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Transforming Growth Factor β 1 (TGF- β 1) Appears to Promote Coronary Artery Disease by Upregulating Sphingosine Kinase 1 (SPHK1) and Further Upregulating Its Downstream TIMP-1

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Background: Transforming growth factor (TGF)- β 1 is involved in the pathogenesis of coronary artery disease (CAD), but the mechanism of its action remains unclear. Our study aimed to investigate the role of TGF- β 1 in CAD and to explore the possible mechanisms.

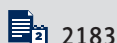
Material/Methods: A total of 60 CAD patients and 54 healthy people were included in this study. Blood samples were drawn from each participant to prepare serum. ELISA was utilized to measure serum level of TGF- β 1. TGF- β 1 expression vector, TGF- β 1 siRNA, and TIMP-1 siRNA were transfected into human primary coronary artery endothelial cell (HCAEC) line cells, and expression of TGF- β 1 sphingosine kinase 1 (SPHK1) and TIMP metalloproteinase inhibitor 1 (TIMP-1) was detected by Western blot. Cell apoptosis was detected by MTT assay.

Results: Serum level of TGF- β 1 was specifically higher in patients with CAD than in healthy controls. Serum levels of active TGF- β 1 can be used to effectively distinguish CAD patients from healthy controls. TGF- β 1 overexpression promoted the apoptosis of HCAEC and TGF- β 1 siRNA silencing inhibited the apoptosis of HCAEC. TGF- β 1 overexpression also promoted the expression of SPHK1 and TIMP-1. SPHK1 overexpression upregulated TIMP-1 but it showed no significant effects on TGF- β 1. TIMP-1 overexpression showed no significant effects on TGF- β 1 or SPHK1. SPHK1 inhibitor and TIMP-1 silencing reduced the enhancing effects of TGF- β 1 overexpression on cell apoptosis.

Conclusions: TGF- β 1 appears to promote CAD through the induction of cell apoptosis by upregulating SPHK1 expression and further upregulating its downstream TIMP-1.

MeSH Keywords: **Coronary Disease • Gene Expression Regulation • Receptors, Transforming Growth Factor beta**

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Background

Coronary heart disease, also known as coronary artery disease (CAD), is caused by the formation of plaque in coronary arteries that are responsible for the supply of oxygen-rich blood to heart muscle [1]. Blockage of coronary arteries leads to insufficient nutrients, blood, and oxygen for the heart. Therefore, CAD is also called ischemic heart disease [2]. CAD is a group of cardiovascular diseases including stable angina, unstable angina, myocardial infarction, and even sudden cardiac death, and is the leading cause of death worldwide, especially in developing countries such as China [2,3]. Over 40% of all deaths are related to CAD [4]. Even worse, the incidence of this disease is predicted to continuously increase due to changes in life style, such as the popularization of Western-style diet [5]. Although progress has been made in treatment of CAD, outcomes are usually poor [6]. Therefore, in-depth investigation of the mechanism underlying this disease may improve the survival of CAD patients.

As a transforming growth factor, TGF- β 1 plays pivotal roles in various normal physiological and pathological processes through the regulation of cell proliferation, growth, differentiation, and apoptosis [7]. The involvement of TGF- β 1 in vascular morphogenesis and dysfunction has been extensively studied previously and TGF- β signaling has been proved to be crucial in cardiovascular disorders [8]. Increased expression level of TGF- β 1 was observed during the development of a variety of human diseases, including CAD [9]. Sphingosine kinases, including SPHK1 and TIMP-1, play pivotal roles in vascular development [9,10]. SPHK1 interacts with different signaling molecules, such as vascular endothelial growth factor, to regulate angiogenesis [10]. Secretion of TIMP-1 promotes the formation of new vessels [11]. However, the function of those proteins in CAD and the underlying molecular mechanism remain unclear. Therefore, our study was carried out to investigate the role of these proteins in CAD and to explore the possible mechanism.

Material and Methods

Subjects

A total of 60 patients with CAD were selected from January 2016 to January 2017 in Qilu Hospital of Shandong University. Those patients were diagnosed using coronary angiography according to the criteria proposed by the American College of Cardiology (ACC)/American Heart Association (AHA) [12]. Patients with other types of cardiovascular diseases, malignancies, or other severe diseases, as well as those with serious infections within 6 weeks before the admission or with active chronic inflammatory disease, were excluded. The enrolled patients included 33 males and 27 females, age 32–73 years,

with an average age of 51 ± 6.9 years. At the same time, 54 healthy people with similar age and sex distributions were selected to serve as a control group. The Ethics Committee of Qilu Hospital of Shandong University approved this study, and all patients signed informed consent.

Serum preparation and enzyme-linked immunosorbent assay

Whole blood (80 ml) was extracted from each participant on the day of admission. Blood samples were kept at room temperature for 2 h, followed by centrifugation at 2000 rpm for 30 min to separate serum. Then, TGF- β 1 ELISA Kit (R&D Systems, Inc., Minneapolis, MN) was used to measure serum TGF- β 1 level with recombinant human TGF- β 1 as calibrator. Acid activation was performed to release biologically active TGF- β 1 [13]. Serum samples were diluted in DPBS buffer with a ratio of 1: 75 and were directly added into the ELISA plate to measure the concentration of active TGF- β 1. Each measurement was performed 3 times and the mean value was calculated.

Cell culture

Human primary coronary artery endothelial cells (HCAEC) were obtained from ATCC (ATCC[®] PCS-100-020[™]). Cells culture was performed according to ATCC protocol. Serum-free culture medium was used in drug treatment. Cells were collected during logarithmic growth phase for subsequent experiments.

Transfection

TGF- β 1 siRNA (catalog# AM16708), TIMP-1 siRNA (catalog# AM16708), and Silencer[®] Negative Control #1 siRNA (catalog# AM4611) were provided by Thermo Fisher Scientific. EcoRI-EcoRI fragments containing full-length TGF- β 1 gene cDNA, SPHK1 gene cDNA, or TIMP-1 cDNA were inserted into pIRSE2-EGFP (Clontech, Palo Alto, CA, USA) vectors to establish TGF- β 1, SPHK1, and TIMP-1 expression vectors, respectively. Empty pIRSE2-EGFP vector was used as a negative control. Before transfection, HCAECs were cultured overnight to reach 70–80% confluence. Lipofectamine 2000 transfection reagent (11668-019, Invitrogen, Carlsbad, USA) was used to transfect 50 nM siRNA or 10 nM vector into 5×10^6 cells.

MTT assay

After transfection, cells were cultured in culture medium containing 10 mM tetraethylammonium (TEA) at a density of 5×10^4 cells/ml to induce cell apoptosis. Then, 100 μ l cell suspension (5×10^3 cells) was added into each well of 96-well plate. Cells were cultured at an incubator (37°C, 5% CO₂) for 6 h, followed by the addition of 10 μ L of MTT. After that, cell culture was performed for another 4 h, and optical density was measured at

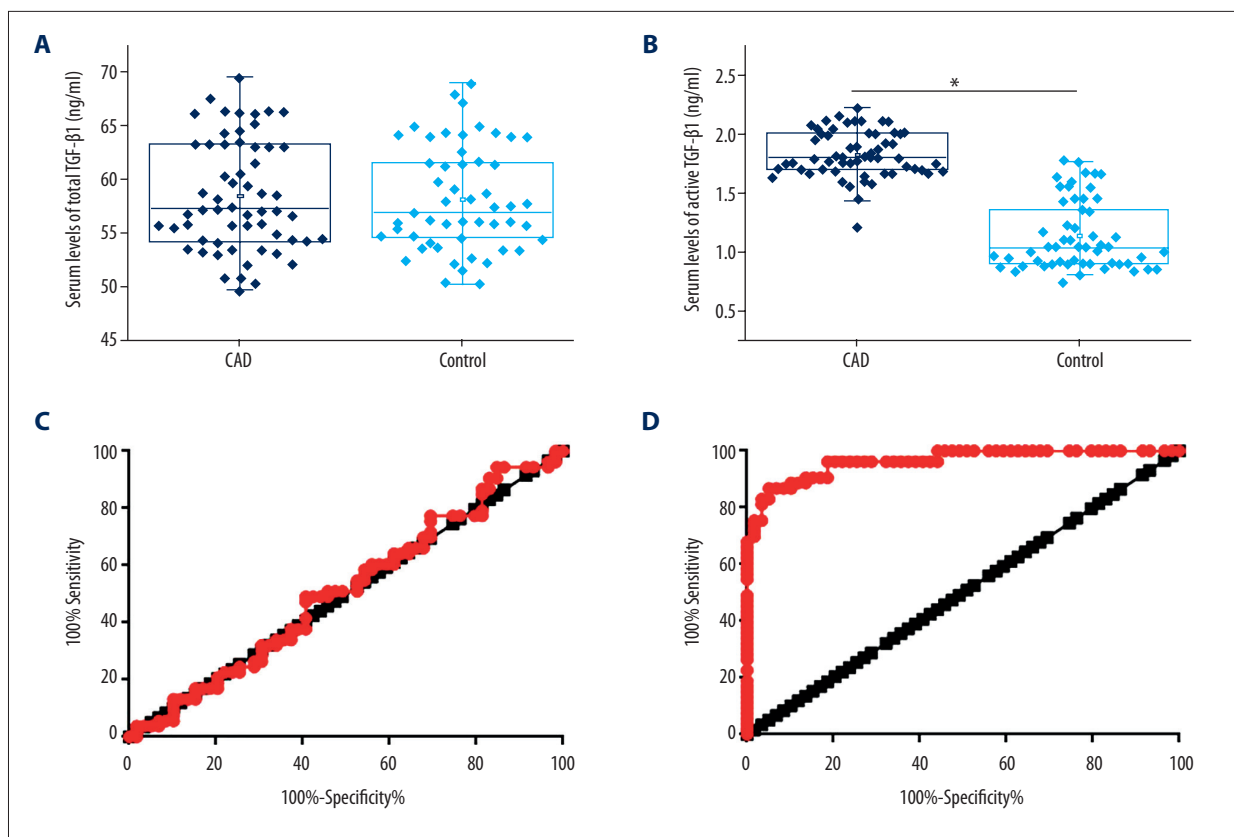


Figure 1. Comparison of serum TGF- β 1 levels between CAD patients and healthy controls and the diagnostic value. **(A)** Comparison of serum total TGF- β 1s level between CAD patients and healthy controls; **(B)** Comparison of serum active TGF- β 1 levels between CAD patients and healthy controls; **(C)** ROC curve analysis of the diagnostic values of serum levels of total TGF- β 1 for CAD; **(D)** ROC curve analysis of serum levels of active TGF- β for CAD. * $p < 0.05$.

570 nm using a Fisherbrand™ AccuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific). Cell apoptosis rate was normalized to the one with the highest apoptotic rate. This experiment was performed in triplicate.

Western blot

RIPA solution (Fisher Scientific) was used to extract total protein from *in vitro* cultured cells, and protein samples were quantified by BCA method. Then, 20 μ g protein was subjected to 10% SDS-PAGE gel electrophoresis, then transferred to a PVDF membrane. Blocking was performed by incubating the membrane with 5% skimmed milk. After that, membranes were washed 3 times with PBS, 10 min each time, followed by incubation with primary antibodies, including rabbit anti-TGF- β 1 (1: 2000, ab92486, Abcam), anti-SPHK1 (1: 2000, ab46719, Abcam), anti-TIMP-1 (1: 2000, ab61224, Abcam), and anti-GAPDH (1: 1000, ab9845, Abcam) overnight at 4°C. The next day, membranes were washed 3 times with PBS, 10 min each time, followed by incubation with anti-rabbit IgG-HRP secondary antibody (1: 1000, MBS435036, MyBioSource) at room temperature for 1 h. After washing with PBS for 15 min, ECL method (Sigma-Aldrich,

USA) was used for signal development. Relative expression levels of TGF- β 1, SPHK1, and TIMP-1 were normalized to endogenous control GAPDH using Image J software. This experiment was performed in triplicate.

Statistical analysis

GraphPad software and Origin software were used for all statistical analyses. Measurement data were recorded by ($\bar{x} \pm s$), and comparisons between 2 groups were performed by *t* test. Comparisons among multiple groups were performed by one-way analysis of variance. Count data were processed using chi-square test. $P < 0.05$ was considered to be statistically significant.

Results

Comparison of serum TGF- β 1 level between CAD patients and healthy controls and the diagnostic value

As shown in Figure 1A, no significant differences in serum levels of total TGF- β 1 were found between CAD patients and healthy

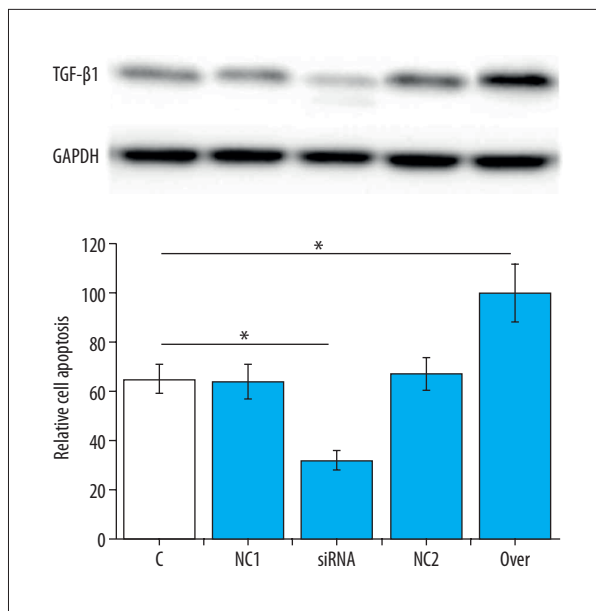


Figure 2. Effects of TGF- β 1 siRNA silencing and overexpression on apoptosis of HCAEC. The upper part shows expression of TGF- β 1 protein and the lower part shows corresponding cell apoptosis. * $p < 0.05$; NC1, negative control 1, cells transfected with negative control siRNA; NC2, negative control 1, cells transfected with empty vector.

controls. However, serum levels of active TGF- β 1 were significantly higher in CAD patients than those in controls ($p < 0.05$, Figure 1B). ROC curve analysis was performed to evaluate the diagnostic values of serum levels of total TGF- β 1 and active TGF- β 1 for CAD. As shown in Figure 1C, the area under the curve (AUC) of serum levels of total TGF- β 1 in the diagnosis of CAD was 0.5109 with 95% confidence interval of 0.4033 to 0.6184 ($p = 0.8429$). AUC of serum levels of active TGF- β 1 in the diagnosis of CAD was 0.9627 with 95% confidence interval of 0.9326 to 0.9929 ($p < 0.0001$, Figure 1D). These data suggest that active TGF- β 1 but not total TGF- β 1 may serve as a potential diagnostic biomarker for CAD.

Effects of TGF- β 1 siRNA silencing and overexpression on apoptosis of HCAEC

TGF- β 1 siRNA silencing and overexpression cell lines were confirmed by qRT-PCR (data not shown). MTT assay was performed to explore the effects of TGF- β 1 siRNA silencing and overexpression on apoptosis of HCAEC. As shown in Figure 2, TGF- β 1 siRNA significantly inhibited cell apoptosis compared with control HCAEC ($p < 0.01$). In contrast, TGF- β 1 overexpression significantly promoted cell apoptosis ($p < 0.01$). These data suggest that TGF- β 1 signaling promotes apoptosis of HCAEC.

TGF- β 1 is a positive upstream regulator of SPHK1 and TIMP-1 in HCAEC

As shown in Figure 3A, TGF- β 1 overexpression significantly upregulated the expression of SPHK1 and TIMP-1 in HCAECs ($p < 0.05$). In contrast, SPHK1 (Figure 3B) and TIMP-1 (Figure 3C) overexpression showed no significant effects on TGF- β 1 expression ($p > 0.05$). These data suggest that TGF- β 1 is a positive upstream regulator of SPHK1 and TIMP-1 in HCAECs.

SPHK1 is a positive upstream regulator of TIMP-1 in HCAEC

As shown in Figure 4A, SPHK1 overexpression significantly upregulated the expression of TIMP-1 in HCAEC ($p < 0.05$). In contrast, TIMP-1 overexpression showed no significant effects on SPHK1 expression ($p > 0.05$, Figure 4B). These data suggest that SPHK1 is a positive upstream regulator of TIMP-1 in HCAEC.

SPHK1 inhibitor and TIMP-1 silencing reduced the enhancing effects of TGF- β 1 overexpression on cell apoptosis

As shown in Figure 5 SPHK 1 Inhibitor 5C (5 μ M, CATALOG #sc-288340, Santa Cruz Biotechnology) treatment for 1 h or TIMP-1 silencing significantly reduced the enhancing effects of TGF- β 1 overexpression on cell apoptosis ($p < 0.05$). In addition, cell apoptosis showed a similar pattern to the expression pattern of TIMP-1.

Discussion

In this study we found that TGF- β 1 is likely involved in the development of CAD. The action of TGF- β 1 in CAD is likely achieved by inducing cell apoptosis through the upregulation of SPHK1 and TIMP-1. Our study provides a potential diagnostic and prognostic biomarker, as well as a therapeutic target, for CAD.

Increased expression level of TGF- β 1 has been treated as an indicator for the development of certain human diseases. In a recent study, Mou et al. reported that serum levels of TGF- β 1 levels were significantly higher in patients with diabetic nephropathy than those in healthy controls, and the increased TGF- β 1 level in serum can be used to effectively distinguish diabetic nephropathy patients from people with normal physiological conditions [14]. In another study, increased serum TGF- β 1 levels were proved to be responsible for the decreased chemotherapy response of non-small cell lung cancer cells, which is a major cause of unsatisfactory treatment outcomes and poor prognosis of those patients [15]. In another study, serum total TGF- β 1 level was found to be 2 times higher in patients with CAD than in normal healthy controls [11]. However,

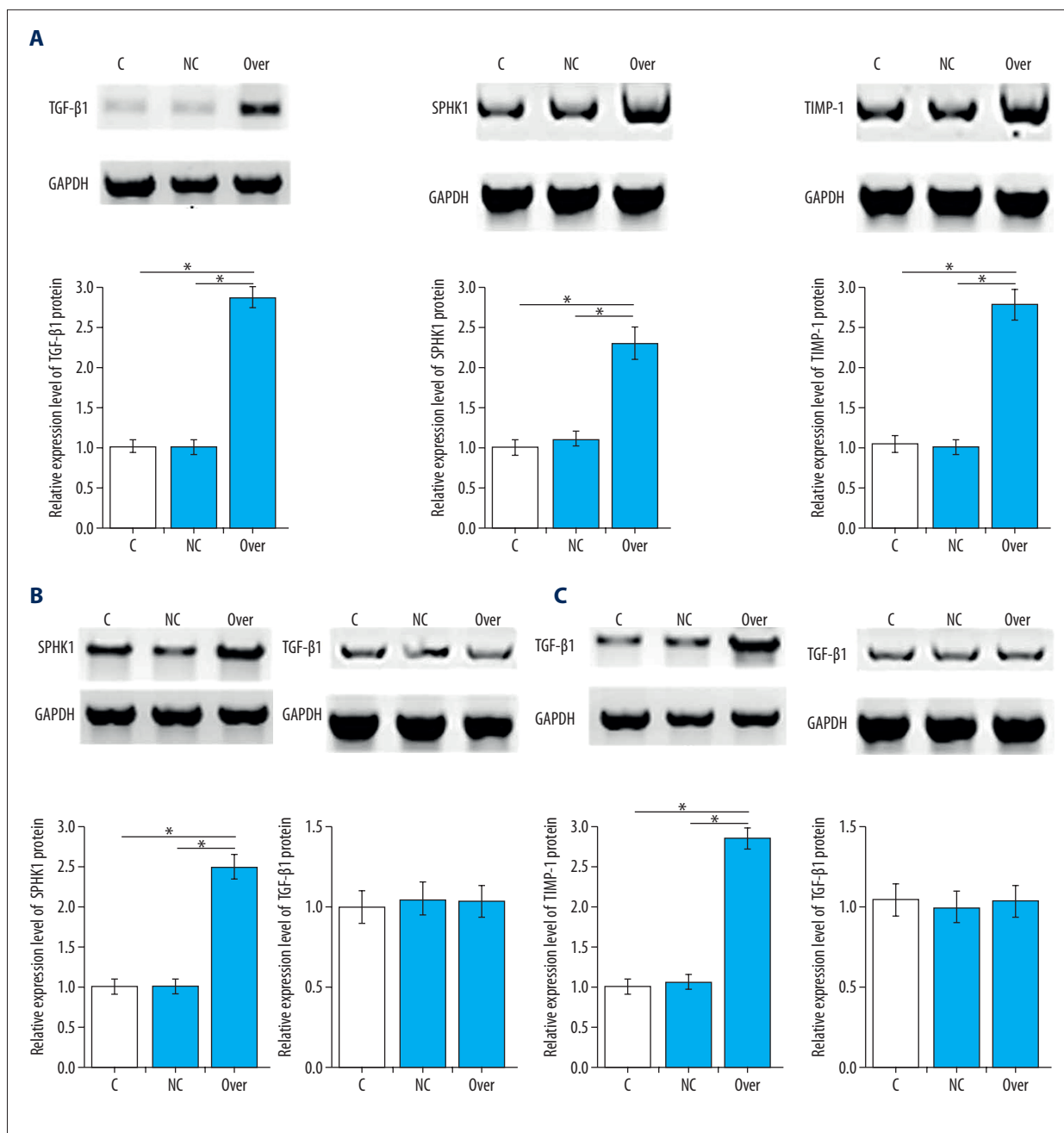


Figure 3. TGF-β1 is a positive upstream regulator of SPHK1 and TIMP-1 in HCAEC. **(A)** Effects of TGF-β1 overexpression on expression of SPHK1 and TIMP-1 in HCAEC; **(B)** Effects of SPHK1 overexpression on expression of TGF-β1 in HCAEC; **(C)** Effects of TIMP-1 overexpression on expression of TGF-β1 in HCAEC. * $p < 0.05$.

one study reported no significant differences in serum total TGF-β1 level between CAD patients and healthy controls, but serum levels of active TGF-β1 were 2 times higher in CAD patients than in healthy controls [16]; consistent with that study, in our study, no significant differences were found between CAD patients and healthy controls in serum levels of total TGF-β1, while serum levels of active TGF-β1 were significantly higher in CAD patients than in healthy controls. In addition,

ROC analysis showed that serum levels of active TGF-β1 but not total TGF-β1 can be used to effectively distinguish CAD patients from normal healthy people.

TGF-β1 is growth factor that plays pivotal roles by affecting cell proliferation, growth, differentiation, and apoptosis [7]. Abnormal expression of TGF-β1 can induce apoptosis of certain types of cells, such as human gingival epithelial cells, through

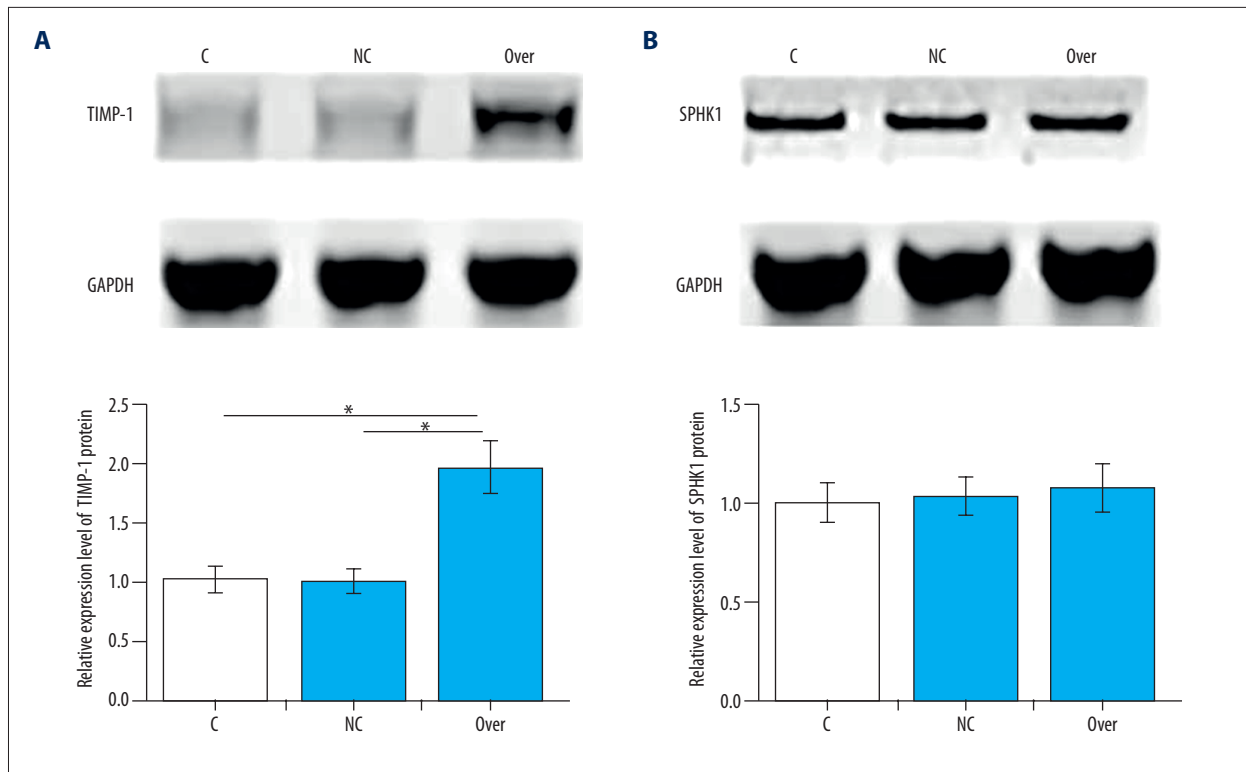


Figure 4. SPHK1 is a positive upstream regulator of TIMP-1 in HCAECs. (A) Effects of SPHK1 overexpression on expression of TIMP-1 in HCAECs; (B) Effects of TIMP-1 overexpression on expression of SPHK1 in HCAECs. * $p < 0.05$.

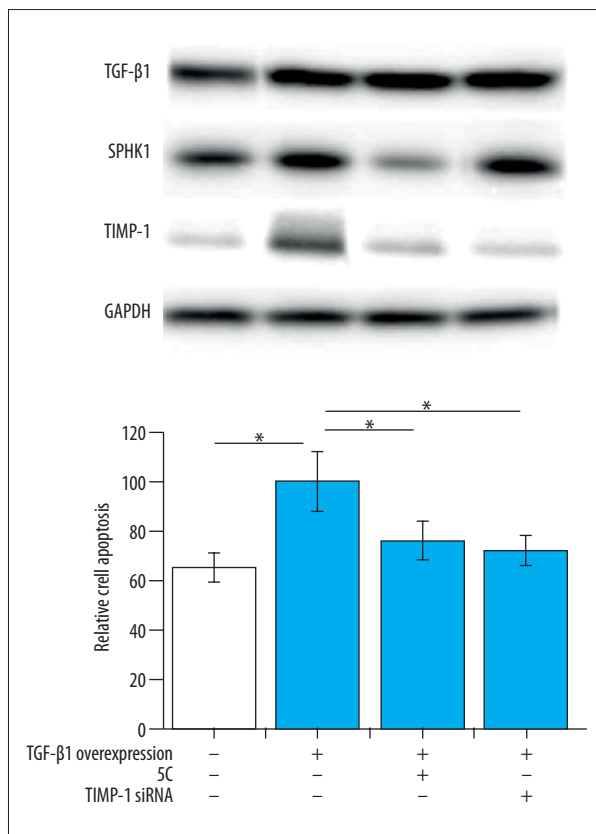


Figure 5. SPHK1 inhibitor and TIMP-1 silencing reduced the enhancing effects of TGF- β 1 overexpression on cell apoptosis. The upper part shows expression of corresponding proteins and the lower part is normalized cell apoptosis. * $p < 0.05$.

multiple pathways [17]. An anti-proliferative role of TGF- β 1 has also been reported in progression of different types of human diseases [7]. Progression of CAD is closely correlated with the apoptosis of heart cells, such as endothelial cells [18]. In our study, TGF- β 1 overexpression significantly promoted apoptosis of HCAEC, while TGF- β 1 siRNA significantly inhibited cell apoptosis. These data suggest that inhibition of TGF- β 1 expression may serve as a target for the treatment of CAD.

Sphingosine kinase 1, or SPHK1, catalyzes the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P) and plays pivotal roles in energy metabolism, which is crucial for the growth and survival of cells [19]. Abnormal expression of sphingosine kinase members has been proved to be involved in the regulation of cell apoptosis [20,21]. TIMP metalloproteinase inhibitor 1 (also known as TIMP1) is a tissue inhibitor of metalloproteinases [22]. The involvement of TIMP1 in cell apoptosis has also been widely studied [23]. It has been reported that TGF- β 1 can regulate the expression of SPHK1 to achieve its biological functions [24]. In addition, SPHK1

mediates the expression of TIMP1 [22]. SPHK1 serves as a mediator of TGF- β signaling to upregulate TIMP-1 expression [25]. Consistent with a previous study [24], in the present study, TGF- β 1 overexpression significantly promoted the expression of SPHK1 and TIMP-1, while SPHK1 and TIMP-1 overexpression showed no significant effects on TGF- β 1 expression. SPHK1 overexpression upregulated the expression of TIMP-1, but TIMP-1 overexpression showed no significant effects on expression of SPHK1. In addition, treatment with SPHK1 inhibitor 5C and TIMP-1 silencing significantly reduced the enhancing effects of TGF- β 1 overexpression on cell apoptosis. These results suggest that TGF- β 1 can promote CAD through the induction of cell apoptosis by upregulating SPHK1 expression and further upregulating its downstream TIMP-1.

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Conclusions

Serum level of TGF- β 1 was higher in patients with CAD than in healthy controls. Serum level of active TGF- β 1 but not total TGF- β 1 can be employed to effectively diagnose CAD. TGF- β 1 overexpression significantly promoted the apoptosis of HCAEC and TGF- β 1 siRNA silencing significantly inhibited the apoptosis of HCAEC. TGF- β 1 overexpression significantly increased the expression levels of SPHK1 and TIMP-1. SPHK1 overexpression upregulated the expression of TIMP-1, but it showed no significant effects on TGF- β 1 expression. TIMP-1 overexpression showed no significant effects on TGF- β 1 and SPHK1. SPHK1 inhibitor and TIMP-1 silencing reduced the enhancing effects of TGF- β 1 overexpression on cell apoptosis. Therefore, we conclude that TGF- β 1 can promote CAD through the induction of cell apoptosis by upregulating SPHK1 expression and further upregulating its downstream TIMP-1.

Conflicts of interests

None.