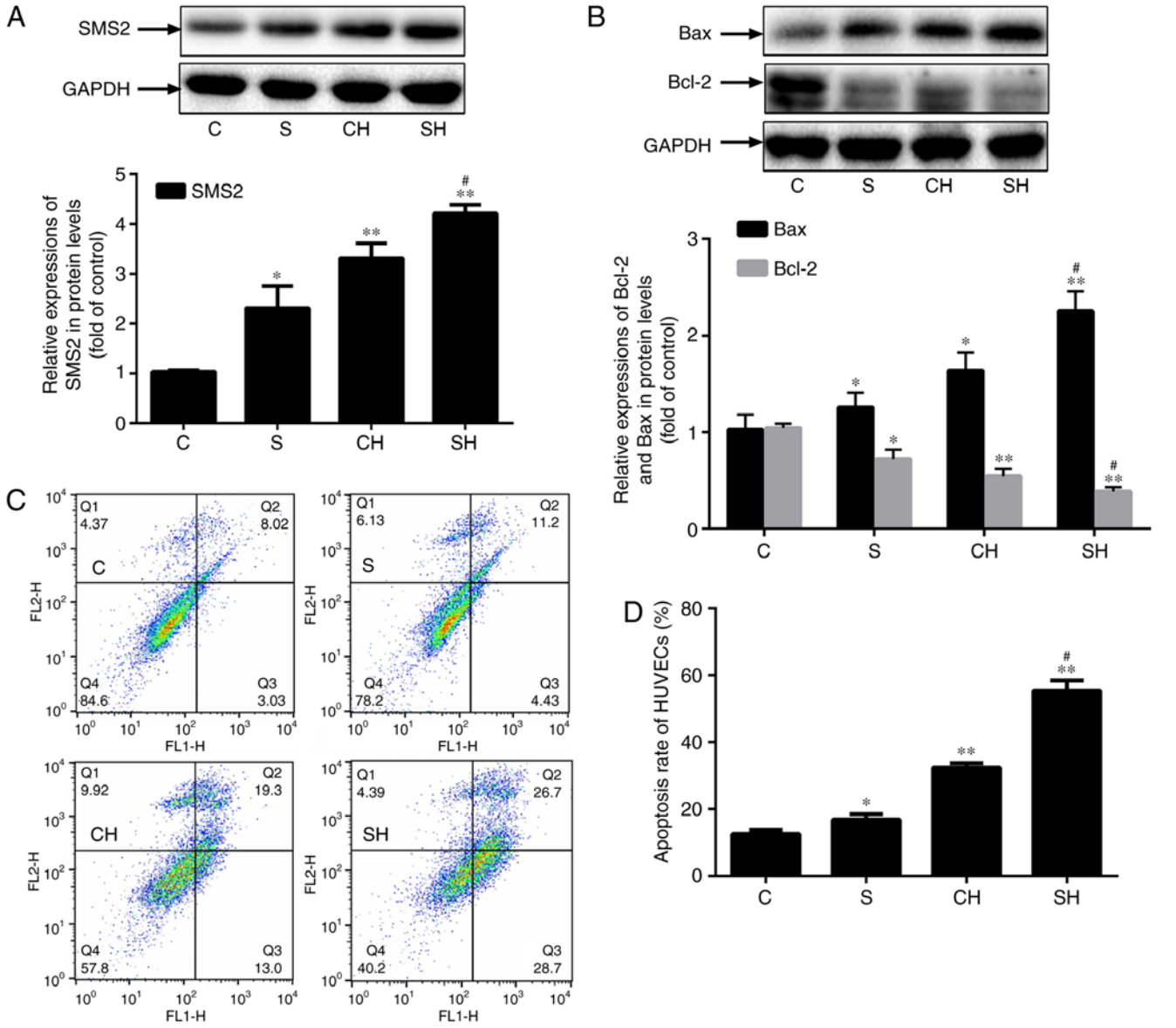

Figure 2. Effect of SMS2 on the proliferation and apoptosis in HUVECs. Effect of SMS2 on the proliferation and apoptosis in HUVECs. Cells were transfected with SMS2 overexpression plasmids and treated with or without H_2O_2 (500 μmol/l) for 24 h. (A) The protein levels of (A) SMS2, and (B) Bax and Bcl-2 were analyzed by western blot analysis. (C) The apoptosis of the C, S, CH and SH groups were analyzed by flow cytometry. (D) The quantified apoptosis rate of HUVECs. Values are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.001 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. CH group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; SMS2, sphingomyelin synthase 2; HUVECs, human umbilical vein endothelial cells; C, control group; S, SMS2 overexpression group; CH, control group treated with H_2O_2 for 24 h; SH, S group treated with H_2O_2 for 24 h.

most significantly decreased by 20 μmol/l Dy105 treatment (P<0.001; n=3). Therefore, the treatment concentration of Dy105 selected for subsequent experiments was 20 μmol/l. From the data presented in Fig. 6B, it was identified that the SMS enzyme activity was markedly decreased compared with the control group; it was decreased by 50.09% compared with control group (P<0.001; n=3). Subsequently, the levels of LDH, MDA and SOD intracellularly and in the culture media were measured, and it was demonstrated that the inhibition of SMS enzyme activity by Dy105 decreased the levels of LDH and MDA, and increased the level of SOD activity (Fig. 6C-E; P<0.05; n=3). However, LiCl reversed these changes (Fig, 6C-E; P<0.001; n=3). These results suggested that the inhibition of SMS enzyme activity may attenuate EC dysfunction. In addition, Fig. 6F indicated that the protein expression of β-catenin was significantly upregulated in the CH, DH, LH and DLH group compared with the control group (P<0.05; n=3), and the content of β-catenin in the LH and DLH groups was increased by 122.03 and 103.05%, respectively, compared with the DH group (Fig. 6F; P<0.05; n=3). In particular, the content of β-catenin was markedly increased in the LH group compared with the DLH group. Concomitantly, the levels of phosphorylated β-catenin were also investigated, and the results were the inverse of those for β-catenin levels (Fig. 6F; P<0.05; n=3). The ratio of phosphorylated-β-catenin/β-catenin (Fig. 6G) was also investigated; the results indicated that the changes in this ratio were coincident with Fig. 6F. These results indicated that Dy105 may decrease the expression of β-catenin to inactivate the Wnt/β-catenin signaling pathway; conversely, LiCl may re-activate it.

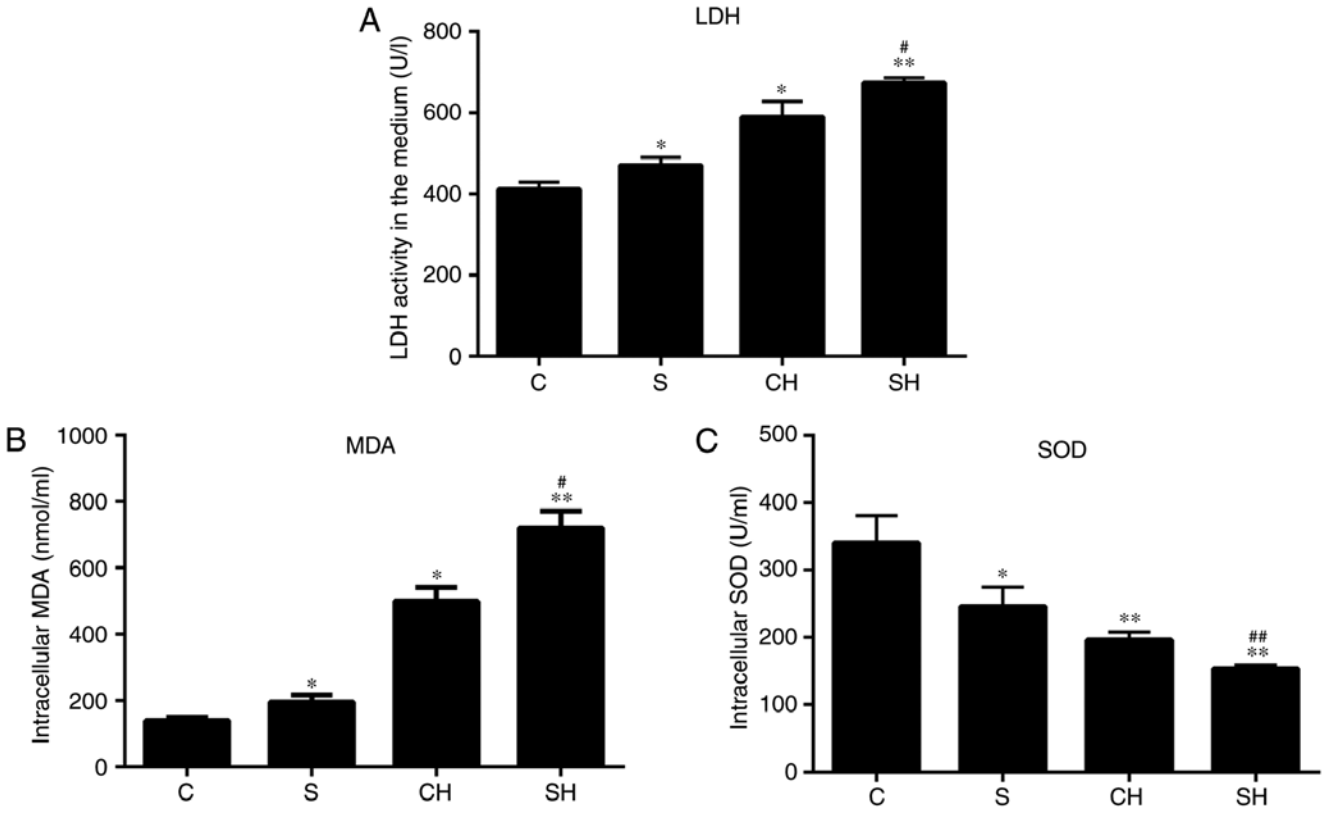

Figure 3. Overexpression of SMS2 increases the LDH content of cell media, and affects intracellular MDA content and SOD activity levels. Cells and supernatant were collected, and the (A) LDH, (B) MDA and (C) SOD levels were measured with assay kits. Values are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.001 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. CH group. LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase; SMS2, sphingomyelin synthase 2; C, control group; S, SMS2 overexpression group; CH, control group treated with H_2O_2 for 24 h; SH, S group treated with H_2O_2 for 24 h.

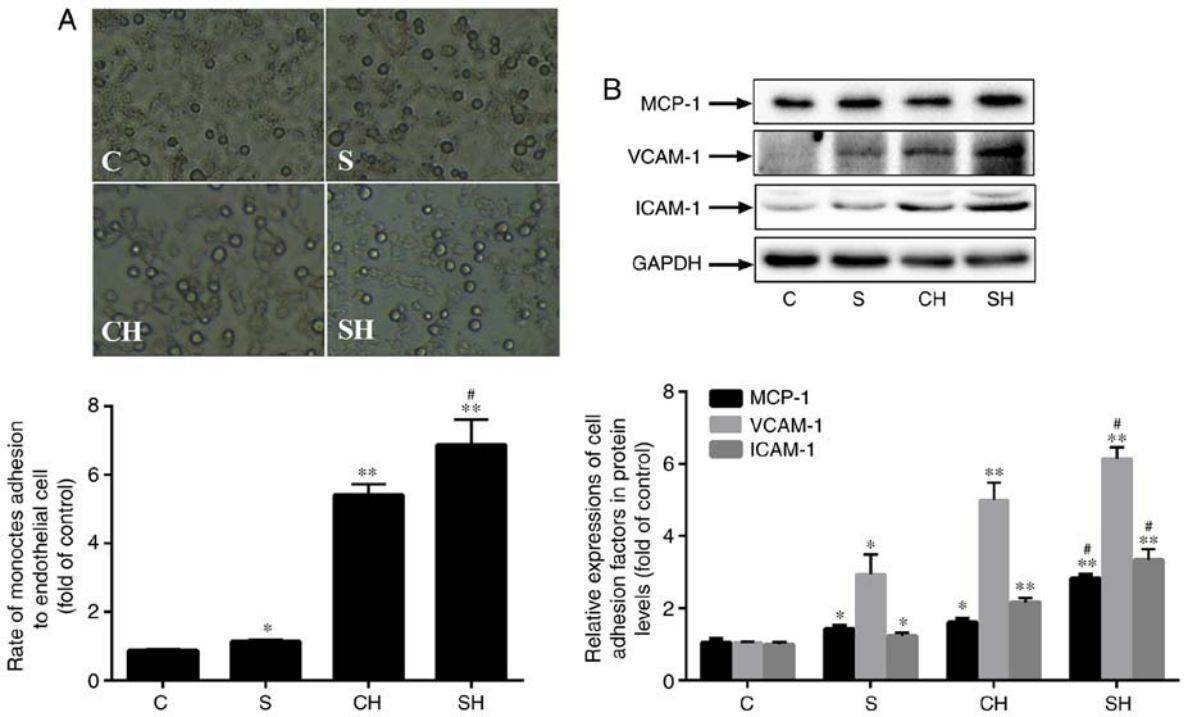

Figure 4. Overexpression of SMS2 increased monocyte adhesion in HUVECs. The overexpression of SMS2 enhances monocyte adhesion in HUVECs. (A) The adhesion rate of HUVECs to THP-1 cells. (B) The protein expression of MCP-1, VCAM-1 and ICAM-1 were detected by western blot analysis. Values are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.001 vs. control group; $^{\#}$P<0.05 vs. CH group. Magnification, x20; HUVECs, human umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intracellular adhesion molecule 1; MCP-1, monocyte chemotactic protein 1; SMS2, sphingomyelin synthase 2; C, control group; S, SMS2 overexpression group; CH, control group treated with H_2O_2 for 24 h; SH, S group treated with H_2O_2 for 24 h.

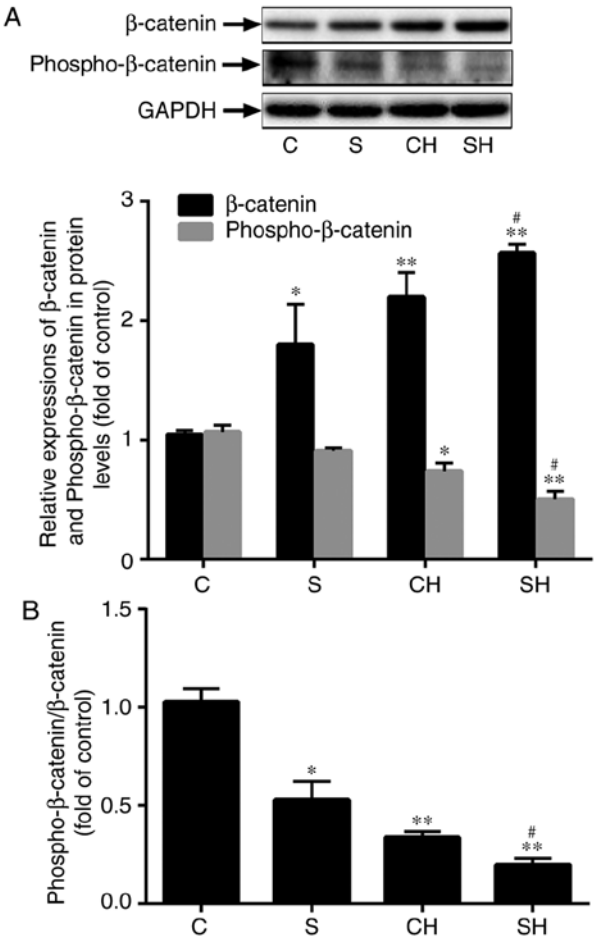

Figure 5. Overexpression of SMS2 activates the WNT/β-catenin signaling pathway. The overexpression of SMS2 activates the WNT/β-catenin signaling pathway. (A) The protein expression of β-catenin and phosphorylation of β-catenin were determined by western blot analysis. (B) Phospho-β-catenin/β-catenin. Values are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.001 vs. control group; $^{\#}$P<0.05 vs. CH group. SMS2, sphingomyelin synthase 2; Phospho, phosphorylated; C, control group; S, SMS2 overexpression group; CH, control group treated with H_2O_2 for 24 h; SH, S group treated with H_2O_2 for 24 h.

Discussion

The results of the present study indicated that H_2O_2 may upregulate the levels of SMS2 in a dose-dependent manner, and that the oxidative stress-induced dysfunction of HUVECs was associated with the activation of β-catenin. In addition, SMS2 may increase the expression of β-catenin and attenuate the phosphorylation of β-catenin, which may augment the oxidative stress-induced dysfunction of HUVECs induced by H_2O_2. Conversely, when the SMS activity was inhibited by Dy105, the Wnt/β-catenin signaling pathway was suppressed and ECs dysfunction was also attenuated; however, LiCl reversed these changes.

It has been established that HUVECs serve a pivotal role in AS occurrence and development (2). However, oxidative stress is one of the major factors causing HUVECs dysfunction when antioxidant mechanisms are overwhelmed by ROS (5,6). H_2O_2 is the primary source of endogenous ROS and has been extensively used to establish *in vitro* models (5,36,37). In the present study, different doses of H_2O_2 were used to treat HUVECs to induce dysfunction, and it was identified that the expression of SMS2 also increased with increasing H_2O_2 concentrations, which suggested that SMS2 expression is closely associated with HUVECs dysfunction. Subsequently, additional data from the present study validated that SMS2 was involved in the oxidative stress-induced dysfunction of HUVECs, as its expression was induced by H_2O_2, and its overexpression augmented HUVECs dysfunction. ECs dysfunction and the upregulation of MCP-1, VCAM-1 and ICAM-1 are critical events in AS (2,36). The results from the present study also indicated that SMS2 overexpression increased the expression of MCP-1, VCAM-1 and ICAM-1, and the adhesion ability of THP-1 cells, which may assist in recruiting macrophages into the intima and contribute to the initiation of AS (38,39). All of these results suggested that SMS2 is involved in AS occurrence and development via promoting HUVECs dysfunction and adhesion ability to THP-1 cells. Although previous studies have verified that SMS contributes to AS occurrence and development through the inhibition of RCT and may lead to lipid deposition (13-15,40,33), to the best of our knowledge, the present study is the first to suggest that SMS2 may promote HUVEC dysfunction and adhesion ability to THP-1 cells, and assist in AS occurrence and development.

It has been previously established that the canonical Wnt/β-catenin signaling pathway is critical in a number of cellular processes (17,18). In these cellular processes, when the Wnt/β-catenin signaling pathways are activated, cytoplasmic β-catenin, the central molecule of the Wnt signaling pathway, is not able to be phosphorylated and is degraded by the action of the destruction complex (axis inhibition protein 1, APC, WNT signaling pathway regulator, casein kinase 1 alpha 1 and glycogen synthase kinase 3β). This effect results in the accumulation of cytoplasmic β-catenin and translocation into the nucleus, where it promotes the expression of a number of key genes involved in different cellular functions via activating the transcription regulation activity of T-cell factors (17). However, the results from the present study and the data from several other studies have suggested that the Wnt/β-catenin signal pathway is associated with the cell dysfunction and apoptosis induced by H_2O_2 (41,42). For example, in cardiomyocytes, H_2O_2 may upregulate dishevelled-1 (Dvl-1), β-catenin and Myc proto-oncogene protein, promote β-catenin nuclear activity, activate Wnt/β-catenin signaling and finally facilitate DNA damage and tumor protein 53-mediated apoptosis (41,42), whereas when secreted frizzled related protein 1 suppressed the expression of Dvl-1 and β-catenin and the activity of the Wnt/frizzled pathway, the H_2O_2-induced apoptosis of cardiomyocytes was decreased (43). However, in ECs, oxidative stress is regarded as a critical pathogenic factor for dysfunction, and the accumulation of ROS may result in EC apoptosis (25,44). Therefore, ox-LDL may serve a critical role in the progression and development of AS via increasing oxidative stress (45). Additional studies have demonstrated that in this process, the Wnt/β-catenin signaling pathway is activated; this observation resulted in the identification of certain genes with expression patterns associated with HUVECs apoptosis (25,44). In the present study, when HUVECs were treated with H_2O_2, the expression of β-catenin was increased, and the phosphorylation of β-catenin was decreased. It is clear that the Wnt/β-catenin signaling pathway is involved in the H_2O_2-induced dysfunction of HUVECs. In addition, the present study identified that the overexpression of SMS2 improved the dysfunction of HUVECs and

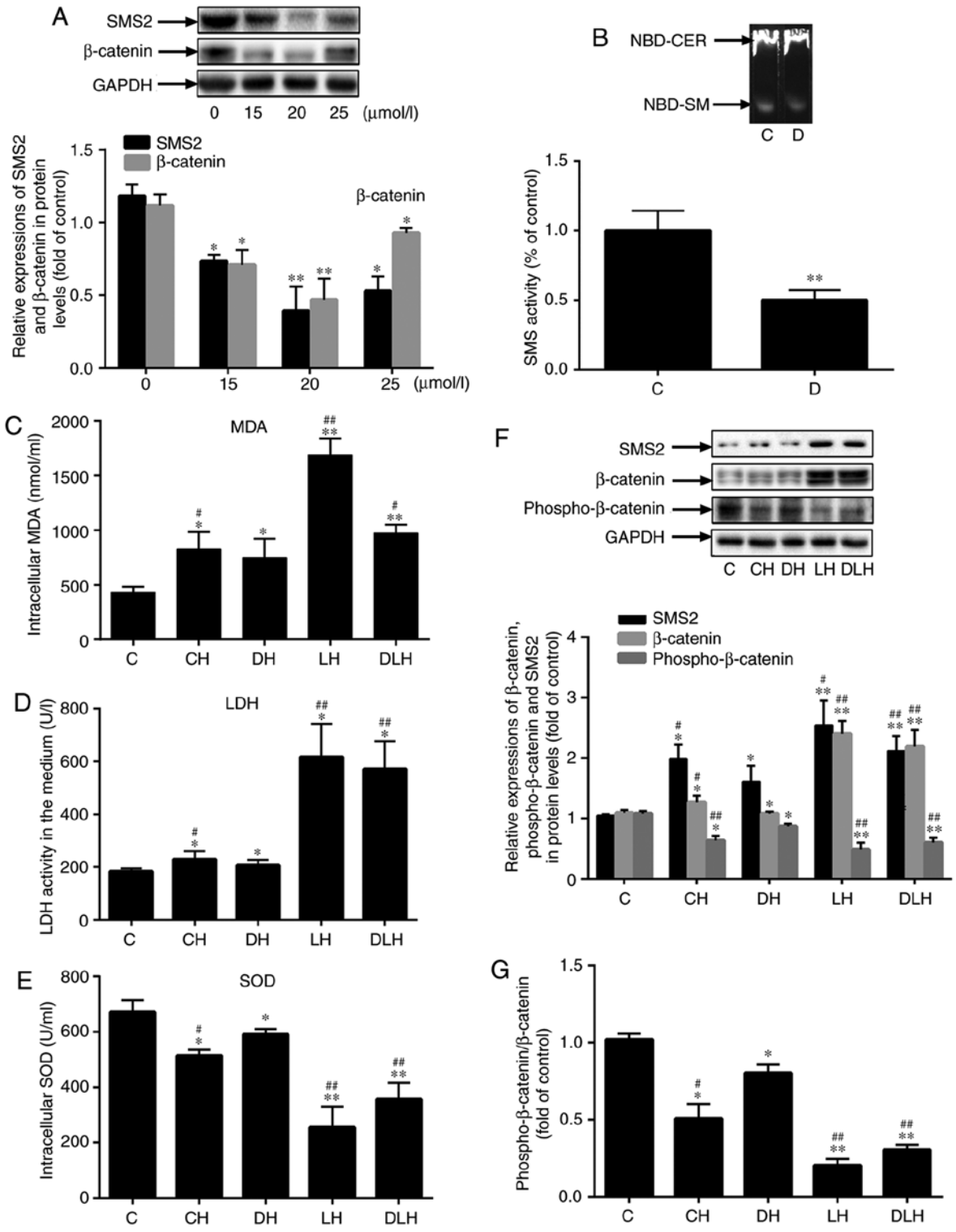

Figure 6. Inhibition of the SMS activity may decrease endothelial cells dysfunction and inactivate the Wnt/β-catenin signaling pathway. (A) The expression of SMS2 and β-catenin was measured by western blot analysis in human umbilical vein endothelial cells treated with Dy105 at different dosages. (B) SMS activity was measured by thin-layer chromatography. Values are presented as the mean ± standard deviation (n=3). (C) The level of MDA. (D) The activity of LDH. (E) The levels of SOD. (F) The protein expression of SMS2, β-catenin and phosphorylation of β-catenin were determined by western blot analysis. (G) Phospho-β-catenin/β-catenin ratio. Values are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.001 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 vs. DH group. SMS2, sphingomyelin synthase 2; LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase; D, Dy105 treatment, NBD-CER, NBD-ceramide, NBD-SM, NBD-sphingomyelin; C, control group, CH, control group treated by H_2O_2 for 24 h; DH, Dy105+H_2O_2 group, LH, LiCl+H_2O_2 group, DLH, Dy105+LiCl+H_2O_2 group.

their adhesion ability to THP-1 cells. The possible mechanism may be that SMS2 overexpression results in the accumulation of cytoplasmic β-catenin and translocation to the nucleus through attenuating phosphorylation of β-catenin, while the inhibition of SMS activity may increase the phosphorylation of β-catenin and decreased the nuclear translocation of β-catenin.

Although SMS has two isozymes, no significant difference was observed in the expression levels of SMS1, but SMS2 expression was altered following treatment with H_2O_2 in the present study. Therefore, the overexpression of SMS2 may promote the dysfunction of HUVECs through activating the Wnt/β-catenin signal pathway. In addition, it has been established that SMS2 is a key enzyme involved in the biosynthesis of SM, and that it is also involved in the production of diacylglycerol (DAG) (46). Yang *et al* (47) hypothesized that DAG may activate β-catenin, where osteoclasts were stimulated by colony-stimulating factor 1. Therefore, we hypothesize that the overexpression of SMS2 increases the content of DAG, which may activate β-catenin and aggravate the dysfunction of HUVECs. However, the detailed mechanism by which SMS2 affects the Wnt/β-catenin signaling pathway requires additional investigation.

Taken together, the results from the present study indicate the following: Oxidative stress induced by H_2O_2 may cause dysfunction in HUVECs via increasing the expression of SMS2 and activation of the Wnt/β-catenin signaling pathway; and Dy105 may inhibit H_2O_2-induced dysfunction of HUVECs through inactivating the Wnt/β-catenin signaling pathway.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

PZ, LH and HH performed the experiments and were major contributors in writing the manuscript. XD and ZH analyzed and interpreted the data and were responsible for the study design and drafting of the manuscript. ML was responsible for statistical analysis. XH prepared the reagents and revised the manuscript. NY designed all of the study and provided funding. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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