Angiotensin II type 2 receptor gene transfer elicits cardioprotective effects in an angiotensin II infusion rat model of hypertension

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 nonhemodynamic 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). Although 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 (3, 19–22, 24–26), 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. (18), which showed that simultaneous administration of an AT2R antagonist with an AT1R antagonist negated the anti-hypertrophic effects of the AT1R antagonist alone.

Despite numerous studies supporting an antihypertrophic role for the AT2R (4, 8, 13, 17), the overall role of the AT2R in cardiac pathophysiology is still unresolved. Studies using transgenic and knockout animals indicate opposing roles for the AT2R in CH. Although some studies indicate that the AT2R has an antihypertrophic effect (1, 4, 7, 8), others show that the AT2R is necessary for the development of CH (10, 11), and still others demonstrate no effects of the AT2R on CH (1, 12, 16). 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. Second, 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 (7). 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 (17). The SHR is a genetic model of hypertension in which 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 nongenetic model of hypertension using an angiotensin II (ANG II) infusion model of hypertension in...
Sprague-Dawley (SD) rats. This ANG II infusion model was chosen to further characterize the CV 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 (6). 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 neomycin resistance gene (Lenti-AT2R) under the elongation factor 1α (EF1α) promoter was created and prepared as previously described (5, 17).

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 3 × 10⁹ multiplicity of infection of Lenti-AT2R (AT2R) were injected into the left cardiac ventricular cavity of the 5-day-old animals as previously described (9, 21, 23). After the virus was administered, the animals were returned to their respective mothers until weaning. At 15 wk of age osmotic minipumps (model 2004 Alzet; Durect, Cupertino, CA) were inserted subcutaneously to deliver 200 ng·kg⁻¹·min⁻¹ ANG II or 0.9% saline at an infusion rate of 0.25 μl/h for 4 wk. 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 ANG II-infusion-induced hypertension by radiotelemetry (Data Sciences). The rats were anesthetized with 2–2.5% isoflurane, and the abdominal cavity was exposed. The cava and the radiotelemetry device was inserted into the abdominal aorta and secured, and the wound was closed. Direct BP was monitored for 1 h at each time point. Datquest IV software (Data Sciences) was used to analyze the raw data, which is expressed as mean arterial pressure. Following these initial studies to establish the ANG II 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 4.7-T Oxford magnet using a Bruker Avance console and Paravision software. The animals were anesthetized with 1.5–2% isoflurane and 1 l/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 100 MHz. The animal and receive coil were inserted into a 22-cm diameter transmit-only quadrature volume coil. Dorsal and sagittal images were acquired using a cardiac gated cineotropic gradient echo sequence with the following parameters: FOV = 70 × 30 mm, matrix = 256 × 128, TR = 12 ms, TE = 2.2 ms, NEX = 4AVG, slice thickness = 1.5 mm, 14 frames with 1 frame per 12 ms. 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 = 40 × 30 mm, TR = 12 ms, TE = 2.3 ms, 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 center point to focus all the lines. Images were spatially calibrated using original FOV and matrix size.

Assessment of cardiac hypertrophy and fibrosis. After 4 wk of ANG II infusion, rats were euthanized, and hearts were removed, blotted, and weighed to determine heart weight to body weight ratios (HW/BW) as previously described (21). The apex was flash frozen and used for quantitative autoradiography while the rest of the heart was fixed in PLP solution (2% paraormaldehyde, 75 mM lysine, 37 mM sodium phosphate, and 10 mM sodium periodate) and used to look at the histology using Masson trichrome staining. Separate sections were taken to access the extent of perivascular and myocardial fibrosis. Images of perivascular fibrosis were focused on 3–4 images located in a similar area of the heart among groups. Myocar- dial 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 AXiplan 2 microscope (Zeiss) and the MCID Elite 6.0 software program, which analyzes data as a ratio of collagen area over total area (Imaging Research).

AT1R and AT2R expression. Quantitative autoradiography was preformed on heart tissues as previously described (2, 23). Frozen apices of the heart were sectioned at a thickness of 20 μm and thaw mounted onto subbed chrome-alum slides. After drying, sections were stored at −20°C for less than 1 wk. 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 NaNO₃ buffer at pH 7.1–7.2) for 30 min. Next, the sections were incubated for 2 h in AM5 buffer containing 500 pM ¹²⁵I-labeled [Sar¹,Ile⁸]ANG II (¹²⁵I-SI-ANG II) and either 3 μM ANG II (nonspecific), 10 μM losartan (AT2R binding), or 10 μM PD123,19 (AT1R binding). Following this incubation, the sections were quickly rinsed two times with distilled water then five times in AM5 buffer for 1 min each and finally two quick distilled water changes. Following this series of rinses, the sections were dried under a stream of cool air for 4 min 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 ¹²⁵I-SI-ANG II to the AT1R and AT2R were quantitated essentially as previously established (2). The values for AT1R and AT2R binding were derived by subtracting nonspecific binding from the respective sections incubated in the presence of PD123,19 (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.

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 ¹²⁵I-SI-ANG II to the AT1R and AT2R in both the control rats infused with saline or ANG II was not distinguishable from background (Fig. 1). However, AT2R- trans-
duced rat hearts demonstrated a significant and robust increase in the AT2R binding, with no changes in AT1R-specific binding (Fig. 1).

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

**Cardiac pathophysiology.** After 2 wk of infusion, the animals were subjected to MRI to characterize the effects of AT2R overexpression on cardiac pathophysiology. ANG II infusion resulted in a 123% increase in end diastolic left ventricular wall thickness (LVWT; Fig. 3). A value of 2.1 ± 0.01 mm was observed compared with 1.7 ± 0.04 mm in control animals infused with saline (Fig. 3A). However, this ANG II-induced increase in LVWT was absent in the AT2R-transduced rats (1.8 ± 0.06 mm). 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 ANG II; 92 ± 4% AT2R ANG II).

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

ANG II infusion caused a 300% increase in myocardial fibrosis (Fig. 5, A, B, and D) from 0.02 ± 0.001 (control saline) to 0.06 ± 0.02 (control ANG II). This effect of ANG II was severely blunted in the AT2R-transduced rats (Fig. 5, C and D; 0.03 ± 0.01 AT2R ANG II). In contrast, the ANG II-infusion-induced increase in perivascular fibrosis (0.24 ± 0.01 control saline; 0.38 ± 0.05 control ANG II; 0.34 ± 0.03 AT2R ANG II) was not significantly attenuated by AT2R transduction (Fig. 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 CV-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 ANG II-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, with use of the same delivery method, the lentiviral vector transduces ~40% of the heart (5). 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
Fig. 5. The effect of AT2R transduction on myocardial fibrosis following ANG II infusion. Following the termination of the experiment, hearts were preserved in PLP solution, stained with Masson trichrome, and quantitated as described in the MATERIALS AND METHODS. Images are representative of the extent of myocardial fibrosis in the control saline (A), control ANG II (B), and AT2R-ANG II (C) animals. The bar graph represents the combined data from 3 animals in each group (D). *P < 0.05 compared with control saline. †P < 0.05 compared with AT2R ANG II. All images were all taken at 5× magnification.

Fig. 6. The effect of AT2R transduction on perivascular fibrosis following ANG II infusion. Heart sections were prepared as described in Fig. 5. Representative photographs of the extent of perivascular fibrosis in the control saline (A), control ANG II (B), and AT2R ANG II (C) rats are shown. The bar graph represents the quantitated data (D); n = 3 per group. *P < 0.05 compared with control saline. All images were all taken at 5× magnification.
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 (15). Thus, if the Lenti-AT2R is targeting these stem-like cells, then 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 trichrome staining of the ANG II-infused hearts indicates that the AT2R prevents myocardial fibrosis while not 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 (5). 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 (28). Second, we are seeing opposing roles for the AT2R in the heart tissue vs. 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 (28). 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 (11, 14, 21). 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 (17). Using the ANG II 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 nongenetic 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 whether the AT2R provides cardioprotective effects against heart failure and myocardial infarction. In addition, because of its versatility the lentivector delivery 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 (27).

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Present address of B. Falcón: Department of Anatomy and the Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA.

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