Regular Article

Effect of Prolonged Infusion of Alamandine on Cardiovascular Parameters and Cardiac ACE2 Expression in a Rat Model of Renovascular Hypertension

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Alamandine is a new member of the angiotensin family. Here, we studied the mRNA and protein expression of cardiac angiotensin-converting enzyme 2 (ACE2) in the chronic phase of a rat model of 2-kidney, 1-clip hypertension (2K1C), and the effects of 2-week alamandine infusion on blood pressure, cardiac indices, and ACE2 mRNA and protein expression in the hearts. The rats were subjected to to sham-operation or placement of plexiglass clips around the left renal artery. Alamandine, at a dose of 600 µg/kg/d, was administered for 2 weeks via an osmotic mini-pump. At 18 weeks, after induction of hypertension, blood pressure and cardiac indices of contractility were measured using a Powerlab Physiograph system. The ACE2 mRNA and protein levels were determined using real time-PCR and Western blotting, respectively. In the hypertensive rats, alamandine caused a significant decrease in systolic blood pressure (p < 0.001), left ventricular end-diastolic pressure (p < 0.001), and left ventricular systolic pressure (p < 0.001) and increase in the maximum rate of pressure change in the left ventricle (dP/dt(max)) (p < 0.05). Also, the ACE2 mRNA expression in the heart increased in the hypertensive rats compared to the normotensive rats (p < 0.05), and alamandine restored this to normal values, although these changes were only seen at the mRNA and not the protein level. Histological analysis of cardiac tissue confirmed that alamandine decreased cardiac fibrosis and hypertrophy in 2K1C hypertensive rats. Our results indicate that alamandine, which acts as a depressor arm of the renin–angiotensin system, could be developed for treating hypertension.

Key words: alamandine; hypertension; renin–angiotensin system; angiotensin-converting enzyme 2 (ACE2); ACE2 expression

INTRODUCTION

The renin–angiotensin system (RAS) plays a crucial role in the regulation of blood pressure and fluid balance. Angiotensinogen, a component of the renin–angiotensin system, is processed in a 2-step reaction by renin and angiotensin-convert ing enzyme 1 (ACE1). The effect of renin on angiotensinogen is processed in a 2-step reaction by renin and angiotensin-converting enzyme 1 (ACE1). The effect of renin on angiotensinogen results in the formation of an inactive decapeptide known as angiotensin I (Ang I). Then ACE1, through its peptidase effect, converts Ang I to an octapeptide called Ang II, which is the central mediator for RAS.

Ang II is a potent vasoconstrictor that decreases the activity of renin. Some studies have shown an increase in the activity of RAS during clinical and experimental heart failure. However, recently and after the discovery of ACE2, the classical view of RAS has been questioned. There is currently greater awareness that other angiotensin peptides such as angiotensin A (Ang A), angiotensin 1–7 (Ang (1–7)) and alamandine are also physiologically important and have significant biological activities. ACE2 is a carboxypeptidase and was initially known by its effect on Ang I, leading to the formation of Ang 1–9. Ang A is also a substrate for ACE2. Ang A can be hydrolyzed by ACE2 to generate alamandine, and alamandine can also be produced directly from Ang (1–7) by decarboxylating its aspartic residue. Alamandine and Ang (1–7) are also structurally similar; the only difference between these 2 peptides is the presence of alanine (in alamandine) in place of an aspartate residue in the amino terminus. They have similarity in most of their biological effects. Alamandine has been shown to produce endothelial-dependent vasorelaxation in aortic rings in mice and rats, an effect that has been previously reported for Ang (1–7). Administration of 50 µg/kg of alamandine once a day to isoproterenol-treated rats resulted in a remarkable decrease in collagen I, collagen III, and fibronectin accumulation. In our previous study, a 15-min infusion of alamandine, in the early phase of 2-kidney, 1-clip (2K1C) renovascular hypertension in rats, produced a dose-dependent biphasic hemodynamic effect. In the chronic stage of 2K1C hypertension, however, an impairment of endothelium-dependent relaxation is seen which may contribute to sustained high blood pressure. Liu et al. showed that long-term administration of alamandine had beneficial effects on hypertension, cardiac hypertrophy left ventricular functions in spontaneously hypertensive rat (SHR). SHR closely simulate primary or essential hypertension that represent normal renin, sodium-independent model of hypertension.

However, renovascular hypertension is the most common cause of secondary hypertension. The hypertension resulting from activation of the renin–angiotensin–aldosterone system and subsequent production of AngII. Increased Ang II levels increase ACE2 activity. ACE2 plays a fundamental role in

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the RAS because it opposes the actions of Ang II. The main function of ACE2 is the degradation of Ang II resulting in the formation of Ang (1–7) and alamandine which oppose the functions of Ang II.10

To date, no studies have examined the effects of 2K1C induced hypertension, an established animal model of human renovascular hypertension, on ACE2 expression. Additionally hemodynamic effects of alamandine and its possible effect on ACE2 expression in chronic stage of renovascular hypertension has not been investigated. Therefore, this study was designed to evaluate these issues.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (180–200 g) were obtained from the Experimental Animal Centre of Fasa University of Medical Sciences. Rats were housed with a 12-h/12-h light/dark cycle with standard rat chow and water ad libitum. Experiments were carried out according to the all applicable international, national, and institutional guidelines for conducting animal studies and were approved (IR.FUMS.RES.1395.52) by the Ethics Committee, Medical Sciences University, Fasa, Iran.

Experimental Groups Osmotic mini-pumps (Alzet, model 2002, Scientific Marketing, London, U.K.) were filled with either isotonic saline or alamandine (Phoenix Pharmaceuticals, Inc., Burlingame, CA, U.S.A.) dissolved in saline. Animals were divided randomly into three groups (with each group consisting of seven animals), namely a normotensive group, rats with SBP <140 mmHg; a hypertensive group, SBP ≥140 mmHg; and s.c. implanted with osmotic pumps containing saline, as control. Animals were anesthetized by chloroform or sodium thiopental (50 mg/kg, intraperitoneally (i.p.)) was given to anesthetize the rats. Then, spectrometry and agarose gel electrophoresis were performed to assess the quality and quantity of RNA in samples. Complementary first-strand DNA (cDNA) was synthesized using a cDNA synthesis kit (TaKaRa, Japan) with oligo (dT) and random hexamer primers, according to the manufacturer’s recommendations. Sybr Green real-time PCR kit (TaKaRa, Japan) and real-time PCR System (ABI, U.S.A.) were used for cDNA amplification and the relative quantification of mRNA levels. GenBank sequences (www.ncbi.nlm.nih.gov/Genbank) were used as guidance for primer design. ACE2 specific primers were: forward 5'-GAC AGCAATGGCACCTG-3' and reverse 5'-CGTTCTCAAAGCAAGCAGG-3', and β-actin specific primers were used as an internal control (forward 5'-CGGTGTTGAACGGATTGGCC-3' and reverse 5'-GTTGAAGGGATCATCTGGAAC-3'), all designed by AlleleID software (Version 7.5).

All PCR reactions consisted of 10 µl of Sybr Green master mix, three µl (150 ng) of synthesized cDNA and 10 µl of each primer in a final volume of 20 µl. The cDNA was amplified according to the following PCR conditions: incubation at 95°C for 3 min followed by 35 cycles of 5 s at 95°C, 10 s at 60°C and 30 s at 72°C. The melt curve protocol followed with 10 s at 95°C and then 10 s each at 0.2°C increments between 60°C and 95°C. Additionally to certify the specificity of the amplicons, the PCR products were electrophoresed on a 1.5% agarose gel. Real-time PCR was performed in duplicate. A negative control (no template control) was included for each run to check any contamination. ACE2 threshold cycle (Ct) value of each sample was normalized by the β-actin Ct value, using the formula: ΔCt(ACE2) = Ct(ACE2) − Ct(β-actin). Then, ACE2 mRNA expression in samples was quantified relative to samples of untreated rats (normotensive group) as follows: ΔΔCt = ΔCt(treated rats) − ΔCt(untreated rats). Fold changes of the expression of ACE2 mRNA in all samples were calculated using the equation 2^{-ΔΔCt}.

Evaluation of ACE2 Protein Expression in Rat Hearts by Western Blotting The heart tissues were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Sigma, U.S.A.). The homogenates were then centrifuged at 14000 rpm for 20 min at 4°C. The Bradford method was used to measure the concentration of protein lysate in samples. Then, 50 µg of extracted proteins were separated on 12% sodium dodecyl sulfate (SDS) polyacrylamide resolving gel with a 3% stacking gel. Proteins were electro-transferred from gels onto an activated polyvinylidene fluoride (PVDF) membrane (GE Healthcare, U.S.A.) using a Mini Trans-Blot Electrophoretic Transfer system (GE Healthcare). Membranes were blocked with 5% of bovine serum albumin (BSA). Sheep anti-rat ACE2 primary antibody (1:200 dilution) (Abcam, U.S.A.) and horseradish peroxidase (HRP)-conjugated goat anti-sheep...
secondary antibody (1:10000 dilution) (Abcam) were used to
detect the ACE2 protein. Furthermore, sheep anti-rat β-actin
(1:200 dilution) (Abcam) and HRP-conjugated goat anti-sheep
antibody (1:10000 dilution) (Abcam) was used for detect-
ing β-actin as an internal control. Electrochemiluminescence
(ECL) (Bio-Rad, U.S.A.) was used as a substrate, and pro-
tein signals were detected by the ChemiDoc imaging system
(Bio-Rad). GelAnalyzer software (Version 2010) was used to
determine the intensities of the protein bands. The intensity
of the ACE2 protein band was normalized to the intensity of
the β-actin band in each sample. Finally, fold-changes of the
expression of the ACE2 protein in all samples relative to the
controls were calculated.

**Histology** For hematoxyl and eosin stain, heart tissue
was fixed for overnight in 4 percent of formalin, dehydrated
and embedded in paraffin. Paraffin embedded samples were
sectioned at 3 µm thickness. Light microscopy was used to
examine the slides.

**Statistical Analyses** Statistical analyses were carried out
using Prism software (GraphPad v7, San Diego, CA, U.S.A.).
All values are presented as mean ± standard deviation (S.D.).
Data were analyzed using one-way ANOVA and the Tukey’s
multiple comparisons test. A p-value less than 0.05 was con-
sidered statistically significant.

**RESULTS**

**Effects of Alamandine on BP, Heart Rate and Cardiac
Contractility of 2K1C Hypertensive Rats** Figure 1 shows
the changes of SBP and diastolic blood pressure (DBP) after
sham operation in normotensive rats or after application of
renal arterial clip in 2K1C hypertensive rats and 2K1C hy-
pertensive rats that received alamandine (600 µg/kg/d) for 2
weeks.

The basal SBP/DBP obtained for normotensive and hy-
pertensive groups after 18 weeks was 121/81 ± 5/8 and
186/134 ± 11/8 mmHg, respectively. Alamandine injection
administered at a dose of 600 µg/kg/d (dose selected accord-

![Fig. 1. Time Course of the Change in Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) after Sham Operation in Normotensive
Rats or after Application of Renal Arterial Clip in 2-Kidney, 1-Clip (2K1C) Hypertensive Rats and 2K1C Hypertensive Rats That Received 2 Weeks
Infusions of Alamandine (600 µg/kg/d)
Results represent mean values ± S.D. of seven animals; *p < 0.05, **p < 0.01, ***p < 0.001 as compared with hypertensive rats.](image-url)
ing to our pilot study) for 2 weeks produced a fall in SBP and DBP (139/101 ± 9/18 mmHg) (p < 0.001) (Fig. 2).

Heart rates in normotensive and hypertensive rats were

Heart rates in normotensive and hypertensive rats were 326.3 ± 67.6 and 332 ± 38.5 beats/min, respectively. No significant changes were observed after alamandine administration (336 ± 43.4 beats/min). In the normotensive and hypertensive rats, +dP/dt(max) was 4144 ± 422 and 3763 ± 416.4 mmHg/s, and −dP/dt(max) was −3600 ± 240.5 and −3492 ± 334.2 mmHg/s, respectively. After 2 weeks of infusion with alamandine, the +dP/dt(max) increased to 4358 ± 405.4 and −dP/dt(max) reached to −3993 ± 321.6 mmHg/s, which were significantly higher than hypertensive rats (p < 0.05) (Fig. 3).

Hypertensive rats had higher basal LVEDP and LVSP compared to the normotensive rats, i.e., (24.2 ± 2.9 vs. 24 ± 1.7 mmHg, p < 0.001) and (183.5 ± 10.5 vs. 121 ± 4 mmHg, p < 0.001), respectively. Moreover, alamandine caused a significant reduction in LVEDP and LVSP levels (8.3 ± 2 vs. 141.3 ± 9.2, respectively) (p > 0.001) (Fig. 4).

Fig. 2. Change in SBP (A) and DBP (B) in 2K1C Hypertensive Rats Induced by a 2-Week Infusion of Saline or Alamandine (600 µg/kg/d), Compared to the Normotensive Group, in Anesthetized Rats

Results represent mean values ± S.D. of seven animals; *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 3. Change in +dP/dt(max) (A) and −dP/dt(max) (B) in 2K1C Hypertensive Rats Induced by 2-Week Infusions of Saline or Alamandine (600 µg/kg/d) Compared to the Normotensive Group in Anesthetized Rats

Results represent mean values ± S.D. of seven animals; *p < 0.05.

Fig. 4. Change in Left Ventricular Systolic Pressure (LVSP) (A) and Left Ventricular End-Diastolic Pressure (LVEDP) (B) in 2K1C Hypertensive Rats Induced by 2-Week Infusions of Saline or Alamandine (600 µg/kg/d) Compared to the Normotensive Group in Anesthetized Rats

Results represent mean values ± S.D. of seven animals; ***p < 0.001.

Fig. 5. Fold-Change in ACE2 mRNA Levels of the Heart in 2K1C Hypertensive Rats Induced by 2-Week Infusions of Saline or Alamandine (600 µg/kg/d) Compared to the Control Group (Normotensive, Normalized as 1)

Results represent mean values ± S.E. of seven animals; *p < 0.05, **p < 0.01.
Effects of Alamandine on ACE2 mRNA and Protein Expression in the Heart of 2K1C Hypertensive Rats

ACE2 mRNA expression levels in heart tissues of the hypertensive rats were significantly increased by 1.5 ± 0.7-fold relative to the normotensive group (p < 0.05) (Fig. 5); Alamandine infusion in hypertensive rats decreased ACE2 mRNA expression to 0.9 ± 0.3-fold relative to the normotensive group, which was significantly lower than the hypertensive group (p < 0.01), but was not significantly different as compared to normotensive rats (Fig. 5).

A non-significant change in ACE2 protein expression was observed in the hypertensive rats (1.2 ± 0.6-fold) and hypertensive rats that received alamandine (1.1 ± 0.5-fold) compared to the normotensive rats (Fig. 6).

Alamandine Attenuated Cardiac Hypertrophy and Fibrosis

To confirm cardiac hypertrophy, fibrosis and fibrillar collagen we performed a hematoxylin and eosin (H&E) stain (Fig. 7). The staining revealed that 2K1C hypertension increased cardiac fibrosis, fibrillar collagen and hypertrophy when compared with the normotensive group which was restored by alamandine administration (600 µg/kg/d) for 2 weeks.

DISCUSSION

In our study, we investigated the effects of prolonged systemic infusion of alamandine on cardiovascular indices and ACE2 expression during the chronic phase of renovascular hypertension in a 2K1C rat model. Our results showed a significant decrease in mean arterial pressure (MAP), LVSP, DBP and SBP when alamandine was infused for two consecutive weeks, with a concomitant increase in +dP/dt(max) and −dP/dt(max). The data were partly consistent with previous
results which indicated that endothelium-dependent vasodilation mediated by alamandine in aortic rings could be attenuated by N-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor).

Our previous work showed that a 15-min infusion of alamandine in the early phase of renovascular hypertension had a biphasic effect, i.e., transient increases in MAP, LVSP, +dP/dt(max) and −dP/dt(max), followed by long-lasting reductions in these parameters, which extended over the rest of the infusion period. However, the present study demonstrated that, in the chronic phase of renovascular hypertension, prolonged alamandine infusion produced only one hypotensive response that was accompanied by an increase in contractility.

The hypotensive effect of alamandine in the chronic phase of renovascular hypertension is consistent with previous studies demonstrating that the drug via Mas-related G protein-coupled receptor D (MrGd), promotes vasodilatory and antihypertensive actions through endothelial nitric oxide synthase (eNOS) activity and nitric oxide generation. Alamandine has a hypotensive effect when microinjected into the caudal ventrolateral medulla of 2K1C hypertensive rats and has a pressor effect when administered to the rostral ventrolateral medulla of Fisher rats. Shen et al. have previously shown that the administration of alamandine to the hypothalamic paraventricular nucleus increased blood pressure and enhanced sympathetic activity via its MrGd receptor and that the cAMP-protein kinase A (PKA) pathway was involved in mediating these effects.

We observed that in the hypertensive rats, +dP/dt(max) and −dP/dt(max) were lower, and LVEDP was significantly higher than normotensive rats. An LVEDP level >16 mmHg has been considered to be a sign of left ventricular diastolic dysfunction. Alamandine decreased LVEDP in hypertensive rats which is most likely a result of improved cardiac function after alamandine using.

Improvement in heart function and cardiomyocyte hypertrophy was seen in Ang-(1–7)-treated mice. Alamandine pretreatment ameliorated cardiac function and hypertrophy associated with hypertension. Even though alamandine and Ang-(1–7) activate distinct receptors, both have an antihypertrophic effect that is related to nitric oxide (NO) production in cardiac cells. It has been found that MrGd expression was higher in the hearts of hypertensive rats compared with those of normotensive rats, and Ang II treatment increased MrGd expression in cardiomyocytes. It has recently been shown that hypertension increased PKA expression in the heart. PKA may be involved in cardiac hypertrophy and LV dysfunction, and alamandine decreased cardiac hypertrophy by inhibiting the PKA pathway. Alamandine has an anti-fibrotic effect in cardiac muscle. It has been shown, a significant decrease in the accumulation of collagen I, collagen III, and fibronectin in isoproterenol-treated rats when they were given alamandine in a dose of 50 µg/kg daily. This is in agreement with our histological results showing increased cardiac hypertrophy, fibrosis and fibrillar collagens in the 2K1C hypertensive rats, as compared to the normotensive group. Myocardial fibrosis may restrict myofibrillar movement, increased myocardial stiffness, depressed systolic function and thus impair overall cardiac function. These effects were attenuated by alamandine administration (600 µg/kg/d) for 2 weeks.

Alamandine does not increase calcium levels in cardiomyocytes, but it increases cardiac contractility by sensitizing the myofilament to calcium by activation of PKC. In a more recent study, de Jesus et al. observed that alamandine, via MrGd receptors, has protective effects on cardiomyocytes. MrGd receptors have an antihypertrophic role in cardiomyocytes via AMP-activated protein kinase (AMPK) activation and NO production. AMPK which plays a key role in protecting the heart is the mediator of alamandine actions in cardiac cells. Alamandine antihypertrophic action goes beyond the RAS system since alamandine antagonized cardiac hypertrophy induced by phenylephrine. Alamandine decreased lipopolysaccharide (LPS)-induced inflammation and apoptosis in the heart. Alamandine pre-treatment inhibited LPS-induced increases in inflammatory cytokines and decreased caspase-3 and caspase-9 levels and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cardiomyocytes in the heart. Also, a-myosin heavy chain (MHC) and β-MHC decreased after LPS administration, and these effects were reversed by alamandine. These results indicated that alamandine improved LPS-induced cardiac dysfunction and increased cardiac contractility. Additionally, alamandine has cardioprotective effects on ischemia–reperfusion Injury by activating the antioxidant and antiapoptotic enzymes via the MrGd receptor. Alamandine improved reperfusion-induced changes in LVEDP, LVDP, ±dP/dt, and coronary blood flow. Thus, in the chronic phase of 2K1C, alamandine may increase contractility by improving cardiac function.

In this study, we also examined the effects of alamandine on ACE2 expression in the heart. ACE2 in the heart is present in various cells including cardiomyocytes and cardiac fibroblasts. Also, ACE2 is largely expressed in the vascular endothelial cells of the heart and is a critical regulator of heart function in vivo. A previous study on ACE2-knockout mice showed abnormal heart function due to the absence of ACE2 protein. In both human and rats, the localization of ACE2 in the heart, and endothelial cells of intra-myocardial blood vessels and smooth muscle cells, indicates that ACE2 plays a role in local vasodilation control.

The ACE2 expression is greatly influenced by various pathological disease conditions. It is possible that, under some circumstances, such as in certain pathophysiologic settings, the counter-regulatory effects of ACE2 increases.

In our study, ACE2 gene expression was increased in the hearts of the hypertensive group. When alamandine was administered for 2 weeks, gene expression of ACE2 returned to the normotensive level. This suggests that the increased ACE2 gene level may be a compensatory response to heart failure and that alamandine could be responsible for the decrease in ACE2 gene expression by improving heart function. However, in this study, there were no significant differences in cardiac protein levels of ACE2 between all groups. The mRNA level of a gene does not usually correlate with its protein level. It has been shown that transcription, mRNA decay, translation, and protein degradation are all important determinants of protein concentrations. One of the main post-translational modification is ubiquitination. Ang II is responsible for internalization and lysosomal degrading of ACE2. Thus, an increase in tissue Ang II level during the chronic phase of 2K1C hypertension may play a role in the degradation of the protein by lysosomes. Thus our finding indicates that in the chronic phase of renovascular hypertension ACE-2 as the...
protective arm of RAS increases. Renin–angiotensin system is composed of both pressor and depressor arms. Imbalances between these opposing arms may be responsible for the development of cardiovascular disorders. There is mounting evidence of the beneficial effects of the depressor arm of RAS in hypertension and different types of disease. Most of the currently existing treatment plans for hypertension is based on suppression of the pressor arm of RAS via ACE inhibitors, or angiotensin II type 1 receptor blockers. However, activation of the depressor arm may have more therapeutic effects.

In conclusion, alamandine showed beneficial effects on the cardiac index in the chronic phase of 2K1C hypertensive rats. Alamandine has a hypotensive and positive inotropic cardiac effect in this hypertension model. Thus, alamandine can be considered as a therapeutic target for the treatment of hypertension in this phase, especially in the failing heart. Further studies are, however, needed to clarify the function of alamandine in the cardiovascular system.

**Conflict of Interest** The authors declare no conflict of interest.

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