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ORIGINAL PAPER

The effect of resveratrol on angiotensin II levels and the rate of transcription of its receptors in the rat cardiac hypertrophy model

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Abstract This study investigated the effect of resveratrol on serum and cardiac levels of angiotensin II and transcription of its main receptors following pressure overload induced-hypertrophy. Rats were divided into untreated (Hyp) and resveratrol treated hypertrophied groups (H + R). Intact animals served as the control (Ctl). Cardiac hypertrophy was induced by abdominal aortic banding. Blood pressure (BP) was recorded via left carotid artery cannula. Fibrosis was confirmed by Masson trichrome staining. Angiotensin II level was measured using an ELIZA test. Gene expression was assessed by a real time PCR (RT-PCR) technique. We observed that in the H + R group BP and heart weight/body weight were decreased significantly ($p < 0.001$, $p < 0.05$, respectively vs Hyp). The cardiac levels of angiotensin II and AT1a mRNA were increased in the Hyp group ($p < 0.01$ vs Ctl). In the H + R group the AT1a mRNA level was decreased significantly ($p < 0.05$ vs Hyp). It could be concluded that resveratrol protects the heart against hypertrophy progression in part by affecting cardiac AT1a transcription.

Keywords Myocardial hypertrophy · Angiotensin II · Resveratrol · AT1a · AT1b · AT2

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Introduction

Hypertension is one of the major risk factors of heart attacks, cardiac ischemia, and progression of cardiac failure. Hypertension-induced heart failure usually develops following the progression of left ventricular hypertrophy (LVH) [1]. The heart adapts itself through induction of hypertrophy in response to a chronic stress such as pressure or volume overload [2]. Adaptation of cardiac hypertrophy by the induction of a genetic program leads to increased protein synthesis and increased size of cardiomyocytes [3]. LVH is accompanied by loss of cardiomyocytes, reorganization of extracellular matrix (ECM), and increased ECM elements [4]. During progression of hypertrophy, the expression profile of numerous genes changes, which ultimately results in impairments in the performance of the heart [5].

According to the research conducted so far, the activity of the renin–angiotensin system (RAS) plays a key role in the pathogenesis of myocardial diseases such as LVH and heart failure. Angiotensin II (Ang II) activates the hypertrophy and ultimately apoptotic paths in cardiomyocytes via vasoconstriction as well as with direct cellular effects [6].

Resveratrol is a phytochemical polyphenol which has attracted the attention of cardiovascular researchers in recent years. Extensive studies support the antioxidant and cardioprotective effects of resveratrol, though its exact mechanisms are not clearly understood yet. Resveratrol exerts its anti-hypertrophic effects via several mechanisms including decrease of blood pressure, activation of the anti-hypertrophic signaling path of AMPK, and inhibition of the prohypertrophic path of Akt [7, 8]. Studies indicated that resveratrol inhibits Ang II-induced cell proliferation, endothelin-1 expression, and ERK phosphorylation in

vascular smooth muscle cells [9]. There is some evidence that resveratrol shows potential cardioprotective effects via Sirtuin-1 (SIRT1) activation [10]. SIRT1 deacetylases histones and also non-histonic proteins such as transcription factors of P53, FOXO, PGC-1α, HIF-1α, and the NF-kappa B [11].

Despite the well-appreciated importance of the RAS in the pathogenesis of cardiovascular diseases and also the role of resveratrol in increasing the cardiac resistance, noticed recently, the mechanisms pertaining to the role of resveratrol and its effect on the RAS are still unclear. Hence, this study aimed at determining the effect of resveratrol on the RAS; specifically on cardiac and serum level of Ang II and the transcription level of the main receptors of Ang II in hypertrophied hearts.

Materials and methods

Experimental design

Male Wistar rats weighing 160–220 g were housed in a controlled room under a diurnal cycle of 12 h light/12 h darkness at an approximate temperature of 25 °C.

The rats were divided into the following groups ($n = 7$):

1. Intact animals which served as the control (Ctl).
2. Rats subjected to abdominal aortic banding to induce hypertrophy (Hyp).
3. A group of rats pretreated with resveratol (Sigma–Aldrich Chemie GmbH, 1 mg/kg/day, intraperitoneally, injection volume was 0.5 ml) for 14 days before aortic banding. Treatment was continued till 21 days after hypertrophy induction (H + R).
4. A group of animals received resveratrol without hypertrophy intervention (R).
5. Animals received DMSO as the resveratrol solvent (H + DMSO).

Animal model of cardiac hypertrophy

To induce hypertension and consequently LVH, animals were subjected to abdominal aortic banding. Briefly, rats were anesthetized with intraperitoneal injection of ketamine (70–90 mg/kg) and Xylazine (10 mg/kg). An incision was made in the left flank. After exposing the suprarenal abdominal aorta, a 21-guage needle was placed beside the artery and a suture was tied around the artery. After ensuring partial banding of artery and not complete occlusion, the needle was removed. Abdominal wall muscles and skin were sutured by absorbable and non-absorbable sutures, respectively. In the hypertrophic groups, tetracycline was injected to animals intramuscularly for 6 days. Three weeks after aortic banding animals were anesthetized again and arterial blood pressure was measured directly by cannulation of the carotid artery connected to a power lab system. At the end of the intervention, in addition to weighing of rats, the cardiac mass was also measured after being washed with cold PBS solution. The serum and heart tissue were collected and kept at −70 °C for further studies [12, 13].

Histology

To investigate cardiac fibrosis in the experimental groups, three samples from each group were collected. The fresh cardiac tissue was immediately fixed in 10 % formaldehyde. After tissue processing and paraffin embedding, cross-sectional slices were obtained. In the last stage, they were stained with Masson trichrome.

Analysis of mRNA expression by real-time PCR

RNA was extracted from the left ventricular tissue using RNx plus (Sinagen, Iran). Quantity and quality of the RNA was evaluated by reading absorption at 260 nm wavelength using the photo absorption nanodrop (Epoch, Box998). A reverse transcription reaction was performed using the enzyme RevertAid^{TM}M-MuLV Reverse Transcriptas (Fermentas, USA). In the presence of specific primers, the cDNA of experimental groups underwent the real-time PCR reaction (RT-PCR) using MasterMix containing SYBR green (Takara, Japan). The sequence of the primers used is given in Table 1 [14, 15]. The β-actin gene was used as the reference. Comparison of gene expression was done using the ΔΔCT method.

Angiotensin II assay by ELIZA test

To measure the tissue level of angiotensin II, the heart tissue was homogenized by the addition of 1 ml of lysine solution (containing PMSF: 10 μ/cc/dl, HEPES: 520 mg/dl, EGTA: 76 mg/dl, EDTA: 37/2 mg/dl, Tris–Hcl: 788 mg/dl, SDS: 100 mg/dl, sodium deoxycholate: 0/25 g/dl, Triton 100x: 1 cc/dl, NaCl: 874 mg/dl, distilled water: 100 cc) using a homogenizer. The obtained homogenous tissue was centrifuged for 45 min at 4 °C at 13,000 rpm. Then, the supernatant along with serum were used for measurement of Ang II using an ELIZA kit (Phoenix, France) according to the kit instructions [16].

Statistical analysis

Blood pressure and HW/BW were analyzed by Kruskal–Wallis test with Dunn's post-test for multiple comparisons. Ang II and receptor transcription levels were assessed by

Table 1 Primer sequences for RT-PCR of the candidate genes

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
AT1a	CCATTCACCCTGCCTCAG	ACGGCTTTGCTTGGTTACTC
AT1b	ATGTCTCCAGTCCCCTCTCA	TGACCTCCCATCTCCTTTTG
AT2	CAATCTGGCTGTGGCTGACTT	TGCACATCACAGGTCCAAAGA
B-actin	AACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG

one-way ANOVA followed by Tukey post test. A value of $p < 0.05$ was considered as statistically significant. Data are presented as mean ± SEM. Statistical analysis was performed using Prism software (version 5).

Results

Effect of resveratrol on blood pressure

According to Table 2, in the Hyp group the systolic blood pressure was 154.2 ± 9.2 mmHg which shows significant increase compared to the Ctl group ($p < 0.001$). In the H + R group systolic blood pressure was 130 ± 8 mmHg, which was significantly different from the Hyp group ($p < 0.01$).

Effect of resveratrol on heart weight/body weight

As shown in Fig. 1, the ratio of HW/BW in the Hyp group was 4.072 ± 19 mg/kg, which shows a significant increase compared to the Ctl group which was 3.384 mg/kg ($p < 0.01$). In the H + R group in which the animals were pretreated with resveratrol, the mean HW/BW was 3.413 ± 19 mg/kg which was significantly different from the Hyp group ($p < 0.05$). In group R, blood pressure did not change significantly.

Effect of resveratrol on serum and tissue levels of angiotensin II

One of the main goals of this study was determination of Ang II level in serum and cardiac tissue of experimental groups. As presented in Fig. 2, there was no significant change in the serum level of Ang II among the experimental groups. Regarding the Ang II level in left ventricular tissue, in the Hyp group, the Ang II level was increased to 260.8 ± 22.1 ng/ml which was significantly different compared with the Ctl group ($p < 0.01$). In resveratrol treated animals the Ang II level did not change significantly.

Effect of resveratrol on cardiac AT1a, AT1b, and AT2 mRNA levels

As displayed in Fig. 3, in the Hyp group the AT1a mRNA level was increased by 81.1 ± 13 % indicating a significant difference compared to the Ctl group ($p < 0.01$), while in the H + R group, the AT1a mRNA reached 22 ± 0.9 % which shows a significant difference compared with the Hyp group ($p < 0.05$). There was no significant difference among the experimental groups regarding AT1b and AT2 mRNA levels.

Discussion

The first part of our findings revealed that following pressure overload-induced hypertrophy, the cardiac levels of Ang II and AT1a receptor increased significantly, while the serum level of Ang II as well as cardiac transcription levels of AT1b and AT2 receptors of Ang II did not change significantly.

There is some evidence supporting the role of the renin-angiotensin system in the pathogenesis of cardiovascular disease such as ischemia reperfusion, cardiac hypertrophy, and heart failure. It seems that the direct action of Ang II on cardiomyocytes may play a significant role in the hypertrophy process [6]. Our data is in agreement with previous studies. Schunkert et al. investigated the effect of left ventricle hypertrophy on ACE activity and mRNA expression in rats and showed an increase of cardiac ACE activity and Ang II in hypertrophied hearts [17]. It should be pointed out that in their study hypertrophy was

Table 2 Changes of blood pressure in experimental groups

Groups	Ctl	Hyp	H + R	R	H + DMSO
Systolic pressure (mmHg)	112.3 ± 8.8	154.2 ± 9.2***	130 ± 8##	110.7 ± 9.4	148.3 ± 8.7
Diastolic pressure (mmHg)	75.8 ± 5.5	104.3 ± 6.6***	81.6 ± 4.2#	71 ± 3.5	110.6 ± 9.1

Systolic and diastolic blood pressure at the end of experiments in the control (Ctl), hypertrophy (Hyp), hypertrophy + resveratrol (H + R), resveratrol (R) and hypertrophy + DMSO (H + DMSO) groups. Data are presented as mean ± SEM. *** $p < 0.001$ vs Hyp; # $p < 0.05$ and ## $p < 0.01$ vs Hyp

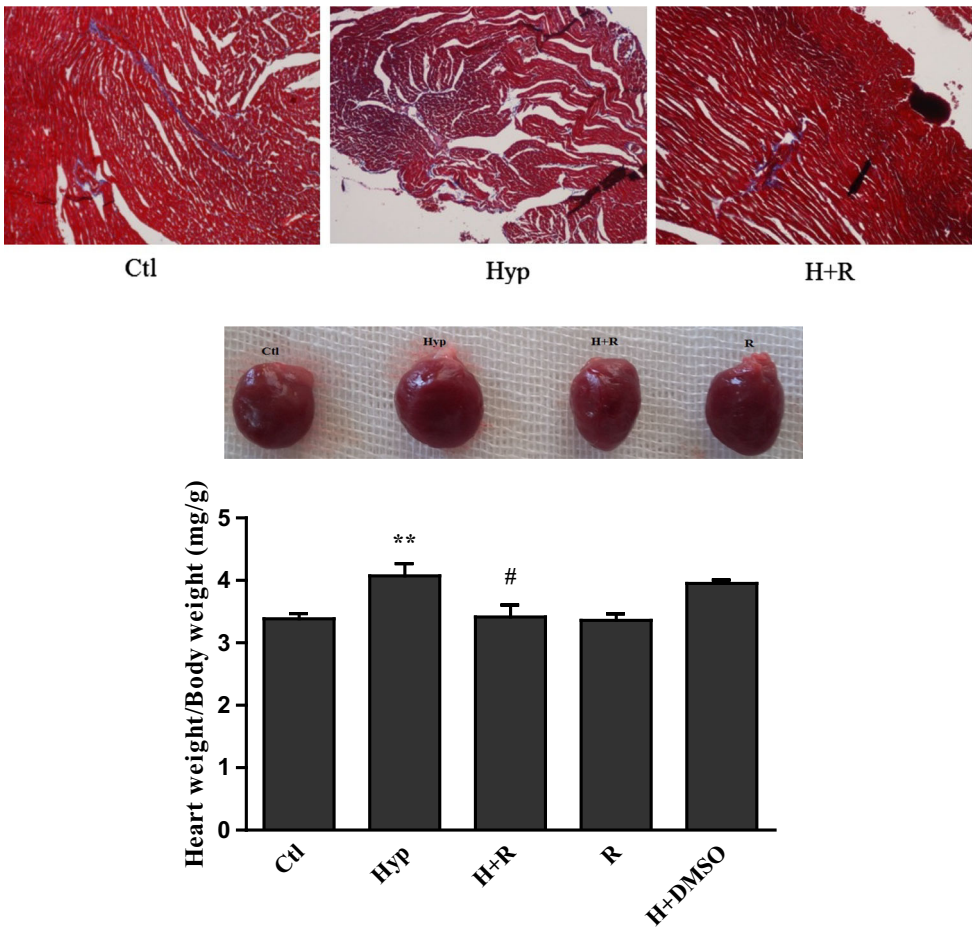

Fig. 1 Heart weight/body weight in experimental groups. As a pivotal marker of cardiac hypertrophy, heart weight/body weight ratio was assessed in the control (Ctl), hypertrophy (Hyp), hypertrophy + resveratrol (H + R) and resveratrol (R) groups. Sections of the left ventricular tissue stained with Masson trichrome shown in *upper panel*. Data are presented as mean ± SEM. $^{**}p < 0.01$ vs Ctl and $^{\#}p < 0.05$ vs Hyp

examined 8 weeks after thoracic aortic banding which is a longer time than the time for hypertrophy induction in our study. Moreover, Liu et al. reported that in rat model of nephrectomy-induced cardiac hypertrophy, serum level of Ang II was increased [18].

It has been shown that the high rate of angiotensinogen expression in cardiomyocytes led to increased cardiac Ang II and ventricular hypertrophy [19]. There is also some evidence indicating that angiotensin II reinforces the hypertrophy of cardiomyocytes and the cellular proliferation of vascular smooth muscles by itself [19, 20].

Some studies reveal that Ang II increases the accumulation of collagen and promotes cardiac fibrosis in hypertrophied hearts, but its function is still unclear [20–23]. AT1a, AT1b, and AT2 are the main receptors of Ang II which are implicated in a variety of effects of Ang II in the cardiovascular system [24]. AT1 is abundantly present in cardiac tissue, blood vessels, kidneys, adrenal gland, liver, brain, and lungs of adults. Ang II manifests its short-term effects via binding to AT1 and activating protein kinase C and calcium calmodulin kinase and subsequently by phosphorylation of proteins. But, it exerts its long-term effects through activating the MAPK path and RSK which ultimately results in left ventricle hypertrophy subsequent to hypertension [25, 26]. Nonetheless, Ang II exerts its beneficial effects such as vascular relaxation, increased arterial remodeling, and increased resistance to reperfusion ischemia through binding to AT2, the expression of which is very high during the fetal stage [22, 27].

Injection of Ang II increases the expression of pro-hypertensive RAS components (ACE and AT1R), while it decreases the anti-hypertensive RAS components (ACE2 and AT2 receptors) in the paraventricular nucleus [28].

In our study the transcription level of AT1a receptor was increased significantly in the left ventricle, suggesting that overexpression of this key receptor of Ang II in response to myocardial hypertrophy may be a principal mechanism for overactivity of Ang II. Regarding the important role of

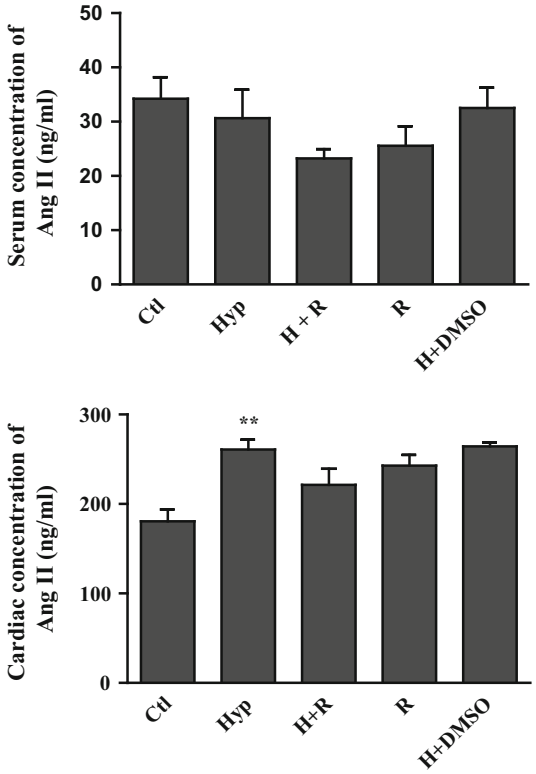

Fig. 2 Angiotensin II levels in serum and cardiac tissue. Using the ELIZA method, serum and cardiac levels of angiotensin II were measured in the control (Ctl), hypertrophy (Hyp), hypertrophy + resveratrol (H + R) and resveratrol (R) treated groups. Data are presented as mean ± SEM. $^{**}p < 0.01$ vs Ctl

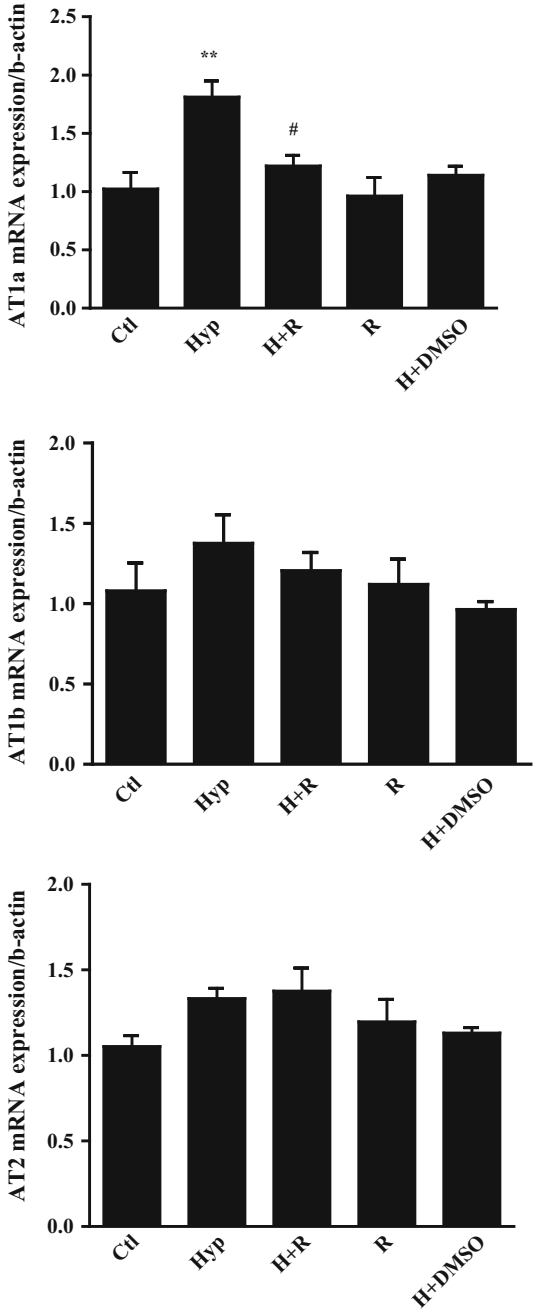

Fig. 3 Cardiac transcription levels of target genes. AT1a, AT1b, AT2 receptor mRNA levels were evaluated in the control (Ctl), hypertrophy (Hyp), hypertrophy + resveratrol (H + R), resveratrol (R) and hypertrophy + DMSO (H + DMSO) groups. Data are presented as mean ± SEM. $^{**}p < 0.01$ vs Ctl, $^{\#}p < 0.05$ vs Hyp

AT1 receptor in the cardiovascular system, there are many studies which have shown the beneficial effects of the blockers of this receptor in cardiac diseases.

Long-term treatment of rats with high stroke-prone blood pressure with AT1 blocker candesartan cilexetil resulted in decreased mass of the left ventricle. It is interesting to note that in higher doses the left ventricle mass was actually smaller than normal pressure rats, reflecting the trophic response of angiotensin II with mediation of AT1 receptors. Reduced cardiac hypertrophy in transgenic hypertensive rats treated with telmisartan and spontaneously hypertensive rats treated with losartan have been reported. Some primary observations of patients with mild to moderate hypertension indicate that the useful effects of antagonists of AT1 receptors improve left ventricle hypertrophy in humans [22].

The result of our study regarding the increase of blood pressure, heart weight to bodyweight ratio and collagen deposition is in agreement with previous studies, which showed an increase of these hypertrophy markers in response to abdominal aortic banding.

The data of the second part of our study demonstrated that resveratrol prevents aortic banding-induced hypertension and normalizes HW/BW and collagen deposition in hypertrophied hearts.

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenol phytoalexin found in plant species like grapes, peanuts, and mulberries. Many studies in the last decade have demonstrated that resveratrol exerts a wide spectrum of protective effects on the cardiovascular system through

its anti-inflammatory, vasodilatory and anti-thrombotic properties [29, 30].

The anti-hypertrophic effects of resveratrol have been reported in previous studies. Wojciechowski et al. reported that treatment with resveratrol interrupts the progression and returning of structural disturbances and cardiac function in pressure overload and volume overload rats [31]. Another pretreatment of rats with resveratrol for 14 days protected the hearts against harmful cardiac hypertrophy [32]. In cultured cardiomyocytes, treatment with resveratrol limits the induced hypertrophic response with phenylephrine [8].

It should be pointed out that in our study resveratrol did not decrease blood pressure at the baseline, suggesting that we used a non-hypotensive dose of resveratrol, and that resveratrol protects the heart against hypertrophy without changing the baseline hemodynamic parameters such as blood pressure. The anti-hypertensive effects of resveratrol have been demonstrated in several animal models. In hypertrophied rat induced by nephrectomy, oral treatment with resveratrol (50 mg/kg/day) led to a significant decrease in systolic pressure and decreased myocardial hypertrophy. These cardioprotective effects are related to a reduction in serum endothelin-1 and an increase in serum levels of NO without significant change in Ang II level [18].

Recently, our team showed that resveratrol in combination with vitamin D protects the heart against myocardial ischemia reperfusion injury characterized by decrease of infarct size and reperfusion-induced arrhythmia [33]. Consequently, regarding the numerous reports, it seems that resveratrol plays an important role in improving the hypertrophy induced by hypertension through several mechanisms.

Moreover, in the last part of our study, we dealt with the investigation of the therapeutic effect of resveratrol on serum and tissue level of Ang II and its main receptors in hypertrophied hearts. Our data show that pretreatment with resveratrol decreased the AT1a transcription level in the left ventricle of hypertrophied rats, suggesting that resveratrol protects the heart against hypertrophy in part by decreasing AT1a mRNA levels. The effect of resveratrol on the RAS components in different cardiovascular diseases was reported previously. For example, Miyazaki et al. showed that the high expression of SIRT1 is accompanied by a decrease of AT1 mRNA and Ang II levels. These findings concluded that resveratrol has suppressed the AT1R expression through activating SIRT1 [34].

Due to our research restrictions, the components of the RAS, and also ACE activity, have not been measured. In future studies it could help to provide more comprehensive and perfect information by exploring all other components of the system in both cardiac and kidney tissues and blood circulation.

Conclusion

Based on the findings of this study, it could be concluded that following LVH, which was characterized by increase of blood pressure, HW/BW and collagen deposition, cardiac levels of angiotensin II and AT1a receptor increased. Resveratrol at a non-hypotensive dose exerts anti-hypertrophic effects in part by normalizing AT1a mRNA levels in the left ventricle of hypertrophied rats.

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Compliance with ethical standards

Ethical approval All animal procedures performed in this study were approved by the Animal Ethics Care and Use Committee of Shahid Sadoughi University of Medical Sciences.

Conflict of interest The authors declare they have no conflict of interest.

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