Acid Sphingomyelinase Deficiency Prevents AdipoRon-induced Transcription Factor EB activation and Differentiation in Arterial Smooth Muscle Cells

by

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A Thesis submitted to the Pharmacological and Pharmaceutical sciences

College of Pharmacy

in partial fulfillment of the requirements for the degree of

Master of Science

in Pharmacology

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November, 2021

ACKNOWLEDGMENTS

Thanks to Dr. Yang Zhang for his guidance on my project.

Thanks to all committee members for their time and valuable feedback.

Thank my family for their support.

ABSTRACT

AdipoRon is a selective agonist of adiponectin receptor and has been reported to protect against vascular remodeling by preventing smooth muscle cell (SMC) dedifferentiation. Our recent studies have demonstrated that activation of transcription factor EB (TFEB) and its downstream autophagy signaling contribute to adipoRon-induced SMC dedifferentiation. This study aims to examine whether acid sphingomyelinase is involved in mediating adipoRon-induced TFEB activation in SMCs. In cultured arterial SMCs isolated from wild-type (Smpd1+/+) mice, adipoRon induced expression of acid sphingomyelinase (ASM; gene symbol Smpd1) and ceramide production, which is accompanied by TFEB nuclear translocation and upregulated transcription of genes involved in autophagy pathway and enhanced autophagic flux pathways. However, such adipoRon-induced ceramide, TFEB activation, and autophagic signaling were suppressed in SMCs from acid sphingomyelinase gene knockout (Smpd1-/-) mice. Consistently, adipoRon suppressed serum-induced cell proliferation and inhibited SMC migration in Smpd1+/+ SMCs as characterized by wound-healing retardation, F-actin reorganization, and matrix metalloproteinase-9 downregulation; whereas these inhibitory effects of adipoRon on proliferation and migration were attenuated in Smpd1-/- SMCs. Mechanistically, adipoRon increased the expression of protein phosphatase 2A (PP2A) and calcineurin in Smpd1+/+ SMCs but not Smpd1-/- SMCs. Pharmacological inhibition of PP2A by okadaic acid (OA) blocked adipoRon-induced TFEB activation and gene expression inSmpd1+/+ SMCs. Furthermore, activation of calcineurin by lysosomal TRPML1 channel agonist ML-SA1 could similarly activate TFEB and downstream autophagic signaling in Smpd1+/+ and Smpd1-/- SMCs. Inhibiting calcineurin by FK506 and cyclosporin A (CsA) prevented AdipoRon-induced TFEB and autophagy signaling activation. Together, these data suggest that adipoRon-induced TFEB signaling in SMCs is dependent on the ASM-mediated activation of phosphatase PP2A and calcineurin. This study provides novel mechanistic insights into understanding the therapeutic effects of adipoRon on TFEB signaling and pathological vascular remodeling.

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LIST OF ABBREVIATIONS

SMC: smooth muscle cell

TFEB: transcription factor EB

ASM: acid sphingomyelinase

OA: okadaic acid

CsA: cyclosporin A

PP2A: protein phosphatase 2A

ECM: extracellular matrix

VSMC: vascular smooth muscle cell

CVD: cardiovascular disease

TLR: Toll-like receptor

CMA: chaperone-mediated autophagy

MITF: microphthalmia

LAMP1: lysosomal-associated membrane protein-1

LAMP2A: lysosomal-associated membrane protein-2A

MMP: matrix metalloproteinases

S1P: sphingosine 1-phosphate

GPCR: G protein-couple receptor

CKD: chronic kidney disease

Introduction

Smooth muscle cells (SMCs) are the major cell type of healthy arteries medial layer and are primarily quiescent and highly differentiated[1]. SMCs are essential for maintaining blood vessel integrity structure and vascular tissue homeostasis and primarily controlling blood pressure and distribution. Based on dynamical phenotype modulation, SMCs are also involved in physiological and pathological vascular remodeling.[2] The contractile phenotype, characterized as spindle-shaped, can switch to a synthetic phenotype characterized as rhomboid in response to local cues, such as vascular injury or atherosclerosis. A dedifferentiated synthetic phenotype exhibit increasing proliferation and remodeling of extracellular matrix (ECM) to promote migration, and reduce SMC marker gene expression compared with differentiated contractile phenotype[2,3]. Increasing studies have shown that vascular smooth muscle cells (VSMCs) with aberrant proliferation and migration develop vascular diseases, such as atherosclerosis[4,5], inflammation[6], restenosis[7], and aortic aneurysm disease[8]. Under these pathophysiological conditions, vascular remodeling forces SMCs to switch from contractile to the synthetic phenotype, contributing to neointima formation through enhancing proliferation and migration to intima[9]. Hence, preventing SMCs from switching to a dedifferentiation phenotype may be a new target.

Cardiovascular disease (CVD) is one of the important factors that threaten human health. About one-third of all deaths are due to cardiovascular disease each year[10]. Therefore, it is urgent to study its pathogenesis mechanism and find effective treatment approaches. Obesity is closely related to CVD, affecting the quality of life and increased mortality. According to clinical follow-up of obese patients, it was found that over two-thirds died of CVD and these numbers continue to rise [11]. The high risk of CVD in obese is due to changing heart structure and function and a strong correlation between

obesity and other major CVD risk factors, such as atherosclerosis, hypertension, and dyslipidemia[12].

Adipokines are biologically active substances produced by adipose tissue, including adiponectin, tumor necrosis factor, etc. Adipokine disorder in obese patients is the main reason for CVD development[13]. Evidence indicates that adipokines regulate SMCs phenotype switching between differentiated with dedifferentiated after various stimulation[14]. Among adipokines, adiponectin, which is consists of 244 amino acid residues and is secreted in large amounts by adipose tissue, has a protective effect that inhibit metabolic disorders and atherosclerosis. Studies show that the level of adiponectin significantly decreased in obesity-inducing myocardial infarction, and overexpressed adiponectin protects the heart from cardiac dysfunction and ischemia-reperfusion injury in mice[15]. There are several ways that adiponectin inhibits CVD. The activation of adiponectin receptors 1 and 2 by adiponectin enhances ceramidase activity and promotes ceramide's decomposition and metabolism to increase the formation of S1P, an anti-apoptotic metabolite. AMPK does not participate in regulation during this process[16]. Adiponectin can also directly activate TRPM4 channels to cause hyperpolarization of VSMCs or stimulate the release of NO from adjacent adipocytes to enhance performance the opening of BKCa channels in VSMCs to achieve the effect of inducing vasodilation[17]. Adiponectin down-regulated Toll-like receptor (TLR) 4 signaling to inhibit inflammatory activation and the interaction of cardiac and immune cells to prevent autoimmune myocarditis inflammation and injury[44]. Taken together, adiponectin is an important target for treating obesity and cardiovascular diseases. However, adiponectin is not suitable for clinical use due to its short half-life and large molecular weight[18]. Therefore, using receptor activators is a good way to mimic the effects of adiponectin.

AdipoRon, an adiponectin receptor agonist, mimics some adiponectin benefit effects[45]. For example, adiponectin level in type 2 diabetic patients is lower than normal patients, and this correlates with increased damage to blood vessels and the development of CVD.

Experiments have shown that increasing the content of adiponectin by taking AdipoRon improves insulin resistance and lipotoxicity of diabetic nephropathy in type 2 diabetic mice, by activating the AdipoR1-AMPK and AdipoR2-PPARα pathways[16,19]. AdipoRon plays an anti-apoptotic effect by partially activating AMPK, protecting the heart from ischemia/reperfusion injury. Therefore, AdipoRon also has a mechanism independent of AMPK signaling to protect the cardiovascular system[20]; AdipoRon activates the PI-3 kinase/Akt metabolic pathway in a concentration-dependent manner, inhibiting the activation of ERK and p38 MAPK mitotic factors reducing VSMC proliferation and migration, and reducing pro-inflammatory factors exhibiting anti-atherosclerosis and anti-inflammatory effects[21]; AdipoRon inhibition of mTOR/p70S6K signaling prevents VSMC proliferation, suppressing neointimal hyperplasia[22]. Recently, our lab showed AdipoRon activation of TFEB relied on intracellular calcium, but was not related with AMPK, ERK1/2, Akt, and mTOR. VSMC proliferation and migration were inhibited to maintain VSMC homeostasis between contractile and synthetic phenotypes[18]. These findings might explain the reason why AdipoRon's cardiovascular protection effect is partially dependent on the AMPK pathway.

Autophagy is a highly evolutionarily conserved, repetitive, and dynamic circulatory system, which is indispensable for maintaining cell homeostasis[23]. Autophagy has three types: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Among them, macroautophagy is the one that has been studied the most and is the one we focus on here. The difference between macroautophagy and microautophagy (capture mechanism) and CMA (chaperones mediated) is that macroautophagy synthesizes autophagosomes to isolate the cargo from the lysosome, and the cargo is not degraded and recovered until the autophagosome fuses with the lysosome to form an autophagolysosome[24]. Therefore, autophagosome fusion with lysosomal is a key step for macroautophagy. Macroautophagy can degrade the cytoplasm and damaged organelles through the fusion of autophagosome and lysosome, thereby promoting nutrient supply and "garbage" removal. Research has shown that

SMCs deficiency of autophagy facilitates the development of atherosclerosis associated with outward arterial remodeling[25]. Accumulating evidence indicates that when the autophagy-lysosomal system is enhanced by genetic or pharmacological means atherosclerosis is reduced[26]. Our lab showed that trehalose, a natural disaccharide, worked as a TFEB activator and autophagy inducer[27]: A activation of TFEB and its autophagy signal axis can reverse high fat diet-induced intimal hyperplasia and neointimal formation [23]. Together, the regulation of SMC autophagy may be a new therapeutic approach for vascular disease.

Transcription factor EB (TFEB), a master gene that regulates lysosomal biogenesis and autophagy, belongs to the microphthalmia transcription factor (MITF) family involved in cellular processes, such as proliferation, metabolism, and organelle biogenesis[28]. The activated TFEB is dephosphorylated by calcineurin and translocated to the nucleus then binds to the common 10-base E box-like nucleotide sequence palindrome to activate the transcription of genes, like p62, that regulate the synthesis of lysosomes and autophagosomes, thereby activating autophagy[29]. Recently, our lab indicated that AdipoRon-induced TFEB activation can inhibit the proliferation and migration of arterial smooth muscle cells. The work reveals that AdipoRon-induced TFEB activation depends on intracellular calcium, not on Akt, ERK1/2, AMPK, and mTOR[18]. Therefore, AdipoRon-induced TFEB-autophagy pathway activation needs further detailed study.

Sphingolipids contain an eighteen-carbon amino-alcohol backbone structure, produced by different enzymes to active products that regulate cell functions and signal transduction[30]. Sphingomyelin is the most abundant sphingolipid in the human cell, metabolized into ceramide and phosphorylcholine by sphingomyelinase catalyzed sphingomyelin phosphodiester bond breakage. Ceramide is involved in the cellular responses caused by a variety of stimulation, such as pathogen infection, stress, disease, etc[46-48]. Ceramidase metabolizes ceramide to sphingosine, and sphingosine kinase phosphorylates sphingosine to sphingosine-1-phosphate(S1P) which binds to a G

protein-coupled receptor to elevate intracellular Ca2+ level by activating inositol trisphosphate IP3-mediated Ca2+ mobilization[31]. Sphingomyelinase deficiency can cause Niemann-Pick, a disease characterized by excessive sphingomyelin accumulation and lysosomal storage. Sphingomyelinase (encoded by Smpd1 gene) plays an optimal role under different pH conditions (acidic, alkaline, neutral), and acid sphingomyelinase (ASM) has been shown closely related to cell activation[32]. A previous study showed that ASM deficiency prevents lysosomal fusion with autophagosome to form autophagolysosomes impairing autophagy, and this is reversed by overexpressing ASM in coronary arterial SMCs[33]. Base on this result, our lab further demonstrated that ASM deficiency causes accumulation of p62/SQSTM1 by inhibiting autophagy, signaling to trigger SMC transition to myofibroblast[34]. Collectively, ASM is required for autophagy signaling and mediates SMCs homeostasis.

Collectively, AdipoRon inhibits SMCs proliferation and migration by TFEB activation depending on intracellular Ca2+. By contrast, blocking TFEB-mediated autophagy signaling promotes SMCs switching to a dedifferentiated phenotype. ASM as an indispensable enzyme in the autophagy signaling pathway is also participated in mediating SMC morphological change. However, whether ASM is involved in AdipoRon-induced TFEB activation and differentiation in arterial SMC has not been identified.

Methodology

1. Arterial SMCs primary culture

As previously described, SMCs were isolated from six-week male C57BL/6J Smpd1-/-(ASM deficiency) mice and Smpd1+/+ (wild-type littermates) mice. In brief, deeply anesthetized by pentobarbital sodium (25 mg/kg) intraperitoneal injection. The heart was excised with an intact aortic arch and immersed in ice-cold Krebs-Henseleit in a petri dish. A 25-gauge needle filled with Hanks' buffered saline solution was inserted into the aortic lumen opening and deep into the heart close to the aortic valve. Hanks' buffered saline solution was injected through the pillow at 0.1 ml/min for 15 minutes, and then replace with a warm enzyme solution. The perfusion fluid was collected at 30, 60, and 90 minutes, and at the last time point, the heart was cut and washed with Venus to ensure that the cells in the ventricle were obtained. The collected perfusate containing cells was centrifuged at 1000 rpm for 10 minutes. The pellet after centrifugation was incubated with Advanced Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, 7892-06) which mixed with 10% fetal bovine serum(Sigma, A3733-500G), 10% mouse serum(Equitech-Bio, SM30-0500), and 2% antibiotics(Gibco, 15-140-122), in six-well plates and incubated at 37°C in 5% CO2. The medium was changed 3 days after the cells were separated, and then changed once or twice a week until the cells grew to confluence.

2. Immunoblotting

The cells were lysed in Laemmli sample buffer (Bio-Rad,161-0737) containing mercaptoethanol (Sigma Aldrich, M3148). The mixture was heated at 95°C for 10 minutes and then placed in an ice-cold water ultrasonic bath for 10 minutes. 20 micrograms of protein were separated by 8-12% SDS-PAGE. The separated proteins were transferred to a PVDF membrane by electrophoresis (35 V) at 4°C for 20-24 hours. The PVDF membrane with protein was blocked with Bovine Serum Albumin (BSA) (Sigma, A3733-500G). After washing with TBST, the membrane was probed

with the indicated primary antibody(LC3: CST, 12741S; P62/ SQSTM1: Abcam, ab109012; PP2A: CST, 2259S; Calcineurin: CST, 2614S; β-actin: CST, 3700S). The membrane was washed again and incubated with the corresponding secondary antibody(IRDye 800CW donkey anti-mouse IgG (H_L):LICOR,926-32212; IRDye 800CW donkey anti-rabbit IgG (H_L):LICOR,926-32213; donkey anti-mouse IgG (H_L), HRP: Thermo Fisher,A16011; stabilized peroxidase-conjugated goat anti-rabbit (H_L): Invitrogen,32460; goat anti-rat IgG-HRP: Thermo Fisher,629520.). The protein bands were visualized and analyzed with the LI-COR Odyssey Fc system.

3. Real-time PCR

Aurum Total RNA Mini Kit (Bio-Rad,732-6820) was used to extract RNA. iScript Reverse Transcription Super Mix (Bio-Rad,1708841) was used for reverse transcription of cDNA. iTaq Universal SYBR Green supermix (Bio-Rad,172512) was used in the Bio-Rad CFX Connect real-time system. The cycle threshold values were converted to relative gene expression levels using the $2^{-\Delta\Delta Ct}$ Ct method. The data were normalized to that of internal control β-actin.

4. Immunofluorescence staining

Approximately 1×10^4 mouse SMCs were added to a 24-well culture plate with a cover glass. After culturing with DMEM for 24 hours, AdipoRon(AdipoGen, AGCR10154M010) was added and continued for 24 hours. The treated cells were quickly washed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After washing 3 times with PBS, the cells were permealized with 3% Triton X-100(Sigma, X100) in PBS for 15 minutes, then incubated with the corresponding primary antibody, overnight at four degrees. The secondary antibody (Alexa Fluor 488: Fisher, I21411; Alexa Fluor 555: Fisher, PIA32816.) was paired with the corresponding primary antibody (TFEB: Bethyl Laboratories, A303-673A; ASM: Novus Biologies, NBP2-45889; Ceramide: Enzo, ALX-804-196-T050.). DAPI was used to stain cell nuclei at room temperature for fifteen minutes, then

fixed with an anti-fluorescence quencher (EMS, 17986-01). The Olympus IX73 imaging system was used to visualize the cells. Image-Pro Plus 6.0 software was used to analyze the Pearson correlation between colocalization efficiency and average fluorescence density.

5. Wound scratch assay of SMC migration.

A 2 mm wide pipette tip was used to make a linear scratch on the SMC covered in a six-well plate. After 24 hours, the Olympus IX73 imaging system was used to image the scratched area of the cells. Image-Pro Plus 6.0 software was used to quantify the average injured area.

6. MMP activity assay

The MMP Activity Kit (Abcam, ab112146) was used here to determine the activity of MMP. In a 96-well plate, SMC was cultured in AdipoRon for 24 hours and then incubated with a universal MMP activity indicator (fifty microliters of fluorescence resonance energy transfer peptide) at room temperature for 30 minutes. An excitation/plate reader fluorescence emission of 485/520nm was used for intensity detection and analysis.

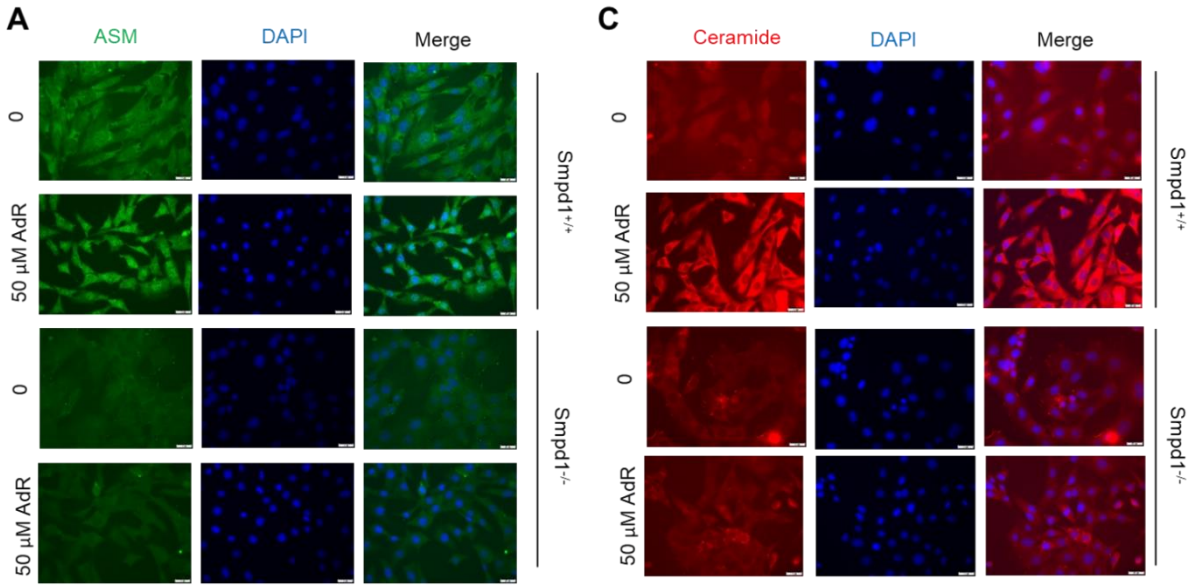
7. Statistics analysis

Student t-test is used to analyze experiments compared between 2 groups. One-way and two-way ANOVA are used to analyze over 1 variable. Graphpad Prism 6.0 software (GraphPad Software, USA) was used to perform statistical analysis. $P<0.05$ was considered statistically significant.

Results

1. AdipoRon induced ASM expression and ceramide production

AdipoRon, as a selective agonist of adiponectin receptor, has been shown to prevent SMC dedifferentiation by increasing TFEB activation[16]. To study whether AdipoRon mediates ASM expression, we first cultured Smpd1+/+ and Smpd1-/- SMCs with or without AdipoRon (50 μM) for 24h to detect ASM expression. As shown in Figs. 1A and B, the ASM fluorescence intensity of Smpd1+/+ SMCs treated with AdipoRon significantly increased compared with Smpd1+/+ SMCs without AdipoRon. However, AdipoRon treated Smpd1-/- SMCs exhibited no difference. This result indicated that AdipoRon regulates ASM expression in SMCs. To confirm, ceramide, produced from sphingomyelin catalyzed by ASM, also be tested shown in Figs. 1 C and D. Consistently, ceramide expression was also markedly higher in Smpd1+/+ SMCs treated with AdipoRon than without. Smpd1-/- SMCs ceramide could not be upregulated by AdipoRon. Together, AdipoRon



increased ASM and ceramide expression in Smpd1+/+, but not in Smpd1-/- SMCs suggest that AdipoRon regulate ASM and ceramide expression.

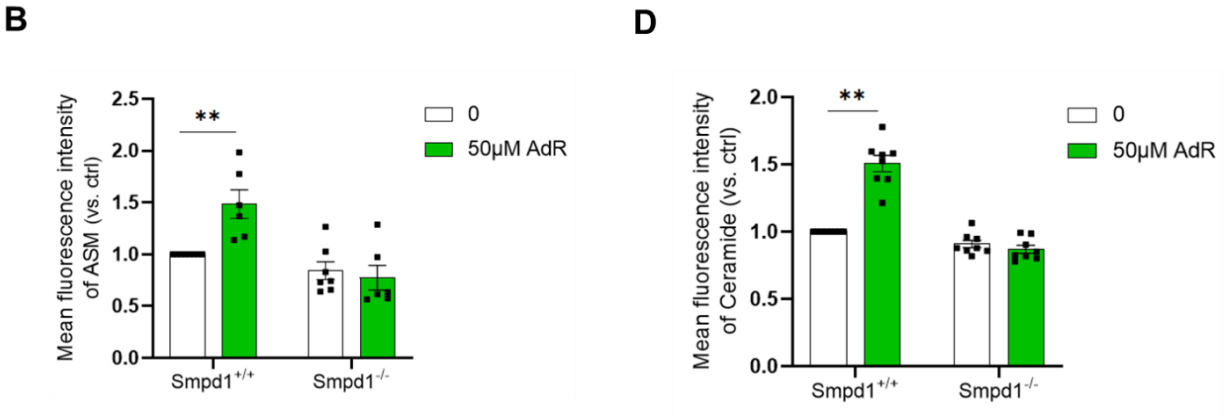
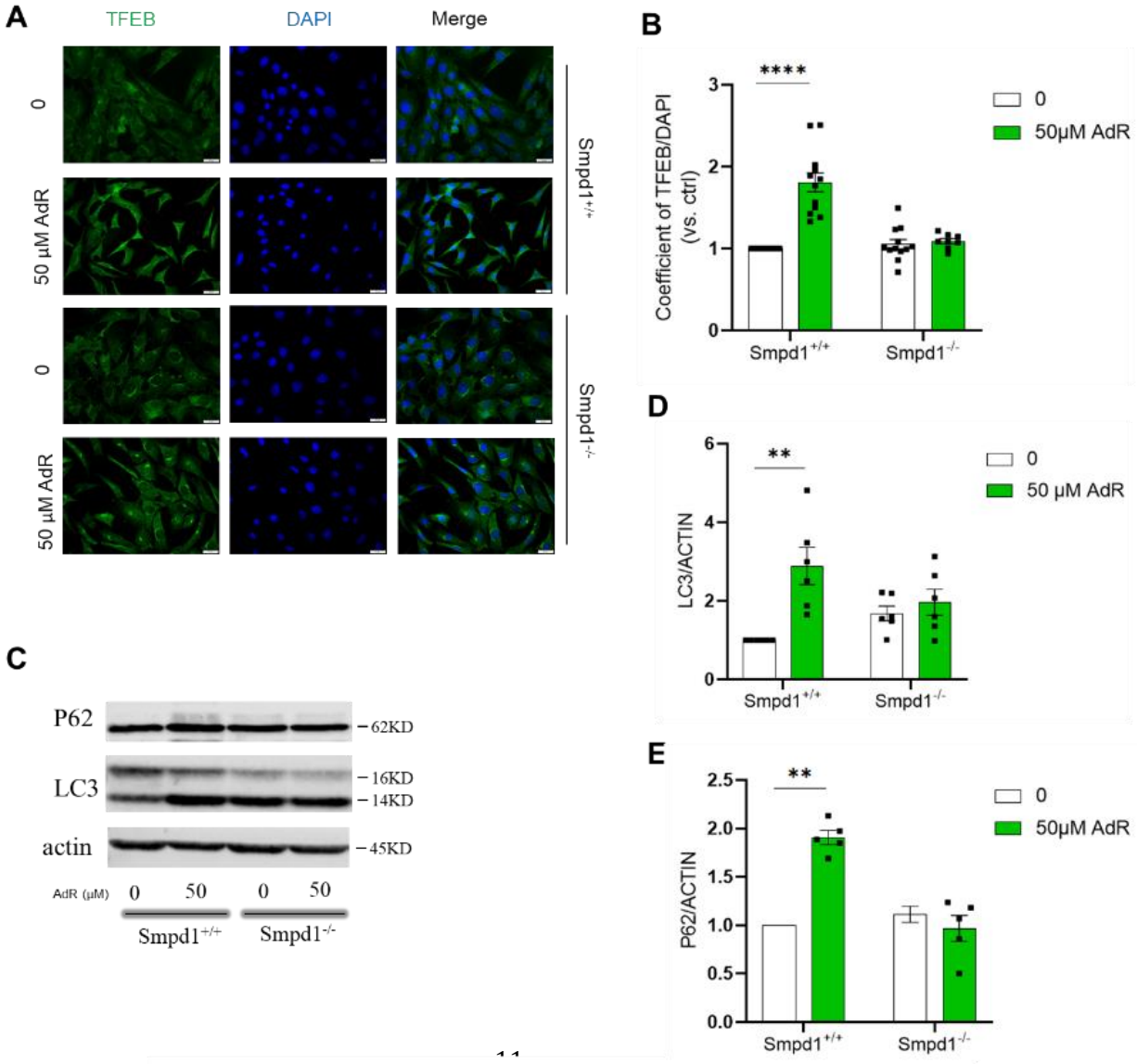


Fig 1. AdipoRon induced ASM expression and ceramide production in Smpd1+/+ SMCs. Smpd1+/+ and Smpd1-/- SMCs cultured with or without 50 μM AdipoRon 24h. A and B: ASM expression were showed in immunofluorescence images. Fluorescence intensity of ASM was quantified. (n=6). C and D: Ceramide expression were showed in immunofluorescence images. Fluorescence intensity of ceramide was quantified. (n=6). **P* < 0.05 vs. Smpd1+/+ SMCs without AdipoRon treatment.

2. Smpd1 gene ablation inhibits AdipoRon-induced TFEB and autophagy signaling activation

We next examined whether ASM is involved in AdipoRon-induced TFEB and autophagy signaling activation. We cultured Smpd1+/+ and Smpd1-/- SMCs with or without AdipoRon. By observing TFEB translocation to the nucleus, we determined whether TFEB was activated. As shown in Figs. 2A and B, TFEB activated by AdipoRon in Smpd1+/+ SMCs, but not in Smpd1-/- SMCs. This result demonstrated that ASM is one necessary step for AdipoRon-induced TFEB activation. Consistently, the TFEB mRNA levels are also confirmed an increased stimulation by AdipoRon in Smpd1+/+ SMCs (Figs 2F).

LC3, an autophagy marker, and p62, an autophagic substrate, reflect that autophagy flux. AdipoRon treated Smpd1+/+ SMCs expressed higher protein levels of LC3 and P62, shown in Figs. 2D and F. Furthermore, the RNA levels of LC3 and p62 are consistent with reduced protein levels (Figs. 2F). AdipoRon also increases lysosomal-associated membrane protein-1 (LAMP1), and the lysosomal-associated membrane protein-2A (LAMP2A) mRNA levels in Smpd1+/+ SMCs, but not Smpd1-/- SMCs. Together with all results, ASM is indispensable for AdipoRon-induced TFEB and autophagy signaling activation.



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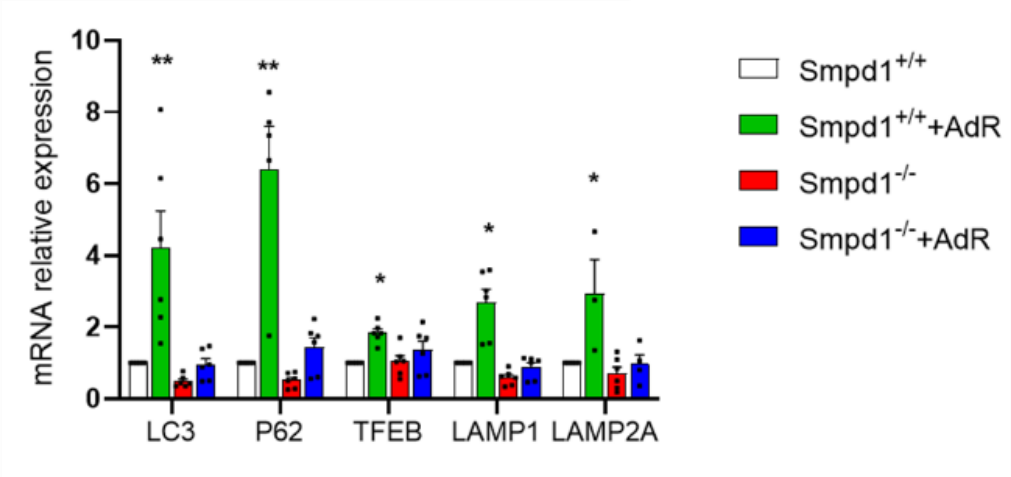
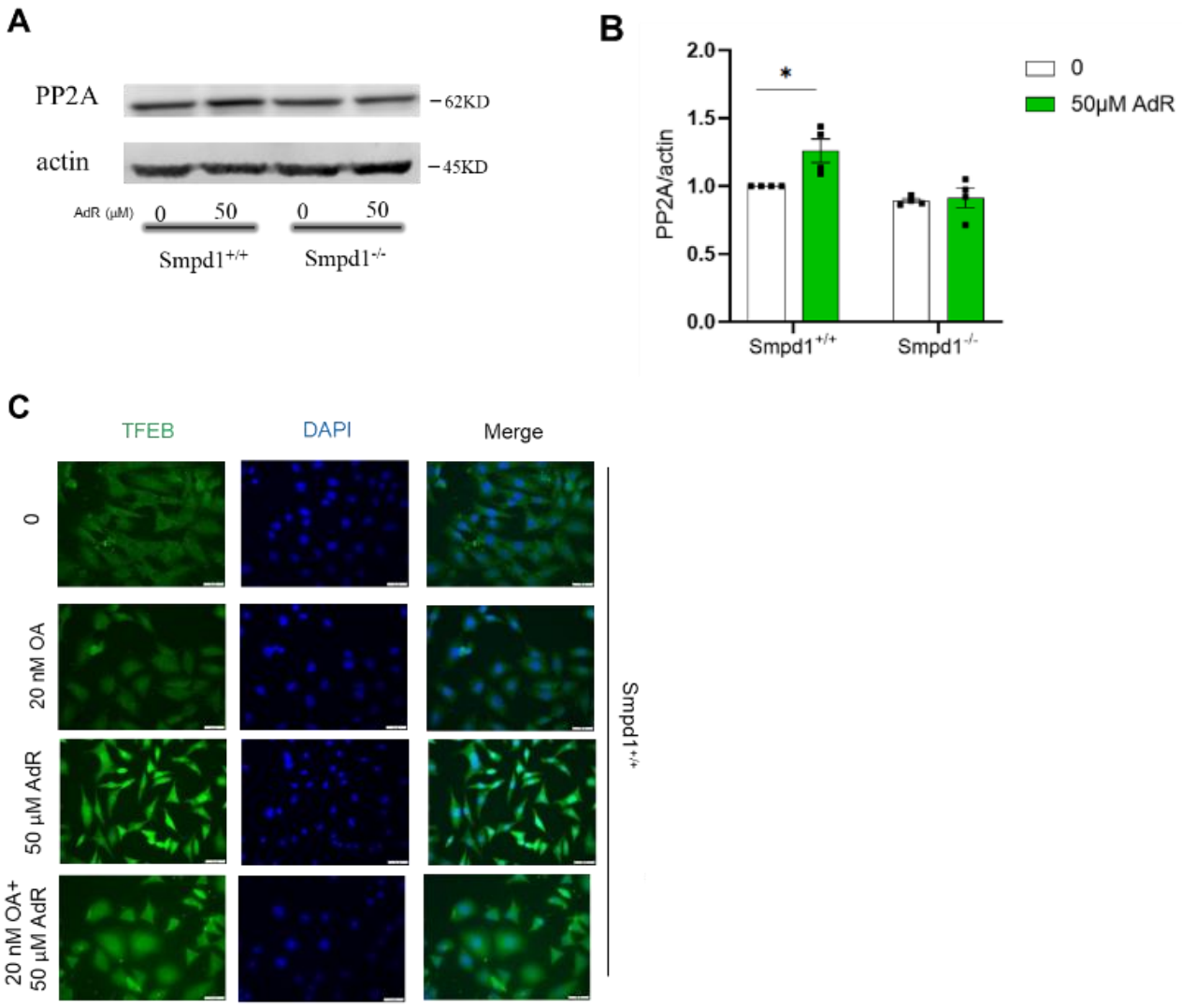


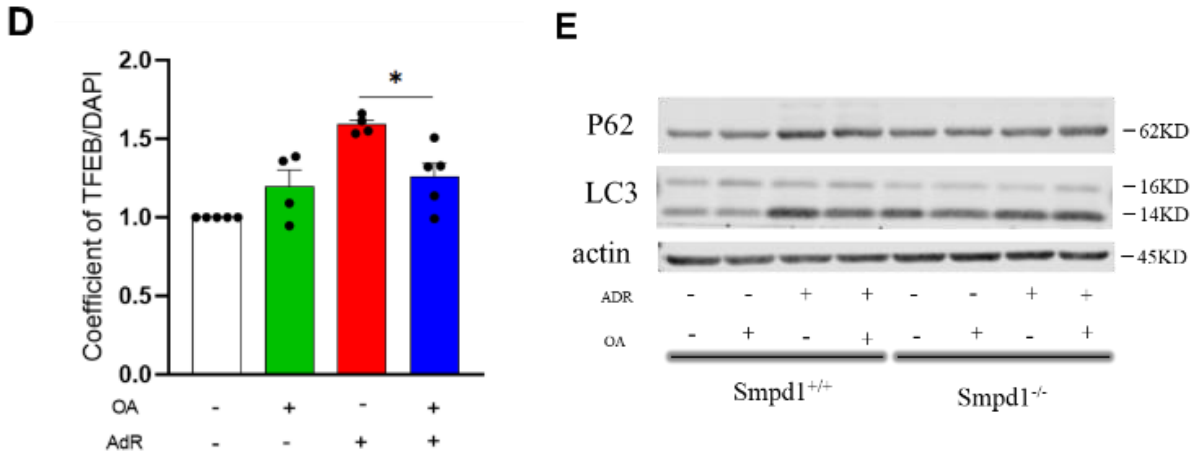
Fig 2. Smpd1 gene ablation inhibits AdipoRon-induced TFEB and autophagy signaling activation. Smpd1+/+ and Smpd1-/- SMCs cultured with 50 μM AdipoRon 24h. A and B: TFEB activation was showed in immunofluorescence images (green: TFEB; blue: nuclei). TFEB nucleus translocation was quantified. (n=6). C, D and E: LC3 and P62/SQSTM1 protein expression were performed in immunoblotting images and quantified. (n=4-6). F: LC3, p62/SQSTM1, TFEB, LAMP1 and LAMP2A mRNA levels were showed in Real-time PCR analysis. (n=6). *P < 0.05 vs. control as indicated.

3. ASM mediates AdipoRon-induced TFEB activation by activating PP2A

PP2A, a serine and threonine phosphatase, targets kinases and effectors to regulate cellular proliferation. Previous studies shows that ceramide increases PP2A activation, contributing to ceramide's anti-proliferation role[35]. To determine the role of PP2A in AdipoRon-induced TFEB activation, the expression of PP2A was measured. As shown in Figs. 3A and B, AdipoRon increases PP2A expression in Smpd1+/+ SMCs, but not in Smpd1-/- SMCs. This data confirmed that PP2A expression increase by AdipoRon was achieved by increasing ASM-ceramide level. AdipoRon-induced TFEB activation in Smpd1+/+ SMCs was inhibited by Okadaic acid (OA) (20 nM), a PP2A inhibitor (Figs. 3C and D), indicating that inhibition of

PP2A prevents AdipoRon-induced TFEB activation. Inhibiting PP2A also reverses AdipoRon-induced autophagy activation. As shown in Figs. 3E, F, and G, the increased expression of LC3 and P62 by AdipoRon were reduced by OA. These results indicated that ASM mediated AdipoRon-induced TFEB and autophagy flux activation via regulating PP2A.

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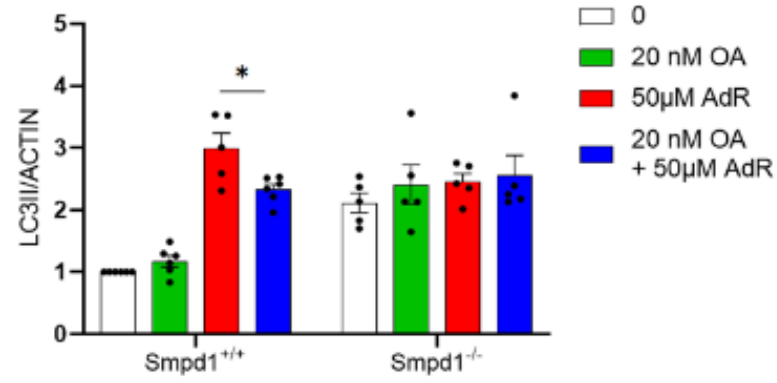
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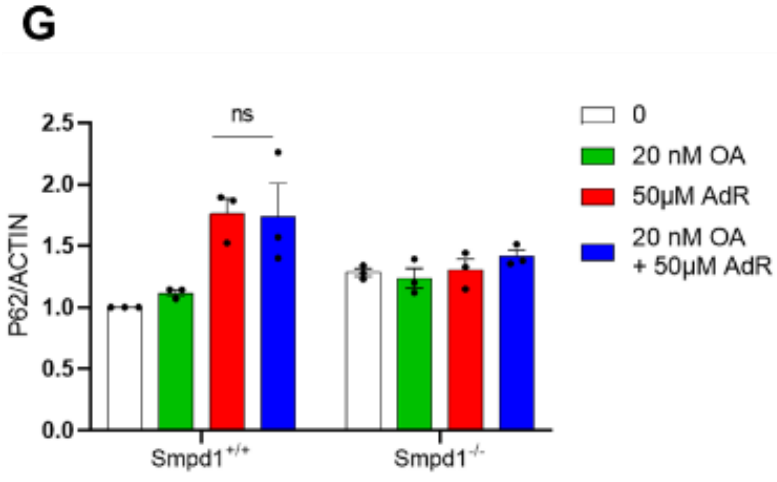
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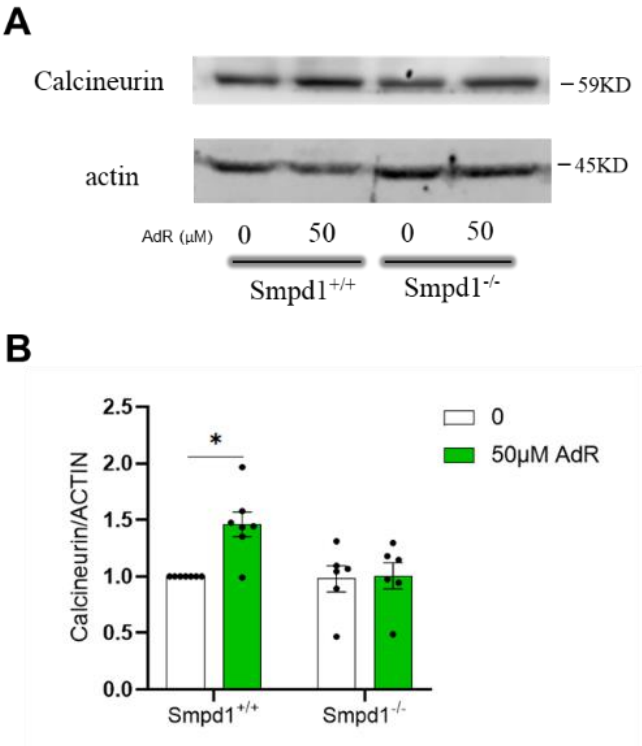
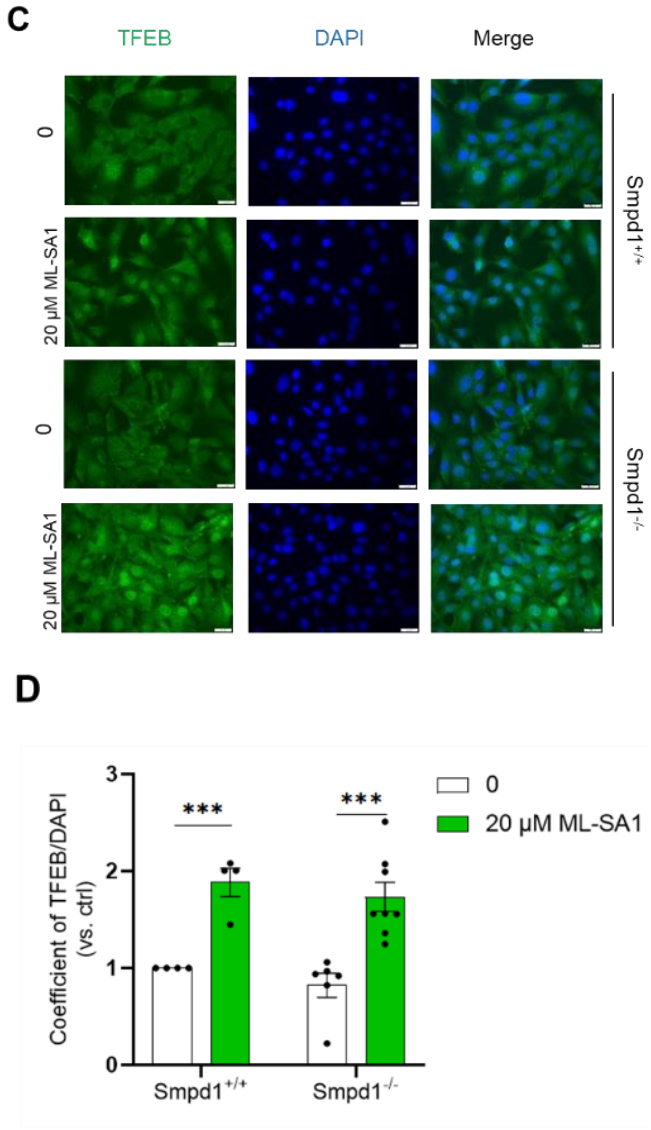
Fig 3. ASM mediates AdipoRon-induced TFEB activation by activating PP2A. Smpd1+/+ and Smpd1-/- SMCs cultured with 50 μM AdipoRon 24h, and OA was added before AdipoRon 1h. A and B: PP2A protein expression was performed in immunoblotting images and quantified. (n=4). C and D: TFEB activation was showed in immunofluorescence images (green: TFEB; blue: nuclei). TFEB nucleus translocation was quantified. (n=4-6). E, F and G: LC3 and P62/SQSTM1 protein expression were performed in immunoblotting images and quantified. (n=4-6). *P < 0.05 vs. control as indicated.

4. ASM mediates AdipoRon-induced TFEB activation by activating calcineurin

Our previous study suggested that AdipoRon-induced TFEB activation depends on intracellular $Ca2^{+}$[16]. Calcineurin, activated by Ca2+ released from lysosomal, is indispensable for TFEB nuclear translocalization[43]. Therefore, we investigated whether calcineurin is involved in AdipoRon-induced TFEB activation. As Figs. 4A and B showed, AdipoRon activates calcineurin in Smpd1+/+ SMCs, but not in Smpd1-/- SMCs. ML-SA1, a TRPML1 agonist,



stimulates Ca2+ release activating calcineurin (Figs. E and F). Figs. 4C shows that ML-SA1 induced TFEB nuclear translocation in both Smpd1+/+ and Smpd1 -/- SMCs. Furthermore, we determined whether ML-SA1 activates autophagy in Smpd1-/- SMCs. As shown in Figs. 4E and F, ML-SA1 significantly upregulated p62 and LC3 protein expression. Together, these data demonstrated that ASM deficiency does not obstruct Ca2+ release, triggered by ML-SA1, still activates calcineurin-induced TFEB activation.



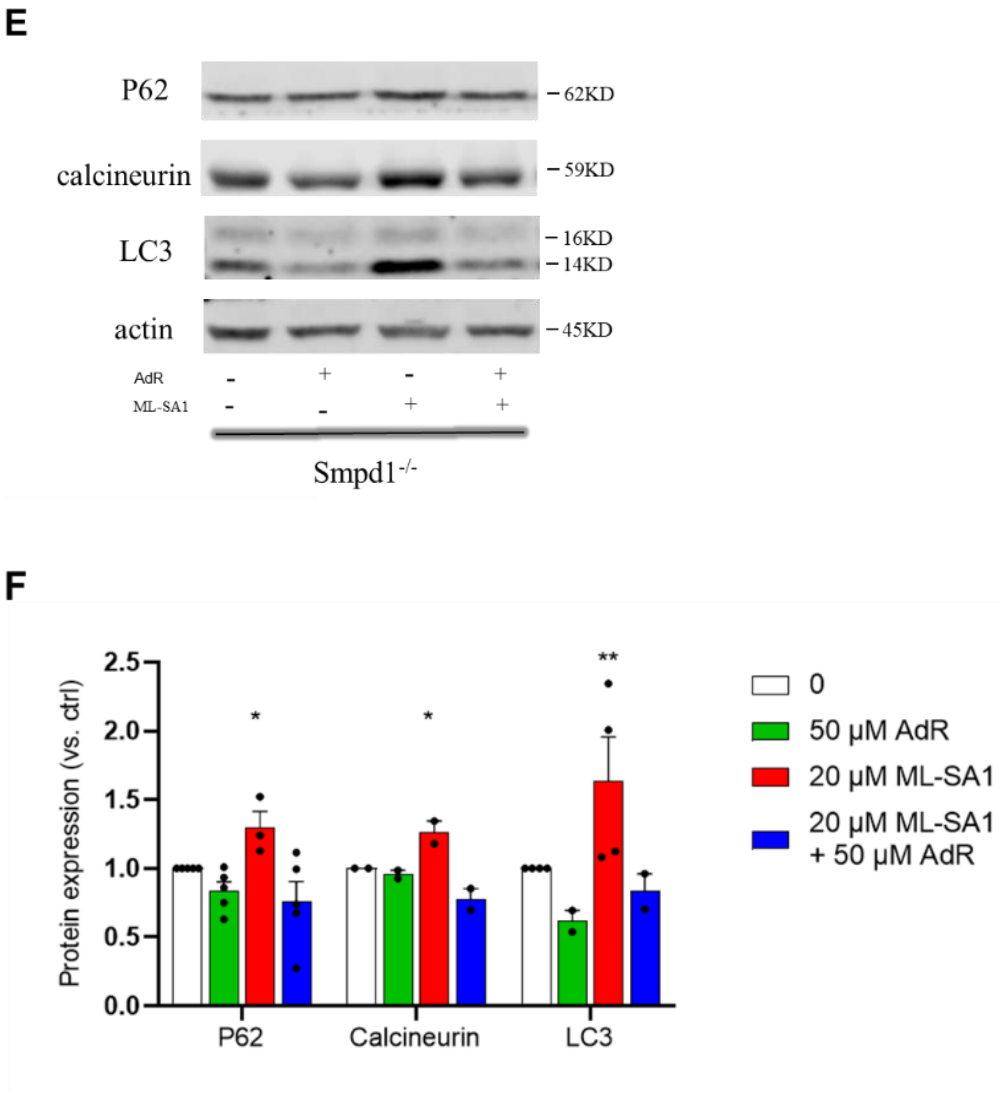
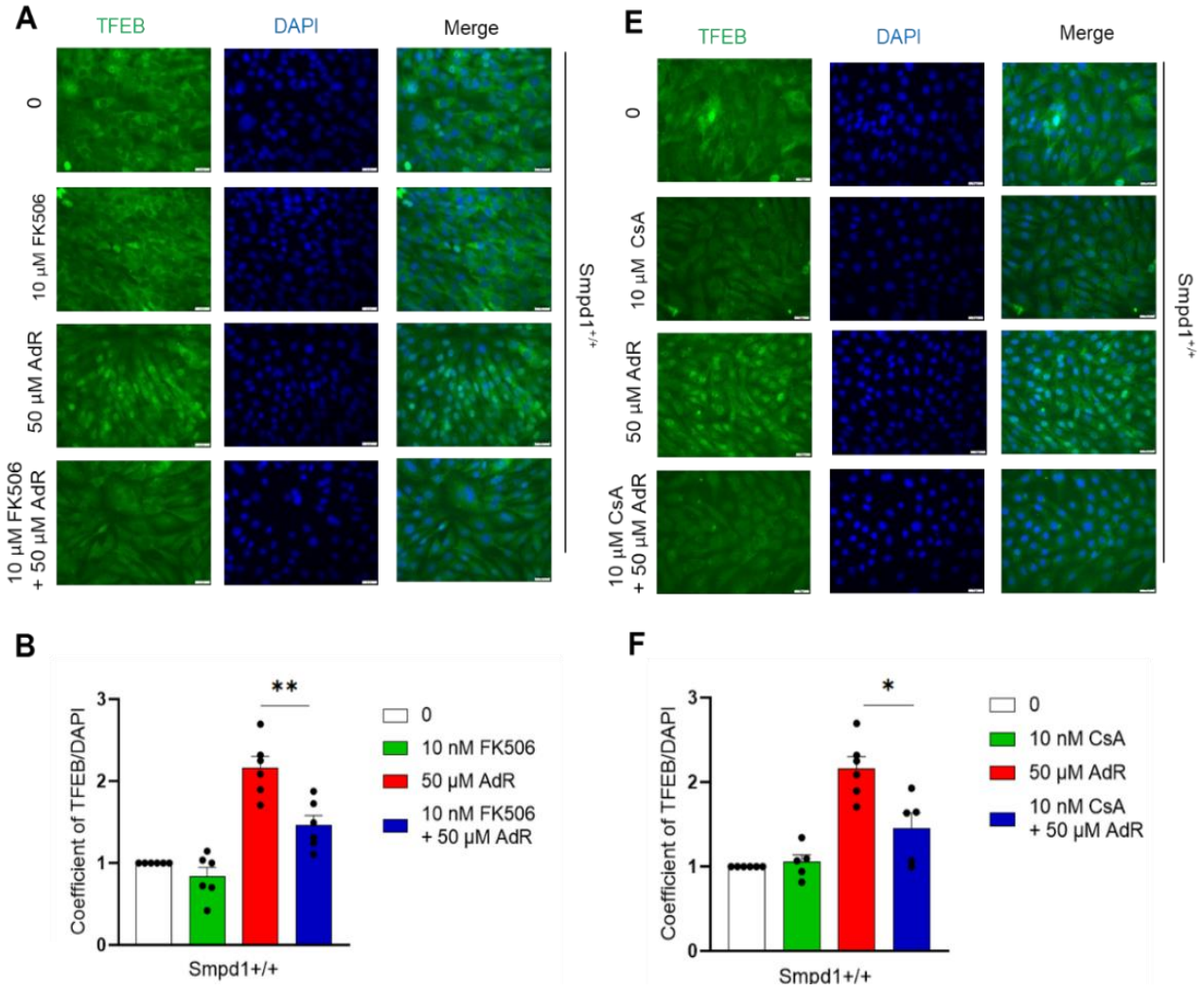


Fig 4. ASM mediates AdipoRon-induced TFEB activation by activating calcineurin. Smpd1+/+ and Smpd1-/- SMCs cultured with 50 μM AdipoRon 24h, and ML-SA1 was added before AdipoRon 1h. A and B: Calcineurin protein expression was performed in immunoblotting images and quantified. (n=6). C and D: TFEB activation after ML-SA1 treatment was showed in immunofluorescence images (green: TFEB; blue: nuclei). E and F: P62/SQSTM1, calcineurin and LC3 protein expression were performed in immunoblotting images and quantified. (n=4-6). *P < 0.05 vs. control as indicated.

5. Calcineurin inhibition decrease AdipoRon-induced TFEB activation

To determine whether calcineurin inhibition reverses AdipoRon-induced TFEB nuclear translocation, we choose two calcineurin inhibitor, FK506 and cyclosporin A. Figs. 5A, B, E and F show that both FK506 and cyclosporin A reduced AdipoRon-induced TFEB nuclear translocation. Calcineurin also reverses AdipoRon-induced autophagy activation.



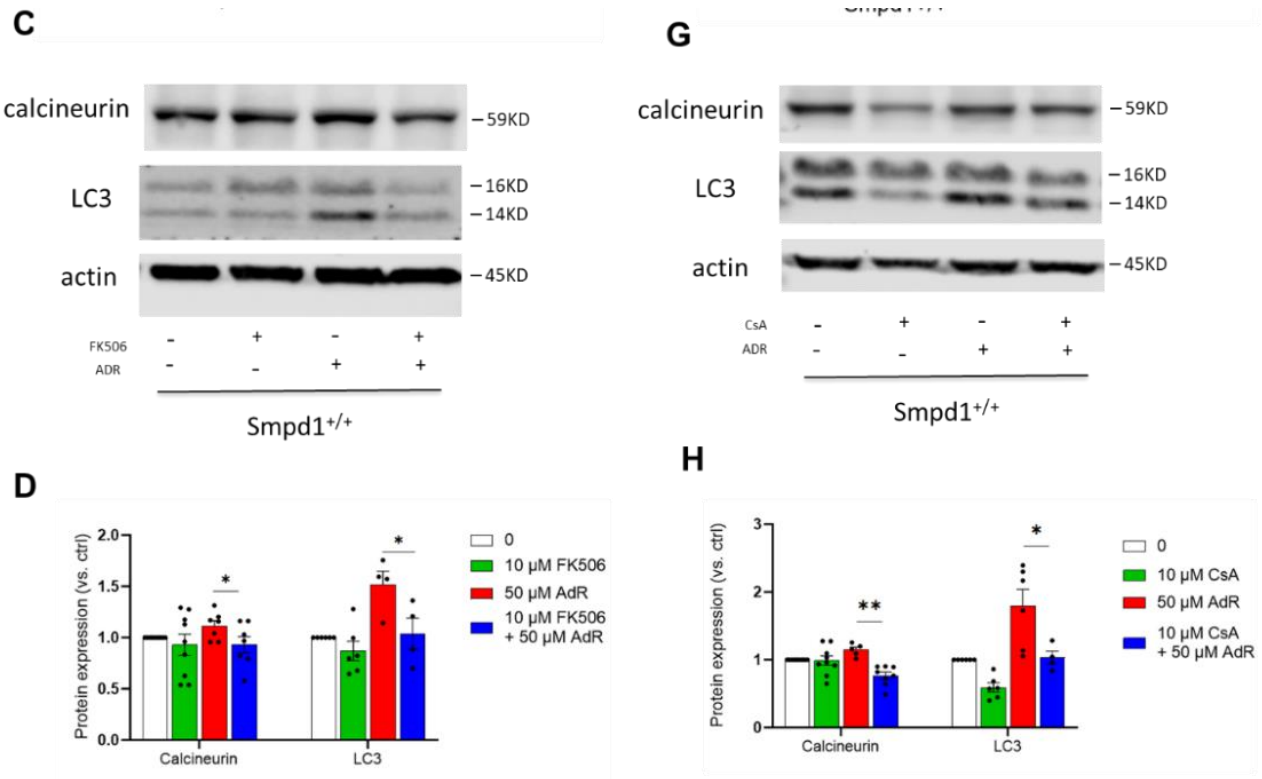


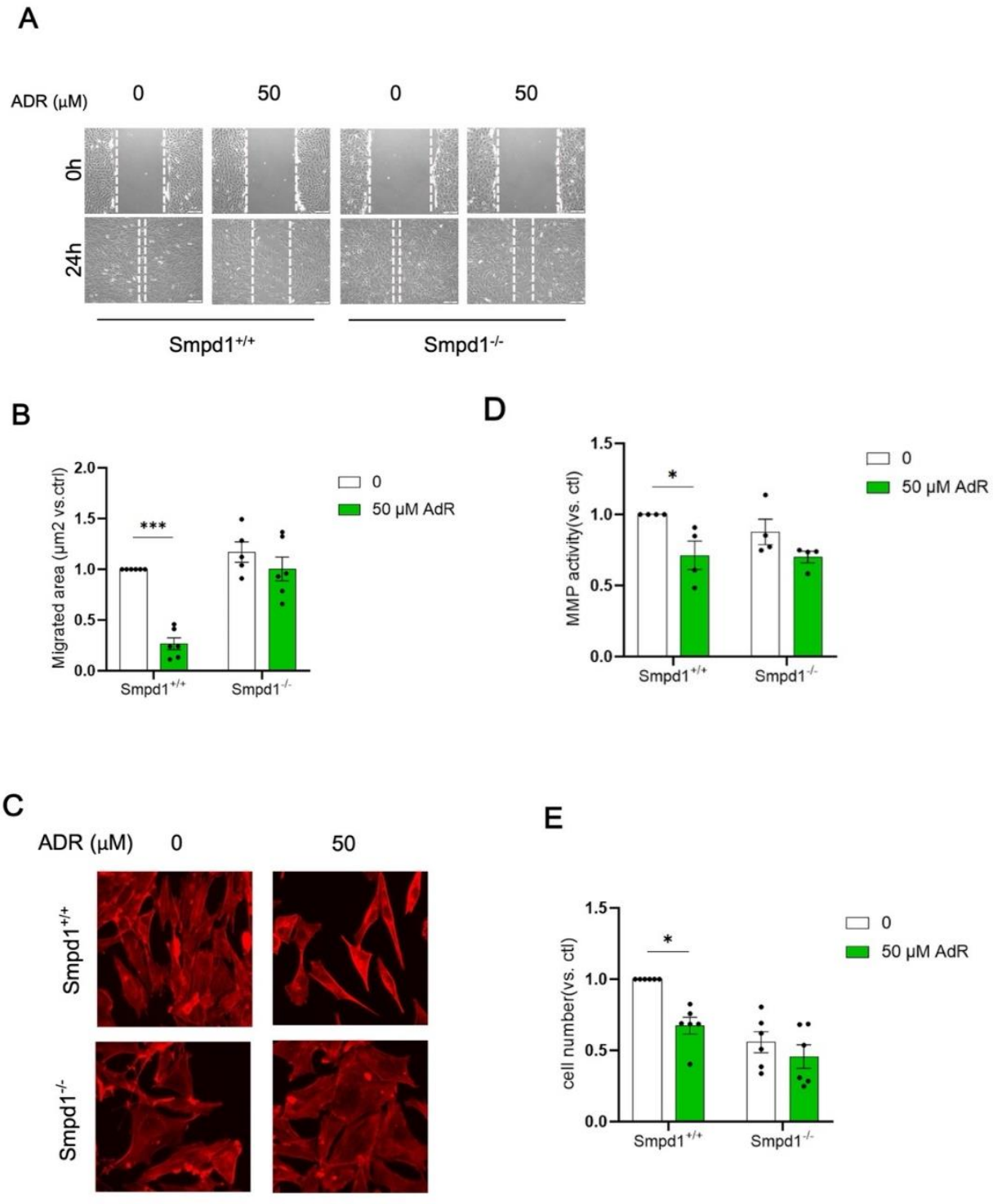
Fig 5. Calcineurin inhibition prohibit AdipoRon-induced TFEB activation. Smpd1+/+ and Smpd1-/- SMCs cultured with 50 μM AdipoRon 24h, and FK506 and CsA was added before AdipoRon 1h. A, B, E and F: TFEB activation inhibited by FK506 and CsA were showed in immunofluorescence images (green: TFEB; blue: nuclei). TFEB nucleus translocation was quantified. (n=6). Nuclei stained by DAPI (blue). C, D, G and H: Calcineurin and LC3 protein expression were performed in immunoblotting images and quantified. (n=6-8). *P < 0.05 vs. control.

As shown in Figs. 5 D and H, both FK506 and cyclosporin A decreased AdipoRon-induced LC3 protein expression increases in Smpd1+/+ SMCs. Interestingly, when cyclosporin A is used alone, it also inhibits the expression of LC3. Taking these together, calcineurin activation remedies ASM deficiency obstructed TFEB activation, and calcineurin inhibition prevents AdipoRon-induced TFEB activation. Therefore, calcineurin plays a necessary and sufficient role in AdipoRon-induced TFEB activation.

6. Smpd1 gene ablation reverse the effects of AdipoRon on SMC migration and proliferation

Our previous study indicated that TFEB activation by Trehalose, a TFEB activator, inhibits SMC migration and proliferation. We next confirmed that AdipoRon-induced TFEB activation also decreases Smpd1+/+ SMCs migration (Figs. 6A and B). However, ASM deficiency inhibited AdipoRon induced SMCs migration effect (Figs. 6A and B). Consistently, matrix metalloproteinases (MMP), are 20 membrane or secreted related peptidase enzymes, controlling SMCs migration in vascular remodeling[49]. AdipoRon inhibited MMP activity in Smpd1+/+, but not in Smpd1-/- SMCs(Figs. 6D). SMCs usually switching to dedifferentiation phenotype facing stimulation. As shown in Figs. 6C, both Smpd1+/+ and Smpd1-/- SMCs exhibited a migrative phenotype. However, only Smpd1+/+ SMCs cultured with AdipoRon convert to contractile phenotype, but Smpd1-/- does not. Moreover, AdipoRon reduced Smpd1+/+ SMCs cell number, but no effect on Smpd1-/- SMCs(fig6e). Detect on ki67 measured cell proliferation, as shown in Figs. 6G and F, AdipoRon significantly decreased Ki67 positive cell numbers in Smpd1+/+, but not in Smpd1-/- SMCs.

Taking these data together, AdipoRon inhibits SMC's migration and proliferation. However, Smpd1 gene ablation abolished the AdipoRon inhibition effect.


A
ADR (μM)
0
50
0
50
0h
24h
Smpd1+/+
Smpd1-/-
B
Migrated area (μm2 vs.ctrl)
2.0
1.5
1.0
0.5
0.0
***
0
50 μM AdR
Smpd1+/+
Smpd1-/-
D
MMP activity(vs. ctl)
1.5
1.0
0.5
0.0
*
0
50 μM AdR
Smpd1+/+
Smpd1-/-
C
ADR (μM)
0
50
Smpd1+/+
Smpd1-/-
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cell number(vs. ctl)
1.5
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0
50 μM AdR
Smpd1+/+
Smpd1-/-

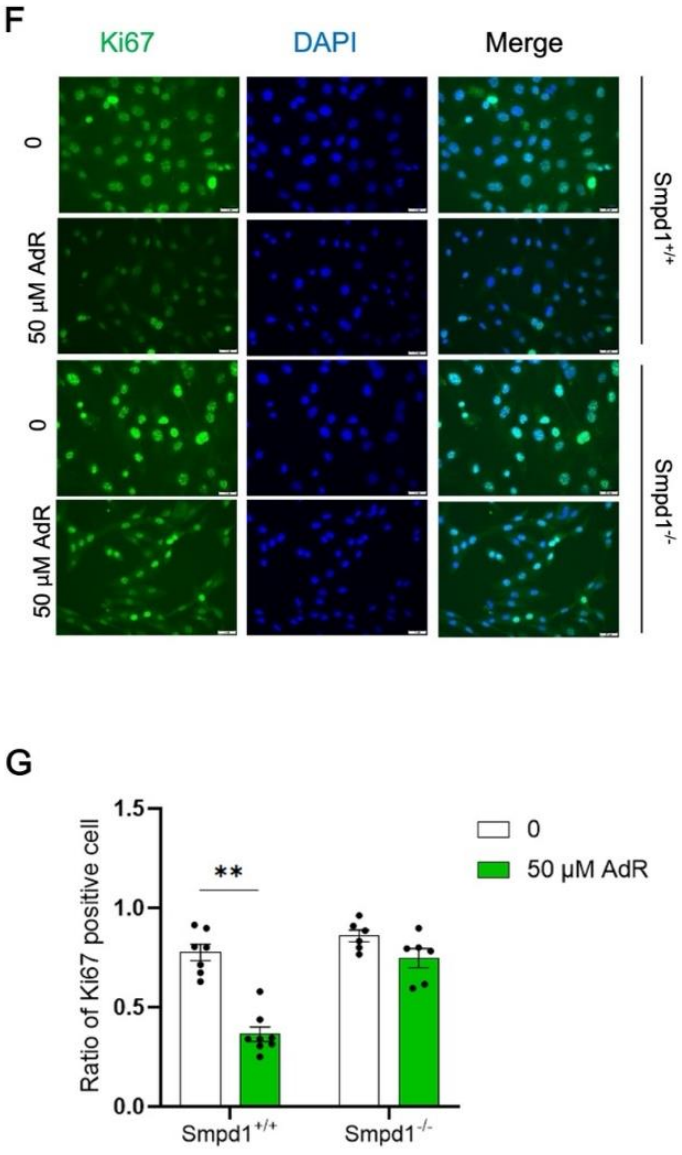

Fig 6. Smpd1 gene ablation reverse AdipoRon-induced SMC migration and proliferation. Smpd1+/+ and Smpd1-/- SMCs cultured with AdipoRon 24h. A and B: scratch assay was used to reflect cell migration. (n=6). C: F-actin filaments arrangement showed in immunofluorescence images (n=4). D: representative MMP activity quantification (n=4). E: cell counting quantified data show cell numbers. (n=6). F and G: Ki67-positive cells level (green) was showed in immunofluorescence images and quantified. (n=6). *P < 0.05 vs. control as indicated.

Discussion

This study aims to explore whether ASM is involved in the AdipoRon protective effect on SMCs. The present study demonstrated that ASM is indispensable for AdipoRon inhibiting SMCs proliferation and migration by activating the TFEB-autophagy signaling pathway. In Smpd1-/- SMCs, AdipoRon-induced TFEB and autophagy signaling activation were abolished, in contrast with Smpd1+/+ SMCs, promoting TFEB nuclear translocation and increasing the expression of LC3 and P62/SQSTM1. Furthermore, the effect of AdipoRon in preventing SMCs from switching to a dedifferentiated phenotype disappeared with the knockout of the Smpd1 gene.

AdipoRon is an active synthetic adiponectin agonist, binding with adiponectin receptor 1/2 (AdipoR1/2) to mimic the adiponectin biological effect[16]. Studies have shown that adiponectin activates ceramidase by binding to receptors and hydrolyzes ceramide to form sphingosine and free fatty acids[36]. Among them, sphingosine phosphorylated by sphingosine kinase produces sphingosine 1-phosphate (S1P), which can be coupled with a G protein-coupled receptor (GPCR) to play an important role as a signaling molecule. Since AdipoRon also acts on AdipoR1/2, it performs the same effect as adiponectin. Ceramide, involved in the development of many diseases, is produced by sphingolipids hydride by sphingomyelinase. According to our study, AdipoRon can increase the activity of ceramidase and promote the production of S1P. However, whether AdipoRon has a regulatory effect on ceramide upstream has not been elucidated.

ASM, a lysosomal ceramide-producing enzyme encoded by the Smpd1 gene, plays an essential role in mediating various stimulations by regulating autophagy[37].
In the current study, we found AdipoRon activated ASM in Smpd1+/+SMCs, with unsurprisingly, no change in Smpd1-/- SMCs. With ASM activation, more sphingolipids are hydrolyzed into ceramide and phosphorylcholine. Our results showed

that AdipoRon upregulated ceramide in Smpd1+/+ SMCs, but not in Smpd1-/-. This result demonstrated that in the presence of ASM, AdipoRon could upregulate the expression of ceramide. Although AdipoRon has been reported to have the function of increasing the activity of ceramidase, these two effects do not conflict but are like a dynamic balance[38]. AdipoRon activates ASM to upregulates the expression of ceramide, increasing the hydrolysis of sphingolipids. Nevertheless, AdipoRon binding with AdipoR1/2 activates ceramidase directly.

Autophagy is a dynamic and repetitive process, degrading and recycling excess proteins and broken organelles through lysosomes. Autophagy plays a significant role in maintaining cell homeostasis and inhibiting the transition of SMC from a differentiated phenotype to a dedifferentiated phenotype. The fusion of autophagosomes with lysosomes is a key step in autophagy maturation. Excessive proliferation and migration of SMC are involved in various vascular diseases, such as atherosclerosis and restenosis[39]. Our previous study indicated that AdipoRon activates TFEB, involved in lysosomal biosynthesis and autophagy signaling pathways, and autophagy markers expression, lc3 p62 [18]. Accumulating evidence indicates that ASM is essential for autophagy maturation and maintaining SMCs homeostasis. However, whether ASM is involved in AdipoRon-induced autophagy was not clear. In the present study, our results show that AdipoRon increase TFEB nuclear translocation in Smpd1+/+ SMCs, with no effect in Smpd1-/- SMCs. Similar, AdipoRon also upregulates TFEB mRNA levels in Smpd1+/+ SMCs, with no effect in Smpd1-/- SMCs. These results indicated that ASM deficiency inhibits AdipoRon-induced lysosomal synthesis by preventing TFEB activation. To confirm whether ASM mediates AdipoRon-induced autophagy activation, we measured both the autophagosome marker (LC3) and the autophagic substrate (P62/SQSTM1) protein expression and mRNA levels. The result shows both LC3 and P62/SQSTM1 protein expression, and mRNA levels markedly increased in Smpd1+/+ SMCs cultured with AdipoRon; whereas ASM deficiency abolished AdipoRon-induced autophagy activation. The mRNA level of LAMP1 and LAMP2A further confirm that AdipoRon promotes autophagy maturation depending on ASM.

Interestingly, the protein expression of LC3 in Smpd1-/- SMCs are higher than Smpd1+/+ SMCs. The study indicated that Smpd1-/- SMCs exist more autophagic vacuoles, not affecting autophagy induction but impairing autophagic flux[34].

Recently, TFEB activated by AdipoRon dependence on intracellular Ca^{2+} was clarified[18]. Researchers found that lysosomal Ca^{2+} release mediated by TRPML1 was markedly reduced in ASM deficiency patients compared with normal patients [40]. Therefore, we hypothesized that ASM mediated AdipoRon-induced TFEB activation was related to intracellular Ca2+ level. Our results show that with the increase of ceramide stimulated by AdipoRon, calcium-dependent phosphatase activity was upregulated in Smpd1+/+ SMCs, such as PP2A and calcineurin. Both PP2A inhibitor (OA) and calcineurin inhibitor (FK506 and CsA) reduceda AdipoRon-induced TFEB nuclear translocation and autophagy flux activation. Taken together, our results demonstrated that ASM mediates AdipoRon-induced TFEB and autophagy flux activation via regulating calmodulin activation, such as PP2A and calcineurin.

SMC homeostasis between differentiated and dedifferentiated phenotypes has a significant effect on vascular disease development. Under various stimulations, SMC switching to a dedifferentiated phenotype to cause abnormal proliferation and migration causing vascular disease, such as atherosclerosis[41]. Therefore, inhibition of SMC proliferation and migration may become a potential therapeutic intervention target for vascular disease. Appropriated autophagy contributes to preventing SMC proliferation and migration[42]. In the present study, we also demonstrated that AdipoRon largely inhibits proliferation, migration, and phenotype switching in Smpd1+/+ SMCs, whereas there was no similar effect in Smpd1-/- SMCs.

In summary, our results highlight the importance of ASM in AdipoRon-induced TFEB and autophagy activation preventing SMCs proliferation and migration. Our findings provide new sight into clear how ASM participates in AdipoRon protect effect for SMCs.

Future study

Our research is dedicated to contributing to the treatment of cardiovascular diseases. Based on the conclusion of this study: the positive effect of AdipoRon on inhibiting SMC proliferation and migration depends on regulating ASM, activating PP2A and calcineurin. Therefore, the future work of this study is focusing on exploring whether the protection of AdipoRon against cardiovascular diseases diminished in Smpd1-/- mice.

Chronic kidney disease (CKD) has been recognized as one of the common causes and an independent risk factor for CVD[50]. Compared with patients without CKD, those patients with CKD exhibit higher rates and more severe CVD[51]. Atherosclerosis associated with CKD increases CVD mortality due to the alterations of vascular[52]. Therefore, we will choose the CKD mice model for further study.

Smpd1+/+ and Smpd1-/- mice with or without CKD surgery are used to examine the role of ASM in the AdipoRon cardiovascular protection effect. We will use echocardiography to detect the cardiac function and the coronary flow velocity reserve of the mice to examine CVD of mice. At the same time, we will also use molecular biology experiments to detect biomarkers' protein and gene levels in specific tissues (such as the heart, aorta, etc.).

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