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Azilsartan ameliorates angiotensin II-induced skeletal muscle atrophy via AT1R-mediated PI3K/AKT/FOXO3a signaling pathway

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ABSTRACT

Background Sarcopenia is a progressive age-related skeletal muscle disorder characterized by the loss of muscle mass, strength, and physical performance. Increasing evidence suggests that activation of the renin–angiotensin system (RAS) contributes to muscle wasting through promotion of protein degradation and impairment of anabolic signaling. Azilsartan (AZL), a highly selective angiotensin II type 1 receptor (AT1R) blocker, has demonstrated pleiotropic effects beyond blood pressure control. However, its effects on skeletal muscle atrophy and the underlying mechanisms remain incompletely understood.

Methods We combined a prospective observational study with mechanistic investigations in animal and cellular models. Older adults with hypertension and sarcopenia received AZL (40 mg/day) or other antihypertensive therapies for 6 months, and muscle strength, gait speed, and appendicular skeletal muscle mass were assessed. Moreover, Ang II-induced skeletal muscle atrophy models were established in mice and C2C12 myotubes to evaluate the effects of AZL on muscle morphology and signaling pathways.

Results AZL treatment was associated with significant improvements in handgrip strength, gait speed, and appendicular skeletal muscle mass in older hypertensive patients. In experimental models, Ang II induced skeletal muscle dysfunction and atrophy, characterized by reduced exercise capacity, decreased grip strength, and muscle fiber loss, were attenuated by AZL treatment. Mechanistically, AZL restored PI3K/Akt/FOXO3a signaling and suppressed the expression of the muscle-specific ubiquitin ligases Atrogin-1 and MuRF1. The protective effects of AZL were abolished by the AT1R agonist TRV120027 in both *in vivo* and *in vitro* models and were further attenuated by the PI3K inhibitor LY294002 in C2C12 myotubes.

Conclusions AZL attenuates Ang II-induced skeletal muscle atrophy through AT1R blockade and subsequent activation of PI3K/Akt/FOXO3a signaling, thereby suppressing ubiquitin-proteasome system-mediated muscle protein degradation. These findings provide mechanistic and translational evidence supporting the potential of AZL as a therapeutic strategy for preserving skeletal muscle health in older adults with hypertension.

Sarcopenia is an age-related syndrome characterized by progressive loss of skeletal muscle mass, strength, and physical function, which significantly increases the risk of falls, disability, and mortality in older adults.[1] Although the mechanisms underlying sarcopenia are multifactorial,[2] increasing evidence suggests that dysregulation of the renin-angiotensin system (RAS) may contribute to age-related

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muscle deterioration.[3] Beyond its established role in blood pressure regulation, the RAS influences skeletal muscle homeostasis through modulation of oxidative stress, inflammation, mitochondrial function, and protein turnover.[3,4]

Angiotensin II (Ang II), the principal effector peptide of the RAS, promotes skeletal muscle wasting primarily through activation of the angiotensin II type 1 receptor (AT1R).[5,6] Experimental studies have demonstrated that Ang II induces oxidative stress, suppresses insulin-like growth factor-1 (IGF-1)/Akt signaling, and activates the ubiquitin–proteasome system (UPS), resulting in increased expression of the muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF1 and subsequent muscle protein degradation.[7,8] Consistent with these findings, both genetic deletion and pharmacological inhibition of AT1R have been shown to improve muscle function and attenuate age-related muscle decline in animal models,[5,9] supporting a pathogenic role of excessive RAS activation in skeletal muscle ageing.

Among angiotensin receptor blockers (ARBs), azilsartan (AZL) is a highly selective AT1R antagonist characterized by sustained receptor occupancy and potent antihypertensive efficacy.[10] In addition to its cardiovascular and renal protective effects, AZL has been reported to exert anti-inflammatory, antioxidative, and metabolic regulatory actions.[11–14] Recently, Prajapati, *et al*.[15] demonstrated that AZL attenuated age-related sarcopenia in naturally aged rats by preserving muscle mass, improving muscle strength, and reducing the transition from fast- to slow-twitch muscle fibers. However, the molecular mechanisms underlying these beneficial effects remain incompletely understood. Moreover, clinical evidence regarding the association between AZL use and skeletal muscle health in older adults is currently lacking.

Therefore, in the present study, we combined clinical and experimental approaches to investigate the relationship between AZL treatment and skeletal muscle health. We first examined the association between AZL use and sarcopenia-related parameters in older adults with hypertension. We then evaluated the effects of AZL on Ang II-induced skeletal muscle atrophy and explored the underlying molecular mechanisms. By integrating clinical observations with mechanistic studies, we sought to provide translational evidence supporting the potential role of AZL in the prevention and treatment of sarcopenia.

METHODS

Study Population

This single-center, prospective observational study enrolled older patients with hypertension combined with sarcopenia admitted to the Department of Geriatrics at Sir Run Run Hospital, Nanjing Medical University, from January 2024 to April 2025. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the hospital (approval number: 2023-SR-001) and registered at the Chinese Clinical Trial Registry (ChiCTR2300071464). Written informed consent was obtained from all participants prior to enrollment. The inclusion criteria include (1) age ≥ 65 years; (2) diagnosis of primary hypertension according to the 2024 Chinese Guidelines for the Prevention and Treatment of Hypertension;[16] (3) diagnosis of sarcopenia based on the Asian Working Group for Sarcopenia (AWGS 2019) criteria,[17] defined as low muscle mass (men < 7.0 kg/m^2, women < 5.7 kg/m^2) accompanied by either low muscle strength (handgrip strength: men < 28 kg, women < 18 kg) or slow gait speed (< 1.0 m/s). The exclusion criteria include (1) acute heart failure, acute coronary syndrome, or severe arrhythmias; (2) active malignancy or recent chemotherapy; (3) severe hepatic or renal dysfunction, or hematological disorders; (4) in use of medications which may affect muscle metabolism (e.g., glucocorticoids, immunosuppressants); and (5) Incomplete clinical data or loss to follow-up. Patients were assigned to the AZL group (40 mg/day) or non-AZL group based on physician's choice and patient preference. The non-AZL group received other types of antihypertensive drugs except for ARBs, including calcium channel blockers (amlodipine, 5–10 mg/day), diuretics (hydrochlorothiazide, 12.5–25 mg/day), and β-blockers (metoprolol 47.5–95 mg/day)). Adherence to medication was monitored by pill count and patient interview at each follow-up visit (months 1, 3, and 6). All participants were community-dwelling older adults with stable daily activities. No structured exercise or rehabilitation program was implemented during the study period.

Assessment of Sarcopenia

Appendicular skeletal muscle mass (ASM) was measured using bioelectrical impedance analysis (BIA; InBody 720), and the Appendicular skeletal muscle index

(ASMI) was calculated as ASM divided by height squared (ASMI = ASM/height^2). Maximal handgrip strength was measured for both hands using a digital dynamometer (Jamar, USA), and the mean of two measurements was recorded. Usual gait speed was assessed using the 6-meter walk test.

Blood Pressure

Seated blood pressure was measured using an automated sphygmomanometer, and the mean of three readings was recorded.

Biochemical Parameters

Fasting blood samples were collected in the morning to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), fasting blood glucose (FBG), creatinine (Cr), blood urea nitrogen (BUN), total cholesterol, triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

Nutritional Status

Nutritional status was assessed using the Malnutrition Universal Screening Tool (MUST), a validated screening tool recommended for identifying malnutrition risk in community-dwelling and outpatient populations.[18]

Mice

Male C57BL/6J mice (8 weeks old, 22–25 g) were obtained from the Experimental Animal Center of Nanjing Medical University and were housed under controlled conditions (22 ± 2 °C, 50–60% humidity) with a 12-hour light/dark cycle and provided with standard chow and water *ad libitum*. After a 1-week acclimation period, mice were randomly assigned to three groups: Control, Ang II, and Ang II + AZL (n = 8 per group). The Control group received intraperitoneal injections of saline, while the Ang II group received continuous subcutaneous infusion of Ang II (1 μg/kg·min) (A9525, Sigma) via a mini-osmotic pump (Alzet, USA) for 28 days to induce skeletal muscle atrophy as previously reported.[19,20] The Ang II + AZL group received the same Ang II infusion combined with oral gavage of AZL (5 mg/kg/day) for 28 days.[21] For receptor-dependence validation, mice were treated with AT1R agonist TRV120027 (1 mg/kg) or AT2R antagonist PD123319 (1 mg/kg) for 28 days. At the end of the experiment, mice were anesthetized and euthanized, and tibialis anterior and gastrocnemius muscles were collected. All animal procedures were approved by the Animal Ethics Committee of Nanjing University (approval number: IACUC-1905027) and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Assessment of Exercise Capacity and Muscle Strength

Exercise endurance was evaluated using a mouse treadmill (Zhishuduobao, DB030, China). The initial speed was 10 m/min and increased by 2 m/min every 3 min until exhaustion, defined as continuous contact with the shock grid for > 5 s. Total running time and distance were recorded. Forelimb grip strength was measured using a mouse grip meter (Softmaze, Shanghai, China). Mice grasped a T-shaped bar while the tail was gently pulled horizontally until release. Each mouse was tested three times, and the highest value was recorded.

Histological Analysis

Fresh muscles were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 4 μm for hematoxylin and eosin (HE) staining. Muscle fiber cross-sectional area (CSA) was assessed by capturing images under a microscope (Zeiss Axio Scope.A1, Germany) and analyzing five randomly selected fields per section using ImageJ software (NIH, USA).

C2C12 Myoblast Culture and Treatment

Mouse C2C12 myoblasts (ATCC, USA) were cultured in high-glucose DMEM supplemented with 10% FBS (Gibco, USA), 80 U/mL penicillin, and 0.08 mg/mL streptomycin at 37 °C in 5% CO_2. At sub-confluence, the medium was replaced with differentiation medium containing 2% horse serum (Biological Industries, Israel) for four days to generate mature myotubes. For Ang II-induced atrophy, mature myotubes were treated with Ang II (1 μmol/L) for 24 h. In intervention experiments, cells were co-treated with Ang II and AZL (1, 5, or 10 μmol/L) for 24 h. For mechanistic studies, on day 4 of differentiation, myotubes were co-treated with either the AT1R agonist TRV120027 (5 μmol/L) or the PI3K inhibitor LY294002 (2.5 μmol/L) for 24 h.

Cell Viability Assay

C2C12 cells were seeded at 1 × 10^4 cells/well in 96-well plates and induced to differentiate. Cell viability

was assessed using a CCK-8 kit (Dojindo, Japan). After 2 h of incubation, absorbance was measured at 450 nm using a microplate reader (Synergy H1, BioTek, USA), and relative viability was calculated according to the manufacturer's instructions.

Immunofluorescence Staining

Differentiated myotubes were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 5% BSA for 1 h. Cells were incubated overnight at 4 °C with an anti-myosin heavy chain (MHC) antibody (MF20, 1:200, DSHB), followed by a 1 h incubation with a Cy3-conjugated secondary antibody and DAPI counterstaining. Images were captured using a fluorescent microscope (Zeiss Axio Scope, Germany), and diameters of ≥ 100 randomly selected myotubes were measured using ImageJ software.

Western Blot Analysis

Total protein was extracted using pre-chilled RIPA buffer (Beyotime, China) and quantified by BCA assay. Equal amounts of protein were separated on 4%-20% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4 °C with primary antibodies, including PI3K (Proteintech, 60225-1-Ig), p-Akt (Cell Signaling Technology, #4060), Akt (Proteintech, 10176-2-AP), p-FOXO3a (Cell Signaling Technology, #9466), FOXO3a (Abcam, ab23683), MuRF1 (Proteintech, 55456-1-AP), and Atrogin-1 (Proteintech, 67172-1-Ig). After a 1.5-h incubation with HRP-conjugated secondary antibodies, proteins were visualized using ECL substrate and imaged with ChemiDoc™ XRS+ (Bio-Rad, USA). Densitometric analysis was performed using Image Lab 6.0 software.

RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted using an RNA extraction kit (TIANGEN, China) and 1 μg RNA was reverse-transcribed to cDNA using the PrimeScript™ RT kit (Takara, Japan). qPCR was performed on a QuantStudio 5 system (Applied Biosystems, USA) using SYBR Green Master Mix (Thermo Scientific, USA). GAPDH was used as the internal reference, and relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.0 and SPSS 27.0. Continuous variables are presented as mean ± SE and tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene). Two-group comparisons were performed using independent-samples *t*-tests or Mann-Whitney *U* tests, and multiple-group comparisons were conducted using one-way ANOVA with Tukey's post hoc test or Kruskal-Wallis with Dunn's *post hoc* test, as appropriate. Categorical variables were analyzed by χ^2 or Fisher's exact tests. Correlations were assessed using Pearson or Spearman methods based on data distribution. Multivariate logistic regression analysis was taken to determine the variables that contributed to the improvement of sarcopenia. Experiments were repeated independently three times. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of the Study Cohort

A total of 317 older patients with hypertension were enrolled in this study (mean age 76.9 ± 5.2 years; 61.9% male), including 163 patients in the AZL group and 154 in the non-AZL group. There was no significant difference of clinical characteristics between the two groups at baseline (all $P > 0.05$; Table 1).

Effects of AZL on Skeletal Muscle Parameters

During the treatment, no serious adverse events were reported. Mild dizziness was noted in three patients in the AZL group and two patients in the non-AZL group, all of which experienced self-remission without specific treatment. After 6 months of treatment, blood pressure was effectively controlled in both groups (Table 2). Both groups achieved similar blood pressure control after treatment (SBP: 123.3 ± 7.3 *vs.* 123.1 ± 7.1 mmHg, $P = 0.782$; DBP: 71.2 ± 4.5 *vs.* 72.1 ± 4.2 mmHg, $P = 0.654$). In the AZL group, skeletal muscle mass, grip strength, and gait speed were all significantly improved ($P < 0.05$), whereas in the non-AZL group, only gait speed showed a statistically significant improvement ($P < 0.001$). Multivariable logistic regression analysis adjusting for age, sex, BMI, diabetes, smoking, baseline ASMI, grip strength, gait speed, nutritional status, and concomitant medications (statins, antiplatelet agents) showed that AZL treatment remained independently associated with

Table 1 The baseline clinical parameters of older hypertensive patients with sarcopenia.

Variables	Azilsartan group (n = 163)	Non-Azilsartan group (n = 154)	P-value
Age, yrs	77.0 ± 4.4	76.9 ± 5.8	0.193
Males	107 (65.5%)	90 (58.4%)	0.186
Diabetes	50 (30.7%)	48 (31.2%)	0.924
Smoke	30 (18.4%)	20 (13.0%)	0.186
BMI, kg/m^2	22.56 ± 3.29	22.43 ± 3.09	0.222
FBG, mmol/L	5.93 ± 1.91	5.90 ± 1.43	0.167
ALT, U/L	20.1 ± 5.9	20.3 ± 6.2	0.254
AST, U/L	25.1 ± 4.8	24.7 ± 5.4	0.334
TBIL, μmol/L	13.4 ± 3.8	14.1 ± 4.4	0.192
eGFR, mL/min per 1.73m^2	75.94 ± 11.15	81.68 ± 25.41	0.153
BUN, mmol/L	5.82 ± 1.48	5.84 ± 1.81	0.104
TC, mmol/L	4.68 ± 0.89	4.85 ± 1.09	0.099
TG, mmol/L	1.43 ± 0.81	1.44 ± 0.79	0.818
LDL-C, mmol/L	2.20 ± 0.43	2.18 ± 0.49	0.417
HDL-C, mmol/L	1.50 ± 0.36	1.50 ± 0.37	0.958
SBP, mmHg	173.7 ± 13.0	171.8 ± 12.9	0.186
DBP, mmHg	90.7 ± 7.9	91.6 ± 8.0	0.728
ASMI, kg/m^2	5.81 ± 0.79	5.72 ± 0.83	0.116
Grip strength, kg	19.3 ± 4.9	19.1 ± 4.9	0.127
Gait speed, m/s	0.88 ± 0.07	0.87 ± 0.06	0.126
MUST score > 2	14 (8.6%)	12 (7.8%)	0.792

Data are presented as mean ± SD or n (%). ALT: alanine transaminase; ASMI: appendicular skeletal muscle mass index; AST: aspartate aminotransferase; BMI: body mass index; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; FBG: fasting blood glucose; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; MUST: Malnutrition Universal Screening Tool (score > 2 indicates malnutrition risk); SBP: systolic blood pressure; TBIL: total bilirubin; TC: total cholesterol; TG: triglyceride.

Table 2 The effect of AZL on blood pressure and skeletal muscle parameters in older hypertensive patients with sarcopenia.

Variables	AZL group (n = 163)		Non-AZL group (n = 154)		P-value
	Baseline	After treatment	Baseline	After treatment	
SBP, mmHg	173.7 ± 13.0	123.3 ± 7.3	171.8 ± 12.9	123.1 ± 7.1	0.782
DBP, mmHg	90.7 ± 7.9	71.2 ± 4.5	91.6 ± 8.0	72.1 ± 4.2	0.654
ASMI, kg/m^2	5.81 ± 0.79	6.02 ± 0.60	5.72 ± 0.83	5.88 ± 0.64	< 0.001
Grip strength, kg	19.3 ± 4.9	20.2 ± 4.5	19.1 ± 4.9	18.9 ± 5.8	0.023
Gait speed, m/s	0.88 ± 0.07	1.01 ± 0.17	0.87 ± 0.06	0.93 ± 0.21	0.089

Data are presented as mean ± SD. ASMI: appendicular skeletal muscle mass index; AZL: Azilsartan; DBP: diastolic blood pressure; SBP: systolic blood pressure. The P value was calculated using independent t-test comparing the two groups after treatment.

improvement in ASMI (adjusted OR = 1.42, 95% CI: 1.12–1.80, P = 0.004) and grip strength (adjusted OR = 1.35, 95% CI: 1.08–1.69, P = 0.009) (Table 3).

AZL Attenuates Ang II-Induced Muscle Atrophy in Mice

Ang II administration resulted in a significant reduction in body weight (Figure 1A) and elevated systolic and diastolic blood pressure (Figure 1B). Exercise capacity was markedly impaired, with shorter exercise distance (214.24 ± 8.63 *vs.* 353.37 ± 9.06 m; Figure 1C-a) and exercise time (15.36 ± 0.75 *vs.* 22.18 ± 0.79 min; Figure 1C-b). Ang II also decreased forepaw grip strength (103.41 ± 2.14 g; Figure 1D) and reduced muscle weight of the gastrocnemius and quadriceps (Figure 1E). Consistently, Ang II increased Atrogin-1 and MuRF1 mRNA and pro-

Table 3 Multivariable logistic regression analysis for the association between AZL treatment and improvements in skeletal muscle parameters.

Outcome	Adjusted OR (95% CI)	*P* value
ASMI improvement	1.42 (1.12–1.80)	0.004
Grip strength improvement	1.35 (1.08–1.69)	0.009
Gait speed improvement	0.89 (0.70–1.13)	0.089

Adjusted for age, sex, BMI, diabetes, smoking, baseline ASMI, grip strength, gait speed, nutritional status, and concomitant medications (statins, antiplatelet agents). ASMI: appendicular skeletal muscle mass index; BMI: body mass index.

tein expression (Figure 1F-1G) and reduced muscle fiber cross-sectional area (Figure 1H).

AZL Ameliorates Muscle Atrophy via Activating the AT1R/PI3K/Akt/FOXO3a Pathway

Ang II markedly increased the mRNA and protein expression of the muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF1, whereas AZL significantly attenuated these responses (Figures 2A, 2B). Notably, co-treatment with the AT1R agonist TRV120027 largely abolished the inhibitory effects of AZL, restoring Atrogin-1 and MuRF1 expression to levels comparable to those observed in the Ang II group (Figures 2A, 2B). Consistent with these findings, Ang II suppressed PI3K expression and reduced the phosphorylation of Akt and FOXO3a, indicating inhibition of the PI3K/Akt/FOXO3a signaling pathway (Figure 2C). AZL treatment effectively restored pathway activation, whereas TRV120027 markedly attenuated the AZL-mediated recovery of PI3K expression and Akt/FOXO3a phosphorylation (Figure 2C). In contrast, blockade of AT2R with PD123319 did not significantly influence the effects of AZL on Ang II-induced Atrogin-1 and MuRF1 expression at either the mRNA or protein level (Figures 2D, 2E). Similarly, PD123319 failed to modify the restoration of PI3K/Akt/FOXO3a signaling induced by AZL (Figure 2F). Together, these findings indicate that the anti-atrophic effects of AZL are predominantly mediated through AT1R blockade, leading to reactivation of PI3K/Akt/FOXO3a signaling and suppression of ubiquitin-proteasome system-dependent muscle protein degradation.

AZL Attenuates Ang II-Induced C2C12 Myotube Atrophy

AZL at concentrations ranging from 1 to 10 μmol/L exhibited no detectable cytotoxicity in differentiated C2C12 myotubes (Figure 3A). Exposure to Ang II (1 μmol/L, 24 h) markedly induced the expression of the atrophy-associated E3 ubiquitin ligases Atrogin-1 and MuRF1 at both the mRNA and protein levels (Figures 3B, 3C). AZL co-treatment significantly attenuated these responses in a dose-dependent manner (Figures 3D, 3E). Consistently, Ang II also induced pronounced myotube atrophy, as evidenced by reductions in myotube diameter and cross-sectional area, which was ameliorated by AZL treatment (Figure 3F). Together, these results indicate that AZL protects against Ang II-induced myotube atrophy by suppressing atrophy-related catabolic signaling and preserving myotube structure, without adversely affecting cell viability.

AZL Attenuates Ang II-Induced C2C12 Myotube Atrophy via AT1R/PI3K/Akt/FOXO3a Signaling

To further elucidate the mechanism underlying the anti-atrophic effects of AZL, pharmacological modulation of the AT1R/PI3K/Akt signaling axis was performed in Ang II-treated C2C12 myotubes. Activation of AT1R with TRV120027 greatly reversed the inhibitory effects of AZL on the expression of Atrogin-1 and MuRF1 (Figures 4A, 4B). In addition, TRV120027 markedly attenuated the AZL-induced restoration of PI3K expression and Akt/FOXO3a phosphorylation (Figure 4C), indicating that activation of PI3K/Akt/FOXO3a signaling by AZL is dependent on AT1R blockade. To further determine the involvement of PI3K signaling, myotubes were treated with the PI3K inhibitor LY294002. Inhibition of PI3K significantly blunted the protective effects of AZL, resulting in re-elevated Atrogin-1 and MuRF1 expression (Figures 4D, 4E). Moreover, LY294002 abolished AZL-induced activation of Akt and FOXO3a despite continued AZL treatment (Figure 4F), confirming that PI3K acts as a critical downstream mediator of AZL signaling. Collectively, these findings provide mechanistic evidence that AZL protects against Ang II-induced myotube atrophy through AT1R-dependent activation of the PI3K/Akt/FOXO3a pathway, thereby suppressing Atrogin-1- and MuRF1-mediated catabolic signaling.

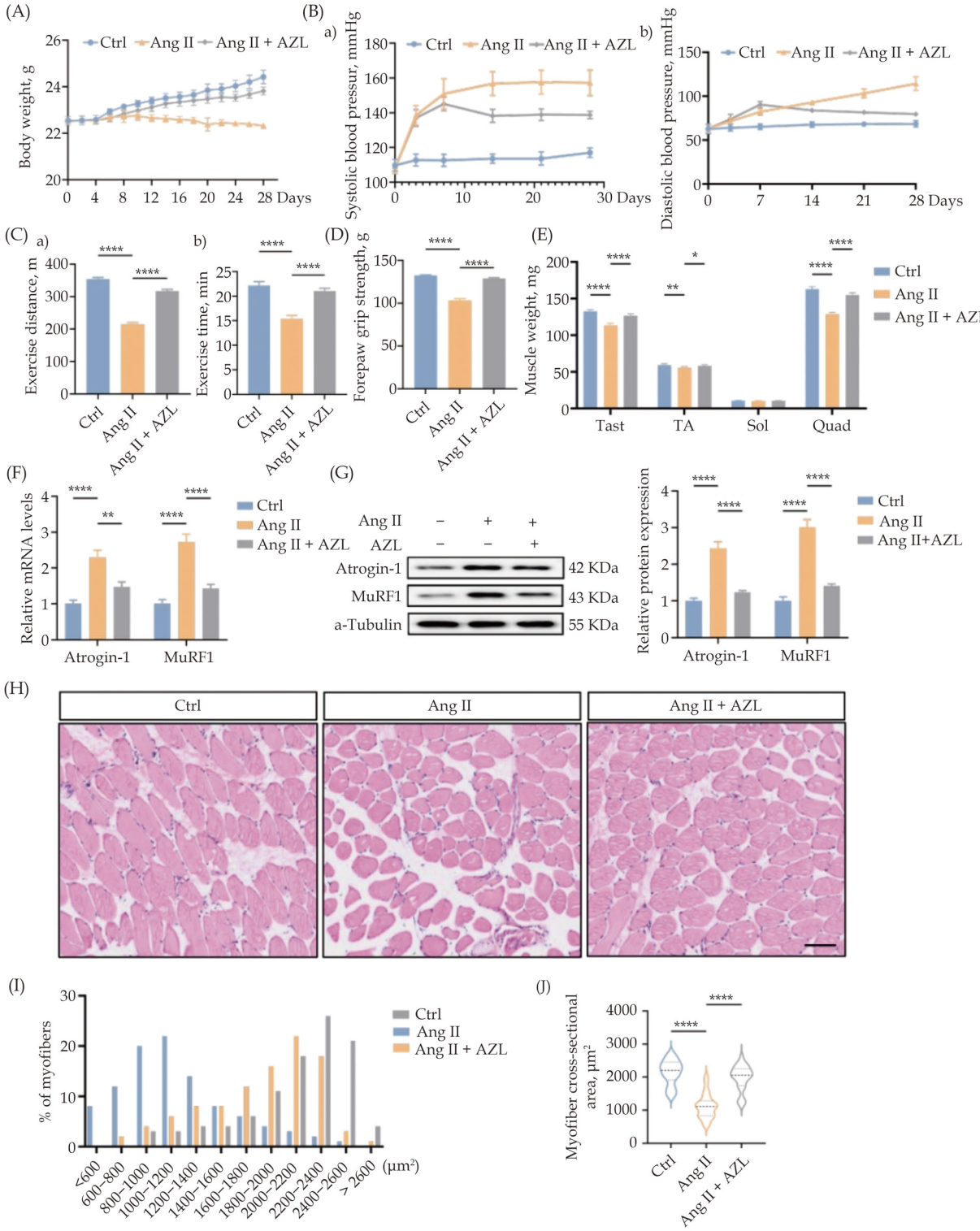


Figure 1 AZL ameliorates Ang II-induced muscle atrophy in mice. (A): Body weight; (B): blood pressure measurements: (a) systolic and (b) diastolic blood pressure; (C): exercise endurance: (a) exercise distance and (b) exercise time; (D: forelimb grip strength; (E): muscle weight of the gastrocnemius (Gast), tibialis anterior (TA), soleus (Sol), and quadriceps (Quad); (F–G): mRNA (F) and protein (G) expression levels of Atrogin-1 and MuRF1 (n = 6). (H): Representative HE-stained sections of gastrocnemius muscle showing muscle fiber cross-sectional area (scale bar = 100 μm). N = 8 in each group unless other indicated. ****P < 0.001.

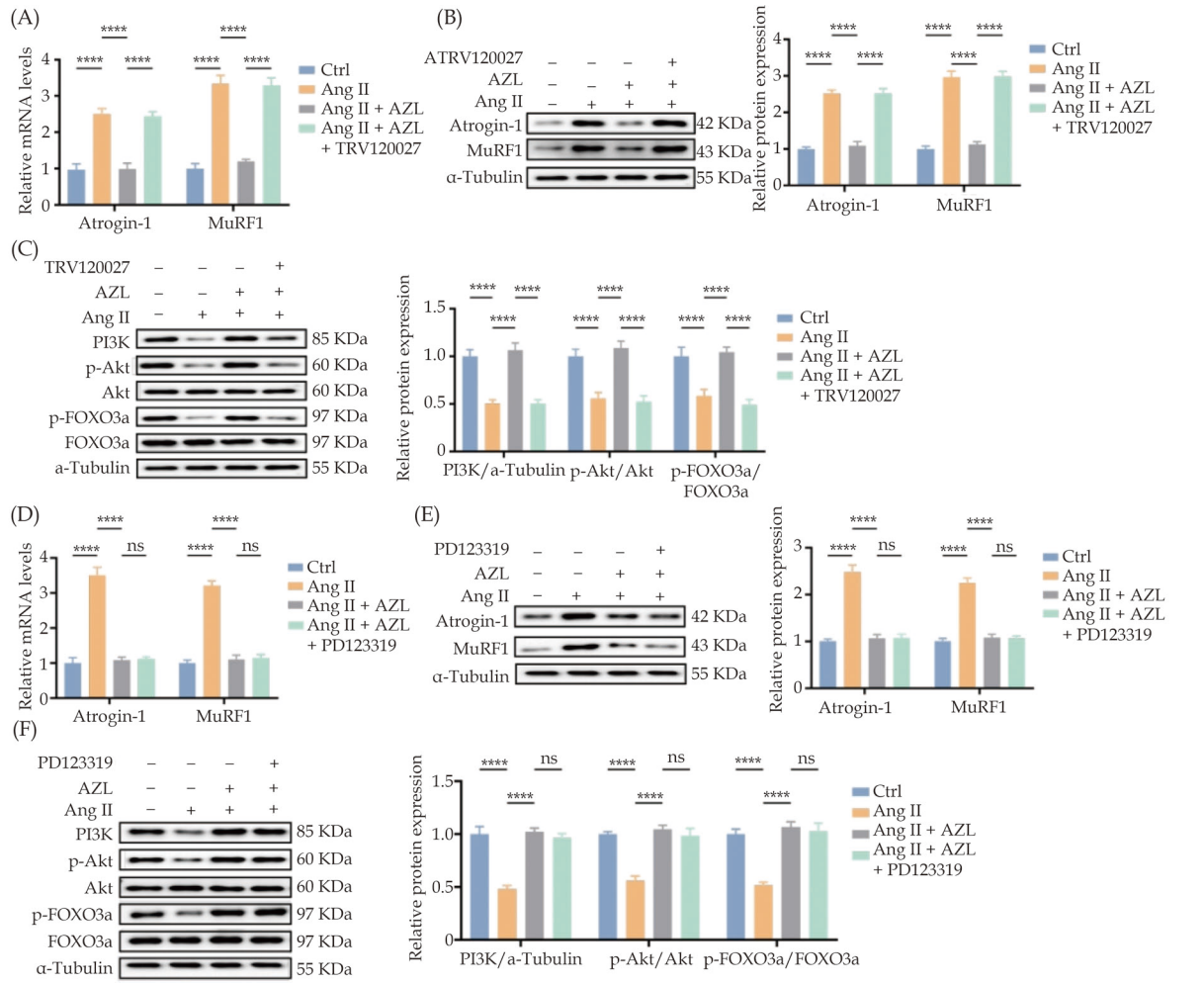


Figure 2 Effects of AT1R activation and AT2R blockade on AZL-mediated regulation of muscle atrophy markers and PI3K/Akt/FOXO3a signaling. (A): qRT-PCR analysis of Atrogin-1 and MuRF1 mRNA in Ang II- and AZL-treated skeletal muscle with or without the AT1R agonist TRV120027; (B): Western blot analysis of Atrogin-1 and MuRF1 protein under the same conditions; (C): Western blot analysis of PI3K, p-Akt/Akt, and p-FOXO3a/FOXO3a following AT1R activation with TRV120027; (D): qRT-PCR analysis of Atrogin-1 and MuRF1 mRNA after AZL treatment with the AT2R antagonist PD123319; (E): Western blot analysis of Atrogin-1 and MuRF1 protein after PD123319 treatment in the presence of AZL; (F): Western blot analysis of PI3K, p-Akt, Akt, and p-FOXO3a/FOXO3a following PD123319 treatment. $N = 6$ in each group. ****$P < 0.001$.

DISCUSSION

This study provides integrated clinical and experimental evidence showing that AZL improves skeletal muscle health in older patients with hypertension. Clinically, AZL increased skeletal muscle mass and grip strength over six months, suggesting a protective effect on muscle independent of blood pressure control. Mechanistic studies further support this observation. AZL inhibited Ang II-induced muscle atrophy by blocking AT1R and restoring PI3K/Akt/FOXO3a signaling, which suppressed the expression of the muscle-specific ubiquitin ligases Atrogin-1 and MuRF1.

The present findings may have important translational implications for older adults with hypertension who are at increased risk of sarcopenia and physical decline. Beyond its established antihypertensive effects, AZL attenuated skeletal muscle atrophy in our Ang II-induced model, suggesting that inhibition of AT1R signaling may confer additional benefits on skeletal muscle homeostasis. Notably, our findings extend previous observations by Prajapati, *et al.*,[15] who demonstrated that AZL alleviated age-related sarcopenia in naturally aged rats, preserving muscle mass, improving muscle strength, and attenuating the transition from fast- to slow-twitch muscle fibers. Together, these findings suggest that AZL may exert muscle-protective effects across dis-

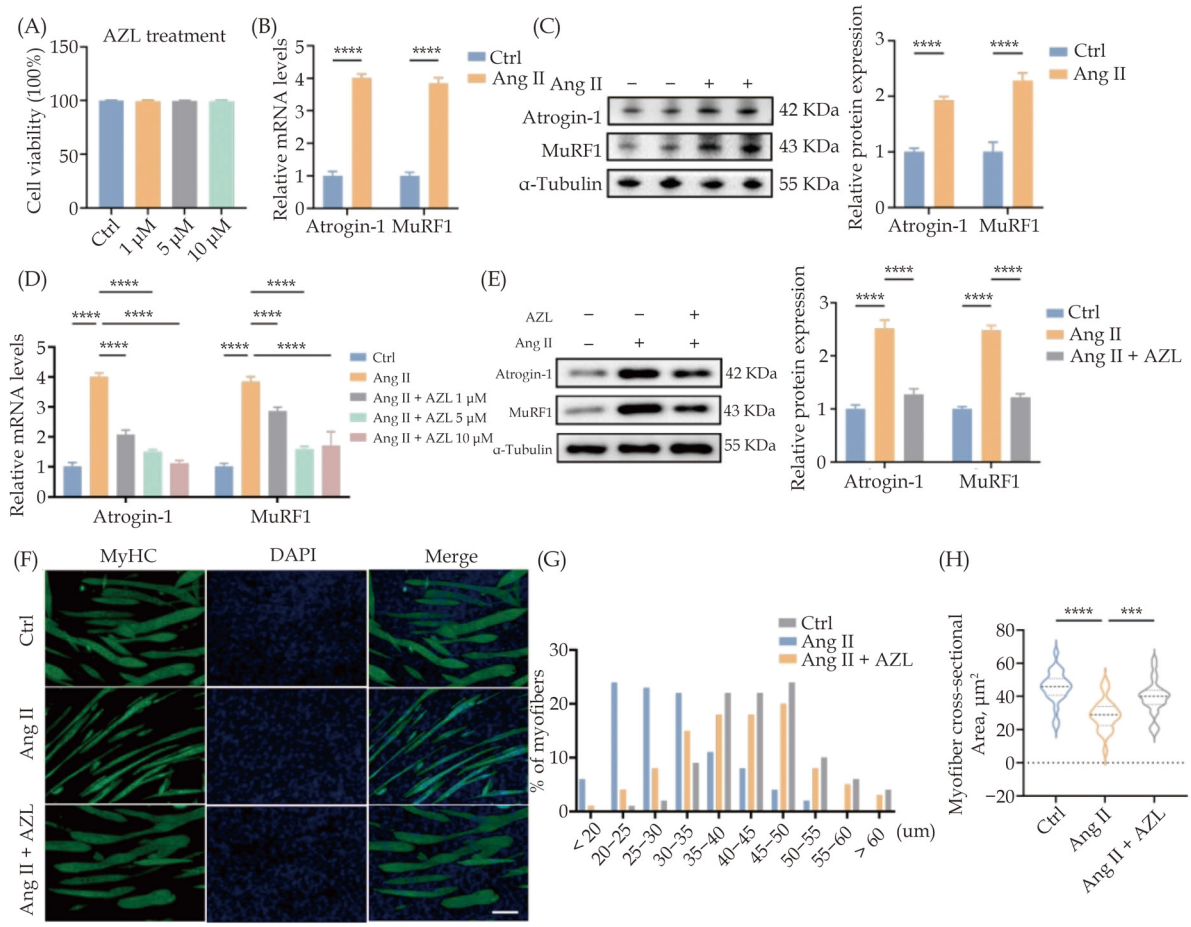


Figure 3 AZL attenuates Ang II-induced atrophy in C2C12 myotubes. (A): Cell viability of mature C2C12 myotubes following 24 h treatment with AZL (1, 5, 10 μmol/L); (B): qRT-PCR analysis of Atrogin-1 and MuRF1 mRNA in myotubes treated with Ang II (1 μmol/L, 24 h); (C): Western blot analysis of Atrogin-1 and MuRF1 protein expression following Ang II treatment (1 μmol/L, 24 h); (D): qRT-PCR analysis of Atrogin-1 and MuRF1 mRNA in myotubes treated with Ang II (1 μmol/L) in the presence of AZL (1, 5, 10 μmol/L); (E): Western blot analysis of Atrogin-1 and MuRF1 protein expression after combined treatment with Ang II and AZL; (F): representative immunofluorescence images showing myotube morphology (diameter and cross-sectional area) under control, Ang II, and Ang II + AZL conditions. All experiments were performed in differentiated C2C12 myotubes. $N = 6$ in each group. ****$P < 0.001$.

tinct pathological settings, including both ageing-associated and Ang II-driven muscle wasting. Nevertheless, the muscle-protective effects observed in the present study should be interpreted within the broader context of the ARB class. Previous experimental studies have demonstrated that other ARBs, including losartan and irbesartan, can attenuate muscle wasting through mechanisms involving suppression of catabolic signaling, reduction of inflammation, and preservation of muscle regenerative capacity.[22–24] Therefore, it remains uncertain whether the benefits observed with AZL reflect a unique pharmacological property or a class effect shared among ARBs. Direct comparative studies evaluating different ARBs under identical experimental conditions will be required to determine whether specific agents provide superior protection against muscle loss. In addition, prospective clinical studies are needed to establish whether the muscle-preserving effects observed experimentally translate into improved muscle mass, strength, and physical performance in older adults with hypertension.

Our study identifies activation of the PI3K/Akt/FOXO3a axis as a key mechanism underlying the anti-atrophic effects of AZL in Ang II-induced skeletal muscle wasting (Figure 5). Consistent with previous reports,[25] Ang II suppressed Akt phosphorylation and promoted FOXO3a nuclear translocation, leading to increased expression of the muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF1, two central mediators of ubiquitin-proteasome system (UPS)-dependent proteolysis.[26] AZL effectively reversed these alterations, restoring Akt activ-

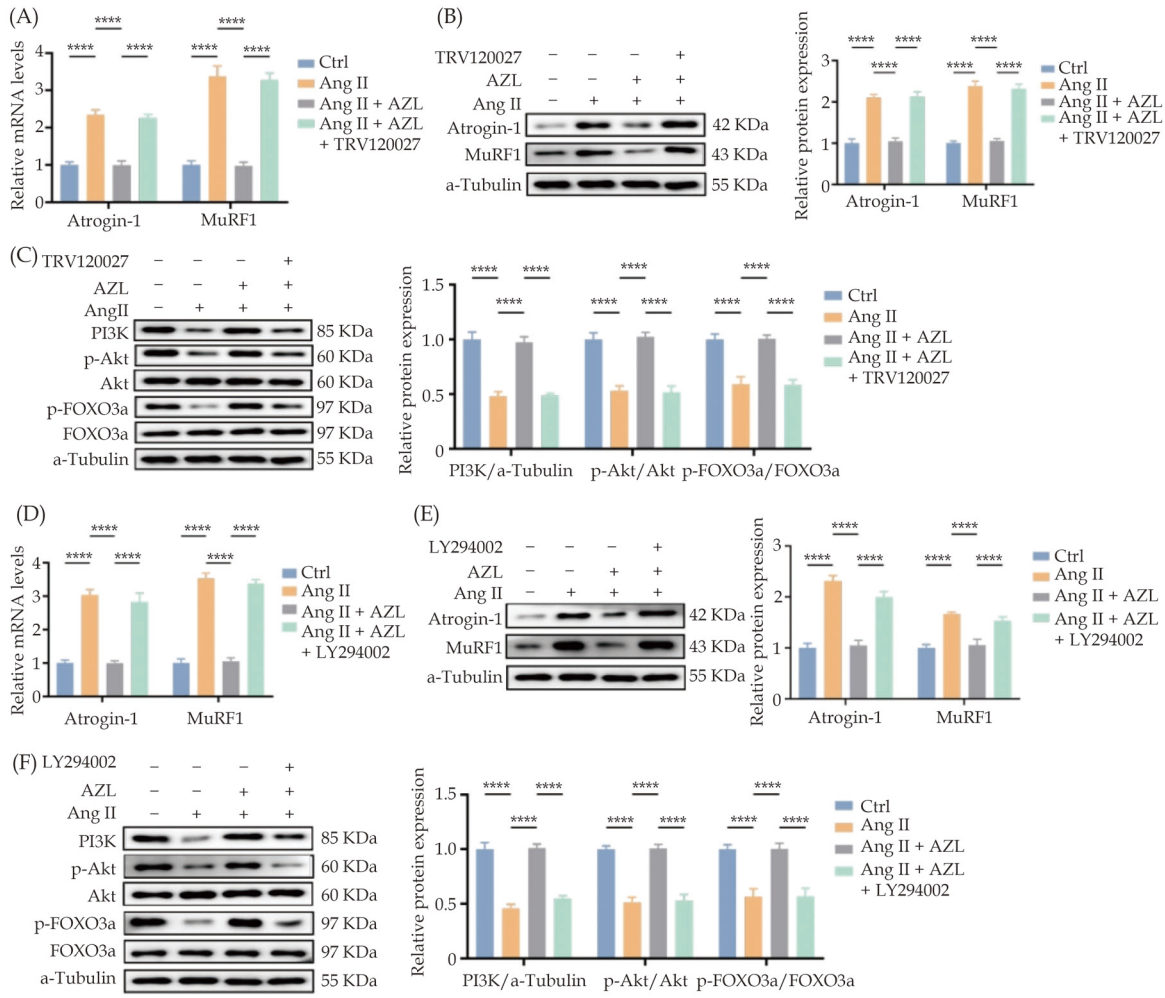


Figure 4 Effects of AT1R activation and PI3K inhibition on AZL-mediated protection against Ang II–induced myotube atrophy. (A): qRT-PCR analysis of Atrogin-1 and MuRF-1 mRNA in C2C12 myotubes treated with Ang II (1 μmol/L), AZL, and/or the AT1R agonist TRV120027; (B): Western blot analysis of Atrogin-1 and MuRF-1 protein under the same conditions; (C): Western blot analysis of PI3K, p-Akt/Akt, and p-FOXO3a/FOXO3a following co-treatment with TRV120027; (D): qRT-PCR analysis of Atrogin-1 and MuRF-1 mRNA in myotubes treated with AZL with or without the PI3K inhibitor LY294002; (E): Western-blot analysis of Atrogin-1 and MuRF-1 protein following LY294002 treatment; (F) Western blot analysis of PI3K, p-Akt/Akt, and p-FOXO3a/FOXO3a after PI3K inhibition. Overall, Ang II suppressed PI3K/Akt/FOXO3a signaling and increased atrophy markers, whereas AZL reversed these effects. Both AT1R activation and PI3K inhibition attenuated the protective action of AZL, indicating that its effects are mediated via AT1R-dependent PI3K/Akt/FOXO3a signaling. $N = 6$ in each group. ****$P < 0.001$.

ity, inhibiting FOXO3a nuclear accumulation, and attenuating the induction of Atrogin-1 and MuRF1. These findings suggest that blockade of AT1R signaling mitigates excessive UPS activation and thereby preserves skeletal muscle mass under catabolic conditions. The regulation of skeletal muscle homeostasis, however, extends beyond the UPS.[27] Multiple interconnected pathways, including mTOR-dependent protein synthesis, autophagy-lysosome-mediated protein degradation, and mitochondrial quality control, contribute to the maintenance of muscle mass and function.[28–30] Dysregulation of these pathways has been implicated in various forms of muscle wasting, including cachexia, sarcopenia, and disuse atrophy.[31] Previous studies have suggested that excessive Ang II/AT1R signaling may impair autophagic flux, promote mitochondrial dysfunction, and enhance oxidative stress, thereby exacerbating muscle degeneration.[32–34] Whether AZL exerts additional protective effects through modulation of these processes remains unclear. As our current study focused primarily on the PI3K/Akt/FOXO3a pathway, the involvement of autophagy, mTOR signaling, mitochondrial homeostasis, and related metabolic adaptations was not directly examined. Future studies incorporating assessments of

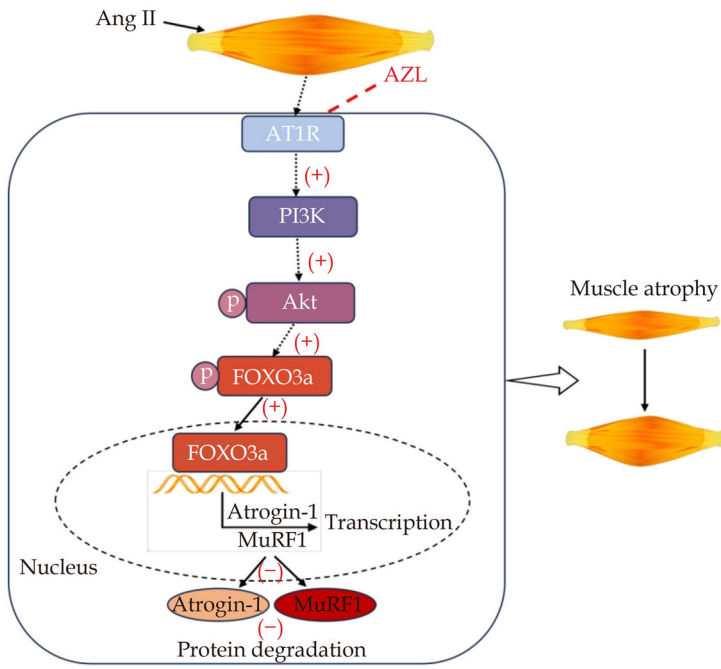


Figure 5 Schematic illustration of the proposed mechanism by which AZL alleviates Ang II-induced myotube atrophy via the AT1R/PI3K/Akt/FOXO3a signaling pathway. Ang II activates AT1R, leading to suppression of PI3K/Akt phosphorylation and subsequent dephosphorylation and nuclear translocation of FOXO3a. Nuclear FOXO3a promotes transcription of muscle-specific ubiquitin ligases Atrogin-1 and MuRF1, accelerating protein degradation and resulting in muscle atrophy. AZL, by selectively blocking AT1R (indicated by a red dashed line), restores PI3K/Akt activation, enhances FOXO3a phosphorylation, retains it in the cytoplasm, and downregulates Atrogin-1 and MuRF1 expression, thereby reducing protein degradation and improving muscle integrity. Red plus (+) and minus (−) signs represent the role of AZL. Ang II: angiotensin II; AZL: azilsartan; AT1R: angiotensin II type 1 receptor.

autophagic activity, mitochondrial function, and protein synthesis signaling will be important to provide a more comprehensive understanding of the mechanisms through which AZL preserves skeletal muscle integrity.

Several limitations should also be acknowledged when interpreting the clinical findings. First, because the clinical component of this study was observational in nature, the possibility of confounding by indication cannot be completely excluded. Patients with more severe hypertension, greater cardiovascular risk, or prior exposure to antihypertensive therapies may have been more likely to receive AZL, potentially influencing the observed associations. Although extensive adjustment for demographic characteristics, comorbidities, and relevant clinical variables was performed, residual and unmeasured confounding may still exist. Therefore, the observed association between AZL use and improved muscle-related outcomes should not be interpreted as evidence of causality. Second, while the mechanistic experiments support a biological rationale linking AZL to muscle preservation, the translational relevance of these findings requires confirmation in prospective clinical studies. Randomized controlled trials specifically designed to evaluate changes in muscle mass, strength, and physical performance will be necessary to establish whether AZL provides clinically meaningful protection against sarcopenia and frailty in older adults with hypertension. Importantly, because sarcopenia is characterized not only by reduced muscle mass but also by impaired muscle strength and physical performance, future studies should integrate functional assessments and patient-centered outcomes. Such investigations may help determine whether the muscle-preserving effects of AZL translate into reduced frailty, disability, hospitalization, and other adverse health outcomes in ageing populations. Finally, although TRV120027 is widely used as a pharmacological tool to selectively activate AT1R and has demonstrated high receptor specificity, the possibility of off-target effects inherent to pharmacological approaches cannot be completely excluded. Therefore, while our findings strongly support a critical role for AT1R in mediating the protective effects of AZL, additional studies employing genetic approaches, such as AT1R knockdown or knockout models, will be necessary to further validate the specificity of this signaling pathway.

CONCLUSION

In conclusion, AZL attenuates Ang II-induced skeletal muscle atrophy through AT1R-dependent activation of PI3K/Akt/FOXO3a signaling. Consistent with these mechanistic findings, AZL use was associated with favorable muscle-related outcomes in older adults with hypertension. Taken together, these results provide mechanistic and translational support for targeting AT1R signaling to preserve skeletal muscle homeostasis and warrant further clinical evaluation of AZL in ageing-related muscle disorders, including sarcopenia.

DISCLOSURE

Clinical Trials Registration Number

ChiCTR2300071464

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Ethics Statement

The study protocol was approved by the Ethics Committee of Sir Run Run Hospital, Nanjing Medical University (Approval No. 2023-SR-001) and registered at the Chinese Clinical Trial Registry (ChiCTR2300071464). All animal experiments were carried out under the approval of the Animal Ethics Committee of Nanjing University (permit number: IACUC-1905027) and complied with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Author Contributions

Conceptualization was performed by Y.C., W.P.L., X.L., and W.G. Methodology was designed by Y.C., W.P.L., Y.Y., P.L., and J.W.L. Investigation was conducted by Y.C., W.P.L., Y.Y., C.Z., and J.S.X. Formal analysis was carried out by W.P.L., Y.Y., and J.W.L. Resources were provided by Z.K.S. and J.S.X. The original draft was written by Y.C. and W.P.L. Review and editing were completed by all authors. Supervision was provided by X.L., W.G., and Z.K.S. Project administration was handled by X.L. and W.G. Funding was acquired by Z.K.S., X.L., and W.G. All authors read and approved the final manuscript.

Conflict of Interest

None.

Availability of Data

The data that support the findings of this study are available upon reasonable request.

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