Published in final edited form as:

Basic Clin Pharmacol Toxicol. 2019 April ; 124(4): 370–384. doi:10.1111/bcpt.13146.

Tricyclic antidepressant amitriptyline inhibits autophagic flux and prevents tube formation in vascular endothelial cells

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Abstract

Amitriptyline is a tricyclic antidepressant and an inhibitor of lysosomal acid sphingomyelinase (ASM). Amitriptyline is well-known for its cardiovascular side effects and toxicity in psychiatric patients. However, the mechanisms underlying the cardiovascular side effects of amitriptyline remain largely undefined. This study aimed to determine the effects of amitriptyline on angiogenic capability of vascular endothelial cells in physiological settings and identify its mechanism of action. The *ex vivo* aortic ring angiogenesis and *in vitro* cultured endothelial cell tube formation assay were used to assess the effects of amitriptyline on endothelial angiogenic capability. It was demonstrated that amitriptyline impaired the angiogenesis of aortic rings, which was similar to that found in aortic rings with haplo-insufficiency of the ASM gene. In cultured mouse microvascular endothelial cells (MVECs), amitriptyline impaired the proliferation and tube formation under basal condition, which were accompanied by attenuated angiogenic signalling pathways such as eNOS, Akt, and Erk1/2 pathways. Mechanistically, amitriptyline inhibited autophagic flux without affecting autophagosome biogenesis at basal condition. ASM gene silencing or autophagy inhibition mimics the inhibitory effects of amitriptyline on endothelial cell proliferation and tube formation. Collectively, our data suggest that amitriptyline inhibits endothelial cell proliferation and angiogenesis via blockade of ASM-autophagic flux axis. It is implicated that the cardiovascular side effects of amitriptyline may be associated with its inhibitory action on physiological angiogenesis.

Amitriptyline is a tricyclic antidepressant and has been extensively used in clinical practice to prevent migraine attacks [1, 2] and alleviate various types of chronic pain [3, 4].

Pharmacologically, amitriptyline is a functional inhibitor of acid sphingomyelinase (ASM)

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Conflicts of interest

The authors declare no conflicts of interest.

[5, 6]. ASM hydrolyzes the sphingolipid sphingomyelin to release ceramide, predominantly in lysosomes but also in secretory lysosomes and on the plasma membrane [7, 8]. Ceramide is further metabolized into sphingosine, which is readily phosphorylated into sphingosine-1-phosphate (S1P) by sphingosine kinase (SphK) [7, 8]. Both ceramide and S1P are critical components of sphingolipid signalling pathway [7, 8]. ASM resides in the lysosome and usually attaches to the inner membrane leaflet by electrostatic forces [9]. Amitriptyline interferes with the binding of ASM to the inner lysosome membrane and results in dissociation of the enzyme from the inner membrane leading to its degradation [9]. It has been well documented about the cardiovascular side effects and toxicity of amitriptyline in psychiatric patients without pre-existing cardiac disease such as stroke, conduction disturbances, arrhythmia and hypotension [10–12]. However, the molecular mechanisms by which amitriptyline exerts its cardiovascular side effects are much less studied. Previous studies showed that high concentrations of amitriptyline disturbed cytoskeletal organization in endothelial cells and vascular smooth muscle cells, resulting in pyknotic, rounded-up and detached or detaching cells point [13]. These findings raise the possibility that cardiovascular side effect of amitriptyline may be linked to de-regulated endothelial cell proliferation and migration and related functions such as angiogenesis.

Angiogenesis is a biological process of forming new blood vessels from pre-existing vessels that plays a vital role in growth and development, as well as in pathological settings such as wound healing [14, 15]. The angiogenesis involves several mechanisms such as degradation of extracellular matrix, disruption of intercellular junctions, and migration, proliferation and capillary tube formation of vascular endothelial cells [16]. Many factors stimulate angiogenesis such as vascular endothelial growth factor (VEGF) as well as protein or lipid mediators such as angiopoietin and prostaglandins [16]. Accumulating evidence has shown that sphingolipid signalling components ceramide and S1P are involved in endothelial cell survival, migration, proliferation and angiogenesis [17–20]. However, the precise role of ASM and its mechanism of action in angiogenesis remain largely undefined.

Autophagy is a dynamic and life-sustaining process of subcellular degradation that is critical for cellular homeostasis [21]. Autophagy initiates with the biogenesis of autophagosomes, which are double membrane-bound vesicles that incorporate various cellular cargos for degradation [21]. Once autophagosomes are formed, they enter into a process known as autophagic flux, in which autophagosomes fuse with lysosomes to form autophagolysosomes and then the cargo carried by autophagosomes and eventually autophagolysosomes themselves are degraded by lysosome hydrolases and proteases [21]. Recent studies have shown that autophagy plays a critical role in modulating angiogenic signalling pathways elicited under low nutrient starvation conditions or by VEGF [22]. Interestingly, our studies have demonstrated that ASM is needed for efficient autophagic flux in vascular smooth muscle cells as ASM deficiency impairs lysosome trafficking and fusion with autophagosomes leading to reduced formation of autophagolysosomes [23, 24]. These previous studies implicate that amitriptyline may influence the ASM-autophagic flux axis that leads to inhibition of endothelial cell angiogenesis. To test this hypothesis, the present study first examined the effects of amitriptyline and ASM gene targeting on migration, proliferation, angiogenic signalling and matrix gel-based tube formation in cultured microvascular endothelial cells (MVECs). Further, the present study determined

whether amitriptyline impairs autophagic flux in MVECs and whether inhibition of autophagic flux could mimic the effects of amitriptyline on angiogenesis.

Material and methods

Animals.

ASM gene haploinsufficiency (*Smpd1*^{+/-}; *Smpd1* is the gene symbol) and wild-type (*Smpd1*^{+/+}) male mice (C57BL6 background) at 12 week of age and 20–25 grams of bodyweight were used as previously described [23]. All experimental protocols were reviewed and approved by the Instructional Animal Care Committee of University of Houston. Mice were housed in a conventional animal facility with a 12-hr light/dark cycle with *ad libitum* access to standard rodent chow.

Aortic ring angiogenesis assay.

Aortic ring angiogenesis measurements were performed as previously described [25]. Briefly, 200 μ L of Matrigel Basement Membrane Matrix (Corning, MA, USA) was coated into a 24-well plate and incubated at 37°C for 30 min. Thoracic aortas were excised and flushed with ice-cold phosphate-buffer saline to remove the blood. Surrounding fibro-adipose tissue was dissected free, and the aortas were sectioned into 1-mm rings and incubated on Matrigel. Rings were incubated with Endothelial Cell Growth Medium EBM2 containing VEGF (Lonza, MO, USA) with or without amitriptyline (Sigma, MO, USA), which was replaced daily. Images were acquired at 5 randomly chosen rings from day 6.

Cell culture and treatments.

The MVEC line EOMA was purchased from ATCC and cultured as described [26]. In brief, MVECs were maintained in DMEM (Thermo, USA) supplemented with 10% FCS (Thermo, USA) and 1% penicillin/streptomycin (Thermo, USA), and incubated at 37°C and 5% CO₂. Cells were treated with indicated dose of amitriptyline, 10 nM bafilomycin B1 (Sigma, MO, USA), 5 μ M chloroquine (Sigma, MO, USA) or 10 μ M spautin-1 (Sigma, MO, USA) for 6 hr.

Cytotoxicity assay.

The cytotoxicity of amitriptyline or autophagy blockers was assessed by lactate dehydrogenase (LDH) release assay. LDH activity in the cell culture medium was determined by Piercetm LDH Cytotoxicity Assay Kit (Thermo, IL, USA) according to the manufacturer's protocol. The absorbance was spectrophotometrically quantified at 490 nm using CLARIOstar microplate reader (BMG Labtech, Germany). The percentage of LDH activity in indicated samples over the maximum LDH activity (1% Triton X-100 treated samples) was used to represent cytotoxicity.

RNA interference.

Small interference RNAs (siRNAs) for ASM or Atg7 were commercially available (Santa Cruz Biotechnology, CA, USA). The sequences for these siRNAs were confirmed to be effective in silencing target genes in different cells by the company and by real-time PCR or

western blotting by our laboratory. The scrambled small RNA has been also confirmed as non-silencing double stranded RNA and was used as a control in this study. Transfection of siRNA was performed with the siLentFect Lipid Reagent (Bio-Rad, CA, USA) according to the manufacturer's instructions and as we previously described [26]. Cells were further incubated for 48 hr post siRNA transfection.

Endothelial cell tube formation assay.

The angiogenic ability of endothelial cells was assessed by *in vitro* Matrigel-based tube formation assay. Briefly, 50 μ L of Matrigel Basement Membrane Matrix was coated into a 96-well plate and incubated at 37°C for 30 min. MVECs (3×10^4 cells) were seeded on the Matrigel and treated as indicated for 6 hr. Images were acquired at 5 randomly chosen microscopic fields. The cumulative tube length was analysed with Olympus IX73 (Tokyo, Japan) at $\times 200$ magnification.

ASM activity assay.

The enzymatic hydrolysis of sphingomyelin to ceramide and phosphocholine by sphingomyelinase was measured at pH 5.0 with the Amplex® Red reaction kit (Invitrogen, Thermo, OR, USA) as described [23]. Briefly, 1×10^6 cells were pelleted by centrifugation at $1,000 \times g$ for 10 min. at 4°C and washed twice with ice-cold PBS. The cell pellet was resuspended in 0.1 mL of lysis buffer containing 100 mM Tris-HCl (pH 8.0), 1% Triton X-100 and $1 \times$ protease inhibitor (Pierce, Thermo, USA) for 30 min. on ice. The lysates were subjected centrifugation at $10,000 \times g$ for 10 min. at 4°C to remove nuclei. ASM activity in the supernatant fractions was assayed in a two-step reaction system by Amplex Red Sphingomyelinase Assay Kit (Thermo OR, USA) as instructed. Amplex Red fluorescence intensity was measured using in CLARIOstar microplate reader (BMG labtech, NC, USA) at excitation and emission wavelengths of 545 and 590 nm, respectively. The enzyme activity of ASM in the protein lysates was normalized to activity (U) of sphingomyelinase from *Bacillus cereus* (provided as a control within the kit). One unit is defined as the amount of sphingomyelinase that will hydrolyse 1 μ mol of TNPAL sphingomyelin/min at pH 7.4 at 37 °C.

Western blotting analysis.

Western blotting were performed as we described previously [27]. Cells were lysed in $2 \times$ Laemmli's sample buffer (Bio-rad, CA, USA) for 20 min. on ice. After boiling for 10 min. at 95°C, equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred at 100 V for 1 hr onto a PVDF membrane (Millipore, USA). The membrane was blocked and incubated with primary antibodies against phospho-eNOS (1:1000), eNOS (1:2000), phospho-Akt (1:1000), Akt (1:2000), phospho-Erk1/2 (1:1000), Erk1/2 (1:2000), Atg7 (1:1000), p62 (1:5000), LC3B (1:4000), or β -actin (1:5000) overnight at 4°C. Antibodies against p62 were purchased from Abcam Inc. (UK) and all others were purchased from Cell Signaling Technology Inc. (MA, USA). Following primary antibody incubation, the membrane was further incubated with 1:15000 secondary antibodies IRDye 800CW donkey anti-rabbit IgG and 680RD goat anti-mouse IgG (Licor, NE, USA). The immunoreactive bands were detected by Licor Odyssey Fc imaging system. Densitometric analysis of images was performed using Licor Image Studio software (NE, USA).

Cell migration assay by electric cell-substrate impedance sensing (ECIS).

ECIS system (Applied Biophysics, NY, USA) was used to conduct migration assay as reported previously [28, 29]. MVEC_s (5×10^4 per well) were cultured in 8W1E+ arrays (Applied Biophysics, NY, USA) and the resistance was subsequently monitored and measured using the ECIS software provided. The cells were incubated until the resistance reached a plateau suggesting a confluent monolayer had formed over the array electrodes. The cell monolayer was subsequently wounded electrically by applying current pulses of 5000 μ A s at 64000 Hz for 30 sec. This wounding procedure created a simultaneous physical break of equal dimensions in the cell monolayer of each well. After wounding, the resistance increased as cells migrated back onto the electrode. The slope of changes in resistance post wounding was used to reflect the cell migration rate.

Immunofluorescence.

Cells grown on glass coverslips were treated as indicated and then fixed in 4% paraformaldehyde for 15 min. and incubated in 0.5% BSA with 0.3% Triton X-100 for 10 min. Cells were washed in phosphate-buffer saline and then incubated with anti-ASM (1:200; Novus, CO, USA), anti-ceramide (1:50; Enzo, NY, USA) and anti-Ki67 (1:100; Abcam, Cambridge, MA, USA) at 4°C for overnight. After incubation with primary antibodies, the slides were washed and labelled with corresponding Alexa Fluor-488 or Alexa Fluor-555 conjugated secondary antibodies (Invitrogen). The slides were mounted in mounting medium with DAPI (Vector, CA, USA) and visualized through an Olympus IX73 fluorescence microscope.

Real-time reverse transcription polymerase chain reaction (RT-PCR).

Total RNA was isolated using PureZOL[™] RNA isolation reagent (Bio-rad, CA, USA) according to the manufacturer's protocol. Afterwards, 1- μ g aliquots of total RNA were reverse-transcribed to cDNA by iScript cDNA synthesis kit (Bio-rad, CA, USA). Equal amounts of the reverse transcriptional products were subjected to PCR amplification on CFX Connect Real-Time System (Bio-rad, CA, USA). The primer sets were synthesized by Eurofins (KY, USA). For ASM: sense ACATTCCCGAGTGCTGCTTATC, antisense GAGGTCACATTGGTTTGTTCGA; β -actin sense TCGCTGCGCTGGTCGTC, antisense GGCCTCGTCACCCACATAGGA.

Electronic spin resonance spectrometric detection of production of superoxide free radicals ($O_2^{\cdot-}$).

Electronic spin resonance detection of $O_2^{\cdot-}$ was performed as we described previously [26]. In brief, 2×10^6 Cells were lysed in 100 μ L sucrose buffer for 30 min. on ice. The lysates were subjected centrifugation at $10,000 \times g$ for 10 min. at 4°C to remove nuclei. 20 μ L cell homogenates were incubated in modified Krebs-HEPES buffer containing deferoxamine (100 μ M; Sigma) and diethyldithiocarbamate (5 mmol/L; Sigma). The samples were then mixed with 1 mM spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Noxygen, Elzach, Germany) with in the presence or absence of 100 units/mL polyethylene glycol (PEG)-conjugated superoxide dismutase (SOD). The reaction mixture loaded in glass capillaries was immediately analysed for $O_2^{\cdot-}$ production for 10 min. using a

Miniscope MS400 spectrometer (Magnettech, Germany). The SOD-inhibitable signals were normalized by protein concentration and used to represent $O_2^{\cdot-}$ production.

Statistics.

All data were presented as mean $\pm$ standard deviation. Significant differences between and within multiple groups were examined using one-way ANOVA test followed by Duncan's multiple-range test. Student's *t* test was used to detect significant difference between two groups. A *P* value <0.05 was considered statistically significant.

Results

Amitriptyline inhibits aortic ring angiogenesis *ex vivo*.

We first determined whether the ASM inhibitor amitriptyline attenuated blood vessel development using a mouse aortic ring assay, an invaluable organ culture model for angiogenesis. The ability of amitriptyline to inhibit endothelial tube outgrowth from small slices of aorta embedded in Matrigel was measured. As shown in Fig. 1A, aortic rings from wild-type (*Smpd1*^{+/+}) mice had profound sprouting and branching of endothelial cell-derived tubes, whereas reduced tube outgrowth or branching was observed when these rings were treated with 5 μ M amitriptyline. Moreover, to mimic the effects of inhibition of ASM activity on endothelial tube formation, we performed aortic ring assay using *Smpd1*^{+/-} mice. Rings from *Smpd1*^{+/-} mice exhibited significantly reduced angiogenesis compared to that in wild-type mice. We also demonstrated that amitriptyline had no cytotoxic effects on endothelial cells that excludes the possibility that amitriptyline inhibits endothelial tube outgrowth from aorta rings via induction of apoptosis (Fig. 1B). Further, amitriptyline can significantly reduce the enzymatic activity of ASM (Fig. 1C). Consistently, amitriptyline decreased ASM expression (Fig. 1D), but did not change ceramide level (Fig. 1E) in these endothelial cells.

Amitriptyline or ASM gene silencing inhibits angiogenesis in MVECs.

We next determined if the reduction in *ex vivo* angiogenesis observed for amitriptyline can be replicated in *in vitro* tube formation Matrigel assay in cultured MVECs. As shown in Fig. 2A, VEGF obviously promoted the tube formation and dose-dependently increased tube length in MVECs. Amitriptyline inhibited endothelial cell tube formation in the presence or absence of 30 ng/mL VEGF (Fig. 2B). To further determine whether inhibition of ASM decreases angiogenesis in MVECs, we knocked down ASM in MVECs by using siRNA. As shown in Fig. 3A and 3B, ASM siRNA decreased the tube formation and tube length in cells treated with or without VEGF compared to those cells transfected with control or scramble siRNA. The successful knockdown was confirmed by showing about significant reduction in ASM mRNA level (Fig. 3C). Together, these results suggest that inhibition of ASM by amitriptyline or gene silencing at basal condition decreases tube formation, signifying that ASM promotes physiological angiogenesis in MVECs. It should be also noted that amitriptyline (Fig. 2B) but not ASM gene silencing (Fig. 3C) abolishes the VEGF-induced component of tube formation. These results may implicate that the effect of amitriptyline on VEGF-induced angiogenesis at pathological condition is independent of ASM activity.

Effects of Amitriptyline on migration, proliferation, and angiogenic signalling pathways in MVECs.

Endothelial cell migration and proliferation are closely related events for modulation of tube formation process. Because ASM can promote physiological angiogenesis, we investigated whether amitriptyline had any effects on angiogenesis related events in MVECs under basal condition. As shown in Fig. 4A, all groups of MVECs treated with or without amitriptyline exhibited similar recovery of resistance following electrical pulse-induced wounding, which suggests that amitriptyline had no effects on the migratory ability of MVECs. In contrast, amitriptyline markedly reduced Ki67-positive cells in a dose-dependent manner indicating that amitriptyline inhibits proliferation of MVECs (Fig. 4B). Endothelial nitric oxide synthase (eNOS), Akt and Erk are major downstream protein kinases that were reported to mediate angiogenesis [22]. Indeed, the phosphorylation of eNOS, Akt, and Erk1/2 in MVECs were significantly attenuated by amitriptyline (Fig. 4C), suggesting that the inhibitory effect of amitriptyline on endothelial tube formation is associated with deactivation of angiogenic signalling pathways. Among the three pathways, Erk1/2 is more pronouncedly reduced by amitriptyline, which indicates the effect of amitriptyline is primarily associated with inhibition of Erk1/2 signalling pathway.

Amitriptyline inhibits autophagic flux but has no effects on autophagic induction.

Microtubule-associated protein 1 light chain β (LC3B) exists in the cytoplasm in a soluble form (LC3B-I) [30]. LC3B-II is a phosphatidylethanolamine-conjugated form of LC3B-I, which is associated with autophagosome membranes and widely used as a highly specific marker for the autophagic vacuoles [21]. Upon fusion with the lysosome, LC3B-II on the inner autophagosomal membrane is degraded. Therefore, the expression of LC3B-II reflects either enhanced autophagosome biogenesis or impaired autophagic flux leading to reduced LC3B-II degradation. In addition to LC3B-II, another two autophagy proteins p62 and Beclin1 are commonly used to assess the status of autophagy. P62 is a selective substrate of autophagy degrading pathway [31]. The accumulation of LC3B-II and p62 upon pharmacological autophagy inhibition is indicative of basal autophagy [32]. Beclin1 is a key activator for autophagosome nucleation and elongation [33], contributes on autophagosome initiation. The expression of Beclin1 is usually up-regulated by autophagy activators. As shown in Fig. 5A, treatment of MVECs with amitriptyline significantly increased the abundance of LC3B-II and p62 but had no effect on Beclin1. These results suggest that amitriptyline may inhibit autophagic flux that the degradation of the substrates in autophagosomes and/or the turnover of these autophagic vacuoles themselves are reduced leading to accumulation of LC3B-II and p62. Fig. 5B shows that amitriptyline had no effects on the mRNA levels of LC3B in MVECs suggesting that amitriptyline does not induce up-regulation of autophagy genes. Moreover, unchanged mRNA and protein levels of Beclin1 also suggest that autophagosome assembly machinery remains functioning and unaltered during amitriptyline treatment. Together, these results support the view that the amitriptyline impairs autophagic flux but does not up-regulate autophagosome biogenesis.

Autophagy inhibition or Atg7 gene silencing prevents the tube formation in MVECs.

We further examined whether autophagy pathway is critical in angiogenesis in MVECs by using three mechanistically distinct inhibitors targeting autophagy pathways. Bafilomycin is a specific H⁺-vATPase blocker that inhibits lysosomal acidification and hence the degradation of autophagosomes [34]. Chloroquine is a weak base that accumulates in lysosomes and increases lysosomal pH thereby inhibits autophagic degradation and/or blocks fusion with the lysosomes [35]. Spautin-1 inhibits autophagosome formation by promoting the ubiquitination-dependent degradation of Beclin1 [36]. As shown in Fig. 6A, all these autophagy inhibitors reduced the tube formation. Fig. 6B confirmed that bafilomycin and chloroquine inhibited autophagic flux and increased LC3II, whereas spautin-1 inhibited autophagosome biogenesis and decreased LC3II. Further, Fig. 6C showed that these inhibitors did not affect the survival of MVECs at indicated concentrations suggesting that the inhibitory effects of these autophagy blockers on tube formation are independent of possible cellular cytotoxicity. Moreover, the effect of these pharmacological inhibitors on angiogenesis was also confirmed by gene silencing that knocks down the expression of Atg7, a key factor in autophagosome biogenesis (Fig. 6D and 6E). These results indicate that the endothelial tube formation is impaired upon blockade of autophagosome biogenesis or autophagic flux.

Effects of autophagy inhibition on migration, proliferation and angiogenic signalling in MVECs.

As shown in Fig. 7A, autophagic flux inhibitors bafilomycin and chloroquine had no effect on migration, whereas autophagy induction inhibitor spautin-1 markedly attenuated endothelial cell migration. It seems that the migration is dependent on autophagosome biogenesis although this possibility needs to be further investigated in future studies. Similar to amitriptyline, all autophagy inhibitors significantly attenuated Ki67-positive proliferating cells (Fig. 7B) and phosphorylation of eNOS, Akt, and Erk1/2 in MVECs (Fig. 7C).

Amitriptyline had no effects on superoxide production.

Reactive oxygen species (ROS) are important stimulators of angiogenesis in endothelial cells [14]. ASM can regulate the endothelial NADPH oxidase activity and production of O₂^{•-}, the precursors of ROS [20, 37]. In this study, the O₂^{•-} level in MVECs was analysed by electron spin resonance spectroscopy, a gold standard method for detecting O₂^{•-}. As shown in Fig. 8A and 8B, amitriptyline had no effects on the O₂^{•-} level in MVECs. This result suggests that the inhibitory effect of amitriptyline on endothelial tube formation is not associated with alteration in ROS.

Discussion

The tricyclic antidepressant amitriptyline has shown cardiovascular side effects and toxicity in psychiatric patients [10–12]. Our findings provide novel evidence that this side effect of amitriptyline may be attributed to its inhibitory action on angiogenesis. Amitriptyline suppresses the endothelial tube formation, accompanied by reduction of ASM activity, cell proliferation and angiogenic signalling including eNOS, Akt and Erk1/2 pathways. Genetic haploinsufficiency or gene silencing of ASM mimics the inhibitory action of amitriptyline

on tube formation. Importantly, ASM impairs autophagic flux and pharmacological inhibitors of autophagic flux suppress proliferation and tube formation in MVECs. Inhibition of ASM-mediated autophagic flux axis might be an important mechanism responsible for the inhibitory effect of amitriptyline on angiogenesis.

Accumulating evidence indicates that the sphingolipid signalling is involved in regulation of angiogenesis [19, 38]. Particularly, neutral sphingomyelinase-2 (NSM2), a sphingomyelinase family member, was shown to modulate angiogenesis triggered by oxidized LDL [19]. However, the precise role of ASM in angiogenesis is not fully characterized. In the present study, the angiogenic capability of endothelial cells was evaluated by a well-established *in vitro* tube formation assay and an *ex vivo* aortic ring sprouting assay. The results from both assays revealed that amitriptyline can inhibit angiogenesis. The lysosomal enzyme ASM usually locates in the inner membrane leaflet of lysosome membrane by electrostatic forces [9]. Once amitriptyline accumulates in lysosomes, it dissociates ASM from the inner membrane that exposes ASM for degradation by lysosomal proteases [9]. ASM is believed to be the major pharmacological target of amitriptyline for its therapeutic effects such as treating major depression [39]. In the present study, this inhibitory effect on ASM activity was mimicked by haploinsufficiency of *Smpd1* gene or gene silencing. These ASM gene manipulations were found to attenuate the angiogenic ability of endothelial cells in a similar manner to amitriptyline. To the best of our knowledge, this is the first report that implicates a role of ASM in amitriptyline-generated effects on angiogenesis.

Physiological angiogenesis has a pivotal role in health and growth. In certain pathological settings, increased release of angiogenic factors such as VEGF causes increased angiogenesis, which could play either beneficial or detrimental role depends on the types of diseases [40]. For instance, increased angiogenesis is beneficial for efficient wound healing and the rescue of ischaemic myocardium after myocardial infarction [41, 42]. On the contrary, increased angiogenesis appears to be detrimental in pathological processes of tumor growth, macular degeneration and atherosclerotic plaque formation [43–45]. The present study showed that amitriptyline impaired endothelial tube formation either at basal condition or with VEGF stimulation. This suggests to us that amitriptyline may affect angiogenesis in both physiological and pathological settings. ASM gene silencing significantly reduced the tube formation at basal condition indicating that ASM is critically involved in physiological angiogenesis. In addition, in pathological settings, amitriptyline may still be able to suppress angiogenesis even in situations of VEGF up-regulation. Cardiovascular side effects and toxicity of amitriptyline were present in psychiatric patients without pre-existing cardiac disease such as stroke, conduction disturbances, arrhythmia, and hypotension [10–12]. Thus, our findings reveal that these cardiovascular side effects could be attributed to inhibition of angiogenesis that may affect development and health or loss of beneficial action such as efficient wound healing and rapid rescue of ischaemic myocardium.

Angiogenesis is a complex physiological process that involves several mechanisms including migration and proliferation of endothelial cells [15]. Surprisingly, the data of amitriptyline effect on endothelial cell migration and proliferation are few in the literature, in contrast to

those reports on either apoptosis or survival. One plausible explanation is that activation of ASM in pathological settings usually leads to apoptosis, and inhibition of ASM by amitriptyline was a common approach to prevent cell death and increase cell survival [8, 17]. The present study monitored the endothelial cell migration by ECIS wound healing assay, in which the cell layer was disrupted by electric signals instead of traditional “scratch” approach. It was found that amitriptyline did not affect endothelial cell migration into wounded area. A recent study found that proliferative endothelial cells expressed higher level of ASM revealing a direct association between ASM activity and proliferation in endothelial cells [46]. In the present study, our immunofluorescence studies clearly demonstrated that amitriptyline markedly reduced the number of proliferative MVECs at basal condition implicating that ASM activity is essential for maintaining proliferation. Several angiogenic signalling pathways are involved in endothelial cell proliferation including eNOS, Akt and Erk1/2 [16, 22, 47]. Indeed, these angiogenic signalling pathways were also more or less attenuated by amitriptyline in MVECs at basal condition. It is plausible that inhibition of ASM by amitriptyline deactivates angiogenic signalling and decreases endothelial cell proliferation leading to impaired angiogenesis.

The role of ASM in health and diseases is mostly linked with changes in ceramide, the hydrolysis product of sphingomyelin by ASM activity [8]. Due to the compartmentalization of ASM in lysosomes, ceramides are predominantly produced in lysosomes but also in secretory lysosomes and on the plasma membrane [7, 8]. Ceramide is further metabolized by ceramidase into sphingosine and readily phosphorylated by SphK into S1P [8]. In addition to sphingomyelinase, ceramide can be produced via *de novo* ceramide synthesis, in which the rate-limiting step is the synthesis of ketosphinganine from palmitoyl CoA and serine by serine palmitoyl transferase (SPT) [47]. Ceramide is known to activate protein phosphatase (PP2A) that in turn deactivates Akt, Erk and eNOS phosphorylation [48–51]. In line with this action of ceramide on PP2A, a previous study demonstrated that ceramides from SPT-mediated *de novo* synthesis pathway inhibited VEGF-induced angiogenesis via ceramide-PP2A axis [47], indicating an anti-angiogenic role of ceramide-PP2A axis. On the contrary, S1P, the downstream metabolite of ceramide, is a critical regulator promoting endothelial cell proliferation, migration and angiogenesis [38, 52, 53]. Indeed, oxidized LDL promotes angiogenesis through NSM2-mediated ceramide production [19]. This pro-angiogenic action of ceramide was attributed to concomitantly activated SphK1 during oxidized LDL stimulation that enhances generation of S1P [19]. S1P is well known to modulate migration of mammalian cells including endothelial cells. It is implicated that the ceramide/S1P ratio correlates with the actual effect of ceramides in angiogenesis, which could be either pro-angiogenic or anti-angiogenic depending on the types of angiogenic factors and/or specific ceramide metabolizing pathways being activated. In the present study, the anti-angiogenic effect of amitriptyline was associated with attenuated angiogenic signalling pathways (Akt, Erk and eNOS) and proliferation but not with migration. Moreover, amitriptyline did not affect the ceramide level in endothelial cells at basal condition. Thus, these results suggest that the inhibitory effect of amitriptyline on angiogenesis may be mediated through an ASM-dependent but ceramide-independent pathway.

Our data further demonstrated that ASM-autophagic flux axis might be the ceramide-independent mechanism involved in the anti-angiogenic effect of amitriptyline. ASM

deficiency causes altered 'sphingolipid rheostat' with abnormal levels of sphingolipid species including accumulation of sphingomyelin in lysosome membranes [54]. Previous studies demonstrated that accumulated sphingomyelin can inhibit the activity of mucolipin transient receptor potential channel 1 (TRPML1), a principle lysosomal Ca^{2+} channel, and thereby block the lysosomal Ca^{2+} -dependent membrane trafficking [55–57]. Indeed, our recent studies demonstrated that inhibition of ASM activity by amitriptyline or genetic ablation of *Smpd1* gene inhibits TRPML1-mediated lysosomal Ca^{2+} release and autophagosome trafficking leading to impaired autophagic flux [23, 24]. Consistently, the present study demonstrated that amitriptyline (2.5–10 μM) inhibited autophagic flux in MVECs without affecting autophagosome biogenesis (Fig. 5). Interestingly, a recent study demonstrated that amitriptyline increased autophagy initiation as an adaptive mechanism of cell survival in human hepatocytes [58]. However, amitriptyline in this study was used at 50 μM that is much higher than IC_{50} (5 μM) for ASM activity, which could cause an off-target effect. So far, there is a lack of reports regarding the role of TRPML1 in endothelial cells. In brain capillary endothelial cells, TRPML1 has been suggested to play a role in endo-lysosome trafficking and fusion with basal membrane to secrete endocytic contents, a final step in transcytosis for brain Fe uptake [59]. However, the role of TRPML1 in lysosomal Ca^{2+} and autophagosome trafficking remains undefined in endothelial cells and are worth further investigation. Nonetheless, it is plausible to propose that inhibition of ASM by amitriptyline results in lysosomal accumulation of sphingomyelin which in turn attenuates TRPML1-mediated lysosomal Ca^{2+} and autophagosome trafficking leading to impaired autophagic flux in endothelial cells.

Autophagy is essential for cell survival, differentiation, proliferation and homeostasis. However, whether autophagy plays a protective or detrimental role in certain disease settings is normally complex because self-eating or pro-survival functions of autophagy can also be fatal. Emerging evidence supports a role of autophagy in both physiology and pathophysiology of angiogenesis. For instance, autophagy is an important promoter for angiogenesis induced by nutrient deprivation and VEGF via activation of Akt [22]. Moreover, Atg7, a key promoter of autophagy, has pro-angiogenic activity in brain [60]. Enhanced autophagy by PDGF was proposed to increase the turnover of non-essential protein or organelle substrates that helps cells adapt to the increased needs for cell growth in vascular smooth muscle cells [61]. Similar mechanism may account for the pro-angiogenic actions of autophagy in endothelial cell proliferation and angiogenesis. On the contrary, autophagy activation in certain pathological settings was linked to angiogenesis suppression. For example, exogenous ceramide at high concentration (50 μM) potently suppressed angiogenesis [62]. Interestingly, the anti-angiogenic capabilities of ceramide rely upon the induction of autophagy but not apoptosis [62]. In diabetes, methylglyoxal, the precursor of advanced glycation endo-products (AGEs), impaired angiogenesis via autophagy-mediated VEGFR2 protein degradation [63]. It seems that excessive autophagy up-regulation leads to angiogenesis suppression in pathological conditions such as diabetes. In the present study, inhibition of both autophagic flux and autophagosome biogenesis were found to attenuate angiogenic signalling, proliferation, tube formation (Fig. 6 and 7). Thus, our findings provide novel evidence that ASM-autophagic flux is pro-angiogenic in endothelial cells.

Finally, the present study excluded a role of ROS in the anti-angiogenic effect of amitriptyline. ROS are known as key signalling molecules to mediate angiogenesis [14]. Amitriptyline inhibits ASM-ceramide-NADPH oxidase-ROS signalling axis in endothelial cells under death receptor stimulation [37, 58, 64]. The previous data suggest that the major source of agonist-stimulated ROS production in endothelial cells is from ASM-ceramide-dependent NADPH oxidase activity. However, in the present study, amitriptyline at dose less than 10 μ M did not alter $O_2^{\cdot -}$ (the precursor of all forms of ROS) production in MVECs at basal condition. These data are also consistent with our finding that amitriptyline did not alter ceramide level at this condition (Fig. 1E). These data indicate that ASM inhibition is not sufficient to affect ceramide-mediated ROS production in physiological angiogenesis.

Provided with the important role of physiological angiogenesis in health and development, we propose that the anti-angiogenic activity of amitriptyline may account for one of its cardiovascular side effects. Mechanistically, amitriptyline inhibits angiogenic signalling pathways and proliferation in endothelial cells thereby reducing physiological angiogenesis, at least partially, through suppression of ASM-autophagic axis, which may represent a potentially new mechanism for angiogenesis impairment in psychiatric patients in amitriptyline therapy.

Acknowledgement

This study was supported by grants from the National Institutes of Health (HL122769 and HL122937).

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Figure 1. Amitriptyline inhibits aortic ring angiogenesis *ex vivo*.

(A) Representative images of angiogenic sprouting from aortic rings derived from wildtype (*Smpd1*^{+/+}) or *Smpd1*^{+/-} mice. The *Smpd1*^{+/+} aortic rings were also cultured in the presence of amitriptyline (Ami, 5 μM). Summarized data show the average number and length of sprouts in *Smpd1*^{+/+} or *Smpd1*^{+/-} mice. * *versus Smpd1*^{+/+} alone ***P*<0.01, n=4; **P*<0.05, ***P*<0.01, ****P*<0.001, n=4. (B-C) MVECs were treated with amitriptyline (Ami, 0–10 μM) for 6 hr. The cytotoxicity of amitriptyline (B), the effects of amitriptyline on acid

sphingomyelinase (ASM) activity (C), ASM expression (D), and ceramide (E) were shown.

* *versus* control (Ami at 0 μ M); * P <0.05, ** P <0.01, *** P <0.001, n=4.

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Figure 2. Amitriptyline inhibits the tube formation in MVECs.

(A) Representative images show the tube formation of MVECs cultured in Matrigel for 6 hr in the absence or presence of VEGF (10–50 ng/ml). Summarized data show the average tube length. **versus* VEGF at 0 μM, * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$, $n = 4$. (B) Representative images show the effect of amitriptyline (0–10 μM) on the tube formation of MVECs in Matrigel for 6 hr in the absence (Ctrl) or presence of VEGF (30 ng/mL). **vs.* Ctrl with Ami at 0 μM, * $P < 0.05$, ** $P < 0.01$; #*versus* VEGF alone (Ami at 0 μM), # $P < 0.05$, ## $P < 0.01$, $n = 4$.

Figure 3. ASM gene silencing blocks the tube formation in MVECs.

MVECs were treated with vehicle control (PBS) or transfected with scramble or acid sphingomyelinase siRNA (si-ASM) for 48 hr and then used for indicated assays. (A) Representative images of tube formation of MVECs cultured in Matrigel for 6 hr in the absence (Ctrl) or presence of VEGF (30 ng/ml). (B) Summarized data of average tube length (n=4). * *versus* scramble Ctrl, * $P<0.05$, *** $P<0.001$; # *versus* scramble+VEGF, ### $P<0.001$. (C) Quantitative analysis of acid sphingomyelinase (ASM) mRNA level in MVECs treated with PBS or transfected with scramble siRNA or si-ASM by real time RT-PCR (n=4). * *versus* Scramble, * $P<0.05$.

Figure 4. Effects of amitriptyline on migration, proliferation and angiogenic signalling in MVECs.

(A) Migration of MVECs were analyzed by ECIS. MVECs were cultured on chamber slides with electrodes until confluency that trans-monolayer electrical resistance reaches a plateau. Cells were then incubated with or without amitriptyline for 1 hr before introduction of electrical pulse for cell wounding. Representative traces show the cell migration as monitored by determining the resistance of wounded area using ECIS. Summarized data show the slope of the recovery resistance curve post wounding which reflects the rate of unwounded cells migrate toward wounded area (n=4). (B) Proliferation of MVECs was examined by immunostaining with Ki67 (green). Nuclei were counter-stained with DAPI

(blue). Representative images and summarized data show the effect of amitriptyline (0–10 μ M, 6 hr) on the percentage of Ki67-positive cells (n=4). (C) Representative western blot images and summarized data show the effect of amitriptyline (0–10 μ M, 6 hr) on phosphorylated eNOS, Akt and Erk1/2 (n=4). * *versus* control (Ami at 0 μ M), * P <0.05, ** P <0.01, *** P <0.001.

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Figure 5. Effects of amitriptyline on autophagic flux or induction in MVECs.

MVECs were stimulated with amitriptyline (0–10 μM for 6 hr. (A) Representative western blotting gel documents and summarized data show the protein expression of LC3B, p62, Beclin1 and β-actin (n=4). (B) Quantitative analysis of mRNA level of Beclin1 and LC3B by real-time RT-PCR (n=4). * *versus* control (Ami at 0 μM), $P < 0.05$, ** $P < 0.01$.

Figure 6. Autophagy inhibition or *Atg7* gene silencing prevents the tube formation in MVECs. (A) Representative images of tube formation of MVECs cultured in Matrigel for 6 hr in the absence (PBS) or presence of bafilomycin B1 (Baf, 10 nM), chloroquine (CQ, 5 μM), or spautin-1 (SP-1, 10 μM) (n=4). (B-C) MVECs were treated with indicated autophagy inhibitors at above-mentioned concentration. Panel B shows data for western blot analysis of LC3B and β-actin (n=4). Panel C shows the cytotoxicity of abovementioned autophagy blockers as measured by LDH release assay (n=3). *versus PBS, * $P<0.05$, ** $P<0.01$. (D-E) MVECs were treated with vehicle control (PBS) or transfected with scramble or *Atg7* siRNA (si-*Atg7*). Panel D shows the western blot analysis of *Atg7* expression (n=4). Panel E

shows the effect of Atg7 siRNA on tube formation (n=4). * *versus* Scramble, * $P<0.05$, *** $P<0.001$.

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Figure 7. Effects of autophagy blockers on migration, proliferation and angiogenic signalling in MVECs.

(A) MVECs were incubated with vehicle control (PBS), bafilomycin B1 (Baf, 10 nM), chloroquine (CQ, 5 µM), or spautin-1 (SP-1, 10 µM) for 1 hr before wounding. Representative traces by ECIS show the effects of indicated autophagy inhibitors on cell migration (n=4). (B-C) MVECs were treated with indicated inhibitors for 6 hr. Panel B shows the proliferation that was measured by immunostaining with Ki67 (green) and DAPI (blue) and summarized data of Ki67-positive cells (n=4). Panel C shows the representative western blot images and summarized data of phosphorylated eNOS, Akt and Erk1/2 (n=4). *versus PBS, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 8. Effect of amitriptyline on superoxide free radical production in MVECs. MVECs were treated with amitriptyline (0–10 μM) for 6 hr. Representative traces of electron spin resonance (ESR) spectrometry (A) and summarized data (B) show the production of superoxide free radical anions ($\text{O}_2^{\cdot -}$).