The Acid Sphingomyelinase Inhibitor Amitriptyline Ameliorates TNF-α-induced Endothelial Dysfunction via the MAPK Signaling Pathway

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Research Article

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Abstract

Purpose

Inflammation associated endothelial cell (ECs) dysfunction is key to atherosclerotic disease. The soluble endo-lysosomal protein, acid sphingomyelinase (ASMase) plays a crucial role in atherosclerosis. However, the mechanism by which ASMase regulates the inflammatory reaction in ECs remains unknown. Numerous studies have demonstrated that in addition to the antidepressant effects, amitriptyline can also effectively inhibit ASMase. Thus, the aim of this study was to investigate whether the effects of amitriptyline protect against inflammation in TNF-α-induced ECs.

Methods

HUVEC were pre-incubated with 2.5 μM amitriptyline (1 h) and subjected to 23 h TNF-α (20 ng/ml). EdU, tube formation, transwell and monocyte adhesion assay were performed to investigate the endothelium function. Vascular tone measurement was used to detected endothelial-dependent relaxation in mice. Protein levels of ICAM-1, VCAM-1, MCP-1, MAPK and NF-κB were detected using western blot.

Results

We demonstrated that preconditioning with the amitriptyline down-regulated TNF-α-induced expression of proinflammatory proteins including ICAM-1, VCAM-1, and MCP-1 in ECs, as well as the secretion of sICAM-1 and sVCAM-1. Additionally, we demonstrated that amitriptyline suppressed TNF-α-induced MAPK phosphorylation as well as the activity of NF-κ B in HUVEC. Results also indicated that with treatment of TNF-α, the aorta of ASMase$^{-/-}$ mice showed elevated endothelial-dependent relaxation compared to wild-type counterparts.

Conclusion

These results suggest that the AMI reduces endothelial inflammation, consequently improving vascular endothelial function. This study showed that amitriptyline might protect ECs from TNF-α-induced inflammation. Thus, identification of amitriptyline as a potential strategy to reverse endothelial function is of importance to preventing vascular diseases.

1. Introduction

Atherosclerosis (AS) is known as a chronic inflammatory disease of the endarterium, and the pathogenic mechanism of atherosclerosis refers to expression of cytokine/chemokine, activation of pro-inflammatory signal pathways and increased oxidative stress [1-3]. Endothelial cells (ECs), as the innermost layer in all vessels, is crucial in regulating tissue homeostasis by controlling the extravasation

of circulating cells into tissues. This is achieved by endothelial cell-driven alteration of cytokine, chemokine, and adhesion molecule production and controlling the movement of immune cells into the site of injury [4, 5]. ECs targeted by the pro-inflammatory TNF-α are regulated in multiple functions including adhesion, thrombosis, and the inflammatory response [6-12]. Endothelial dysfunction promotes adhesion and migration of macrophage, which are widely recognized as critical to the initiation and progression of atherosclerotic lesions[13, 14].

Sphingolipids are key structural cell membrane lipid compounds. Ceramide (CER) is generated from sphingomyelin (SM) which hydrolyzedby sphingomyelinase (SMase), which triggers a cascade of bioactive lipids. SMase activation occurs in different types of cardiovascular cells such as cardiomyocytes(CMs), ECs, and vascular smooth muscle cells (VSMCs) to mediate cell proliferation, cell death, cardiac fibrosis[15], and contraction of cardiomyocytes [16, 17].

SMaseincludes three isoforms based on their optimum pH (acid, neutral, and alkaline) [18]. The direct metabolitesof SMase are recognized as second messengers in various specific biological activities such as oxidized lipoproteins (ox-LDL)-induced cell proliferation [19, 20] and TNF-α[21, 22] induced expression of adhesion molecules.ASMaseis expressed by most human tissues, and is mediated by proinflammatory cytokines, lipopolysaccharide (LPS), and cytotoxic agents [23-25]. Blocking the production of CER by suppressing ASMaseis a potential therapeutic strategy against cardiovascular diseases[26, 27]. Numerous studies have shown that different types of SMase inhibitors, as well as SMase deficiency, can relieve atherosclerosis symptoms [24, 28].Inhibition of ASMase has been used as a treatment against ischemia reperfusion injury and atherosclerosis[16]. However, the design of bioavailable specific ASMase inhibitors remains a challenge.

Amitriptyline (AMI) is a secondary amine tricyclic antidepressant that mainly inhibits noradrenaline uptake and lysosomal acid sphingomyelinase(ASMase)[29]. Zhongyang Lu etal. demonstrated that AMI is an effective inhibitor in both nonalcoholic steatohepatitis (NASH) in LDL receptor-deficient ($LDLR^{-/-}$) mice with type 2 diabetes mellitus (T2DM)[30]. However, the underlying mechanism by which AMI attenuates atherosclerosis remains undiscovered.

In this study, we aimed to determine whether AMI or ASMase knockout could upregulate the phosphorylation of endothelial nitric oxide synthase (eNOS) and activity of ECs *in vivo* and *in vitro,* inhibit TNF-α induced reactive oxygen species (ROS) production, inflammation, and cell adhesion. We also explored the underlyingmechanisms.

2. Materials And Methods

2.1. Animals

Procedures of animal handing and experiments were in accordance with the NIH regulations for the care and use of animals in research, and were approved by the Animal Ethics and the Use Committee of the

Second Affiliated Hospital of Guangzhou Medical University. The 8–12-week-old wild-type (WT) C57BL/6J male mice were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). ASMase knockout ($ASMase^{-/-}$) mice on a C57BL/6 background were obtained from Cyagen Biosciences Inc. As previously described, all the mice were housed in a 12 h dark/light cycle with unlimited access to food and water. After an initial adaptation period, the mice were randomly assigned to five groups: 1. WT mice(Sham group), 2. WT mice with TNF-α treatment (TNF-α group), 3. WT mice with TNF-α and amitriptyline treatment (TNF-α-AMI group), 4. $ASMase^{-/-}$ mice ($ASMase^{-/-}$ group), and 5. $ASMase^{-/-}$ mice with TNF-α treatment ($ASMase^{-/-}$-TNF-α group) (n = 5 per group). The mice in group 3 received 5.0 mg/kg of amitriptyline by intragastric administration for 30 days. During the last four days, mice in groups 2, 3, and 5 were intraperitoneally injected with recombinant mouse TNF-α protein (50349 Sino Biological, Beijing, China) at a dose of 30 mg/kg body weight for 4 consecutive days. Saline was used as a negative control during the same period. After the experiment, all mice were fasted for 12 h and anesthetized by inhalation of 2.0% isoflurane followed by cervical dislocation.

2.2. Vascular tone measurement

The thoracic aortic rings (2–3 mm in length) were removed immediately after the mice were sacrificed and surrounding tissues gently removed and immersed in oxygenated modified Physiological Saline Solution (PSS) containing (in g/L) NaCl, 7.598; $MgSO_4$, 0.29; KCl, 0.35; KH_2PO_4, 0.26; $NaHCO_3$, 1.25; glucose, 1.0; $CaCl_2$, 0.178; and Na_2-EDTA, 0.01 (pH 7.4). The rings were then cut and mounted onto a 4-channel Wire Myograph System (610M; DMT, Aarhus. Denmark) in a 5-mL organ chamber filled with PSS and gassed with 95% O_2 and 5% CO_2 at 37°C. The artery ring was stretched to a resting tension of 9 mN and acclimated for 30 mins; the PSS was replaced every 15 min. The solution was replaced twice with 60 mmol/L of High potassium Physiological Saline Solution (KPSS) containing (in g/L) NaCl, 4.37; $MgSO_4$, 0.29; $CaCl_2$, 0.178; KCl, 4.47; KH_2PO_4, 0.26; $NaHCO_3$, 1.25; glucose, 1.0; and Na_2-EDTA, 0.01 (pH 7.4) to test for maximal contraction. After adequate washing with PSS and equilibration, the aortic tissues were examined for endothelium-dependent relaxation under cumulative concentrations of acetylcholine (10^{-10} to 10^{-6} mol/L) and endothelium-independent relaxation under cumulative concentrations of sodium nitroprusside (SNP, 10^{-11} to 10^{-7} mol/L) after submaximal contraction with phenylephrine (0.1 μmol/L). To explore the potential roles of NOS in changes of relaxation, the NOS blocker L-NAME (10 μmol/L) was added to the bath for 30 min before the application of Ach, and then Ach-induced vasodilatation was re-examined.

2.3. Cell culture and treatment

HUVEC were isolated from human umbilical veins as previously described[31]. Human THP-1 monocytes were grown in suspension at a cell density between 10^5 and 10^6 cells/mL in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. HUVEC were cultured in EBM-2 as described previously [32]. After HUVEC reached 70–80% confluence, they were starved in FBS-free EBM-2 for 12 h and then preincubated with

AMI (0.625–2.5 μM) for 1 h before cells were incubated with TNF-α (20 ng/ml) for 24 h and then subjected to further analysis.

2.4. Acid sphingomyelinase activity assay

An ASMase assay kit (ab190554, Abcam) was used to assay ASMase activity in HUVEC. Briefly, the cells were lysed with 1X Mammalian Cell Lysis Buffer (ab179835, Abcam) and then the samples were reacted with ASMase assay regents according to the protocol recommended by the manufacturer. After the incubation, fluorescence from each sample was detected at Ex/Em = 540/590 nm using a microplate reader.

2.5. Cell viability assay

The viability of HUVEC was determined using the Cell Counting Kit-8 (CCK-8, Dojindo, CK04-11). HUVEC were seeded into 96-well plates and allowed to reach 70% confluence. After treatment with AMI for 24 h, the cells were incubated in 100 μL fresh serum-free medium containing 10 μL CCK-8 reactive solution for 3 h. The absorbance of the supernatant was detected at 450 nm according to the manufacturer's instructions.

2.6. Western blotting

The protein levels of ICAM-1, VCAM-1, MCP-1, phosphor-eNOS, phosphor- transcription factor nuclear factor-κB (NF-κB), phosphor-p44/42 mitogen-activated protein kinase (MAPK), phosphor-SAPK/JNK MAPK, and p-P38 MAPK were analyzed using western blotting analysis. As described previously [33], cells were washed with PBS twice and then lysed in 65 μL ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher, USA). Total protein (10–20*μg*) was separated by SDS-PAGE and transferred to PVDF membranes (0.45*μm, Millipore*, USA). Then, the membranes were blocked with 5% non-fat milk in Tris-buffered saline Tween (TBST) at room temperature for 1 h and incubated overnight at 4°C with primary antibodies for VCAM-1 (1:1000, Cell Signaling Technology, USA, 13662), ICAM-1 (1:1000, Cell Signaling Technology, USA, 67836), MCP-1 (1:1000, Cell Signaling Technology, USA, 39091), phosphor-eNOS (Ser1177, 1:1000, Cell Signaling Technology, USA, 9570), phospho-p44/42 (1:1000, Cell Signaling Technology, USA, 4320), phospho-SAPK/JNK (1:1000, Cell Signaling Technology, USA, 4668), phosphor-P38 (1:1000, Cell Signaling Technology, USA, 4511), phosphor-NF-κB (1:1000, Cell Signaling Technology, USA, 3033), ASMase (1:1000, ABclonal, China, A6743), β-actin (1:1000, Cell Signaling Technology, USA, 4970), and GAPDH (1:1000, Cell Signaling Technology, USA, 3261). After incubation with the secondary anti-rabbit antibody (1:5000, Jackson, USA, 144208), immune complexes were detected with an ECL Western Blotting Substrate (Affinity, USA, KF005). Densitometry was performed using ImageJ (1.52v, USA) software.

2.7. Nitric oxide (NO) detection

The NO level in the supernatant was measured by the nitrate reductase method according to the instructions of the NO determination kit (No. A012, Nanjing Jiancheng, China). Absorbance was detected using a spectrophotometer at a wavelength of 550 nm.

2.8. Proliferation assay

The effects of AMI on cell proliferation were determined by EdU staining (Riobio, C10310-3). Cells (5×10^3/well, five replicates per group) were seeded into 96-well plates, and incubated with 10 nM EdU for 4 h. Cells were washed, fixed and stained according to the manufacturer's instructions. Cells were observed and counted using a fluorescence microscope.

2.9. Adhesion of monocytes assay

HUVEC were cultured in 24-well glass chamber slides. THP-1 cells were labeled with 5 μg/ml calcein-AM in EBM-2 medium containing 5% FBS. Calcein-AM-labeled THP-1 cells (4.0×10^5 cells/mL) were seeded on AMI-treated and TNF-α-treated ECs and co-incubated for 45 min. Images were obtained using a fluorescence microscope.

2.10. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed on the cell supernatant to determine the levels of ceramide (11268, RUIXIN, China), sICAM-1 (1113542, Dakota, China), and sVCAM-1 (1114062, Dakota, China), according to the manufacturer's instructions.

2.11. Transwell assay

The ECs migration assay was performed using Costar transwell cell culture chambers (354234, Corning, USA) according to the manufacturer's instructions. HUVEC were preconditioned and seeded (4×10^4 cells per well) in the top chambers with 8.0-μm pore polycarbonate membrane inserts. Cells were then incubated in serum-free medium in the upper layer, and medium with 3% FBS was added to the bottom chambers. Cells were incubated for 16 h and cells in the lower layer were washed, fixed, stained with crystal violet solution (Sigma-Aldrich) and observed under a microscope.

2.12. Tube formation

Matrigel was used to coat the 96-well plates and allowed to solidify (37°C, 45 min), prior to seeding with HUVEC. Cells were incubated at 37°C for 6 h in serum free EBM-2 medium.

2.13. Detection of ROS Generation by DHE Fluorescence Staining

The ECs were incubated with dihydroethidium (DHE, 20 μM) in EBM-2 for 45 min, followed by washing three times with PBS. Quantification of fluorescent images was performed with ImageJ software. (1.52v, USA).

2.14. Statistical analysis

Data are presented as mean ± standard Error of Mean (SEM). Statistical analysis was performed using SPSS 21.0 software (IBM Corporation, Armonk, NY, USA). Student's t test was employed to compare data

between two group or multigroup comparisons were analyzed through one-way analysis of variance, followed by a post hoc Tukey test. A $P < 0.05$ denoted a statistically significant difference.

3. Results

3.1. Amitriptyline inhibited TNF-α-induced ASMase activation

To explore the effect of TNF-α and amitriptyline on ASMase, we assessed the protein expression and activity of ASMase and the release of ceramide. In ECs treated with TNF-α (5 ng/ml to 30 ng/ml), the protein expression of ASMase as well as the release of ceramide were increased in a dose-dependent manner, which was most significant at a dose of 20 ng/ml (Fig. 1a-1c). Moreover, 2.5 μM amitriptyline significantly inhibited the activity of ASMase (Fig. 1d) and the release of ceramide (Fig. 1e). Thus, amitriptyline was regarded as an active inhibitor of ASMase.

3.2. Amitriptyline enhanced the function of endothelial cells

To measure AMI-induced NO release in endothelial cells, western blotting was performed to identify the phosphorylation of eNOS. Results showed that the phosphorylation degree of eNOS increased following AMI treatment in a dose-dependent manner (Fig. 2a). The release of NO from endothelial cells was also measured via nitrate reductase levels. In accordance with the western blotting results, AMI also elevated the amount of nitrite oxide released into the medium of HUVEC (Fig. 2b). To determine the effect of AMI on ECs, we performed multiple functional assays including viability and proliferation. As shown in Fig. 2c and 2d, HUVEC incubated with AMI showed a marked increase in proliferation (75% with EdU staining) and cell viability (69% in the CCK-8 assay) compared to the control group. Therefore, these results indicated that AMI increased ECs proliferation, viability and the release of NO.

3.3. Amitriptyline inhibited ICAM-1 and VCAM-1 expression and monocyte adhesion in TNF-α-induced endothelial cells

To determine whether AMI regulates the adhesion of monocytes, we first explored the effect of AMI on adhesion molecule expression induced by TNF-α in HUVEC by western blotting. As shown in Fig. 3a, AMI alone did not cause any significant effects. However, the protein expression of adhesion molecules was significantly increased after the administration of TNF-α (20 ng/ml) compared with the control group. AMI (2.5 μM) decreased the enhanced expression of ICAM-1, VCAM-1, and MCP-1 (Fig. 3b). ELISA assay showed similar results (Fig. 3b, 3c).

To further identify the role of AMI in TNF-α-induced monocyte recruitment on the vascular endothelium, we performed cell adhesion assays using THP-1 monocytes and HUVEC (Fig. 3d). A small number of monocytes adhered to HUVEC in the absence of TNF-α. However, after treatment with TNF-α for 24 h, the number of adherent THP-1 cells dramatically increased. In addition, pretreatment of cells with AMI

inhibited TNF-α-induced monocyte adhesion to endothelial cells. No significant difference in THP-1 cell count was observed between the AMI alone and Control groups. These results implied that AMI blocked monocyte migration in response to inflammatory mediators by inhibiting the expression of endothelial adhesion molecules.

3.4. Amitriptyline prevented TNF-α-mediated ECs dysfunction

We then investigated the effect of AMI on the function of ECs induced by TNF-α. TNF-α is an important risk factor for atherosclerosis as it impairs endothelial cell function [34–36]. First, the effect of AMI on angiogenic characteristics including proliferation, migration and tube formation in ECs were examined. A transwell coculture system (8 μm) was used to detect the migration of ECs. We found that TNF-α stimuli decreased the migration of HUVEC while pretreatment with AMI clearly increased migration (Fig. 4a, b). As shown in Fig. 4c and 4d, HUVEC treated with TNF-α exhibited lower EdU staining intensity in comparison to the Control group, indicating inhibition of cell proliferation. However, HUVEC preincubated with AMI showed markedly improved proliferation compared with the TNF-α group. We next determined whether AMI improved blood vessel development using a Matrigel assay in cultured HUVEC, TNF-α strongly suppressed tube formation in normal HUVEC, where pre-treatment with AMI restored angiogenesis (Fig. 5a, 5b). Therefore, these data indicated that AMI prevented TNF-α-induced endothelial dysfunction.

3.5. Amitriptyline prevented TNF-α-stimulated ROS production

Oxidative stress is a key factor contributing to the pathogenesis of atherosclerosis [37–39]. After incubation with TNF-α for 24 h, the level of ROS was significantly augmented (Fig. 5c). However, AMI pretreatment significantly mitigated TNF-α-induced ROS generation in HUVEC. These data suggested that AMI prevented TNF-α-stimulated ROS production.

3.6. Amitriptyline reversed TNF-α-induced increased MAPK activity

MAPK signaling is activated by TNF-α binding to the receptor [40]. Therefore, we investigated whether AMI inhibits TNF-α induced proinflammatory effects in ECs through the MAPK signal transduction pathway. Western blotting results showed that TNF-α significantly enhanced the phosphorylation of NF-κB, p38 MAPK, p44/42 MAPK, and JNK MAPK following various treatments. However, this increased phosphorylation was significantly attenuated by treatment with AMI (Fig. 6a, b). No difference in MAPK activation was observed between the AMI alone and control groups. Therefore, the results indicate that AMI treatment attenuated MAPK and NF-κB activation in TNF-α-induced ECs.

3.7. The knockout and inhibition of ASMase improved vascular function in intraperitoneal injection of TNF-α mice

To explore the role of ASMase and amitriptyline in arterial relaxation after intraperitoneal injection of recombinant mouse TNF-α, we measured the vasodilative response of isolated aortic segments to endogenic and exogenic NO. PE-precontracted aortas from ASMase$^{-/-}$ and WT mice were analyzed. As shown in Fig. 7(a), the diastolic response to acetylcholine of the TNF-α group was far weaker than that of the Sham group, indicating that endothelium-dependent diastolic function was impaired under TNF-α. In the ASMase$^{-/-}$-TNF-α group and the TNF-α-AMI group, the aortic rings were much more sensitive to acetylcholine than the TNF-α group. Treatment with the NO inhibitor L-NAME (10 μM) suppressed acetylcholine-induced endothelium-mediated vasodilation, and there was no significant difference in vasodilation among different groups of mice (Fig. 7b). However, these results were not found under SNP-induced endothelium-independent vasodilation, which represents the relaxation function of smooth muscle (Fig. 7c). Thus, the results showed that ASMase impaired NO release to regulate endothelium-mediated vasodilation and vascular tone. Knockout and inhibition of ASMase enhanced NO bioavailability and secondary vasodilation after treatment with TNF-α.

4. Discussion

This study revealed that AMI, an inhibitor of lysosomal acid sphingomyelinase, elevated eNOS phosphorylation and endogenous NO production in HUVEC. Preincubation with AMI prevented TNF-α-induced endothelial dysfunction. AMI also decreased the activation of MAPK signaling when treated by TNF-α via inhibiting the activity of ASMase and the release of ceramide, in turn reducing the expression levels of adhesion molecules and ROS. After treatment with TNF-α, endothelial-dependent relaxation of aorta in ASMase$^{-/-}$ mice and mice treated with amitriptyline was increased compared with wildtype counterparts.

ECs, as a crucial structural component of blood vessels, also play an important role in vessel functions such as regulating vascular tone by producing vasoactive factors. ECs dysfunction, including the imbalance of vasodilation and vasoconstriction, increased ROS generation, pro-inflammatory cytokine production, and insufficient NO bioavailability, contribute to atherosclerosis [41, 42]. Mainly located in the lysosomal sections ASMase hydrolyzes SM to phosphocholine and CER, which is linked with atherosclerosis [43].

AMI has been widely used in clinical practice as an anti-depressive therapy, however, its therapeutic action may not merely benefit people with depression [44]. AMI can antagonize many receptors such as the serotonin receptor Further, AMI is a functional inhibitor of ASMase [29]. Therefore, it is likely that AMI may regulate cellular function through multiple biological pathways. In LDLR$^{-/-}$ mice with T2DM, AMI was reported to significantly alleviate NASH by inhibiting the ASMase-CER system[30]. We hypothesized that improved symptoms might be attributed to AMI inhibition of ASMase activity and proinflammatory

cytokine production. Additionally, Yuxiang Dai et al. revealed that AMI attenuated cardiomyocyte apoptosis induced by Hypoxia/ reoxygenation [45]. However, the role of AMI in ECs remains unknown. Previous research reported that TNF-α increased the production of acid sphingomyelinase, thus aggravating the atherogenic process [46]. In this study, we discovered that TNF-α significantly increased the expression of acid sphingomyelinase and the release of ceramide in ECs. After incubation with AMI, ASMase activity and the release of ceramide were significantly inhibited. In addition, our study also found that AMI promoted ECs proliferation and viability and upregulated the phosphorylation of eNOS and endogenous NO production.

The original event in atherosclerosis is accumulation of monocytes from the peripheral blood to the intima of the vascular wall [43]. In this study, we discovered that AMI significantly inhibited TNF-α-induced expression of adhesion molecules in ECs. In addition to protein expression, we further discovered that AMI effectively down-regulated the secretion of sICAM-1 and sVCAM-1. More importantly, we found that TNF-α-induced monocyte adhesion to endothelial cells was significantly inhibited after pretreatment with AMI. In this study, we discovered a novel role for AMI in TNF-α-induced monocyte-endotheliocyte adhesion. AMI inhibited the expression of TNF-α-mediated adhesion molecules, in turn reducing monocyte-endotheliocyte adhesion.

The dysfunction of ECs has become a novel and significant frontier in studies of cardiovascular disease prevention [32, 47–49]. Effective ECs repair after injury is crucial to preventing atherosclerosis and vascular remodeling [50]. To determine if AMI improved TNF-α induced ECs functions, we first found that TNF-α significantly inhibited ECs function such as proliferation, migration, and tube formation *in vitro*. However, preincubation with AMI prevented TNF-α-induced endothelial dysfunction. Therefore, AMI exhibited a protective effect by preventing endothelial dysfunction.

ROS are crucial modulators of vascular tone, autophagy, and cytotoxicity and play an important role in the progression of cardiovascular disease [13, 51]. In *vitro* studies using ECs found that ASMase plays a role in TNF-α-induced mitochondrial ROS production [52]. Previous study also showed that inhibition of ASMase decreased the production of ROS derived from CER in RAECs induced by palmitic acid [53]. As we expected, AMI significantly reduced TNF-α-induced production of ROS. Thus, AMI may improve impaired endothelial function by reducing ROS production.

Endothelial inflammation plays a crucial role in the progression of atherosclerosis. The MAPK/NF-κB signaling pathway is considered to be a key factor in regulating ECs inflammation and plays a vital role in TNF-α-induced production of proinflammatory mediators in ECs [54]. It is noteworthy that NF-κB was also reported to regulate atherosclerotic lesion formation [55]. The expression of chemokines and adhesion molecules associated with endothelial inflammation in atherosclerosis has also been shown to be regulated by NF-κB[56]. Previous studies have shown that TNF-α activated phosphatidylcholine-phospholipase C (PC-PLC) leading to the production of 1,2- diacylglycerol (DAGs) and triggering of protein kinase C (PKC) and ASMase[57]. ASMase hydrolyzes SM to CER, resulting in the activation of NF-κB[22]. We also found that the phosphorylation of NF-κB, p38, JNK, and p42/44 significantly increased in

HUVEC following treatment with TNF-α. These processes were normalized by pretreatment with AMI. These results indicate that AMI inhibited the activation of NF-κB and MAPK, alleviating TNF-α-induced ECs inflammation, a potentially vital mechanism by which AMI regulates the production of adhesion molecules.

It was reported that reduced ASMase induced vasodilation, however, the underlying mechanism is not fully understood. In this study, we explored the function of ASMase in mouse arterial dilation after intraperitoneal injection of recombinant mouse TNF-α. We noted that the endothelium-dependent dilation of mouse aorta was impaired under the influence of TNF-α. Additionally, downregulation and inhibition of ASMase enhanced NO bioavailability and improved vasodilation in mice after injection of TNF-α [58].

To summarize, we discovered that preincubation with AMI ameliorated TNF-α-induced ASMase/CER/MAPK activation, which efficiently inhibited monocyte/ECs interaction induced by TNF-α and reduced subsequent endothelial inflammation and endothelial dysfunction. Therefore, our results demonstrate that AMI has beneficial effects on endothelial dysfunction which are mediated by its anti-inflammatory properties. These findings identify a novel understanding of AMI and provide a possible strategy for the prevention of vascular diseases.

Declarations

Data Availability

The data supporting the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Yang Ji, Jing Chen, and Lihua Pang contributed equally to this work.Shiming Liu and Yun Zhong designed the study;Yang Ji, Jing Chen, Lihua Pang, Changnong Chen, Jinhao Ye, Hao Liu, HuanzhenChen, Chuanfang Cheng participated the experiment.Yang Ji, Jing Chen, Lihua Pang,Changnong Chen,Songhui Zhang, Shaojun Liu, Shiming Liu and Yun Zhong analyzed the date; and Yang Ji, Jing Chen, Li-hua Pang, Hao Liu,Shiming Liu and Yun Zhong wrote the paper.

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Figures

Figure 1

Amitriptyline inhibited TNF-α-induced ASMase activation (a) Western blotting data showing effects of TNF-α on expression levels of ASMase in HUVEC. (b) Statistical results of relative protein expression. (c) The effect of TNF-α on ceramide levels in the supernatant. (d) Activity of ASMasein HUVEC. (e) The effect of AMI (0-2.5 μM)on ceramide levels in the supernatant of TNF-α-activated HUVEC as measured by ELISAData are represented as the mean±SEM (n=3–6) *P<0.05, **P<0.01, ***P<0.001 vs control; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs TNF-α.

Figure 2

Amitripty line enhanced the function of endothelial cells(a) The effect of AMI on HUVEC proliferation. (b) Effects of AMI (0-10 μM, 24 hours) on the viability of HUVEC. (c) Representative western blotting images and summarized data showing the effect of AMI (0-10 μM, 24 hours) on phosphorylated eNOS. (d) NO

release from endothelial cells induced by AMI (0–10 μM, 24 hours). Bar: 400 μm in (a). Data are represented as the mean±SEM; *P<0.05, **P<0.01, ***P<0.001 vs control.

Figure 3

Amitriptyline inhibited ICAM-1 and VCAM-1 expression and monocyte adhesion in TNF-α-induced end othelial cells(a) Western blotting data showing effects of AMI on expression levels of VCAM-1, ICAM-1, and MCP-1 in TNF-α stimulated HUVEC. (b,c) The effect of AMI (0,0.625,1.25,2.5 μM) on sVCAM-1 levels in the supernatant (b), sICAM-1 (c) in TNF-α-activated HUVEC as measured by ELISA. (d) The effect of AMI on THP-1 monocyte adhesion to TNF-α-activated HUVEC. Bar: 400 μm in (d). Data are represented as the mean±SEM(n=3–6)*P<0.05, **P<0.01, ***P<0.001 vs control;$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs TNF-α.

Figure 4

Amitripty line prevented TNF-α-mediated ECs dysfunction (a) AMI attenuated TNF-α effects on HUVEC inthe transwell (8μM) assay. (b) Quantitative analysis of cell migration in (a). (c) AMI attenuated the effect of TNF-α in HUVEC as measured by EdU staining. (d) Quantitative analysis of the proliferation rates in (c) (n =5). Bar: 200 μm in (a),400 μm in (c). Data are represented as the mean±SEM(n=5)*P<0.05, **P<0.01, ***P<0.001 vs control;$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs TNF-α.

Figure 5

Treatment with AMI improved tube formation and decreased oxidase-dependent ROS generation in HUVEC mediated by TNF-α(a) AMI alleviated the inhibitory role of TNF-α on angiogenesis in HUVEC.(b) Quantification of total branch. (c,d) Representative fluorescent images of DHE staining (c).summary data (d) showing the roles of AMI in the production of ROS in HUVEC treated with TNF-α. Bar: 200 μm in (a), 400 μm in (c). Data are represented as the mean±SEM(n=5)*P<0.05, **P<0.01, ***P<0.001 vs control;$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs TNF-α.

Figure 6

Amitriptyline reversed TNF-α-induced increased MAPK and NF-κ B activity Western blotting and (a) statistical analysis (b) of expression of phosphor-NF-κ B, phosphor-ERK, phosphor-P38 and phosphor-JNKin TNF-α treated HUVEC. Data are represented as the mean±SEM(n=3)*P<0.05, **P<0.01, ***P<0.001 vs control;$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs TNF-α.

Figure 7

The role of ASMase in arterial relaxation after intraperitoneal (i.p.) injection of recombinant mouse TNF-α(a,b) Cumulative concentration-dependent Ach-induced relaxation of mouse aortic vessels in the absence (a) and presence of L-NAME (b). (c) The relaxation concentration-response curve to SNP was attained in mouse aortic vessels. (d) Maximum relaxation of aortic vessels with or without L-NAME in response to 10-6 M Ach.Data are represented as the mean±SEM(n=5)*P<0.05, **P<0.01, ***P<0.001 vs WT;$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs WT- TNF-α;

Figure 8

Diagram of the signaling cascades involved in the effect of AMI on TNF-α signaling pathways in ECsTNF-α actives PC-PLC by binding toTNF-α receptors. Activated PC-PLC produced 1,2-DAGs which triggers two enzymes, PKC and ASMase. AMI suppressed ASMase hydrolyzes SM, which results in decreaseof NF-kB and MAPK activity, alleviate monocytes adhesion and improve proliferation, angiogenesis and migration in ECs. PC: phosphatidylcholine; PC-PLC: phosphati dylcholine-phospholipase C; PKC: protein kinase C; DAG:diacylglycerol.

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