Dopamine 5 receptor mediates Ang II type 1 receptor degradation via a ubiquitin-proteasome pathway in mice and human cells

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Hypertension is a multigenic disorder in which abnormal counterregulation between dopamine and Ang II plays a role. Recent studies suggest that this counterregulation results, at least in part, from regulation of the expression of both the antihypertensive dopamine 5 receptor (D5R) and the prohypertensive Ang II type 1 receptor (AT1R). In this report, we investigated the interaction in vivo and in vitro between these GPCRs. Disruption of the gene encoding D5R in mice increased both blood pressure and AT1R protein expression, and the increase in blood pressure was reversed by AT1R blockade. Activation of D5R increased the degradation of glycosylated AT1R in proteasomes in HEK cells and human renal proximal tubule cells heterologously and endogenously expressing human AT1R and D5R. Confocal microscopy, Förster/fluorescence resonance energy transfer microscopy, and fluorescence lifetime imaging microscopy revealed that activation of D5R initiated ubiquitination of the glycosylated AT1R at the plasma membrane. The regulated degradation of AT1R via a ubiquitin/proteasome pathway by activation of D5R provides what we believe to be a novel mechanism whereby blood pressure can be regulated by the interaction of 2 counterregulatory GPCRs. Our results therefore suggest that treatments for hypertension might be optimized by designing compounds that can target the AT1R and the D5R.

Introduction

Hypertension is associated with sodium retention caused by decreased renal sodium excretion, which is tightly regulated by both natriuretic and antinatriuretic hormones. Dopamine and Ang II are among those hormones that have counterregulatory effects on cellular signal transduction, production of reactive oxygen species, renal sodium excretion, and blood pressure via renal dopamine D1-like receptors (dopamine 1 receptor [D1R] and D2R subtypes), D2-like receptors (D2R, D3R, and D4R subtypes), and Ang II type 1 receptors (AT1R; Rs) (1–4). The D2R is widely expressed in the rodent kidney, specifically in the proximal and distal tubules, cortical collecting ducts, medullary ascending limbs of Henle, arterioles, glomeruli, juxtaglomerular cells, and macula densa (5). The AT1R is also widely expressed in the kidney, specifically in the proximal and distal tubules, cortical collecting ducts, medullary ascending limbs of Henle, arterioles, glomeruli, juxtaglomerular cells, and macula densa (2, 6–8).

Recent evidence shows that fenoldopam (Fen), a D1-like receptor agonist, decreases AT1R protein expression in rat renal proximal tubules (RPTs) and in vascular smooth muscle cells (9). Gene knockdown studies using antisense oligonucleotides that target either the D2R or D3R transcript point to D2R, not D3R, as the negative regulator of AT1R protein expression in human RPT cells (10). Furthermore, renal AT1R protein expression is greater in D3R knockout (Drd5–/–) mice than in wild-type (Drd5+/+) littermates (9), and renal D3R protein expression is increased in Agr1a1a1 mice, underscoring the important downstream regulatory function of D3R on AT1R protein expression, and vice versa. Moreover, Drd5–/– mice are hypertensive and salt sensitive (11), while Agr1a1a1 mice have decreased blood pressure (12), indicating the importance of dopamine receptors (5) and regulators (e.g., GPCR kinase 4; ref. 13) in the regulation of blood pressure.

In this report, we investigated the interaction in vivo and in vitro between D2R and AT1R and explored the mechanisms involved in this interaction. We showed that the high blood pressure of Drd5–/– mice, which was associated with increased AT1R protein expression, was normalized by AT1R blockade. Activation of the D3R decreased AT1R protein level by increasing AT1R degradation.

Nonstandard abbreviations used: AT1R, Ang II type 1 receptor; Chlor, chloroquine; CLBL, clasto-lactocystin β-lactone; D2R, dopamine 5 receptor; Fen, fenoldopam; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster/fluorescence resonance energy transfer; LAMP 1, lysosome-associated membrane protein 1; Met/Cys, methionine/cysteine; RAS, renin-angiotensin system; ROI, region of interest; RPT, renal proximal tubule; τ, quench time; Ub, ubiquitin.

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in proteasomes via a ubiquitin/proteasome pathway; the degrading action was initiated by the ubiquitination of the glycosylated AT\textsubscript{1}R at the plasma membrane. Stimulation of the D\textsubscript{2}R dissociated the interaction of the D\textsubscript{2}R with the AT\textsubscript{1}R and increased the proteasomal degradation of the ubiquitinated AT\textsubscript{1}Rs. We believe our results demonstrate a novel paradigm of GPCR-mediated cellular responses and regulation of degradation of one GPCR by another and moreover provide a novel mechanism for the regulation of blood pressure by the dopaminergic system.

Results

Elevated blood pressure in Drd5\textsuperscript{−/−} mice is not associated with alterations in renal or plasma catecholamines. We previously reported that high blood pressure of anesthetized Drd5\textsuperscript{−/−} mice was, at least in part, due to increased central nervous sympathetic tone, based on the greater percentage reduction in blood pressure with \(\alpha\)-adrenergic blockade but similar percentage reduction in blood pressure after adrenalectomy in Drd5\textsuperscript{−/−} mice relative to their wild-type littermates (11). Here, renal and plasma levels of norepinephrine, epinephrine, and \textsubscript{L}-DOPA were not different between the 2 mouse strains (Supplemental Table 2). However, the renal dopamine level increased in Drd5\textsuperscript{−/−} mice relative to their wild-type littermates (Supplemental Table 2). Therefore, the elevated blood pressure in Drd5\textsuperscript{−/−} mice was not caused by increased peripheral catecholamines or a deficiency in renal dopamine levels.

Activation of D\textsubscript{2}R decreases AT\textsubscript{1}R protein expression in vitro. Renal cross-transplantation experiments have previously shown that the kidney contributes about 50% of blood pressure regulation (14). The RPT and thick ascending limb of Henle are the sites of increased sodium reabsorption in human essential polygenic hypertension (15), whereas the distal nephron is the site of increased sodium transport in monogenic hypertension (16). Therefore, we next studied the effect of D\textsubscript{2}R-like receptor stimulation on AT\textsubscript{1}R protein expression in human RPT cells, which endogenously expressed D\textsubscript{2}R (data not shown), Drd5\textsubscript{−/−} (Supplemental Figure 2), and AT\textsubscript{1}R (Supplemental Figure 2). In human RPT cells, Fen (1 \(\mu\)M for 20 min) decreased AT\textsubscript{1}R protein expression (Figure 2). The effect of Fen on AT\textsubscript{1}R protein expression was observed for the approximately 70–90 kDa band, but not for the approximately 40–60 kDa band; presumably, the former represents the glycosylated form of AT\textsubscript{1}R (17, 18). The observed change in AT\textsubscript{1}R expression was specifically caused by D\textsubscript{2}R-like receptor stimulation, because cotreatment with SCH23390, a D\textsubscript{2}R-like receptor antagonist that had no effect by itself, abrogated the decrease in AT\textsubscript{1}R protein expression (Figure 2). These data indicate that a D\textsubscript{2}R-like receptor, presumably the D\textsubscript{2}R (9, 10), negatively regulates AT\textsubscript{1}R protein expression in human RPT cells.

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only affects the expression of the glycosylated species of AT1R. Moreover, the rapid D3R-mediated decrease of this band, but not the approximately 65–85 kDa AT1R band (Figure 3A), indicates that the inhibitory effect does not result from the downregulation of transcription or translation, but rather may be the result of increased AT1R protein degradation.

Characterization of D3R-mediated AT1R degradation. To characterize the D3R-mediated AT1R turnover, we performed pulse-chase experiments by [35S]methionine/cysteine (Met/Cys) labeling of AT1R/D3R HEK293 cells treated with vehicle, Fen, or Ang II. Stimulation of the D3R with Fen resulted in degradation of the AT1R more rapid than that observed with vehicle treatment, but slower than that with Ang II treatment (Figure 4). Unlike Ang II, which increased degradation of both the glycosylated and the nonglycosylated AT1R, Fen increased the degradation of only the glycosylated AT1R (Figure 4A), and the amount of the approximately 100–120 kDa AT1R band decreased as a function of time (0.365 ± 0.618−0.22), which suggests a first-order decay.

Protein degradation in mammalian cells occurs primarily in either lysosomes or proteasomes (22, 23). Spontaneous or Ang II–induced AT1R degradation occurs in lysosomes (24, 25). To explore the sites of D3R-mediated AT1R degradation in AT1R/D3R HEK293 cells, we examined the colocalization of the AT1R with marker proteins for proteasomes or lysosomes using laser scanning confocal microscopy. Under basal conditions, a substantial fraction of AT1Rs (43.47%) were localized at the plasma membrane (Supplemental Figure 3 and Supplemental Table 3), consistent with the previous observation that the mouse AT1R mainly localizes at dopamine receptors (20, 21). To investigate the mechanism of the regulation of AT1R protein expression by the D3R, we transfected human full-length AT1R tagged at the C terminus with EGFP into HEK293 cells expressing the D3R. Flow cytometry (Supplemental Figure 1A) and immunoblot analyses (Supplemental Figure 1B) of the AT1R-EGFP transfected confirmed the stable heterologous expression of AT1R-EGFP in D3R HEK293 cells. Radioligand binding assays (Supplemental Figure 1C) using [3H]-SCH 23390 and [125I]-I-Sar Ang II as ligands for the D1-like receptors (here, only the D3R) and the AT1R, respectively, also showed the expression of both the D3R and the AT1R. Ang II increased the phosphorylation of ERK1/2 in a dose-dependent manner (Supplemental Figure 1D), indicating that the EGFP tag did not impair AT1R function in the transfected cells.

We next tested whether activation of the D3R decreased AT1R protein expression in the transfected cells (designated AT1R/D3R HEK293 cells). Fen decreased the approximately 100–120 kDa band, which presumably represents the glycosylated AT1R plus the EGFP tag, but not the approximately 65–85 kDa AT1R band, which presumably represents the nonglycosylated AT1R plus the EGFP tag, as early as 20 minutes after treatment (Figure 3A). Because the D3R is not expressed in HEK293 cells (20, 21), these results indicate that the effect of Fen on AT1R protein expression is solely the result of D3R activation.

Treatment with PNGase F (Figure 3B) greatly decreased the intensity of the approximately 100–120 kDa AT1R band, which indicates that this band represents the glycosylated form of the receptor. Thus, the D3R

![Figure 2](http://www.jci.org) Effect of Fen on AT1R protein expression. Immortalized human RPT cells were incubated with vehicle (lane 1), Fen (1 μM for 20 min; lane 2), or SCH 23390 (1 μM for 50 min) in the absence (lane 3) or presence (lane 4) of Fen. AT1R protein expression in total cell lysates was determined by immunoblotting with anti-AT1R (top panel); 2 bands were observed, denoted a and b, which presumably represent the glycosylated and nonglycosylated forms of the AT1R, respectively (17, 18). Immunoblotting for actin (bottom panel) showed equal sample loading in each lane. Shown is 1 immunoblot from 3 experiments. The effect of vehicle, Fen, or SCH 23390 in the presence or absence of Fen on AT1R protein expression was also quantified. n = 3. *P < 0.05 versus vehicle, ANOVA, Student-Newman-Keuls test. Data are mean ± SEM.

![Figure 3](http://www.jci.org) Effect of D3R stimulation on the expression of the glycosylated form of the AT1R protein. (A) AT1R/D3R HEK293 cells were incubated with Fen (1 μM) for the indicated times; total cell lysates were electrophoretically separated, transferred onto nitrocellulose membranes, and immunoblotted with anti-GFP (band a, ~100–120 kDa; band b, ~65–85 kDa) to detect AT1R protein expression. Immunoblotting for actin was used to monitor the sample protein loading. n = 3. *P < 0.05 versus 0 min, ANOVA, Student-Newman-Keuls test. (B) Protein samples (50 μg) from AT1R/D3R HEK293 cells were incubated with 10 μM PNGase F at 37°C for the indicated times, electrophoretically separated, transferred onto nitrocellulose membranes, and immunoblotted with anti-GFP antibody. Band S is sensitive to PNGase F and corresponds to the glycosylated form of the AT1R (band a). Band R is resistant to PNGase F and corresponds to the nonglycosylated form of the AT1R (band b). The ratio of band S intensity to band R intensity was determined by densitometry in 3 separate experiments. n = 3. *P < 0.05 versus 0 h, ANOVA, Student-Newman-Keuls test. Data are mean ± SEM.
the plasma membrane (26). Lysosome-associated membrane protein 1 (LAMP 1), a marker of lysosomes, and p44S\(^{10}\)R, a marker of proteasomes, were scattered throughout the cytoplasm with much less expression at the plasma membrane (Supplemental Figure 3). In the cytoplasm, there was some colocalization of the AT,R and LAMP 1 (23.19%) and of the AT,R and p44S\(^{10}\)R, albeit to a lesser degree (11.26%; Supplemental Figure 3 and Supplemental Table 4).

Stimulation with Fen resulted in considerable internalization of AT,Rs with patches and clusters of AT,R fluorescence in both plasma membrane and cytoplasm. Fen treatment increased the colocalization of AT,R with p44S\(^{10}\)R from 11.26% to 56.12%, whereas the treatment did not affect the percentage of colocalization between AT,Rs and LAMP 1 (Supplemental Figure 3 and Supplemental Table 4). In contrast, treatment with Ang II increased the colocalization between AT,Rs and LAMP 1 without affecting that between AT,R and p44S\(^{10}\)R (Supplemental Figure 3). These results strongly suggest that upon activation, the D\(_{1}\)R directs the degradation of the AT,R to proteasomes and not to lysosomes. The Fen-induced changes in the intracellular distribution of the AT,R did not occur in the cells incubated with SCH23390 prior to Fen treatment (Supplemental Figure 3 and Supplemental Tables 3 and 4), which indicates that the effect of Fen was through the activation of the D\(_{1}\)R.

\(\text{D}_{1}\)R-mediated AT,R degradation occurs in proteasomes, but not lysosomes. To investigate further the \(\text{D}_{1}\)R-mediated AT,R degradation in AT,R/D\(_{1}\)R HEK293 cells, we used clasto-lactocystin \(\beta\)-lactone (CLBL), a proteasome inhibitor, and chloroquine (Chlor), a lysosome inhibitor, in \([^{35}\text{S}]\text{Met/Cys}\) metabolic experiments. Fen-stimulated AT,R degradation was greatly reduced in the presence of CLBL but not Chlor; the half-life of the AT,R in the presence of CLBL was 149.5 min (decay rate, 0.537), while it was 43.8 min (decay rate, 0.665) in the presence of Chlor (Figure 5A). Morphological observation showed that CLBL, but not Chlor, abrogated the Fen-stimulated colocalization of AT,R and p44S\(^{10}\)R (Figure 5B and Supplemental Table 4). In the presence of CLBL, image analysis showed that 42.40% of AT,R resided in the plasma membrane in spite of the presence of Fen (Supplemental Table 3). Flow cytometry data also showed that CLBL, but not Chlor, inhibited the Fen-stimulated degradation of the AT,R (Figure 5C). Chlor alone slightly increased the AT,R fluorescence (Figure 5C), consistent with previous observations that spontaneous degradation of the AT,R occurs in lysosomes (24, 25). Again, inhibition of proteasome but not lysosome activity abrogated \(\text{D}_{1}\)R-mediated AT,R degradation, which confirmed that this degradation occurs in proteasomes.

To corroborate the biochemical and morphological studies indicating that exogenously and endogenously expressed AT,Rs are directed to proteasomes after Fen stimulation, proteasomes were isolated from human RPT cells treated with vehicle, Ang II, or Fen. Using proteasome affinity beads, AT,Rs were identified in proteasomes isolated from Fen-treated human RPT cells, but not in those isolated from vehicle- and Ang II–treated cells (Figure 5D), which indicates that Fen stimulation directs endogenously expressed AT,R to proteasomes in human RPT cells.

AT,R interacts with ubiquitin and is ubiquitinated at the plasma membrane. Ubiquitin (Ub) modification plays an important role in targeting proteins for degradation (23, 27–32). To determine the role of ubiquitination in the degradation of the AT,R, we first studied the colocalization of Ub (and ubiquitinated proteins), p44S\(^{10}\)R, and the AT,R in vehicle- and Fen-treated cells (Figure 6A). In vehicle-treated cells, Ub and p44S\(^{10}\)R were scattered throughout the cytoplasm, and minimal—if any—colocalization was observed among the 3 proteins (Figure 6A). In contrast, Fen treatment induced significant colocalization of the AT,R and p44S\(^{10}\)R, the AT,R and Ub, and p44S\(^{10}\)R and the AT,R, Ub, and p44S\(^{10}\)R at the plasma membrane (Figure 6A and Supplemental Table 5).

To validate the morphological findings observed with confocal microscopy, Förster/fluorescence resonance energy transfer (FRET) microscopy (33) was used to detect the interaction between the AT,R and endogenous Ub in AT,R/D\(_{1}\)R HEK293 cells. Fen stimulation markedly increased the energy transfer of the AT,R and Ub at the plasma membrane from 3.6% ± 1.2% in unstimulated cells to 34.1% ± 2.4% (Figure 6B). At the same time, the energy...

Figure 4
Characterization of AT,R degradation by \([^{35}\text{S}]\text{metabolic labeling. AT,R/D}_{1}\R HEK293 cells were treated with vehicle, Ang II (100 nM for 30 min), or Fen (1 \(\mu\)M for 2 h), pulsed with \([^{35}\text{S}]\text{Met/Cys, and then chased with cold amino acids for the indicated times. The cell lysates were immunoprecipitated with anti-GFP antibody, and the immuno-}


complexes were subjected to SDS-PAGE. Dried gels were exposed to X-ray films. (A) Autoradiographs of AT,R degradation with vehicle, Ang II, and Fen treatment. (B) Quantification of AT,R degradation. Values are mean ± SEM of arbitrary density units normalized to the time-0 value (n = 3). The decrease in AT,R protein in the presence of vehicle followed a first-order exponential curve, with a half-life of 127.6 min. The decrease in AT,R protein with Ang II or Fen treatment also followed a first-order exponential curve; treatment with Ang II decreased the half-life of AT,R protein to 16.8 min, while treatment with Fen resulted in a half-life of 37.7 min. P < 0.05, Fen versus vehicle and versus Ang II, repeated-measures ANOVA. No error bar is visible when the SEM is smaller than the symbol.
transfer had no obvious increase in the cytoplasm, which indicates that Fen stimulation increased the AT1R and Ub interaction initially at the plasma membrane but not in the cytoplasm.

Fluorescence lifetime imaging microscopy (FLIM) allows quantitative monitoring of protein interaction by measuring the change in lifetime of the donor molecule, which does not require any spectral bleed-through correction and is independent of change in fluorophore concentration and excitation intensity (34). To verify the above observations obtained with FRET microscopy, FLIM was used to measure the interaction between the AT1R and Ub at the plasma membrane by monitoring the quench time (τ) of the AT1R (as a donor) in the presence or absence of Ub (as an acceptor). As shown in Figure 7A, the average τ of the AT1R was 2.32 ns in the absence of the acceptor and 2.33 ns in vehicle-treated cells in the presence of the acceptor. Fen stimulation shortened the average τ1 of the AT1R to 1.68 ns because of the occurrence of FRET between the AT1R and Ub, which suggests their interaction upon Fen stimulation. There was no interaction between them in the basal state, consistent with the confocal and FRET microscopy observations.

To determine directly whether the AT1R is ubiquitinated, human RPT (Figure 7B) and AT1R/D5R HEK293 (Supplemental Figure 4) plasma membrane fractions were isolated by biotinylation, immunoprecipitated with anti-AT1R or anti-GFP, and immunoblotted with Ub antibody (clone FK1). Fen caused the polyubiquitination of both endogenous (Figure 7B) and heterologously overexpressed (Supplemental Figure 4) AT1R, which was not observed in the basal state. The Fen-stimulated ubiquitination of AT1R was similar in the presence (RPT cells) or absence (AT1R/D5R HEK293 cells) of the D5R, which indicates that the D5R was not involved in D3R-mediated AT1R ubiquitination. The Fen-stimulated polyubiquitination of the endogenous AT1R was

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**Figure 5**

Fen-stimulated AT1R degradation occurs in proteasomes but not lysosomes. (A) AT1R/D5R HEK293 cells treated with Fen (1 μM for 2 h) and either Chlor (100 μM for 3 h) or CLBL (4 μM for 3 h) were pulsed with [35S] Met/Cys and chased with cold amino acids for the indicated times. Shown is 1 autoradiograph of each treatment. Data (mean ± SEM) followed a first-order exponential curve for both treatments. The AT1R protein half-life was 43.8 min for Fen plus Chlor and 149.5 min for Fen plus CLBL. n = 3. (B) Subcellular distribution of AT1R in AT1R/D5R HEK293 cells treated as in A. Green, AT1R-EGFP; red, lysosomes or proteasomes (detected by Alexa Fluor 633–conjugated anti-LAMP 1 or anti-p44S10); yellow, colocalization. Scale bars: 10 μm; 2.5 μm (insets). (C) AT1R/D5R HEK293 cells were treated with vehicle, Fen (1 μM for 6 h), Chlor (100 μM for 10 h), CLBL (4 μM for 10 h), Chlor plus Fen, or CLBL plus Fen. Nontransfected D5R HEK293 cells are shown as a control. Right: MFI of AT1R-EGFP. n = 3. *P < 0.05, **P < 0.001 versus vehicle, ANOVA, Student-Newman-Keuls test. Data are mean ± SEM. (D) Human RPT cells were treated with vehicle, Ang II, or Fen; cell lysates were incubated with control or proteasome-affinity beads for 4 hours at 4°C. After washing, the matrix was suspended in SDS sample buffer, separated by SDS-PAGE, and transferred onto nitrocellulose. Membranes were immunoblotted for either AT1R or p44S10. Experiments were repeated twice with similar results.
The AT$_1$R is ubiquitinated, and the ubiquitination of the AT$_1$R is initiated at the plasma membrane. AT$_1$R/D$_5$R HEK293 cells were treated with vehicle or Fen (1 μM for 5 min). (A) Immunostaining for p44S$_{10}$ or Ub (clone P4D1). Distance calibration was based on objective and zoom of the images taken: 1 pixel equals 0.133 μm. Green, EGFP-tagged AT$_1$R; red, p44S$_{10}$; blue, Ub; yellow, colocalization of AT$_1$R and p44S$_{10}$ (green and red); cyan, colocalization of AT$_1$R and Ub (green and blue); magenta, colocalization of p44S$_{10}$ and Ub (red and blue); white, colocalization of AT$_1$R, Ub, and p44S$_{10}$ (red, green, and blue). Scale bars shown for vehicle-treated cells also apply to Fen-treated cells. (B) The image of AT$_1$R-EGFP (as donor fluorophore) was used for drawing ROIs, which were divided into plasma membrane (rectangles) and cytoplasm (ovals) to determine the spatial interaction between the AT$_1$R and Ub (clone P4D1, Alexa Fluor 555; as acceptor fluorophore). The pure FRET and 2-dimensional distribution images of energy transfer efficiency (E%) were generated as described in Methods. Graphs show mean ± SEM energy transfer efficiency and distance ($r$) of ROIs in the plasma membrane (Mem) and cytoplasm (Cyt) in the corresponding AT$_1$R images processed from 6–8 cells. *P < 0.05 versus vehicle, Student’s t test. Distances beyond experimental limitations (>90 Å) are given a 0 value, indicating no occurrence of FRET.

Figure 6
Glycosylation is necessary for the ubiquitination of AT_1R at the plasma membrane. (A) AT_1R/D_3R HEK 293 cells were treated with vehicle or Fen (1 μM for 5 min). Shown are FLIM images, histograms, and decay graphs of cells treated in the absence (top) or presence (middle and bottom) of acceptor (Ub). The left shift (arrow) of AT_1R lifetime in Fen-treated samples demonstrated the occurrence of FRET. The lifetime of AT_1R (donor) in the absence of acceptor was 2.32 ± 0.2 ns; it was 2.33 ± 0.2 ns in the presence of acceptor with vehicle treatment, indicating no energy transfer. With Fen treatment in the presence of acceptor, the donor lifetime had 2 peaks: the first was quenched at 1.68 ± 0.2 ns (τ_1) because of the occurrence of FRET, with a corresponding energy transfer efficiency of 27.6% ± 3.7%; the second was 2.24 ± 0.3 ns (τ_2), which represented the unquenched AT_1R. The FLIM patterns observed in the basal state and after Fen were similar in 4 different experiments. Quantification was performed in 16–30 cells from 1 of the 4 experiments. Data are mean ± SEM. (B) Human RPT cells were treated with vehicle, Fen (1 μM for 5 min), tunicamycin (Tunica; 10 μg/ml for 12 h), or tunicamycin plus Fen. Cell membrane fractions isolated by biotinylation were immunoprecipitated with normal mouse Ig M/G (lane 1), normal rabbit IgG (lane 2), anti-AT_1R mAb (lane 3), or anti-D_3R antibody (lane 4) and immunoblotted with anti-Ub (clone FK1) or anti-AT_1R mAb. (Ub)n, polyubiquitin chain; HC, heavy chain.

Discussion

Our results demonstrate what we believe to be a novel mechanism of the regulation of the AT_1R protein expression, which is physiologically important in vivo. The D_3R mediates the degradation of glycosylated AT_1R through the Ub/proteasome pathway, a process initiated at the plasma membrane, in contrast to either spontaneous or Ang II–mediated degradation of the AT_1R, which occurs in lysosomes.

Ubiquitination plays important roles in the regulation of proteins destined for lysosome or proteasome degradation (28, 35). A number of GPCRs in mammalian cells have been reported to be ubiquitinated, such as the β_2-adrenergic receptor (36), chemokine receptor CXCR4 (37, 38), platelet-activating factor receptor (39), and protease-activated receptors 1 and 2 (40, 41). However, to our knowledge, no previous studies have suggested that the AT_1R is ubiquitinated following its stimulation. Indeed, the agonist-mediated endocytosis of the rodent AT_1A/R does not require ubiquitination of the receptor (42).

Lysine is the target amino acid for ubiquitination. The human AT_1R contains many lysine residues, especially in its intracellular domains: in 19% (10 of 53) of its carboxy terminus, 20% (4 of 20) of its third intracellular loop, 7% (1 of 15) of its second intracellular loop, and 20% (2 of 10) of its first intracellular loop. Even though ubiquitination is not required for rodent AT_1A/R internalization either in the basal state or following Ang II stimulation in CHO cells (42), human AT_1R ubiquitination can still occur under certain conditions. Our results showed that following short-term D_3R stimulation, Ub accumulated at sites where the AT_1R was located at the plasma membrane (Figure 6, A and B, and Figure 7A), where it was initially ubiquitinated (Figure 7B). The ubiquitinated AT_1Rs were then directed to proteasomes for subsequent degradation (Figures 5–7).

N-glycosylation is a common posttranslational modification of GPCRs and plays roles in the formation of their active conformation, targeting to the cell surface and intracellular signaling (43). Human bradykinin B2 receptor requires N-glycosylation for its maturation at the cell surface (44); certain sites of β_2-adrenergic receptor N-glycosylation can alter receptor targeting to the degradation pathway (45). Both dopamine receptors (46, 47) and the AT_1R (17, 18) are N-glycosylated. Glycosylation is required for the D_3R to be functionally expressed at the cell surface (46). N-glycosylation of the AT_1R is important for its proper folding, targeting, and cell surface display (18). Our results showed that glycosylation was required for the interaction of AT_1R with D_3R (Figure 7B), which is consistent with previous observation that glycosylation is important for their homologous (44) or heterologous (47) interaction with their binding partners to accomplish a series
of intracellular functions. Glycosylation may also serve as a signal for AT$_1$R ubiquitination upon Fen stimulation and subsequent degradation in proteasomes (refs. 48–50, Figure 7B, and Supplemental Figure 4), which is consistent with a previous observation that N-linked high-mannose oligosaccharides recognized by Ub ligase lead to the ubiquitination of N-glycosylated proteins (49). Therefore, we speculate that ubiquitination of the glycosylated AT$_1$R expressed at the plasma membrane initiates its degradation in proteasomes (Figures 6 and 7). Additionally, glycosylation may protect the nascent AT$_1$R from proteolytic degradation (6, 7, 48). However, the protection is superseded by activation of the D$_3$R, resulting in targeting of the receptor to proteasomes for degradation (Figures 6 and 7).

Both the D$_3$R and the AT$_1$R are GPCRs that are important in the regulation of sodium balance and blood pressure. The RAS regulates blood pressure by renal and nonrenal mechanisms (2, 6, 7, 14, 51). The paracrine regulation of sodium transport in the proximal tubule by the RAS is exerted via several angiotensin receptor subtypes (AT$_1$R, AT$_2$R, and AT$_3$R) (2, 6, 7). The activation of AT$_1$R by Ang II increases sodium transport, whereas the activation of AT$_2$R and AT$_3$R decreases sodium reabsorption. Under physiologic conditions, the major effect of Ang II is to stimulate sodium transport via the AT$_1$R (2, 6, 7). In the hypertensive state, the AT$_1$R function is increased, which may or may not be associated with increased expression (2, 6–8). However, the mechanism underlying this phenomenon in RPTs is not completely clear. Assessment of the in vivo expression levels of D$_3$R and AT$_1$R following dopaminergic or AT$_1$R stimulation may clarify this issue. Dopamine and its receptors have been reported to participate in the regulation of the RAS, including Ang II receptors (5, 9, 52, 53). D$_3$-like receptors have been reported to increase the AT$_1$R (53) and decrease the AT$_1$R (9, 52) expression in RPTs. The D$_3$-like receptor subtype regulating the expression of a particular angiotensin receptor subtype has not been established.

Second-generation D$_3$S$^{-/-}$ mice in a mixed B129Sv and C57BL/6 background (11, 21) and F6 D$_3$D$^{-/-}$ mice in a C57BL/6 (>98% congenic) background (9, 21) have high blood pressure (9, 11, 21) and increased AT$_1$R protein expression (9). Renal or plasma catecholamine levels (Supplemental Table 2) could not explain the increased blood pressure and AT$_1$R protein expression observed in D$_3$S$^{-/-}$ mice. Renal blood pressure was not different between D$_3$S$^{-/-}$ and D$_3$D$^{-/-}$ littermates (Figure 1C). Renin is mainly synthesized and secreted by juxtaglomerular cells, which express AT$_1$R and D$_3$R, but not D$_3$R (11). Because D$_3$R is not expressed in juxtaglomerular cells, one would expect no change in AT$_1$R expression or alteration in renin levels via the AT$_1$R/renin feedback loop. Although D$_3$R can increase renin secretion, D$_3$R protein expression in the brain and kidney is not altered by knocking out the gene encoding D$_3$R (11). This indicates that the juxtaglomerular AT$_1$R/renin feedback loop is not altered by knocking out the D$_3$R gene. Consistent with previous reports (54), AT$_1$R blockade with losartan treatment increased renin expression in D$_3$S$^{-/-}$ mice. Interestingly, this effect was not observed in D$_3$D$^{-/-}$ mice, the mechanism of which remains to be determined. Nonetheless, our results showed that the increased blood pressure in D$_3$S$^{-/-}$ mice was probably not caused by increased production of renin and/or Ang II.

The results of the present study show that the hypertension resulting from the absence of the gene encoding D$_3$R is caused, at least in part, by the increase in AT$_1$R protein expression, because of the absence of the counterregulatory effect of the D$_3$R on the AT$_1$R. Activation of the D$_3$R initiated AT$_1$R ubiquitination, which directed the AT$_1$R to proteasomal degradation (Figures 5–7). Glycosylation was required for the ubiquitination of the AT$_1$R (Figure 7). These results provide evidence for a mechanism by 2 posttranslational modifications crucial in the regulation of the AT$_1$R, a class B GPCR, mediated by the D$_3$R, a class A GPCR. The negative regulation of the AT$_1$R expression by the D$_3$R has physiologic consequences, because chronic administration of the AT$_1$R blocker losartan normalized the elevated blood pressure of D$_3$S$^{-/-}$ mice (Figure 1A). Inhibition of the RAS is an important component of the treatment of essential hypertension, but the control of hypertension remains suboptimal (55). Our results showing that the antihypertensive effect of D$_3$-like receptors counteracted the prohypertensive effects of the RAS, in part by D$_3$R-mediated degradation of the AT$_1$R, provides opportunities for designing specific drugs that can target the AT$_1$R and/or the D$_3$R for the optimization of hypertension therapy.

**Methods**

Antibodies and reagents; cell lines and transfection; radioligand binding; radioligand binding autoradiography; biotinylation and immunoprecipitation; deglycosylation; proteasome isolation; flow cytometry; and determination of catecholamine levels. See Supplemental Methods.

D$_3$S$^{-/-}$ mice and blood pressure measurement. The animal protocols were reviewed and approved by the Georgetown University Animal Care and Use Committee. For details, see Supplemental Methods.

**Pulse-chase.** AT$_1$R/D$_3$R HEK293 cells were labeled by 35S-labeled Met/Cys as described previously (56). Briefly, cells were starved in Met/Cys-free DMEM (Invitrogen) containing 10% dialyzed FBS for 2 h and pulsed for 3 h with medium containing 300 μCi/ml 35S-labeled Met/Cys. Cycloheximide (10 μg/ml) was added to inhibit de novo protein synthesis and then chased for varying durations in full culture medium containing 3 mM unlabeled Met/Cys. At the end of each individual chase period, the cells were washed with PBS 3 times to remove unbound label. Cell pellets were lysed with lysis buffer, and the AT$_1$R was immunoprecipitated using anti-GFP antibody. Immunoprecipitated protein complexes were eluted with protein sample buffer at 85°C for 15 minutes and resolved by SDS-PAGE. Dried gels were exposed to X-ray films for autoradiography. The quantified AT$_1$R bands were normalized to the time-0 value. The mean and SEM of 3 experiments were plotted, and the exponential decays were calculated (OriginPro; OriginLab Corp.). The decay rate constants and the half-lives of the AT$_1$R protein, which represent the rates of AT$_1$R degradation, were calculated based on individual decay equations.

**Confocal immunofluorescence microscopy.** AT$_1$R/D$_3$R HEK293 cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with PBS, the fixed cells on coverslips were incubated overnight at 4°C with Alexa Fluor 633–conjugated (Invitrogen) polyclonal rabbit LAMP 1 (2 μg/ml) or polyclonal goat p448S–conjugated (Invitrogen) monoclonal mouse Ub antibody (2 μg/ml). Coverslips were mounted in SlowFade mounting medium (Invitrogen) and sealed onto glass slides. Samples were imaged using an Olympus Fluoview FV300 laser scanning confocal microscope equipped with a x60/1.4 NA objective. Quantitative analysis was conducted by using MetaMorph 6.1 (Molecular Devices). Background was subtracted from each image, after which the images were thresholded to identify specific protein fluorescence. Whole cells, plasma membrane, or cytoplasmic areas were identified by creating regions of interest (ROIs). Fluorescence in each (integrated intensity) was determined and expressed as percent of total fluorescence for cytoplasm and plasma membrane. For determination of colocalization, the function “Colocalization” in MetaMorph software was used where percent overlap...
(integrated intensity measurement) was determined for AT-R over LAMP 1, or AT-R over p44S, Ubt over AT-R, and Ubt over p44S.

FRET microscopy and data processing. The FluoroPhore pairs used for FRET imaging in this study were AT-R-EGFP (as donor dipole) and Alexa Fluor 555 (as acceptor dipole; Invitrogen) conjugated with Ub antibody. Seven images were acquired for each FRET experiment, as described previously (33), with an Olympus Fluoview FV300 laser scanning confocal microscope equipped with a x60/1.4 NA objective, argon (488 nm) and HeNe (543 nm) laser, emission filters 515/50 nm and 590 nm LP. Either single-labeled donor or acceptor samples or double-labeled samples were acquired under the same condition throughout the image collection. The uncorrected FRET images (uFRET) were acquired by donor excitation in acceptor channel, which contained pure FRET (pFRET) and contaminations from both donor and acceptor spectral bleed-through (SBT). pFRET images were generated using a previously described algorithm (33) for pixel-by-pixel removal of donor and acceptor SBT on the basis of matched fluorescence levels between the double-labeled specimen and single-labeled reference specimens.

ROIs were selected in the uFRET images (33). In this study, we used image e (donor excitation in donor channel of double-labeled specimen) as the reference image for selection of ROIs to separate plasma membrane and cytoplasm fluorescence. The percentage of energy transfer (%E) was processed on a pixel-by-pixel basis by using the equation %E = 1 - [(Ida/Ida + pFRET × [Pd/Pa] × [Sd/Sa] × [Qd/Qa])]; where Pd and Pa (photo multiplier tube gain of donor and acceptor channels, respectively) set to the same when images were acquired; Sd and Sa (spectral sensitivity of donor and acceptor channels, respectively) provided by the manufacturer; Qd and Qa (donor and acceptor quantum yield, respectively) measured by spectrophotometer as described previously (57); Ida is the image of donor excitation in donor channel of double labeling samples after removing of background; and pFRET is the processed or corrected FRET. The calculation of distance of donor and acceptor (r) was based on the following previously described equation (33): (33) \( r = \frac{R_0}{1 + \frac{1}{6}} \), where E is the efficiency of energy transfer. Förster’s distance (R0) in this study was 67.5 (33).

FLIM. Cell preparation and fluorophores of specimens were performed as described above. Time-domain FLIM was performed with a 120-s acquisition in a 2-photon BioRad Radiance 2100 microscope with x60 NA 1.4 objective lens at the M.W. Keck Center for Cellular Imaging of the University of Virginia, as described previously (34). The AT-R was fused with EGFP as the donor fluorophore, and the acceptor fluorophore was Ub (clone P4D1), conjugated with Alexa Fluor 555. The single exponential decay of lifetime was processed in the donor alone samples, and the double exponential decay of lifetime was performed in the presence of acceptor fluorophores. Donor fluorophores (AT-R-EGFP) were excited at 790 nm by a femtosecond pulse from a coherent Ti:sapphire laser system. Emissions filtered with a center of 515 nm were collected by a high-speed photomultiplier tube (MCP R3809; Hamamatsu), and fluorescence lifetime imaging capability was incorporated, with addition of a fast time-correlated single photon counting acquisition board (SPC 730; Becker and Hickl). Fluorophore lifetimes were fitted to 1- or 2-exponential decay curves on Spclmage 2.60 (Becker and Hickl). The non-FRETing population, measured as the donor lifetime in the absence of an acceptor, is represented as \( t_0 \); the FRETing population (\( t_1 \)) is represented in the figures. The energy transfer efficiency is calculated as 1 - \( (t_0/t_1) \), where \( t_0 \) and \( t_1 \) are the donor excited state lifetimes in the presence and absence, respectively of the acceptor. Cell images and lifetime distributions are representative examples. The FLIM patterns observed in the basal state and after Fen were similar in 4 different experiments. Quantification was performed in 16–30 cells from 1 of the 4 experiments.

Results. Results are expressed as mean ± SD or SEM as indicated. Significant differences among groups were determined by factorial or repeated ANOVA (Holm-Sidak or Student-Newman-Keuls test) for multiple comparisons and Student’s test for 2 comparisons. P < 0.05 was considered statistically significant (SigmaStat 3.0; SPSS Inc.).

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