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PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330
Int J Hum Genet, 24(1): 97-108 (2024)
DOI: 10.31901/24566322.2024/24.01.874

TRIM26 Accelerate Oxidative Stress Injury of Vascular Endothelial Cell in Model of AS by NTH1 Function

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KEYWORDS AS. NTH1. Oxidative Stress. Tripartite Motif 26. Vascular Endothelial Cell

ABSTRACT AS (AS) is an important pathological process of the most common cardiovascular diseases. This study investigated the role and mechanism of TRIM26 in patients with AS. TRIM26 mRNA expression was upregulated in patients and mice with AS. In the mice model, TRIM26 was found to accelerate oxidative stress and AS. Overexpression of TRIM26 induced TH1 expression to promote oxidative stress in the in vitro model through inhibiting NTH1 expression. Conversely, si-TRIM26 induced NTH1 expression to reduce oxidative stress in the in vitro model. TRIM26 protein was found to interact with the NTH1 protein, and TRIM26 increased the ubiquitination of the NTH1 protein. In summary, TRIM26 was found to contribute to oxidative stress injury in AS, particularly under oxidative stress conditions. Therefore, TRIM26 may act as a critical factor in the induction of the AS model.

INTRODUCTION

With changes in social progress and lifestyle, the incidence and early onset of Atherosclerosis (AS) have shown an upward trend (Kong et al. 2021). AS is a chronic inflammatory disease. At present, the total population of cardiovascular disease alone in the world has exceeded 587 million. In clinical practice, the treatment for AS mainly includes drug therapy and revascularization therapy. However, due to the complex pathogenesis of AS, existing treatment methods focus on improving clinical symptoms and reducing the risk of adverse events, and there is no radical cure available. AS can cause diseases like stroke and myocardial infarction (Martos-Rodríguez et al. 2021), making the active treatment of AS crucial for preventing and treating these diseases (Mohammadkarim et al. 2021). AS is a chronic vascular inflammation mediated lesion. AS is a disease caused by cholesterol accumulation. AS involves the changes of various cell physiological states.

Cardiovascular and cerebrovascular diseases rank first in morbidity and mortality in developed Western countries (Cheng et al. 2017), and AS is closely associated with oxidative stress (Kattoor et al. 2017). ROS can lead to damage in DNA, RNA, protein and lipids, causing cell dysfunction of related illnesses (Förstermann et al. 2017; Khosravi et al. 2019). Oxidative stress refers to a pathological process in which the body is stimulated to increase ROS in the body, leading to an imbalance between the oxidation and antioxidant systems, thereby damaging tissue cells (Zhang et al. 2022). ROS includes superoxide, hydrogen peroxide, hydroxyl radicals, peroxy radicals, singlet oxygen, and ozone. There are multiple ROS enzyme production systems in the vascular wall, including reduced coenzyme II (NADPH) oxidase (NAPP Hoxidase, NOX), xanthine oxidase Mitochondrial respiratory chain enzymes and uncoupled endothelial nitric oxide synthase (eNOS). Moderate concentrations of ROS play an important signaling role under physiological conditions (Liu et al. 2022). Endothelial dysfunction is a key early stage of atherosclerotic lesion formation (Cheng et al. 2017). After endothelial cell injury, low-density lipoprotein enters the subendothelium and is oxidised, promoting monocyte migration and adhesion, promoting foam cell formation, and accelerating the progression of AS (Cheng et al. 2017). At present, the related factors of carotid AS are the focus of clinical research.

A large amount of oxidative free radicals can aggravate the oxidative stress damage of endothelial cells, and also regulate downstream nuclear transcription factor (NF-êB) signalling pathways to promote cell apoptosis (Kattoor et al. 2017).

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(Kattoor et al. 2017). ox-LDL participates in the pathogenesis of AS by damaging vascular endothelial cells, directly targeting vascular endothelial cells, promoting mitochondrial apoptosis pathway, and promoting upregulation of ROS levels to induce endothelial cell apoptosis (Kattoor et al. 2017). Vascular endothelial cells are monolayer endothelial cells arranged along the surface of the vascular lumen, separating the vascular lumen from vascular smooth muscle. Endothelial cell integration stress response (ISR) caused by complex stress environment in cardiovascular system and endothelial cell injury caused by inflammatory reaction are the causes of AS. Compared with mitochondria in muscle tissue cells, the primary function of mitochondria in endothelial cells is to participate in signal transduction and maintain the integrity of cell function, followed by energy metabolism. Mitochondrial oxidative stress is an important cause of mitochondrial damage in endothelial cells, which leads to abnormal intracellular signal transduction and metabolic disorders, leading to severe cell damage.

The tripartite motif (TRIM) family is part of the E3 ubiquitin ligase family (Zhao et al. 2021). An overview of the TRIM family's functions and significant molecular regulatory mechanisms in DNA receptor-mediated innate signalling pathways can offer novel insights into regulating innate immune signalling pathways (Xie et al. 2022).

TRIM26 plays an important role in respiratory diseases, autophagy, innate immunity, and tumor development and as a susceptibility gene for schizophrenia. Previous literature has reported that TRIM26 targets the degradation of IRF3 in the nucleus and negatively regulates antiviral responses. TRIM26 promotes the formation of TBK1 NEMO complex through ubiquitination at its K27 position. TRIM26 and Mule simultaneously catalyze the ubiquitination of NEIL1 and induce its degradation. Recent studies have shown that TRIM26 mediates ubiquitination at K27 of HCV Viral protein NS5B, thereby promoting HCV replication and proliferation.

Objectives

This study assessed the impact of TRIM26 in individuals with AS and its potential mechanisms.

METHODOLOGY

Animals and Drugs

Patients with AS and normal healthy volunteers were obtained at Changzhou Wujin People's Hospital. This study was approved by the Ethics Committee of Changzhou Wujin People's Hospital. Peripheral blood from patients or healthy volunteers was collected.

Mice Experiment

The ApoE"/" mice (Institute of Model Animals, Nanjing University) were kept at 23± 1°C and humidity of 55 to 60 percent. The study protocol was approved by the ethics committees of Changzhou Wujin People's Hospital.

Mice were fed with a HFD-high-fat diet (10% lard, 4% milk powder, 2% cholesterol, and 0.5% sodium cholate) for 12 weeks. Next, WT and TRIM26 -/- mice were fed with a HFD-high-fat diet (10% lard, 4% milk powder, 2% cholesterol, and 0.5% sodium cholate) for 12 weeks. Then, mice were anesthetised with 50 mg/kg pentobarbital sodium and killed for measurements of aortic lesion size and biochemical analysis.

Quantitative PCR

Total RNA was extracted using a TRIZOL reagent (Life Technologies Inc.). qRT-PCR assays were performed using LightCycler® 480 SYBR Mix (Roche, Germany). The expression levels of mRNA was normalised to the GAPDH expression using the 2–ÄÄct method.

Cell Culture and RNA Interference

Human aortic endothelial cells (HAECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA), grown in Endothelial Cell Medium (ECM, Carlsbad, CA, USA) supplemented with 10 percent FBS, 1 percent (v/v) penicillin/streptomycin, and 1 percent endothelial cell growth factors at 37°C with 5 percent CO2 and 95 percent air. Plasmids were transfected into HAECs using Lipofectamine 2000. The cells were exposed to 60 ìg/mL of ox-LDL after transfection for 24 hours.

ELISA Kits Experiment

SOD, MDA, GSH, GSH-px and ROS levels were performed according to the instructions of the ELISA kit (Beyotime Institute of Biotechnology, China). The absorbance value was quickly read using the microplate reader at a detection wavelength of 450 nm. ROS level was measured with 2,7-dichlorodihydrofluorescein diacetate (DCF-DA; Sigma-Aldrich, St. Louis, MO).

Western Blot Analysis

Total protein was extracted using Radio Immunoprecipitation Assay (RIPA) and PMSF reagent (Beyotime, Beijing, China). Protein lysates were separated based and transferred onto a Polyvinylidene Fluoride (PVDF, Millipore) membrane. The membrane was incubated with ant-TRIM26 antibody, anti-NTH1 antibody and anti-â-actin antibody at 4°C overnight. Membranes were incubated with the secondary antibody for 2 hours at room temperature. The bound antibodies were detected using enhanced chemiluminescence (ECL) with â-actin used as a control.

Statistical Analysis

Graphad Prism 6 was used for the statistical analysis. All values are expressed as means ± SEM unless specified. $P < 0.05$ was considered statistically significant. The differences between groups were analysed using Student's *t*-test.

RESULTS

TRIM26 Expression in Patients with AS and Mice Model of AS

To investigate TRIM26's role in the development of AS, the expression of TRIM26 in patients with AS and a mouse model of the disease was examined. The results showed that TRIM26 expression was significantly increased in the serum of AS patients compared to the control group (1.234±0.825 vs. 5.810±4.846, p<0.05) (Fig. 1A). In addition, there was a positive correlation between serum TRIM26 mRNA and IL-1â levels in AS patients, with a ROC value of 0.9549 (Figs. 1B-1C). Furthermore, both TRIM26 mRNA (1.014 ± 0.172 vs. 5.457 ± 0.917, p<0.01) and protein (1.173 ± 0.248 vs. 3.267 ± 0.046, p<0.01) were upregulated in mice with AS (Figs. 1D-1E).

TRIM26 Accelerated Oxidative Stress Injury of Vascular Endothelial Cells in AS Model

Next, the study then investigated the possible function of TRIM26 in an AS model. In vitro experiments using TRIM26 mimics and si-TRIM26 were performed to regulate TRIM26 mRNA expression. TRIM26 mimics increased the level of TRIM26 mRNA (1.028 ± 0.253 vs. 7.332 ± 2.892, p<0.01), leading to an increase in ROS production (1 ± 0.056825757 vs. 2.2125 ± 0.133853153, p<0.01) and MDA level (62.16666667 ± 2.778888667 vs. 133.6666667 ± 6.98013053, p<0.01), as well as a decrease in cell growth level (1.31 ± 0.08 vs. 0.65 ± 0.02, p<0.01), SOD level, GSH-PX level, and GSH level (85.66666667 ± 5.792715732 vs. 20.66666667 ± 1.247219129; 283.3333333±6.236095645 vs. 163.3333333±43.65266951;45.33333333± 4.921607687 vs. 13.33333333±1.699673171; all p<0.01) in an in vitro AS model (Fig. 2A-2G, Table 2). On the other hand, si-TRIM26 inhibited the expression of TRIM26 mRNA (1.008±0.128 vs. 0.405 ±0.037 vs. 0.499 ± 0.077 vs. 0.34±0.056, p<0.01), ROS production (1.004219409 ± 0.068816483 vs. 0.641350211 ± 0.285364362, p<0.01), and MDA level (61.5 ± 12.57643299 vs. 18.83333333 ± 3.324989557, p<0.01), while promoting cell growth (0.86 ± 0.04 vs. 1.72 ± 0.04, p<0.01) as well as SOD level (92 ±5.354126135 vs. 230.3333333 ± 4.988876516, p<0.01), GSH-PX level (303.3333333±41.29837231 vs. 713.3333333± 33.24989557, p<0.01), and GSH level (49.66666667± 7.039570694 vs. 128.3333333 ± 8.178562764, p<0.01) in an in vitro AS model (Fig. 2H-2N).

TRIM26 Accelerated AS in Mice Model

To further investigate the role of TRIM26 in AS, researchers induced the disease in TRIM26-/- mice. The results showed that these mice had increased plaque area in the aortic sinus (341.6666667±37.93268892 vs. 651.6666667 ± 12.47219129, p<0.01) and brachiocephalic branch (114.3333333±1.699673171 vs. 228.3333333 ± 4.109609335, p<0.01). Additionally, there was a reduction in thrombus area, total plasma cholesterol (7.866667 ± 0.20548 vs. 3.666667 ± 0.903081, p<0.01), HDL cholesterol (0.508333 ± 0.052652 vs. 0.146667 ± 0.020138, p<0.01), and LDL cho-

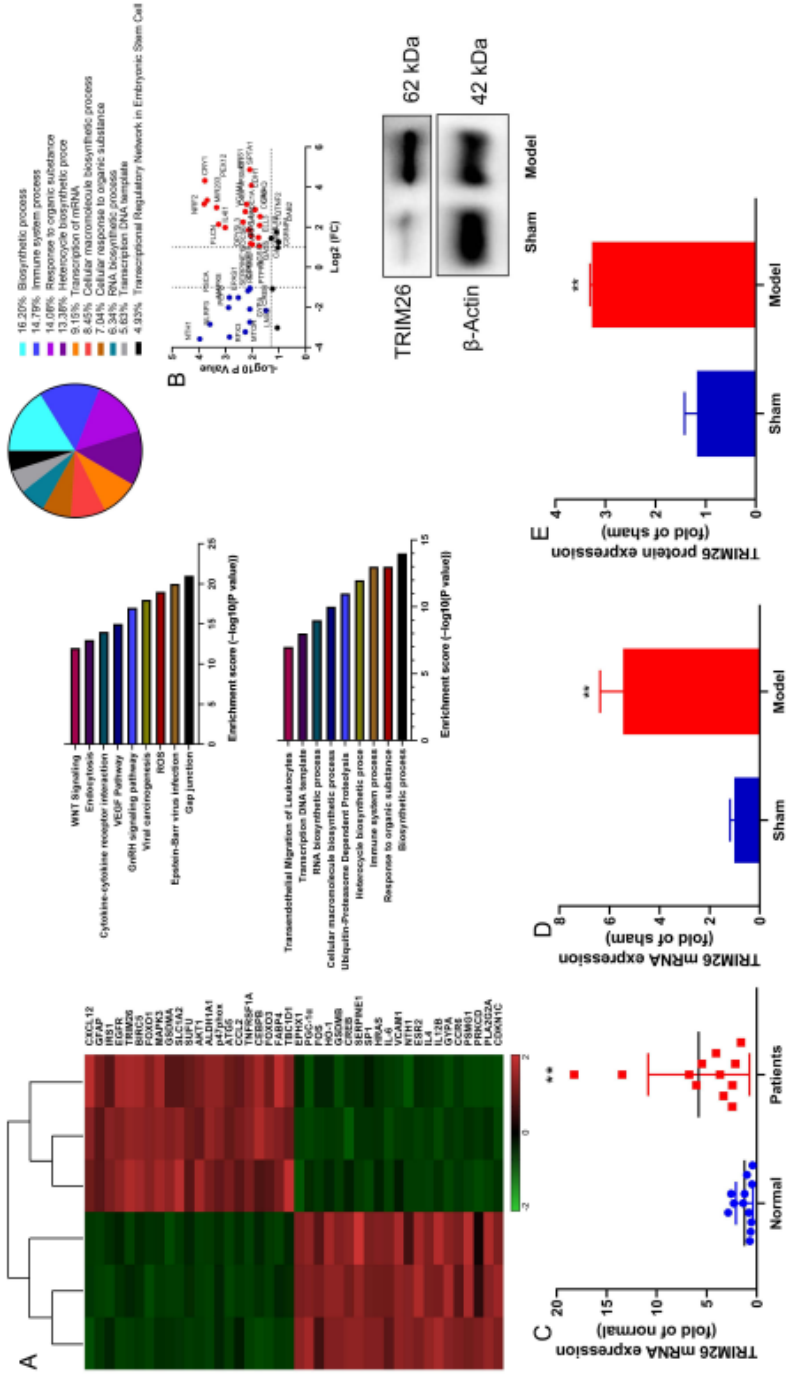

Fig.1. TRIM26 expression in model of AS
Heat map, signaling pathway and analysis results (A), Volcano map (B), TRIM26 mRNA expression (C) in patients with AS; TRIM26 mRNA (D) and protein (E) expression in mice with AS;
****p<0.01 compared with normal volunteers group or sham control group**

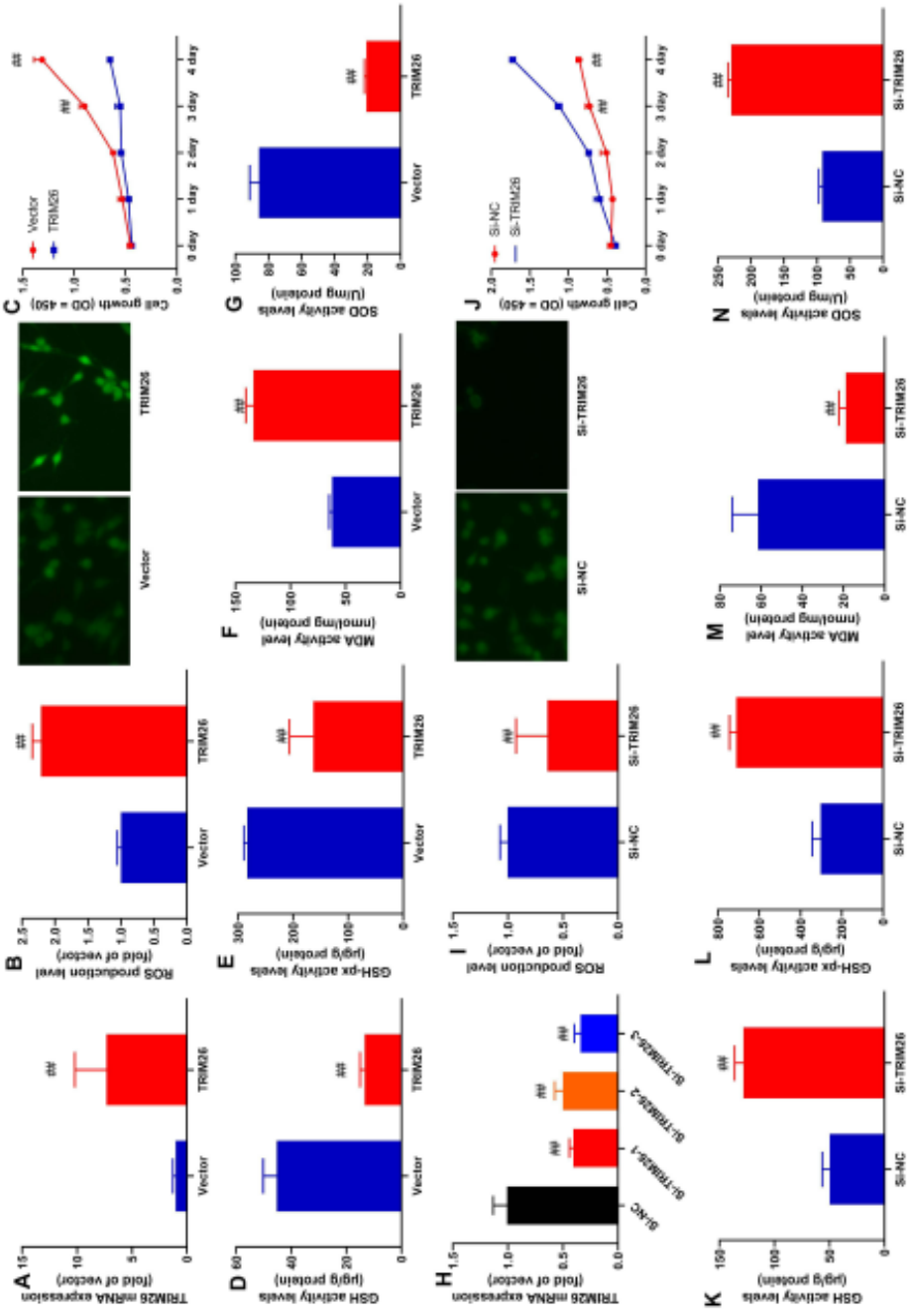

Fig. 2. TRIM26 expression in model of AS
TRIM26 mRNA expression (A), ROS production levels (B), cell growth (C), GSH (D), GSH-px (E), MDA (F), SOD (G) levels in vitro model by TRIM26 group;
TRIM26 mRNA expression (H), ROS production levels (I), cell growth (J), GSH (K), GDH-px (L), MDA (M), SOD (N) levels in vitro model by si-TRIM26 group.
##p<0.01 compared with control negative group or si-negative group

lesterol (9.566667 ± 1.592343 vs. 3.8 ± 0.294392, p<0.01, Fig. 3A-3H). Also, the levels of GSH, GSH-px, and SOD were inhibited (63 ±3.265986324 vs. 130 ± 5.099019514; 341.6666667 ± 37.93268892 vs. 651.6666667 ± 12.47219129; 114.3333333 ± 1.699673171 vs. 228.3333333 ± 4.109609335; all p<0.01), while MDA level was induced (64.5 ± 3.265986324 vs. 21.33333333 ± 3.681787006, p<0.01), as shown in Figures 3G-3J.

TRIM26 Regulated NTH1 Function in Mice Model of AS and In Vitro Model of AS

The study validated TRIM26's role in an AS model, where the overexpression of TRIM26 plasmid at 24 and 48 hours suppressed the mRNA expression levels of NTH1 in the in vitro AS model (1.098 ± 0.427 vs. 0.599 ± 0.377 vs. 0.276 ± 0.116 vs. 0.147 ± 0.028, p<0.01) (Fig. 4A). Additionally, TRIM26 plasmid reduced NTH1 protein expression levels (1.134± 0.144 vs. 0.394 ± 0.044, p<0.01) while inducing TRIM26 protein expression (1.159 ± 0.151 vs. 2.449±0.399, p<0.01) (Fig. 4B-4C). Next, si-TRIM26 mimics increased NTH1 mRNA expression levels (1.007± 0.12 vs. 1.055± 0.15 vs.3.868± 0.103 vs. 7.037 ± 1.675, p<0.01) at 24 and 48 hours in the *in vitro model of AS* (Fig. 4D). Si-TRIM26 mimics suppressed TRIM26 protein expression (1.004 ± 0.017 vs. 0.294 ± 0.056, p<0.01), and induced NTH1 protein expression levels (1.276 ± 0.206vs.3.353± 0.391, p<0.01) in the *in vitro model of AS* (Fig. 4E-4F, Table 4). There was the inhibition of NTH1 mRNA (1.009± 0.137 vs. 8.965 ± 2.313, p<0.01) and protein expression levels (1.219 ± 0.178 vs. 3.311 ± 0.042, p<0.01) in TRIM26$^{-/-}$ mice model of AS (Fig. 4G-4H). Furthermore, NTH1 mRNA and protein expression levels were inhibited in TRIM26-/- mice model of AS. The study also confirmed the TRIM26 and NTH1 protein association using Co-IP, and overexpression of TRIM26 led to increased NTH1 ubiquitination, while down-regulation of TRIM26 reduced NTH1 ubiquitination in the in vitro AS model.

TRIM26 Regulated NTH1 Function in Mice with AS

In an in vitro model, TRIM26 increased the expression of NTH1 (3.434 ± 0.221 vs. 1.119 ± 0.16, p<0.01), resulting in a decrease in NTH1 protein expression. Si-NTH1 inhibited the expression of NTH1 (1.02 ± 0.07 vs. 0.335 ± 0.076, p <0.01) induced by the overexpression of TRIM26, leading to a reduction in NTH1 protein expression. The transfection of NTH1 plasmid resulted in an increase in ROS production (0.99 ± 0.065319726 vs. 0.303333333 ± 0.054365021, p<0.01) and MDA level (65.33333333 ± 3.472111109 vs. 16.66666667 ± 1.027402334, p<0.01), and a reduction in cell growth (2.05 ± 0.09 vs. 1.26 ± 0.04, p<0.01), SOD level (173.3333333 ± 14.05544576 vs. 107.6666667 ± 5.436502143, p<0.01), GSH-PX level (690 ± 32.40370349 vs. 298.3333333 ± 23.92116682, p<0.01), and GSH level (88.33333333 ± 3.681787006 vs. 56.33333333 ± 5.734883511, p<0.01) through the overexpression of TRIM26 (Fig. 5A-5H, Table 5). Si-NTH1 reduced ROS production (1.810526316 ± 0.070350658 vs. 1.010526316 ± 0.060162906, p<0.01) and MDA level (125.3333333 ± 3.299831646 vs. 24 ± 1.779513042, p<0.01), and increased cell growth (0.65 ± 0.02 vs 1.31 ± 0.08, p<0.01), SOD level (38.33333333 ± 4.109609335 vs. 112 ± 6.377042157, p<0.01), GSH-PX level (150 ± 17.79513042 vs. 381.6666667 ± 46.42796092, p<0.01), and GSH level (24 ± 4.546060566 vs. 84 ± 4.320493799, p<0.01) through the overexpression of TRIM26 (Fig. 5I-5N).

DISCUSSION

AS is a common disease characterized by the formation of atherosclerotic plaques in arterial intima, and it is also a common cause of cardiovascular critical disease. AS is a chronic inflammatory disease characterized by lipid streaks, fibrous plaques and atherosclerotic plaques. Cardiovascular disease caused by AS is still the most important cause of death in the world. At present, the pathogenesis of AS has not been clarified. The formation process of AS is mainly the damage of vascular endothelial cells leading to the decline of endothelial cell function, the formation of early lipid streaks and atherosclerotic plaques with lipid rich necrotic cores, the proliferation of fibrous tissue and calcium salt deposition leading to vascular stenosis or blockage, and the degradation, thinning and instability of atherosclerotic plaque fiber caps lead to plaque rupture and thrombosis (Patel et al. 2021). Lipid infiltration, platelet activation, thrombosis, intimal damage, inflammatory responses are

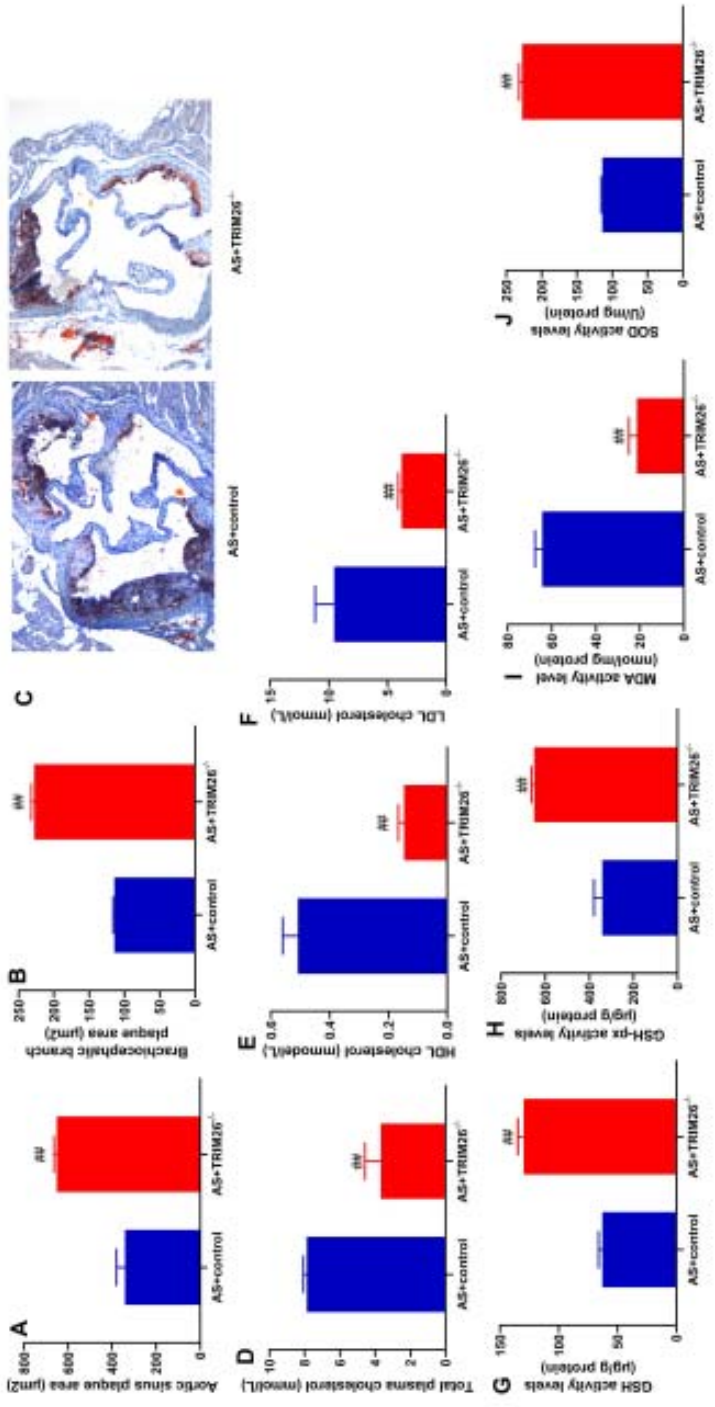

Fig. 3. TRIM26 accelerate AS in mice model
Aortic sinus plaque area (A), brachiocephalic branch plaque area (B), AS (C), total plasma cholesterol (D), HDL level (E), LDL level (F), GSH (G), GDH-px (H), MDA (I), SOD (J) levels
AS+control, WT mice model of septocemia; AS+TRIM26-/-, TRIM26 mice model of septocemia with Jmjd3.
##p<0.01 compared with WT mice model of septocemia group

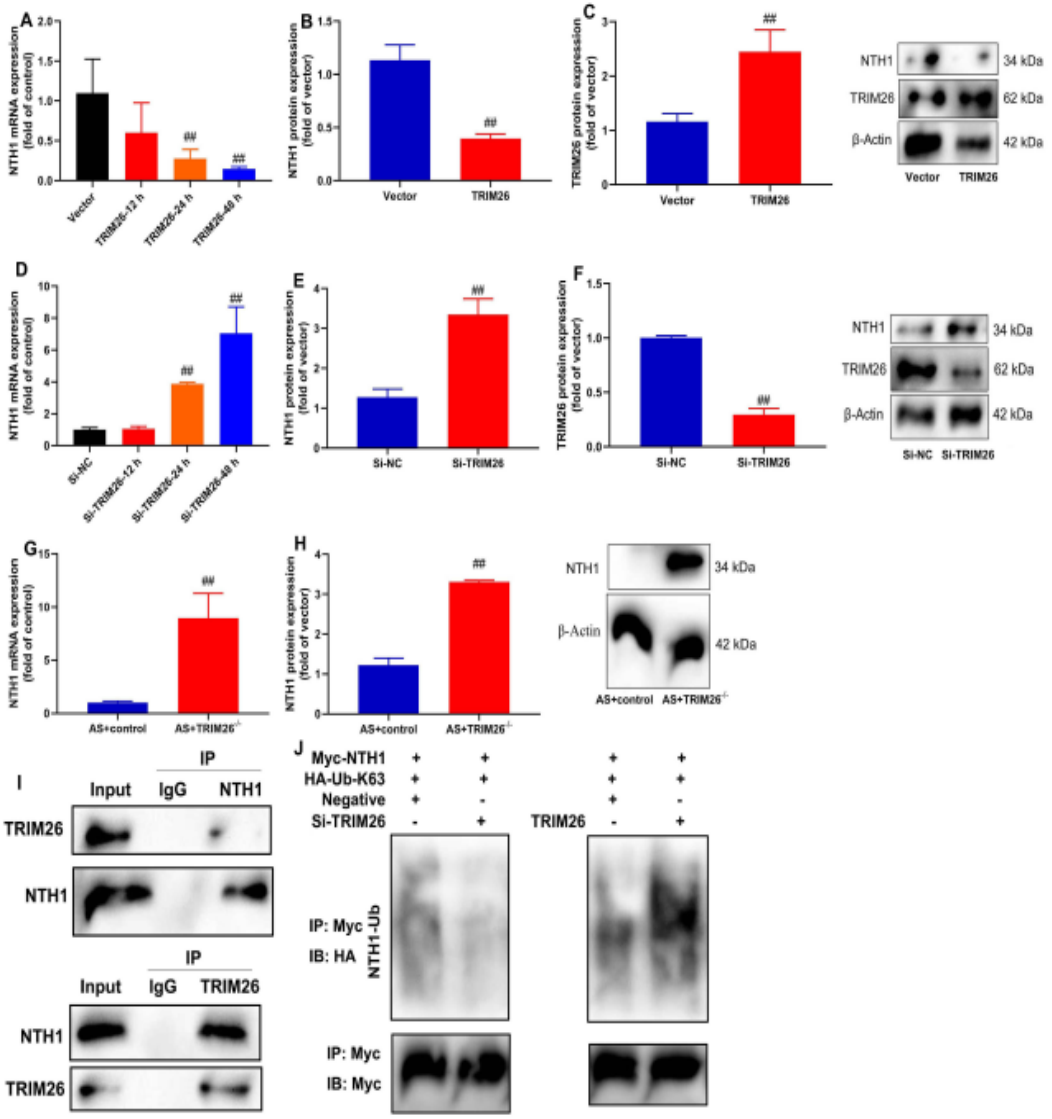

Fig. 4. TRIM26 regulated NTH1 function in mice of AS
NTH1 mRNA expression (A), NTH1 and TRIM26 protein expression (B and C) in vitro model of AS by TRIM26;
NTH1 mRNA expression (D), NTH1 and TRIM26 protein expression (E and F) in vitro model of AS by si-TRIM26;
NTH1 mRNA and protein expression (G and H) in mice with AS;
The association between NTH1 and TRIM26 proteins by Co-IP (I);
NTH1 ubiquitination in vitro model (J).
AS+control, WT mice model of septocemia; AS+TRIM26-/-, TRIM26 mice model of septocemia with Jmjd3
##p<0.01 compared with control negative group or si-negative group
##p<0.01 compared with WT mice model of septocemia group

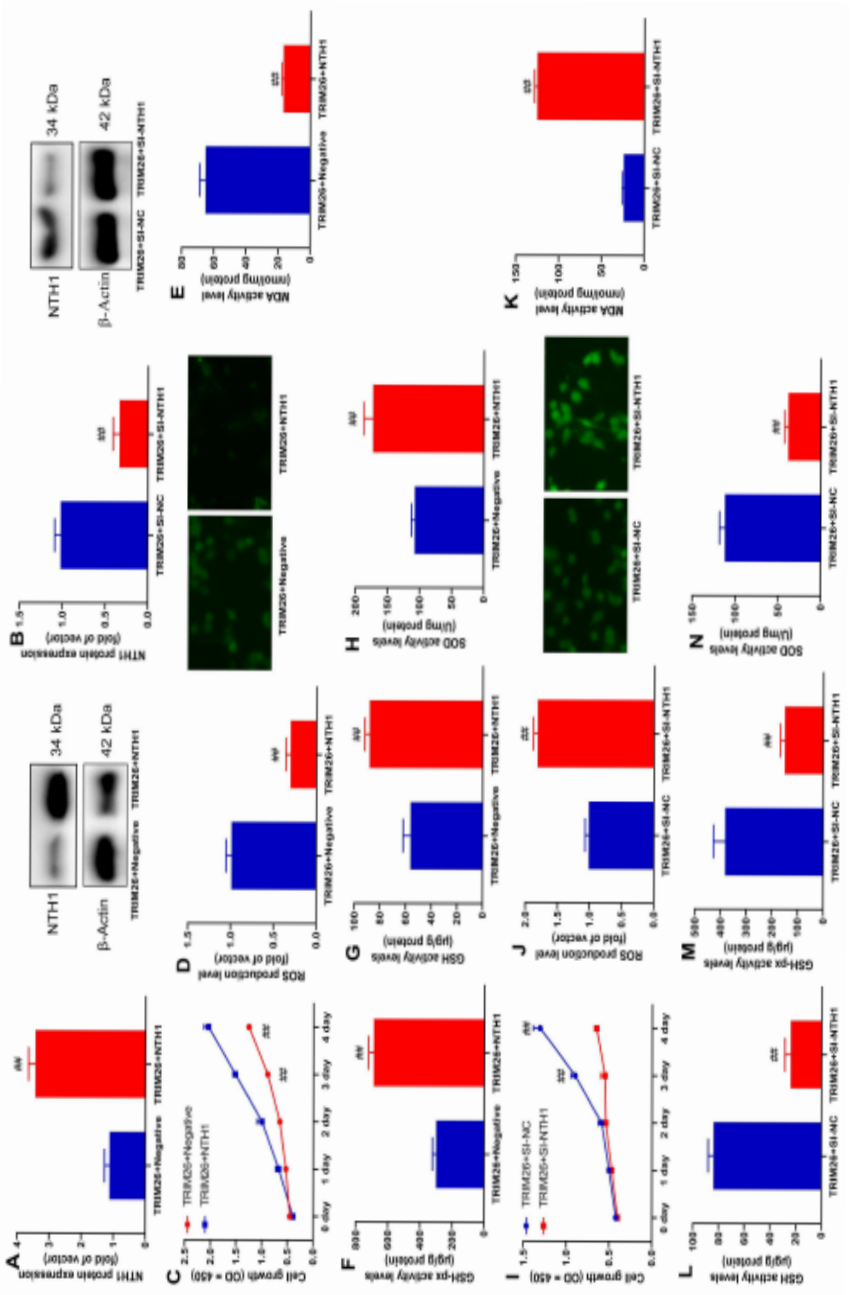

Fig. 5. NTH1 control the effects of TRIM26 in model of septicemia
NTH1 protein expression in vitro model of TRIM26 and NTH1 (A)/ TRIM26 and si-NTH1 (B);
Cell growth (C), ROS production (D), MDA (E), GDH-px (F), GSH (G), SOD (H) levels in vitro model of TRIM26 and NTH1;
Cell growth (I), ROS production (J), MDA (K), GDH-px (L), GSH (M), SOD (N) levels in vitro model of TRIM26 and si-NTH1.
##p<0.01 compared with control negative group or si-negative group

the development of AS (Sanna et al. 2021). Lipid deposits on the vascular wall, triggering inflammation and autophagic apoptosis of AS related cells. Studies have shown that macrophages phagocytize large amounts of lipids and transform into foam cells rich in lipids, which can produce reactive oxygen species and induce inflammation, thereby promoting the progression of AS (Yan et al. 2021). The pathological mechanisms mainly include endothelial dysfunction, differentiation of monocytes into macrophages and formation of foam cells (Wei et al. 2021).

In summary, this study observed increased serum TRIM26 expression in patients with AS. Additionally, both mRNA and protein levels of DCAF1 were up-regulated in mice with AS. Dhawan et al. (2021) indicated that the expression of TRIM26 was increased in model of HSV-2 infection. The expression of DCAF1 mRNA and protein were up-regulated in mice with AS. Zhu et al. (2021) showed that TRIM26 expression levels were increased in the model of liver fibrosis. Taken together, these results suggest a gradual up-regulation of TRIM26 in AS.

Research has shown that oxidized low density lipoprotein (ox-LDL) can participate in AS by promoting cell apoptosis, regulating miRNA, activating platelets, inducing mitochondrial dysfunction, damaging endothelial cells, inducing macrophage oxidative stress, and promoting inflammatory response. In recent years, the correlation between ox-LDL and AS has become a research hotspot, and ox-LDL has gradually gained scholars' attention by acting on organelles, ribonucleic acids, and cell receptors to participate in the process of AS (Wu et al. 2018; Yuan et al. 2019). AS is characterized by lipid accumulation in carotid intima, which leads to hardening and thickening of vascular wall and formation of plaque. When the lesion of carotid AS narrows to a certain extent or the plaque ruptures, it may lead to thrombosis, thus blocking the carotid artery or its branches, which may lead to stroke. AS is a chronic cardiovascular disease characterised by lipid deposition-driven and plaque accumulation in the arterial wall, leading to arterial stenosis, and is an important factor causing high mortality and disability rates in patients (Wu et al. 2018; Yuan et al. 2019). The formation and development of atherosclerotic plaques involve multiple cells and molecules and are a complex chronic degenerative process (Zhu et al. 2021).

AS is a chronic cardiovascular disease driven by lipid deposition and characterized by the accumulation of atherosclerotic plaques in the arterial wall leading to arterial stenosis, which is an important factor leading to high mortality and disability rates of patients (Wang et al. 2019). The study found that TRIM26 accelerates oxidative stress injury of vascular endothelial cells in an AS mouse model. This was supported by Zhu et al.'s (2021) findings that TRIM26 induces ferroptosis and ROS levels in a model of liver fibrosis, and Williams and Parsons' (2018) report that TRIM26 regulates oxidative stress. Overall, the results show that TRIM26 enhances the oxidative stress injury of vascular endothelial cells in an AS model.

Suppression of the Nth1 gene increases backup repair activity for oxidative DNA lesions (Jun et al. 2020; Williams and Parsons 2018). The study revealed that TRIM26 plasmid reduced NTH1 protein expression levels and increased NTH1 ubiquitination in vitro in an AS model. Williams and Parsons (2018) demonstrated that TRIM26 regulates NTH1 for the cellular response to oxidative stress. Together, the findings suggest that TRIM26 inhibits NTH1 protein expression levels by promoting NTH1 ubiquitination in an AS model.

CONCLUSION

In conclusion, TRIM26 accelerates oxidative stress injury of vascular endothelial cells in an AS model through NTH1 inhibition. Therefore, the overexpression of TRIM26 may represent a potential therapeutic target for reducing excessive oxidative stress injury in AS.

RECOMMENDATIONS

To treat AS, the active suppression of TRIM26 expression should be considered as a therapeutic approach to prevent and manage the disease.

AUTHORS' CONTRIBUTIONS

Ruke Tang was dedicated to the study design, and manuscript editing; Jianguo Ji was contributed to the guarantor of integrity of experimental studies, data analysis, manuscript

preparation, statistical analysis and manuscript review; Hao Ding, Guoping Zhu and Yong Li carried out the data acquisition. All authors approved this paper.

CONSENT FOR PUBLICATION

This study was approved by the Ethics Committee of Changzhou Wujin People's Hospital.

DATA AVAILABILITY STATEMENT

All data analysed during this study are included in this paper and its online supplementary files. Further enquiries can be directed to the corresponding author

ACKNOWLEDGEMENT

N/A

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The written informed consents were obtained from all the subjects and this study was approved by the Ethics Committee of Changzhou Wujin People's Hospital.

CONFLICT OF INTEREST

The authors declare that they have no competing interest.

FUNDING

N/A

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Paper received for publication in August, 2022
Paper accepted for publication in June, 2023