Regulation of nonclassical renin-angiotensin system receptor gene expression in the adrenal medulla by acute and repeated immobilization stress

Regina Nostramo, Lidia Serova, Marcela Laukova, Andrej Tillinger, Chandana Peddu, and Esther L. Sabban
Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York
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Nostramo R, Serova I, Laukova M, Tillinger A, Peddu C, Sabban EL. Regulation of nonclassical renin-angiotensin system receptor gene expression in the adrenal medulla by acute and repeated immobilization stress. Am J Physiol Regul Integr Comp Physiol 308: R517–R529, 2015. First published January 14, 2015; doi:10.1152/ajpregu.00130.2014.—The involvement of the nonclassical renin-angiotensin system (RAS) in the adrenomedullary response to stress is unclear. Therefore, we examined basal and immobilization stress (IMO)-triggered changes in gene expression of the classical and nonclassical RAS receptors in the rat adrenal medulla, specifically the angiotensin II type 2 (AT2) and type 4 (AT4) receptors, (pro)renin receptor [(P)RR], and Mas receptor (MasR). All RAS receptors were identified, with AT2 receptor mRNA levels being the most abundant, followed by the (P)RR, AT1A receptor, AT1 receptor, and MasR. Following a single IMO, AT2 and AT4 receptor mRNA levels decreased by 90 and 50%, respectively. Their mRNA levels were also transiently decreased by repeated IMO. MasR mRNA levels displayed a 75% transient decrease as well. Conversely, (P)RR mRNA levels were increased by 50% following single or repeated IMO. Because of its abundance, the function of the (P)RR was explored in PC-12 cells. Prorenin activation of the (P)RR increased phosphorylation of extracellular signal-regulated kinase 1/2 and tyrosine hydroxylase at Ser31, likely increasing its enzymatic activity and catecholamine biosynthesis. Together, the broad and dynamic changes in gene expression of the nonclassical RAS receptors implicate their role in the intricate response of the adrenomedullary catecholaminergic system to stress.

The adrenal medulla is an important mediator of the stress response, releasing catecholamines and peptides to activate the “fight or flight” response (8) to allow the organism to respond and handle the threat to homeostasis. With prolonged or repeated exposure to stress, the adrenal medulla displays an increased capacity to synthesize and release catecholamines (reviewed in Refs. 33, 56, and 72). Although beneficial in the short term, prolonged or repeated exposure to these and other stress hormones elicits an increase in allostatic load, causing the stress response to become maladaptive (41, 43). Consequently, chronic stress is a major contributor to the development of cardiovascular, immune, and neuropsychiatric disorders and can adversely influence the progression of diseases such as diabetes and cancer (reviewed in Refs. 9, 18, 42, 60, and 61).

Many pathways are involved in orchestrating the enhanced biosynthetic capacity of adrenomedullary cells in response to stress (12, 33). For example, stress triggers the production of circulating and adrenal angiotensin II (ANG II), the main effector component of the renin-angiotensin system (RAS) (25, 78), which acts as a potent secretagogue of adrenomedullary epinephrine and norepinephrine (15) and elevates the gene expression of the catecholamine biosynthetic enzymes (63). These effects, however, depend on which ANG II receptor subtype is activated. ANG II stimulation of the ANG II type 1 (AT1) receptor enhances, whereas activation of the ANG II type 2 (AT2) receptor inhibits, catecholamine biosynthesis in the adrenal medulla and cultured chromaffin cells (2, 52, 55, 66, 67).

The AT1 and AT2 receptors are part of the classical RAS, in which liver-derived angiotensinogen is cleaved by renin (EC 3.4.23.15) to form angiotensin I (ANG I), which is subsequently converted to ANG II by angiotensin I-converting enzyme (ACE; EC 3.4.17.23). Although much work has aimed to understand adrenomedullary regulation by the classical RAS, the involvement of the more recently identified nonclassical components of this system remains to be determined. The nonclassical RAS includes multiple additional active angiotensin peptides as well as receptors that bind these ligands (reviewed in Ref. 17). For example, ACE2 [the enzyme that catalyzes the conversion of ANG I or ANG II to ANG-(1–9) or ANG-(1–7), respectively]-catalyzed formation of ANG-(1–7), either directly or indirectly from ANG II or ANG I, respectively, activates the Mas receptor (MasR), which is a seven-transmembrane domain G protein-coupled receptor like the AT1 and AT2 receptors. Conversely, aminopeptidase-mediated cleavage of amino acids from the NH2-terminus of ANG II leads to the formation of ANG III and ANG IV. ANG IV binds to the AT4 receptor, which is a member of the M1 family of aminopeptidases. Moreover, c-met is another target for ANG IV that belongs to the type I tyrosine kinase receptor family and is associated with a broad range of physiological processes (reviewed in Ref. 74). An additional RAS receptor is the (pro)renin receptor [(P)RR], which was cloned and identified as a full-length protein in 2002 (51). (P)RR binds both renin and its proenzyme prorenin [herein referred to as (pro)renin when they can be used interchangeably]. The binding of renin to the (P)RR leads to an increase in catalytic efficiency, whereas the binding of prorenin, which is up to 100-fold more abundant in the plasma (13), elicits a conformational change in the proenzyme, shifting the 43-amino acid prosegment out of its enzymatic cleft, allowing prorenin to be enzymatically active via a nonproteolytic and reversible process. The (P)RR is a multifunctional protein functionally linked to the vacuolar H+-ATPase, which regulates the pH of cellular and intracellular vesicles, as well as canonical Wnt-β-catenin signaling and noncanonical β-catenin-independent Wnt signal transduction pathways that are involved in many cardiac pathologies. (Pro)renin stimula-
tion of the (P)RR can also activate extracellular signal-regulated kinase 1/2 (ERK1/2), which induces upregulation of profibrotic genes such as transforming growth factor-β1, plasminogen activator inhibitor-1, collagens, and fibronectins (reviewed in Ref. 48).

Much like the classical adrenomedullary RAS, the nonclassical RAS components may also play a role in the regulation of the adrenal medulla under basal conditions and in response to stress. Therefore, the focus of the current study was to characterize nonclassical RAS receptor expression in the adrenal gland and to determine the effect of single and repeated stress on their mRNA levels in the medulla. Our results demonstrate that mRNA for all of the nonclassical RAS receptor genes are found in the adrenal medulla, and their gene expression is modulated by stress exposure. Based on the abundance of the (P)RR in catecholamine-synthesizing chromaffin cells, the functional significance of the changes in (P)RR gene expression was further explored.

MATERIALS AND METHODS

Animals. All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the New York Medical College Institutional Animal Care and Use Committee. All efforts were made to minimize pain and discomfort. Male Sprague-Dawley rats (250–320 g) were purchased from Taconic Farms (Germantown, NY), housed three per cage, and maintained under controlled conditions (23 ± 2°C, 14:10-h light-dark cycle, lights on from 6:00 A.M. to 8:00 P.M.) with food and water ad libitum. They were allowed to acclimate to the animal facility for 7–10 days before exposure to the stress.

Stress. Immobilization stress (IMO), a strong noninvasive physical stressor with psychological components, was performed as previously described (37, 47). The rats were immobilized on a metal board by taping the limbs with surgical tape and restricting the motion of the head as originally described by Kvetnansky and Mikulaj (32). IMO was performed at the same time of the day (between 8:00 A.M. and noon) for all experiments. Rats were randomly divided into groups and subjected to various repetitions of IMO. For single IMO (1× IMO), rats were immobilized one time for 2 h and subsequently killed either immediately (0 h) or 3 h after termination of the stress. For repeated IMO, rats were immobilized for 2 h daily for 6 consecutive days (6× IMO) and killed either immediately (0 h) or 3 h after termination of the stress. One group of rats (the adapted control group) was immobilized for all but the last IMO and killed 1 day later (5× IMO + 24 h) to isolate the effect of the sixth IMO and elucidate the sustained effects of repeated stress exposure. Absolute controls were not exposed to stress.

Following IMO, the rats were killed by decapitation. Each animal was brought individually into the procedure room, and the time between the transfer of the rat from its cage until decapitation was 35–40 s on average. Left and right adrenals were dissected. In some experiments, the adrenal medulla was isolated. A small incision was made at the edge of the cortex, and the medulla was gently squeezed out. Any cortex tissue adhering to the adrenal medulla was gently removed. We previously estimated that the medulla prepared this way is >95% pure (36). The left and right adrenal medullas from each individual animal were frozen separately in liquid nitrogen and kept at −80°C. Alternatively, adrenal glands were fixed in paraformaldehyde solution and processed for immunohistochemistry or immunofluorescence.

PC-12 cell culture and treatment. Rat adrenomedullary-derived PC-12 cells were grown in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 5% horse serum (Gemini Bio-Products), and 50 μg/ml of streptomycin with 50 IU/ml penicillin (0.5%; Invitrogen) and maintained at 37°C in a humidified incubator with 5% CO2, with media changed every other day, as previously described (52). Before treatment, cells were plated at about 50% density. Cells were treated with 20 nM human prorenin (Molecular Innovations, Novi, MI) or vehicle for various times. Alternatively, cells were treated with 1 μM ANG II (Sigma-Aldrich), dissolved in distilled water. In all experiments, controls were treated with vehicle.

Isolation of RNA and quantification of changes in mRNA levels. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and then quantitated using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Subsequently, RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) using the oligo(dT) primer. For quantitative Real-Time PCR, 2 μl of cDNA product (30 ng) were mixed with 12.5 μl of FastStart Universal SYBR Green Master Roche Roche, Indianapolis, IN) and 1 μl of the following primer pairs: AT1A receptor, AT3 receptor, AT2 receptor, MasR, (P)RR, tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), or glyceroldehyde-3-phosphate dehydrogenase (GAPDH; Integrated DNA Technologies, Coralville, IA), with sequences shown in Table 1. Quantitative Real-Time PCR results were analyzed on an ABI7900HT Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA). Data were normalized to GAPDH mRNA levels and expressed as the relative fold change vs. control, calculated using the difference in ΔCt values (ΔΔCt) method (38). GAPDH mRNA levels were not altered by any of the experimental conditions.

Western blot analysis. Protein from PC-12 cells and various rat tissues (adrenal medulla, heart, liver, lungs, brain, and spleen) was isolated with the Qproteome Mammalian Protein Preparation Kit (Qiagen), according to the manufacturer’s protocol. The tissues were homogenized using a Polytron knife (Kinematica). For the PC-12 cells and adrenal medulla, a sonication step followed by centrifugation through QIAshredder spin columns (Qiagen) was used after addition of the lysis buffer supplemented with Halt protease and phosphatase inhibitor (Thermo Scientific). Protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA). Western blot was performed using primary antibodies for AT1 receptor (1:3,000, catalog no. 6918; Cell Signaling Technology, Danvers, MA), (P)RR performed using primary antibodies for AT4 receptor (1:3,000, catalog no. ab40790; Abcam, Cambridge, MA), phospho (p)ERK1/2 (1:1,000; catalog no. 9101L; Cell Signaling Technology), or (p)MasR (1:3,000; catalog no. 6300S; Cell Signaling Technology), or (p)AT2R (1:3,000; catalog no. 6301S; Cell Signaling Technology), (p)AT1R (1:3,000; catalog no. 3027; Cell Signaling Technology), (p)AT3R (1:3,000; catalog no. 3028; Cell Signaling Technology), or antigens of interest, followed by secondary antibodies (1:20,000; Cell Signaling Technology, Danvers, MA). Membranes were exposed to X-ray film, and protein expression was quantitated using ImageJ software. Western blot analysis of GAPDH was performed as a loading control. Protein from PC-12 cells and various rat tissues (adrenal medulla, heart, liver, lungs, brain, and spleen) was isolated with the Qproteome Mammalian Protein Preparation Kit (Qiagen), according to the manufacturer’s protocol. The tissues were homogenized using a Polytron knife (Kinematica). For the PC-12 cells and adrenal medulla, a sonication step followed by centrifugation through QIAshredder spin columns (Qiagen) was used after addition of the lysis buffer supplemented with Halt protease and phosphatase inhibitor (Thermo Scientific). Protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA). Western blot was performed using primary antibodies for AT1 receptor (1:3,000, catalog no. 6918; Cell Signaling Technology, Danvers, MA), (P)RR performed using primary antibodies for AT4 receptor (1:3,000, catalog no. ab40790; Abcam, Cambridge, MA), phospho (p)ERK1/2 (1:1,000; catalog no. 9101L; Cell Signaling Technology), or pSer11 TH (1:1,000; catalog no. AB5423; Cell Signaling Technology).
followed by incubation with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology). Immunoactive bands were visualized by chemiluminescence detection (SuperSignal West Pico Chemiluminescent Kit; Thermo Fisher Scientific). After visualization of proteins, the membranes were stripped and reprobed with antibody for GAPDH (1:5,000, catalog no. 2118; Cell Signaling) and/or HRP-conjugated β-actin antibody (1:20,000, catalog no. sc-47778; Santa Cruz Biotechnology).

**Immunofluorescence and immunohistochemistry.** The adrenals were fixed overnight in 4% paraformaldehyde and 0.05 M sodium orthovanadate in 0.1 M sodium phosphate buffer, pH 7.4 at 4°C, and subsequently transferred to 15% and then 30% sucrose in 0.1 M sodium phosphate buffer, pH 7.4 at 4°C. The adrenals were embedded in OCT (Ted Pella, Redding, CA) and kept at −80°C before sectioning 16 μm thick on a cryostat (Leica, Buffalo Grove, IL) and mounted onto glass slides coated with 0.5% gelatin.

For immunofluorescence, the slides were treated with 50% ethanol for 30 min, washed in Tris-buffered saline (TBS) and then TBS-0.05% Triton X-100 (Tx). Sections were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in 3% BSA in TBS-Tx for 1 h at 4°C and then washed in TBS-Tx. The sections were incubated with primary antibody against the (P)RR (1:100; Abcam) in 10% normal donkey serum in 3% BSA in TBS-Tx overnight at 4°C. This antibody was synthesized using a synthetic peptide derived from the COOH-terminus of the human (P)RR. After being washed in TBS-Tx, slides were incubated in primary mouse antibody against TH (1:500; catalog no. 22941; ImmunoStar, Hudson, WI) in 10% normal donkey serum in 3% BSA in TBS-Tx overnight at 4°C. After being washed in TBS-Tx, the sections were incubated in Alexa Fluor secondary antibodies (Life Technologies, Grand Island, NY) to rabbit (Alexa Fluor donkey anti-rabbit 594, catalog no. A21207) or mouse (Alexa Fluor donkey anti-mouse 488, catalog no. A21202) diluted 1:200 in 1% normal donkey serum in 3% BSA in TBS-Tx for 1 h at room temperature in the dark. After being washed in TBS, the slides were covered with a cover slip in Vectashield Hard Set antifading medium (Vector Laboratories, Burlingame, CA). The sections were visualized using a Zeiss Axiosmiager Microscope with an Axiocam Digital Camera.

For immunohistochemistry, the slides were incubated with 50% ethanol for 30 min, washed in TBS, and incubated in 1% H2O2 in TBS for 20 min. After being washed, the sections were blocked for 90 min and incubated in primary antibody for (P)RR (1:100) or insulin regulated aminopeptidase (IRAP, for the AT4 receptor, 1:250; Cell Signaling) overnight as described above. The sections were washed with TBS and incubated in anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) in 0.8% BSA in TBS for 90 min. Sections were again washed in TBS and incubated in Vectastain ABC reagent (Vector Laboratories) for 1 h. After being washed, sections were incubated in 3,3′-diaminobenzidine for 5 min. Sections were washed in TBS, allowed to dry, and then dipped in xylene before covered with a cover slip with permount. Sections were imaged as described above.

**Demonstration of AT4 receptor and (P)RR antibody specificity by antigen blocking.** For blocking/competition, the antibody against (P)RR or IRAP (for AT4 receptor subtype) was combined with a 10-fold (by weight) excess of blocking peptide in 300 μl of blocking buffer. To neutralize IRAP receptor antibody, we used 17-amino acid peptide from rat IRAP/leucyl-cystinyl aminopeptidase (catalog no. IRAP1P-1; Alpha Diagnostic International). To block (P)RR antibody, human ATP6P2 peptide (catalog no. ab41522; Abcam) was used. The same amount of antibody was added to the control tubes (no peptide), and the volume was adjusted with corresponding blocking buffer. All tubes were mixed gently and incubated at 37°C for 2 h. Immune complexes were removed by centrifugation for 15 min (4°C, 12,000 revolutions/min) to avoid high background. The supernatant was carefully removed and verified for effective neutralization of antibodies. Before incubation, the volume was adjusted according to antibody concentration consistently giving a positive signal for the rat adrenal in Western blot or immunohistochemistry.

**Statistical analysis.** All data are expressed as means ± SE, with n = 5–8/group for animal experiments and n = 4–6/group for experiments in PC-12 cells. Differences were analyzed by analysis of variance (ANOVA) followed by Bonferroni’s post hoc analysis (if more than two groups) using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA). A value of P ≤ 0.05 was considered significant.

**RESULTS**

**Localization and expression of the nonclassical RAS receptors in the rat adrenal gland.** The relative expression of the mRNAs for the classical and nonclassical RAS receptors was determined in rat adrenal medulla using Real-Time RT-PCR analysis by calculating the difference in Ct values (ΔCt) between each gene of interest (GOI) and the housekeeping gene GAPDH (ΔCt = [CtGOI] − [CtGAPDH]) (Fig. 1). One-way ANOVA revealed significant differences in their abundance (F = 30.0, P < 0.0001), with AT2 receptor mRNA levels higher than the others analyzed (P < 0.001). Levels of AT1A receptor mRNA, the only other classical RAS receptor subtype expressed in the adrenal medulla, were approximately fourfold less than the AT2 receptor. All of the nonclassical RAS receptor genes were also found in the adrenal medulla. Of these, the (P)RR was the most abundant, although its levels were twofold less than the AT2 receptor. Relative levels of AT4 receptor and MasR mRNAs were one and two orders of magnitude, respectively, lower than the AT2 receptor.

The presence and localization of the nonclassical RAS receptors, specifically the (P)RR and AT4 receptors, in the rat adrenal gland were examined by immunohistochemistry. MasR expression was not analyzed because of the low levels of mRNAs for the classical and nonclassical RAS receptors was determined in rat adrenal medulla using Real-Time RT-PCR analysis by calculating the difference in Ct values (ΔCt) between each gene of interest (GOI) and the housekeeping gene GAPDH (ΔCt = [CtGOI] − [CtGAPDH]) (Fig. 1). One-way ANOVA revealed significant differences in their abundance (F = 30.0, P < 0.0001), with AT2 receptor mRNA levels higher than the others analyzed (P < 0.001). Levels of AT1A receptor mRNA, the only other classical RAS receptor subtype expressed in the adrenal medulla, were approximately fourfold less than the AT2 receptor. All of the nonclassical RAS receptor genes were also found in the adrenal medulla. Of these, the (P)RR was the most abundant, although its levels were twofold less than the AT2 receptor. Relative levels of AT4 receptor and MasR mRNAs were one and two orders of magnitude, respectively, lower than the AT2 receptor.

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![Fig. 1. Gene expression profile of renin-angiotensin system (RAS) receptors in the rat adrenal gland. RNA was isolated from rat adrenal medullas, and the relative abundance of each receptor was determined by Real-Time RT-PCR by calculating the average difference in cycle threshold values (ΔCt) between each gene of interest and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The differences in ΔCt values (ΔΔCt) for each gene of interest and the angiotensin type 2 (AT2) receptor (R), selected because it is the most abundant of the receptors, were then calculated, and the fold change relative to the AT2-R was determined using the formula 2−ΔΔCt. Results replicated in two separate experiments with 4–6 rats each. AT1A-R, angiotensin type 1A receptor; AT4-R, angiotensin type 4 receptor. Data are presented as means ± SE with mRNA levels of AT2-R taken as 1. n = 5–8/group. ***P < 0.001 compared with AT2-R. #P < 0.05 and ##P < 0.01 compared with (pro)renin receptor [(P)RR].](http://ajpregu.physiology.org/}

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expression at the mRNA level (Fig. 1). The (P)RR was localized throughout the adrenals although there was higher signal intensity in the medulla than in the cortex (Fig. 2, C and D). The AT4 receptor was also located predominantly in the adrenal medulla, whereas only low signal intensity was observed in the cortex (Fig. 2, E and F).

To test the specificity of the antibodies used, we performed Western blots with several other rat tissues (Fig. 3). The (P)RR and AT4 receptor antibodies used in this study revealed a similar expression pattern, as previously demonstrated by others (28, 51). Both receptors were detected in heart, lung, brain, and spleen but barely visible in the liver. The Western blot with the antibody to the AT4 receptor showed selective signal of expected size (~165 kDa, 140 kDa in brain) (28). The (P)RR antibody selectively detected a protein of the expected size of about 42 kDa, with a doublet seen in brain samples, probably because of differences in (P)RR glycosylation (62). PC-12 cells, which originate from rat pheochromocytoma, also expressed both types of receptors. The AT4 receptors were more abundant in PC-12 cells than in the adrenal medulla (Fig. 4A).

The specificity of antibodies used for Western blots and immunohistochemical analyses was further verified in experiments in which blocking peptides were applied before incubation with the primary antibody (Fig. 4A, A and B). Preincubation with the (P)PR blocking peptide completely prevented binding with primary antibody, as shown by Western blot analysis (Fig. 4A). Similar results were obtained with immunohistochemistry of the adrenal (Fig. 4B). A similar concentration of the AT4 receptor blocking peptide visibly reduced, but did not entirely block, the signal on the Western blot (Fig. 4A) and abolished most of the staining in the adrenal medulla (Fig. 4B).

Modulation of gene expression of nonclassical RAS components in the adrenal medulla by single and repeated IMO. Because the nonclassical RAS receptors are expressed in the adrenal medulla, they may play a role in the response of the adrenomedullary catecholaminergic system to stress. There-
fore, the stress-triggered changes in $\text{AT}_{1A}$ receptor, $\text{AT}_{2}$ receptor, $\text{(P)RR}$, and MasR gene expression were determined in the rat adrenal medulla using the well-characterized IMO. Rats were immobilized for 2 h one time (1 IMO) or for 2 h for 6 consecutive days (6 IMO) and killed either immediately or 3 h afterward. These time points were chosen based on the optimal times to observe changes in mRNA levels of the catecholamine biosynthetic enzymes (44, 47). An additional group, referred to as the adapted control group, was exposed to 5 IMO and killed 1 day later (i.e., on the 6th day) to isolate the effect of the sixth IMO and to demonstrate the prolonged effects of repeated stress exposure.

In response to stress, changes in $\text{AT}_{4}$ receptor mRNA levels displayed a biphasic response (Fig. 5B), similar to those observed previously with the $\text{AT}_{2}$ receptor (Ref. 52 and Fig. 5A). $\text{AT}_{4}$ receptor mRNA levels decreased by $\sim50\%$ 3 h after 1 IMO. However, 1 day after repeated exposures (adapted controls) $\text{AT}_{4}$ receptor mRNA levels were elevated by 50% compared with unstressed absolute controls. Exposure to an additional sixth immobilization elicited a decrease in $\text{AT}_{4}$ receptor mRNA levels, declining to nearly 50% of unstressed basal control levels immediately after the stress exposure. This reduction was more transient than with the first IMO and did not continue to decrease 3 h afterward.

MasR mRNA levels were also reduced in response to stress exposure. MasR mRNA levels decreased by $\sim70-80\%$ immediately after 1 IMO and 6 IMO compared with unstressed controls (Fig. 5C). However, the decline was transient, returning to unstressed control levels 3 h after the stress.

Conversely, $\text{(P)RR}$ mRNA levels displayed a different response to IMO (Fig. 5D). Three hours after exposure to a single 2-h IMO, $\text{(P)RR}$ mRNA levels were elevated nearly 50% compared with unstressed absolute controls. In the adapted controls, $\text{(P)RR}$ mRNA levels declined below basal control levels. However, exposure to an additional IMO again increased $\text{(P)RR}$ mRNA levels compared with unstressed absolute controls. In fact, $\text{(P)RR}$ mRNA levels are double those observed before the sixth IMO.

In addition to altering the gene expression of RAS receptors, IMO may also alter the biosynthesis of various angiotensin peptides. Changes in gene expression of angiotensinogen (the precursor for angiotensin peptides), ACE (the enzyme that catalyzes the formation of ANG II from ANG I), and ACE2 were measured 3 h after single and repeated IMO. Following 1 IMO, there was no change in mRNA levels of angiotensinogen or ACE (Fig. 5E). However, 6 IMO elicited an increase in both angiotensinogen and ACE mRNA levels. Angiotensinogen mRNA levels were elevated 70%, whereas ACE mRNA levels were double unstressed control values. Conversely, ACE2 mRNA levels were significantly decreased following 1 IMO and 6 IMO (Fig. 5E).

Role of (pro)renin receptor signaling on the regulation of adrenomedullary catecholamine biosynthesis. Because the $\text{(P)RR}$ is the most abundantly expressed gene of the nonclassical RAS receptor members and displays a marked upregulation at the mRNA level in response to stress, we focused on this receptor and its potential role in the adrenomedullary catecholaminergic response to stress. First, to determine whether the $\text{(P)RR}$ is expressed in catecholamine-synthesizing cells within the adrenal medulla, colocalization of the $\text{(P)RR}$ with the first and rate-limiting enzyme of the catecholamine biosynthetic pathway, TH, was determined using immunofluorescence. Almost all of the TH-positive cells appear to express the $\text{(P)RR}$, suggesting that the $\text{(P)RR}$ is expressed in catecholamine-synthesizing cells (Fig. 6).

The function of the $\text{(P)RR}$ in the adrenal medulla is not currently known; however, its expression in catecholamine-synthesizing cells and its increased mRNA levels with stress suggest that the $\text{(P)RR}$ may play an important role in regulating stress-triggered catecholamine biosynthesis and/or release. Stimulation of the $\text{(P)RR}$ has been reported to activate various intracellular signaling pathways, such as ERK1/2 (22, 24, 58), which is also activated in response to IMO (57). Therefore, we next examined the effects of $\text{(P)RR}$ activation on phosphorylation of ERK1/2 using rat adrenomedullary-derived PC-12 cells. Human prorenin was used to activate the $\text{(P)RR}$. Prorenin was chosen over renin because it is the preferred agonist for the $\text{(P)RR}$, with a $K_d$ of 6-8 nM for prorenin and $>20$ nM for renin (4, 46), and its concentration in the plasma is at least 10 times higher than renin (13). Additionally, the human form of prorenin can bind to the rat $\text{(P)RR}$ (6), as expressed in PC-12 cells (Fig. 4A), but reportedly does not interact with rat angiotensinogen (3, 5).

PC-12 cells were treated with 20 nM human prorenin for various times up to 2 h, and subsequently phosphorylation of ERK1/2 was determined by Western blot analysis. One-way ANOVA revealed significant effects of treatment on $\text{pERK1/2}$
expression in PC-12 cells ($F = 8.1, P < 0.0001$). Treatment with 20 nM human prorenin for 15, 30, and 60 min led to an increase in ERK1/2 phosphorylation ($P < 0.01$ compared with 0 time point), which returned toward basal levels by 2 h (Fig. 7). These results suggest that prorenin activation of the (P)RR elicits activation of the ERK1/2 MAP kinase pathway.

However, the effects of prorenin on ERK1/2 phosphorylation could also be the result of (P)RR-mediated increases in prorenin activity and subsequently ANG II production, although this possibility is unlikely since rat angiotensinogen is reportedly not a substrate for human prorenin (3). To exclude the involvement of ANG II in prorenin-mediated phosphorylation of ERK1/2, we pretreated PC-12 cells with ANG II. Treatment with $10^{-9}$ M ANG II for 5 min significantly decreased ($P < 0.01$), rather than increased, ERK1/2 phosphorylation in PC-12 cells (Fig. 8, A and B) as expected, since PC-12 cells express the AT2 receptor and almost undetectable AT1 receptor subtype mRNA (Fig. 8 C). Together these results indicate that phosphorylation of ERK1/2 in response to human prorenin treatment is not the result of a (P)RR-mediated increase in ANG II production. In fact, in the unlikely event that human prorenin can elicit an increase in ANG II production in rat PC-12 cells, the observed effect of human prorenin-mediated ERK1/2 phosphorylation would be underestimated.

Phosphorylation of ERK1/2 can alter gene expression by activating several transcription factors such as cAMP response element-binding protein (CREB) and the activator protein 1 family of transcription factors Jun, Fos, and ATF1 (19, 70), which have been shown to enhance TH and/or DBH gene expression (reviewed in Ref. 33). Therefore, we assessed the ability of human prorenin to modulate TH and DBH mRNA levels in PC-12 cells. Following 3–24 h of treatment with 20 nM human prorenin, TH and DBH mRNA levels were similar to time-matched vehicle-treated controls (Fig. 9, A and B), suggesting that prorenin activation of the (P)RR does not alter catecholamine biosynthetic enzyme gene expression.

However, catecholamine levels are also regulated by activity of their biosynthetic enzymes, particularly the first and rate-limiting enzyme TH. The activity of TH is regulated by phosphorylation at several serine residues, mediated by different kinases (reviewed in Ref. 14). Phosphorylation of Ser$^{31}$ increases TH activity and is mediated by ERK1/2 (21). Treatment of PC-12 cells with human prorenin had a significant effect on TH phosphorylation at Ser$^{31}$ ($F = 10, P < 0.001$).
Phosphorylation of Ser$^{31}$ was evident after 30 min and remained for as long as 2 h (longest time point tested) after prorenin treatment (Fig. 9C), indicating a role for prorenin in stimulating catecholamine biosynthesis in adrenomedullary cells.

**DISCUSSION**

This study demonstrates that the genes for all of the nonclassical RAS receptors are expressed in the adrenal medulla and display apparent differences in abundance. (P)RR mRNA was the most abundant and appeared to be localized to catecholamine-synthesizing chromaffin cells. Activation of this receptor could elevate TH enzymatic activity by ERK-dependent phosphorylation of Ser$^{31}$. In response to stress, the gene expression profiles of all the nonclassical RAS receptors exhibited dynamic changes, and these receptors likely play an important role in the fine tuning of the adrenomedullary catecholaminergic system, especially under stress conditions.

The adrenomedullary $AT_4$ receptor. $AT_4$ receptor mRNA was found in the adrenal medulla, but at levels 10-fold lower
than the abundantly expressed AT₂ receptor. The AT₄ receptor was predominantly found in the adrenal medulla as well as in the outermost layer of the adrenal cortex. This is consistent with a previous report of AT₄ receptor expression, as identified by radioligand binding assay, in the bovine adrenal glomerulosa and medulla (31). We also confirmed the presence of the AT₄ receptor in PC-12 cells.

The role of the AT₄ receptor in the adrenals remains unknown. A previous study indicates that ANG IV can stimulate adrenocortical cell proliferation most likely via the AT₄ receptor (54). Within the adrenal medulla, this receptor could be involved in the regulation of catecholamine release, as has been previously demonstrated in the brain. Local infusion of ANG IV and binding to the AT₄ receptor elicits a dose-dependent release of dopamine from the striatum (64, 65). As an aminopeptidase, the AT₄ receptor could also change the peptide profile in the adrenal medulla by cleaving the NH₂-terminal amino acid from numerous substrates known to exist in the adrenal gland such as ANG III, lys-bradykinin, oxytocin, vasopressin, dynorphin A, neurokinin A, neuromedin B, Met-
enkephalin, and somatostatin (reviewed in Refs. 1 and 68). Although it remains to be determined whether these peptides are substrates in vivo, cleavage of these peptides could lead to degradation or create alternative active peptides. For example, cleavage of the NH₂-terminal amino acid of ANG III and lys-bradykinin yields ANG IV and bradykinin, respectively (reviewed in Ref. 34).

The adrenomedullary MasR. Although MasR mRNA was detected in the adrenal medulla, levels were greater than two orders of magnitude less than the AT₂ receptor. To the best of our knowledge, this is the first report of MasR gene expression in this tissue. Because of the low mRNA levels, MasR protein was not assessed, and thus it remains to be determined whether the modest expression of MasR mRNA is reflected by low levels of protein expression.

The physiological significance of the MasR in the adrenal medulla is not only currently unknown but also questionable given the very low levels of MasR mRNA expression. The rapid decline in MasR mRNA levels suggests, however, that the effects mediated by this receptor may oppose the effects mediated by the adrenomedullary stress response, possibly those mediated by the AT₁ receptor. This is supported by multiple lines of evidence. First, mRNA levels of ACE2 are also decreased by single and repeated IMO. This would likely decrease the formation of ANG-(1–7), the main ligand for the MasR, particularly if ANG II levels are elevated in response to stress, as would be expected (78). Second, the MasR antagonizes AT₁ receptor-mediated signaling. This is mediated by constitutive activity of the MasR, which activates protein kinase C-dependent phosphorylation of the AT₁ receptor and subsequently AT₁ receptor desensitization (7). The MasR has been reported to heterodimerize with the AT₁ receptor; however, it is unclear whether heterodimerization is required for antagonism of AT₁ receptor-mediated signaling (reviewed in Ref. 40). Last, MasR knockout (KO) mice display increased vasoconstriction in response to ANG II in mesenteric microvessels (29).

The adrenomedullary (pro)renin receptor. Of the nonclassical RAS receptors, mRNA for the (P)RR was the most abundant. Expression of this receptor was detected not only in chromaffin cells, but to a lesser extent in the cortex as well. This finding is in contrast to the work by Yamamoto et al. (75), which demonstrated more (P)RR immunostaining in the cortex of the nonneoplastic human adrenal gland. Furthermore, up-regulation of the (P)RR has been observed in aldosterone-producing adenomas (75). Thus, we suggest that the health status and interspecies differences may play a role in (P)RR expression and distribution within the adrenal gland, similarly as for AT₄ receptor expression in the same tissues of different animal species (73). We also confirmed the presence of (P)RR protein in PC-12 cells, which has been reported to be localized primarily in the Golgi and endoplasmic reticulum in nerve growth factor-induced PC-12 cells (10).

The widespread expression of the (P)RR in the adrenal is not surprising since the (P)RR contains an essential second function, originally characterized in the adrenal medulla, in which the COOH-terminal region associates with the vacuolar H⁺-ATPase, an enzyme involved in vesicle acidification (39). This alternative form of the (P)RR is produced via proteolytic cleavage of the extracellular (pro)renin-binding NH₂-terminus and is termed ATP6AP2. Unlike the NH₂-terminal domain, the transmembrane and COOH-terminus of the (P)RR gene is conserved not only in mammals and vertebrates but also in invertebrates, which do not possess a functional RAS. Because the mRNA and protein analyses performed in these studies cannot differentiate between the (P)RR and ATP6AP2, the low-intensity staining of the inner layers of the adrenal cortex could reflect ATP6AP2, whereas the intense staining of the adrenal medulla likely reflects full-length membrane-associated (P)RR as well as ATP6AP2.
In response to single or repeated stress, (P)RR mRNA levels were increased in the medulla by ~50% 3 h after the exposure, consistent with a stress-triggered rise in plasma renin levels (25). Adrenal (pro)renin levels may also be elevated by stress, although this remains to be determined. However, transgenic rats with excessive adrenal renin gene expression secrete large amounts of prorenin and adrenal gland steroids in the circulation and have maximal response to ACTH stimulation in regard to urinary excretion of corticosterone. These data raise the possibility that adrenal (pro)renin acting via the (P)RR may have effects on steroid production (59).

Elevated expression of the (P)RR has been observed in other pathological conditions, such as cardiac tissues of stroke-prone spontaneously hypertensive rats on a high-salt diet (23) and in the clipped kidney of Goldblatt hypertensive rats (30). (P)RR overexpression has numerous pathophysiological roles in the regulation of the cardiovascular and renal systems via ANG II-independent mechanisms, such as increasing blood pressure, aldosterone levels, and cyclooxygenase-2 expression (reviewed in Ref. 50). Furthermore, the association between elevated plasma prorenin levels and diabetic complications, including diabetic retinopathy, led to the proposal that prorenin may have a pathogenic role in diabetes (71).

The (P)RR in the adrenal medulla may regulate catecholamine biosynthesis and/or release under basal conditions and in response to stress by two (pro)renin-dependent mechanisms. First, (pro)renin stimulation of the (P)RR can elicit an increase in ANG II production since the localization of the (P)RR at the membrane may bring (pro)renin in close proximity with membrane-bound ACE and ANG II receptors for efficient ANG II production and receptor binding (49). The increase in ANG II synthesis with stress is supported by an increase in angiotensinogen and ACE mRNA levels with repeated stress exposure and a decrease in ACE2 mRNA levels with both durations of stress. Accordingly, ACE2 expression plays a key role in the regulation of ANG II levels, since ACE2 KO mice display increased levels of plasma and tissue ANG II as a result of decreased plasma ANG II metabolism and increased tissue ANG I levels (11, 20, 76).

Additionally, (pro)renin stimulation of the (P)RR can induce intracellular signaling cascades. This is the first evidence of (P)RR signaling in adrenomedullary-derived cells. Human prorenin increased phosphorylation of ERK1/2 in PC-12 cells in an ANG II-independent manner, indicating that these effects are mediated by activation of the (P)RR. Stimulation of the (P)RR has been previously shown to activate ERK1/2 phosphorylation in a range of cell types (22, 51, 58). Interestingly, treatment of PC-12 cells with human prorenin did not alter mRNA levels of TH or DBH, even though ERK1/2 activates transcription factors, such as CREB, c-Fos, and c-Jun, that are known regulators of TH and DBH gene expression (reviewed in Ref. 33). Conversely, human prorenin...
was found to induce phosphorylation of TH at Ser31, a modification that increases TH activity (14). Ser31 phosphorylation is likely mediated by ERK1/2, since these are two of only three kinases known to phosphorylate TH at Ser31 (14), the other kinase being cyclin-dependent kinase 5 (27). Accordingly, phosphorylation of ERK1/2 and TH at Ser31 has been previously observed in the adrenal medulla in response to stress (53, 57). Thus, the (P)RR in the adrenal medulla may function in stimulating catecholamine biosynthesis, and this process may be enhanced by stress exposure.

In addition to playing a role in catecholamine biosynthesis, (P)RR signaling may also modulate other processes. For example, in vascular smooth muscle cells, (P)RR-mediated activation of ERK1/2 stimulates cell proliferation and hypertrophy (35). This could contribute to adrenomedullary hypertrophy in response to prolonged or repeated stress exposure.

Because in vivo activation of the (P)RR has the potential to modulate adrenomedullary function, it is important to consider whether (pro)renin-(P)RR interactions could actually occur in the adrenal medulla. Phosphorylation of ERK1/2 was observed when PC-12 cells were treated with 20 nM human prorenin. This is consistent with concentrations known to activate ERK1/2 phosphorylation in other cell types (5, 16). However, 20 nM prorenin is ~4,000-fold higher than basal plasma prorenin levels. According to Batenburg et al. (5), prorenin levels would need to be two to three orders of magnitude greater than basal plasma levels for the formation of ANG II from the prorenin-(P)RR interaction, whereas prorenin or renin levels would need to be four to five or three to four orders of magnitude greater, respectively, to activate (P)RR-mediated signaling. Therefore, in tissues that do not synthesize prorenin, it is unlikely that prorenin-(P)RR interactions would occur. However, the adrenal gland does produce prorenin. In fact, outside of the kidney, the adrenal gland contains the highest levels of renin (45). Thus, if (pro)renin-(P)RR interactions do occur in vivo, it would likely happen in the adrenal gland. Considering the stress-triggered increase in adrenal angiotensinogen mRNA levels, as we observed, and ANG II levels (78), adrenal (pro)renin levels may also increase, allowing (P)RR signaling to occur specifically, or to a greater extent, in response to stress.

Changes in the gene expression of nonclassical RAS components in the adrenal medulla may orchestrate catecholamine production in response to stress. The stress-triggered changes in gene expression of the nonclassical RAS receptors displayed an overall biphasic effect. Within a few hours of exposure to either a single or repeated IMO, adrenomedullary AT2 and AT4 receptor mRNA levels were decreased. However, 1 day after repeated stress exposure, AT2 and AT4 receptor mRNA levels were elevated above unstressed control levels but dropped again following another stress session. In fact, the kinetics of the changes in AT2 and AT4 receptor mRNA levels were nearly identical. Conversely, (P)RR mRNA levels were increased following single or repeated stress, but decreased below unstressed control levels 1 day after repeated stress exposure. These changes may be an adaptive response of the adrenal medulla to stress. We have previously shown that the adrenal medullary catecholaminergic system responds differently to acute and repeated IMO (33, 56).

Based on the known physiological roles of the RAS receptors, the stress-triggered downregulation in AT2 receptor, AT4 receptor, and MasR may decrease signaling through these "protective" receptors and facilitate increased signaling via the AT1 receptor, particularly with acute stress exposure, leading to increased catecholamine biosynthesis. The effects mediated by elevated AT1 receptor may be further bolstered by increased signaling via the (P)RR. The restoration of AT2 and AT4 receptor mRNAs to basal levels 3 h after 6× IMO but not after a single stress session may be an adaptive and more rapid response to repeated stress. Conversely, the increase in AT2 and AT4 receptor expression and decrease in (P)RR following prolonged exposure to stress in adapted controls might represent a protective mechanism. Accordingly, AT2 receptor upregulation mediates neuro-, vaso-, and renoprotective effects in numerous pathologies such as hypertension, stroke, and diabetes, as well as with aging (reviewed in Ref. 26). In the adrenal medulla, these changes may play a role in opposing AT1 receptor-mediated stimulation of catecholamine biosynthesis in the recovery period following repeated stress exposure.

Previous reports revealed that acute and prolonged exposure to various stressors induces ANG II formation in the circulation and adrenal gland (77). Accordingly, elevation in the gene expression of angiotensinogen, ACE, and (P)RR with repeated IMO suggests that local ANG II production may be especially important with prolonged stress. To further support this, ACE2 mRNA is decreased with both single and repeated stress, which may decrease the production of non-ANG I, non-ANG II peptides to facilitate the formation of increased ANG II. This may help mediate the changing demand to adapt to repeated exposures to stress.

**Perspectives and Significance**

Overall, these data indicate complex regulation of the adrenomedullary RAS by stress, which includes many of the nonclassical RAS components. Because of the downregulation of the AT2 receptor, MasR, and AT4 receptor, stress may remove inhibitory factors in catecholamine biosynthesis and trigger an increase in AT1A receptor activation, which would enhance catecholamine biosynthesis and release. These changes are already observed upon exposure to even a single episode of IMO. The increase in angiotensinogen, ACE, and (P)RR mRNA levels and decrease in ACE2 mRNA levels support an increase in adrenal ANG II synthesis that is known to be derived almost entirely from local synthesis (69). Most of these responses required repeated exposures to the stress. Together, these changes likely comprise a crucial layer of the intricate response of the adrenomedullary catecholaminergic system to stress.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

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