Upregulation of Angiotensin-Converting Enzyme 2 by All-trans Retinoic Acid in Spontaneously Hypertensive Rats

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Abstract—There is increasing evidence that all-trans retinoic acid (atRA) influences gene expression of components of renin-angiotensin system (RAS), which plays a pivotal role in the pathophysiology of essential hypertension. To further validate effects of atRA on the RAS and to assess the possibility that atRA affects the activity of angiotensin-converting enzyme 2 (ACE2), gene, and protein expression of ACE2 have been examined by real-time polymerase chain reaction and Western blot methods in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Rats were treated with atRA (10 or 20 mg·kg⁻¹·day⁻¹) or placebo given as daily intraperitoneal injection for 1 month. ACE2 expression was markedly decreased in placebo-treated SHR when compared with WKY rats. However, in atRA-treated SHR, a significant upregulation of ACE2 expression was observed in heart and kidney. In conclusion, chronic atRA treatment increases gene and protein expressions of ACE2, resulting in the reduction of blood pressure and the attenuation of myocardial damage in SHR, which suggests that atRA may be an attractive candidate for the potential prevention and treatment of human essential hypertension. (Hypertension. 2004;44:907-912.)

Key Words: hypertension, essential ■ angiotensin-converting enzyme ■ renin-angiotensin system ■ rats

It is well-recognized that essential hypertension is a genetically complex metabolic and cardiovascular disorder controlled by both genetic and environmental factors.† The ideal aim of modern antihypertensive therapy is not only a reduction of blood pressure but also the prevention or amelioration of its complications, such as myocardial remodeling at the molecular genetic level.‡,³ Research advances on the key transcription factors and nuclear hormone receptors provide insight into the development of novel transcription-modulating antihypertensive drugs, among which is all-trans retinoic acid (atRA), a biologically active metabolite of vitamin A.² atRA modulates gene transcription and exerts its other effects by binding the retinoic acid receptor (RAR) and retinoid X receptor (RXR)⁴,⁵ and interfering with some key transcription factors such as the activated protein-1.⁶,⁷ Studies have demonstrated that atRA influences the activities of genes responsible for the pathogenesis of hypertension and its complications.²,³,⁶,⁸ Dechow et al⁹ reported that atRA reduced blood pressure in the renal damage model. Spontaneously hypertensive rats (SHR) are recognized as genetically hypertensive rats, which resemble, in many aspects, humans with essential hypertension.⁹ Chronic atRA treatment prevented medial thickening of intramyocardial and intrarenal arteries and ventricular fibrosis in SHR.³ However, whether atRA reduces arterial pressure and attenuates myocardial damage in SHR deserves further study.

atRA has been shown to regulate the gene expression of components of renin-angiotensin system (RAS), including renin, angiotensin-converting enzyme (ACE), angiotensin (Ang) II, and its type 1 (AT₁) receptor.⁶,⁷,¹⁰ RAS is critically involved in regulating blood pressure as well as fluid and electrolyte balance, and has been exhibited to be a far more complex system than initially thought with the discovery of ACE2, an enzyme similar to ACE.¹¹–¹³ ACE2 may counteract the function of ACE by metabolizing the Ang II to generate Ang 1-7,¹,¹⁴,¹⁵ which modulate blood pressure through antagonization of Ang II actions and potentiation of nitric oxide (NO) release.¹⁶–¹⁸ Thus, ACE2 may play a potential role in maintaining cardiorenal function and blood pressure homeostasis.¹¹,¹⁹–²¹ The addition of ACE2 to the complexities of the RAS may open new possibilities for the treatment of essential hypertension.¹¹,²⁰,²¹

In view of the effects of atRA on ACE and other RAS activities, we speculated that atRA might influence the activity of ACE2 and therefore elicit some beneficial effects in hypertension. The present study was designed to examine the possibility that atRA influences the expression and action of ACE2 in SHR. To this end, we investigated the effects of atRA on the ACE2 messenger RNA (mRNA) and protein levels in SHR and performed transmission electron microscope (TEM) technique, aiming to analyze the putative actions of atRA on the myocardial morphology in SHR.

Methods

Animal and Tissue Processing

Twelve-week-old male Wistar-Kyoto (WKY) rats and SHR (obtained from the Shanghai Institute of Hypertension, China) were

Received April 23, 2004; first decision May 25, 2004; revision accepted September 17, 2004.
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Hypertension is available at http://www.hypertensionaha.org
DOI: 10.1161/01.HYP.0000146400.57221.74

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randomly assigned to 5 treatment groups: WKY-C (WKY control treated with vehicle 1 mL·kg⁻¹·day⁻¹), WKY-R (WKY treated with atRA 10 mg·kg⁻¹·day⁻¹), SHR-C (SHR control treated with vehicle 1 mL·kg⁻¹·day⁻¹), SHR-L (SHR treated with low-dose atRA 10 mg·kg⁻¹·day⁻¹), and SHR-H (SHR treated with high-dose atRA 20 mg·kg⁻¹·day⁻¹). Fresh suspensions of atRA (Sixth Pharmaceutical Company, Shanghai, China) in vehicle were prepared in a darkened room each day. Rats received daily intraperitoneal injection of atRA or vehicle (50% atrial fluid, 45% saline, and 5% ethanol) for 1 month. All rats were weighed and their blood pressures were measured by the tail-cuff method once per week. Rats were housed in a room with a 12:12-hour light–dark cycle and fed with a standard diet and water ad libitum. At the end of the 1-month treatment period, the heart and kidney were removed carefully after the rat was decapitated. The middle part of the left ventricle was immediately cut into small pieces and immersed in 2.5% glutaraldehyde for TEM analysis. Serum and tissue samples were flash-frozen in liquid nitrogen (LN₂) and stored at −70°C until assay. All experiments were performed in accordance with the national animal protection law.

Isolation of Total RNA and Synthesis of cDNA
Total RNA was isolated from heart and kidney tissues using Trizol Reagent (Invitrogen). Complementary DNAs (cDNA) were synthesized by standard techniques (Superscript First Strand Synthesis System for RT-PCR; Invitrogen). An aliquot of the resulting single-strand cDNA was used in the real-time polymerase chain reaction (PCR) experiments as described below.

Quantitative Real-Time PCR
Primers and probes were designed for rat ACE2: 5′-primer (5′-ACCTCTTACATCACGCTCTCCTGGTCTGTTGAT-TAMRA), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-primer (5′-GACATGCCGCGCTGGAAAGAC), 3′-primer (5′-AGCCCCAGATGCCCTTTGATT), and probe (FAM-5′-CCTCGGGCCGCGCTTACACCA-TAMRA). Real-time PCR was performed in a 25-μL reaction mixture prepared with a TaqMan PCR core reagent kit (Applied Biosystems [ABI]) containing an appropriately diluted cDNA solution, 0.2 μM each primer, 0.2 μM probe, and 0.2 μM/μL ROX Reference Dye (Invitrogen) under the following conditions: at 50°C for 10 minutes, at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. To obtain a calibration curve, we amplified a known amount of a plasmid (pGEM-T easy vector; Promega) with atRA 10 mg·kg⁻¹·day⁻¹, and SHR-H (SHR treated with high-dose atRA 20 mg·kg⁻¹·day⁻¹). Fresh suspensions of atRA (Sixth Pharmaceutical Company, Shanghai, China) in vehicle were prepared in a darkened room each day. Rats received daily intraperitoneal injection of atRA or vehicle (50% atrial fluid, 45% saline, and 5% ethanol) for 1 month. All rats were weighed and their blood pressures were measured by the tail-cuff method once per week. Rats were housed in a room with a 12:12-hour light–dark cycle and fed with a standard diet and water ad libitum. At the end of the 1-month treatment period, the heart and kidney were removed carefully after the rat was decapitated. The middle part of the left ventricle was immediately cut into small pieces and immersed in 2.5% glutaraldehyde for TEM analysis. Serum and tissue samples were flash-frozen in liquid nitrogen (LN₂) and stored at −70°C until assay. All experiments were performed in accordance with the national animal protection law.

Measurement of Serum atRA Levels
Under minimal light, blood was drawn from the tail vein 2 hours after administration of atRA or placebo. Serum (0.2 mL) was mixed with 0.2 mL of 0.25 mol/L ammonium acetate (pH 3.8), 0.6 mL of acetonitrile (Sigma), and 1 mL of hexane. The resulting supernatant with 0.2 mL of 0.25 mol/L ammonium acetate/acetonitrile (150/80/5) was washed 3 times with Tris-buffered saline/Tween and incubated with alkaline phosphatase-labeled secondary antibody (1/5000, Santa Cruz) for 1 hour at room temperature. The positive bands representing ACE2 protein were developed with the use of the Western Blue alkaline phosphatase-labeled secondary antibody (1/5000, Santa Cruz) for 1 hour at room temperature. The positive bands representing ACE2 protein were developed with the use of the Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega) and quantified with an Automated Imaging System (Bio-Rad).

TEM Analysis
Samples of rat left ventricle tissues were washed 3 times with phosphate-buffered saline, followed by postfixation with 1% osmium tetroxide for 1 hour, stained with 2% aqueous uranyl acetate, and dehydrated in a graded series of ethanol. After infiltration and polymerization, ultra-thin sections were cut, flattened with xylene vapor, collected on nickel grids, and observed on a HITACH-300 electron microscope (HITACH, Japan).

Statistical Analysis
All values are shown as mean±SEM. Statistical analyses were performed by ANOVA, followed by Student-Newman-Keuls test. P<0.05 was considered as statistically significant.

Results
Effects of atRA on Blood Pressure and Body Weight in SHR
Figure 1 illustrates a marked increase of systolic blood pressure (SBP) in SHR compared with WKY-C rats (P<0.01, respectively). SBP did not differ among SHR groups before and 1 and 2 weeks after treatment. However, a significant reduction of SBP was shown in SHR-L and SHR-H 3 and 4 weeks after atRA treatment (P<0.05, respectively). There was no statistically significant change in SBP between WKY-C and WKY-R rats. No significant differences across groups were shown in body weight changes in SHR and WKY rats before and 1, 2, 3, and 4 weeks after treatment,
indicating that chronic atRA treatment reduced blood pressure in SHR but had no effect on body weight.

Effects of atRA on ACE2 mRNA Expression in SHR
A good linear relationship was shown between the amount of rat ACE2 standard and the threshold cycle number from the standard curve ($r=0.987$; Figure 2). Figure 3 reveals that both heart and kidney ACE2 mRNA expression were markedly depressed in placebo-treated SHR compared with WKY-C rats ($P<0.01$, respectively), whereas in atRA-treated SHR, ACE2 mRNA expression was significantly enhanced in heart and kidney ($P<0.05$, respectively). ACE2 mRNA expression did not differ among WKY rats.

Effects of atRA on ACE2 Protein Expression in SHR
As shown in Figure 4, ACE2 protein expression was decreased by $\sim 30\%$ in both heart and kidney in placebo-treated SHR ($P<0.01$, respectively). By contrast, in atRA-treated SHR, ACE2 protein was significantly increased in both heart and kidney and nearly approached that seen in WKY-C rats ($P<0.05$, respectively). However, there was no statistically significant change of ACE2 protein expression between WKY-C and WKY-R rats.

Effects of atRA on Myocardial Morphology in SHR
Figure 5C shows severe myocardial damage of the left ventricle in placebo-treated SHR, characterized with myocardial mitochondria swelling, crest disruption, and myofilaments derangement, whereas Figure 5A and 5B show normal myocardial mitochondria and myofilaments of WKY rats. However, in atRA-treated SHR (Figure 5D and 5E), the myocardial damage of left ventricle was obviously attenuated, especially in the high-dose group.

Discussion
The present study showed that both heart and kidney ACE2 mRNA and protein expressions were markedly decreased in placebo-treated SHR when compared with WKY rats. In atRA-treated SHR, there was significant upregulation of ACE2 mRNA and protein expression in heart and kidney,
accompanied by a reduction of blood pressure and an attenuation of myocardial damage. However, chronic atRA treatment had no effect on ACE2 expression and blood pressure in WKY rats. These results have confirmed and extended the effects of atRA on the RAS and opened the intriguing possibility that atRA may reduce blood pressure and attenuate myocardial damage in SHR. Our findings also suggested that the increase of ACE2 might be involved in the antihypertensive and cardioprotective effects of atRA in SHR.

There are many known ways in which atRA controls gene expression and protein production. But in terms of molecular mechanisms, a single, predominant, classical pathway has emerged: ligand involvement (atRA) plus receptor dimerization (RAR/RXR), DNA binding, and RAR response element (RARE) regulation. Initially, atRA activates a heterodimer RAR/RXR, which further recognizes and binds to RARE consensus sequence, thereby activating or repressing target gene transcription. Here, in the relative absence of ACE2 in SHR, atRA can upregulate the ACE2 expression, which may be mediated by this pathway via the RAR/RXR-mediated signaling. However, in WKY rats in which ACE2 are expressed normally, atRA did not show its regulatory action on ACE2 expression. Further studies are required to elucidate the molecular mechanism of the effects of atRA on ACE2 expression and the reason for its different role in hypertensive and normotensive rats.

Although the relation between ACE2 and blood pressure remains controversial, there is increasing evidence that ACE2, acting as a negative regulator of ACE and the RAS, may function to potentiate the vasodilating and antihypertensive effect. The RAS is a coordinated hormonal cascade that governs blood pressure, in which ACE2 is a rate-limiting enzyme and plays a key role. The possibility that ACE2 is involved in blood pressure modulation was suggested when ACE2 was mapped to the quantitative trait loci on the X chromosome in SHR, which contains a particular region that is important in the heritability and induction of raised blood pressure. In addition, recent research has demonstrated that baseline blood pressure in knockout mice lacking the ACE2 gene (ace2/ace2) is higher than that in normal mice (ace2/ace2). Spontaneous hypertension develops in SHR that exhibit a relative lack of ACE2 level, whereas in WKY rats that remain normotensive, higher ACE2 expression could account for the “resistance” to hypertension. In a state in which ACE2 is expressed in excess, hypertension may develop. Accordingly, it is reasonable to surmise that the increase of ACE2 expression in atRA-treated SHR in the present study may be responsible for the antihypertensive action of atRA.

Besides limiting the production or antagonizing the vasoconstrictive effect of Ang II, facilitating formation of Ang 1-7 contributes to the beneficial effect of ACE2 on blood pressure. Crackower et al found that the ace2 knockout mice displayed a significant increase in Ang II levels, which might aggravate the vasoconstriction and hypertension and impair NO-mediated vascular relaxation. Contrarily, the increase of ACE2 can antagonize Ang II-induced vasoconstriction through the vasodilator effect of Ang 1-7 and the release of NO. Evidence that ACE2 can augment the expression of Ang 1-7 in heart or kidney has been obtained from relevant investigations. Ang II may be directly converted to Ang 1-7 by ACE2. ACE2 can also cleave Ang I to generate Ang 1-9, subsequently subjected to enzymatic cleavage by ACE, resulting in the formation of Ang 1-7. Ang 1-7 functions as an important regulator in NO formation and blood pressure homeostasis through various mechanisms such as antagonization of Ang II and AT1 receptor action and potentiation of crosstalk between ACE and bradykinin receptor type 2 (B2).
receptor on membranes. Thereby, the increase of ACE2 is a potential reason for the antihypertensive effect of atRA in SHR by attenuating the vasoconstrictive effect of Ang II, promoting the formation of Ang 1-7 and subsequently facilitating the release of NO.

Target organ protection is the “endpoint” aim of hypertension treatment. In the present study, the cardioprotective effect of atRA was shown, at least in part, from the TEM. Experimental and clinical studies suggest that the Ang II–NO imbalance causing oxidative stress may be crucially involved in the occurrence and persistence of high blood pressure and the abnormal myocardial remodeling in hypertension. The loss of ACE2 results in an increase of Ang II and a decrease of NO levels, which are associated with severe cardiac damage in SHR via induction of oxidative stress.

The relative absence of ACE2 level observed in the placebo-treated SHR might be responsible for severe myocardial damage in SHR (from TEM). Accordingly, the increase of ACE2 might contribute to the attenuation of myocardial damage in atRA-treated SHR, suggesting that the cardioprotective effect of atRA results from the increase of ACE2. ACE2 can directly counteract the Ang II-mediated production of reactive oxygen species via AT1 receptor. Furthermore, ACE2 may promote the activity of B3 receptor via the increase of Ang 1-7, leading to the formation of the reactive oxygen species-insensitive AT1-B3-heterodimer and the mitigation of myocardial oxidative damage by repressing AT1 receptor homodimer. These findings provide new insights regarding the potential role of atRA on the attenuation of myocardial damage in SHR by resisting oxidative stress.

The effects of atRA could be different depending on the dose, the duration, and the administration route. The dose of atRA (10 or 20 mg·kg\(^{-1}\)·day\(^{-1}\)) in the present study was selected according to a previous experiment that showed an efficient reduction of blood pressure. Recently, Liu et al. did not find the antihypertensive effect of atRA treatment in their study, although there was an obvious trend to decreased blood pressure in atRA-treated SHR. Some possible explanations for this may be the suboptimal dose (5 mg·kg\(^{-1}\)·day\(^{-1}\)), the oral administration, and the different age and source of SHR. More importantly, the concentration of atRA was not measured in their study. In the present work, a sustained elevation of serum atRA was observed in atRA-treated rats, which is consistent with previous research. Data suggest that higher doses of atRA may evoke some side effects or even retinoid toxicity. Hence, it is necessary to define a minimal effective dose of atRA that favorably acts on hypertension and its complications in future studies.

In summary, chronic atRA treatment increases the expression of ACE2 in both mRNA and protein levels, resulting in the reduction of blood pressure and the attenuation of myocardial damage in SHR, which suggests that atRA may be an attractive candidate for the potential prevention and treatment of human essential hypertension.

Perspectives

Drugs that specifically influence the ACE2 expression may have promising clinical value in the prevention and treatment of essential hypertension. The upregulatory action of atRA on ACE2 expression in SHR is appealing. However, the molecular mechanisms involved require further study. Moreover, whether any effects of atRA observed here may predict outcome in more complex clinical patients with essential hypertension remains to be elucidated. The present study provides a platform for further inquiry relating to the possible effects of chronic treatment with atRA on blood pressure and hypertension-induced pathological changes.

Acknowledgments

This study was supported by National Natural Science Foundation of China (grant 30370340). We gratefully appreciate Drs Ji-Kai Jiang and Xiao-Shan Liu for their helpful advice.

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Hypertension. 2004;44:907-912; originally published online October 11, 2004; doi: 10.1161/01.HYP.0000146400.57221.74

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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