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Falcón, Beverly L.; Stewart, Jillian M.; Bourassa, Erick; Katovich, Michael J.; Walter, Glenn; Speth, Robert C.; Sumners, Colin; and Raizada, Mohan K., "Angiotensin II type 2 receptor gene transfer elicits cardioprotective effects in an angiotensin II infusion rat model of hypertension" (2004). *HPD Articles*. 109.
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Articles in PresS. Physiol Genomics (September 21, 2004). doi:10.1152/physiolgenomics.00170.2004

ANGIOTENSIN II TYPE 2 RECEPTOR GENE TRANSFER ELICITS CARDIOPROTECTIVE EFFECTS IN AN ANGIOTENSIN II INFUSION RAT MODEL OF HYPERTENSION

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Running Title: AT2R Prevents Cardiac Pathophysiology

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ABSTRACT

The role of the angiotensin II type 2 receptor (AT2R) in cardiovascular physiology remains elusive. We have developed an *in vivo* lentiviral vector-mediated gene transfer system to study the physiological functions of the AT2R. Our objectives in this study were to determine if the AT2R influences cardiac hypertrophy and myocardial and perivascular fibrosis in a non-genetic rat model of hypertension. Lenti-viral vector containing the AT2R or saline was injected intracardially in five-day old Sprague Dawley rats. This resulted in a persistent overexpression of the AT2R in cardiac tissues. At 15 weeks of age, animals were infused with either 200ng/kg/min of angiotensin II or saline by implantation of a 4-week osmotic minipump. This resulted in an increase in blood pressure that reached maximal by 2 weeks of treatment, and was associated with a 123% increase in left ventricular wall thickness and a 129% increase in heart weight to body weight ratios. In addition, the increase in cardiac hypertrophy was associated with a 300% and 158% increase in myocardial and perivascular fibrosis, respectively. Cardiac transduction of the AT2R resulted in an 85% attenuation of left ventricular wall thickness, 91% attenuation of HW/BW and a 43% decrease in myocardial fibrosis induced by angiotensin-infusion. These improvements in cardiac pathology were observed in the absence of attenuation of high blood pressure. Thus, our observations indicate that long-term expression of the AT2R in the heart attenuates cardiac hypertrophy and fibrosis in a non-genetic rat model of hypertension.

Keywords: cardiac hypertrophy, myocardial fibrosis, gene therapy, lentivirus

INTRODUCTION

The conventional concept of cardiac hypertrophy (CH) is that it is an enlargement of the heart in response to increased cardiac workload such as increased blood pressure (BP). While CH itself is asymptomatic, if it is left untreated it can lead to the development of cardiac fibrosis and further stiffening of the heart, which can eventually lead to heart failure. In addition, recent evidence indicates that other non-hemodynamic factors such as the local renin-angiotensin system (RAS), the sympathetic nervous system and genetics also play an important role in the development of CH independent of BP.

There are two major receptors of the RAS, the angiotensin II type 1 receptor (AT1R) and type 2 receptor (AT2R). While numerous studies have shown that activation of the AT1R leads to the development of CH and inhibition of this activation by AT1R antagonists or antisense gene therapy, leads to a reduction in CH (Black et al, 1996, Oka-Akagi et al, 2002, Pachori et al, 2001, Pachori et al, 2002, Phillips et al, 1997, Senbonmatsu et al, 2000, Shibasaki et al , 2002, Shikata et al, 2003), the role of the AT2R in CH remains elusive. It is believed however, that the AT2R opposes AT1R activation and that it is the unopposed activation of the AT2R that plays an important role in the protection against CH when patients are given AT1R blockers. This theory is supported by a study performed by Mukawa et al, which showed that simultaneous administration of an AT2R antagonist with an AT1R antagonist negated the antihypertrophic effects of the AT1R antagonist alone (Mukawa et al, 2003).

Despite numerous studies supporting an antihypertrophic role for the AT2R (Brede et al, 2003, Gross et al, 2004, Lako-Futo et al, 2003, Metcalfe et al, 2004), the overall role of the AT2R in cardiac pathophysiologies is still unresolved. Studies using transgenic and knockout animals indicate opposing roles for the AT2R in CH. While some studies indicate that the AT2R has an antihypertropic effect (Akishita et al, 2000, Brede et al, 2003, Gallinat et al, 2000, Gross et al, 2004), others show that the AT2R is necessary for the development of CH (Ichihara et al, 2001, Kurisu et al, 2003), and still other demonstrate no effects of the AT2R on CH (Akishita et al, 2000, Kurisu et al, 2003, Masaki et al, 1998). These conflicting observations in knockout and transgenic animals could be attributed to a number of factors. First, the observed differences could be due to differences in genetic background and strains of the animals. Secondly, the conflicting observations could be a result of inherent issues associated with the role of the AT2R in cardiovascular (CV) development. The expression of the AT2R is highest during embryonic development and decreases after birth (Gallinat et al, 2000). Thus altering AT2R expression during fetal life may inadvertently result in improper CV development.

To circumvent these inherent problems associated with transgenic and knockout animals, our laboratory has developed a lentiviral vector to deliver the AT2R following natural embryonic development. Previous studies in our laboratory have shown that using this system in the spontaneously hypertensive rat (SHR) prevented the development of CH (Metcalfe et al, 2004). The SHR is a genetic model of hypertension where the manifestation of the disease has

multigenetic origins. The present study was designed to determine the physiological effects of the AT2R on CH in a non-genetic model of hypertension using an angiotensin II (AngII)-infusion model of hypertension in Sprague Dawley (SD) rats. This AngII-infusion model was chosen to further characterize the cardiovascular role of the AT2R for several reasons. First, the hypertensive and normotensive rats have the same genetic background. Second, the time of onset, duration, and severity of the hypertension can be carefully controlled. Third, like human hypertension, this model has both neural and peripheral manifestations in the expression of this disease (Cox and Bishop, 1991). Thus, this present model is physiologically more relevant than a multigenetic rat model to human disease because CH is dependent on the RAS without the confounding genetic determinants associated with the SHR.

MATERIALS AND METHODS

Animal Procedures and Treatment with the Lentivirus Containing the AT2R

A lentiviral vector that bicistronically expresses the AT2R and the neomyocin resistance gene (Lenti-AT2R) under the elongation factor 1 α (EF1α) promoter was created and prepared as previously described (Coleman et al, 2003, Metcalfe et al, 2004).

SD rats were purchased from Charles River Laboratories (Wilmington, MA). At 5 days of age, rats were lightly anesthetized with methoxyflurane (Pittman Moore). A single bolus of either viral resuspension buffer (Control) or $3x10^8$ multiplicities of infection of Lenti-AT2R (AT2R) were injected into the left cardiac ventricular cavity of the 5-day old animals as previously described (Huentelman et al, 2002, Pachori et al, 2002, Raizada et al, 1984). After the virus was administered, the animals were returned to their respective mothers until weaning. At 15 weeks of age osmotic mini-pumps (Model 2004 Alzet®, Durect Corporation, Cupertino, CA) were inserted subcutaneously to deliver 200ng/kg/min AngII or 0.9% at an infusion rate of 0.25μl/hr for 4 weeks. All animal procedures were conducted under the approval of our Institutional Animal Care and Use Committee and adhered to the guidelines for the care and use of laboratory animals.

BP Measurements

Direct BP measurements were carried out to establish the conditions of AngII-infusion-induced hypertension by radiotelemetry (Data Sciences, Inc). The rats were anesthetized with 2-2.5% isoflurane and the abdominal cavity was

exposed. The cannula of the radiotelemetry device was inserted into the abdominal aorta, secured, and the wound was closed. Direct BP was monitored for one hour at each time point. Dataquest IV software (Data Sciences, Inc) was used to analyze the raw data, which is expressed as mean arterial pressure. Following these initial studies to establish the AngII-infusion conditions, indirect BP was monitored on a regular basis in subsequent studies using the tail-cuff method. This indirect method of BP monitoring was chosen in subsequent studies to prevent damage to the radiotelemetry devices caused by Magnetic Resonance Imaging (MRI).

Physiological Measurements

MRI of the rat cardiac cycle was performed at the University of Florida, McKnight Brain Institute's Advanced Magnetic Resonance Imaging and Spectroscopy Facility. Animals were imaged on a 4.7T Oxford Magnet using a Bruker Avance console and Paravision software. The animals were anesthetized with 1.5-2% isoflurane and 1L/min oxygen and monitored using the Small Animal Instrument (SAI) monitoring and gating system for respiration rate and cardiac triggering. The heart was centered in a custom built receive-only quadrature saddle surface coil tuned to 200 Mhz. The animal and receive coil were inserted into a 8.8cm diameter transmit-only quadrature volume coil. Dorsal and sagittal images were acquired using a cardiac gated cinetographic gradient echo sequence with the following parameters: FOV=70x30mm, matrix=256x128, TR=12msec, TE=2.2msec, NEX=4AVG, slice thickness=1.5mm, 14 frames with one frame per 12ms. Based on the sagittal and dorsal views, short axis images

were prescribed from base to apex and collected with the Cine-GE sequence described above except with FOV=40x30mm, TR=12msec, TE=2.3msec, and 14 frames to capture the entire cardiac cycle. Wall thickness was determined based on the magnitude value of the complex MR images using the NIH Image-J analysis program. Briefly, papillary muscles were used as indicators of the same area of the heart. In the papillary muscle region, the heart was still-framed in end diastole and 10 different measurements of left ventricular, right ventricular and septal wall thickness were taken using an imaginary centerpoint to focus all the lines. Images were spatially calibrated using original FOV and matrix size.

Assessment of Cardiac Hypertrophy and Fibrosis

After 4 weeks of Ang II infusion, rats were euthanized, hearts were removed, blotted and weighed to determine heart weight to body weight ratios (HW/BW) as previously described (Pachori et al, 2002). The apex was flash frozen and used for quantitative autoradiography while the rest of the heart was fixed in PLP solution (2% paraformaldehyde, 75 mM lysine, 37 mM sodium phosphate, and 10 mM sodium periodate) and used to look at the histology using Masson's trichrome staining. Separate sections were taken to access the extent of perivascular and myocardial fibrosis. Images of perivascular fibrosis were focused on 3-4 arteries located in a similar area of the heart among groups. Myocardial fibrosis was analyzed by focusing on areas of hearts where no vessels were observed. Thus, perivascular fibrosis assessment was not included

in the estimation of myocardial analysis. The extent of fibrosis i.e. blue staining, was determined using an Axioplan 2 microscope (Zeiss) and MCID Elite 6.0 software program which analyzes data as a ratio of collagen area over total area (Imaging Research, Inc).

AT1R and AT2R Expression

Quantitative autoradiography was preformed on heart tissues as previously described (Bagby et al, 2002, Raizada et al, 1984). Frozen apices of the heart were sectioned at a thickness of 20 microns and thaw mounted onto subbed chrome-alum slides. After drying, sections were stored at -20° C for less than one week. On the day of the autoradiography procedure the sections were thawed to room temperature and incubated with AM5 buffer (150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, and 50 mM NaPO4 buffer at pH 7.1-7.20) for 30 minutes. Next, the sections were incubated for 2 hours in AM5 buffer containing 500 pM ^{125}I-sarcosine^{1}-isoleucine^{8}-Ang II (SI-Ang II) AngII and either 3 μM AngII (non-specific), 10 μM losartan (AT2R binding) or 10 μM PD123,319 (AT1R binding). Following this incubation, the sections were quickly rinsed 2 times with distilled water then 5 times in AM 5 buffer for one minute each and finally 2 quick distilled water changes. Following this series of rinses, the sections were dried under a stream of cool air for 4 minutes and exposed to Biomax MR-1 (Kodak) film in X-ray cassettes for an appropriate exposure duration. A set of iodine-125 calibration standards (Microscales (RPA-522, Amersham) were included with each film for densitometric quantitation.

Specific binding of ^{125}I-SI-AngII to the AT1R and AT2R were quantitated essentially as previously established (Bagby et al, 2002). The values for AT1R and AT2R binding were derived by subtracting non-specific binding from the respective sections incubated in the presence of PD123,319 (AT1R expression) or losartan (AT2R expression). A thresholding technique was used to enable quantitation of irregularly shaped loci of high binding density in the AT1R and AT2R binding sections. The procedure set a lower limit for detectability of signal exceeding arbitrarily assigned steps to measure the density of AT2R binding in loci expressing high levels of AT2R bindings.

Statistics

All results are expressed as mean ± standard error. Data were analyzed by ANOVA followed by the Fisher's or Bonferroni's post-hoc tests. Values of $p<0.05$ were considered statistically significant.

RESULTS

AT2R Gene Transfer by Lenti-AT2R in the Heart

Relative levels of AT1R and AT2R were measured by quantitative autoradiography in the hearts of control and AT2R-transduced rats. Binding of ^{125}I-SI-AngII to the AT1R and AT2R in both the control rats infused with saline or AngII was not distinguishable from background (Figure 1). However, AT2R-transduced rat hearts demonstrated a significant and robust increase in the AT2R binding, with no changes in AT1R specific binding (Figure 1).

Blood Pressure Effects

Radiotelemetric devices were used to determine the blood pressure profile of control and AT2R transduced SD animals infused with either saline or AngII (Figure 2). AngII infusion resulted in an increase in BP that reached a maximum level within two weeks (94 ± 3 mmHg Control Saline vs 172 ± 5 mmHg Control AngII; Figure 2). This AngII-induced increase in BP was not affected in the AT2R-transduced rats (160 ± 8 mmHg; Figure 2B). Finally, similar effects were observed on BP at the conclusion of the study prior to pathophysiological measurements.

Cardiac Pathophysiologies

After 2 weeks of infusion, the animals were subjected to MRI to characterize the effects of AT2R overexpression on cardiac pathophysiology. AngII infusion resulted in a 123% increase in end diastolic left ventricular wall thickness (LVWT; Figure 3B). A value of 2.1 ± 0.01mm was observed compared to 1.7±0.04mm in control animals infused with saline (Figure 3A). However, this

AngII-induced increase in LVWT was absent in the Lenti- AT2R-transduced rats (Figure 3C: 1.8±0.06mm). In contrast to LVWT, there were no significant differences in right ventricular and septal wall thickness between any of the groups (data not shown). In addition, ejection fraction was also comparable among all three groups of rats (86 ± 3% Control Saline; 91 ± 3% Control AngII; 92 ± 4% AT2R AngII).

HW/BW revealed that with AngII-infusion Lenti-AT2R-transduction reduced the HW/BW (Figure 4: 3.2±0.08 mg/g) compared to the control rats infused with AngII, (Figure 4: 3.5±0.07 mg/g). This reduction by AT2R transduction, however, was not a complete prevention of the AngII action, as the control animals infused with saline (Figure 4: 2.7±0.1 mg/g) were significantly lower than both of the AngII-infused animals (Figure 4).

AngII infusion caused a 300% increase in myocardial fibrosis (Figures 5A,B,D) from 0.02 ± 0.001 (Control Saline) to 0.06 ± 0.02 (Control AngII). This effect of AngII was severely blunted in the AT2R-tranduced rats (Figures 5C-D; 0.03 ± 0.01 AT2R AngII). In contrast, the AngII infusion induced increase in perivascular fibrosis (0.24 ± 0.01 Control Saline; 0.38 ± 0.05 Control AngII, 0.34 ± 0.03 AT2R AngII) was not significantly attenuated by AT2R-transduction (Figure 6) of coronary arterioles.

DISCUSSION

Through the use of a lentiviral vector, we were able to effectively overexpress the AT2R in the heart on a long-term basis following a single intraventricular injection of Lenti-AT2R at 5 days of age. Although this method results in the overexpression of the AT2R in many tissues, heart is the only cardiovascular-relevant tissue that is predominantly transduced. In addition, it overcomes the inherent problems with transgenic and knockout animals. Our observations show that AT2R expression prevents the development of CH and myocardial fibrosis without influencing BP.

AT2R transduction significantly attenuated AngII-mediated CH as observed by both MRI and HW/BW ratios. In addition, AT2R overexpression significantly reduced the extent of myocardial fibrosis. All of these effects were seen despite limited transduction of cardiac tissue. Previous observations have shown that using the same delivery method, the lentiviral vector transduces ~40% of the heart (Coleman et al, 2003). In addition, this present study demonstrates a significant level of AT2R expression in cardiac tissue, although the expression was not uniformly distributed throughout the tissue. Despite this, we observed dramatic cardioprotective effects. There are several ways to explain this phenomenon. Recent evidence indicates that during the development of CH the number of new myocytes forming from stem-like cells is enhanced (Mangi et al, 2003). Thus, if the Lenti-AT2R is targeting these stem-like cells the overall effect of the AT2R will be improved. Secondly, there could be some unknown paracrine or autocrine factors that propagate the effect of the

AT2R. As more and more studies are performed it will be interesting to see if we can identify AT2R transduction in these stem-like cells or identify these unknown paracrine and autocrine factors.

Masson's trichrome staining of the AngII-infused hearts indicates that the AT2R prevents myocardial fibrosis while having any protective effect on perivascular fibrosis. These observations are fascinating for several reasons. First, despite a low transduction efficiency of cardiac fibroblasts, which secrete collagen, we see a tremendous prevention of myocardial fibrosis. Previous studies in our laboratory have shown that a majority of transduced cells in the heart exhibit cardiac myocyte morphology (Coleman et al, 2003). Thus, either low levels of AT2R expression in the fibroblasts are sufficient to prevent the development of myocardial fibrosis or AT2R expression in the cardiomyocytes promotes cross-talk with other cell types to create a global effect despite limited transduction. An example of similar cross-talk would be how the AT2R in vascular smooth muscle cells are able to send signals to the endothelial cells and back again to cause vasodilation (Tsutsumi et al, 1999). Secondly, we are seeing opposing roles for the AT2R in the heart tissue versus the vasculature. This could indicate that either the AT2R has different roles in various cell types, or a more likely possibility would be a low transduction efficiency of the vasculature (Tsutsumi et al, 1999). The relevance of this observation remains to be explained and may require the use of endothelial or vascular smooth muscle cell-specific promoters to deliver the AT2R.

A number of studies indicate that all of the components of the RAS exist in the heart and it is this tissue RAS that appears to regulate cardiac function. Inhibitors to multiple components of the RAS has been shown to reduced CV pathophysiologies independent of BP (Kaneko et al, 1996, Linz et al, 1992, Pachori et al, 2002). This study indicates that AT2R overexpression in the heart is exerting its effects on CH and myocardial fibrosis independent of BP. These effects are similar to the effects our laboratory has previously observed in the SHR model of hypertension (Metcalfe et al, 2004). Using the AngII-infusion model of hypertension, however, we were better able to define the effect of the local RAS on the prevention of cardiac pathophysiologies without the confounding genetic restrictions associated with the SHR.

We have now been able to show that the AT2R provides cardioprotective effects against CH in both a genetic and non-genetic model of hypertension. Both of these studies indicate that the protective effects are through the local RAS. In addition, in the present study we were able to extend these results to show that the AT2R also prevents the development of myocardial fibrosis. Thus, they set the stage for future studies as our method of AT2R overexpression is not only therapeutically relevant, but also allows for the study of the AT2R without the confounding developmental problems associated with transgenic and knockout animals. It will be interesting to use this paradigm to determine if the AT2R provides cardioprotective effects against heart failure and myocardial infarction. In addition, because of its versatility the lentiviral vector system can be used to drive AT2R expression with specific promoters, such as oxygen

sensitive response elements to investigate the role of this receptor in ischemia-induced heart damage. This strategy has recently been used to control ischemia-induced heart damage (Tang et al 2004).

ACKNOWLEDGEMENTS

The authors would like to thank Jasenka Zubcevic for her help with the indirect blood pressure measurements and Nichole Herring for her secretarial assistance. This work was supported by NIH grants HL 56921 and HL 68085 (MKR, CS). Beverly Falcón was a predoctoral fellow of the AHA Florida/Puerto Pico Affiliate.
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FIGURE LEGENDS

Figure 1 Quantitative autoradiography of Angiotensin receptors in the AT2R-transduced heart.

Autoradiography was performed to determine the expression of the AT1R and AT2R in the hearts of control and Lenti-AT2R transduced rats. Hearts were isolated, the apex was frozen and quantitative autoradiography was performed as described in the Materials and Methods. Representative autoradiograms are presented (A) showing the nonspecific binding in the first column, AT1R specific binding in the second, and AT2R specific binding in the third column. Bar graphs represent the quantitation of autoradiograms for the AT1R (B) and the AT2R (C). n=3/group; * = $p<0.05$ compared to Control Saline, ‡ = $p<0.05$ compared to Control AngII.

Figure 2 Effect of AT2R transduction on BP following AngII infusion.

(A) BP was monitored by telemetry as described in the Materials and Methods. (B) Quantitation of average BP data (n = 4) for saline and 3 each for AngII and AngII plus ACE 2. *Significant different ($p<0.05$) from control saline groups.

Figure 3 MRI of AT2R-transduced control and AngII infused rat hearts.

MRI was used to determine the effect of AT2R transduction on the development of AngII-mediated increases in left ventricular wall thickness. Wall thicknesses of the rats were examined by MRI following 2 weeks of infusion of AngII as described in the Materials and Methods. Representative mid-ventricle short axis images of the Control Saline (A), Control AngII (B), and AT2R AngII (C) are shown. Quantitation of all of the images from the animals is presented

graphically (D). * = $p<0.05$ compared to Control Saline, † = $p<0.05$ compared to AT2R AngII.

Figure 4 The effect of AT2R transduction on cardiac hypertrophy following AngII infusion.

HW/BW were determined as described in the Materials Methods. * = $p<0.05$ compared to Control Saline, † = $p<0.05$ compared to AT2R AngII, ‡ = $p<0.05$ compared to Control Saline.

Figure 5 The effect of AT2R transduction on myocardial fibrosis following AngII infusion.

Following the termination of the experiment, hearts were preserved in PLP solution, stained with Masson's trichrome and quantitated as described in the Materials and Methods. Representative images of the extent of myocardial fibrosis in the Control Saline (A), Control AngII (B), and AT2R-AngII (C) animals. A bar graph represents the combined data from 3 animals in each group (D). * = $p<0.05$ compared to Control Saline, † = $p<0.05$ compared to AT2R AngII. All images were all taken at 5x magnification.

Figure 6 The effect of AT2R transduction on perivascular fibrosis following AngII infusion.

Heart sections were prepared as described in Figure 5. Representative photographs of the extent of perivascular fibrosis in the Control Saline (A), Control AngII (B), and AT2R AngII (C) rats are shown. A bar graph is used to represent the quantitated data (D). n = 3 per group; * = $p<0.05$ compared to Control Saline. All images were all taken at 5x magnification.

Figure 1

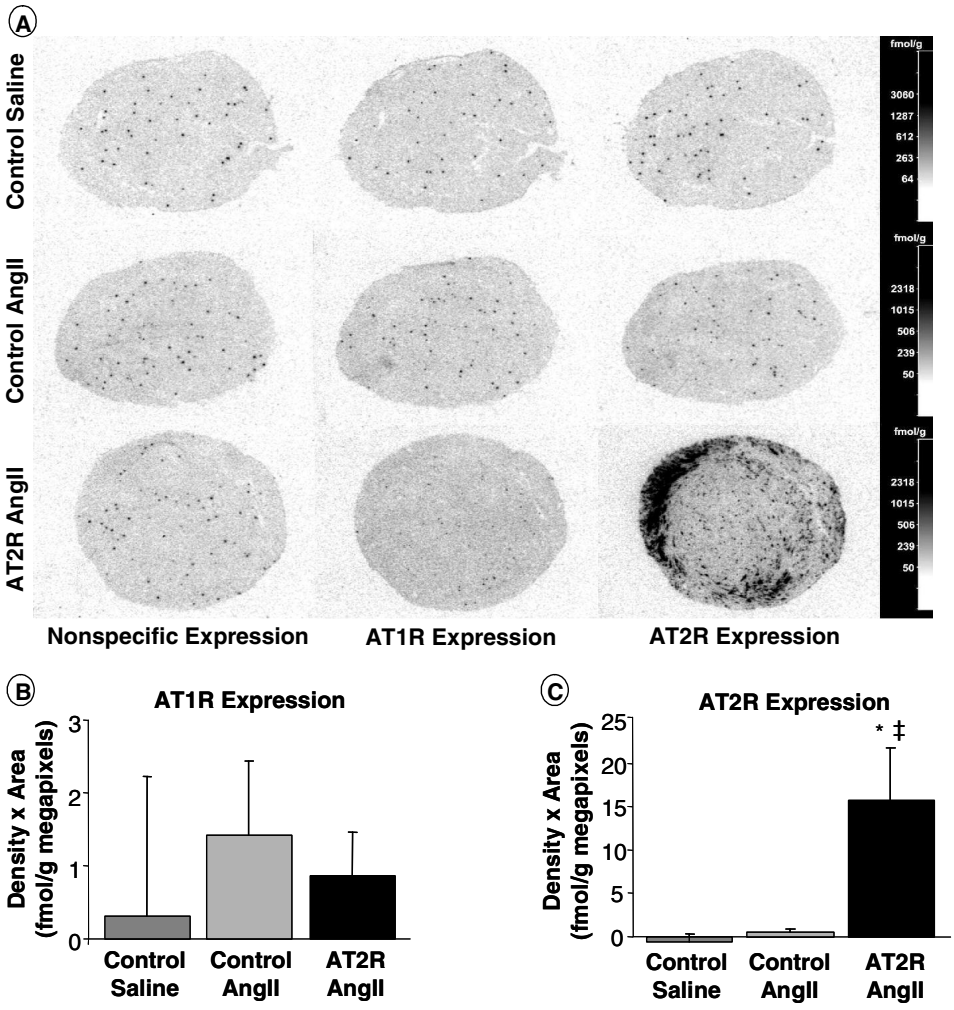

A
Control Saline
Control AngII
AT2R AngII
fmol/g
3080
1287
612
263
64
fmol/g
2318
1015
506
239
50
fmol/g
2318
1015
506
239
50
Nonspecific Expression
AT1R Expression
AT2R Expression
B
AT1R Expression
Density x Area (fmol/g megapixels)
3
2
1
0
Control Saline
Control AngII
AT2R AngII
C
AT2R Expression
Density x Area (fmol/g megapixels)
25
20
15
10
5
0
* ‡
Control Saline
Control AngII
AT2R AngII

Figure 2

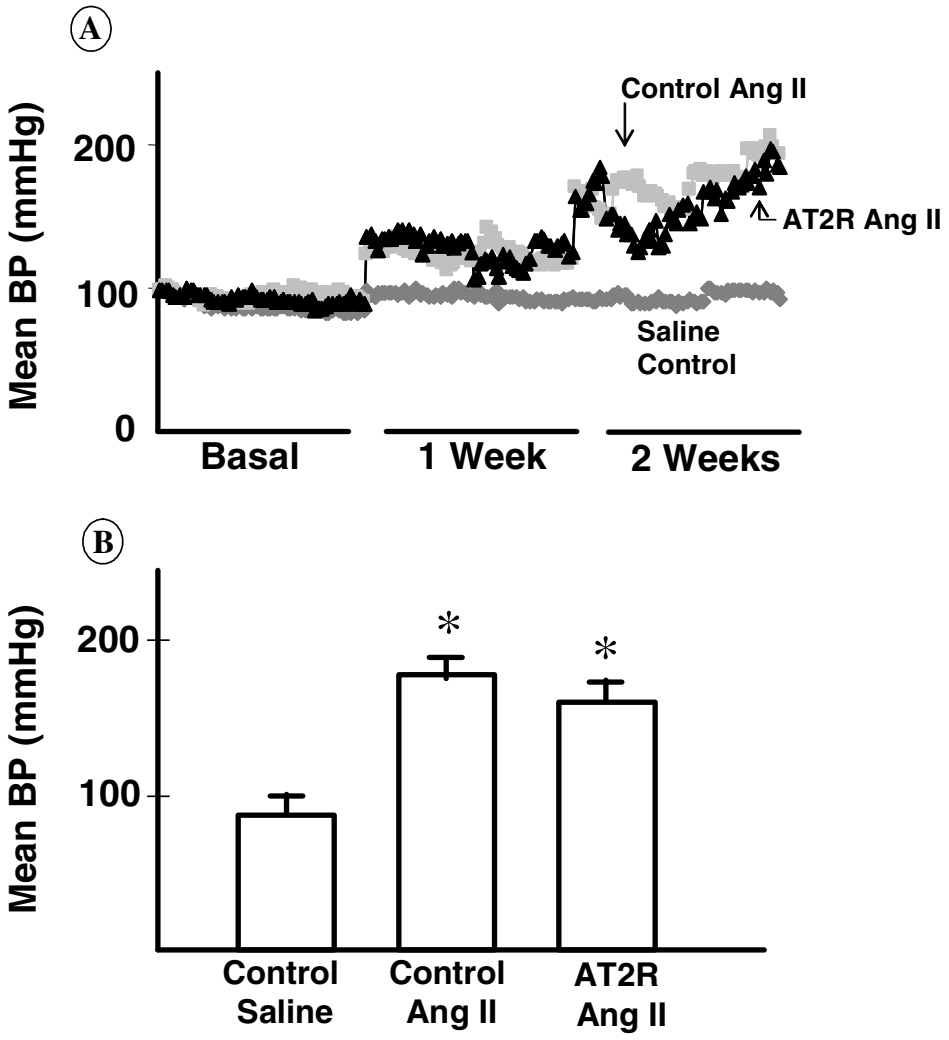

A
Control Ang II
AT2R Ang II
Saline
Control
Mean BP (mmHg)
200
100
0
Basal
1 Week
2 Weeks
B
Mean BP (mmHg)
200
100
*
*
Control
Saline
Control
Ang II
AT2R
Ang II

Figure 3

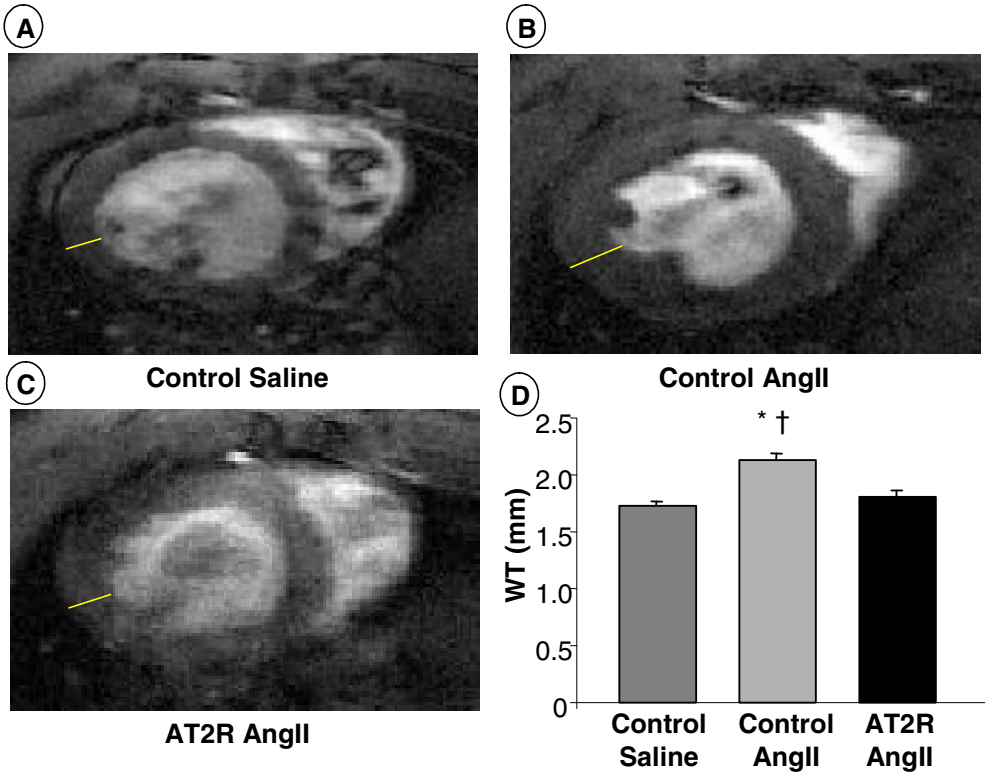

A
B
Control Saline
Control AngII
C
D
AT2R AngII
* †
WT (mm)
2.5
2.0
1.5
1.0
0.5
0
Control Saline
Control AngII
AT2R AngII

Figure 4

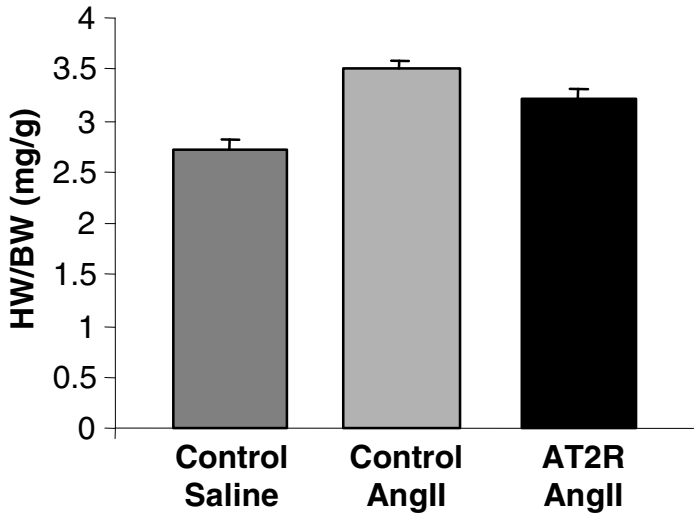

HW/BW (mg/g)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control Saline
Control AngII
AT2R AngII

Figure 5

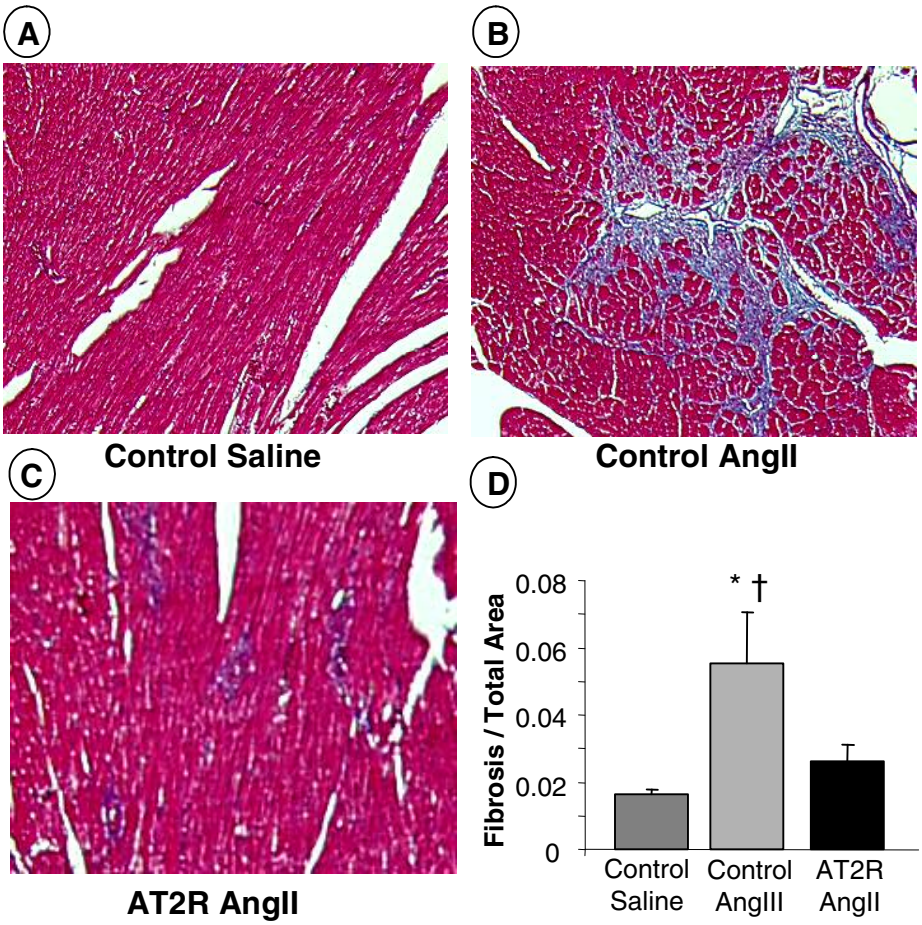
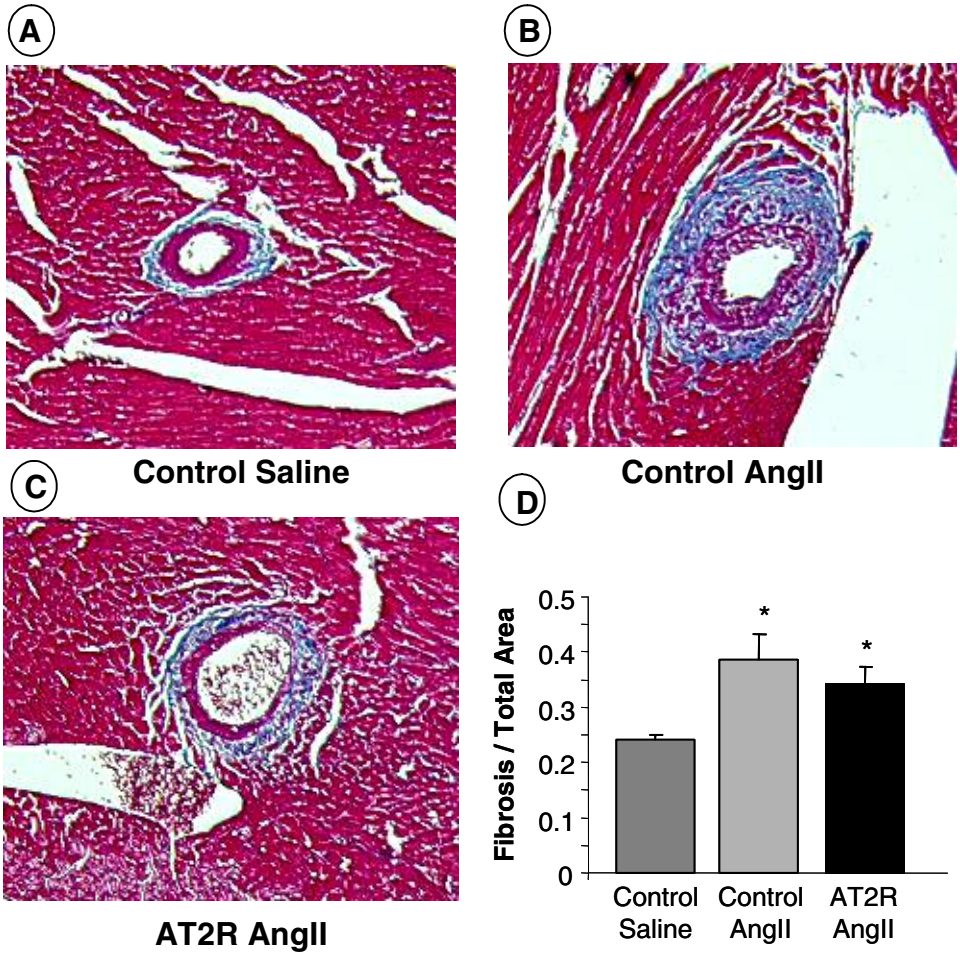

A
B
Control Saline
Control AngII
C
D
AT2R AngII
Fibrosis / Total Area
0.08
0.06
0.04
0.02
0
* †
Control Saline
Control AngIII
AT2R AngII

Figure 6


A
B
Control Saline
Control AngII
C
D
AT2R AngII
Fibrosis / Total Area
0.5
0.4
0.3
0.2
0.1
0
*
*
Control Saline
Control AngII
AT2R AngII