Lack of Specificity of Commercial Antibodies Leads to Misidentification of Angiotensin Type 1 Receptor Protein

Marcela Herrera, Matthew A. Sparks, Adolfo R. Alfonso-Pecchio, Lisa M. Harrison-Bernard, Thomas M. Coffman

Abstract—The angiotensin II type 1 receptor (AT₁R) mediates most hypertensive actions of angiotensin II. To understand the molecular regulation of the AT₁R in normal physiology and pathophysiology, methods for sensitive and specific detection of AT₁R protein are required. Here, we examined the specificity of a panel of putative AT₁R antibodies that are commonly used by investigators in the field. For these studies, we carried out Western blotting and immunohistochemistry with kidney tissue from wild-type mice and genetically modified mice lacking the major murine AT₁R isoform, AT₁ₐA (AT₁ₐA KO), or with combined deficiency of both the AT₁ₐA and AT₁ₐB isoforms (AT₁ₐB/A KO). For the 3 antibodies tested, Western blots of protein homogenates from wild-type kidneys yielded distinct bands with the expected size range for AT₁R. In addition, these bands appeared identical in samples from mice lacking 1 or both murine AT₁R isoforms. Additionally, the pattern of immunohistochemical staining in kidneys, liver, and adrenal glands of wild-type mice was very similar to that of AT₁ₐB/A KO mice completely lacking all AT₁R. We verified the absence of AT₁,R subtypes in each mouse line by the following: (1) quantitative polymerase chain reaction documenting the absence of mRNA species, and (2) functionally by assessing angiotensin II–dependent vasoconstriction, which was substantially blunted in both AT₁ₐA KOs and AT₁ₐB/A KOs. Finally, these antibodies failed to detect epitope-tagged AT₁ₐR protein overexpressed in human embryonic kidney cells. We conclude that anti-AT₁R antibodies available from commercial sources and commonly used in published studies exhibit nonspecific binding in mouse tissue that may lead to erroneous results. (Hypertension. 2013;61:253-258.)

Key Words: angiotensin II type 1 receptor ■ Western blot ■ cross-reactivity ■ AT₁ₐA ■ AT₁ₐB

The type 1 angiotensin receptor (AT₁R) is a key component of the renin–angiotensin system. AT₁R mediates most of the classically recognized actions of angiotensin II. Activation of AT₁,R stimulates vascular contractility and renal sodium retention1–4 playing a crucial role in regulating blood pressure in health and disease. In addition to these hemodynamic effects, the AT₁R also mediates cell proliferation, fibrosis, and end organ damage.5–7 Because of the key role of these receptors in physiology and pathophysiology, accurate detection of the AT₁R protein is paramount for investigations aimed at understanding molecular mechanisms of health and disease. Although several AT₁R antibodies are available commercially, and others,4 had major concerns about their specificity.

Humans have only 1 AT₁R isoform; however, 2 isoforms exist in rodents, termed AT₁ₐA and AT₁ₐB. These receptor subtypes are products of 2 different genes: Agtr1a is located on mouse chromosome 13 (17 in rats), and Agtr1b on mouse chromosome 3 (2 in rats). They both encode a 375-amino acid protein with a predicted molecular weight of 42 kDa.9–11 They exist in rodents, termed AT₁ₐA and AT₁ₐB. These receptor subtypes are products of 2 different genes: Agtr1a is located on mouse chromosome 13 (17 in rats), and Agtr1b on mouse chromosome 3 (2 in rats). They both encode a 375-amino acid protein with a predicted molecular weight of 42 kDa.9–11 They share 94% identity and are indistinguishable pharmacologically. Based on bioinformatics predictions (http://www.cbs.dtu.dk/services/NetNGlyc/), these receptors are presumed to undergo post-translational glycosylation. Accordingly, using a plasmid expressing the AT₁R tagged to a myc epitope, Deslauriers and colleagues reported massive AT₁R glycosylation in transfected COS-7 cells.12 In that study, the molecular size of the glycosylated AT₁R form was estimated to be ≈100 to 150 kDa. Although the degree of glycosylation of proteins is a tissue-specific process, it is difficult to predict the molecular mass of these receptors under different tissues or experimental conditions, and published data clarifying this issue are lacking.

During the last decade, anti-AT₁R antibodies have been widely used in scientific reports related to AT₁R signaling and functions. However, their specificity has not been thoroughly investigated in the medical literature. In preliminary studies using mice with targeted deletion of AT₁R genes that were generated in our laboratory, we became concerned about the specificity of these antibodies. Accordingly, we carried out a systematic evaluation of a panel of anti-AT₁R antibodies that were purchased from commercial vendors, focusing on their utility and specificity for Western blot analysis and immunohistochemistry.
Materials and Methods

Please see online-only Data Supplement.

Results

We first performed Western blot analysis using homogenates of cortex (C) and medulla (M) from kidneys of wild-type (WT) mice comparing band patterns produced by 3 anti-AT1R antibodies. As shown in Figure 1, antibodies 1 and 2 each generated a single band between 38 and 48 kDa, which would be around the expected 41-kDa size of the AT1R.9–11 Although each of these antibodies identified a single band, the molecular weight of these bands was slightly different. In contrast, antibody 3 produced multiple bands of a broad range of sizes (Figure 1; n=3). Between the 3 antibodies, the patterns of reactivity were very different with no common bands seen within the predicted molecular size range for the AT1R.

To test the specificity of each antibody for the AT1R, we performed additional Western blot analyses but now using protein homogenates from kidneys of mice genetically deficient in 1 or both AT1R subtypes. As shown in Figure 2, there were no apparent differences in the pattern of reactivity of each of the antibodies between protein extracts of kidneys from WT mice, compared with those from mice lacking the major AT1R isoform, AT1A (AT1AKO). Furthermore, the patterns of antibody reactivity were virtually identical in the kidneys of WT and AT1AKO (Figure 2; n=3). To be certain that we were not missing a band corresponding to AT1R protein that was obscured or low abundance, we overexposed the X-ray film to the membrane for up to 60 minutes, but did not detect additional bands.

We also tested the polyclonal AT1R antibody 2 for immunohistochemical staining. As shown in Figure 3A–3B, tissue sections from WT kidney in the absence (Figure 3A) of primary antibody demonstrate minimal background staining compared with sections incubated with the AT1R antibody (Figure 3B). Smooth muscle cells of the renal and arculate arteries, interlobular (Figure 3B and 3F) and afferent arterioles (Figure 3C, 3E–3G), liver (Figure 3H), and adrenal gland (data not shown) were stained positively with the AT1R antibody on WT and AT1ABKO tissues. Prominent anti-AT1R immunostaining was also visualized in the proximal tubule brush border and basolateral membranes (Figure 3B–3E and 3G) of WT and AT1AKO. Distal tubules, cortical, and medullary collecting ducts (Figure 3B–3E and 3G) also exhibited immunoreactivity. Thus, similar to the Western blotting data, patterns and localization of immunostaining with the AT1R antibody were identical in the kidneys of WT mice (Figure 3B–3D) and the AT1AKO completely lacking AT1R (Figure 3E–3G).

To verify that the AT1R knockout mice used in the studies were truly deficient in receptors, we measured AT1R expression and functional responses to angiotensin II. By quantitative real-time–polymerase chain reaction, AT1A mRNA receptor abundance in WT kidneys was similar in the cortex and medulla (10.7±0.4 and 12.5±1.5 arbitrary units for cortex and medulla, n=4; n.s.). In contrast, AT1A receptor transcript was undetectable in either cortex or medulla of both AT1AKO and AT1ABKO (Figure 4A). Additionally, AT1B receptor mRNA was detected in adrenal glands from WTs and AT1AKO but undetectable in AT1ABKO (Figure 4B). Furthermore, to confirm physiological absence of functional AT1R in the knockouts, we tested the ability of angiotensin II to cause acute increases in blood pressure. Bolus infusions of 10 μg/kg angiotensin II increased blood pressure by 34±4 mm Hg in WT mice. However, this response was significantly blunted in AT1AKO and completely absent in AT1ABKO mice (change: 4±1 and 0.5±0.8 mm Hg for AT1AKO and AT1ABKO; P<0.001 versus WTs, n=3–4; Figure 4C). These experiments confirm the lack of each AT1R subtype expression and activity in our knockout mouse lines and indicate that the anomalous results obtained by Western blotting and immunohistochemistry are likely a result of antibodies cross-reacting with unknown proteins other than the AT1R.
To explore the possibility that the lack of specificity of anti-AT$_1$R antibodies was attributable to insufficient sensitivity, we overexpressed the AT$_1$R in human embryonic kidney (HEK) cells by transfecting them with DNA encoding the mouse AT$_1$AR. To verify appropriate targeting of the AT$_1$R protein to the plasma membrane, we used a plasmid encoding the AT$_1$A receptor fused with the mCherry fluorescence protein (AT$_1$A–mCherry) and imaged the receptor by live cell–fluorescence confocal microscopy. We found that, after 24 hours of transfection, a significant amount of the total AT$_1$AR pool was present at the plasma membrane (Figure 5A, I). In addition, we also observed that the nonplasma membrane–associated AT$_1$R pool does not colocalize with the endoplasmic reticulum–associated protein Calreticulin (Figure 5A, II and III), but it partially colocalizes with the trans-Golgi network–associated protein GalNac-T (Figure 5A, IV–VI).

We next tested the ability of the commercial antibodies to detect AT$_1$AR increments at different levels of over-expression. For this, we subcloned the AT$_1$AR sequence from plasmid AT$_1$A-mCherry into the pcDNA3.1 His vector in frame with the His epitope (AT$_1$A-His), and different amounts of plasmidic DNA were transfected into HEK cells. For detection of the exogenous His-tagged proteins by Western blot, an anti-His antibody was used. The anti-His antibody detected multiple bands close to 39 kDa in cells transfected with 2 $\mu$g plasmid. The intensity of these bands (relative to GAPDH) increased by 83±40% and 193±39% (n=3) in cells transfected with 4 and 8 $\mu$g DNA (lower red box on Figure 5B). In contrast, this pattern was not reproduced by utilizing any of the anti-AT$_1$R antibodies (Figure 5B).

**Discussion**

In this study, we tested 3 different rabbit polyclonal commercial antibodies that have been used in published reports to detect AT$_1$R protein. Antibodies 1 and 2 were raised against a short sequence (15 amino acids) of the extracellular amino terminus of the AT$_1$R protein to the plasma membrane, we used a plasmid encoding the AT$_1$A receptor fused with the mCherry fluorescence protein (AT$_1$A–mCherry) and imaged the receptor by live cell–fluorescence confocal microscopy. We found that, after 24 hours of transfection, a significant amount of the total AT$_1$R pool was present at the plasma membrane (Figure 5A, I). In addition, we also observed that the nonplasma membrane–associated AT$_1$R pool does not colocalize with the endoplasmic reticulum–associated protein Calreticulin (Figure 5A, II and III), but it partially colocalizes with the trans-Golgi network–associated protein GalNac-T (Figure 5A, IV–VI).

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proteins other than the AT1R. Experiments using kidney tissue from mice with genetic deficiencies of the major murine AT1R isoform (AT1A) revealed that all 3 antibodies showed the same pattern of bands on Western blots whether the proteins were derived from WT or AT1AKO mice. Similar findings using the single AT1AKO were recently reported.12a Inclusion of samples from the double knockout (AT1ABKO) eliminated the possibility that the positive signal in the AT1AKO was attributable to upregulation of the homologous AT1BR. This suggests that none of these antibodies recognize the AT1R protein with sufficient specificity in kidney tissue by Western blot.

Our immunohistochemical staining studies revealed apparent AT1R localization in the renal vasculature and proximal and distal tubules. However, identical staining patterns were observed when kidneys from mice lacking both AT1R subtypes (AT1ABKO) were used. The positive AT1R immunostaining in the renal microvasculature of the AT1ABKO kidneys was unexpected because these tissues lack renal vasconstrictor responses to angiotensin II in vitro and in vivo.13,14 Additionally, the apparent positive AT1R immunostaining was also observed in the liver vasculature of AT1ABKO mice, indicating that the nonspecific positive antibody staining observed in the vascular smooth muscle of the AT1ABKO is not restricted to the kidney. The identity of the proteins recognized by these antibodies remains elusive. We conducted immunoprecipitation of kidney homogenates (using antibodies 1 and 2) followed by mass spectrometry and were unable to identify either the AT1R or any other protein in the 41-kDa range. Two possibilities may explain these inconclusive results: (1) the antibodies are not suitable for immunoprecipitation experiments; and (2) the nonspecific protein has yet to be reported and, therefore, does not exist in the current peptide database. Nonetheless, despite our inability to identify the specific protein or proteins identified by these antibodies, the cumulative results from our studies provide compelling evidence that this protein is not the AT1R.

We were confident that these findings were intrinsic to the antibodies and not to the presence of AT1R in our knockout lines because we verified the absence of AT1R mRNA expression and functional responses to angiotensin II in randomly selected mice from our colony. Based on the strategy of gene disruption used to generate our KO mice14,15 where many stop codons were introduced into the early portions of the coding sequence of each gene, it is theoretically possible that very small, truncated forms of the receptors might be generated. However, it is highly unlikely that these protein fragments would reach the cell surface. Moreover, if present, they should appear as unique bands in the knockout mice with sizes substantially smaller than that predicted for the native AT1R protein.14,15

We used HEK cells over-expressing AT1A receptors to test the antibodies again. Our confocal fluorescence images in living HEK cells show proper plasma membrane expression of the AT1A receptor. In addition, AT1A-mCherry fluorescence
was not significant in the endoplasmic reticulum, indicating that processed AT1R proteins were properly sorted to the Golgi apparatus for further secretion, as shown by colocalization with the marker for this organelle. These findings indicate that HEK cells are able to transcribe, translate, and normally sort the AT1AR, which localizes normally to the plasma membrane. Accordingly, we used HEK cells transfected with the AT1AR–His plasmid. Using an anti-His antibody, we detected distinct amounts of His-tagged AT1AR proportional to the amount of DNA transfected under each experimental condition. Bands of molecular weights in the \( \approx 39 \) kDa and \( \approx 65 \) kDa range were detected only in cells transfected with the AT1AR–His plasmid (red boxes on Figure 5B). A single band at \( \approx 51 \) kDa was present in all samples, including the mock-transfected cells, and likely represents endogenous His proteins expressed by HEK cells (marked with an asterisk on Figure 5B). Nevertheless, the \( \approx 39 \) kDa band is consistent with the nonglycosylated form of the AT1R, even though one may question the apparent molecular weight of AT1R "monomer" because this band appears to be slightly smaller than the predicted 41 kDa mass of this protein. This small apparent difference in size could be accounted for by an altered migration velocity due to the positively charged histidines added to the carboxy-terminal tail. Furthermore, the multiple bands apparent at \( \approx 39 \) kDa could also be proteolytic degradation products of the AT1R protein. Additionally, although the multiple banding at \( \approx 65 \) kDa is consistent with the different degrees of AT1R glycosylation reported for the rat kidney, it is also possible that these bands represent ubiquitinated receptor which is the mechanism whereby the AT1R is degraded. Nevertheless, in contrast to the anti-His antibody, these bands were not identified by any of the anti-AT1R antibodies. Although we do not observe specific reactivity of the antibodies to AT1R protein expressed at high levels in HEK cells, the failure of the antibodies to detect the AT1R in Western blots does not appear to be an issue of insufficient sensitivity. It is important to note that the nonspecific bands recognized by the anti-AT1R antibodies in HEK cells are different in number and size compared with those observed in the kidney tissues (Figure 2), which provides additional evidence for the lack of specificity of these antibodies. Although the inability of the commercial antibodies to recognize the AT1R protein could be explained by potential masking of the epitope by the His tag, we believe this is unlikely to occur because of the denaturing conditions used to unfold the proteins in our Western blotting technique. Additionally, the His tag is attached at the carboxy terminus region of the protein, and at least 2 of the antibodies tested are raised against the amino terminus sequence.

Other investigators attempted to generate custom-made antibodies without success. The Daugherty laboratory generated several antisera against potential new antigenic sites within the AT1R receptor, and they were not able to demonstrate any specific interaction to the AT1A or AT1B Receptors by either Western blotting or immunostaining of tissue sections. In a different report by Hoffmann et al., investigators generated antibodies using the last 20 amino acids of the carboxy terminus tail of each AT1A and AT1B receptors. These antibodies yielded bands of different molecular mass in neural cells compared with mammalian tissues. Although these bands were absent when preincubating the antibodies with the immunizing synthetic peptides, this strategy alone is insufficient to demonstrate specificity for the intact receptor protein, and thus the specificity of such antibody remains elusive. Finally, using a monoclonal antibody (6313/G2) raised against and N-terminal peptide (residues 8–17), we previously localized the AT1R protein in renal vascular smooth muscle cells, proximal tubule, and more distal nephron segments in kidneys of adult rats. The specificity of this antibody was confirmed by Western blotting of COS-7 cells transfected with an AT1R–expressing plasmid. Thus, it is possible that identification of the AT1R by antibody-based techniques is suitable for rat but not mouse tissues. In contrast, the Bernstein laboratory raised a polyclonal antibody against N-terminal peptide residues 15–24, which stained mainly vascular smooth muscle cells, brush border, and thick ascending limbs. Unlike our report, this antibody failed to demonstrate immunoreactivity in distal collecting ducts. The origin of this discrepancy is still unknown, but it may be explained by some degree of antibody cross-reactivity. Unfortunately, the identity of the peptide being recognized in such studies has not been verified likely because AT1KO mice were not available at the time.

**Perspectives**

We report here that commercially available antibodies are erroneous tools to detect the AT1R protein. Generation of highly specific antibodies for G protein–coupled receptors has reportedly been difficult, and the reasons behind this are yet to be understood. One explanation is potential differences in structure and charge between the glycosylation (naturally carried in the AT1R) and the synthetic peptides used when raising these antibodies. Successful strategies to generate reliable anti-AT1R antibodies are urgently needed. Investigators should use alternative methods such as ligand-binding, epitope-tagging, Northern blot, or quantitative real-time–polymerase chain reaction when studying the biology of AT1R. Interpretations of previously published work relying solely on quantitative and qualitative assessments of the AT1R protein using these antibodies should be viewed with extreme caution.

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**Disclosures**

None.

**References**


**Novelty and Significance**

*What Is New?*

- Antibody-based detection of AT₁R is unreliable as a result of the lack of specificity of commercial antibodies that recognize proteins different than the AT₁R.

*What Is Relevant?*

- The AT₁R mediates the hypertensive actions of angiotensin II. Understanding AT₁R protein tissue distribution, abundance, and interaction with other molecules is crucial to elucidate the pathophysiology of cardiovascular and renal diseases and develop new pharmacologic tools for their treatment.

- Many investigators have published studies using these unreliable antibodies.

*Caution must be used when interpreting such reports and designing experimental approaches to understand the biology of these receptors.*

**Summary**

Our studies indicate that commercially available antibodies may not always be suitable for detecting AT₁R protein. Inclusion of appropriate positive and negative controls is essential when using antibody-based techniques. Investigators should be wary of using these tools when designing new experimental approaches to understand the biology and pathophysiology of AT₁ receptors.
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ONLINE SUPPLEMENT

Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor (AT₁R) protein

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Materials and Methods

Animals- Mice with targeted deletion of the Agtr1a and Agtr1b genes were generated as previously described.\textsuperscript{1, 2} Wild-type 129 SvEv (WT) mice were used as controls. For these experiments, 2- to 3-month old animals were used. All mice were maintained at the animal facility of the Durham Veterans Affairs Medical Center (VAMC), studies approved by the Duke University and Durham VAMC Institutional Animal Care and Use Committees and experiments carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western blots- Western blotting was performed as previously described.\textsuperscript{3, 4} Briefly, mice were anesthetized with 4% isoflurane (Webster veterinary, Devens, MA) and perfused via the left ventricle with 20 mL ice-cold physiological saline. Kidneys were removed and dissected under a microscope. Tissue (or HEK cells) were homogenized in a buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.3 mol/L sucrose, 1.0% NP-40, 0.1% sodium dodecyl sulfate and 1:100 dilution of a proteases inhibitor cocktail (Sigma, St. Louis, MO). Equal amount of proteins were denatured for 10 min in sample buffer containing 1.6 % sodium dodecyl sulfate and 100 mM dithiothreitol (Sigma) at room temperature. For kidney tissue, 40 µg homogenates were loaded onto the same 4-12% polyacrylamide gel whereas for HEK cells only 5 µg were used. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen, Grand Island, NY). After blocking for 30 min, membranes were incubated over-night at 4°C with primary antibodies as indicated. For detection of the AT\textsubscript{1}R the following commercial rabbit polyclonal antibodies were used: antibody #1: Alomone Labs Cat # AAR-011 lot AN-03 (1:1,000); antibody #2: Santa Cruz Cat # sc-1173 N-10 lot L0209 (1:500); antibody #3: Abcam Cat # ab18801 lot GR13003-3 (1:1,000). Anti Penta-His Qiagen Cat # 34660 (1:1,000) was used to detect His-tagged AT\textsubscript{1}AR and anti GAPDH conjugated Abcam Cat # ab9385 (1:3,000). After incubation for 1 hr at room temperature using a 1:3,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody, reaction products were detected with the Super Signal West Pico chemiluminescence kit (Thermo Scientific, Rockford, IL). The signal was detected by exposure to Kodak RX film and band intensities quantified by densitometry. Detection of GAPDH as a protein loading control was performed in the same membranes without re-stripping, except the blot from Figure 5B first panel, for which samples for GAPDH were loaded in separate lanes. This was due the similar molecular weight of the AT\textsubscript{1}R-HIS protein with GAPDH. The ~51kDa non-specific band revealed by the anti HIS antibody served as second evidence for equal protein loading in these particular experiments

Imunohistochemical staining: Kidneys, livers and adrenal glands were harvested from pentobarbital sodium (50 mg/kg, ip) anesthetized adult AT\textsubscript{1AB}KO (7) and WT (n=6) mice. Kidneys were prepared by immersion fixation in 10% buffered formalin, processed for paraffin embedding, sectioned at a thickness of 3 µm, and stained by the immunoperoxidase technique as we have previously described.\textsuperscript{5, 6} Kidney sections were incubated with the AT\textsubscript{1}R rabbit polyclonal antibody (1:300; sc-1173, N-10, antibody #2). Representative color micrographs were obtained from tissue sections
using X100 objectives from AT1ABKO and WT kidneys. Control experiments were performed by omitting the primary or secondary antibodies. Liver tissues were prepared in a similar manner as described above. Slides were imaged using an Olympus DP72 Digital Camera System mounted to an Olympus BX51 TRF Microscope.

Quantitative RT-PCR- Relative levels of mRNA for the AT1AR were measured from the kidney and for the AT1BR from the adrenal gland, where these receptors are most abundant. RNA was isolated and reverse transcription performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). SYBR Green-based quantitative PCR was carried out using the SYBR Green PCR Master Mix (AppliedBiosystems, Carlsbad, CA). At the end of PCR cycling, melting curve analyses were performed, and representative PCR products were run on agarose gel and visualized by SYBR® safe staining (Invitrogen). For AT1AR expression, the amount of target gene relative to endogenous control was determined by the ΔCT method and for the AT1B receptor expression was visualized by agarose gel electrophoresis. The following primer sequences were used:

AT1a Forward- 5’-ACTCACAGCAACCCTCCAAG-3’,
AT1a Reverse-5’-ATCACCACCAAGCTGTTCCTCCC-3’ (Amplicon size: 236bp);
AT1b Forward-5’- ATGGGGAGCAGCCAAGAGGC-3’,
AT1b Reverse: 5’-CAGGGCAAGATTCAGAAGGA-3’ (Amplicon size: 306bp);
GAPDH Forward-5’-TCACCACCATGGAGAAGGC-3’,
GAPDH Reverse-5’-GCTAAGCAGTTGGTGTTGCA-3’ (Amplicon size: 168).

Controls tubes lacking cDNA and containing RNA were included in each run.

Pressor effect of angiotensin II- Mice were anesthetized with 2% isoflurane and instrumented as previously described 7. After a 30-min equilibration period, during which 0.9% NaCl was administered continuously (280 µl/min/Kg), 10 µg/kg of angiotensin II or vehicle (0.9% NaCl) was given as a bolus (1µl/gr). Baseline pressure was taken just before the administration of angiotensin II and peak response at the maximal pressure attained after angiotensin II administration.

Molecular constructs- The Plasmid pAA356 encoding the sequence of the mouse AT1AR fused with the mCherry (AT1A-mCherry) fluorescent protein, was generated by PCR amplification from the plasmid pCMV-SPORT6-AT1 (clon ID #4989471, Open Biosystems, Lafayette, CO) using Platinum Polymerase (Invitrogen) and the direct primers forward: GCTAGCATGGCCCTTAACTCTTCTACTGAAG and reverse: GGATCCTCCACCTCAGAACAAGCGCAGGC containing NheI and BamHI sites for directional cloning. A resulting 1.1 kb PCR product was purified from an agarose gel and ligated into the cloning vector pCR-Blunt II-TOPO (Invitrogen). After digestion with NheI and BamHI (Promega, Madison, WI) the released fragment was subcloned into the mammalian expression vector mCherry-N1 (Clontech). To generate the plasmid pAA357 encoding the epitope tagged AT1A-His protein (AT1A-His), the same insert from pAA356 was sub-cloned into pcDNA 3.1/myc-His (Invitrogen) within the same restriction sites. All constructions were confirmed by sequencing at the Hartwell center for Biotechnology at St. Jude Children’s Research Hospital.

Expression of AT1A receptors in Human Embryonic Kidney (HEK) cells- The HEK 293 human cell line was obtained from the American Type Culture Collection (ATCC,
Cells were cultured at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 U/ml streptomycin (Invitrogen). For imaging experiments, cells were seeded in 4-well chamber slides (Lab-Tek II-CC2 treated, Nunc, Rochester, NY) at low density. Transfections where performed with 0.25–0.5 µg total DNA/chamber using Lipofectamine 2000 (Invitrogen). Cells were used after 24 hours at approximately 70%-80% confluence. For Western blot experiments, approximately 2 x 10⁶ cells were seeded in 60 mm plates and the following day were transfected with 0, 2, 4 or 8 µg of pAA357 plasmid DNA as indicated. Mock DNA (empty vector) was utilized to deliver equal amount of plasmid per transfection.

**Live cell confocal imaging-** HEK cells were co-transfected with pAA356 plasmid (AT₁A-mCherry) and the endoplasmic reticulum-specific marker Calreticulin fused with enhanced yellow fluorescence protein (Calreticulin-EYFP) or, the trans-Golgi network-specific marker galactosaminyltransferase fused with EYFP (GalNAcT-EYFP), for approximately 24 hours. Cells were transferred to an environmentally controlled chamber and maintained at ~37°C in a 5% v/v CO₂ and humidified atmosphere. Images were acquired using a Nikon C1si inverted laser scanning confocal microscope (60X apochromatic, 1,45 NA objective) using the EZ-C1 3.20 Viewer (Nikon Corporation). Pictures were exported as TIFF, deconvoluted using ImageJ and pseudo-colored and assembled using Adobe Photoshop CS4.

**REFERENCES**


