Original Paper

Tetrahydroxystilbene Glycoside Improves Microvascular Endothelial Dysfunction and Ameliorates Obesity-Associated Hypertension in Obese ZDF Rats Via Inhibition of Endothelial Autophagy

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Key Words

Obesity • Hypertension • Endothelial dysfunction • Tetrahydroxystilbene glycoside • Autophagy

Abstract

Aims: Obesity is a major risk for hypertension. Endothelial dysfunction contributes to increased peripheral vascular resistance and subsequent hypertension. Autophagy regulates endothelial function, however, whether autophagy is related to hypertension in obesity remains largely unclear. We wished to ascertain: (i) the role of autophagy in obesity-induced hypertension and the underlying mechanisms; (ii) if tetrahydroxystilbene glycoside (TSG) influences endothelial dysfunction and obesity-associated hypertension. **Methods:** (TSG-treated) male Zucker diabetic fatty (ZDF) rats and cultured human umbilical vein endothelial cells (HUVECs) were used. Blood pressure was measured non-invasively with a tail-cuff system. Western blotting was performed to determine the expression of autophagy-associated proteins. Autophagy flux was assessed by transfection HUVECs with the Ad-mGFP-RFP-LC3. **Results:** Compared with their lean counterparts, obese ZDF rats exhibited hypertension and endothelial dysfunction, along with impaired Akt/mTOR signaling and upregulated expression of autophagy-associated proteins beclin1, microtubule-associated protein 1 light chain 3 II/I, autophagy protein (ATG)5 and ATG7. Two-week TSG administration restored blood pressure and endothelial function, reactivated Akt/mTOR pathway and decreased endothelial autophagy in ZDF rats. Rapamycin pretreatment blocked the hypotensive effect of TSG in ZDF rats. Suppression of Akt/mTOR expression with siRNA significantly blunted the anti-autophagic effect of TSG in HUVECs as evidenced by abnormal autophagic flux and increased expression of autophagy-associated proteins. **Conclusion:** Endothelial dysfunction in ZDF rats is partially attributable to excessive

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autophagy. TSG improves endothelial function and exerts hypotensive effects *via* regulation of endothelial autophagy.

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Introduction

Numerous epidemiologic studies have suggested that obesity is a major risk for cardiovascular diseases (including hypertension) [1]. According to the Global Burden of Disease Study [2], between 1980 and 2013 worldwide the proportion of men who were overweight or obese increased from 28.8% to 36.9%, and the respective proportion of women increased from 29.8% to 38.0%. Obese patients have a six-fold increased risk of hypertension compared with people of average size [3]. Despite these alarming statistics, the molecular mechanisms underlying the obesity-associated increase in arterial blood pressure (BP) are not known, and the number of effective drug interventions is limited.

Several main factors have been reported to mediate hypertension in obesity in the last few years including endothelial dysfunction [3], sympathetic discharge [4], increase in peripheral vasoconstriction [5], disorders of sodium and water balance [5], release of pro-inflammatory factors and oxidative stress [6-7]. Of great concern is observation on endothelial dysfunction. Vascular endothelial dysfunction has been identified as a key element in the development of obesity-related cardiovascular diseases [8-10]. Obesity impairs vasodilating properties of the vascular endothelium, which promotes changes in the patterns of the flow and pressure of blood, thereby leading to endothelial dysfunction and obesity-related hypertension [11-13].

Autophagy, an evolutionarily conserved catabolic process, refers to the delivery and degradation of cytoplasmic material, which is considered a cellular recycling mechanism. Autophagy is indispensable for cellular homeostasis and organism longevity, and it is in balance with metabolic cycles [14]. Several studies have illustrated that autophagy has a pivotal role in the pathogenesis of myocardial lesions in obesity and the metabolic syndrome [15]. High-fat diet (HFD)-induced obesity disrupts maturation of myocardial autophagosomes. Chronic application of calcium-channel blockers and anti-hypertension drugs can suppress fusion between autophagosomes and lysosomes to impede autophagic flux [16]. Previously, we demonstrated that excessive autophagy in the mesenteric arterioles of young normotensive spontaneously hypertensive rats (SHRs) at least in part conducted to peripheral vascular endothelial dysfunction [17]. However, the specific molecular connection between autophagy and obesity-induced hypertension and impaired vascular endothelial dysfunction remains largely undefined.

Furthermore, effective drug intervention could help to attenuate obesity-related increased BP and impaired endothelial function. 2, 3,5, 4'-tetrahydroxystilbene-2-O- β -D-glycoside (TSG) is the main bioactive ingredient extracted from the traditional Chinese medicinal herb *Polygonum multiflorum* Thunb which has been used safely in China for approximately two millennia, and has been reported to have a wide spectrum of pharmacologic functions. TSG has been reported to help people live longer [18], protect impaired neural networks [19], improve blood flow [20], attenuate inflammatory responses [21], prevent the production of oxygen radicals and protect against oxidized LDL-induced endothelial dysfunction [22]. Previously, we demonstrated that TSG inhibits lysophosphatidylcholine (LPC)-induced apoptosis of human umbilical vein endothelial cells (HUVECs) by activating glutathione peroxidase and superoxide dismutase, clearing intracellular reactive oxygen species (ROS), and reducing lipid peroxidation [21, 23]. Nonetheless, little is known about the effects of TSG on endothelial dysfunction in obesity.

The present study was designed to investigate the role of abnormal autophagy in impaired endothelial dysfunction and increased BP in obesity. Also, we wished to ascertain if

TSG improves vascular endothelial function and subsequent hypertension in Zucker diabetic fatty (ZDF) rats.

Materials and Methods

Ethical approval of the study protocol

The experimental protocol was undertaken according to ethical guidelines set by the Helsinki Declaration. Procedures involving animals were approved by the Animal Care and Welfare Ethics Committee of the Fourth Military Medical University (Xian, China). Employment of the HUVEC line was approved by the Ethics Committee of Xijing Hospital (Fourth Military Medical University). Written informed consent was provided by individual volunteers donating umbilical cords as samples for this research.

Animals and diets

Twelve-week-old male obese ZDF (^{fa/fa}) rats and lean ZDF (^{fa/+}) rats were obtained from Charles River Laboratories (Beijing, China). Animals were housed in inlet valve-closed cages (two per cage) at 25°C with a 12-h light-dark cycle (lights on at 07:00) with free access to standard rat chow and water. ZDF (^{fa/fa}) rats were fed Purina 5008 (15% fat, 56% carbohydrate, 26% protein; LabDiet, Saint Louis, MO, USA), whereas ZDF (^{fa/+}) rats were fed basic chow. Before treatment, the first measurements of body weight, heart rate and BP were taken. Afterwards, ZDF (^{fa/fa}) rats were randomized into two groups and treated daily for 2 weeks by gavage with physiologic (0.9%) saline (model group) or TSG (100 mg/kg/day; TSG group). Lean ZDF (^{fa/+}) rats served as the control group. Each group contained six rats.

The body weight, heart rate and BP of all animals were measured at the end of treatment. None of animals died before the experimental endpoint.

Reagents

TSG (C₂₀H₂₂O₉; molecular weight, 406.4; purity, 98%) was provided by the Department of Natural Medicine in the School of Pharmacy of the Fourth Military Medical University. Phenylephrine, acetylcholine (ACh), sodium nitroprusside (SNP), and rapamycin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Insulin-like growth factor (IGF)-1 was obtained from R&D Systems (Minneapolis, MN, USA).

BP measurement

Systolic blood pressure (SBP) was measured non-invasively with a tail-cuff system (BP-2010A; Softron Beijing, Inc., Beijing, China) in accordance with standard procedures. Rats were allowed to acclimatize on a heating mantle at 39°C for ~10 min before measurement.

Intraperitoneal glucose tolerance test (IPGTT)

After a 16 h fast, alert rats were challenged with a glucose load of 2 g/kg body weight administered I.P. for the glucose tolerance test. Tail blood was taken 0, 30, 60, 90, 120 min after administration of the glucose load, and glucose measurements were determined by using an ACCU-CHEK optium blood glucose meters (Roche, China).

Experiments undertaken in organ chambers

After 2-week administration, animals were anesthetized with 3% pentobarbital sodium. The third branch of mesenteric arterioles of ZDF rats were dissected immediately after killing. Then, they were mounted in ice-cold fresh physiologic salt solution (PSS, pH 7.4) with continuous gassing of a mixture of 95% O₂-5% CO₂.

Mesenteric arteries were cut into rings of length 1 mm. Myographic studies were carried out in a small-vessel wire myograph system containing PSS and aerated with 95% O₂-5% CO₂ at 37°C. The PSS (at pH 7.4) consisted of (in mM): NaCl, 119.0; KCl, 4.7; NaHCO₃, 25.0; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5, glucose, 5.5; EDTA, 0.026. The rings were stretched to an optimal basal tension of 2.0 mN (according to the results of the preliminary experiments), which was based on the length-tension relationship. After equilibration for 60 min, the rings were pre-contracted with phenylephrine (10⁻⁶ mol/L) to stimulate a developed force. To weed out those devitalized artery segments, the rings were contracted with 60 mmol/L KCl solution before the

use of phenylephrine. Once a stable contraction was achieved, an endothelium-dependent vasodilator, ACh (10^{-10} – 10^{-5} mol/L) was added to the perfusate to determine endothelial function. After stabilization of the cumulative response, the rings were washed and allowed to equilibrate to the baseline. The procedure was repeated with the endothelium-independent vasodilator SNP (10^{-10} – 10^{-5} mol/L) to determine the function of vascular smooth muscle. The reaction at each concentration of agents added was measured by Powerlab Chart v8.1 (AD Instruments, Australia). Relaxation in response to each drug was indicated as the percentage of phenylephrine contraction, and 100% relaxation denoted when the active tone returned to the baseline level. After assessment of the procedures described above, substances were washed out thoroughly and vessels were subjected to western blotting.

Cell culture and treatments

HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin in an incubator containing 5% CO₂ at 37°C. Experiments using HUVECs were carried out at passages 3–8. When cells were ~70–80% confluent, new medium was added before drug treatment. HUVECs were synchronized in serum-free DMEM for 6 h before treatments. Then, they were exposed to palmitate (500 µM; Sigma–Aldrich) or bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA) for 24 h. In the TSG experiments, cells were treated with 100 µM TSG for 24 h. In the rapamycin experiments, cells were pretreated for 1 h with the autophagy-inducer rapamycin (100 nM) and then treated with 100 µM TSG for 24 h.

Transfection with the adenovirus-red fluorescent protein–green fluorescent protein–microtubule-associated protein 1 light chain 3 (Ad-mRFP–GFP–LC3) expression vector and Akt/mTOR knockdown

HUVECs were transfected with the Ad-mGFP–RFP–LC3 adenovirus (multiplicity of infection = 50) and, 36 h later, the plates were pre-incubated for 24 h in an incubator (5% CO₂, 37°C). The cells were imaged under a confocal microscope. When red and green fluorescence images merged, then the yellow spots that appeared represented autophagosomes. Red spots represented autophagic lysosomes. In normal phagosome–lysosome fusions, there will be more red fluorescence than yellow fluorescence. In impeded autophagy (in which phagosome–lysosome fusion does not occur), yellow fluorescence is dominant.

In addition, cells were transfected with mechanistic target of rapamycin (mTOR) small interfering RNA (siRNA; GenePharma, Beijing, China) by Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA). The siRNA sequences (sense and antisense, respectively) were: homo AKT1, 5'-GCACUUUCGGCAAGGUGAUTT-3' and 5'-AUCACCUUGCCGAAAGUGCTT-3'; scrambled AKT1 siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'; homo mTOR, 5'-CCACCCGAAUUGGCAGAUUTT-3' and 5'-AAUCUGCCAAUUCGGGUGGTT-3'; scrambled mTOR siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. siRNA was transfected according to manufacturer instructions. The efficiency of gene knockdown was detected using western blotting 48 h after transfection.

Western blotting

Mesenteric artery rings were homogenized and solubilized in RIPA lysis buffer. Protein quantification was done to ensure equal loading (60 µg) in each lane using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Proteins were separated on sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) [24]. Membranes were blocked with 5% bovine serum albumin and incubated overnight at 4°C with antibodies directed against beclin1 (1:1000, ID = 3738; Cell Signaling Technology, Danvers, MA, USA), p62 (1:1000, ab91526; Abcam, Cambridge, UK), microtubule-associated protein 1 light chain 3 II/I (LC3) (1:1000, 2775; Cell Signaling Technology), autophagy protein (ATG) 5 (1:1000, AB108327; Abcam), ATG7 (1:500, bs-2432R; Bioss Antibodies, Woburn, MA, USA), phosphorylated (p)Akt (1:1000, 9271, Cell Signaling Technology), Akt (1:1000, 9272, Cell Signaling Technology), (p)mTOR (1:500, 2971; Cell Signaling Technology), mTOR (1:1000, 2972; Cell Signaling Technology), or β-actin (1:1000, 4970; Cell Signaling Technology). After washing blots to remove excess bound primary antibody, blots were incubated for 1 h with a secondary antibody (1:10000). Antibody binding was detected *via* enhanced chemiluminescence (Millipore), and membranes were scanned with ChemiDocXRS (BioRad Laboratories, Hercules, CA, USA). The intensity of immunoblot bands was analyzed with ImageLab software. Measured intensities were corrected using the internal control (β-actin).

Statistical analyses

Data are the mean $\pm$ SEM and were analyzed by a two-tailed Student's *t*-test between two groups or by one-way ANOVA if three or more groups were compared. In all statistical comparisons, $P < 0.05$ was considered significant. All statistical tests were undertaken with GraphPad Prism v6.0 (Graph Pad, San Diego, CA, USA)

Results

Body mass and the impaired vasorelaxation in mesenteric-artery segments from obese ZDF rats

When compared with their age-matched lean counterparts, obese ZDF rats demonstrated significant differences in body weight ($P < 0.001$, Fig. 1A), SBP (Fig. 1B) and blood glucose tolerance ($P < 0.001$, Fig. 1C) [25]. ACh (endothelium-dependent vasodilator) and SNP (endothelium-independent vasodilator) induced vasorelaxation of mesenteric arterioles in lean ZDF rats and obese ZDF rats. Obese ZDF rats partially lost the ability to undergo ACh-induced relaxation but SNP-mediated vasodilatation was preserved (Fig. 1D, 1E), suggesting impaired endothelial function in vessels showing peripheral resistance in obese ZDF rats.

Autophagy was excessive and impeded in microvascular tissues from obese ZDF rats

Our previous study confirmed that excessive vascular autophagy contributes (at least in part) to microvascular endothelial dysfunction in pre-hypertensive SHR. However, the mechanism responsible for impaired endothelial function and obesity-related hypertension is not known. To determine the mechanisms of endothelial dysfunction in obese ZDF rats, western blotting was carried out.

Mesenteric arterioles from obese ZDF rats did not exhibit a measurable difference in expression of Akt or mTOR compared with mesenteric arterioles from lean ZDF rats (Fig. 2). However, obese ZDF rats displayed a marked decline of phosphorylation of Akt and mTOR. The well-established Akt/mTOR signaling pathway regulates cell autophagy. Hence, our results demonstrated that the expression of two classic autophagy-associated markers beclin1 and LC3, as well as expression of ATG5 and ATG7, increased significantly ($P < 0.05$), suggesting a higher level of autophagosome formation in vascular tissues from obese ZDF rats. In addition, p62 (a selective ubiquitinated substrate of autophagy) expression was also up-regulated, suggesting inhibition of lysosomal fusion to the autophagosome. These results suggested that increased autophagy and impeded autophagy existed in the mesenteric arteries of obese ZDF rats.

Fig. 1. The ACh-induced vasorelaxation was impaired in obese ZDF rats. The body weights (A), systolic blood pressures (B) and intraperitoneal glucose tolerance test (IPGTT) (C) of Lean ZDF rats and ZDF rats. Dose-response curves for ACh-induced endothelium-dependent vasorelaxation (D) and SNP-induced endothelium-independent vasorelaxation (E) were obtained from mesenteric arterioles of Lean ZDF rats and ZDF rats. Data are expressed as percentage of the contraction to phenylephrine (PE). All values are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Lean ZDF (n=6-8).

Fig. 2. A, Representative western blots showing the expressions of phosphorylation of (p-) Akt, Akt, p-mTOR, mTOR, Atg5, Atg7, Beclin1, p62, and LC3II/I. Quantity densitometry of the ratio of p-Akt/Akt and p-mTOR/mTOR(B), Atg5 and Atg7(C), Beclin1, p62, and LC3II/I (D) in mesenteric arteries from Lean ZDF rats and ZDF rats. Data obtained from quantity densitometry are the mean $\pm$ SEM of at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Lean ZDF (n=3-6).

Fig. 3. A, The chemical structure of tetrahydroxystilbene (TSG). The body weights (B), systolic blood pressures (C) and intraperitoneal glucose tolerance test (IPGTT) (D) of Lean ZDF rats and ZDF rats. Dose-response curves for endothelium-dependent vasorelaxation (E) and endothelium-independent vasorelaxation (F) were obtained from mesenteric arterioles of ZDF rats and ZDF+TSG rats. All values are presented as mean $\pm$ SEM. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. ZDF (n=6-8).

TSG treatment reduced BP and improved vasorelaxation in the mesenteric arterioles of obese ZDF rats

TSG is a bioactive compound extracted from *Polygonum multiflorum* Thunb. Our previous research showed that TSG administration can improve mesenteric vascular endothelial function in young normotensive SHR rats significantly.

In the present study, we measured the body weight and BP of obese ZDF rats before and

Fig. 4. A, Representative western blots showing the expressions of p-Akt, Akt, p-mTOR, mTOR, Atg5, Atg7, Beclin1, p62, and LC3II/I. Quantity densitometry of the ratio of p-Akt/Akt and p-mTOR/mTOR(B), Atg5 and Atg7(C), Beclin1, p62, and LC3II/I (D) in mesenteric arteries from ZDF rats and ZDF+TSG rats. Data obtained from quantity densitometry are the mean $\pm$ SEM of at least 3 independent experiments. # P <0.05, ## P <0.01 vs. ZDF (n=3-6).

after TSG administration. TSG treatment had significant effects on body weight, SBP (P <0.05, Fig. 3B, 3C), whereas no significant difference in glucose tolerance was found (P >0.05, Fig. 3D). In addition, after 2-week administration of TSG, ACh-induced vasodilatation was restored (at least in part) in mesenteric-artery segments from obese ZDF rats, but SNP-induced dilatation changed in a non-significant manner (P >0.05, Fig. 3E). These results suggested that obese ZDF rats lost 15%–20% of their weight after 2 weeks of TSG treatment, with partially reduced SBP. Also, TSG administration restored microvascular endothelium-dependent vasodilation significantly in obese ZDF rats (P <0.05, Fig. 3F).

TSG administration inhibited the excessive mesenteric vascular autophagy in obese ZDF rats

Western blotting revealed that TSG administration caused marked enhancement in the activation of Akt and mTOR, whereas no significant change was observed in expression of total Akt or mTOR (P >0.05), in the mesenteric arterioles of obese ZDF rats (Fig. 4). More importantly, expression of the autophagy-associated proteins beclin1, LC3, ATG5, and ATG7 in microvascular tissues was reduced significantly (P <0.05). In addition, TSG treatment reduced the up-regulated expression of p62 in mesenteric-artery segments from obese ZDF rats, suggesting that impeded autophagic flux was corrected (at least in part). Collectively, the findings mentioned above illustrate (for the first time) that TSG treatment causes activation of the Akt/mTOR signaling pathway and down-regulation of autophagy, in the microvascular tissues of obese ZDF rats.

TSG treatment suppressed the expression of autophagy-associated proteins and unblocked the autophagic flux in HUVECs

To investigate further if Akt/mTOR-mediated autophagy contributes to endothelial dysfunction in high fat (HF) conditions, we cultured HUVECs in complete medium and saturated sodium palmitate (500 μ mol/L; a common fatty acid). After HF incubation for 18 h, the phosphorylation of Akt and mTOR was down-regulated significantly (P <0.01, Fig. 5A). In addition, the expression of the autophagy-associated proteins ATG5, ATG7, beclin1 and LC3II/I was up-regulated notably, and p62 expression was increased, suggesting excessive autophagy and impeded autophagy after HF incubation. Furthermore, a potent activator of Akt/mTOR signaling, IGF-1, was used as a positive control, and rapamycin served as an mTOR antagonist. Upon treatment with TSG (100 μ g/mL) or IGF-1 (100 ng/mL) for 24 h, the

Fig. 5. The effects of TSG incubation on cell autophagy and Akt/mTOR pathway in human umbilical vein endothelial cells (HUVECs). A, Representative western blots showing the expressions of phosphorylation of (p-) Akt, Akt, p-mTOR, mTOR, Atg5, Atg7, Beclin1, p62, and LC3II/I and quantity densitometry of the ratio of p-Akt/Akt and p-mTOR/mTOR (B), Atg5 and Atg7 (C), Beclin1, p62, and LC3II/I (D) in mesenteric arteries from ZDF rats and ZDF+TSG rats. E, HUVECs were transfected with mRFP-GFP-LC3 adenovirus, and the adenovirus allows distinction of autophagosomes (GFP + RFP + yellow puncta) and autolysosomes (GFP – RFP + red puncta) as GFP fluorescence is quenched in the acidic autolysosomes. Data obtained from quantity densitometry are the mean $\pm$ SEM of at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. Control, # $P < 0.05$, ## $P < 0.01$ vs. HF, \$ $P < 0.05$ vs. HF+TSG (n=3-6).

expression of Akt and mTOR was activated and was accompanied by down-regulation of the expression of ATG5, ATG7, beclin1 and LC3II/I. However, pretreatment with rapamycin almost completely abolished the anti-autophagic effects of TSG administration. More importantly, we transfected HUVECs with the mRFP-GFP-LC3 adenovirus; hence, autophagosomes (GFP + RFP + yellow puncta) could be distinguished from autolysosomes (GFP – RFP + red puncta). Sodium palmitate induced autophagy (red fluorescence) and accumulation of autophagosomes (increased yellow fluorescence) compared with control (Fig. 5E), suggesting that p62 accumulated due to the blocked fusion of autophagosomes with lysosomes in a HF condition *videlicet*, as well as excessive autophagy and impeded autophagy. After treatment with IGF-1 or TSG, red and yellow fluorescence decreased to different extents, respectively, whereas rapamycin pretreatment blocked the effects of TSG (increased red fluorescence). These results suggested that incubation with TSG restored excessive autophagy to a normal level and regulated the impeded autophagic flux to unimpeded autophagic flux in HUVECs.

Silencing of Akt or mTOR blunted TSG incubation-induced protective effects in HUVECs

To ascertain directly if TSG treatment improved vascular endothelial function *via* the Akt/mTOR pathway, siRNAs targeting Akt and mTOR were applied in HUVECs. The basal expression of Akt and phosphorylation of Akt were decreased notably by siRNA targeting Akt, but not by scrambled siRNA (Fig. 6A), so the level of phosphorylated mTOR was reduced.

Fig. 6. Effects of TSG incubation on autophagy and Akt/mTOR pathway in HUVECs with or without small interfering RNA (siRNA). Representative western blots showing the expressions of phosphorylation of (p-) Akt, Akt, p-mTOR, mTOR, Atg5, Atg7, Beclin1, p62, and LC3II/I in HUVECs with or without small interfering RNA (siRNA) targeting Akt (A) and mTOR (E).

Fig. 7. ZDF+TSG rats pretreated with rapamycin showed hypertension and endothelial dysfunction. The body weights (A) and systolic blood pressures (B) of ZDF+TSG rats and ZDF+RAPA+TSG rats. Dose-response curves for ACh-induced vasorelaxation (C) and SNP-induced vasorelaxation (D) were obtained from mesenteric arterioles. E, Representative western blots showing the expressions of phosphorylation of (p-) Akt, Akt, p-mTOR, mTOR, Atg5, Atg7, Beclin1, p62, and LC3II/I. Quantity densitometry of the ratio of p-Akt/Akt and p-mTOR/mTOR (F), Atg5 and Atg7 (G), Beclin1, p62, and LC3II/I (H) in mesenteric arteries from ZDF+TSG rats and ZDF+RAPA+TSG rats. All values are presented as mean ± SEM. RAPA, rapamycin. *P<0.05, **P<0.01, ***P<0.001 vs. ZDF+TSG (n=6-8).

Fig. 8. Schematic model summarizing the findings of the TSG ameliorates obesity-associated hypertension in ZDF rats. Excessive autophagy induces vascular endothelial dysfunction in obesity, and that TSG treatment ameliorates endothelial dysfunction and subsequent obesity-increased arterial BP (at least in part) via a Akt/mTOR autophagy pathway.

The anti-autophagic effects of TSG incubation were blocked significantly by siRNA targeting Akt ($P < 0.05$). Silencing mTOR expression also blunted TSG-induced anti-autophagic effects in HUVECs (Fig. 6E). Collectively, TSG administration exerted a positive influence on endothelial function very likely via the Akt/mTOR pathway.

Rapamycin blocked TSG treatment-induced autophagy and endothelial dysfunction in the peripheral vessels of ZDF rats

Having demonstrated that TSG administration in ZDF rats activated the Akt/mTOR signaling pathway, inhibited excessive autophagy, and improved endothelial function, we undertook an additional experiment to ascertain the functional importance of TSG-induced vasoprotection and antihypertensive therapy in obesity. In ZDF rats treated with rapamycin and TSG for 2 weeks, the antihypertensive effects of TSG were attenuated remarkably, but their body weights did not change significantly ($P > 0.05$, Fig. 7A–B). Rapamycin treatment also blunted the ACh-induced endothelial protection afforded by TSG therapy (Fig. 7C). More importantly, the phosphorylation of mTOR was reduced significantly and, subsequently, the levels of autophagic-associated proteins such as ATG5, ATG7, beclin1, LC3II/I and p62 were decreased after rapamycin treatment in the mesenteric arterioles of ZDF rats compared with the ZDF+TSG group (Fig. 7E–H). These results suggested that TSG-induced vasoprotection and antihypertensive therapy in obesity are dependent upon mTOR.

Discussion

The present study elicited three main findings. First, we demonstrated that excessive autophagy induces vascular endothelial dysfunction and subsequent elevated BP in obese ZDF rats. Second, TSG treatment improves endothelial dysfunction and reduces obesity-increased SBP directly in obese ZDF rats. Third, the partial reactivation of impaired Akt/mTOR signaling and suppression of excessive autophagy contributes (at least in part) to the hypotensive and vasoprotective effects of TSG in obese ZDF rats. Taken together, we demonstrated, for the first time, that the microvascular endothelial dysfunction observed in obesity-related hypertension is attributable (at least in part) to excessive autophagy, and that TSG may improve endothelial function and subsequently exert a hypotensive effect via suppression of autophagy.

In the present study, obese ZDF rats were genetic models used to study metabolic diseases and their complications. Obese ZDF rats are characterized by the homozygous mutated leptin receptor that induces hyperphagia, hyperlipidemia, obesity, and hypertension [26, 27]. The endothelium secretes various vasoconstrictors and vasodilators. The imbalance between the vasodilator NO and vasoconstrictor endothelin-1 in the endothelium contributes to endothelial dysfunction and increased peripheral vascular resistance and, subsequently, to hypertension [28-30].

Several studies have explored the underlying mechanisms between obesity and endothelial dysfunction. Schafer and colleagues suggested that endogenous aldosterone and exogenous aldosterone in obesity lead to endothelial dysfunction by inducing expression of ROS-generating enzymes and activation of the cyclo-oxygenase-1 signaling pathway in HFD mouse models [31]. It has been reported that autophagy deficiency and subsequent mitochondrial dysfunction can restore expression of fibroblast growth factor-21, and together can promote protection from obesity-induced insulin resistance [32]. Interestingly, Emilie and colleagues observed endothelial dysfunction in 4-week ZDF rats due to excessive production of ROS whereas, in 1-year-old ZDF rats, cyclo-oxygenase-2 derivatives were associated with impaired endothelial function and excessive production of ROS [33]. The present study demonstrated that impeded autophagy and excessive autophagy account (at least in part) for obesity-induced hypertension and endothelial dysfunction. Taken together, obesity-related increased BP and endothelial dysfunction might be corrected (at least in part) by improved autophagic flux *via* alleviation of oxidative stress [34, 35].

Accumulating evidence has demonstrated the pivotal role of autophagy in the metabolic syndrome and its complications [36]. Omental adipose tissues from obese patients with type-2 diabetes mellitus have shown a reduction in expression of mammalian target of rapamycin complex-1 with a simultaneous increase in autophagy and impaired mitochondria [37]. Increased levels of autophagy induced by obesity in adipocytes increase the degradation of adiponectin and insulin receptors. Thus, enhanced autophagic degradation has been purported to play a part in insulin resistance [38]. In addition, *ATG7* knockout mice show more brown adipose tissues and increased numbers of mitochondria. These autophagy-compromised mice are slimmer as well as being resistant to HFD-induced obesity and insulin resistance [39].

Discrepant experimental data have revealed abnormal autophagy in metabolic syndrome-associated cardiac injury. For instance, in HFD mouse models, myocardial autophagy is unchanged but cardiac function is suppressed [40]. In contrast, impeded cardiac autophagic flux has been reported in HFD-induced mouse models of obesity and metabolic syndrome, and results in cardiac hypertrophy and functional anomalies [41]. In another study, autophagy suppression was demonstrated to protect against diabetic cardiomyopathy because of the deterioration of cardiac anomalies by beclin1 overexpression but reduction of cardiac injury by deficiency of beclin1 and *ATG16* [42]. However, few researchers have focused on whether abnormal autophagy has a role in obesity-related endothelial dysfunction. In the present study we found, for the first time, that the ratio of LC3B II/LC3B I expression was increased significantly, whereas p62 accumulation was enhanced in the mesenteric arterioles from obese ZDF rats and HFD mice compared with their normal counterparts, respectively. Hence, it is important to take other factors into consideration when trying to ascertain if obesity-induced changes in vascular endothelial function can be influenced by disturbed autophagy: different experimental animal models; the dose and duration of action of drugs; autophagic flux. Interestingly, McCarthy [8] demonstrated that autophagic proteins Beclin-1, *ATG5*, and LC3II/I were all decreased in SHR aorta compared with WKY, and in their study, male and female WKY and SHRs aged 12-15 weeks were used. However, in our previous study in SHRs, we found that excessive autophagy contributed to mesenteric vascular endothelial dysfunction in prehypertensive SHRs (aged 4 weeks), but autophagy appeared not to be responsible for the aortic endothelial dysfunction. In addition, our present study showed excessive autophagy in mesenteric arterioles from ZDF rats. It is widely recognized that the elastic contraction of the main artery is a prerequisite for the formation of arterial blood

pressure, however, the dysfunction and angiosclerosis in peripheral resistant vessels always happen earlier (than in aorta). Autophagy usually serves as a survival mechanism in response to stress conditions, but excessive autophagy results in cell death. Therefore, it is possible that the endothelial dysfunction is the result of the variation of basal vascular autophagy with the aging changes.

Polygonum multiflorum Thunb is an edible medicinal plant used widely in China because it can enhance cognition and delay senescence. Zhou and colleagues [19] showed that TSG administration reduced the levels of ROS and IGF-1 and increased levels of superoxide dismutase and Klotho protein in the serum, improved the memory and regulated the body weight of D-galactose-induced aging mice. These data suggest that TSG had a promising anti-aging effect by regulation of *Klotho* expression [19]. TSG is a free radical-scavenger [22], and exerts anti-aging effects in mammals *via* a neural insulin/IGF-1 signaling pathway [19]. TSG exhibits strong anti-oxidative and anti-inflammatory effects, as well as protecting mammals from cerebral ischemia and/or reperfusion injury [23, 43]. The ability for TSG to induce proliferation of cardiac stem cells as well as the transcription and differentiation of cardiomyocyte genes has been validated. These new cardiomyocytes could repair damaged cardiac muscles after myocardial infarction [43]. Cell damage induced by LPC has been attributed to activation of Notch signaling, and TSG has been shown to protect HUVECs from LPC-induced damage by suppression of the activation of Notch signaling [21]. Previously, we showed that TSG administration exhibits vasodilatation directly on mesenteric arteries in a dose- and endothelium-dependent manner under a hypertensive condition in young SHR [17]. However, direct evidence of the effects of TSG treatment on BP is lacking. In the present study, we have provided the first direct proof that TSG treatment reduces obesity-increased SBP in obese ZDF rats. Although TSG failed to lower BP in essential hypertension, it did attenuate the rise in SBP in obesity-induced hypertension, that is, TSG could be a potent hypotensive drug aimed at obesity-related hypertension. In addition, the obese ZDF rats showed higher blood glucose and impaired glucose tolerance than their lean counterparts, while there was no difference in these metabolic parameters between obese rats treated with and without TSG. The results suggested that TSG may decrease SBP and improve endothelial function in a glucose-independent manner. Interestingly, the body weights of obese ZDF rats were reduced after TSG administration. TSG has been shown to reduce the serum concentrations of free fatty acids, triglycerides, and total cholesterol, and to control the extent of liver hypertrophy effectively [44]. Therefore, why TSG exhibited hypotensive effects on obese ZDF rats, but not healthy or non-diabetic young SHR, and whether the hypolipidemic effect of TSG works in concert with anti-autophagic and hypotensive effects, must be determined.

It has been reported that TSG administration can reduce levels of LC3 II and beclin1, alleviate injury to the function of endoplasmic reticulum induced by neurotoxicity to β -amyloid protein, as well as improve learning and memory in *APP695V717I* transgenic mice (models of Alzheimer's disease) [45]. Previously, we demonstrated that TSG administration inhibited excessive mesenteric vascular autophagy by activating the Akt/mTOR pathway in normotensive young SHR, and further improved endothelial function, but could not attenuate hypertension effectively [17]. Nonetheless, in that study, we found a specific molecular link connecting TSG treatment to excessive autophagy under obesity-induced hypertension. Furthermore, there is no significant structural similarity between TSG and other autophagy-associated inhibitors such as hydroxychloroquine and 3-Methyladenine. Our investigation suggests that TSG is more inclined to have a role in obesity-associated hypertension by regulation of the autophagic flux and expression of Akt/mTOR signaling proteins, which has been evidenced by previous and the present study. TSG treatment also attenuated increased BP and endothelial dysfunction *via* the Akt/mTOR autophagy pathway in aortae from HFD-induced obese mice, similar to that in genetically obese ZDF rats. These results provide direct evidence that TSG administration exerts hypotensive and anti-autophagic effects on congenital and acquired models of fat rodents.

Conclusion

We demonstrated, for the first time, that excessive autophagy and impeded autophagy induce vascular endothelial dysfunction in obesity, and that TSG treatment ameliorates endothelial dysfunction and subsequent obesity-increased arterial BP (at least in part) *via* a Akt/mTOR autophagy pathway (Fig. 8). Therefore, TSG could be an anti-hypertension drug candidate used for the protection and prevention of endothelial dysfunction in obesity-associated cardiovascular diseases.

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Disclosure Statement

The authors had no conflicts of interest to declare in relation to this article.

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