Parallel Changes in Neuronal AT1R and GRK5 Expression Following Exercise Training in Heart Failure


Abstract—Although exercise training (ExT) is an important therapeutic strategy for improving quality of life in patients with chronic heart failure (CHF), the central mechanisms by which ExT is beneficial are not well understood. The angiotensin II type 1 receptor (AT1R) plays a pivotal role in the development of CHF and is upregulated in a number of tissues owing, in part, to transcription factor nuclear factor kappa B (NF-κB). In addition, AT1R is marked for internalization and recycling via G-protein–coupled receptor kinase (GRK) phosphorylation. Because previous studies have shown that the beneficial effects of ExT in CHF rely on a reduction in angiotensin II, we hypothesized ExT would decrease AT1R, GRK5, and NF-κB protein expression in the paraventricular nucleus and rostral ventrolateral medulla of CHF rats. Following infarction by coronary artery ligation, animals were exercised 4 weeks postsurgery on a treadmill at a final speed of 25 miles per minute for 60 minutes, 5 days per week for 6 weeks. Western blot analysis of paraventricular nucleus and rostral ventrolateral medulla micropunches revealed an upregulation of AT1R, GRK5, and NF-κB in the infarcted group that was reversed by ExT. Furthermore, the relative expression of phosphorylated AT1R and AT1R/GRK5 physical association was increased in the CHF sedentary group and reversed by ExT. Overexpression of GRK5 in cultured CATH.a neurons blunted angiotensin II-mediated upregulation of AT1R and NF-κB; conversely, silencing of GRK5 exacerbated angiotensin II-mediated AT1R and NF-κB upregulation. Taken together, increased GRK5 may regulate AT1R expression in CHF, and ExT mitigates AT1R and its pathway components. (Hypertension. 2012;60:00-00.) • Online Data Supplement

Key Words: angiotensin II ■ GRK5 ■ NF-κB ■ receptor turnover

A hallmark of the chronic heart failure (CHF) state is increased sympathetic outflow, which can be correlated to both disease severity and mortality; however, the mechanisms underlying the increased sympathoexcitation are not completely understood. A good deal of work has focused on the role of the angiotensin H (Ang II) type 1 receptor (AT1R) regulation in the development of CHF and as a driver of sympathoexcitation.

The paraventricular nucleus (PVN) of the hypothalamus is an integrative center for endocrine, hormonal, and neural control. The PVN contains separate neuronal cell populations: the parvocellular subgroup, which projects to the rostral ventrolateral medulla (RVLM) and the intermediolateral cell column of the spinal cord, influencing sympathetic nerve activity; and magnocellular neurons, which project to the posterior pituitary to release vasopressin. Neurons from the RVLM, in turn, project to the sympathetic preganglionic motor neurons, whose activity is responsible for blood pressure regulation and baroreflex function and are the primary drivers of sympathetic tone. Previous data from this laboratory and others suggest that, in rats with CHF, the neurons in these nuclei are hyperactive.

Exercise training (ExT) in humans with CHF was originally contraindicated owing to fear of worsening cardiac function combined with the assumption that patients with CHF would have limited capacity for ExT and increased risk for exercise-related mortality. It is becoming increasingly accepted that ExT in CHF is safe and can increase survival, decrease complications, and abrogate increases in muscle-sympathetic nerve activity; however, the mechanism by which ExT exerts these beneficial effects in the CHF state and its effect on AT1R expression and signaling are not well-understood.

On binding of Ang II to the AT1R, a G-protein–coupled receptor, the Gq signaling cascade is activated, leading to a wide range of downstream consequences, including increased intracellular calcium, inositol trisphosphate, phospholipase A2, increased NAD(P)H oxidase activity, Janus kinase, c-Jun

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From the Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE (K.K.V.H., C.W.E., E.P., I.I.P., K.P.P., I.H.Z.);

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Correspondence to Irving H. Zucker, PhD, Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, NE 68198-5850. E-mail izucker@unmc.edu

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N-terminal kinase, extracellular signal-related kinase and protein kinase C activity. In CHF, increased circulating Ang II can lead to long-term activation of many of these signaling events via the AT1R. In addition, similar to other enhanced sympathoexcitatory disease conditions such as hypertension, the AT1R is upregulated in both central and peripheral tissues. Recent data from our laboratory has also shown that, in a neuronal cell line, Ang II-mediated upregulation of AT1R is, in part, driven by nuclear factor kappa B (NF-κB) and activator protein 1, 2 transcription factors that regulate the expression of many genes during CHF and other clinical pathologies.

On ligand binding, most G-protein–coupled receptors exhibit a rapid loss of responsiveness following activation, leading to receptor internalization. This desensitization is mediated, in part, by G-protein–coupled receptor kinases (GRKs), which phosphorylate the active receptor. The phosphorylated receptor is then bound by β-arrestin and sequestered into endosomal vesicles for either recycling or degradation. Previous work has shown that the AT1R can be phosphorylated by both GRK5 and/or GRK2 (BARK1) in a tissue-specific manner. GRK2, or BARK1, not only plays a role in promoting β-arrestin binding to GPCRs, but it also phosphorylates non-GPCR substrates and is implicated in signal transduction. GRK5 is a membrane-associated kinase and is robustly expressed in heart and lung tissue, smooth and skeletal muscle, and brain. However, the role that GRK5 plays in AT1R regulation in the brain has not yet been elucidated. In the current study, we hypothesized that central GRK5 and p65 NF-κB, 2 proteins involved in AT1R expression, are also increased during CHF and normalized by ExT.

Methods

Animals

Male Sprague-Dawley rats, weighing 220 to 280 g (Sasco Breeding Laboratories), were fed and housed according to institutional guidelines. Protocols were approved by the University of Nebraska Institutional Animal Care and Use Committee and were in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Laboratory Animals. Rats were given rat chow and water ad libitum and were housed in a room with a 12-hour light-dark cycle. Rats were allowed to acclimatize for 1 week before cardiac surgery.

Induction of Heart Failure

Rats were randomly assigned to either the Sham-operated control group or the CHF group. CHF was induced by ligation of the left coronary artery as has been described previously. The expanded methods are available in the online-only Data Supplement.

Metabolic Cage Assessment and Measurement of Urinary Norepinephrine Excretion

Please see the online-only Data Supplement for metabolic cage assessment and measurement of urinary norepinephrine excretion (NE).

Exercise Training Protocol

Four weeks following coronary artery ligation surgery, rats were randomly assigned to either ExT or Sedentary (Sed) groups to produce 4 total experimental groups: Sham-Sed, HF-Sed, Sham-ExT, and HF-ExT. ExT was carried out on a motor-driven treadmill (Columbus Instruments) at a final speed of 20 to 25 miles per minute, 60 minutes per day, and 5% to 10% incline for a period of up to 6 weeks, according to a protocol modified from Musch and Terrell. Additional protocol details can be found in the online-only Data Supplement.

Micropunch of the PVN and RVLM and Isolation of Protein for Western Blot Measurements

Micropunches of the PVN and RVLM were isolated as described previously. Additional protocol details can be found in the online-only Data Supplement.

Cell Culture and Maintenance

CATHa catecholaminergic neurons were maintained in RPMI-1640 with 15% horse serum and 5% fetal bovine serum and an antibiotic cocktail. Cells were differentiated 48 to 72 hours before experimentation by serum starvation, as performed previously. Please see the online-only Data Supplement for additional details.

Western Blot Measurement of AT1R and GRK5 Proteins

Western blot measurements are detailed within the online supplement.

Statistical Analysis

Data are presented as mean±SE. The data were subjected to 1-way ANOVA followed by comparison for individual group differences using the Newman-Keuls test or Bonferroni correction. Statistical significance is indicated by a value of P<0.05.

Results

Heart weight data and echocardiographic values are shown in the online-only Data Supplement Table S1. Briefly, there was a significant decrease in ejection fraction and fractional shortening and a significant increase in cardiac dimensions in the CHF animals compared to Sham; however, there was no effect of ExT on any of these parameters, consistent with previous findings. To confirm an ExT effect, the soleus of some animals was removed and analyzed for citrate synthase activity. As expected, citrate synthase levels were significantly elevated in both Sham and CHF-ExT groups (Sham-ExT: 13.0±1.7 μmol/g/min; CHF-ExT: 12.3±0.9 μmol/g/min compared with the Sed groups: Sham-Sed: 8.3±1.5 μmol/g/min; CHF-Sed: 8.4±0.87 μmol/g/min; P<0.03 comparing Sed with ExT for each group), demonstrating a significant effect of ExT.

To assess the effects of ExT on a more global index of sympathetic tone, we assessed urinary NE in the 4 groups of animals. Compared with Sham-Sed animals, CHF-Sed animals excreted significantly more NE (1.95±0.46 g NE/24 hours; P<0.05). On the other hand, CHF ExT animals excreted significantly less NE compared with HF-Sed animals (0.55±0.04 versus 4.11±1.40 μg NE/24 hours; P<0.05). Sham-ExT animals excreted approximately the same NE as Sham-Sed animals (1.94±0.46, 1.95±0.57, respectively). Taken together, these data suggest that ExT decreases sympathoexcitation in rats with CHF.

Given that one of the hallmarks of CHF is increased circulating Ang II concentrations, we hypothesized that this would lead to an increase in AT1R expression in the PVN and RVLM of CHF-Sed animals and a subsequent decrease in
Figure 1. AT1R (A), GRK5 (B), p65 NF-κB (C) and β-arrestin (D) are increased in the PVN (solid bars) and RVLM (open bars) of CHF animals and normalized by ExT. GRK2 (E), another kinase implicated in regulating AT1R expression, is unchanged in the PVN during both CHF and ExT. *P<0.05 versus Sham-Sed. †P<0.05 versus CHF-Sed; n=5 to 7.
investigated changes in the phosphorylated form of NF-

AT1R protein following ExT. We performed Western blot analysis on lysed PVN and RVLM micropunches and probed for AT1R expression (Figure 1A). There was a significant increase in AT1R protein expression in the CHF-Sed animals compared with the Sham groups in both nuclei. Following ExT, AT1R expression in CHF animals was decreased to Sham levels.

Because AT1R expression is altered in CHF animals, we next considered the role of GRK2 and GRK5 in the regulation of AT1R during CHF and its modulation by ExT. We performed Western blotting on the aforementioned micropunch lysates and immunoblotted for GRK5 and GRK2 (Figure 1B; Figure 1E). GRK5 protein was increased in CHF-Sed animals by 50% compared with Sham-Sed animals. This elevated GRK5 protein was normalized by ExT, thereby following the same trend observed for AT1R protein. These fluctuations in GRK5 and AT1R were also mimicked by changes in β-arrestin in the PVN (Figure 1D). Although β-arrestin was also significantly greater in the CHF-Sed animals compared with Sham in the RVLM, ExT did not normalize β-arrestin expression. In contrast, GRK2 was unchanged across animal groups (Figure 1E).

Given that NF-κB activation has been implicated in the upregulation of a number of genes in the CHF state and that previous studies from our laboratory have shown that, in a mouse catecholaminergic CATH.a neuronal cell line, Ang II stimulation leads to increased NF-κB levels, we also investigated changes in the phosphorylated form of NF-κB in CHF and ExT animals. Examination of PVN and RVLM micropunch lysates for the p65 subunit of NF-κB indicate that this transcription factor is elevated in CHF animals and is normalized following ExT (Figure 1C). Interestingly, in the PVN of CHF-ExT animals, there was significantly lower p65 NF-κB protein expression compared with Sham-Sed animals. These data imply a relationship between GRK5 and AT1R and potential involvement of NF-κB.

To further examine the relationship between GRK5 and AT1R, we performed coimmunoprecipitation experiments in the same PVN micropunch total cell lysates (Figure 2A). We hypothesized there would be increased GRK5/AT1R interaction in the CHF-Sed group compared with Sham animals and that this interaction would be decreased in the CHF-ExT group. Indeed, there was a 4-fold increase in GRK5/AT1R association in CHF-Sed animals compared with Sham groups, which was normalized by ExT. To confirm a functional effect of GRK5 on AT1R, we examined the amount of phosphorylated AT1R by immunoprecipitating using an anti-AT1R antibody and immunoblotting with an anti-phosphothreonine antibody (a site of GRK5 action) (Figure 2B). There was a 100% increase in phosphorylated AT1R in CHF-Sed animals compared with Sham-Sed. ExT in CHF animals exhibited a degree of phosphorylated AT1R that was similar to Sham-Sed animals. Because GRK2 levels do not change in the face of either CHF or ExT, we wanted to confirm the GRK5-AT1R interaction is specific by repeating coimmunoprecipitation experiments in these same lysates immunoblotting for GRK2. As shown in online-only Data Supplement Figure 2A, GRK2 and AT1R do not associate. As a control for any nonspecific protein-protein interaction, we performed experiments in the same lysates pulling down for the α2C adrenergic receptor (α2C-AR), which has been shown previously to not be modified by GRK5 (online-only Data Supplement Figure 2B). Indeed, in all lysates tested, there was no interaction between the α2C-AR and GRK5, but we were able to detect both GRK5 and GAPDH in the total lysates on the same blot.

To further explore the interaction between AT1R and GRK5, we next used the CATH.a neuronal cell line to examine changes in AT1R expression following manipulation of GRK5 either by overexpression or knockdown. We first characterized the changes in AT1R, GRK5, and p65 NF-κB protein expression, following stimulation with 100-nmol/L Ang II for 4 hours (Figure 3A–3C). As expected, stimulation with Ang II led to an upregulation of all proteins; conversely, stimulation with 100-nmol/L losartan (Los), an AT1R antagonist, or costimulation with Ang II plus Los did not provoke any changes in protein abundance. In addition, these experiments were repeated at 1-nmol/L Ang II, which evoked similar results (see online-only Data Supplement Figure S3). Importantly, the upregulation of AT1R and p65
NF-κB following Ang II stimulation was completely prevented following transient transfection of GRK5 plasmid (Figure 3A through 3C). These data further suggest that GRK5 plays a role in the regulation of AT1R, potentially via modulation of NF-κB. To confirm this finding, we performed the inverse experiments in which we silenced GRK5 expression in CATH.a neurons with siRNA knockdown (Figure 4A–4C). Knockdown of GRK5 led to a 3-fold increase in AT1R protein levels before stimulation, and following the addition of Ang II, AT1R was upregulated further.

**Discussion**

The main finding in the current study was that the expression of AT1R, GRK5 and p65 NF-κB were augmented in the PVN and RVLM of animals with CHF, and this augmentation was abrogated following a regimen of ExT for 6 weeks. Furthermore, we demonstrated that ExT is associated with normalization in AT1R protein expression in the PVN. Importantly, this normalization was concomitant with a decrease in GRK5, a protein responsible for marking the AT1R for internalization in AT1R protein expression in the PVN. Importantly, this normalization was concomitant with a decrease in GRK5, a protein responsible for marking the AT1R for internalization and degradation, and NF-κB, a transcription factor involved in the upregulation of AT1R.

The observation that ExT reduces AT1R expression is consistent with the idea that this maneuver is capable of impacting protein expression to this and, potentially, other G-protein-coupled receptor kinases. This and other laboratory studies have shown that central AT1R expression is upregulated in CHF and hypertension. We have previously described a transcriptional pathway by which the AT1R is regulated in CHF and in response to exogenous Ang II.

It is still unclear if ExT impacts all of the downstream proteins that are involved in this pathway. Unfortunately, almost nothing is known about AT1R regulation outside of its transcription in the brain and the control of autonomic outflow in disease states following interventions such as ExT. We sought to explore the role of the G protein regulatory pathway in these observations. In this regard, an interesting aspect of the current study was that GRK5 was also upregulated in CHF-Sed animals, and this increase was normalized by ExT. Functionally, our data suggest that GRK5 and AT1R are interacting and that GRK5 may play a role in the regulation of AT1R (ie, increase in receptor phosphorylation; Figure 2). Subsequent studies in the CATH.a neurons indicated that silencing of GRK5 exacerbates AT1R and p65 NF-κB increase. Taken together, we propose that the ancillary increase in GRK5 in the PVN and RVLM is a compensatory mechanism responding to the primary increase in AT1R seen in CHF. The AT1R behaves in a nonclassical manner as compared with other GPCRs; instead of a ligand-induced receptor desensitization and internalization, AT1R expression increases with continued Ang II presence. Although initially counterintuitive that GRK5, a regulatory kinase would also be increased with AT1R and p65 NF-κB, our data would suggest that, in fact, GRK5 upregulation, seen either in CHF-Sed animals or in CATH.a neurons stimulated with Ang II, is a means by which the cell prevents even further increases in Ang II. In essence, GRK5 is serving as a
brake for the Ang II/AT1R feed-forward axis. This scenario is certainly not unprecedented. For instance, a similar example also seen in CHF is the response of Atrial Natriuretic Peptide (ANP). One would expect that the increase in ANP secretion would evoke natriuresis as a way of normalizing volume in CHF; however, this does not happen because of the many antinatriuretic factors that are activated in CHF.29 Similarly, we propose here that GRK5 is upregulated to counterbalance, even further, increases in AT1R expression. Work by Ishizaka et al has shown that GRK5 is upregulated in vascular smooth muscle cells in an Ang II-dependent manner and is also upregulated in the aorta of hypertensive animals.26 We hypothesize that, in the absence of GRK5, the upregulation of AT1R seen in CHF would be further exacerbated. GRKs have previously been suggested as potential therapeutic targets for the treatment of CHF.40,41 In a study by Sorriento et al., an adenovirus encoding the amino terminus of GRK5 was injected into the cardiac wall of spontaneously hypertensive rats (SHRs) and decreased left ventricular hypertrophy via a kinase-independent stabilization of IkB.32 In light of our previous data in the CATH.a neuronal cell line that indicated NF-κB protein was upregulated in an Ang II-dependent manner,20 it is possible that there are physical and functional interactions of central GRK5, NF-κB and IkB in both normal and CHF animals. Whether ExT has an effect on the stabilization of the GRK5/1kB/NF-κB complex and if there is a protective effect of GRK5 overexpression in the brain of animals with increased sympathoexcitation is not clear at the present time. It is of note that p65 NF-κB protein decreased in CHF-ExT animals to levels lower than that of Sham. Because p65 NF-κB is a transcription factor on which a number of signaling cascades besides AT1R converge, it is likely that some of the other beneficial effects of ExT in CHF animals that we did not examine in this study are also being altered, thereby leading to a redundant (or additional) decrease in p65 NF-κB. GRK5 can also translocate to the nucleus of cardiac myocytes and serve as a histone deacetylase kinase (HDAC kinase).43 It will be important to investigate this novel aspect of GRK5 signaling in the brain.

The role of ExT as a useful therapeutic paradigm in the setting of CHF has been examined in several clinical trials. Belardinelli et al clearly demonstrated that a supervised ExT regimen not only reduced cardiovascular events but also increased survival.11 This study was carried out in a relatively small cohort, and neurohormonal data were not provided. A larger multicenter clinical trial is ongoing to evaluate the effects of ExT in patients with CHF. The Heart Failure: A Controlled Trial Investigating Outcomes of Exercise Training (HF-ACTION) trial has so far indicated an enhanced quality of life and a reduction in hospitalization, as well as a reduction in all-cause mortality for patients with CHF ExT.44 Unfortunately, these clinical studies do not address mechanisms. In a study by Adams et al., it was clearly shown that ExT enhanced endothelial function in the forearm vasculature by a NO-dependent mechanism.45 It is not clear if this contributes to central alterations as well. In a seminal study by Roveda et al.,14 direct recording of muscle-sympathetic nerve activity (MSNA) in patients with CHF provided strong support for the inhibition of peripheral sympathetic out-

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**Figure 4.** GRK5 knockdown with siRNA leads to greater increases in AT1R and p65 NF-κB protein following Ang II stimulation. Values are expressed as a ratio of protein to GAPDH and normalized to no ligand. A. AT1R. B. p65 NF-κB. C. GRK5. *P<0.05 versus no ligand. †P<0.05 versus non-siRNA Ang II stimulation. ‡versus non-siRNA; n=4 to 6.
flow following ExT, which is consistent with that observed in the current study. In fact, our data suggest a more global reduction in sympathetic outflow based on the decrease in NE.

**Perspectives**

We show here that ExT normalizes the upregulated AT1R, GRK5, and p65 NF-kB protein seen in the PVN and RVLM of CHF animals. We speculate that GRK5 upregulation in the CHF state may be one mechanism to prevent further increases in AT1R expression. These data suggest that ExT and central RAS modulation may be highly interactive in various disease states characterized by sympathoexcitation. Although the question of how ExT mediates beneficial effects in CHF remains unclear, the correlative decreases in AT1R and p65 NF-kB may be one potential mechanism. It is possible that targeting GRK5 as an alternate means of downregulating AT1R in the CHF state may provide a novel strategy for potential therapy applicable to a variety of sympathoexcitatory states, including hypertension.

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**Disclosures**

None.

**References**


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Parallel Changes in Neuronal AT1R and GRK5 Expression Following Exercise Training in Heart Failure

Karla K.V. Haack, Christopher W. Engler, Evlampia Papoutsi, Iraklis I. Pipinos,
Kaushik P. Patel, and Irving H. Zucker

Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5850
Supplemental Methods

Induction of Heart Failure. Briefly, all rats were anesthetized with isoflurane (0.5-2% in oxygen) intubated, and mechanically ventilated. Under sterile conditions, a left thoracotomy was performed through the fifth intercostal space. Subsequently, the pericardium was opened and the heart was exteriorized. The left anterior descending coronary artery was ligated with a 6–0 prolene suture that was passed through the superficial layers of myocardium, between the pulmonary artery outflow tract and left atrium. Following ligation, the heart was placed in its original position, and the thorax was closed. The air within the thorax was evacuated, allowing the rats to resume spontaneous respiration and recover from anesthesia. Analgesia (Buprenorphine, Reckitte Benckiser, Hull, UK; 0.1 mg/kg, sc) was administered post-surgically. Sham-operated rats were prepared in the same manner but did not undergo coronary artery ligation. Left ventricular dysfunction was assessed using hemodynamic and anatomic criteria. Echocardiograms were performed (Vevo 770; Visualsonics, Inc.) before, during, and after the six week ExT period. Rats with ejection fraction as determined by echocardiogram of less than 50%, were considered to be in HF.

Exercise Training Protocol. Initially, a low speed (10 m/min) and grade (0%) and short duration (10 min/day; 5 days/week) was used to familiarize the rats with running on the treadmill. The speed, duration, and grade were gradually increased to 20-25 m/min, 60 min/day, and 5-10%, respectively, to ensure that a significant endurance effect was produced. This level of exercise is considered moderate for the sham rats. Only rats that ran steadily with little or no prompting were used in the study. To ensure a similar level of ExT between groups and to document a training effect, in some animals, citrate synthase activity assays on the soleus muscle were performed following the protocol of Srere. In a subgroup of animals, an ExT effect was determined by the duration of time the animal could run continuously without lagging or stopping. This testing was performed both pre- and post-ExT or Sed periods.

Metabolic Cage Assessment and Measurement of Urinary Norepinephrine Excretion. Rats were placed in a metabolic cage to measure water and food intake, urine and fecal excretion, and body weight for 72-96 hour intervals pre- and post-ExT. The urine was collected under mineral oil and frozen (–80°C) until it was used for the measurement of norepinephrine concentration. This measurement was done using a Norepinephrine Enzyme Immunoassay kit (Labor Diagnostika Nord GmbH & Co KG, Montreal, Quebec) according to the manufacturer’s instructions.

Micropunch of the PVN and RVLM and isolation of protein for Western blot measurements. After euthanization, brains were removed and quickly frozen on dry ice. Coronal sections were cut through the hypothalamus and medulla at the level of the PVN and RVLM respectively using a cryostat and, following the Palkovits technique, the nuclei were bilaterally punched using a diethylpyrocarbonate (DEPC)-treated blunt 18-gauge needle attached to a syringe. Punches were lysed in 200 µL of RIPA buffer with fresh protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and total protein concentration was measured using a BCA Assay kit.
**Cell Culture and Maintenance.** For overexpression experiments, a GRK5 in pcDNA3 plasmid (a generous gift from Dr. Jeffrey Benovic) was transfected into confluent CATH.a cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent according to manufacturer’s instructions. GRK5 knockdown using siRNA (Santa Cruz) transfection were also performed using Lipofectamine 2000 according to manufacturer’s instructions.

**Western blot measurement of proteins.** Samples were adjusted to contain the same concentration of total protein, and then equal volumes of 2X 4% SDS sample buffer were added. The samples were boiled for 3 min and then loaded onto a 7.5% SDS-PAGE gel (40 µg/20 µl per well). Gels were subjected to electrophoresis at 115 V/gel for 60 min. The fractionated proteins on the gel were electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA) at 50 V for 90 min. The membrane was probed with any of the primary antibodies overnight: rabbit anti-phosphothreonine (1:500, Abcam, Cambridge, MA), goat or rabbit anti-AT1R, rabbit anti-GRK5, rabbit anti-GRK2, mouse anti-p65 NF-κB, and/or mouse anti-GAPDH (1:500-1:1,000, Santa Cruz). Although the commercial availability of the antibodies should imply specificity, we performed blocking peptide experiments for the GRK5 antibody in both PVN and CATH.a lysates (Supplemental Figure 1). Following thorough washes with PBST, the samples were then probed with the appropriate secondary antibodies (Li-Cor Biosciences, Lincoln, NE). Blots were developed using a Li-Cor Odyssey scanner and quantitative analysis of band densitometry was performed using the Li-Cor Odyssey software. The relative abundance of proteins of interest was calculated as the ratio of intensity of the band relative to the intensity of GAPDH. Graphs summarizing individual experiments are shown as a fold change compared to the Sham Sed animals or fold change compared to non-stimulated cells. Preparation of samples and co-immunoprecipitation experiments can be found in the online supplement.

**Co-immunoprecipitation.** In order to determine the interaction between AT1R and GRK5 co-immunoprecipitation (co-IP) experiments were carried out. In co-IP experiments, equivalent amounts of PVN total protein lysate were tumbled with Roche Protein G beads (Roche, Indianapolis, IN) and pre-cleared for three hours. After centrifugation (20 seconds, 12,000 x g), the pre-cleared lysate was allowed to tumble with appropriate antibody (either goat anti-AT1R or rabbit anti-GRK5, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr prior to the addition of Protein G beads. The conjugated beads and lysate were tumbled overnight at 4°C. The beads were washed with two separate washing buffers (Buffer 1: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tween-20, 0.05% Na Deoxycholate; Buffer 2: 50 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.1% Tween-20) with protease inhibitor cocktail added on the day of experimentation. Protein was eluted from the beads using 50µL of 2X 4% SDS sample buffer with β-mercaptoethanol (2.5% final concentration) added fresh and heated to 100°C prior to western blotting.


Table S1. Echocardiographic, baseline body weight, and heart weight data.

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<th>parameter</th>
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<td>FS%</td>
<td>39.9 ± 5.3</td>
<td>43.5 ± 4.1</td>
<td>23.2 ± 2.2</td>
<td>23.6 ± 3.6</td>
</tr>
<tr>
<td>BW (g)</td>
<td>443 ± 10</td>
<td>388 ± 17 †</td>
<td>432 ± 11</td>
<td>386 ± 11 †</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.3 ± 0.2</td>
<td>4.3 ± 0.5</td>
<td>4.4 ± 0.1 †</td>
<td>4.4 ± 0.3 †</td>
</tr>
</tbody>
</table>

Data are means±SE. * p<0.05 compared to sham, † p<0.05 compared to sed, ‡ p=0.0514 compared to sham. LVDd, Left ventricular end diastolic diameter; LVDs, left ventricular end systolic diameter; LVd Vol, left ventricular end diastolic volume; LVs Vol, left ventricular end systolic volume; EF%, Ejection Fraction; FS%, fractional shortening; HW/BW, heart weight/body weight ratio.
Figure S1. GRK5 antibody is specific in both PVN micropunch lysates and CATH.a neurons.
Figure S2. GRK2 and AT1R α2c-AR and GRK5 do not physically associate. A. In co-immunoprecipitation experiments in PVN micropunches, lysates were pulled down with an anti-AT1R antibody (AT), and IP samples were run alongside total protein lysates. Immunoblotting with an anti-GRK2 antibody (G) indicates that AT1R and GRK2 do not physically associate, as no positive bands were detected in any of the IP lanes. Both GRK2 (shown in red) and GAPDH (shown in green) were detected in all lanes containing total lysate. B. Lysates were pulled down with an anti-α2c-AR antibody and IP samples were run alongside total protein lysates. α2c-AR and GRK5 do not physically associate, but both GRK5 (shown in green) and GAPDH (shown in red) are detected in total lysate.
Figure S3. Lower dose of Ang II elicits same changes in AT1R, p65 NF-κB and GRK5. Western blots of CATH.a neuron total lysates stimulated with 1 nM Ang II, 100 nM Los, or 1 nM Ang II plus 100 nM Los. Stimulation with 100-fold lower dose of Ang II still increases AT1R, p65 NF-κB and GRK5 proteins.