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Angiotensin II and AT_1 receptor in hypertrophied ventricles and aortas of sinoaortic-denervated rats[1]

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ABSTRACT

AIM: Angiotensin II and AT_1 receptor are the major effector components of renin-angiotensin system (RAS), and also the main growth-stimulating factors in cardiovascular system. The present study was to observe these two factors in the hypertrophied ventricles and aortas of sinoaortic-denervated rats. **METHODS:** Rats were examined at 2, 10, and 16 weeks after sinoaortic denervation (SAD). The hypertrophy was evaluated by the ratio of organ weight to body weight. Angiotensin II concentration and AT_1 receptor mRNA expression were measured by radioimmunoassay and RT-PCR respectively, using a positive control of candesartan treatment. **RESULTS:** Aortic hypertrophy existed in 2-, 10-, and 16-week SAD rats, left ventricular hypertrophy in 10- and 16-week SAD rats, and right ventricular hypertrophy in 16-week SAD rats. In all three kinds of examined SAD rats, plasma angiotensin II levels remained unchanged, indicating circulating RAS is at normal level in the chronic phase of SAD. However, cardiovascular tissue RAS was activated, as evidenced by increase of aortic angiotensin II concentrations at 10 and 16 weeks after SAD, and up-regulation of aortic and left ventricular AT_1 receptor mRNA expressions at 16 weeks after SAD. **CONCLUSION:** The activated tissue RAS is secondary to the hypertrophy, and probably involved in the maintenance of cardiovascular hypertrophy following SAD.

INTRODUCTION

Arterial baroreflex (ABR) is a major regulatory mechanism in cardiovascular system. To study ABR function, one method is interruption of ABR by sinoaortic denervation (SAD)[1,2]. Using this method, we and others have found that chronic SAD can produce cardiovascular hypertrophy[1-8], directly demonstrating the pathological significance of ABR dysfunction. It has also been revealed that high blood pressure variability (BPV) following SAD plays an important role in SAD-induced aortic and left ventricular hypertrophy[4,8]. However, in addition to hemodynamics, little is known about other mechanisms involved in the cardiovascular hypertrophy of SAD rats.

Accumulating evidence suggests that neurohumoral factors (especially growth-regulatory factors) are involved in the cardiovascular hypertrophy of certain diseases. It has been shown that SAD causes neurohumoral changes in rats. For instance, renal sympathetic activity[9,10], plasma renin activity[11], plasma vasopressin[12] and adrenomedullary catecholamine synthesis[13] are increased in the acute phase of SAD, but they return to

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normal levels in the chronic phase. We consider that these transient changes may not be very important in the SAD-induced cardiovascular hypertrophy (because they are not parallel in time), and that unknown persistent changes of growth-regulatory factors may be involved in the SAD-induced cardiovascular damage. Among growth-regulatory factors/systems, renin-angiotensin system (RAS) is the most important in cardiovascular system, and tissue RAS activation is involved in cardiovascular remodeling in hypertension and heart failure[14-16]. Although it was reported that rat plasma renin activity was increased on d 1 after SAD, and it was normal on d 10 after SAD[11], little is known about tissue RAS in SAD rats, which may be associated with SAD-induced cardiovascular damage.

Therefore, in the present study, tissue RAS was observed to explore the potential role of possible activated RAS in cardiovascular damage produced by chronic SAD. We focused on the aortic and ventricular hypertrophy as our measures of cardiovascular damage, because they are the typical pathological changes in SAD rats, and they have been the most frequently used measures of hypertensive target organ damage in human and animal studies. Rats with different times after SAD were used to know the time course of the changes.

MATERIALS AND METHODS

SAD operation and experimental groups Male Sprague-Dawley rats were purchased from the Sino-British SIPPR/BK Lab Animal Ltd (Certificate No 02-25-3). At the age of 8-10 weeks, SAD was performed according to the procedure described by Krieger[17] with minor modification. Briefly, rats were anaesthetized with a mixture of ketamine (50 mg/kg, ip) and diazepam (5 mg/kg, ip) and were then medicated with atropine sulfate (0.5 mg/kg, ip) and procaine benzylpenicillin (60000 U, im). After a midline neck incision and bilateral isolation of the neck muscles, aortic baroreceptor denervation was carried out bilaterally by cutting the superior laryngeal nerves near the vagi, removing the superior cervical ganglia including a small section of the sympathetic trunk, and sectioning aortic depressor nerves. The carotid sinus baroreceptors were denervated bilaterally by stripping the carotid bifurcation and its branches followed by the application of 10 % phenol (in 95 % thanol) to the external, internal, and common carotid arteries and the occipital artery. In control rats, sham operation (Sham) was performed with the midline neck incision and bilateral isolation of the neck muscles. Phenol was also applied in Sham rats to each common carotid artery to control for any potential effects of phenol in the SAD group[7]. After operation, rats were housed with controlled temperature (23-25 °C) and lighting (8:00-20:00 light, 20:00-8:00 dark) and with free access to standard chow and tap water, and brought up for 2, 10, and 16 weeks to prepare 2-, 10-, and 16-week SAD rats and time-matched Sham rats. An additional group of SAD rats was fed with rat chow containing candesartan cilexetil (donated by Dr Peter MORSING at AstraZeneca, Molndal, Sweden), an AT_1 receptor antagonist, for 16 weeks. The average dose of candesartan cilexetil was about 6 $mg \cdot kg^{-1} \cdot d^{-1}$. The dose is sufficient to block AT_1 receptor, according to the previous study[18]. This treatment group was as a positive control for measurement of angiotensin II and AT_1 receptor mRNA levels, since it has been demonstrated that when the AT_1 receptors are sufficiently and chronically blocked by candesartan, the plasma angiotensin II levels and the tissue AT_1 receptors are upregulated[19].

Gross detection and sample collection The animal was weighed and killed by decapitation. The blood sample (2 mL) was rapidly collected into prechilled tube containing 2 g/L sodium edetic acid, cooled in ice-water bath, and centrifuged at 4 °C for isolation of the plasma. The aorta and heart were immediately excised and rinsed in cold physiological saline. Then the atria and vessels were removed from the ventricles. The right ventricular free wall was separated from the left ventricle and septum, and they were blotted and weighed separately. At the same time, the aorta was cleaned of adhering fat and connective tissue. Just below the branch of the left subclavicular artery a 22-mm-long segment of thoracic aorta was harvested, blotted, and weighed. Immediately after measurement of ventricular and aortic weights, 50-100 mg of each ventricle (near to the apex of heart) and abdominal aorta were separately frozen in liquid nitrogen. Thereafter, ventricular wall thickness was measured from the middle section of ventricular wall, at three points on the free wall of left and right ventricle and at the midpoint of the interventricular septum, using a vernier[7, 20]. As an index of ventricular hypertrophy, the ratio of ventricular weight to body weight was determined, and as an index of aortic hypertrophy, the aortic weight (22-mm-long) and the ratio of aortic weight to body weight were used[4,7,21].

Measurement of angiotensin II concentration Angiotensin II concentration was determined using the radioimmunoassay kit (provided by China Institute of Atomic Energy). The plasma was mixed with enzyme inhibitors and stored at -80 ºC before assay. Tissue angiotensin II was measured with the similar method described by Yu *et al*[16]. Briefly, the frozen tissues of aortas and ventricles were thawed, homogenized in hydrochloric acid 0.1 mol/L and centrifuged at 4 ºC. The supernatant was mixed with enzyme inhibitors and stored at -80 ºC before assay. During radioimmunoassay, a standard curve with known quantities of angiotensin II was generated at first, and the sample angiotensin II values were then automatically obtained from the standard curve.

Measurement of AT_1 receptor mRNA expression Total RNA was prepared using the RNA isolation kit (provided by Shanghai Hua Shun Bioengineering Ltd). Reverse transcription-polymerase chain reaction (RT-PCR) was performed according to RNA PCR Kit Ver. 2.1 Manual (Takara Biomedicals, Japan) with 1 μg of total RNA. cDNA was synthesized with random primers and AMV reverse transcriptase. PCR amplification was performed with synthetic gene-specific primers for rat AT_1 receptor[22] and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)[23]. GAPDH was as internal control. The primers for AT_1: 5' sense primer, 5' AC AGC TTG GTG GTG ATT GTC 3' (137-156); 3' antisense primer, 5' AT GAT GCA GGT GAC TTT GGC 3' (452-433). The primers for GAPDH: 5' sense primer, 5' TCC CTC AAG ATT GTC AGC AA 3' (421-440); 3' antisense primer, 5' AG ATC CAC AAC GGA TAC ATT 3' (728-709). The amplification profile involved denaturation at 94 ºC for 45 s, annealing at 55 ºC for 45 s, and extension at 72 ºC for 90 s for 30 cycles. After amplication, PCR products were electrophoresed on a 1.5 % agarose gel containing ethidium bromide. Bands corresponding to RT-PCR products were visualized by ultraviolet light, and their intensities were measured by densitometry. The amount of AT_1 mRNA was expressed as ratio of optical density of AT_1 mRNA to GAPDH mRNA from the same RNA sample[24].

Statistical analysis Data are reported as mean±SD. The differences between two groups were determined by unpaired *t* test. The differences among three groups were evaluated by using analysis of variance (ANOVA) followed by unpaired *t* test. Statistical significance was judged at $P<0.05$.

RESULTS

Body and organ weights in SAD rats There were no significant differences in initial body weights at the time of the operation between SAD and time-matched Sham controls (Tab 1). However, the final body weights were significantly lower in SAD than Sham rats at 2, 10 and 16 weeks after operation. For the ventricles, the absolute weight and wall thickness of each ventricle were not significantly changed after SAD, whereas the normalized weight of left ventricle was increased by 12 % at 10 and 16 weeks after SAD, and the normalized weight of right ventricle was increased by 11 % at 16 weeks after SAD. For the aortas, both absolute and normalized weights of aorta were increased in all three groups of rats, at 2-, 10-, and 16-week SAD, when compared with time-matched Sham controls.

Angiotensin II concentrations in SAD rats In plasma, left ventricles, and right ventricles, the angiotensin II concentrations were not significantly changed at 2, 10 and 16 weeks after SAD (Tab 2). In aortas, the angiotensin II concentrations at 2 weeks after SAD tended upwards, but were not significantly different from the Sham controls. However, the aortic angiotensin II concentrations were significantly increased at 10 and 16 weeks after SAD, when compared with time-matched Sham controls. Also, the plasma angiotensin II levels were obviously higher in candesartan-treated SAD group (836±515 ng/L, $n=6$, $P<0.01$) than untreated SAD group (110±87 ng/L, $n=14$) and Sham group (143±102 ng/L, $n=11$).

AT_1 receptor mRNA expressions in SAD rats The left ventricular and aortic AT_1 receptor mRNA expressions were examined at 16 weeks after operation (Fig 1). GAPDH mRNA expression as an internal control was not significantly different in Sham, SAD, and candesartan-treated SAD groups. AT_1 receptor mRNA expressions in left ventricles and aortas were significantly up-regulated in SAD groups, as compared with Sham groups. In positive control groups with long-term treatment of candesartan, AT_1 receptor mRNA expressions were markedly increased, when compared with Sham and untreated SAD groups.

DISCUSSION

Ventricular and aortic hypertrophy in SAD rats One of the most commonly used parameters to

Tab 1. Body weight, ventricular weight and wall thickness, and aortic weight in SAD and Sham rats at 2, 10, and 16 weeks after operation. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* time-matched Sham.

	2-week		10-week		16-week	
	Sham (*n*=12)	SAD (*n*=10)	Sham (*n*=5)	SAD (*n*=8)	Sham (*n*=12)	SAD (*n*=14)
BW						
Initial (g)	293±13	298±8	222±22	219±15	294±12	294±10
Final (g)	307±18	280±15[c]	438±32	377±22[c]	514±63	470±41[b]
Ventricular weight						
LVW (mg)	698±65	673±84	908±78	879±70	974±99	1004±107
RVW (mg)	204±20	189±24	263±42	232±25	277±22	281±30
VW (mg)	902±79	862±103	1171±113	1111±92	1251±116	1285±135
LVW/BW (mg/g)	2.27±0.12	2.39±0.18	2.07±0.05	2.33±0.18[c]	1.91±0.12	2.14±0.16[c]
RVW/BW (mg/g)	0.67±0.06	0.67±0.07	0.60±0.07	0.61±0.06	0.54±0.04	0.60±0.04[c]
VW/BW (mg/g)	2.94±0.15	3.07±0.22	2.67±0.09	2.95±0.22[b]	2.45±0.15	2.74±0.19[c]
LVW/RVW	3.43±0.27	3.58±0.33	3.49±0.39	3.82±0.24	3.52±0.25	3.58±0.18
Ventricular wall thickness						
LVWT (mm)	3.33±0.22	3.33±0.25	-	-	3.46±0.27	3.61±0.22
RVWT (mm)	1.15±0.16	1.10±0.09	-	-	1.13±0.11	1.18±0.15
SWT (mm)	2.64±0.49	2.5±0.3	-	-	2.59±0.34	2.59±0.31
LVWT/RVWT	2.96±0.55	3.0±0.4	-	-	3.1±0.4	3.08±0.25
Aortic weight						
AW (mg)	21.4±1.7	25.8±2.5[c]	23.1±1.7	26.5±2.4[c]	24.4±1.7	27.6±3.0[c]
AW/BW (mg/g ×100)	6.97±0.65	9.0±0.9[c]	5.3±0.5	7.0±0.7[c]	4.8±0.4	5.9±0.7[c]

Sham indicates sham operation; SAD, sinoaortic denervation; BW, body weight; LVW, left ventricular weight; RVW, right ventricular weight; VW, LVW+RVW; LVWT, left ventricular wall thickness; RVWT, right ventricular wall thickness; SWT, septum wall thickness; and AW, aortic weight.

Tab 2. Plasma, ventricular and aortic angiotensin (Ang) II levels in SAD and Sham rats at 2, 10, and 16 weeks after operation. Mean±SD. [b]*P*<0.05 *vs* time-matched Sham. *n* is indicated in parentheses.

	Plasma Ang II/ ng· L^{-1}	Left ventricular Ang II/ pg· g^{-1} tissue	Right ventricular Ang II/ pg· g^{-1} tissue	Aortic Ang II/ pg· g^{-1} tissue
2-week				
Sham	150±131 (6)	194±63 (8)	191±80 (8)	199±63 (10)
SAD	157±105 (8)	240±68 (8)	221±78 (8)	245±70 (10)
10-week				
Sham	146±104 (5)	191±155 (5)	185±135 (5)	141±100 (5)
SAD	143±126 (7)	192±33 (7)	179±66 (7)	340±162 (7)[b]
16-week				
Sham	143±102 (11)	152±80 (12)	147±55 (10)	190±80 (7)
SAD	110±87 (14)	131±66 (11)	131±47 (13)	346±142 (9)[b]

Sham indicates sham operation; SAD, sinoaortic denervation.

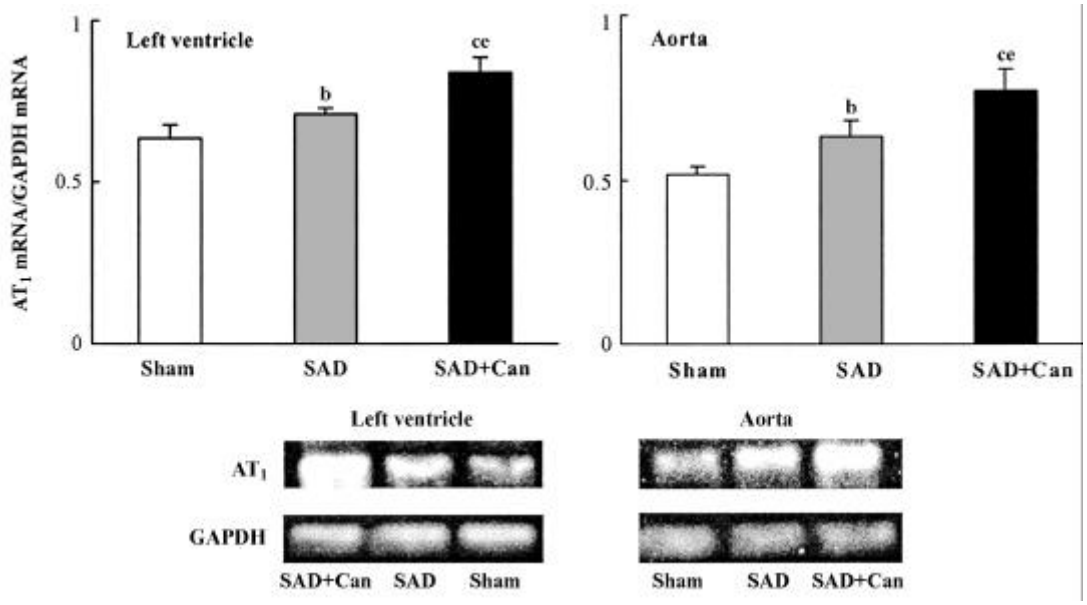

Fig 1. RT-PCR analysis of AT_1 receptor mRNA expression in left ventricles and aortas of SAD and Sham rats 16 weeks after operation. In SAD+Can group, SAD rats were treated with candesartan cilexetil (Can) for 16 weeks. Upper: The amount of AT_1 mRNA was measured by scanning and expressed as ratio of optical density of AT_1 mRNA to GAPDH mRNA. Mean±SD. *n*=3 in each group. $^bP<0.05$, $^cP<0.01$ *vs* Sham; $^eP<0.05$ *vs* SAD. Lower: Typical electrophoretic bands of RT-PCR products.

evaluate the cardiovascular hypertrophy is the ratio of organ weight to body weight[4,7,21]. It is also called the relative weight, the normalized weight, or hypertrophy index[4,7,21]. From the hypertrophy index, there existed aortic hypertrophy in 2-, 10-, and 16-week SAD arts, left ventricular hypertrophy in 10- and 16-week SAD rats, and right ventricular hypertrophy in 16-week SAD rats. The time course indicates that the hypertrophy of the aorta, left ventricle, and right ventricle occurs one by one.

Our present results of aortic and left ventricular hypertrophy are consistent with the previous data reported by our lab[4] and others[6,7]. However, the right ventricular hypertrophy is much less in the present study (no right ventricular hypertrophy at 2 and 10 weeks after SAD, and 11 % increase in the hypertrophy index at 16 weeks after SAD) compared with the literature (39 % increase in the hypertrophy index at 6 weeks after SAD)[7], although both studies did not find significant differences in absolute values of ventricular weight and wall thickness between SAD and Sham rats. At the present time, there is no proper explanation for the discrepancy. Perhaps, it is due to the different rats used in these two studies; Long-Evans rats in the literature study[7] and Sprague-Dawley rats in the present study.

Compared with spontaneously hypertensive rats (SHR), the left ventricular hypertrophy is modest in SAD rats; 12 % increase in the hypertrophy index in 16-week SAD rats at the age of 26 weeks, and 30 %[25], 36 %[26] and 42 % (our unpublished data) increase in the hypertrophy index in SHR at the age of 15, 26 and 50 weeks respectively. However, aortic hypertrophy occurs earlier in SAD rats than SHR, since aortic hypertrophy was not found in SHR at the age of 15 and 26 weeks[21,26], but existed at the age of 50 weeks (our unpublished data).

RAS and hypertrophy in SAD rats RAS is an important system in regulating cardiovascular structure and function. Its effector hormone, angiotensin II, mainly acts through 2 receptor subtypes, AT_1 and AT_2. Most effects of angiotensin II, including vasoconstriction, aldosterone and vasopressin release, renal salt and water retention, sympathetic facilitation, and cell growth, are mediated by AT_1[27]. The functions of AT_2 are complicated and remain to be further clarified, although it has been reported that the actions of AT_2 appear to be opposite in some cases to those of AT_1[19, 27]. In addition to circulating RAS, local tissue RAS plays a role in the maintenance of cardiovascular function and structure. It is considered that the circulating RAS mediates acute effects of angiotensin, including vasoconstriction, renal salt and water homeostasis, and cardiac rhythmicity. The tissue RAS, in contrast, may exert long-term

effects of angiotensin involved in cell growth and differentiation and has been implicated in processes such as nephrosclerosis in the kidney and hypertrophy of the heart and blood vessels under certain cardiovascular diseases[14,27].

In the present study, we examined the major effector components of RAS, angiotensin II and AT_1 receptor, at different time points after SAD. In methodology, candesartan treatment was designed as a positive control, since it has been demonstrated that plasma angiotensin II levels and tissue AT_1 receptors were upregulated following sufficient and chronic blockade of AT_1 receptors with candesartan[19], which is further supported by present results from candesartan-treated rats. It was found that the plasma angiotensin II levels remained unchanged at 2, 10, and 16 weeks after SAD. These indicate that circulating RAS is not activated in the chronic phase of SAD, which is in accordance with previous data[11]. However, the local tissue RAS was activated, as evidenced by increase of aortic angiotensin II concentrations at 10 and 16 weeks after SAD and upregulation of aortic and left ventricular AT_1 receptor mRNA expressions at 16 weeks after SAD, indicating that certain components of tissue RAS are involved in the SAD-induced cardiovascular hypertrophy. Also, we found that tissue RAS activation following SAD was different in ventricles and aortas, ie, the angiotensin II concentrations were unchanged in ventricles in all examined SAD rats, but elevated in aortas at 10 and 16 weeks after SAD, suggesting that tissue angiotensin II concentration appears not important for SAD-induced ventricular hypertrophy.

Regarding the time course of hypertrophy and RAS activation following SAD, the present study showed that hypertrophy occurred earlier than RAS activation. Many previous studies have shown that the most immediate and prominent change after SAD is a substantial increase in BPV, and the increased BPV is persistent during acute and chronic phase of SAD and plays a role in SAD-induced cardiovascular hypertrophy[4,8]. Taken together, BPV increasing, cardiovascular hypertrophy and RAS activation occurs one by one following SAD. These suggest that increased BPV is an initial factor for causing cardiovascular hypertrophy and RAS activation, and cardiovascular tissue RAS activation is secondary to an increased BPV and hypertrophy. Nevertheless, the tissue RAS activation may be involved in the maintenance or aggravation of SAD-induced cardiovascular hypertrophy, which is supported by the following evidence. First, from a vast amount of data in other models of cardiovascular diseases that tissue RAS activation may exert long-term effects on cell growth and be involved in the cardiovascular hypertrophy remodeling[14-16,27,28], it may be deduced that tissue RAS activation in the SAD model may play a role in SAD-induced cardiovascular hypertrophy. Second, SAD-induced cardiovascular hypertrophy can be efficiently prevented by long-term treatment with captopril, an angiotensin converting enzyme inhibitor, or candesartan, an AT_1 receptor antagonist[29,30].

It should be noted that the components of RAS involved in cardiovascular hypertrophy may be different in various cardiovascular diseases, and sometimes dependent on the stage of the disease. Generally speaking, in most of cardiovascular models such as hypertension and myocardial infarction, both angiotensin II and AT_1 receptor are involved[14-16,27,28,31]. However, in heart failure, although it has been reported that angiotensin II is elevated and involved in the cardiac hypertrophy[14,32], the AT_1 receptors are down-regulated in human ventricles with idiopathic dilated cardiomyopathy but not with ischemic cardiomyopathy[33]. The down-regulation of the AT_1 receptors may not be disease specific but may correlate to the severity of heart failure[33]. Significant down-regulation only in severely impaired left ventricles may imply that AT_1 receptors are determinants for the shift to end-stage heart failure[32]. The beneficial or deleterious effects due to the down-regulation of the AT_1 receptors are unknown and remain to be studied[32]. Our present study showed that both angiotensin II and AT_1 receptors were up-regulated in aortas 16 weeks after SAD, and only AT_1 receptors rather than angiotensin II were up-regulated in left ventricles 16 weeks after SAD. These results exclude the effect of tissue angiotensin II on SAD-induced left ventricular hypertrophy, and are consistent with the result of mild left ventricular hypertrophy following SAD.

Regarding mechanisms underlying SAD-induced hypertrophy, our previous study has reported that high BPV following SAD plays a major role through the direct mechanical effects and possibly the indirect effects from neurohumoral factors[4]. The current study provided the new findings that certain components of tissue RAS, *ie*, aortic angiotensin II and AT_1 receptors and left ventricular AT_1 receptors, were activated in chronic SAD rats, and this tissue RAS activation was secondary to increased BPV and cardiovascular

hypertrophy, and may play an additional role in the maintenance of cardiovascular hypertrophy following SAD. Other growth-regulatory factors that may have the effects on SAD-induced hypertrophy, such as AT_2 receptor (another component of RAS) and NO, remain to be studied in the future.

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