Differences in Cardiovascular Responses to Alamandine in Two-Kidney, One Clip Hypertensive and Normotensive Rats

Ava Soltani Hekmat, PhD; Kazem Javanmardi, PhD; Amin Kouhpayeh, PhD; Ehsan Baharamali, MD; Mojtaba Farjam, PhD

Background: Alamandine is a newly discovered component of the renin-angiotensin system, which regulates blood pressure. In this study, the effect of alamandine on cardiovascular parameters in two-kidney, one clip (2K1C) hypertensive rats and normotensive rats, and the possible roles of the angiotensin II type 1 receptor (AT1R) and the PD123319-sensitive receptors in mediating this effect was investigated.

Methods and Results: The cardiovascular parameters were monitored for 10 min before the infusion of the drugs or saline, and for 30 min afterward. In the 2K1C hypertensive rats, alamandine caused brief increases in mean arterial pressure (MAP), left-ventricular systolic pressure (LVSP) and maximum rate of pressure change in the left ventricle (dP/dt(max)). This was followed by decreases in these parameters, which extended throughout the remainder of the infusion period. Losartan, an AT1R blocker, abolished alamandine’s initial pressor effect and PD123319, which can block AT2R and Mas-related G protein-coupled receptor D (MrgD) receptors, partially decreased the late depressor effect. Left ventricular end-diastolic pressure (LVEDP) decreased during alamandine infusion; this effect was reduced by PD123319. In the normotensive rats, alamandine increased MAP, LVSP, dP/dt (max), and it decreased LVEDP during the infusion period. These effects of alamandine were reduced by losartan.

Conclusions: The results of this investigation suggest that, under normal conditions, alamandine acts via AT1R, but in pathological conditions such as hypertension, its effect on PD123319-sensitive receptors masks its effect on AT1R.

Key Words: Alamandine; Hypertension; Renin-angiotensin system; 2K1C

Despite major progress in the research on the pathogenesis and management of hypertension and other aspects of cardiometabolic disease, these conditions underlie a significant number of cases of morbidity and mortality due to cardiovascular and chronic kidney disease. It is predicted that there will be an increase in the hypertension prevalence rate in the United States (US). Currently, this condition affects 29% of the population in this country; that is, ~65 million individuals.1

The significance of the renin-angiotensin system (RAS) in the development of hypertension and cardiovascular disease is well known.2 Chronic RAS activation is a significant causal factor associated with the gradual malfunction of organs such as blood vessels, the kidneys and the heart.2 These consequences of RAS activation have encouraged the development of medicines that can deactivate the RAS and thereby prevent the associated pathologies. The first class of drugs aimed at RAS inhibition are known as angiotensin-converting enzyme (ACE1) inhibitors, and they came on the market in the late 1970s.3 A second class of drugs that target the RAS are known as angiotensin II type 1 receptor (AT1R) blockers. These drugs block angiotensin II (Ang II) – the primary active peptide in the RAS, which has many effects on blood vessels, the heart, the brain and the kidneys – from activating AT1R. AT1R is one of the two well-characterized receptors that Ang II activates (in addition to angiotensin II type 2 receptor (AT2R)).4 Losartan is an angiotensin receptor blocker,4 which mainly antagonizes AT1R, while PD123319 can block AT2R and Mas-related G protein-coupled receptor D (MrgD) receptors.5

Recently, numerous researchers have shown that the RAS has additional complex multifunctional peptides, enzymes and receptors. The discovery of the ACE2/angiotensin-(1–7) [Ang-(1–7)]/Mas receptor axis,6,7 which counterbalances several functions of the ACE/Ang II/AT1R axis, has highlighted the complexity of the RAS. The ACE2/Ang-(1–7)/Mas receptor axis acts as a counter-regulatory axis.7 It involves the heptapeptide peptide known as Ang-(1–7), which acts through a G-protein-coupled receptor called Mas.8 Ang-(1–7) can be hydrolyzed from Ang II by the action of peptidases such as ACE2 and prolyl endopeptid-
Alamandine, a heptapeptide that is formed by the removal of the C-terminal phenylalanine residue (Phe8) from Ang A, is another newly discovered component of the RAS. It binds to MrgD receptors. The only difference betweenalamandine and Ang-(1–7) is the existence of an N-terminal alanine residue (Ala1) in alamandine. Alamandine can be formed in the rat heart after perfusion with Ang-(1–7). However, the mechanism by which it is synthesized remains unidentified. Alamandine produce endothelium-dependent vasodilation of aortic rings. This vasodilation can be diminished by the nitric oxide (NO) synthase inhibitor, N-nitro-L-arginine methyl ester.

A previous study showed that alamandine reduced blood pressure when it was injected into the caudal ventrolateral medulla of Fischer rats. Alamandine has been shown to exhibit cardiac antifibrotic properties in Sprague-Dawley rats.

As in rat plasma, alamandine is found in human plasma, and it has been shown to increase in patients with end-stage renal disease. However, the role of alamandine in humans is unknown.

As RAS contains both a pressor and depressor arm, and the role of alamandine on cardiovascular functions is not fully understood, this study was carried out in order to assess the effects of alamandine on cardiovascular parameters in an acute model of renovascular hypertension, and the role of AT1R and PD123319-sensitive receptors in mediating these effects.

**Methods**

Alamandine was obtained from Peptide Institute, Inc. (Osaka, Japan), PD 123319 from Tocris Bioscience (Bristol, UK) and losartan from Merck (Darmstadt, Germany). Male Sprague-Dawley rats (180–230 g) were obtained from the Experimental Animal Centre of Fasa University of Medical Sciences. Only rats with a systolic arterial pressure of >160 mmHg were included in the study. They were exposed to a 12-h light:dark cycle and they were given standard rat chow and water ad libitum. The experiments conformed to the national guidelines for conducting animal studies, and the study was approved by the Ethics Committee at Fasa University of Medical Sciences.

The animals were divided into 2 main groups: the 4-weeks’ 2K1C hypertensive rats (Group I) and the normotensive rats (Group II).

The first part of the study examined the effects of alamandine on cardiovascular parameters in the 2K1C hypertensive rats, and the possible roles of AT1R and PD123319-sensitive receptors in mediating these effects. The rats in Group I (n=56) were randomly allocated to 8 subgroups. Three of these subgroups were assigned intravenous infusions of alamandine in one of the following doses: 25 µg/kg (Alamandine 25), 50 µg/kg (Alamandine 50) or 75 µg/kg (Alamandine 75). One subgroup was assigned intravenous infusions of alamandine (75 µg/kg) plus PD 123319 (30 µg/kg) (PD123319+Alamandine 75), one was assigned alamandine (75 µg/kg) plus losartan (10 mg/kg) (Losartan+Alamandine 75), one was assigned PD123319 (30 µg/kg) alone (PD123319), and one was assigned losartan (10 mg/kg) alone (Losartan). The control group was assigned intravenous infusions of saline.

The second part of the study examined the effects of alamandine on cardiovascular parameters in the normotensive rats. The rats in Group II (n=28) were randomly allocated to 4 subgroups. One subgroup was assigned intravenous infusions of alamandine (75 µg/kg) plus PD 123319 (30 µg/kg) (PD123319+Alamandine 75), one was assigned alamandine (75 µg/kg) plus losartan (10 mg/kg) (Losartan+Alamandine 75), the control group was assigned intravenous infusions of saline. All the infusions were carried out over a 15-min period in volumes of 1.5 mL.

In order to prepare the 2K1C rats, the animals were anesthetized using intraperitoneal injections of ketamine (80 mg/kg) and xylazine (10 mg/kg). A 4-cm longitudinal incision was made in the skin and in the abdominal wall of the left flank of each rat. The left renal artery of each rat was exposed and separated from the renal vein, and a solid Plexiglas clip with a 0.2 mm cleft diameter was placed around it. Procaine penicillin-G powder (30,000 U) was applied to the incisions. The skin and abdominal layers were sutured using 4-0 silk threads and 5-0 chromic catgut, respectively. After the animals recovered from the anesthesia, they were kept in individual cages under standard conditions.

After 4 weeks, the 2K1C rats, along with the normotensive rats, were anesthetized using sodium thiopental (50 mg/kg, i.p.). A polyethylene catheter (PE-50) was placed in the right carotid artery, leading to the left ventricle of each rat. The right femoral artery was also cannulated. The catheters were attached to pressure transducers, which were utilized for the measurement of the left ventricular pressure and the arterial blood pressure. The left jugular vein was also cannulated to allow for intravenous injections. The heart rate (HR), mean arterial pressure (MAP), maximal rate of increase of left ventricular pressure during systole (+dP/dt(max)) and maximal rate of decrease of pressure during diastole (−dP/dt(max)), left-ventricular systolic pressure (LVSP) and left ventricular...
Cardiovascular Responses to Alamandine

PD 123319 partially blocked alamandine’s depressor effect, but not the pressor effect (Figure 2B.D). In the normotensive rats, alamandine (70 μg/kg) induced an increase in MAP and LVSP during the infusion period and for a few minutes afterward (Figure 3A,B). This effect was eliminated by

end-diastolic pressure (LVEDP) were recorded using a 4-channel Power Lab physiology system (ADInstruments, Australia). The cardiovascular parameters were monitored for 10 min before the injection of the drugs or saline, and for 30 min afterward.

Statistical Analysis
For the 7 rats in each group, the mean of each parameter and the standard error of the mean (SEM) of each parameter were calculated. The variations in the parameters with regard to comparisons between the treatment groups and the control group were analyzed using analysis of variance (ANOVA) followed by the Holm-Sidak multiple comparison test, using Sigma Plot software (version 11.0). The significance level was set at P<0.05.

Results
In each main group before the infusion period, the cardiovascular parameters, HR, MAP, +dP/dt (max), −dP/dt (max), LVSP and LVEDP were comparable between the subgroups. The HR was not significantly altered by alamandine. Figures 1–8 show the time-courses of changes in MAP, LVSP, +dP/dt (max), −dP/dt (max) and LVEDP in 2K1C hypertensive rats and normotensive rats.

In the 2K1C hypertensive rats, alamandine produced a dose-dependent biphasic arterial and left ventricular pressure response; that is, a brief increase in MAP and LVSP followed by a decrease that continued for the remainder of the infusion period. After the infusion period, an increase in MAP and LVSP was observed for a few minutes (Figure 1A,B). Losartan abolished the pressor component of the response to alamandine (Figure 2A,C). In contrast,
For a few minutes after the infusion period, non-significant increases in +dP/dt(max) and −dP/dt(max) were observed. Losartan abolished alamandine's initial pressor effect (Figure 5A, C), and PD 123319 partially decreased alamandine's late depressor effect (Figure 5B, D). In contrast, in the normotensive rats, alamandine increased +dP/dt(max) and −dP/dt(max) during the infusion period and for a few minutes afterward. Losartan, but not PD 123319, significantly decreased this effect (Figure 6A, B).

Compared to the normotensive rats, the 2K1C hypertensive rats had markedly higher basal +dP/dt (max) and −dP/dt (max) values. In the 2K1C hypertensive rats, alamandine induced a significant dose-dependent increase in +dP/dt (max) and −dP/dt (max) in comparison to the control group. Subsequently, the values of these 2 parameters decreased significantly compared to the control group, and this decrease continued during the infusion period (Figure 4A, B). For a few minutes after the infusion period, non-significant increases in +dP/dt(max) and −dP/dt(max) were observed. Losartan abolished alamandine’s initial pressor effect (Figure 5A, C), and PD 123319 partially decreased alamandine’s late depressor effect (Figure 5B, D). In contrast, in the normotensive rats, alamandine increased +dP/dt (max) and −dP/dt (max) during the infusion period and for a few minutes afterward. Losartan, but not PD 123319, significantly decreased this effect (Figure 6A, B).
Cardiovascular Responses to Alamandine during the infusion period and for a few minutes afterward, and PD 123319 reduced this effect. Previous studies have demonstrated that some components of the renin-angiotensin system have similar biphasic properties. An example is the ANG II biphasic pressure response; that is, an increase followed by a decrease that has been observed in rabbits.

Additionally, Scheuer and Perrone showed that in anesthetized rats, administration of ANG III produced a much more consistent biphasic pressure response compared with ANG II. They also demonstrated that in anesthetized rats, AT1 and AT2 receptors mediated the pressor and the depressor phase of the response, respectively. In agreement with these data are the results that revealed the pressor actions of Ang II are increased in the AT2-null mice model, and this pressor action is increased with chronic administration of the AT2R antagonist, PD123319, in rats. In addition, Ang-(1–7), which is structurally and functionally very similar to alamandine, also has biphasic effects on rat aortic rings.

In our study, the HR of the rats was not significantly influenced by alamandine. In addition, the early pressor effects of alamandine during the infusion period and for a few minutes afterward caused a decrease in LVSP, +dP/dt(max) and −dP/dt(max), which were abolished by losartan. The brief increases were followed by long-lasting reductions in these parameters, which extended over the rest of the infusion period and were reduced by PD 123319. Following the infusion period, non-significant increases in these parameters were observed for a few minutes. In contrast, LVEDP decreased during the infusion period and for a few minutes afterward, and PD 123319 reduced this effect.

**Discussion**

The present study demonstrated that, in renovascular hypertensive rats, alamandine produced a dose-dependent biphasic hemodynamic effect. This involved brief increases in MAP, LVSP, +dP/dt (max) and −dP/dt(max), which were abolished by losartan. The brief increases were followed by long-lasting reductions in these parameters, which extended over the rest of the infusion period and were reduced by PD 123319. Following the infusion period, non-significant increases in these parameters were observed for a few minutes. In contrast, LVEDP decreased during the infusion period and for a few minutes afterward, and PD 123319 reduced this effect.

Previous studies have demonstrated that some components of the renin-angiotensin system have similar biphasic properties. An example is the ANG II biphasic pressure response; that is, an increase followed by a decrease that has been observed in rabbits. Additionally, Scheuer and Perrone showed that in anesthetized rats, administration of ANG III produced a more consistent biphasic pressure response compared with ANG II. They also demonstrated that in anesthetized rats, AT1 and AT2 receptors mediated the pressor and the depressor phases of the response, respectively. In agreement with these data are the results that revealed the pressor actions of Ang II are increased in the AT2-null mice model, and this pressor action is increased with chronic administration of the AT2R antagonist, PD123319, in rats. In addition, Ang-(1–7), which is structurally and functionally similar to alamandine, also has biphasic effects on rat aortic rings.

In our study, the HR of the rats was not significantly influenced by alamandine. In addition, the early pressor
response to alamandine was accompanied by falls in LVEDP, which is most likely a result of an increase in ventricular depletion in the inotropic state due to the effect of alamandine. Thus, HR and preload are not determinants of the initial pressor response to alamandine.

Muscle contractility has been shown to be closely connected with ion currents.18 Alamandine is a member of the angiotensin family, and angiotensin has been shown to change the ion currents.19 Considering that the functional properties of alamandine seem to be similar to Ang-(1–7), and it has been shown that Ang-(1–7) has an agonistic effect on AT1R,20 we hypothesize that the pressor response may result from the activation of AT1R. In this study, the pressor effect was abolished by losartan. One of the mechanisms by which AT1R mediates its effect is activation of phospholipase C (PLC). This activation can result in the generation of inositol triphosphate (IP3) and the subsequent mobilization of Ca2+, or the production of diacylglycerol and the resultant activation of protein kinase C (PKC).21 When inotropy is increased, the Frank-Starling curve shifts up and to the left, indicating an increase in stroke volume and a reduction in LVEDP.22

Alamandine does not increase levels of Ca2+ in cardiomyocytes, but it may regulate cardiac contractility by sensitizing the myofilament to Ca2+ via activation of PKC.23 Changes in the affinity of the contractile elements for Ca2+ (as well as changes in the magnitude of the Ca2+ transient) can help to regulate the contraction of cardiomyocytes.18 Further studies are needed to elucidate alamandine’s mechanism of action regarding cardiac contractility.

Alamandine’s depressor effects were significantly larger than its pressor effects. The hypotensive effect of alamandine in the depressor phase is consistent with previous than its pressor effects. The hypotensive effect of alamandine was blocked by PD123319. This suggests that PD123319 is also an antagonist for MrgD.5 So when we talk about the receptors that are blocked by PD123319, it would be more appropriate to cite them as PD123319-sensitive receptors rather than AT2 receptors. This term covers both AT2 and MrgD receptors. However, this should be taken into account, that in this study, PD123319 did not completely block the depressor phase of response in 2K1C hypertensive rats, implying that other receptors may also be involved. This result needs to be investigated further.

MrG is the additional receptor for angiotensin (1–7). MrG leads to an increase in adenyl cyclase activity, consequently increasing cAMP levels, thereby activating protein kinase A, and leading to cAMP response element-binding protein phosphorylation.24 Additionally, it has been shown that alamandine, via the MrG receptor, can promote the stimulation of eNOS and the subsequent generation of NO, and thereby exerts its vasodilatory and anti-hypertensive actions.5

The AT2R receptor stimulates various signaling pathways, which can be categorized into 3 broad types: regulation of protein phosphorylation, activation of phospholipases, and regulation of NO/cyclic guanosine monophosphate (cGMP).25,26

Thus, the depressor phase may be due to the activation of PD123319-sensitive receptors and subsequent vasodilation, which can cause reduced peripheral resistance and even venous return.

It seems that both AT1 and PD123319-sensitive receptors were activated simultaneously during alamandine infusion. The force generation can be altered by 2 mechanisms. The first mechanism is the changes in inotropy (length-independent activation) and the second one is changes in fiber length (preload; length-dependent activation). When AT1 receptors are stimulated, a positive inotropic effect is induced (first mechanism). However, venous return to the heart is decreased by the vasodilator effect of alamandine through the PD123319-sensitive receptors. This causes a gradual decrease in preload (LVEDP) of the ventricle and results in a reduction in contractility (second mechanism). Gradual decline in LVEDP and contractility produced by PD123319-sensitive receptors overcome the contractility stimulation generated by AT1 receptors. This causes the pressor response to switch to the depressor response.

In the normotensive rats, only alamandine’s presser effect was observed. MAP, LVSP, +dP/dt(max) and −dP/dt(max) increased, and LVEDP decreased during the infusion period and for a few minutes afterwards. Losartan, but not PD123319, significantly reduced this effect, indicating the

Figure 8. Time-course of changes in LVEDP in normotensive rats induced by 15-min infusions of saline, alamandine (75µg/kg), alamandine (75µg/kg) plus PD123319 (30µg/kg), and alamandine (75µg/kg) plus losartan (10mg/kg). The values represent the mean±SEM. The significance tests all involve comparisons with the control group: *P<0.05. Abbreviations as in Figures 2,7.5

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Alamandine 75</th>
<th>Alamandine 75 + PD123319</th>
<th>Alamandine 75 + Losartane</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.5</td>
<td>3.0</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>2.5</td>
<td>2.8</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>2.0</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td>10</td>
<td>2.0</td>
<td>1.5</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>15</td>
<td>1.5</td>
<td>1.0</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>30</td>
<td>1.0</td>
<td>0.5</td>
<td>0.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Cardiovascular Responses to Alamandine

role of AT1R in mediating the pressor response in normotensive rats.

It has been shown that situations of intensified RAS activity cause the downregulation of AT1 receptors, whereas a reduction in the activity of the RAS upregulates the AT1 receptor. In transgenic rats that overexpress the renin gene, cardiac and vascular AT1 receptor expression is downregulated. The AT1 receptor downregulation may be a compensatory protective response to the activated RAS.

Additionally, expression of the AT2 receptors within the heart under normal conditions is extremely low; however, during hypertension, its expression increases to the degree that its action predominates over that of the AT1 receptors. AT2R messenger ribonucleic acid (mRNA) is upregulated in the aorta of mice with early 2K1C hypertension, and AT2R activation limits Ang II-induced aortic contractions in 2K1C hypertensive rats (via the phosphorylation of endothelial NO synthase and the increased production of vascular cGMP). Thus, the increased expression of AT1 receptors and the decreased expression of PD123319-sensitive receptors in the normotensive rats shows that AT1R upregulated in the aorta of mice with early 2K1C hypertension and the decreased expression of PD123319-sensitive receptors in the normotensive rats may be responsible for the alamandine’s pressor effect that was observed in the normotensive rats.

It is possible that alamandine’s pressor effect masks the depressor response in normotensive rats. As there could be substantial alterations in the relative numbers of AT1R and PD123319-sensitive receptors during the development of hypertension, it would be useful to determine the effects of AT1R and PD123319-sensitive receptor blockades during different stages of hypertension.

In summary, our results indicate that, in renovascular hypertensive rats, alamandine produced a biphasic hemodynamic effect. This involved a brief pressor effect mediated by AT1R, followed by a long-lasting depressor response mediated, in part, by PD123319-sensitive receptors. In normotensive rats, only alamandine’s pressor effect was observed, which was mediated by AT1R. In addition, the data suggest that the RAS is composed of both pressor and depressor systems. Impulses between these opposing systems may contribute to the development of hypertension. There is a growing body of research for beneficial effects of the depressor arm of the RAS axis in hypertension and several other disorders. Most of the currently existing treatment strategies of hypertension is based on suppression of the pressor arm of RAS with ACE inhibitors and AT1R blockers. However, it is time to consider that simultaneous activation of the depressor arm may have more beneficial effects.

Our results indicate that alamandine, as a counterbalancing novel RAS, may be developed as a therapeutic target for the treatment of hypertension.

Acknowledgments

This research has been supported by Fasa University of Medical Sciences.

Conflicts of Interest

There are no conflicts of interest.

References