Chronic Losartan Treatment Up-Regulates AT$_1$R and Increases the Heart Vulnerability to Acute Onset of Ischemia and Reperfusion Injury in Male Rats

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Abstract

Inhibition of angiotensin II type 1 receptor (AT$_1$R) is an important therapy in the management of hypertension, particularly in the immediate post-myocardial infarction period. Yet, the role of AT$_1$R in the acute onset of myocardial ischemia and reperfusion injury still remains controversial. Thus, the present study determined the effects of chronic losartan treatment on heart ischemia and reperfusion injury in rats. Losartan (10 mg/kg/day) was administered to six-month-old male rats via an osmotic pump for 14 days and hearts were then isolated and were subjected to ischemia and reperfusion injury in a Langendorff preparation. Losartan significantly decreased mean arterial blood pressure. However, heart weight, left ventricle to body weight ratio and baseline cardiac function were not significantly altered by the losartan treatment. Of interest, chronic in vivo losartan treatment significantly increased ischemia-induced myocardial injury and decreased post-ischemic recovery of left ventricular function. This was associated with significant increases in AT$_1$R and PKC$\delta$ expression in the left ventricle. In contrast, AT$_2$R and PKC$\varepsilon$ were not altered. Furthermore, losartan treatment significantly increased microRNA (miR)-1, -15b, -92a, -133a, -133b, -210, and -499 expression but decreased miR-21 in the left ventricle. Of importance, addition of losartan to isolated heart preparations blocked the effect of increased ischemic-injury induced by in vivo chronic losartan treatment. The results demonstrate that chronic losartan treatment up-regulates AT$_1$R/PKC$\delta$ and alters miR expression patterns in the heart, leading to increased cardiac vulnerability to ischemia and reperfusion injury.

Introduction

Heart disease is the leading cause of morbidity and mortality in the United States. In addition to other risk factors, clinical and animal studies have shown an association between angiotensin II receptor (ATR) expression on cardiomyocytes and increased risk of ischemic heart disease
and reduced cardiac recovery after ischemic injury [1–5]. Myocardial ischemia is a common form of cardiopathology. The heart may experience prolonged ischemia under a variety of conditions, including cardiomyopathy, endothelial dysfunction and coronary arterial disease, vascular dysfunction and hypotension. Animal studies suggest a link between pre-conditioning the heart with angiotensin II type 1 receptor (AT₁R) blockers and cardiac protection in ischemia and reperfusion (IR) injury [6–8]. AT₁R is predominately found in the adult heart and its expression is up-regulated after IR injury [1]. The AT₂R is predominately found in the fetal and neonatal heart and its expression declines as the heart matures. AT₁R plays an important role in the regulation of blood pressure, fluid, electrolyte balance, and is involved in pathological conditions such as cardiac remodeling and inflammation [5]. While the effect of AT₂R is considered to be the opposite of AT₁R, the role of AT₂R in the heart is less clear. However, one study indicates AT₂R is a direct antagonist by binding to AT₁R forming heterodimerization [9].

Angiotensin II is the main activator of AT₁R and AT₂R. The systemic renin-angiotensin system (RAS) plays an important role in the regulation of angiotensin II levels in the circulation. In addition, the local and tissue RAS also contributes significantly to the angiotensin II production and function [10, 11]. In the heart, the local production of angiotensin II by the cardiac RAS, as well as other alternative pathways, plays a key role in the maintenance of an appropriate cellular milieu balancing stimuli inducing and inhibiting cell growth and proliferation, as well as mediating adaptive responses to myocardial stress, for example, after myocardial ischemic injury [10].

Studies in rats have shown that ischemia leads to dysregulation of ATRs in the heart and that acute pretreatment with AT₁R blockers prior to ischemia may lead to both cardioprotective and deleterious effects [3, 8, 12, 13]. In one study, acute AT₁R blockade impaired post-ischemic left ventricular recovery and increased AT₁R mRNA, but did not change AT₁R or AT₂R protein levels [13]. However, in an in vivo study of acute IR, AT₁R blockade provided improved recovery of left ventricular function, which was dependent on AT₂R [14]. These data suggest that the outcome of cardiac recovery in IR injury is dependent on the expression levels and activity of AT₁R and AT₂R. The expression profiles of AT₁R and AT₂R are, in turn, influenced by the time and duration of AT₁R blockade as well as IR injury [15, 16].

Thus, the effects of AT₁R on the acute onset of myocardial ischemia and reperfusion injury and the recovery of ventricular function immediately after ischemic injury in the heart remain controversial, depending on systemic vs. local blockade, as well as chronic vs. acute blockade of AT₁R. The present study addresses the question of whether chronic in vivo inhibition of AT₁R by losartan modulates cardiac AT₁R and AT₂R expression, cardiac vulnerability to the acute onset of myocardial ischemia and reperfusion injury, and the recovery of ventricular function after ischemic injury in male rats. Because protein kinase C delta (PKCδ) and protein kinase C epsilon (PKCe) play pivotal roles in the regulation of myocardial ischemic injury and the mechanisms for increased heart susceptibility to IR injury involve the up-regulation of PKCδ and down-regulation of PKCe expression in the heart as demonstrated in previous studies, [17–19] we investigated the effects of AT₁R blockade on PKCδ and PKCe expression in the left ventricle. In addition, given the recent findings that altered microRNA (miR) expression profiles are involved in cardiovascular disease including ischemic heart disease,[20–23] and that studies have shown the implication of dysregulation of miR-1, -15a-5p, -15b, -21, -24, -92a, -133a, -133b, -210, -214, -320, and -499 in the development of cardiopathology including arrhythmia, cardiac remodeling, angiogenesis, and regulation of cardiomyocyte survival [24–26]. The present study also determined the effects of chronic in vivo inhibition of AT₁R by losartan on miR expression profiles in the heart. Herein, we present evidence in male rats that chronic AT₁R blockade up-regulates AT₁R/PKCδ and alters miR expression profiles in the left
ventricle, leading to increased cardiac vulnerability to acute onset of myocardial ischemia and reperfusion injury and decreased recovery of left ventricular function immediately after ischemic injury.

**Materials and methods**

**Experimental animals**

Six month old male Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). There were two experimental protocols. In protocol 1, animals were randomly divided into two groups: saline control and losartan (Sigma, 10 mg/kg/day) treatment, continuously administered by osmotic pumps (Alzet model 2ML4, Durect Co) at 60 μl/day for 14 days. The hearts were then isolated and treated with ischemia and reperfusion (IR) in the absence of losartan in the perfusate. In protocol 2, animals were treated with saline or losartan for 14 days, and the hearts were treated with IR in the continuous presence of losartan (1 μmol/L) in the perfusate. Each group had 4–6 animals. Rats were anesthetized with isoflurane (5% for induction and 3% for maintenance) and osmotic pumps with saline or losartan were implanted subcutaneously between the shoulder blades. Femoral arteries were catheterized by polyethylene tubing (PE-10) to measure blood pressure (BP) during the treatment. The adequacy of anesthesia was determined by the loss of a pedal withdrawal reflex and any other reaction from the animal in response to pinching the toe, tail, or ear of the animal. After 14 days of treatment, hearts were isolated for functional studies and protein and miR measurements. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University, and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Hearts subjected to ischemia and reperfusion**

Hearts were isolated and retrogradely perfused with Krebs-Heinseleit buffer (in mmol/L: NaCl 118.5, NaHCO3 25, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, Glucose 11, and CaCl2 2, pH 7.4) via the aorta in a modified Langendorff apparatus with a pressure transducer connected to a saline-filled balloon inserted into the left ventricle to assess ventricular function [18]. After the 30 minutes of baseline recording, hearts were subjected to 20 minutes of global ischemia by stopping the flow, followed by 60 minutes of reperfusion in the absence or presence of losartan (1 μmol/L) before ischemia during the baseline recording and throughout the period of ischemia and reperfusion (Fig 1). Left ventricular developed pressure (LVDP), heart rate (HR), dP/dtmax, dP/dtmin, and LV end diastolic pressure (LVEDP) were continuously recorded.

**Measurement of myocardial infarct size**

Myocardial infarct size was measured as described previously [18]. In brief, at the end of reperfusion, left ventricles were collected, cut into four slices, incubated with 1% triphenyltetrazolium chloride solution for 15 minutes at 37°C, and immersed in formalin for 30 minutes. Each slice was then photographed separately, and the areas of myocardial infarction in each slice were analyzed by computerized planimetry (Image-Pro Plus), corrected for the tissue weight, summed for each heart, and expressed as a percentage of the total left ventricle weight.

**Western immunoblotting**

Protein was isolated from left ventricles, and AT1R, AT2R, PKCε, and PKCδ protein abundance was measured with Western blot analysis using the primary antibodies (Santa Cruz) against AT1R (1:1000), AT2R (1:2000), PKCε (1:3000) and PKCδ (1:500), as described previously [3].
Each experimental group had samples from five animals. To assure equal loading and minimize variability among gels, samples were normalized to GAPDH.

Measurement of miRs by real-time qRT-PCR

Mature miRs were analyzed by miScript II RT kit (Qiagen) and miScript SYBR Green PCR kit with miScript Primer Assay kit (Qiagen) according to manufacturer’s instructions. Briefly, RNA was isolated from left ventricles and cDNA for mature miR profiling was prepared using the miScript II RT kit. Mature miRa were determined by real-time PCR using the miScript SYBR Green PCR kit (Qiagen). cDNA template was diluted to 1 ng/μl in RNase free water. Two nanograms of template cDNA were used for miRs quantification in a final volume of 25 μl system containing specific primers and QuantiTect SYBR Green PCR master mix following manufacturer’s instructions. Primers included miScript Universal Primer, rat-specific miScript mature miRNA primers and SNORD61 miScript Primer Assay (Qiagen). Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification. The real-time PCR was performed in triplicate and threshold cycle numbers were averaged for each sample using IQ5 real-time PCR (BioRad). The expression levels of each mature miR in control and losartan-treated heart tissues were computed following the method described by Livak and Schmittgen,[27] and expressed as fold of SNORD61.

Data analysis

Data are expressed as mean ± S.E.M. Statistical analysis (P<0.05) was determined by repeated measure ANOVA or Student’s t test, where appropriate.

Results

Effect of chronic in vivo losartan treatment on mean arterial blood pressure, heart rate, heart weight and heart to body weight ratio

As shown in Fig 1, losartan administration by subcutaneous osmotic implantation caused a significant decrease in systolic, diastolic and mean arterial blood pressure throughout 14 days of the treatment. There were no significant differences between saline control and losartan-treated groups in baseline heart rate (saline, 257.0 ± 63.0 vs. losartan, 245.0 ± 47.0, P>0.05), heart weight (saline, 1.7 ± 0.1g vs. losartan, 1.6 ± 0.2g, P>0.05), body weight (saline, 439.5 ± 26.2g vs. losartan, 465.6 ± 22.3g, P>0.05) and the left ventricle to body weight ratio (saline, 3.1 ± 0.3 mg/g vs. losartan, 2.9 ± 0.4 mg/g, P>0.05) after 14 days of treatment (Table 1).
Effect of chronic in vivo losartan treatment on heart susceptibility to acute onset of ischemic injury

Hearts were isolated from animals treated with saline or losartan for 14 days, and were studied in a Langendorff preparation. There were no significant differences in left ventricle developed pressure (LVDP), heart rate (HR), dP/dt\text{max} and dP/dt\text{min} at the baseline between the two groups (Table 1). Global ischemia for 20 minutes caused a significant impairment in LV function in both groups. As shown in Fig 2, compared with the control group, there were significant decreases in post-ischemic recovery of LVDP, dP/dt\text{max} and dP/dt\text{min} in the losartan treatment group. Recovery of HR was not significantly different between the control and losartan groups (Fig 2). As shown in Fig 3, global ischemia caused myocardial infarction and increased left ventricle end diastolic pressure (LVEDP). Compared with the control group, chronic in vivo losartan treatment significantly increased post-ischemic LVEDP and myocardial infarct size (Fig 3).

Effect of chronic in vivo losartan treatment on AT1R, AT2R and PKC isozyme expression

The protein abundance of AT1R, AT2R, PKCε and PKCδ in the left ventricle was determined by Western blot analysis. As shown in Fig 4, AT1R levels were significantly increased in the losartan treatment group than those in the saline control (P<0.05). Unlike AT1R, AT2R protein abundance was not significantly altered by the losartan treatment. Differential expression of PKC isozyme proteins was also identified in the left ventricle. Whereas there was no significant difference in PKCε protein levels between saline control and losartan-treated animals, the level of PKCδ was significantly greater in the losartan treatment group than those in the saline control (P<0.05) (Fig 4).

Effect of chronic in vivo losartan treatment on miR expression profiles in the left ventricle

Although several recent studies have reported the signature of miR expression profiles in IR-related injuries, the results have varied depending on the duration of ischemia and direct correlation between ATR activity and miR expression in the heart remains largely elusive [20, 28]. As shown in Fig 5, miR-1, -15b, -92a, -133a, -133b, -210, and -499 levels were significantly increased in the left ventricle of animals with chronic in vivo losartan treatment, as compared to the saline control group (P<0.05). In contrast, miR-21 expression was significantly increased in the left ventricle of animals with chronic in vivo losartan treatment, as compared to the saline control group (P<0.05).
decreased in the left ventricle (P<0.05). The losartan treatment had no significant effect on the expression of miR-15a-5p, -24, -214, and -320 in the heart.

Effect of chronic in vivo losartan treatment on heart susceptibility to acute onset of ischemic injury in the presence of losartan during ex vivo perfusion of the heart

We further determined the functional significance of the in vivo losartan treatment-induced increase in AT1R in modulating heart vulnerability to acute onset of ischemic injury by blocking AT1R with losartan during the ex vivo perfusion of the heart in a Langendorff preparation. As shown in Table 2, there were no significant differences in baseline LV function between the saline control and in vivo chronic losartan treatment groups in the presence of losartan (1 μmol/L) during the ex vivo perfusion of the heart in the isolated heart preparation. Of importance, continued blockade of AT1R with losartan during ex vivo perfusion of the heart blocked the in vivo chronic losartan treatment-induced increase in heart susceptibility to acute onset of ischemic injury. As shown in Fig 6, in the presence of losartan during the heart perfusion in a Langendorff preparation, there were no significant differences in post-ischemic recovery of LVDP, HR, dP/dt max and dP/dt min between the saline control and in vivo chronic
Losartan treatment groups. Consistent with these findings, there were no significant differences in myocardial infarct size and LVEDP between the two groups (Fig 7).

**Fig 3. Effect of chronic losartan treatment on myocardial ischemic injury.** Losartan (10 mg/kg/day) or saline were administered to male rats via osmotic pumps for 14 days. Hearts were isolated and were subjected to 20 minutes of ischemia and 60 minutes of reperfusion in a Langendorff preparation. Left ventricular end diastolic pressure (LVEDP) was measured during reperfusion (A). Myocardial infarct size (B) was determined at the end of reperfusion and expressed as a percentage of the total left ventricle weight, as described in Methods. LVEDP data were analyzed by repeated measure ANOVA, and infarct size by t-test. *P < 0.05, losartan vs. control; n = 4.

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**Fig 4. Effect of chronic losartan treatment on protein expression in the left ventricle.** Losartan (10 mg/kg/day) or saline were administered to male rats via osmotic pumps for 14 days. Protein abundance of AT₁R, AT₂R (A), PKCδ, PKCe (B) in the left ventricle was determined by Western blot analyses. Data were analyzed by t-test. *P < 0.05, losartan vs. control; n = 6.

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Discussion

The present study demonstrates that in vivo chronic AT₁R blockade with losartan leads to a significant increase in AT₁R and PKCδ protein expression as well as an ischemic-sensitive signature of miR expression in the left ventricle, resulting in an increase in the heart vulnerability.

Table 2. Effect of chronic losartan (10 mg/kg/day) treatment on heart and body weight and pre-ischemic left ventricle function in the continued presence of losartan in ex vivo heart perfusion.

|               | Baseline | Saline     | Losartan |
|---------------|----------|------------|----------|
| HR (b.p.m)    | 275.0±33.0| 259.0±11.0 |          |
| BW (g)        | 470.5±25.1| 445.5±13.6 |          |
| HW (g)        | 2.0±0.1   | 1.8±0.1    |          |
| LVW (g)       | 1.5±0.1   | 1.3±0.0    |          |
| LVW/BW (mg g⁻¹) | 3.1±0.3   | 2.9±0.4    |          |
| dP/dt_max (mmHg s⁻¹) | 4367.0±83.6| 4401.0±263.3|       |
| dP/dt_min (mmHg s⁻¹) | 2654.0±54.5| 2511.0±108.6|       |
| LVDP (mmHg)   | 110.1±5.6  | 108.9±3.2  |          |

HR, heart rate; BW, body weight; HW, heart weight; LVW, left ventricular weight; LV, left ventricle; LVDP, left ventricular developed pressure; Data are mean ± SEM, n = 4.

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to acute onset of ischemic and reperfusion injury and a decrease in post-ischemic left ventricular function recovery in an isolated heart preparation. In addition, this heightened heart susceptibility to acute ischemic injury induced by chronic *in vivo* losartan treatment is inhibited by the continued blockade of AT1R during *ex vivo* heart perfusion, suggesting a role for the increased AT1R expression in the left ventricle.

In studies involving *acute* losartan treatment, inhibition of AT1R lead to improved cardiac recovery, but the potential gain in function was abrogated by AT2R inhibition [14, 29]. This suggests that during AT1R inhibition angiotensin II concentrations increase, resulting in increased activation of the AT2R, conferring cardioprotection via production of nitric oxide [29, 30]. In the present study, chronic *in vivo* losartan treatment did not increase AT2R density, but increased AT1R expression. It has been shown that cardiac overexpression of the AT2R attenuates left ventricle remodeling after myocardial infarction [31]. Therefore, in addition to our findings, it is plausible that AT2R acts as a fail-safe switch to counteract the AT1R during increased levels of angiotensin II. However, there may be a limit to which AT2R can compensate and the increase in AT1R activity may overwhelm the AT2R ability to antagonize AT1R.

Previous studies with chronic antagonism of AT1R reported varying results. In one study, 11 and 3 weeks treatment with losartan or UP269-6, a noncompetitive AT1R blocker, increased AT2R protein, but caused no change in AT1R [32]. In addition, while losartan was able to preserve, but not improve, post-ischemic LV function, UP269-6, considered a more effective AT1R antagonist, resulted in a significant decrease in LV recovery [32]. Other studies have

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**Fig 6. Effects of *ex vivo* AT1R blockade on chronic *in vivo* losartan-modulated post-ischemic recovery of LV function.** Losartan (10 mg/kg/day) or saline were administered to male rats via osmotic pumps for 14 days. Hearts were isolated and were subjected to 20 minutes of ischemia and 60 minutes of reperfusion in a Langendorff preparation in the continued presence of losartan (1 μmol/L). \( \frac{dP}{dt_{\text{max}}}(A) \), \( \frac{dP}{dt_{\text{min}}}(B) \), Left ventricular developed pressure (LVDP) (C), and heart rate (beats per minute) (D) were determined. Data were analyzed by repeated measure ANOVA.

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shown that chronic AT1R antagonism decreases infarct size, increases AT1R and AT2R mRNA, and increases PKCε protein, but no functional results were reported [15, 33]. Cardiac recovery following acute pretreatment of AT1R blockers in animal models of IR injury is variable [3, 8, 13, 34]. Thus, even subtle protocol differences may explain these variable findings. In addition, the present finding suggests that the outcome of cardiac recovery immediately after IR injury is dependent on the expression levels and activity of AT1R, which are also sensitive to protocol differences, i.e., blockade of AT1R before, during and after the ischemic insult, as well as systemic vs. direct local heart blockade or chronic vs. acute blockade of AT1R.

In the present study, the heart became more vulnerable to acute onset of ischemic injury after the chronic losartan treatment. While this finding is somewhat surprising and may appear contrary to the view that AT1R hyperactivity is implicated in ischemic injury of the heart, it indeed is likely due to the up-regulation of AT1R in the heart by chronic blockade of AT1R. Since losartan is a competitive AT1R blocker, in vivo chronic administration leads to an increase in AT1R abundance in the heart in a negative feedback manner. This increased AT1R density and activation in subsequent acute onset of ischemic insult in the isolated heart preparation was unopposed, which led to increased myocardial infarction and decreased post-ischemic recovery of left ventricular function. This notion is supported by the finding that the post-ischemic left ventricular function was preserved in in vivo chronic losartan treated group with continued losartan presence in ex vivo perfusion of the heart. Although this finding suggests that increased AT1R density in the heart may be detrimental to the heart in response to acute onset of ischemic injury, previous studies of losartan on the recovery of left ventricular function in the setting of ischemia and reperfusion injury in isolated rat hearts suggested a detrimental effect of the acute AT1R blockade [3, 12]. These findings are intriguing and suggest that the local and direct activation of cardiac AT1R in the regulation of heart response to acute onset of ischemic insult is much more complex than was previous thought and is likely to be context-specific. Thus, maintaining the normal level of AT1R activity in the heart appears

**Fig 7. Effects of ex vivo AT1R blockade on chronic in vivo losartan-modulated myocardial ischemic injury.** Losartan (10 mg/kg/day) or saline were administered to male rats via osmotic pumps for 14 days. Hearts were isolated and were subjected to 20 minutes of ischemia and 60 minutes of reperfusion in a Langendorff preparation in the continued presence of losartan (1 μmol/L). Left ventricular end diastolic pressure (LVEDP) was measured during reperfusion (A). Myocardial infarct size (B) was determined at the end of reperfusion and expressed as a percentage of the total left ventricle weight, as described in Methods. LVEDP data were analyzed by repeated measure ANOVA, and infarct size by t-test.

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important and either increased or decreased AT₁R activation is likely to be detrimental to the heart in the setting of acute ischemia and reperfusion injury.

The finding of increased PKCδ in the left ventricle provides a mechanistic insight into the understanding of losartan-mediated up-regulation of AT₁R and increased heart vulnerability to ischemic injury. Whereas PKCε has been implicated in cardioprotection, [35] increased PKCδ activity has coincided with a reduction in cardiac recovery in the setting of acute ischemia and reperfusion injury [36]. It has been shown that PKCδ inhibits glyceraldehyde-3-phosphate dehydrogenase and promotes the accumulation of mitochondria near lysosomal-like structures, which leads to an increase in mitochondrial permeability [37]. Several recent studies demonstrated that PKCδ played a key role on AT₁R-mediated inflammation, oxidative stress and cardiac remodeling with cardiac fibroblasts [38–40].

In addition, the present study demonstrates that chronic in vivo losartan treatment alters miR expression profiles in the left ventricle with a signature of heightened cardiac vulnerability to ischemic injury. Up-regulation of miR-1, -15b, -92a, -133a, and -133b have been shown to be involved in the regulations of multitude of genes in the heart, contributing to the development of arrhythmia, hypertrophy, fibrosis, and suppression of angiogenesis, and cell death and survival [41–48]. In comparison, the elevated expression of miR-210 and miR-499 are more in favor with cell survival. MiR-210 induces angiogenesis and miR-499 stimulates cardiac stem cells to commit into mature working cardiomyocytes, [49–51] albeit cardiomyocytes are terminally differentiated in mature adult hearts and there are minimal cardiac stem cells to be stimulated. In addition, miR-1, miR-15 and miR-21 can directly influence the survival of cardiomyocytes. Both miR-1 and miR-15b target Bcl-2 down-regulation [47, 48] and thus, increase the cardiomyocyte susceptibility to apoptosis in the setting of ischemia and reperfusion injury. In contrast, miR-21 has been shown to promote cardioprotection by targeting PDCD4 [52]. Thus, the finding that chronic losartan treatment significantly increased miR-1 and, particularly miR-15b of 9-fold, as well as decreased miR-21 by 50% in the left ventricle, suggests a novel mechanism of miRs in angiotensin receptor-modulated vulnerability of the heart in response to acute onset of ischemic injury. The question of whether this chronic losartan-induced, ischemic-sensitive signature of miR expression in the heart is caused by AT₁R blockade or by an unopposed activation of cardiac AT₂R remains to be determined. Future studies are needed to determine how AT₁R interaction with PKCδ and miR mediate heart susceptibility in the presence of losartan during ex vivo perfusion of the heart.

In perspective of clinical significance, inhibition of AT₁R is an important pharmacological therapy in the management of hypertension, particularly with long-term benefits in the treatment of patients in post-myocardial infarction period and at risk for ischemic heart disease. Yet the role of cardiac AT₁R in acute onset of myocardial ischemia and reperfusion injury and left ventricular function recovery immediately after the ischemic insult still remains controversial. In our first experimental protocol, animals were pretreated with losartan or saline for two weeks duration. However, losartan was absent during the IR treatment of the heart. In clinics, losartan is used in conjunction with β-blockers and diuretics in patients with high risk for coronary artery disease and patients with prior ischemic events. The first experimental protocol was designed to replicate prolonged use of losartan followed by withdrawal from losartan, which is seen in patients transitioning to angiotensin-converting-enzyme inhibitor (ACEi), non-compliance, or reduction in risk factors. Our results demonstrated that the prolonged treatment of losartan significantly increased AT₁R abundance in the myocardium, which was associated with increased IR-induced myocardial injury and decreased cardiac functional recovery. To determine whether this increased injury was indeed AT₁R-mediated, in our second experimental protocol, animals were pretreated with losartan or saline for two weeks, followed by IR treatment of the heart in the continuous presence of losartan in the perfusate. The
results showed that the presence of losartan during the IR treatment blocked the losartan pre-treatment-induced increase in myocardial injury, providing the evidence of a causal role of AT₁R in ischemic myocardial injury. Taken together in clinical perspective, these findings suggest that patients who are on prolonged losartan treatment significantly increase AT₁R in the heart and activation of elevated AT₁R in the acute setting of IR is detrimental to cardiac recovery. Therefore patients are more susceptible to ischemic heart injury during the initial withdrawal of losartan and gradual losartan withdrawal should be considered to allow sufficient time to decrease AT₁R in the myocardium. It should be noted that the present study was conducted in animals with healthy hearts. Thus, it is unclear whether chronic losartan treatment in patients who have experienced prior myocardial ischemia and infarction elevates AT₁R and increases heart susceptibility to IR. Given that remote ischemic preconditioning (RIPC) has clinical potential to minimize myocardial infarction in patients with high risk [53] and that RIPC mediates protection indirectly via remote humoral conditioning including adenosine, bradykinin, opioids, and HIF but minimal correlation with AT₁R [54, 55], the present finding may not suggest the exclusion of RIPC for patients with sartans. ARBs are an important group of antihypertensive drugs, the present finding is of critical importance in the clinical perspective, and suggests a potential serious side effect of abrupt withdrawal in the ARB treatment in an increased risk of myocardial infarction in the setting of acute ischemic injury. Whereas the present study was conducted in male rats, the previous study demonstrated that the acute and direct effects of AT₁R and AT₂R on modulating acute ischemia and reperfusion injury in rats were in a gender-independent manner [3]. Nonetheless, the effect of chronic AT₁R blockade on the heart sensitivity to acute onset of ischemic injury in females remains to be determined.

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Author Contributions
Conceived and designed the experiments: MS LZ. Performed the experiments: MS CD. Analyzed the data: MS CD LZ. Contributed reagents/materials/analysis tools: LZ. Wrote the paper: MS LZ.

References
1. Yang BC, Phillips MI, Ambuehl PE, Shen LP, Mehta P, Mehta JL. Increase in angiotensin II type 1 receptor expression immediately after ischemia-reperfusion in isolated rat hearts. Circulation. 1997; 96 (3):922–6. Epub 1997/08/05. PMID: 9264502.
2. Yan X, Price RL, Nakayama M, Ito K, Schuldtt AJ, Manning WJ, et al. Ventricular-specific expression of angiotensin II type 2 receptors causes dilated cardiomyopathy and heart failure in transgenic mice. American journal of physiology Heart and circulatory physiology. 2003; 285(5):H2179–87. Epub 2003/07/19. doi:10.1152/ajpheart.00361.2003 PMID: 12869376.
3. Xue Q, Dasgupta C, Chen M, Zhang L. Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. Cardiovascular research. 2011; 89(2):300–8. Epub 2010/09/28. doi: 10.1093/cvr/cvq303 PMID: 20870653; PubMed Central PMCID: PMC3020132.
4. Raff U, Ott C, Ruilope LM, Menne J, Haller H, Schmieder RE. Prevention of electrocardiographic left ventricular remodeling by the angiotensin receptor blocker olmesartan in patients with type 2 diabetes. Journal of hypertension. 2014; 32(11):2267–76; discussion 76. Epub 2014/10/03. doi: 10.1097/hjh.0000000000000313 PMID: 25275251.
5. Dasgupta C, Zhang L. Angiotensin II receptors and drug discovery in cardiovascular disease. Drug discovery today. 2011; 16(1–2):22–34. Epub 2010/12/15. doi: 10.1016/j.drudis.2010.11.016 PMID: 21147255; PubMed Central PMCID: PMC3022115.
6. Flynn JD, Akers WS. Effects of the angiotensin II subtype 1 receptor antagonist losartan on functional recovery of isolated rat hearts undergoing global myocardial ischemia-reperfusion. Phamacotherapy. 2003; 23(11):1401–10. Epub 2003/11/19. PMID: 14620386.
7. Ozer MK, Sahne A, Birincioglu M, Acet A. Effects of captopril and losartan on myocardial ischemia-reperfusion induced arrhythmias and necrosis in rats. Pharmacological research: the official journal of the Italian Pharmacological Society. 2002; 45(4):257–63. Epub 2002/05/29. doi: 10.1006/phrs.2002.0965 PMID: 12030787.

8. Sato M, Engelman RM, Otani H, Maulik N, Rousou JA, Flack JE 3rd, et al. Myocardial protection by preconditioning of heart with losartan, an angiotensin II type 1-receptor blocker: implication of bradykinin-dependent and bradykinin-independent mechanisms. Circulation. 2000; 102(19 Suppl 3):i346–51. Epub 2000/11/18. PMID: 11082412.

9. AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U. The angiotensin II AT2 receptor is an AT1 receptor antagonist. The Journal of biological chemistry. 2001; 276(43):39721–6. Epub 2001/08/17. doi: 10.1074/jbc.M105253200 PMID: 11507095.

10. Urata H, Nishimura H, Ganten D. Chymase-dependent angiotensin II forming systems in humans. American journal of hypertension. 1996; 9(3):277–84. Epub 1996/03/01. PMID: 8695029.

11. Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physiological reviews. 2005; 86(3):747–803. Epub 2006/07/04. doi: 10.1152/physrev.00036.2005 PMID: 16816138.

12. Ford WR, Clanachan AS, Judgutt BI. Opposite effects of angiotensin AT1 and AT2 receptor antagonists on recovery of mechanical function after ischemia-reperfusion injury in isolated working rat hearts. Circulation. 1996; 94(12):3087–9. Epub 1996/12/15. PMID: 8989113.

13. Xu Y, Kumar D, Dyck JR, Ford WR, Clanachan AS, Lopaschuk GD, et al. AT(1) and AT(2) receptor expression and blockade after acute ischemia-reperfusion in isolated working rat hearts. American journal of physiology Heart and circulatory physiology. 2002; 282(4):H1206–15. Epub 2002/03/15. doi: 10.1152/ajpheart.00839.2000 PMID: 11893553.

14. Parlakpinar H, Ozer MK, Acet A. Effects of captopril and angiotensin II receptor blockers (AT1, AT2) on myocardial ischemia-reperfusion induced infarct size. Cytokine. 2011; 56(3):688–94. Epub 2011/10/07. doi: 10.1016/j.cytto.2011.09.002 PMID: 21975128.

15. Xu Y, Menon V, Judgutt BI. Cardioprotection after angiotensin II type 1 blockade involves angiotensin II type 2 receptor expression and activation of protein kinase C-epsilon in acutely reperfused myocardial infarction in the dog. Effect of UP269-6 and losartan on AT1 and AT2-receptor expression and IP3 receptor and PKCepsilon proteins. Journal of the renin-angiotensin-aldosterone system: JRAAS. 2000; 1(2):184–95. Epub 2002/04/23. doi: 10.3317/jraas.2000.024 PMID: 11967812.

16. Xu Y, Clanachan AS, Judgutt BI. Enhanced expression of angiotensin II type 2 receptor, inositol 1,4, 5-trisphosphate receptor, and protein kinase cepsonal during cardioprotection induced by angiotensin II type 2 receptor blockade. Hypertension. 2000; 36(4):506–10. Epub 2000/10/21. PMID: 11040227.

17. Patterson AJ, Chen M, Xue Q, Xiao D, Zhang L. Chronic prenatal hyperoxia induces epigenetic programming of PKCepsilon gene repression in rat hearts. Circulation research. 2010; 107(3):365–73. Epub 2010/06/12. doi: 10.1161/circresaha.110.221259 PMID: 20538683; PubMed Central PMCID: PMC2919213.

18. Bae S, Zhang L. Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. The Journal of pharmacology and experimental therapeutics. 2005; 315(3):2115–20. Epub 2005/08/16. doi: 10.1111/j.1529-8057.2005.00125.x PMID: 16108769.

19. Kumar D, Menon V, Ford WR, Clanachan AS, Judgutt BI. Effect of angiotensin II type 2 receptor blockade on mitogen activated protein kinases during myocardial ischemia-reperfusion. Molecular and cellular biochemistry. 2004; 254(1–2):211–8. Epub 2004/03/20. PMID: 15030186.

20. D’Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. European heart journal. 2010; 31(22):2765–73. Epub 2010/06/11. doi: 10.1093/eurheartj/ehq167 PMID: 20534597; PubMed Central PMCID: PMC2980809.

21. Meder B, Keller A, Vogel B, Haas J, Sedaghat-Hamedani F, Kayvanpour E, et al. MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction. Basic research in cardiology. 2011; 106(1):13–23. doi: 10.1007/s00395-010-0123-2 PMID: 20886220.

22. Pan Z, Sun X, Ren J, Li X, Gao X, Lu C, et al. mir-1 exacerbates cardiac ischemia-reperfusion injury in mouse models. PloS one. 2012; 7(11):e50515. Epub 2012/12/12. doi: 10.1371/journal.pone.0050515 PMID: 23226300; PubMed Central PMCID: PMC3511560.

23. Song MA, Paradis AN, Gay MS, Shin J, Zhang L. Differential expression of microRNAs in ischemic heart disease. Drug discovery today. 2014. Epub 2014/12/03. doi: 10.1016/j.drudis.2014.10.004 PMID: 25461956.

24. Kukreja RC, Yin C, Salloum FN. MicroRNAs: new players in cardiac injury and protection. Molecular pharmacology. 2011; 80(4):558–64. Epub 2011/07/09. doi: 10.1124/mol.110.073528 PMID: 21737570; PubMed Central PMCID: PMC3187527.
25. Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, et al. MicroRNA-133 controls cardiac hypertrophy. Nature medicine. 2007; 13(5):613–8. doi: 10.1038/nn1582 PMID: 17468766.

26. Fiedler J, Jabzutyte V, Kirchmaier BC, Gupta SK, Lorenzen J, Hartmann D, et al. MicroRNA-24 regulates vascularility after myocardial infarction. Circulation. 2011; 124(6):720–30. Epub 2011/07/27. doi: 10.1161/circulationaha.111.039008 PMID: 21788589.

27. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protocols. 2008; 3(6):1101–8.

28. Nabialek E, Wanha W, Kula D, Jadczyk T, Krajewska M, Kowalowka A, et al. Circulating microRNAs (miR-423-5p, miR-208a and miR-1) in acute myocardial infarction and stable coronary heart disease. Minerva cardioangiologica. 2013; 61(6):627–37. Epub 2013/11/21. PMID: 24253456.

29. Jalowy A, Schulz R, Dorge H, Behrends M, Heusch G. Infarct size reduction by AT1-receptor blockade through a signal cascade of AT2-receptor activation, bradykinin and prostaglandins in pigs. J Am Coll Cardiol. 1998; 32(6):1787–96. Epub 1998/11/20. PMID: 9822110.

30. Bove CM, Yang Z, Gilson WD, Epstein FH, French BA, Berr SS, et al. Nitric oxide mediates benefits of angiotensin II type 2 receptor overexpression during post-infarct remodeling. Hypertension. 2004; 43(3):680–5. Epub 2004/01/21. doi: 10.1161/01.hyp.0000115924.94236.91 PMID: 14732725.

31. Yang Z, Bove CM, French BA, Epstein FH, Berr SS, DiMaria JM, et al. Angiotensin II type 2 receptor overexpression preserves left ventricular function after myocardial infarction. Circulation. 2002; 106(1):106–11. Epub 2002/07/03. PMID: 12093778.

32. Moudgil R, Xu Y, Menon V, Jugdutt BI. Effect of chronic AT(1) receptor antagonism on postischemic functional recovery and AT(1)/AT(2) receptor proteins in isolated working rat hearts. Journal of cardiovascular pharmacology and therapeutics. 2001; 6(2):183–8. Epub 2001/08/18. PMID: 11509925.

33. Lange SA, Wolf B, Schober K, Wunderlich C, Marquetant R, Weinbrenner C, et al. Chronic angiotensin II receptor blockade induces cardioprotection during ischemia by increased PKC-epsilon expression in the mouse heart. Journal of cardiovascular pharmacology. 2007; 49(1):46–55. Epub 2007/01/31. doi: 10.1097/JFC.0b013e31802c277f PMID: 17261963.

34. Xia Q, Patterson AJ, Xiao D, Zhang L. Glucocorticoid Modulates Angiotensin II Receptor Expression Patterns and Protects the Heart from Ischemia and Reperfusion Injury. PloS one. 2014; 9(9):e106827. Epub 2014/09/30. doi: 10.1371/journal.pone.0106827 PMID: 25265380; PubMed Central PMCID: 3690065.

35. Baines CP, Song CX, Zheng YT, Wang GW, Zhang J, Wang OL, et al. Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. Circulation research. 2003; 92(8):873–80. Epub 2003/03/29. doi: 10.1161/01.res.0000069215.36389.8d PMID: 12663490; PubMed Central PMCID: 3691672.

36. Murriel CL, Mochly-Rosen D. Opposing roles of delta and epsilonPKC in cardiac ischemia and reperfusion: targeting the apoptotic machinery. Archives of biochemistry and biophysics. 2003; 420(2):246–54. Epub 2003/12/05. PMID: 14654063.

37. Yogalingam G, Hwang S, Ferreira JC, Mochly-Rosen D. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) phosphorylation by protein kinase Cdelta (PKCdelta) inhibits mitochondria elimination by lysosomal-like structures following ischemia and reoxygenation-induced injury. The Journal of biological chemistry. 2013; 288(26):18947–60. Epub 2013/05/09. doi: 10.1074/jbc.M113.466870 PMID: 23653351; PubMed Central PMCID: 3696670.

38. Wong SL, Lau CW, Wong WT, Xu A, Au CL, Ng CF, et al. Pivotal role of protein kinase Cdelta in angiotensin II-induced endothelial cyclooxygenase-2 expression: a link to vascular inflammation. Atherosclerosis, thrombosis, and vascular biology. 2011; 31(5):1169–76. Epub 2011/02/12. doi: 10.1161/atvbaha.110.216044 PMID: 21311042.

39. Lu P, Miao SB, Shu YN, Dong LH, Liu G, Xie XL, et al. Phosphorylation of smooth muscle 22alpha facilitates angiotensin II-induced ROS production via activation of the PKCdelta-P47phox axis through release of PKCdelta and actin dynamics and is associated with hypertrophy and hyperplasia of vascular smooth muscle cells in vitro and in vivo. Circulation research. 2012; 111(6):697–707. Epub 2012/07/17. doi: 10.1161/circresaha.112.272013 PMID: 22798525.

40. Olson ER, Shamhart PE, Naugle JE, Meszaros JG. Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase Cdelta and intracellular calcium in adult rat cardiac fibroblasts. Hypertension. 2008; 51(3):704–11. Epub 2008/01/16. doi: 10.1161/hypertensionaha.107.098459 PMID: 18195168.

41. Osbourne A, Calway T, Broman M, McSharry S, Earley J, Kim GH. Downregulation of connexin43 by microRNA-130a in cardiomyocytes results in cardiac arrhythmias. Journal of molecular and cellular cardiology. 2014; 74:53–63. Epub 2014/05/14. doi: 10.1016/j.yjmcc.2014.04.024 PMID: 24819345.
42. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, et al. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nature medicine. 2007; 13(4):486–91. Epub 2007/04/03. doi: 10.1038/nm1569 PMID: 17401374.

43. Hinkel R, Penzkofer D, Zuhlke S, Fischer A, Husada W, Xu QF, et al. Inhibition of microRNA-92a protects against ischemia/reperfusion injury in a large-animal model. Circulation. 2013; 128(10):1066–75. Epub 2013/07/31. doi: 10.1161/circulationaha.113.001904 PMID: 23987866.

44. Dong DL, Chen C, Huo R, Wang N, Li Z, Tu YJ, et al. Reciprocal repression between microRNA-133 and calcineurin regulates cardiac hypertrophy: a novel mechanism for progressive cardiac hypertrophy. Hypertension. 2010; 55(4):946–52. Epub 2010/02/24. doi: 10.1161/hypertensionaha.109.139519 PMID: 20177001.

45. Karakikes I, Chaanine AH, Kang S, Mukete BN, Jeong D, Zhang S, et al. Therapeutic cardiac-targeted delivery of miR-1 reverses pressure overload-induced cardiac hypertrophy and attenuates pathological remodeling. Journal of the American Heart Association. 2013; 2(2):e000078. Epub 2013/04/25. doi: 10.1161/jaha.113.000078 PMID: 23612897; PubMed Central PMCID: PMCPmc3647279.

46. Hullinger TG, Montgomery RL, Seto AG, Dickinson BA, Semus HM, Lynch JM, et al. Inhibition of miR-15 protects against cardiac ischemic injury. Circulation research. 2012; 110(1):71–81. Epub 2011/11/05. doi:10.1161/circresaha.111.244442 PMID: 22052914; PubMed Central PMCID: PMCPmc3354618.

47. Fasanaro P, D’Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. The Journal of biological chemistry. 2008; 283(23):15878–83. Epub 2008/04/18. doi: 10.1074/jbc.M800731200 PMID: 18417479; PubMed Central PMCID: PMCPmc3259646.

48. D’Ascenzo F, Moretti C, Omede P, Cerrato E, Cavallero E, Er F, et al. Cardiac remote ischaemic preconditioning reduces periprocedural myocardial infarction for patients undergoing percutaneous coronary interventions: a meta-analysis of randomised clinical trials. EuroIntervention: journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology. 2014; 9(12):1463–71. Epub 2014/04/24. doi: 10.4244/eijv9i12a244 PMID: 24755386.

49. Hausenloy DJ, Yellon DM. Remote ischemic preconditioning: Underlying mechanisms and clinical application2008.