Prevention of Cardiac Hypertrophy by Angiotensin II Type-2 Receptor Gene Transfer

Beverly L. Metcalfe, Matthew J. Huentelman, Leonard D. Parilak, David G. Taylor, Michael J. Katovich, Harm J. Knot, Colin Sumners, Mohan K. Raizada

Abstract—The role of the angiotensin II type-2 receptor (AT₂R) in cardiac hypertrophy remains elusive despite its demonstrated involvement in cardiovascular development. We have previously shown that a lentiviral vector gene delivery system is able to transduce cardiac tissue with high efficiency in vivo. Using such an approach, our objectives in the present study were 2-fold: (1) to overexpress the AT₂R in cardiac tissue after completion of natural embryonic development of the heart and (2) to determine the effects of this overexpression on cardiac hypertrophy and basal blood pressure (BP). A lentiviral vector encoding the AT₂R (lenti-AT₂R) was administered (1.5×10⁹ transducing units) into the left ventricular space of 5-day-old spontaneously hypertensive rats (SHRs). AT₂R transgene expression increased in these animals and persisted for 30 weeks. In contrast, the expression of the angiotensin II type-1 receptor remained unchanged following lenti-AT₁R treatment. At 21 weeks following gene transduction, the lenti-AT₁R–treated SHRs exhibited decreased left ventricular wall thickness compared with control animals. In contrast, basal BP did not differ between the two SHR groups. Finally, heart weight to body weight ratios indicated a significant decrease in lenti-AT₁R-treated SHRs compared with SHR controls. Our data indicate that AT₂R overexpression attenuates cardiac hypertrophy in the SHR. This beneficial outcome was observed despite the existence of elevated BP. (Hypertension. 2004;43:1233-1238.)

Key Words: genes receptors, angiotensin II hypertrophy

Left ventricle hypertrophy (LVH) is an adaptive response of the heart to preserve cardiac function. Chronic hypertrophy of the heart is a major risk factor for heart failure. Accumulating evidence indicates that both hemodynamic and nonhemodynamic factors are critical in the development of cardiac hypertrophy.¹⁻⁹ The local renin-angiotensin system (RAS) is one such nonhemodynamic factor, the dysregulation of which plays a role in the development of cardiac hypertrophy.

Binding of angiotensin II (Ang II) to the Ang II type-1 receptor (AT₁R) increases left ventricular hypertrophy, and blocking this interaction with an AT₁R antagonist results in a regression in cardiac hypertrophy in a variety of animal models.²⁻⁸ This view is further supported by gene transfer of the AT₁R antisense (AT₁R-AS).⁹ These investigations indicated that AT₁R-AS transduction prevents the development of cardiac hypertrophy in the spontaneously hypertensive rat (SHR) on a long-term basis. In addition, clinical studies have demonstrated that treatment with an AT₁R antagonist results in a decrease of LVH.¹⁰,¹¹ Although the mechanism by which AT₁R antagonists reduce LVH is still speculative, it has been suggested that unopposed Ang II stimulation of the Ang II type-2 receptor (AT₂R) may contribute to its effectiveness. Evidence for this hypothesis is illustrated in a study conducted by Mukawa et al, which showed that simultaneous administration of an AT₁R antagonist with an AT₂R antagonist negated the antihypertrophic effects of the AT₁R blocker alone.¹² In addition, the ratio of cardiac AT₁R/AT₂R levels increases during cardiac hypertrophy, indicating the relative importance of AT₁R stimulation in this disease.¹³ Studies performed in cultured cardiomyocytes and in hypertrophied hearts provide further support of the role of the AT₁R in the prevention or reversal of cardiac hypertrophy.¹⁴⁻¹⁶

Despite this support for a role of the AT₁R in the regression of LVH, the role of the AT₂R in cardiac hypertrophy remains controversial. Studies from transgenic and knockout animals imply conflicting roles of the AT₂R in cardiac hypertrophy. Inagami’s group has shown that the absence of the AT₂R in knockout animals prevents the development of cardiac hypertrophy when induced by Ang II infusion and by pressure overload.¹⁷,¹⁸ In contrast to these studies, other transgenic studies showed no effects of either AT₁R knockout or overexpression on cardiac hypertrophy.¹⁹⁻²¹

Received February 3, 2004; first decision February 23, 2004; revision accepted March 24, 2004.
From the Departments of Physiology and Functional Genomics (B.L.M., M.J.H., M.K.R.), Pharmacology (D.G.T., H.J.K.), and Cardiovascular Medicine (L.D.P.), College of Medicine and the Evelyn F. and William L. McKnight Brain Institute; and the Department of Pharmacodynamics (M.J.K.), College of Pharmacy, University of Florida, Gainesville.
The first 2 authors contributed equally to this work.
Correspondence to Mohan K. Raizada, Department of Physiology and Functional Genomics, University of Florida, College of Medicine, PO Box 100274, Gainesville, FL 32610. E-mail mraizada@phys.med.ufl.edu
© 2004 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org
DOI: 10.1161/01.HYP.0000127563.14064.FD

1233
These conflicting observations may be due to inherent issues related to the involvement of the AT_2 R in cardiovascular (CV) development. AT_2 R expression is highest during fetal life with a concomitant decrease after birth. Altering AT_2 R expression levels during embryonic development may result in improper CV development. In order to circumvent these developmental issues, we established an efficient method of delivering genes to cardiac tissue after normal development of the CV system. Thus, the present study was designed to determine the efficacy of this viral vector gene delivery system to transfer the AT_2 R in vivo and to determine the consequences of AT_2 R overexpression on LVH and high blood pressure (BP).

Materials and Methods

Lentiviral Construction and Preparation

Lentiviral vectors were created as previously described. A vector was created to express the human placental alkaline phosphatase (hPLAP) gene (lenti-PLAP) to determine the efficacy of transduction. Other lentiviral vectors were created to bicistronically express the AT_2 R and the neomycin resistance gene (lenti-AT_2 R-I-NeoR) or the hPLAP gene (lenti-AT_2 R-I-PLAP). Finally, a control vector was created for in vitro testing (lenti-I-NeoR).

The AT_2 R was cloned into previously described pTYF salt-inactivating lentiviral constructs. The AT_2 R cDNA was a kind gift from Dr. Jeffrey Harrison (University of Florida, Gainesville, Fla). Viral concentration and titration of viral particles were performed as previously described.

Cell Culture

Chinese hamster ovary (CHO) cells transfection with the AT_2 R were a generous gift from Dr. Peter Sayeski (University of Florida). Cells were grown in Ham F-12 media supplemented with 10% fetal bovine serum (Cellgro, Herndon, Va). Cells were transduced with lentiviral vectors at a multiplicity of infection (MOI) of 1 in the presence of 8 μg/mL polybrene (Sigma, St. Louis, Mo). All cells were grown for 3 days before being used in the experiments.

RNA Isolation and Quantification

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to measure expression of the AT_2 R in cell culture and in isolated cardiac myocytes. Real-time RT-PCR was also used to determine the AT_2 R expression levels in the cardiomyocytes. Total RNA was extracted from cell lines using Ambion RNAqueous-4-PCR and from myocytes by using RNAeasy Fibrous Tissue Mini Kit (Invitrogen). Two-step RT-PCR was performed according to the protocols of the manufacturer with an ABI Prism 7000 HT Detection System (Applied Biosystems). The primers and probe used were as follows: AT_2 R (forward): 5'-CCGCAATTATACGTCACACA-3' (reverse): 5'-ATGCCTGATGAGAAAGAGAATGCT-3'; (probe): 5'-FAMCCGCGAGATACG-MGBNFQ-3'. AT_2 R (forward): 5'-CCATCGTCCACCCAAAAGAG-3' (reverse): 5'-GTGACTTGTGGCCACCACCATG-3'; (probe): 5'-FAMCTCGCTTCGCCGCA-MGBNFQ-3'. Relative quantitation was performed using the comparative method as described in Applied Biosystems User Bulletin 2 using ribosomal RNA (18S) as an endogenous control. No reverse transcriptase and no template controls were used to monitor for any contaminating amplification.

Ligand Binding Assay

Specific binding of 125 I-sarcosine 1, isoleucine 8 angiotensin II (125 I-SI-Ang II) to the AT_2 R was performed as previously described. Protein content in each culture well was determined by the method of Lowry et al.

Delivery of the Lentiviral Vector in Vivo

All animals were purchased from Charles River Laboratories (Wilmington, Mass). In the initial experiments, Sprague-Dawley rats were used to establish the transduction efficiency of the lentiviral vector. A single bolus of 30 μL of either the viral resuspension buffer or 1.5×10⁷ colony-forming units of lenti-PLAP were injected into the left ventricle as described. Five-day-old SHR offspring were used to study the effect of lenti-AT_2 R on cardiac hypertrophy. Offspring were removed from their mothers, and the animals were divided into 2 groups: lenti-AT_2 R (experimental) or viral resuspension buffer (control). The left ventricular chamber of each animal was injected as described above. Animal procedures were conducted with the approval of our Institutional Animal Care and Use Committee.

Physiological Measurements

Indirect BP was monitored by the tail-cuff method at 21 weeks of age as previously described. At 31 weeks of age, rats were weighed and euthanized; their hearts were removed, blotted, and weighed, as previously described.

Animals for echocardiographies (ECHOs) were used at 12 and 21 weeks. Briefly, rats were anesthetized, and ECHOs were performed using a Hewlett-Packard Sonos Model 5500 with a 12-MHz transducer. Parastral long- and short-axis images were obtained and end diastolic diameter, end systolic diameter, ejection fraction (EF), and wall thickness were obtained.

Tissues were processed for histochemical staining from the animals injected with lenti-PLAP as described. The same tissues were also frozen in Tissue Tek (Sakura Finetek), sectioned at 30 μm, and stained for hPLAP and 4’,6-diamidino-2-phenylindole.

Statistics

All results are expressed as mean±SE. All data were analyzed by ANOVA, and ECHOs were analyzed by repeated-measures ANOVA using the Student/Newman-Keuls method for all pairwise multiple comparisons. Values of P<0.05 were considered statistically significant.

Results

In Vitro Characterization of AT_2 R Gene Transfer

CHO cells were transduced with a lentiviral construct containing either the AT_2 R transgene or a control viral vector. Real-time RT-PCR analysis indicated an increase in the levels of AT_2 R mRNA in the AT_2 R-transduced cells (Figure 1A). This was associated with a significant increase in the AT_2 R binding. The total binding activity for the AT_2 R-transduced cells was 4.85 pmol/mg of protein with a Kd of 0.82 nmol/L (Figure 1B and 1C). These results indicate that the lentiviral vector can efficiently transfer the AT_2 R in vitro and that transduced CHO cells express the AT_2 R, the binding characteristics of which are similar to those found in other cell types.

In Vivo Gene Transfer

We first wanted to verify that a single intracardiac injection of lenti-PLAP would yield significant expression in the heart. Tissues examined at 3 weeks of age exhibited robust transduction of the heart. Representative pictures are shown in Figure 2. We found that delivery of viral resuspension buffer yielded no hPLAP staining (Figure 2A). However, animals injected with lenti-PLAP show robust hPLAP expression indicated by dark purple staining (Figure 2B through 2D). Figure 2C illustrates the heart cut in half to show that the lentiviral vector transduces throughout the tissue, and Figure...
2D shows a thin-cut section indicating that the transduced cells have myocyte characteristics. These results are consistent with our previous studies, which have shown that this gene delivery method transduces approximately 40% of the cardiac cells, and of the transduced cells, 90% to 95% exhibited cardiac myocyte morphology.

Next, we studied the effect of lenti-AT 2 R transduction on the expression of both the AT 2 R and AT 1 R. Real-time RT-PCR was performed on isolated myocytes from the hearts of 31-week-old rats injected with either lenti-AT 2 R or viral resuspension buffer at 5 days of age. Using AT 2 R-specific primers and probe, there were negligible levels of the AT 2 R detected in the control rats, whereas the AT 2 R mRNA levels were significantly higher in the virus-treated animals (Figure 3A). In contrast to this, there was robust expression of the AT 1 R that did not differ between the control-treated and the lenti-AT 2 R–treated animals (Figure 3B).

Pathophysiology
SHR were administered either lenti-AT 2 R or viral resuspension buffer at 5 days of age. Previous unpublished studies in our laboratory have indicated that there is no difference in heart weight to body weight ratios (HW/BWs) in animals injected with a control lentiviral vector (lenti-I-Neo) versus animals injected with viral resuspension buffer (data not shown). At 12 and 21 weeks of age, the rats were subjected to ECHO to characterize cardiac pathophysiology and the effects of the lenti-AT 2 R transgene. At 12 weeks of age, a control ECHO was performed. This initial measurement of left ventricular wall thickness (LVWT) indicated that neither group of animals exhibited cardiac hypertrophy (SHR control 1.4±0.03 mm versus SHR AT 2 R 1.36±0.03 mm). ECHOs performed at 21 weeks revealed a significant increase in LVWT of the control-treated SHR of 2.0±0.11 mm (Figure 4A). However, no such increase was observed in the lenti-AT 2 R animals, which displayed an LVWT of 1.54±0.09 mm (Figure 4A). The ECHO measurements of LVWT of the Lenti-AT 2 R SHR were similar to that of an age-matched Wistar-Kyoto (WKY) control (1.52±0.09 mm). In contrast, no significant differences were seen in the EF between the control and AT 2 R–treated SHRs. This observation on EF is presumably because at this age, the SHR are not in heart failure. In conclusion, AT 2 R transduction in the SHR prevents the development of cardiac hypertrophy.

Lenti-AT 2 R treatment in SHR showed no significant effects on indirect BP at 21 weeks (193±14 mm Hg) versus control (187±13 mm Hg) (Figure 4B). However, when compared with age-matched WKY control (128±5 mm Hg), both lenti-AT 2 R–treated and control SHR exhibit a hypertensive state. HW/BWs of lenti-AT 2 R–treated animals (3.7±0.02 mg/g) revealed a significantly lower ratio than control animals (4.0±0.10 mg/g) (Figure 4C). These data indicate that AT 2 R transduction of cardiac myocytes alters cardiac hypertrophy without influencing high BP.

Discussion
Through the use of a lentiviral vector system delivered directly into the ventricular space at 5 days of age, we were able to overcome the inherent problems with transgenic and
knockout mice and found that AT$_2$R transduction in the SHR prevents the development of cardiac hypertrophy without influencing high BP.

These findings are exciting for 4 major reasons: (1) the AT$_2$R can induce a beneficial effect on cardiac hypertrophy independent of AT$_1$R effects; (2) the local RAS seems to be the key factor in this effect; (3) transduction of only 40% of cardiac cells seems to produce significant attenuation of hypertrophy, which suggests that some paracrine/endocrine mechanism must exist that propagates signals from AT$_2$R-transduced cells to the entire heart. This proposal is consistent with the observation and conclusions from the cell therapy experiments in which a significant improvement in cardiac functions can be accomplished by implantation of only a few thousand transduced stem cells into the heart$^{27}$; and (4) because the lentiviral vector integrates into the host genome, this system may provide a novel therapeutic option for long-term prevention of cardiac hypertrophy.

It is generally believed that cardiac hypertrophy arises from a growth or enlargement of existing myocytes. However, recent evidence indicates that new myocytes can form from stemlike cells, which are markedly enhanced during cardiac hypertrophy.$^{28}$ In addition, there could be some yet unknown autocrine/paracrine factor that may help to propagate these effects in the heart. Therefore, even with a transduction efficiency of 40%, almost complete inhibition of cardiac hypertrophy can be achieved. Future experiments must address whether these effects are caused by changes in the size of the myocytes, decreased myocyte cell number, or some unknown paracrine/autocrine factor.

A previous study has shown an increase in the AT$_2$R in response to cardiac hypertrophy,$^{13}$ yet our results do not show an increased level of AT$_2$R mRNA in control SHR. There could be many explanations for this difference. In our study, we examined the mRNA levels for the AT$_2$R in the cardiomyocytes, whereas the previous study was looking at AT$_2$R binding in membranes from the whole heart. Therefore, increases in the AT$_2$R observed in these studies could be occurring either at a post-transcriptional level or in cell types other than the cardiomyocytes, such as the fibroblasts or endothelial cells. It is also likely that the compensatory response to increase AT$_2$R does not reach high enough levels to exert these antihypertrophic actions. Thus, it would be important to determine whether AT$_2$R overexpression in adult SHR would result in a reversal of cardiac hypertrophy.

Accumulating evidence indicates that all of the components of the RAS exist in the heart. This local RAS and not the systemic RAS appears to be key in the control of normal cardiac functions. Angiotensin-converting enzyme inhibitors, AT$_1$R antagonists, and AT$_2$R-AS have been used to show that a reduction in left ventricular mass by these inhibitors is independent of changes in arterial pressure.$^{7–9,29}$ In this study, an AT$_2$R- and BP-independent effect of AT$_2$R transduction on cardiac hypertrophy is shown for the first time, thus indicating that in our system, the AT$_2$R is acting at the local rather than a systemic level.

This study presents a fascinating finding, considering the array of studies suggesting opposing roles for the AT$_2$R. Many transgenic and knockout animal experiments indicate that the AT$_2$R either does not play a role in cardiac hypertrophy at all or the AT$_2$R plays a fundamental role in the development of LVH. We believe that our gene transfer model has an advantage over the transgenic models because the genetic manipulation does not occur until after cardiac development has occurred. However, it raises an interesting question as to whether the beneficial effect of AT$_2$R transduction prevents hypertrophy or simply delays it. There is no conclusive evidence to support either situation at the present time. However, we believe that the effect may be a prevention of hypertrophy. This view is based on the fact that at 21 weeks of age, AT$_2$R-treated SHRs have LVWT comparable with that seen in normotensive controls. It will be interesting to determine whether this gene transfer protocol of overexpression will carry over to other models of CV pathophysiology and to investigate the long-term effects of the AT$_2$R transgene on other cardiac pathophysiologies such as heart failure.

Perspectives
A better understanding of the role of the AT$_2$R in cardiac hypertrophy is critical in the future treatment of cardiac pathophysiology. This study provides evidence that a direct delivery of the AT$_2$R in the heart of neonatal SHR prevents the development of cardiac hypertrophy, an effect that is independent of BP. This observation provides a means to determine whether AT$_2$R transduction would lead to a beneficial outcome in other CV pathophysiologies such as heart
failure. In addition, it would be important to determine whether this AT$_2$R gene transfer protocol has the potential to reverse cardiac pathophysiologies. If this can be achieved, this gene delivery system has the potential to be a novel therapy to treat pathophysiologies of the heart without any effects on BP.

**Acknowledgments**

This work was supported by National Institutes of Health grants HL 56921 and HL 68085 (M.K.R., C.S.) and an American Heart Association (AHA) Scientist Development Grant (H.J.K.). B.L.M. and D.G.T. are predoctoral fellows, and L.D.P. is a postdoctoral fellow of the AHA Florida/Puerto Rico Affiliate. We thank Laura Dixon for real-time RT-PCR primer design and optimization and Michael Anthony Cometa for indirect blood pressure measurements.
References


Prevention of Cardiac Hypertrophy by Angiotensin II Type-2 Receptor Gene Transfer

Beverly L. Metcalfe, Matthew J. Huentelman, Leonard D. Parilak, David G. Taylor, Michael J. Katovich, Harm J. Knot, Colin Sumners and Mohan K. Raizada

Hypertension. 2004;43:1233-1238; originally published online April 19, 2004;
doi: 10.1161/01.HYP.0000127563.14064.fd

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/43/6/1233

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/