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A Non-peptide Angiotensin II Type 2 Receptor Agonist Does Not Attenuate Post-Myocardial Infarction Left Ventricular Remodeling in Mice

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Abstract

Cardiac overexpression of the angiotensin II type 2 receptor (AT₂R) attenuates left ventricular (LV) remodeling after myocardial infarction (MI) in transgenic mice. We hypothesized that a novel nonpeptide AT₂R agonist, Compound 21 (C21), would attenuate post-MI LV remodeling. Fifty nine mice were studied for 28 days after 1 hour surgical occlusion-reperfusion of the left anterior descending coronary artery. Immediately thereafter, 23 mice received 0.3 mg/kg/d of C21 via Alzet osmotic mini-pump, 16 received 10 mg/kg/d of the AT₁R antagonist candesartan in drinking water and 20 were untreated controls. Cardiac magnetic resonance imaging (CMR) measured ejection fraction (EF), LV end-systolic and end-diastolic volumes (ESVI, EDVI) indexed to weight serially post-MI. Infarct size was measured on day 1 by late gadolinium-enhanced CMR. At baseline, heart rate, blood pressure, EDVI, ESVI, and EF were similar between groups. Mean infarct size (42–45% of LV mass) was similar between groups. C21-treated animals demonstrated adverse LV remodeling (increased EDVI and ESVI at all post-MI time points) compared to control. Candesartan-therapy preserved LVEF at day 28 compared to the C21-treated group. Thus, direct stimulation of the AT₂R by C21 at 0.3 mg/kg/d does not attenuate post-MI LV remodeling in reperfused MI in mice.

Keywords

myocardial infarction; remodeling; magnetic resonance imaging; angiotensin II; angiotensin receptors

Introduction

The deleterious role of increases in angiotensin II (Ang II) in cardiovascular disease is well established¹. The role of the Ang II receptors in post-myocardial infarction (MI) left ventricular (LV) remodeling has been an object of intense study in recent years. Antagonism

of type 1 receptors (AT₁R) is known to be beneficial post-MI as an alternative to angiotensin converting enzyme inhibition (ACE-I)², whereas the role of the AT₂R is less clear. Cardiac magnetic resonance imaging (CMR) data from our laboratory in a transgenic mouse of cardiac overexpression of the AT₂R demonstrated that baseline LV function is improved and post-MI remodeling is attenuated³. Pharmacologic antagonism of AT₁Rs with losartan was not additive to AT₂-R overexpression, whereas genetic knockout of AT₁Rs (AT_{1A}Rs) further attenuated remodeling⁴. This implies that upregulation of the AT₂R may be responsible for much of the benefits of AT₁R antagonism. Treatment with the nitric oxide synthase inhibitor L-NAME abrogated the AT₂R mediated protection against LV remodeling, suggesting that nitric oxide may underlie this benefit⁵. AT₂R's protective effect was not directly mediated via the bradykinin subtype 2 receptor (B₂R) pathway⁶.

Translating this potential benefit into larger animals including humans has suffered from the lack of an adequate receptor agonist. There are very few selective AT₂R agonists available and those that are, such as CGP42112A, are peptides that are readily metabolized and are partial agonists/antagonists at high concentrations. Another pharmacological AT₂R antagonist, PD123319, has suffered from low selectivity when applied at high doses, leading to contradictory findings⁷. However, a selective AT₂R agonist, Compound 21 (C21) is now available that is 25,000 times more selective for the AT₂R than for the AT₁R, which allows investigators the opportunity to specifically stimulate the AT₂R in *in vitro* and *in vivo* settings⁸. Previous studies in a rat model suggest potential benefit of this compound in the post-MI setting⁹.

We hypothesized that direct AT₂R stimulation with C21 would attenuate remodeling in the post-MI mouse heart. To test this hypothesis, we used CMR to measure LV size and global function post-MI in control mice, C21-treated mice, and mice treated with the AT₁R inhibitor candesartan beginning after the infarction. Infarct size was measured on day 1 to be certain these therapies were not differentially affecting infarct size. Thus, any subsequent effect would be on LV remodeling and the progression to congestive heart failure.

Methods

Mouse Model

Animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996) and were approved by the University of Virginia Animal Care and Use Committee. Seventy seven male wild type C57BL/6 mice, age 8 to 14 weeks, were studied before and after MI for a period of 28 days. Eighteen mice died post-operatively, leaving 59 mice in the study including 20 control, 23 C21-treated mice, and 16 candesartan-treated mice. The surgical infarction and reperfusion techniques have been previously described¹⁰. Briefly, the coronary artery was occluded for 60 minutes by tying down silk suture over a short length of PE-20 tubing with successful occlusion verified by myocardial color change and ECG changes. After 60 minutes, reperfusion was achieved by cutting the suture. In previous studies, this length of occlusion leads to an infarct that subtends approximately 65% of the risk area¹¹. The chest wall was closed in layers and mice recovered with 100% oxygen.

Magnetic Resonance Imaging

Anesthesia was induced with 3.0% inhaled isoflurane and maintained with 1.0–1.25% isoflurane administered via nosecone during imaging at baseline before (day 0) and days 1, 7, and 28 post-MI (Figure 1). Imaging was performed on a 7-Tesla ClinScan (Bruker/Siemens, Erlangen, Germany) with an MRI-compatible physiological monitoring and gating system designed for small animals (SA Instruments, Inc., Stony Brook, NY). All mice were

imaged using a 30 mm diameter cylindrical birdcage radiofrequency (RF) coil. A 2D fast, low-angle shot (FLASH) sequence was used to obtain orthogonal long-axis images. For simultaneous assessment of LV size, global and regional LV function, and infarct size, continuous true-short-axis gradient echo (blackblood) cine MRI was performed from the base to the apex with an ECG-triggered 2D cine FLASH sequence¹² (Figure 1). The echo time (TE) was 1.9 ms, and the repetition time (TR) was adjusted continuously to obtain 16 equally spaced phases through each cardiac cycle. Other parameters include a 15° flip angle, 25.6 × 25.6 mm field of view (FOV), matrix of 128 × 128 pixels, 4 averages and slice thickness of 1.0 mm. This yields a spatial resolution of 200 × 200 × 1000 μm. For late gadolinium enhanced (LGE) imaging of myocardial infarct size on day 1 post-MI (Figure 2), 0.3 mmol/kg of Gd (Gadodiamide, Omniscan, GE Healthcare) was infused intraperitoneally 20 minutes prior to imaging. An inversion recovery sequence was used, with a 180° inversion pulse, TR of 3.9 ms, TE of 1.9 ms, 30° flip angle and 25.6 mm FOV.

Therapy—Twenty three mice received 0.3 mg/kg/d of C21 delivered by surgically placed Alzet osmotic mini-pumps from immediately after the time of infarction to day 28 post-MI. This dose has previously been shown to attenuate post-MI remodeling in Wistar rats⁹. Sixteen mice received candesartan 10 mg/kg/d in drinking water, a dose validated in prior post-MI studies¹³, from after the time of infarction to day 28 post-MI. The remaining controls (n=20) did not have an Alzet minipump implanted nor anything added to their drinking water.

Invasive Hemodynamics—Invasive measurement of LV filling pressures was performed on all mice after the day 28 post-MI MRI. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium 50 μg/g body weight enhanced by local subcutaneous injection of 0.5% Bupivacaine 100 μl. LV pressure was obtained by direct puncture of the LV via left 5th interspace with a 27G needle connected to PE-50 tubing and pressures as well as heart rate were recorded by a MacLab recording System.

AT₂R Protein Levels—Plasma membranes from whole mouse hearts were isolated using a triton solubilized 100,000 × G membrane preparation based upon the method of Nagamatsu et al with slight modifications¹⁴. Protein concentrations were quantified using a bicinchoninic acid protein (BCA) assay (Pierce) and subjected to Western blot analysis. Sodium dodecylsulfate (SDS) samples were then prepared, separated by SDS-PAGE (10% polyacrylamide gels), and transferred onto a nitrocellulose membrane by electroblotting. Membranes were incubated overnight with AT₂R antibody (Chemicon 1:200) at 4°C and subsequently incubated with infrared secondary antibody (anti-rabbit IRDye 800nm; 1:15,000, Licor Biosciences). Immunoreactivity and quantitative assessment of band densities were performed using the Odyssey Infrared Imaging System (Licor Biosciences). The blots were then incubated with SimplyBlue SafeStain (Invitrogen) in order to stain total protein loaded per lane. Immunoreactivity was performed using the Odyssey Infrared Imaging System and quantitative assessment of band densities was performed using MacBiophotonics ImageJ software. Results from 14 controls, 11 candesartan-treated, and 18 C21-treated were reported as a ratio of densitometric units of AT₂R to total protein loaded.

Image Analysis

Quantitative image analysis was performed using the ARGUS (Siemens Healthcare, Princeton, NJ) image analysis program to quantify end-diastolic (EDV) and end-systolic volumes (ESV) and ejection fraction (EF). EDV and ESV were indexed to body weight in grams (EDVI, ESVI). Infarct size quantification, also using the ARGUS program, was performed on the day 1 LGE images¹⁵ (Figure 2). For this calculation, LGE regions were planimeted and the total infarct area calculated as a percentage of LV mass.

Statistical Analysis

Parameters of LV size and function over time were compared between 3 groups using repeated measures analysis of variance (ANOVA) using Sigma Stat (v. 2.0 for Windows, SPSS, Chicago, IL). Infarct size, hemodynamic parameters, and AT₂R protein levels were compared between groups by one way ANOVA. All values are presented as mean $\pm$ S.D; $P < 0.05$ was considered significant.

Results

Baseline LV Size and Function

LV EDVI, ESVI, and EF were similar amongst the 3 groups at baseline as shown in Table 1, Figures 3 and 4.

Infarct Size

MI size as defined by LGE CMR on day 1 post-MI was similar between control, C21-treated and candesartan-treated mice ($45 \pm 12\%$, $45 \pm 11\%$, $42 \pm 13\%$; respectively, $p = \text{NS}$) as illustrated in Figure 2.

Post-MI LV Size and Function

In all 3 groups, EDVI and ESVI increased stepwise during the course of post-MI remodeling with most of the dilatation occurring by day 7 (Table 1). Similarly, EF fell between baseline and day 1, further by day 7 and then stabilized in all groups. EDVI was higher in C21 than control at each time point after infarction. ESVI was higher in C21 than control at day 7 and day 28 post-MI. No difference in EDVI or ESVI was noted between candesartan-treated and control mice. EF was best preserved in the candesartan-treated group such that at day 28 LVEF was $37 \pm 10\%$, higher than C21 ($27 \pm 12\%$) but not statistically different than control mice ($29 \pm 11\%$).

Invasive Hemodynamics—LV end-systolic pressure was lower in the candesartan-treated group at day 28 post-MI compared to controls (Table 2) due to the vasodilatory properties of AT₁R antagonism. C21 was blood pressure neutral (Table 2) as has been true in past studies^{8,9}. Otherwise, heart rate and LV end-diastolic pressure and were similar between groups.

AT₂R Protein Levels—AT₂R protein levels, expressed as a ratio of densitometric units of AT₂R to total protein loaded (Figure 5), were higher in C21-treated mice (1.21 ± 0.17 , mean $\pm$ S.E., $p < 0.05$) than control (0.72 ± 0.06) suggesting AT₂R upregulation by AT₂R agonism in the post-MI setting. No difference was found between and the candesartan-treated group (1.11 ± 0.16) and control.

Discussion

In the present study, 59 infarcted mice were studied over 28 days post-MI, 20 of which were untreated controls, 23 treated with a novel non-peptide agonist of the AT₂R (C21) and 16 treated with the AT₁R inhibitor candesartan. As expected, AT₁R antagonism attenuated the decline in LV ejection fraction post-MI. In contrast, AT₂R agonism and receptor upregulation were not beneficial and, in fact, were associated with adverse parameters of LV remodeling compared to control as evidenced by the increase in LVEDVI and LVESVI. In this study, direct stimulation of the AT₂R by C21, at the 0.03mg/kg/d dose, did not attenuate post-MI LV remodeling.

LV remodeling or the increase in LV size over time post-MI, is associated with adverse outcomes including congestive heart failure (CHF) and death. A principal goal of pharmacologic therapy post-MI is to prevent this process. ACE-I remain the standard of care in patients with post-MI LV dysfunction while AT₁R antagonists are commonly used substitutes in ACE-I intolerant patients. Both therapies have been shown to be beneficial in this setting², with the edge to ACE-I.

It is well established that Ang II in the heart causes vasoconstriction, cellular hypertrophy and interstitial fibrosis¹⁶. However, most of the recognized effects of Ang II are mediated through the AT₁R, while physiologic functions of the AT₂R in myocardium remain under scrutiny. The AT₂R is generally thought to inhibit ventricular growth and remodeling¹⁷ and induce vasodilatation¹⁸. A 2–3-fold increase in AT₂R mRNA has been demonstrated in non-infarcted and infarcted regions of the 1-week post-MI rat heart¹⁹. Antagonism of the AT₂R with selective antagonists reduces endothelial and fibroblast DNA synthesis in the heart and negatively impacts cardiac output and stroke volume in a rat model of MI²⁰. Our group has shown that overexpressing the AT₂R in the heart leads to attenuation of post-infarct LV remodeling³ and that this process is mediated by nitric oxide⁵, but not by bradykinin⁶. Overexpression of AT₂Rs was equivalent to AT₁R antagonism⁴, suggesting that much of the benefit of AT₁R antagonism could be due to AT₂R upregulation. Thus, there is significant potential for a new class of agents that are direct AT₂R agonists that could be additive to therapy in post-MI LV dysfunction.

Studying the effects of the AT₂R remains challenging, primarily due to the lack of reliable tools to directly activate the AT₂R. Previously, the only available AT₂R agonists were metabolically unstable peptides (CGP42112A and p-amino-Phe⁶)^{21–23} while the only available AT₂R antagonist, PD123319, is nonselective when used at higher doses⁷. The emergence of the first non-peptide AT₂R agonist, C21, has allowed investigators to selectively stimulate this receptor in both *in vitro* and *in vivo* settings⁸.

Recently, it was reported that the use of C21 in post-MI Wistar rats may attenuate post-myocardial remodeling, leading to reduced scar volumes as assessed by MRI, improved hemodynamic parameters as assessed by Millar catheter, and improved LV structural changes as assessed by transthoracic echocardiography⁹. In fact, remodeling attenuation was reported at both the 0.03 and 0.3 mg/kg/d doses. While data from this study are encouraging, limitations in study design create potential problems with interpretation of the data. For example, induced MI's led to small overall infarct scar sizes on day 7, ranging from 14.6 ± 1.4% in the C21-treated group to 20.5 ± 1.5% in controls⁹. Measuring infarct size on day 7 rather than on day 1 is potentially problematic because the therapy itself may have altered infarct size and remodeling of noninfarcted myocardium, leading to incorrect sizing of the infarcts by that time point. It would have been important to ensure that infarct sizes were similar on day 1 post-MI. In addition, infarct size drives subsequent LV remodeling, so if infarct size is different, it follows that LV remodeling will be attenuated in the group with the smaller infarct. Thus, the main conclusion that can be drawn from the study of Kaschina et al is that C21 attenuates infarct size. Its effect on LV remodeling cannot be separated from the latter effect.

Benefits of AT₂R agonism on infarct size have been shown in the cerebrovascular bed. When administered centrally, the AT₂R agonist CGP42112A reduced infarct volume in the setting of stroke in a conscious rat model and minimized the loss of AT₂R expression in the infarcted region²⁴. The authors suggested that AT₂Rs reduce oxidative stress during ischemia based on an inverse relationship between superoxide production and AT₂R expression. In this study there was no tonic benefit of AT₂R expression as the AT₂R inhibitor PD123319 had no effect on outcomes. A major difference between this study and

the present study was that the stimulation of the AT₂R occurred prior to the initial injury whereas in the present experiments, therapy was not begun until after the reperfused infarction had occurred.

There are several potential reasons for the lack of benefit and tendency towards adverse effects of C21. Firstly, the 0.3mg/kg/d dose may not be the ideal dose in mice despite its proven benefits in the rat model⁹. Secondly, mean infarct sizes in the current study were quite large (ranging from 42–45%). The larger infarct sizes could have overridden any potential benefit of C21 at the dose studied. Future studies might include not only a range of infarct sizes, but also a range of C21 doses. It may be that the amount of increased activity with AT₂R overexpression in mice that we have shown to be beneficial in this setting which is likely to be an increase of more than 50-fold³ is greater than can be achieved with direct agonism. In addition, it may be that the prime effect of AT₂R agonism in this setting is reduction in infarct size as it was in prior studies in MI⁹ and stroke²⁴ models.

In conclusion, AT₂R activation with Compound 21 at the dose studied is ineffective in attenuation of post-infarct LV remodeling in the mouse model, especially when compared to the AT₁R inhibitor candesartan. Although previous studies suggest that C21 is associated with reduction in infarct size, the present study does not support the concept that C21 reduces LV remodeling, raising doubt regarding its potential efficacy in the heart failure setting.

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Figure 1.

Representative end-systolic black blood cine imaging at baseline, day 0, (top row) and day 28 (bottom row) in a control mouse (left), C21-treated mouse (center), and candesartan-treated mouse (right.) Note the cavity dilatation and regional wall thinning at end-systole at day 28 most pronounced in the control and C-21 treated example.

Figure 2.

Representative day 1 late gadolinium enhancement (LGE) images of the mid-LV in a control (left), C21-treated mouse (center), and candesartan-treated mouse (right). LGE infarct areas (bright regions) are seen in the distribution of the left coronary artery in the anterolateral free wall in all mice with similar sizes. The black arrowheads point to the border between the bright infarcted tissue and the dark normal myocardium. Mean infarct sizes by LGE CMR, when summed over the entire LV, were similar between groups.

Figure 3.
LV end systolic volume index at each time point for all 3 groups. Data shown are mean $\pm$ S.D.. * $P < 0.05$ vs. control

Figure 4. Ejection fraction at each time point for all 3 groups. Data shown are as mean $\pm$ S.D.. †p<0.05 vs. C21

Figure 5.

Top: Representative western blots measuring the AT₂R band at 42 KD as well as total protein for each treatment group. Data represent mean ± SE. **Bottom:** Western blot analysis of whole cell membranes from day 28 post-MI mouse hearts (control n=14, candesartan-treated n=11, and C21 treated n=18) demonstrating higher AT₂R protein expression following C-21 treatment than control. Data is presented as the ratio of AT₂R protein expression over total protein loaded.

Table 1

LV end-diastolic and end-systolic volume indexed to body weight in grams and ejection fraction

	EDVI ($\mu\text{L/g}$)	ESVI ($\mu\text{L/g}$)	EF (%)
Day 0			
Control (n=20)	1.56 $\pm$ 0.35	0.62 $\pm$ 0.23	62 $\pm$ 7
C21 (n=23)	1.84 $\pm$ 0.45	0.74 $\pm$ 0.30	61 $\pm$ 8
Candesartan (n=16)	1.73 $\pm$ 0.39	0.74 $\pm$ 0.29	58 $\pm$ 10
Day 1			
Control	1.98 $\pm$ 0.50	1.17 $\pm$ 0.46	42 $\pm$ 10
C21	2.39 $\pm$ 0.44 [*]	1.50 $\pm$ 0.34	37 $\pm$ 7
Candesartan	2.32 $\pm$ 0.40	1.47 $\pm$ 0.44	37 $\pm$ 9
Day 7			
Control	2.63 $\pm$ 0.57	1.82 $\pm$ 0.60	32 $\pm$ 11
C21	3.08 $\pm$ 0.69 [*]	2.30 $\pm$ 0.71 [*]	27 $\pm$ 11
Candesartan	2.82 $\pm$ 0.70	1.95 $\pm$ 0.66	33 $\pm$ 9
Day 28			
Control	2.77 $\pm$ 0.85	1.96 $\pm$ 0.78	29 $\pm$ 11
C21	3.34 $\pm$ 0.82 [*]	2.43 $\pm$ 0.74 [*]	27 $\pm$ 12
Candesartan	3.03 $\pm$ 0.78	1.96 $\pm$ 0.77	37 $\pm$ 10 [†]

All results expressed as mean $\pm$ S.D.^{*} $P < 0.05$ vs. control,[†] $P < 0.05$ vs. C21

Table 2

Invasive Hemodynamic Parameters on Day 28

	HR (bpm)	LVESP (mmHg)	LVEDP (mmHg)
Control	521 ± 38	101 ± 8	4 ± 3
C21	552 ± 67	94 ± 8	6 ± 5
Candesartan	559 ± 25	80 ± 14 [*]	5 ± 8

HR, Heart Rate; LVESP, LV End-Systolic Pressure; LVEDP, LV End-Diastolic Pressure;

*
p<0.01 vs. control,