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Activation of the Mas receptors by AVE0991 and MrgD receptor using alamandine to limit the deleterious effects of Ang II-induced hypertension

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Abstract

The MrgD receptor agonist, alamandine (ALA) and Mas receptor agonist, AVE0991 have recently been identified as protective components of the renin-angiotensin system. We evaluated the effects of ALA and AVE0991 on cardiovascular function and remodeling in angiotensin (Ang) II-induced hypertension in rats. Sprague Dawley rats were subject to 4-week subcutaneous infusions of Ang II (80 ng/kg/min) or saline after which they were treated with ALA (50 μg/kg), AVE0991 (576 μg/kg), or ALA+AVE0991 during the last 2 weeks. Systolic blood pressure (SBP) and heart rate (HR) values were recorded with tail-cuff plethysmography at 1, 15, and 29 days post-treatment. After euthanization, the heart and thoracic aorta were removed for further analysis and vascular responses. SBP significantly increased in the Ang II group when compared to the control group. Furthermore, Ang II also caused an increase in cardiac and aortic cyclophilin-A (CYP-A), monocyte chemoattractant protein-1 (MCP-1), and cardiomyocyte degeneration but produced a decrease in vascular relaxation. HR, matrix metalloproteinase-2 and -9, NADPH oxidase-4, and lysyl oxidase levels were comparable among groups. ALA, AVE0991, and the drug combination produced antihypertensive effects and alleviated vascular responses. The inflammatory and oxidative stress related to cardiac MCP-1 and CYP-A levels decreased in the Ang II+ALA+AVE0991 group. Vascular but not cardiac angiotensin-converting enzyme-2 levels decreased with Ang II administration but were similar to the Ang II+ALA+AVE0991 group. Our experimental data showed the combination of ALA and AVE0991 was found beneficial in Ang II-induced hypertension in rats by reducing SBP, oxidative stress, inflammation, and improving vascular responses.

KEYWORDS
alamandine, angiotensin II, AVE0991, cyclophilin A, hypertension, NADPH oxidase

1 | INTRODUCTION

Hypertension (HT) is a common chronic disease associated with an extensive global burden of cardiovascular disease.[1] Several experimental HT models are studied to investigate new treatment targets and new therapeutic drugs to improve the pathophysiology of the disease beyond empirical treatment in HT.[2–5]

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Angiotensin (Ang) II plays a crucial role in the pathophysiology of HT via its hemodynamic and non-hemodynamic mechanisms, including endothelial dysfunction, cardiac and vascular remodeling, left ventricular hypertrophy, vascular smooth muscle hypertrophy, constriction of afferent arterioles, and renal Na^+ uptake.[6–10] Therefore, the Ang II-induced HT model is among the most widely used experimental HT models, particularly in rodents.

Alamandine (ALA) is produced by decarboxylation of the Asp1 residue from Ang (1-7) to the Ala1 residue at the N-terminus. ALA exerts its vasodilator and antifibrotic effects similar to Ang (1-7) but through Mas receptor-associated G-protein coupled receptor (MrgD).[11] Various studies have been published showing the effect of ALA-induced MrgD activation on blood pressure (BP). In renovascular hypertensive rats, ALA showed a biphasic hemodynamic effect after infusion.[12] Oral administration of ALA with β-cyclodextrin showed a prolonged antihypertensive effect in spontaneous hypertensive rats (SHR) and an antifibrotic effect in isoproterenol-treated rats.[11] ALA (25 mcg/kg/h) infusion also suppressed the levels of H_2O_2 and NADPH oxidase-4 (NOX-4)-dependent oxidative stress pathway in a carbon tetrachloride-induced hepatic fibrosis model.[13] Li et al. showed that ALA regulated BP values by increasing eNOS and iNOS expressions and decreased lipopolysaccharide-induced myocardial inflammation, apoptosis, and autophagy in a sepsis-associated cardiac dysfunction model.[14] Additionally, Song et al. investigated the protective efficacy of ALA in a myocardial ischemia–reperfusion model, and the observation was ALA protected rats from myocardial injury by reducing the levels of NF-κB, TNF-α, IL-1β and IL-6 and also activating c-Jun N-terminal kinase.[15]

AVE0991 (5-formyl-4-methoxy-2-phenyl-1-[[4-[2-ethyl-aminocarbonylsulfonamido-5-isobutyl-3-thienyl]-phenyl]-methyl]-imidazole) is a non-peptide and orally active analog of Ang (1-7).[16,17] AVE0991 demonstrated protective efficacy against end-organ damage in the l-nitro-arginine methyl ester (L-NAME)-induced HT as well as in SHR.[18] Similarly, AVE0991 administration regulates left ventricular contractility and post-ischemia–reperfusion coronary blood flow in SHR, also attenuated L-NAME-induced elevated BP by decreasing vasoconstrictor response in the mesenteric artery.[19] In another study, AVE0991 decreased the incidence of abdominal aortic aneurysm development by decreasing MCP-1, TNF-α, MMP-2, and MMP-9 levels in ApoE *knockout* mice in Ang II-induced aneurysm model.[20] Ebermann et al. showed that AVE0991 regulated the systolic BP, dP/dtmax, an indicator of left ventricular contraction response, and dP/dtmin, an indicator of the left ventricular relaxation response in streptozotocin-induced diabetic rats.[21]

Here, we aimed to investigate the effects of ALA, AVE0991, and their combination on systolic BP and vascular contraction-relaxation responses, vascular proliferation, cardiac remodeling, inflammation, and oxidative stress in rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Forty male Sprague Dawley rats (12–16 weeks), weighing 300–400 g, were obtained from İnönü University Experimental Animal Production and Research Center. The rats were housed under a 12:12 h light/dark cycle in temperature (22 ± 1°C) and humidity (55 ± 5%) controlled rooms. Standard pellet feed and water were provided ad libitum to the animals. All experiments and procedures in this study were performed under the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines.[22] This study was approved by the Animal Experiments Ethics Committee of İnönü University (approval detail: 2020/5–3 and HAYBIS 9739).

2.2 | Experimental protocol

Rats were divided into five groups ($n = 8$) by the simple randomization technique. The Control, Ang II, Ang II+ALA, Ang II+AVE0991, and Ang II+ALA+AVE0991 groups. Before the experiment, mini osmotic pumps (Alzet® mini-osmotic pump, model 2004, Durect Corporation, Cupertino, CA, USA) were filled with Ang II (80 ng/kg/min for 40 days). After incubating at 37°C for 48 h, osmotic pumps were placed in the interscapular region under 50 mg/kg ketamine and 10 mg/kg xylazine anesthesia, and tail hemodynamic measurements were recorded at the 1, 15, and 29 days post-implantation. From the 15th day of the experiment, 0.4 ml of 3% DMSO was applied to the rats as in the Control and Ang II groups, whereas ALA (50 mcg/kg), AVE0991 (576 mcg/kg/min), and their combination were given intraperitoneally (ip) for 2 weeks as of group names. On the 29th day, the experiment was terminated and the heart and thoracic aorta were harvested for further analysis.

2.3 | HT induction

Ang II (Angiotensin II (human), vasoconstrictor peptide, ab120183, Abcam, Cambridge, UK) solution was filled into mini osmotic pumps to release 80 ng/kg/min following the manufacturer's instruction. Before the surgical procedure, rats were anesthetized with 10 mg/kg xylazine and 50 mg/kg ketamine ip. Anesthesia was controlled by the toe pinch reflex. Then, the area between the two scapulae was gently shaved with an electric razor, exposing the surgical area before

cleaning with povidone-iodine solution. Then, a horizontal incision was made in line with the mid-scapular region. The skin and muscle plane were separated by blunt dissection with surgical scissors to create a subcutaneous pocket in which the mini-osmotic pump will be placed. The incision was then closed using 4.0 polyglactin-coated vicryl sutures (W9442, Ethicon®).

2.4 | Cardiac hemodynamic measurements

Systolic BP and heart rate were measured by tail-cuff plethysmography using the non-invasive BP system (MAY NIBP-250, Commat Ltd Inc., Ankara, Türkiye). An infrared sensor (RXTCUF-SENSOR13, Biopac System Inc.) was used to detect blood flow pulsations. The corresponding value was obtained by taking the mean value of each rat's last five data of at least 10 consecutive readings. The rats were utilized after a 7-day acclimatization period for BP measurements.[23,24] Further details of the measurement technique can be found in Appendix S1.

2.5 | Isolated organ bath system

At the end of the experiment, the dissected thoracic aorta tissues were quickly placed in horizontal containers containing Krebs–Henseleit solution and were carefully cleaned of perivascular adipose tissues. The thoracic aortic rings (approximately 4 mm) were placed on tungsten wire hooks in a 25 ml isolated organ bath containing Krebs–Henseleit solution heated to 37°C and gassed with carbogen. The upper hook was attached to force-displacement transducers to record isometric contraction responses. Recordings were made with MP100-A (Model MP100-A, Biopac, Goleta, CA, USA) polygraph. The thoracic aortic rings suspended in the isolated organ bath were rested for 30 min under pre-defined loading tension. The Krebs–Henseleit solution in the isolated organ bath was renewed every 15–20 min to prevent the accumulation of metabolic end products. Vascular contractile and relaxation responses were evaluated with phenylephrine (Phe, 3×10^{-9}–10^{-4} mol/L) and acetylcholine (Ach, 10^{-9}–10^{-5} mol/L), *respectively* (Appendix S1).[25]

2.6 | Cardiac and aortic tissue homogenization

Tissue homogenate buffer was prepared by adding 150 μl of protease inhibitor (Protease inhibitor cocktail-EDTA free, Abcam; ab201111) at a ratio of 1:20 to 30 ml of PBS (50 mM, pH 7.4); 750 μl of this buffer was taken and placed in separate Eppendorf tubes; 25 mg of heart tissue taken from the middle portion of the left ventricle was added to the prepared 750 μl buffer and homogenized for 30 s at 13500 rpm with a homogenizer (4 times in total) (Ultra-Turrax T25, IKA-Werke). After the 1st round of homogenization, 50 μl of LOX was taken into a separate tube, and 450 μl of extraction buffer (6 M urea, 10 mM trips pH 7.4) was added and then diluted 10× RIPA (10× RIPA buffer, Abcam; ab156034) buffer to 1× concentration to 0.25 ml per 25 mg tissue. Tissue homogenate prepared for LOX was homogenized for one more round for 15 s at 13500 rpm. Then, the prepared tissue homogenates were centrifuged at 15000 rpm for 10 min at +4°C and divided into three Eppendorf tubes of 150 μl. Total protein content was determined in the remaining 50 μl supernatant using the Bradford method.[26] The dilution rate was determined for each homogenate, and the samples were kept at +4°C till the next day for biochemical analysis.

2.7 | Biochemical assay

Following the manufacturer's instruction of each ELISA kit, standard curves were created using the calculated absorbance values and concentration values of ACE-2 (Rat Angiotensin Converting Enzyme 2 ELISA Kit, Cat. No: E0968Ra, Shanghai, China), CYP-A (Rat Cyclophilin A, ELISA Kit, Cat. No: E1936Ra, Shanghai, China), MCP-1 (Rat MCP1 ELISA Kit (CCL2), ab100778, Abcam, Cambridge, UK), MMP-2 (Rat Matrix Metalloproteinase 2/Gelatinase A, MMP-2 ELISA Kit, Cat. No: E0315Ra, Shanghai, China) MMP-9 (Rat Matrix Metalloproteinase 9/Gelatinase B, MMP-9 ELISA Kit, Cat. No: E0321Ra, Shanghai, China) and NOX-4 (Rat NADPH Oxidase 4, NOX4 ELISA Kit, Cat. No: E1298Ra, Shanghai, China) were calculated using the formula "=TREND()" function in Microsoft Excel for Mac v.16.48 program (Appendix S1).

The semiquantitative analysis was performed for LOX (Lysyl Oxidase Activity Assay Kit (Fluorometric), ab112139, Abcam, Cambridge, UK). The stop solution of the LOX kit was accepted as one-fold, and the values obtained from the next wells were calculated by proportioning the values of the stop solution. Fluorescence increases were analyzed in a microplate reader (Biotek, Synergy H1 microplate reader, China). Fluorescent diffractions were evaluated according to the kit manufacturer's instructions "Ex/Em = 530–570/590–600 nm (maximum Ex/Em = 540/590 nm, 570 nm)".

2.8 | Histopathological assays

At the end of the experiment, the heart and thoracic aorta samples were fixed in 10% formaldehyde solution. Cardiac and aortic sections (usually 4–5 μm

thickness) were stained with the hematoxylin–eosin (H-E) to determine the general morphological structure, whereas the orcein staining method for elastic lamella on aortic sections.

Heart sections were examined for interstitial edema and cardiomyocyte degeneration (dense eosinophilic cytoplasm, pycnotic nucleus) with blinded from the experimental groups. Ten randomly selected fields at ×40 magnification are intact according to the severity of histopathological changes; 0, light; 1, medium; 2 and severe; 3.[27]

The histopathological changes (loss of myofibrils in smooth muscle cells, elastic fiber degeneration) observed in the aortic wall by examining the entire area are intact according to their severity; 0, light; 1, medium; 2 and severe as 3 at ×40 magnification. In addition, intimal-media thickness (IMT) was measured by randomly selecting 10 areas at ×20 magnification from each section in which the H-E staining method was applied.

Analyzes were performed using the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK) with a Leica DFC-280 microscope.

2.9 | Statistical analysis

Statistical analyzes were performed with the Jamovi program, while the analyzes of aortic contraction-relaxation responses were performed using GraphPad Prism ver.8. Post-hoc Dunnett T3 tests were used after a one-way ANOVA with Welch correction for multiple comparisons between groups. Heart rate and systolic BP were compared using two-way ANOVA with time and group as covariates via GraphPad Prism (v9.). Data were presented as mean (95% Confidence Interval, lower bound-upper bound). A p-value less than 0.05 was considered statistically significant.

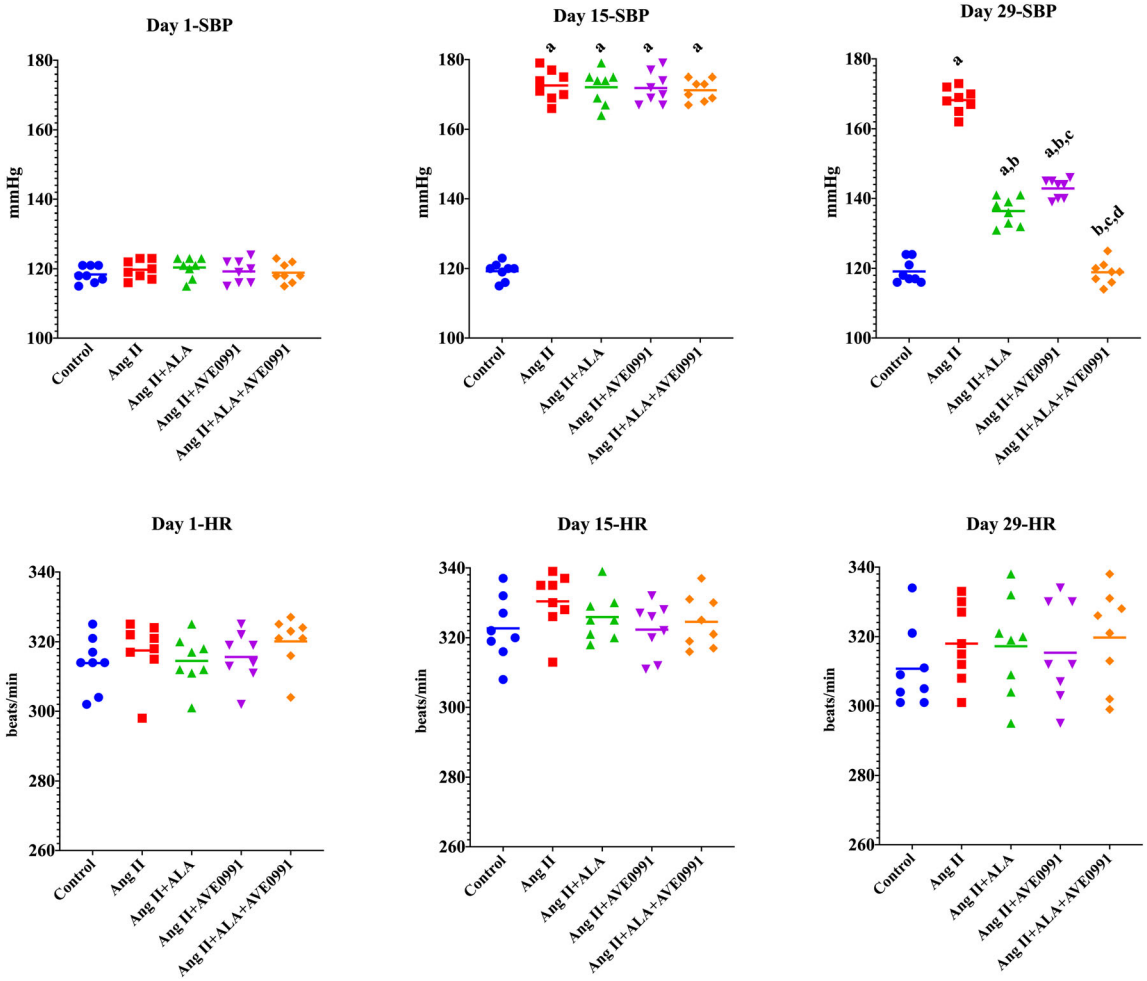


FIGURE 1 Timeline of systolic blood pressure and heart rate changes. Each symbol represents one individual value in the study. $p < 0.05$ vs. Control group, $^{b}p < 0.05$ vs. Ang II group. $^{c}p < 0.05$ vs. Ang II+ALA group, $^{d}p < 0.05$ vs. Ang II+AVE0991 group. Ang II, Angiotensin II; ALA, alamandine; SBP, systolic blood pressure; HR, heart rate

3 | RESULTS

No statistically significant difference was found between the groups when the rat weights were examined before and after the experiment ($p > 0.05$). In addition, there was no statistically significant difference in the weights of the whole heart tissue removed at the end of the experiment and the heart/rat body weights indicators ($p > 0.05$) (Appendix S2).

One rat had developed torticollis-like symptoms, which caused hyperactive behaviors seen all day, and was replaced with another rat in the study. There were no deaths or side effects among groups during the experiment.

TABLE 1 Two-way repeated measures ANOVA for systolic blood pressure and heart rate with time and group as covariates

	F	df	MS	*p* value
Systolic blood pressure				
Time (day)	1914	2	17 867	<0.0001
Group	228.6	4	4016	<0.0001
Time × Group	201.2	8	1878	<0.0001
Heart rate				
Time (day)	10.42	2	1044	<0.0001
Group	1.731	4	160.3	0.17
Time × Group	0.3578	8	35.87	0.94

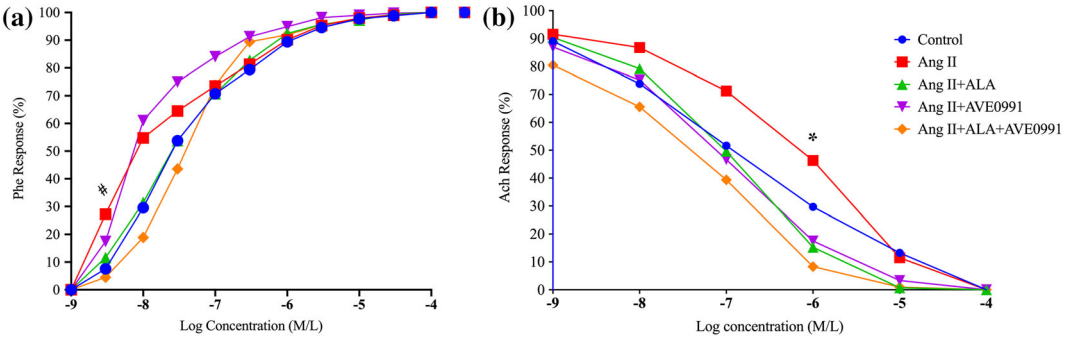
Abbreviations: F, ratio of the two variances that are a measure of dispersion, or how far the data are scattered from the mean; df, degrees of freedom; MS, mean square.

3.1 | Hemodynamics

BP values were recorded by tail-cuff plethysmography. Throughout the experiment, the systolic BP was similar at day 1 among groups. Ang II infusion significantly increased systolic BP in the period of 2–4 weeks ($p < 0.05$) but had no significant effects on HR ($p > 0.05$). As shown in Figure 1, the combination of ALA and AVE0991 reduced systolic BP more than their single used groups ($p < 0.05$) and reached similar levels to the Control group ($p > 0.05$). Furthermore, two-way repeated measures ANOVA models for systolic BP including time and group variables were statistically significant but not heart rate (Table 1).

3.2 | Vascular responses

Phe related pD_2 value was statistically significantly higher in the Ang II+AVE0991 group than the other groups except for the Ang II group ($p < 0.05$). The E_{max} values of the contractile responses were similar among the groups ($p > 0.05$) (Figure 2c). However, it was remarkable that the curve shifted to the left (Figure 2a)



(c)

Groups	Phenylephrine		Acetylcholine	
	pD_2	E_{max} (g)	pD_2	E_{max} (g)
Control	7.47±0.08	2493±212	6.93±0.58	1327±135
Ang II	7.83±0.10	1966±135	6.29±0.22	1273±190
Ang II+ALA	7.52±0.07	1746±103	7.08±0.21[b]	1280±136
Ang II+AVE0991	8.01±0.07[a,c]	1951±151	7.17±0.23[b]	1566±186
Ang II+ALA+ AVE0991	7.41±0.09[d]	1621±178	7.52±0.20[b]	1331±150

Values are expressed as mean±standart error.

[a]: p<0.05 *vs.* Control group, [b]: *vs.* Ang II group. [c]: *vs.* Ang II+ALA group, [d]: *vs.* Ang II+AVE0991 group.

Abbreviations: Ang II: Angiotensin II, ALA: Alamandine, pD_2: -log(EC_{50}), E_{max}: Maximal response.

FIGURE 2 Vascular responses of phenylephrine (a) and acetylcholine (b) on thoracic aorta samples and E_{max} and pD_2 values (c). #: $p < 0.05$ Control vs. Ang II group, Ang II vs. Ang II+ALA+AVE0991 group, Ang II+ALA vs. Ang II+ALA+AVE0991 group at the specified concentration value. *$p < 0.05$ Ang II vs. Ang II+ALA+AVE0991 group at the specified concentration value. [a]$p < 0.05$ vs. Control group, [b]$p < 0.05$ vs. Ang II group, [c]$p < 0.05$ vs. Ang II+ALA group, [d]$p < 0.05$ vs. Ang II+AVE0991 group. Ang II, angiotensin II; ALA, alamandine; Phe, phenylephrine; Ach, acetylcholine

in all Phe concentrations in the Ang II+AVE0991 group compared to the Ang II group. However, there was no significant difference between the groups when compared in mg and percentage ($p > 0.05$). Phe-related contraction responses of the groups were presented separately as mg and percentage by weight in the Appendix S2.

There was no significant difference between the groups in terms of E_{max} values, which define the maximum relaxation response caused by Ach in the thoracic aorta during the isolated organ bath ($p > 0.05$) (Figure 2c). When the pD_2 values, which reached half of the maximum relaxation response, were examined, a significant decrease was observed in the Ang II group compared to the Control group; A statistically significant increase was found in Ang II+ALA, Ang II+AVE0991, and Ang II+ALA+AVE0991 groups compared to Ang II group ($p < 0.05$) (Figure 2b). Although the pD_2 value in the Ang II+ALA+AVE0991 group increased compared to the Ang II+ALA and Ang II+AVE0991 groups, this increase did not reach a statistically significant difference ($p > 0.05$). Relaxation responses are given in mg and percent as a table (Appendix S2).

3.3 | Biochemical results

3.3.1 | ACE-2 levels

In our study, ACE-2 levels were determined by ELISA from the heart and thoracic aorta tissues to evaluate the effects of ALA and AVE0991 applications on ACE-2

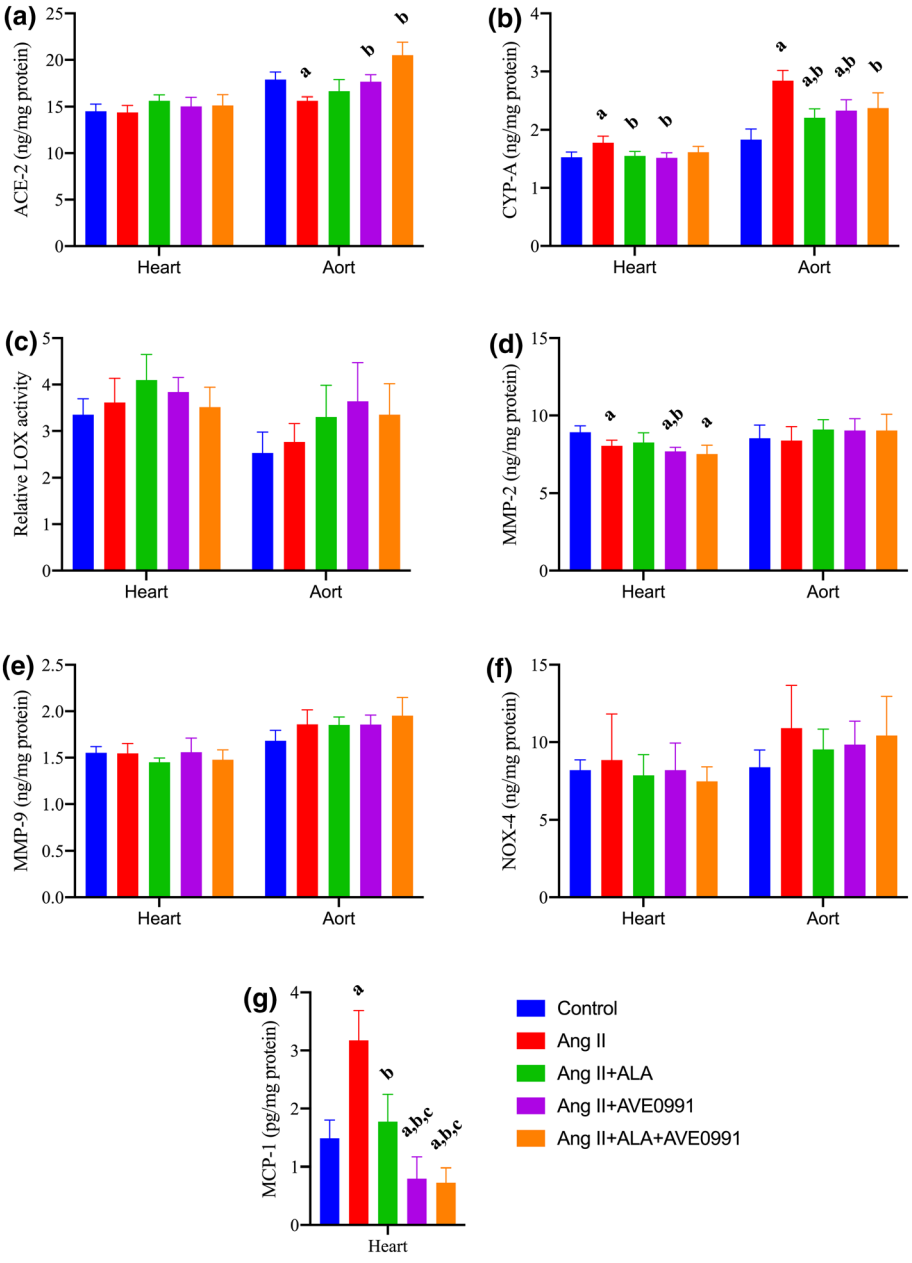


FIGURE 3 Cardiac and vascular protein levels of ACE-2, CYP-A, LOX, MMP-2, MMP-9, NOX-4, and cardiac MCP-1. Data are means (95% CI upper bound-lower bound), $n = 8$ for each group. [a]$p < 0.05$ vs. Control group, [b]$p < 0.05$ vs. Ang II group, [c]$p < 0.05$ vs. Ang II+ALA group, [d]$p < 0.05$ vs. Ang II+AVE0991 group. Ang II, angiotensin II; ALA, alamandine; ACE-2, angiotensin-converting enzyme-2; CYP-A, cyclophilin-A; LOX, lysyl oxidase; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; NOX-4, NADPH oxidase-4; MCP-1, monocyte chemoattractant protein-1

levels (Figure 3a). Cardiac ACE-2 levels were similar in the Ang II group compared to the Control group ($p > 0.05$). In the thoracic aortic tissue, ACE-2 levels in the Ang II group resulted in a statistically significant decrease compared to the Control group ($p < 0.05$).

3.3.2 | CYP-A levels

An intracellular protein released by endothelial cells and vascular smooth muscle cells, CYP-A levels are increased in the presence of inflammation and oxidative stress[28] and were analyzed by ELISA assay (Figure 3b). CYP-A levels in the heart and thoracic aorta tissues in the Ang II group were significantly higher than Control group ($p < 0.05$). Cardiac and aortic CYP-A levels of Ang II+ALA and Ang II+AVE0991 groups were significantly decreased compared to the Ang II group ($p < 0.05$). The combination of ALA and AVE0991 resulted in a significant decrease in aortic CYP-A levels but not cardiac levels compared to the Ang II group ($p > 0.05$).

3.3.3 | LOX levels

The semiquantitative measurement results of the LOX enzyme, which provides the cross-linked stable state of the extracellular matrix proteins and plays an essential role in the intact vessel structure,[29,30] were given in Figure 3c. Our study showed that 80 ng/kg/min Ang II infusion did not cause a statistically significant change in LOX activities compared to the Control group ($p > 0.05$).

3.3.4 | MMP-2 levels

The levels of MMP-2, an important gelatinase in heart and aortic tissues, were determined by the ELISA method. Cardiac MMP-2 levels decreased statistically significantly in the Ang II group compared to the Control group ($p < 0.05$), and this decrease was found to be higher in Ang II+AVE0991 and Ang II+ALA+AVE0991 groups ($p < 0.05$). On the other hand, the aortic MMP-2

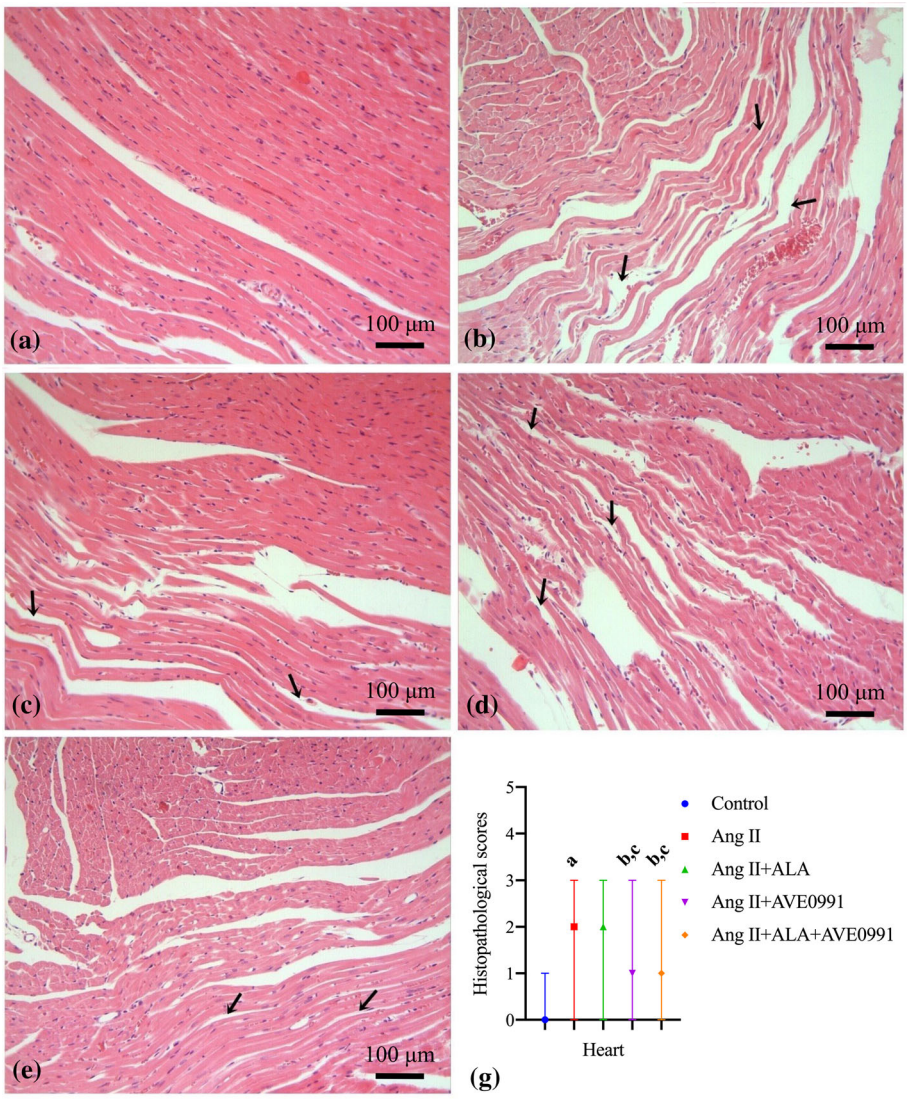


FIGURE 4 The histopathological appearance and scores of myocardium in the control (a), Ang II (b), Ang II+AVE0991 (c), Ang II+ALA (d), and Ang II+ALA+AVE0991 (e) groups. [a]$p < 0.05$ vs. Control group, [b]$p < 0.05$ vs. Ang II group, [c]$p < 0.05$ vs. Ang II+ALA group. Arrows point to interstitial edema, H-E; ×20. Ang II, angiotensin II; ALA, alamandine.

levels were similar among the groups ($p > 0.05$) (Figure 3d).

3.3.5 | MMP-9 levels

Cardiac and aortic MMP-9 levels were not statistically significant differences among groups ($p > 0.05$) (Figure 3e).

3.3.6 | NOX-4 levels

NOX-4 is one of the primary sources of oxidative stress in many tissues such as the brain and heart, especially in vascular tissues.[31] There was no statistically significant difference in terms of NOX-4 among the groups ($p > 0.05$) (Figure 3f).

3.3.7 | MCP-1 levels

The elevation of cardiac MCP-1 levels is accepted as one of the responsible mechanisms in many cardiological diseases, especially HT.[32] In our study, cardiac MCP-1 levels of the Ang II group were found to be significantly increased ($p < 0.05$). Cardiac MCP-1 levels in Ang II+ALA, Ang II+AVE0991, and Ang II+ALA+AVE0991 groups showed a statistically significant decrease compared to the Ang II group ($p < 0.05$). The reduction in Ang II+AVE0991 and Ang II+ALA+AVE0991 groups reached a statistically significant difference when compared with the Control and Ang II+ALA groups ($p < 0.05$) (Figure 3g).

3.4 | Histopathological findings

3.4.1 | Heart

Cardiac sections were evaluated for interstitial edema and cardiomyocyte degeneration in the myocardium. Except for mild changes in the Control group, the myocardium was normal histologically (Figure 4a). On the other hand, cardiomyocyte degeneration and

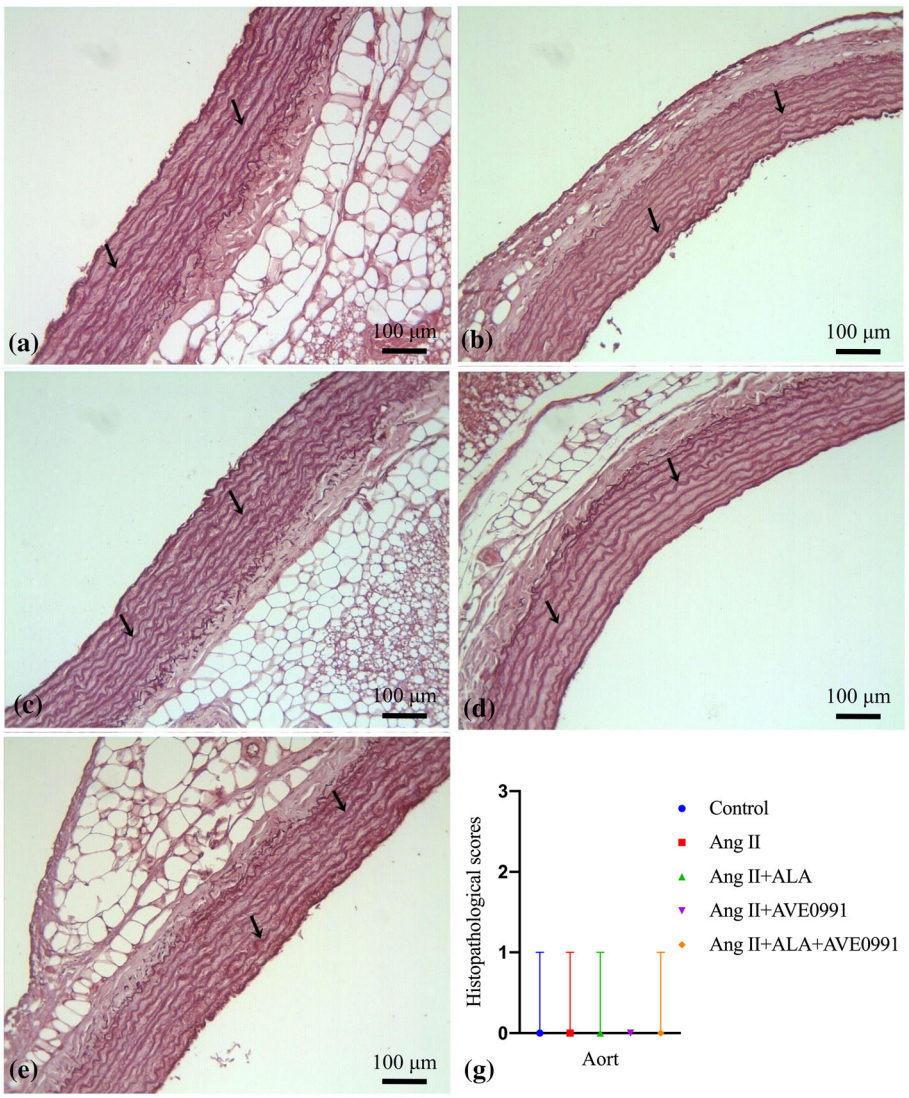


FIGURE 5 The histopathological appearance and scores of the thoracic aorta wall in the control (a), Ang II (b), Ang II+AVE0991 (c), Ang II+ALA (d), and Ang II+ALA+AVE0991 (e) groups. Arrows point to elastic lamellas, Orcein ×20. No statistical difference was observed among groups. Ang II, angiotensin II; ALA, alamandine.

interstitial edema were noted in the Ang II group. These changes observed in the Ang II group (Figure 4b) were found to be statistically higher than the Control group ($p < 0.0001$). Histopathological changes were significantly decreased in Ang II+AVE0991 and Ang II+AVE0991+ALA groups compared to the Ang II group ($p < 0.05$) (Figure 4c,e). Interstitial edema and cardiomyocyte degeneration were observed in the ALA group, similar to the Ang II group (Figure 4d). The histopathological changes observed in the Ang II+ALA group were statistically higher than Ang II+AVE0991 and Ang II+AVE0991+ALA groups ($p < 0.05$). Ang II+AVE0991 and Ang II+AVE0991+ALA groups were found to be similar in terms of histopathological changes. Histopathological evaluation results of the myocardium are given in Figure 4g.

3.4.2 | Aorta

The thoracic aortic wall was histologically normal in all groups, except for the non-significant loss of myofibrils in muscle cells (Figure 5a–e). IMT values were also similar in all groups, pointing out the absence of vascular hypertrophy (Figure 6a–e). The histopathological evaluation results of the aortic wall and IMT values were given in Figure 5g and Figure 6g, respectively.

4 | DISCUSSION

Oral administration of ALA and AVE0991 had been shown antihypertensive effects in several experimental studies.[11,33] The present study showed that subacute systemic administration of ALA and AVE0991 co-therapy also ameliorated HT and the pathophysiological mechanisms, including CYP-A-dependent oxidative stress and MCP-1-related inflammation (Figure 7).

In our study, Ang II (80 ng/kg/min) induced HT model was preferred to achieve the targeted high BP values. When published studies were examined, the Ang II-induced HT model is one of the most widely used drug-induced HT models.[7] However, its

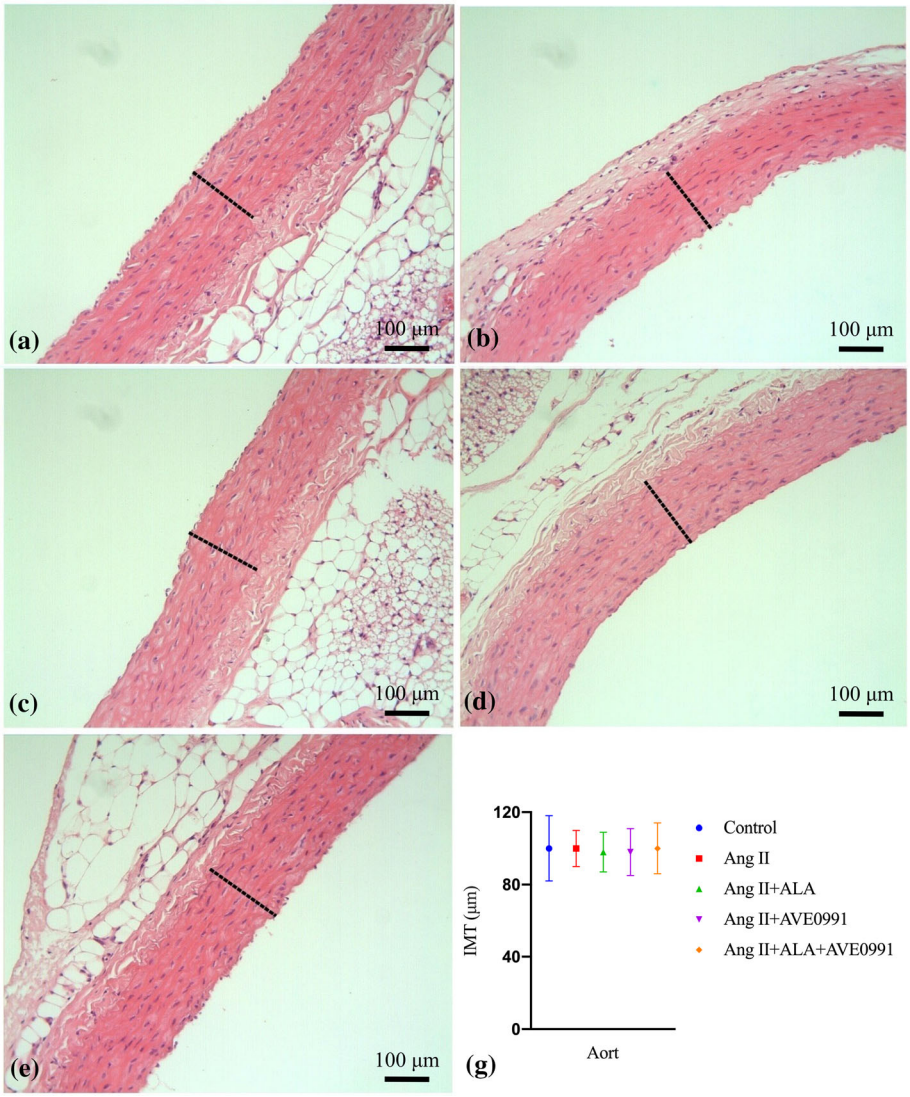


FIGURE 6 Intima-media thickness of the thoracic aorta wall in the control (a), Ang II (b), Ang II+AVE0991 (c), Ang II+ALA (d), and Ang II+ALA+AVE0991 (e) groups. The dashed lines point to IMT. H-E, ×20. No statistical difference was observed among groups. Ang II, angiotensin II; ALA, alamandine; IMT, intima-media thickness.

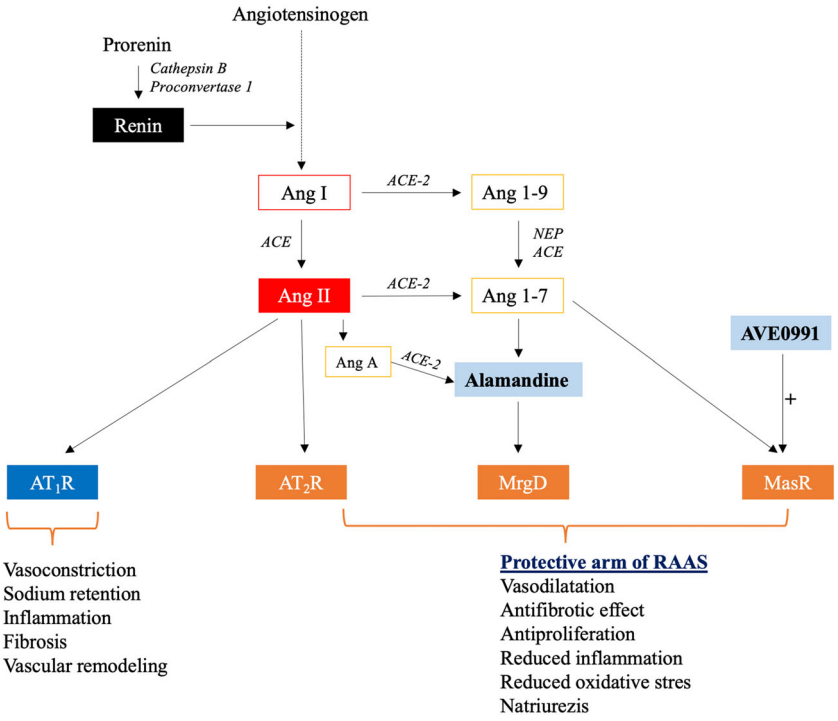


FIGURE 7 ACE2/Ang(1–7)/MAS axis and Ang 1–7/ALA pathway of RAAS.

cardiovascular effects differ with the dose and duration-dependent administration. For instance, Zhuo et al. reported that systolic BP was increased from 115 mmHg to 186 mmHg after Ang II administration for 14 days at an infusion rate of 80 ng/kg/min.[34] In another study, Ang II had been given at an infusion rate of 4.2 ng/kg/min for 3 weeks, and systolic BP increased to 145 mmHg from 115 mmHg.[9] In another recent study, systolic BP values in Sprague Dawley rats administered Ang II with an osmotic pump for 2 weeks at an infusion rate of 200 ng/kg/min was 201 mmHg in the Ang II group while 144 mmHg in the Control group.[35] In an experimental aneurysm study by Phillips et al., Ang II had been given at an infusion rate of 1000 ng/kg/min and the systolic BP value measured on the 14th day was 210 mmHg.[36]

One of the most important results of the study is that systolic BP in the Ang II+ALA+AVE0991 group decreased statistically significantly more than in the Ang II+ALA and Ang II+AVE0991 groups compared to the Control group indicates that the MrgD receptor provides an additional BP reduction beyond the Mas receptor. Besides the dosage of the drugs, this difference may be due to the activation of the AMPK/NO pathway by ALA, unlike AVE0991, following the study of Jesus et al.[37] In this study, ALA-related AMPK/NO pathway activation of the MrgD receptor occurred independently of the Mas receptor of AVE0991. The AMPK/NO pathway of ALA was preserved in myocytes with deleted Mas gene, and the Mas antagonist A-779 could not abolish this effect of ALA. When all the data in our study are examined together, it supports our hypothesis that ALA is associated with a new and crucial protective pathway through the MrgD receptor in the renin angiotensin aldosteron system (RAAS) and the process that started with the discovery of ALA by Lautner et al.[11]

In the present study, the elevation of systolic BP in Ang II administered rats occurred with a statistically insignificant increase in HR, which provides an independent effect from the chronotropy of the heart. This indicates that the mechanism causing the elevated BP is due to stroke volume and/or systemic vascular resistance.

In our study, when Phe contraction responses were examined, a possible explanation for the statistically significant increase in pD_2 value in the Ang II+AVE0991 group compared to the Ang II group might be that AVE0991 has partial agonistic effects in the presence of Ang II. To confirm this hypothesis, Ang II+AVE0991 application and a different experimental HT model such as SHR, DOCA+salt can be performed with similar conditions, and Phe responses should be compared.

Apart from the vascular endothelium, ACE-2 is also found in cardiomyocytes and cardiac fibroblasts in the heart.[38] ACE-2 gene and enzyme have essential roles in the cardiovascular system of humans and experimental animals.[39] Also, recent clinical studies showed the ACE-2 levels could change in many diseases such as hypertension, heart failure, obesity, and connective tissue disease.[40,41] In the study of Crackower et al. on ACE-2 knockout mice, the contractile functions of the heart were impaired due to the absence of the ACE-2

protein.[42] In the renovascular HT model study of Hekmat et al., 600 mcg/kg/day ALA infusion was administered for 2 weeks. It was reported that ACE-2 gene expression decreased to normotensive levels in the study. However, in the same research, ALA did not cause any change in the protein levels of ACE-2.[43] In the experimental liver cirrhosis study by Klein et al., the relationship between AVE0991 and ACE-2 was investigated and reported that AVE0991 reduced ACE-2 levels in the liver.[44] Our study is also important as it is the first study to reveal the relationship between AVE0991 and ACE-2 protein levels in the presence of HT. In addition, the dose and application method of ALA used in the relevant studies are different from our research. In our research, ACE-2 protein levels in heart tissues were similar in the Ang II-induced HT group compared to the Control group. In contrast, these values in aortic tissues showed a statistically significant decrease. However, there was a relative increase in the Ang II+ALA group compared to the Ang II group, not resulting in a significant increase as in the Ang II+-AVE0991 and Ang II+ALA+AVE0991 groups. It might be because of the mRNA level, which is an indicator of activation of a gene, and the protein level is not always proportional. It is a well-known fact that many factors such as transcription, translation, or protein degradation will affect protein concentrations.[45] Therefore, we believe that in our 28-day HT model, increased Ang II concentrations may be decreased by the degradation of ACE-2 protein by lysosomes. Considering that both AVE0991 and ALA+AVE0991 increase ACE-2 values, a substantial therapeutic effect can be obtained in the treatment of HT with the activation of the cardioprotective pathway of RAAS.

Oxidative stress plays an essential role in the pathogenesis of many cardiovascular diseases, including HT, due to increased ROS production. Ang II has been used in many studies to induce inflammation and ROS-mediated oxidative damage by stimulating cytokines such as IL-6, TNF-α and stimulating the migration of neutrophils and macrophages to the affected tissues in animal models.[46,47] NOXs are considered the primary enzyme system that regulates ROS production in vascular smooth muscle cells in response to Ang II.[48] However, Soe et al. showed in their study that CYP-A is a precursor and necessary in the NOX-dependent oxidative stress pathway[49] and showed that CYP-A might be an important therapeutic target not only in HT but also in diseases in which oxidative stress plays a role.[28,50] For example, NOX is composed of cytosolic (p47phox, p67phox, rac1) and membrane (NOX family) subunits, and the regulatory subunit p47phox must be located at the plasma membrane (especially caveola) and associate with other subunits of NOX for its full activation. Then, CYP-A was transferred to CYP-A knockout vascular smooth muscle cells by lentivirus, and ROS production increased significantly due to Ang II administration. Then, to determine the role of p47phox in CYP-A-mediated ROS production, hemagglutinin-p47phox was applied to CYP-A knockout vascular smooth muscle cells but did not increase ROS production. Furthermore, ROS production was markedly increased compared to CYP-A transduced cells in p47phox and CYP-A co-transferred smooth muscle cells, suggesting that CYP-A is a precursor and necessary for Ang II-induced ROS production in vascular smooth muscle cells. In addition, NOX inhibition has been shown to significantly reduce Ang II-induced ROS production, proving that NOX is the main enzyme involved in the production regulated by p47phox and CYP-A.[49] In our study, the significant increase in CYP-A levels in the heart and aortic tissues in the Ang II group compared to the Control group showed that the oxidative stress pathway was stimulated in the formation of HT. Still, the NOX-4 levels in both cardiac and vascular tissues were similar among groups in our study. In addition to NOX-4, other NOX subtypes, especially NOX-2, have been studied many times and considered their role in the pathogenesis of HT.[51–53] In future studies, examining the levels of other subtypes will elucidate the oxidative stress mechanism that occurs in Ang II administration at an infusion rate of 80 ng/kg/min. This is the first study showing that ALA and AVE0991 decreased CYP-A levels in an Ang II-induced HT study.

ROS activation resulting from CYP-A elevation due to Ang II administration may lead to cardiac fibrosis by increasing MMP-2 and MMP-9 expressions. However, in our study, unlike the literature, no significant increase was found in the levels of MMP-2 and MMP-9 in the heart and aortic tissues in the Ang II group. A possible explanation for this situation can be the Ang II dose used in our study -although it was chosen based on the literature created by the relevant model-[34] compared to many studies.[53–56] When the aortic tissues of all groups in our study were examined, the similarity of elastin structures and IMT values as an indicator of vascular hypertrophy among groups supports this situation.

LOX plays an important role in ECM composition by mediating collagen cross-linking and elastin fibers through oxidative deamination of lysine and hydroxylysine residues.[57] Therefore, its function remains unclear in cardiovascular diseases, including HT and heart failure.[58–60] LOX produces H_2O_2, which causes an increase in oxidative stress in vascular and cardiac tissues.[29,61] In our study, LOX determination was analyzed semiquantitatively in heart and aortic tissues compared to the control containing the basal LOX amount in the kit. As a result of this measurement, a 3-fold increase was observed in the heart tissues of the Control group compared to the basal values. However, no statistically significant difference was found among the groups regarding LOX data in the heart and vascular tissues obtained from the groups in our study. In this

context, contrary to the data in our research, LOX levels in SHRs significantly increased compared to normotensive Wistar rats (96). In another study of high-dose Ang II-induced HT, LOX contributed to the development of HT in mice by decreasing vascular compliance in vivo. In the same study, vascular remodeling that develops in high-dose Ang II-induced HT is prevented when LOX inhibition is performed.[62] When the findings from our study were assessed as a whole, we think that the unaffected LOX-dependent oxidative stress pathway and ECM composition may be related to the Ang II dose and experimental model we used in our study. However, further scientific studies are needed to understand this relationship better.

Ang II infusion is known to induce monocyte chemotaxis by increasing the expression of both adhesion molecules and chemokines such as MCP-1 from monocytes in heart tissue and aortic tunica adventitia and increasing ROS production.[32] In the cardiovascular remodeling study of Koyanagi et al., in which was induced by HT induced by giving L-NAME to Wistar-Kyoto rats, the application of monoclonal neutralizing antibody specific to MCP-1 effectively prevented the accumulation of macrophages in the heart tissues of rats.[63] In a study of stroke-prone SHR and rat cardiac hypertrophy induced by a high-salt, high-fat diet, AT1 receptor blocker eprosartan significantly reduced cardiac hypertrophy by suppressing the expression of MCP-1 at the mRNA and protein level, reducing monocyte and macrophage infiltration.[64] In our study, a statistically significant increase was found in cardiac MCP-1 levels in the Ang II group, which is consistent with the literature. Although elevated MCP-1 and macrophage levels are associated with cardiac damage, there is a decrease in MCP-1 in hypertensive rats, with increased CD4+T lymphocyte infiltration in macrophage-deficient heart tissue and diffuse cardiomyocyte loss, resulting in hypertensive cardiomyopathy and end-stage heart disease.[65] This might be because HT also plays a role in regeneration processes such as macrophage activation and cardiac hemostasis, so its levels may change in acute and chronic disease processes.[66] In the literature, many studies showed that AVE0991 reduces MCP-1 levels in different experimental models, consistent with the data in our research. For example, AVE0991 has been shown to reduce increased inflammation and macrophage migration in the bronchial epithelium by reducing MCP-1 via the c-Jun-N-terminal kinase pathway in an ovalbumin-induced asthma model.[67] In another study, after 1000 ng/kg/min Ang II infusion given to mice with ApoE gene deleted, a significant decrease in macrophage migration in the abdominal aorta was demonstrated in a reduction of MCP-1 levels and histopathologically.[20] Jawien et al. found that AVE0991 strongly suppressed inflammation in atherosclerotic C57BL/6 mice by suppressing MCP-1, IL-6, and IL-12.[68]

It has been reported that ALA alleviated inflammation and macrophage migration in the ascending aorta by reducing TNF-α, IL-1β, and MCP-1 in the transverse aortic constriction model.[69] In another study, ALA suppressed the expression of MCP-1 at gene and protein level in a dose-dependent manner in an in vivo pleura model and cell culture research created by intrapleural injection of *Escherichia coli* into Balb/c mice.[70] MCP-1 levels were significantly decreased in Ang II+ALA, Ang II+AVE0991, and Ang II+ALA+AVE0991 groups compared to the Ang II group in our study is also in line with hemodynamic and biochemical data in our research. Our study is important as it is the first study showing that ALA reduces the levels of cardiac MCP-1 protein in the presence of HT.

The histopathological effects of Ang II on cardiac tissue vary depending on the dose and the time. For example, left ventricular hypertrophy,[71] cardiac fibrosis,[72] interstitial edema, cardiomyocyte degeneration, inflammation, and hemorrhage[73,74] have been reported in several studies. Considering the Ang II dose and duration of administration in our study, a statistically significant increase was observed in cardiomyocyte degeneration and interstitial edema in the Ang II groups, consistent with the literature. However, contrary to our study, a recent experimental renovascular HT study[43] reported that ALA given at an infusion rate of 600 mcg/kg/day alleviated cardiomyocyte degeneration in the cardiac tissue. A possible reason for this may be the dose-dependent varying response of ALA and its administration by continuous infusion. AVE0991 has been shown in many studies in the literature to significantly reduce cardiomyocyte degeneration in Ang II-induced cardiac hypertrophy and renovascular HT model studies.[75,76]

Similarly, the effects of Ang II on aortic tissue vary according to the dose administered. For example, when Ang II was administered at an infusion rate of 1000 ng/kg/min for 28 days, it was reported that fibrosis, elastin loss, collagen deposition, and vascular hypertrophy developed in the aortic tissue.[77] Gomolak and Didion[78] investigated the histopathological effects of Ang II administration on the thoracic aorta at an infusion rate of 50, 100, 200, 400, or 1000 ng/kg/min for 14 and 28 days that resulted in vascular hypertrophy. Still, the vascular structure is preserved in 50 and 100 ng/kg/min of Ang II administration applications. The fact that IMT, which was measured as an indicator of vascular hypertrophy, was not affected by Ang II administration in our study is consistent with the literature.

5 | CONCLUSION

In this study, the therapeutic effects of the MrgD receptor agonist ALA and Mas receptor agonist

AVE0991 were investigated in the Ang II-induced HT model. Our experimental data showed that the ALA and AVE0991 co-therapy effectively reduced systolic BP, CYP-A-dependent oxidative stress, cardiac MCP-1 levels, cardiomyocyte degeneration, interstitial edema, and increased aortic ACE-2 levels and vascular-relaxation responses. Despite all this, well-designed further studies are needed to support our results.

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CONFLICTS OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ETHICS STATEMENT

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of İnönü University (approval detail: 2020/5–3 and HAYBIS 9739).

AUTHOR CONTRIBUTIONS

Lokman Hekim Tanriverdi: Conceptualization, Software, Visualization, Data curation, Writing- Original draft preparation, Writing- Reviewing and Editing. **Onural Özhan**: Investigation, Data curation, Validation, Investigation, Methodology. **Ahmet Ulu**: Investigation, Methodology, Visualization. **Azibe Yıldız**: Investigation, Methodology, Visualization. **Burhan Ateş**: Investigation, Methodology, Visualization. **Nigar Vardı:** Investigation, Methodology, Visualization, Writing- Reviewing and Editing. **Hacı Ahmet Acet:** Supervision, Writing- Reviewing and Editing. **Hakan Parlakpinar:** Supervision, Writing- Reviewing and Editing.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article (and its supplementary information files). This study is a part of the corresponding author's PhD thesis finished in December 2021 and deposited in his university's repository (abakus.inonu.edu.tr). Language of the main text is Turkish. Only the abstract is available in English.

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SUPPORTING INFORMATION

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