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Dopamine and Angiotensin Type 2 Receptors Cooperatively Inhibit Sodium Transport in Human Renal Proximal Tubule Cells

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Abstract

Little is known regarding how the kidney shifts from a sodium and water reclaiming state (antinatriuresis) to a state where sodium and water are eliminated (natriuresis). In human renal proximal tubule cells (RPTCs), sodium reabsorption is decreased by the dopamine D_1-like receptors (D_1R/D_5R) and the angiotensin type 2 receptor (AT_2R), while the angiotensin type 1 receptor increases sodium reabsorption. Aberrant control of these opposing systems is thought to lead to sodium retention and subsequently hypertension. We show that D_1R/D_5R stimulation increased plasma membrane AT_2R 4-fold via a D_1R-mediated, cAMP-coupled, and PP2A-dependent specific signaling pathway. D_1R/D_5R stimulation also reduced the ability of angiotensin II to stimulate phospho-ERK, an effect that was partially reversed by an AT_2R antagonist. Fenoldopam did not increase AT_2R recruitment in RPTCs with D_1Rs uncoupled from adenylyl cyclase, suggesting a role of cAMP in mediating these events. D_1Rs and AT_2Rs heterodimerized and cooperatively increased cAMP and cGMP production, PP2A activation, sodium-potassium-ATPase internalization and sodium transport inhibition. These studies shed new light on the regulation of renal sodium transport by the dopaminergic and angiotensin systems and potential new therapeutic targets for selectively treating hypertension.

Keywords

Angiotensin type 2 receptor; dopamine receptors; renal proximal tubule cells; PP2A; cAMP; cGMP; NaKATPase; sodium transport

INTRODUCTION

The kidney is a key organ responsible for regulating sodium and water balance and ultimately blood pressure. The intrarenal balance between the natriuretic effect of dopamine and the antinatriuretic effect of angiotensin II (Ang II) is a key factor in whether or not there is a net increase or decrease in sodium excretion.[1] During low or normal sodium intake, basal renal sodium transport is principally regulated by renal Ang II, [2] with dopamine playing a relatively minor role. Under conditions of moderate excess sodium intake,

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however, renal dopamine levels increase and inhibit sodium transport. [3] Under these conditions, more than 50% of sodium excretion is due to the intrarenal actions of dopamine in dogs[4] and rats.[5] The natriuretic effect of dopamine is also increased in salt-loaded humans. [6]

The orchestration of the ultimate effects of Ang II and dopamine results from a complex integration of their corresponding G protein-coupled receptors (GPCRs) and intracellular effectors. The D_1-like receptors (D_1R and D_5R in mammals) are the primary dopaminergic receptors involved in sodium transport inhibition. Selective stimulation of the D_1R acts through a cAMP-mediated mechanism involving G protein receptor coupled kinase type 4 (GRK4) to decrease both NHE3, the primary sodium transporter in the brush border (apical) membrane, and NaKATPase, the sodium pump in the basolateral membrane. [7,8] Selective stimulation of the D_5R inhibits the antinatriuretic activity of the angiotensin type 1 receptor (AT_1R).[9] Ang II stimulates two GPCRs in the kidney: the AT_1R, which is directly stimulated by Ang II, and the angiotensin type 2 receptor (AT_2R) [1,10] which is stimulated only after Ang II is converted to Ang III. [11] Thus, sodium transport may be increased or decreased depending on the concentration of Ang II and dopamine and the state of sodium balance. [1,10,12]

There is little information on the autologous and heterologous regulation of angiotensin and dopamine receptors, particularly in humans. It is known that AT_1Rs, which are highly expressed in the plasma membrane (PM), respond to Ang II stimulation by a relatively rapid ($T_{1/2}$ = 20 seconds) downregulation. [13,14] In contrast, D_1Rs, which are in relatively low abundance in the PM, respond to dopamine stimulation by a relatively rapid ($T_{1/2}$ = 60 seconds) upregulation or recruitment to the PM from the cytoplasm. [15–17] D_5R and AT_1R can decrease each other's expression in rodents[18,19] and humans [9,20] while the D_1R, D_3R or D_5R can all physically interact with AT_1R to inhibit its function. [20–22]

AT_2Rs are in low abundance in the PM compared to AT_1Rs, and are not downregulated in response to Ang II stimulation. [13,23] Little is known about Ang III-stimulated AT_2Rs and their transregulation by dopamine receptors in humans. In rodents, D_1-like receptor stimulation causes AT_2Rs to translocate to the brush border of the rat renal proximal tubule and the subsequent natriuretic response is blocked by the AT_2R antagonist PD-123319 (PD). [17] This suggests that AT_2Rs are necessary for D_1-like receptor-mediated natriuretic effects. AT_2Rs are upregulated following AT_1R blockade and thus may provide significant counterbalancing effects for AT_1Rs. [24]

The current study tests the hypothesis that stimulation of the D_1R upregulates AT_2Rs via translocation to the PM, physical heterodimerization with D_1Rs, and cooperative functionality. Since inhibitors of post-transcriptional protein expression (siRNA and antisense oligonucleotides) are not 100% effective, we utilized human renal proximal tubule cells (RPTCs) that express a coupling defect between the D_1R and the $G\alpha_S$ proteins that stimulate adenylyl cyclase. [25] These uncoupled cells (uRPTCs) allow us to study the effect of the D_5R in the absence of stimulatory effects from the D_1R. We investigated whether this uncoupling defect has an effect on D_1R/AT_2R transregulation, in comparison to RPTCs that are normally coupled to $G\alpha_S$ and adenylyl cyclase, labelled nRPTCs.

MATERIALS AND METHODS

Cell lines

Human kidneys from fresh surgical specimens came from patients who had unilateral nephrectomy due to renal carcinoma or trauma. A university institutional review board (IRB)-approved protocol was used according to the Declaration of Helsinki, using the recent

version of Title 45, Part 46, U.S. Code of Federal Regulations. This study used the same two cell lines characterized extensively in a previous study [7](nRPTC line i22 and uRPTC line i19). Details about these cells and positive control HEK-293 cells (stably transfected with human D_1R and AT_2R cDNA) are listed in the Online Data Supplement (please see http://hyper.ahajournals.org).

PM protein expression

Detergent-free cell surface membrane sheets (plasma membrane) were isolated as previously reported [9] and outlined in the Online Data Supplement. Briefly, RPTCs were biotinylated with the amine-reactive cleavable biotin labeling reagent (sulfo-NHS-S-S-biotin) and lysed. Biotinylated membranes were isolated with streptavidin sepharose beads. The effects of FEN or DMSO vehicle control (VEH) on D_1R, D_5R and AT_2R protein levels were evaluated using a multi-blot multi-transfer method.

D_1R and AT_2R co-immunoprecipitation

nRPTC and HEK-293 lysates were immunoprecipitated with a rabbit D_1R antibody (Santa Cruz H109) and detected using D_1R rat monoclonal (Sigma D2944) and AT_2R goat polyclonal (Santa Cruz N19) antibodies as described in the Online Data Supplement. Endogenous D_1R and AT_2R protein-protein interaction was confirmed by immunoprecipitating AT_2R with 4 μg rabbit AT_2R antibody (Santa Cruz H143), with subsequent detection using the same two antibodies used for D_1R immunoprecipitation.

Sensitized emission FRET microscopy

Microscopy was performed on fixed non-permeabilized nRPTCs using extracellular epitope-specific and directly, fluorescently-labeled antibodies to D_1R and AT_2R (Alexa 488 and 555, respectively). Details are in the Online Data Supplement.

Ang II peptide binding

RPTCs were stimulated (30-min) with VEH, FEN, or FEN plus LE300, as described in the Online Data Supplement. Briefly, cells were cooled, incubated (1-hr) with fluorescently-labeled Ang II in the presence of losartan, PD123319 or a combination of LOS+PD.. Ang II +PD represent total AT_1R binding and Ang II+LOS represent total AT_2R receptor binding.

ICUE3 and cGMP FRET biosensors

RPTCs were transfected with 4 μg/mL of each biosensor using a Gene Pulser Mx Cell 96-well electroporation system (Bio-Rad) as reported previously [7] and in the Online Data Supplement. CFP/YFP ratiometric imaging on a 96-well Olympus IX81 automated confocal microscope was performed over 20-min as previously described.[7,8]

PP2A assay

RPTCs at 80% confluence were serum-starved and exposed to agonists (30-min). Cells were lysed according to kit instructions (Millipore, Cat# 17-313), with details in the Online Data Supplement.

phospho-ERK measurement

nRPTCs and uRPTCs were cultured, serum-starved and exposed to VEH or FEN (30-min) prior to addition of Ang II or antagonists. Cell lysates were loaded onto nitrocellulose in a 96-well dot-blot apparatus, as described in the Online Data Supplement.

TIRF (total internal reflectance) microscopy

Monensin was added (30-min) to serum-starved nRPTCs followed by FEN or FEN+Ang III (additional 30-min). Cells were fixed, blocked and stained with NaKATPase-α subunit antibody using an Alexa-488-labeled donkey anti-mouse secondary antibody for detection. Imaging details are in the Online Data Supplement.

siRNA to GRK4, D_1R, and D_5R

50 nmol/L GRK4 siRNA or scrambled control was transfected in uRPTCs (4-hr). 48 hr later, agonists were added. siRNA to D_1R and to D_5R were used in separate experiments with nRPTCs to identify which receptor was involved in the FEN-mediated AT_2R recruitment. Details are in the Online Data Supplement.

NaKATPase-mediated sodium efflux

NaKATPase activity was measured as ouabain-sensitive intracellular sodium efflux, using an intracellular sodium sensitive dye (SBFI) as described previously[7] and in the Online Data Supplement. Briefly, RPTCs were labeled with SBFI and incubated in potassium-free media followed by incubations with FEN, Ang III, or both. To initiate sodium efflux, EIPA [(5-(N-ethyl-N-isopropyl) amiloride] and potassium were added to simultaneously block sodium influx and allow NaKATPase to begin active sodium transport. Time-lapse ratio imaging was conducted using an automated confocal microscope.

Statistics

The data are expressed as mean ± SE. Comparisons withinand among 3 or more groups were made by repeated measures or factorial ANOVA, respectively, followed by Student-Newman Keuls or Duncan's test. T-test was used for two-group comparisons. A value of $P<0.05$ was considered significant.

RESULTS

Simultaneous western "multi-blot" analysis of RPTC protein expression in compared the relative amounts of PM receptor expression, minimizing inter-experiment variability (Figure 1). In nRPTCs, FEN (1 μmol/L, 30 min) increased PM expression of D_1R (as published [7]) and AT_2R (499.3±35.2% of control), while D_5R expression was unaffected (1 A, B, C). FEN decreased AT_1R expression in nRPTCs and uRPTCs. Basal AT_1R expression was higher in uRPTCs than nRPTCs (1 D). In uRPTCs, FEN (1 μmol/L, 30 min) had no effect on the PM expression of AT_2R and D_1R; this is likely due to a desensitization of the D_1R by GKR4. [25,26]

We established time-course and concentration-response curves for the D_1R- mediated recruitment of AT_2R (Figure 2). FEN stimulated a 3.96±0.17-fold increase in AT_2R PM recruitment (10-min, 2 A) but only in nRPTCs. A maximal response continued for 30-min and declined at 60-min. The D_1-like receptor antagonist SCH 23390 blocked the 30-min AT_2R recruitment induced by FEN.

Concentration-dependent FEN-stimulated responses were measured at 30-min in RPTCs (2 B). FEN significantly stimulated AT_2R PM recruitment at 100nmol/L (EC_{50} of 29.2±3.2 nM, N=4), but only in nRPTCs. Both SCH (5 μmol/L) and okadaic acid (OA, PP2A inhibitor, 10 nmol/L) blocked the FEN effect (neither SCH nor OA alone had an effect). Forskolin (FSK, adenylyl cyclase agonist, 10 μmol/L) increased AT_2R 4-fold in nRPTCs and uRPTCs (N=4, #$P<0.05$ vs. VEH).

The next 2 panels show siRNA studies: 2 C shows FEN stimulation of uRPTCs, which have impaired membrane recruitment of the D_1R due to increased GRK4 activity. [7, 8] Compared to the scrambled oligonucleotide control (SCR CON), SCR FEN did not affect AT_2R PM expression in uRPTCs, indicating that functional D_1Rs are required for FEN recruitment of AT_2Rs. However, GRK4 siRNA rescued the FEN (1 μmol/L, 30-min)-mediated increase in AT_2R PM expression by 92.9±14.0%. This is similar to our previous reports that GRK4 siRNA rescued the FEN-dependent increase in cAMP[8] and also the PM recruitment of D_1R. [7] Here, GRK4 siRNA alone had no constitutive effect on AT_2R expression and its effect is via D_1R, which is not constitutively active in this setting. The efficacy of this GRK4 siRNA has been published (having produced a 70.6±5.6% decrease in GRK4 expression).[7]

Figure 2D shows siRNA in nRPTCs to separate out the roles of the two D_1-like receptors. AT_2R membrane recruitment following FEN stimulation (5.0±0.3-fold vs. control) was decreased by D_1R siRNA by 84.3±5.9%, but not by D_5R siRNA. Silencing the expression of both D_1R and D_5R did not alter the magnitude of the D_1R siRNA response, indicating that the D_1R works without the D_5R to recruit PM AT_2Rs. Neither D_1R nor D_5R scrambled (SCR) oligonucleotide controls had any effect on AT_2R PM expression (data not shown). We reproduced these findings using antisense (same D_1R and D_5R antisense and scrambled oligonucleotide controls as previously published, data not shown).[9]

We performed co-immunoprecipitation to investigate a physical association between D_1Rs and AT_2Rs (Figure S1). In S1A, nRPTC lysates were immunoprecipitated with rabbit anti-AT_2R antibody or non-specific IgG and analyzed by western blot. Both D_1R and AT_2R were detected in the AT_2R immunoprecipitate but not in the non-specific IgG immunoprecipitate. In S1B, a rabbit anti-D_1R antibody was substituted for the AT_2R immunoprecipitating antibody used in S1A, producing similar results. The same procedures as used in S1B were repeated on lysates from D_1R- and AT_2R stably-transfected HEK-293 cells and a similar result was found (S1C). This suggests that the D_1R/AT_2R association is not cell type-specific as long as both proteins are found within the same cell.

We examined D_1R/AT_2R co-localization in the PM using fluorescence microscopy of labeled extracellular epitope-specific antibodies (Figure S1D). Cell surface D_1R and AT_2R were found co-localized in live nRPTCs using fluorescently-labeled extracellular epitope-specific antibodies. An Alexa-555-labeled D_1R polyclonal antibody and an Alexa-488-labeled AT_2R monoclonal antibody were used. Corrected FRET (cFRET) calculations showed a 1.96±0.25-fold increase vs. control in D_1R/AT_2R association following FEN stimulation.

We measured cAMP accumulation (30-min) using an intracellular FRET-based biosensor that is responsive to cytoplasmic cAMP (Figure 3A) as previously described. [7] In nRPTCs, Ang III alone had no effect, but FEN stimulation of cAMP was enhanced by Ang III, and this was blocked by the AT_2R antagonist PD123319 (PD). This suggests interaction between D_1R and AT_2R in the production of cAMP. PD did not completely block the stimulatory effect of FEN on cAMP but addition of the D_1R antagonist SCH23390 did (data not shown), indicating that Ang III stimulation of AT_2R positively influences the D_1R but D_1R is upstream of AT_2R.

We also examined the previously established link between AT_2Rs and guanylyl cyclase activity[27] (Figure 3B). Neither FEN (1 μmol/L, 20-min) nor Ang III (10 nmol/L, 20-min) increased cGMP accumulation in nRPTCs or uRPTCs. However, the combination of FEN +Ang III markedly increased intracellular cGMP as measured by FRET, but only in nRPTCs. There was no effect in uRPTCs.

We measured protein phosphatase 2A(PP2A) activity as a possible mechanism for the FEN-induced AT_2R recruitment (Figure 4). Ang II decreased PP2A activity while Ang III had no effect. FEN caused an increase in PP2A activity, but only in nRPTCs. In the presence of FEN, both Ang II and Ang III stimulated PP2A activity above FEN alone, but only in nRPTCs. Forskolin (FSK) served as a positive control for the stimulation of cAMP production. The FSK-stimulated increase in PP2A activity agrees with previous reports that the uncoupling of the D_1R is proximal to its coupling to adenylyl cyclase. [26,28]

PM AT_2Rs are in low abundance in basal conditions. Thus, we examined the relative abundance of AT_2R and AT_1R before and after FEN stimulation (Figure S2). We used selective antagonists to measure Ang II-649 binding to AT_1Rs (Ang II+PD) or AT_2Rs (Ang II+LOS). Non-specific binding was determined by measuring Ang II-binding in the presence of PD+LOS and it approximated autofluorescent levels. With FEN stimulation, AT_1R levels decreased in both nRPTCs and uRPTCs. This effect was blocked by LE300 (D_1R/D_5R antagonist), which agrees with previous reports that AT_1R is mediated by a D_5R-selective pathway and is not different between nRPTCs and uRPTCs, [9] and GRK4 does not desensitize the D_5R. [1,18] The AT_2R peptide binding was initially 4-fold lower than AT_1R density, but when stimulated with FEN, the AT_2R peptide binding became almost equal to basal AT_1R expression in nRPTCs. Again, LE300 blocked this effect. In uRPTCs, there was no equalization of the two receptor types.

AT_1R stimulates phosphorylation of p44 and p42 MAP kinase (pERK1/2) and AT_2R inhibits pERK1/2 and AT_1R. [29,30] Since peptides in short-term stimulation are unable to penetrate the outer membrane of cells, we used pERK1/2 as a relative functional readout of cell surface AT_1R and AT_2R stimulation (Figure S3). In the basal state, addition of Ang II to nRPTCs or uRPTCs caused a 4-fold increase in pERK1/2 that was completely inhibited by the AT_1R inhibitor LOS but not the AT_2R inhibitor PD. Addition of FEN decreased pERK1/2 in nRPTCs. FEN addition before Ang II stimulation dramatically changed the nRPTC response to Ang II in a manner consistent with the changes in cell surface expression seen in S2. Ang II stimulation of pERK1/2 was dramatically reduced after the nRPTCs were stimulated with FEN, but less so in uRPTCs. Co-administration of LOS further reduced pERK1/2, an indication of residual AT_1R on the cell surface. In nRPTCs, PD's reversal of the inhibition shows that newly recruited AT_2Rs were partially responsible for the change in responsiveness to Ang II, but in uRPTCs no reversal was seen.

The sodium pump NaKATPase is one of the principal regulators of sodium transport. We therefore examined the effect of Ang III and/or FEN on NaKATPase expression in the PM (Figure 5) via TIRF microscopy (5A) and also measured ouabain-sensitive sodium transport (5B). In 5A, the NaKATPase basolateral membrane expression was measured in nRPTCs using mouse anti-NaKATPase-α and imaged at 70nm above the coverslip surface. NaKATPase internalization was seen as a loss of TIRF fluorescence intensity. In 5B, neither Ang III nor FEN (FEN at suboptimal concentration of 100 nmol/L) decreased sodium efflux. However, the combination of FEN+Ang III significantly reduced sodium efflux. This effect was blocked by the AT_2R antagonist PD, signifying a synergistic inhibition of NaKATPase by D_1R and AT_2R. Figure S4 shows a model depicting the known intracellular pathways that are involved with dopaminergic reduction of sodium transport in a renal proximal tubule cell. Figure S5 shows a model of AT_1R signaling to pERK1/2, and D_1R/D5R and AT_2R effects on this signaling in nRPTCs and uRPTCs.

DISCUSSION

Trafficking and downregulation are two key regulatory mechanisms in the regulation of cell surface expression of GPCRs. [31,32] In cell models like HEK-293 with overexpressed

receptors, AT_1Rs rapidly desensitize and internalize following Ang II stimulation, while AT_2Rs do not. [13] Endogenous AT_2Rs in rodent[33] and in human RPTCs (current data) appear to behave differently. Our studies demonstrate that D_1R/D_5R stimulation caused a recruitment of AT_2Rs to the PM of human RPTCs, similar to the effect of D_1R/D_5R stimulation on D_1R recruitment. Using selective siRNA, we demonstrated that the receptor responsible was the D_1R and not the D_5R. D_1R stimulation recruited PM AT_2Rs from very low basal levels to levels comparable to AT_1Rs. This AT_2R recruitment was not seen in uRPTCs, which have a D_1R that is uncoupled from its effector proteins.

The D_1R-mediated recruitment of AT_2R in nRPTCs was over 4-fold with a $T_{1/2}$ of 5 min, which is consistent with the relatively rapid trafficking of GPCRs[34] following agonist stimulation. [11] The EC_{50} for the D_1R-mediated recruitment of AT_2R was in the high nanomolar range, which is in agreement with the affinity of D_1-like receptors in the renal proximal tubule. [1,16,35] Maximal recruitment of AT_2R to the PM occurred at 10 min and declined at 60 min, implying D_1R desensitization between 30 and 60 min. These data demonstrate that AT_2R cell surface translocation is necessary for its various functions, one of which is to inhibit AT_1R signaling to pERK 1/2 (a surrogate marker for AT_1R function). By inhibiting the cell surface-translocated AT_2R with the PD compound, the inhibitory influence of AT_2R is reversed and AT_1R can then signal more strongly to pERK1/2.

uRPTCs offer a unique model of D_1R dysfunction because the D_1R is uncoupled from effector proteins and adenylyl cyclase. [1,26,28,35,36] In previous studies, we demonstrated that inhibition of GRK4 by 3 methods (intracellular introduction of heparin, which inhibits all GRK isoforms; selective inhibition of GRK4 by GRK4 antisense oligonucleotides; and GRK4-specific siRNA) all blocked the constitutively-increased GRK4 activity in uRPTCs and thus restored D_1R coupling to adenylyl cyclase and D_1R PM recruitment in these cells. [7,8,28] The current report shows similar findings for AT_2Rs, suggesting a key role for GRK4 in modulating their recruitment to the PM as well. We hypothesize this role for GRK4 since it has been shown to directly regulate D_1R and D_3R function. [1,12,26,28,30,37,38]

The current studies extend our previous observations that D_5R stimulation reduces AT_1R function and expression in rodents[18] and humans, [9] and D_1R stimulation increases PM D_1R and AT_2R expression while reducing AT_1R. [21] In the current studies, D_1R/D_5R stimulation reduced Ang II-stimulated phospho-ERK expression by over 80%. Nearly 50% of this signal was reversed by AT_2R blockade, suggesting that the other 50% of the inhibition was mediated by D_5R inhibition of AT_1R.

We have determined that D_1R stimulation of AT_2R recruitment is cAMP and PP2A (specific inhibitor, okadaic acid at 10 nmol/L)-dependent. D_1-like receptor stimulation increases PP2A activity in rodents, but only in WKY and not SHR cells. [37] Here we extend these studies to demonstrate that in humans, Ang II inhibits PP2A activity while Ang III does not. This can in part be explained by Ang III having a 5-fold increase in affinity for the AT_2R over the AT_1R. [39] FEN pre-stimulation shifts the cells' sensitivity to an Ang III-mediated increase in PP2A activity. These results, along with FSK recruitment of AT_2Rs and activation of PP2A even in uRPTCs, suggest a novel pathway commencing with D_1R stimulation of cAMP production, followed by an activation of PP2A and subsequent membrane recruitment of AT_2R.

Renal interstitial production of cGMP is necessary for pressure-natriuresis and NaKATPase internalization in rodents. [40] Renal interstitial infusion of Ang III induces natriuresis in normotensive WKY (but not SHR) rats, an effect that is blocked by an AT_2R inhibitor. [41] Our current report is the first in human RPTCs to show there is a robust increase in cGMP and maximal inhibition of NaKATPase when the D_1R and the AT_2R are stimulated

simultaneously. It is yet to be determined if the local conversion of Ang II to Ang III is also regulated by local dopamine production under conditions of increased sodium load.

PERSPECTIVES

In summary, dopaminergic stimulation may shift the tubule from its normally antinatriuretic state under conditions of a low or normal sodium load to a natriuretic state under a moderate sodium load. The relatively high concentrations of Ang II in low or normal sodium load conditions would stimulate the relatively high levels of AT_1R, favoring sodium reabsorption. Upon dopaminergic stimulation with moderate sodium load, the AT_1R would be downregulated/deactivated and the natriuretic AT_2R, D_1R, D_3R, and D_5R would be upregulated/activated, leading to a net increase in sodium excretion. Our data suggest that use of an AT_2R agonist and a method to restore D_1R coupling by inhibiting GRK4 expression or providing an increase in proximal tubule cAMP may provide selective anti-hypertensive therapy. Similarly, the combination of a D_1R agonist and an AT_1R antagonist may provide an alternative therapeutic approach.

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Novelty and Significance

This paper is the first to show how the dopaminergic system shifts the renal proximal tubule from a sodium-retaining state to a sodium- eliminating state. This shift is mediated by the simultaneous stimulation of the AT_2R (via Ang III) and dopamine receptors, thus unifying the activity of these pathways that were previously thought to be independent. We feel that this paper demonstrates how defects in either of these two pathways are relevant to the etiology of hypertension.

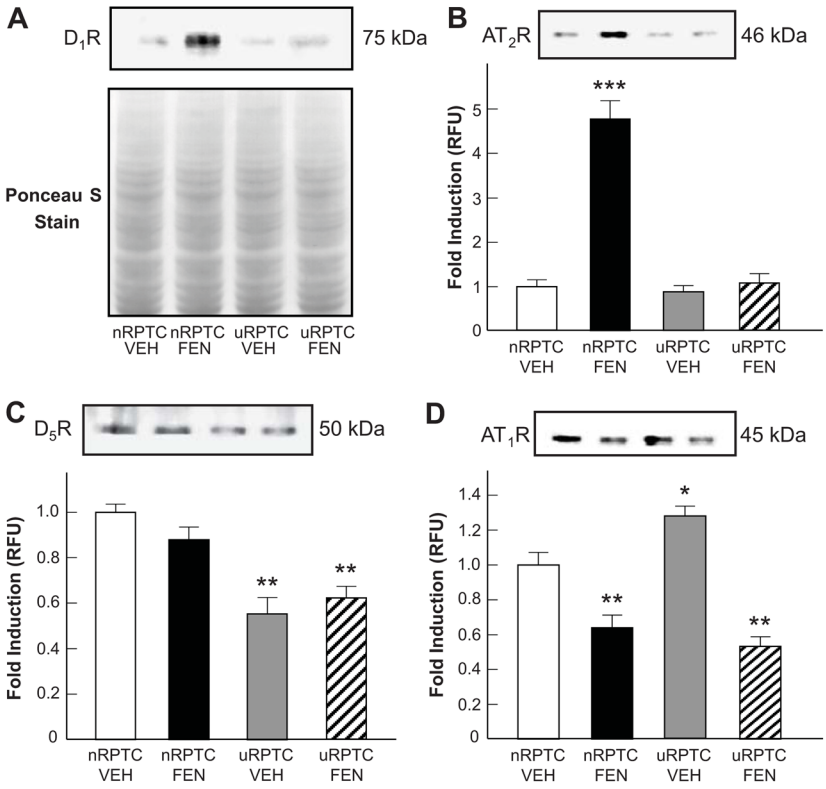

Figure 1. Western multiblot of D_1R, AT_2R, D_5R and AT_1R

DMSO vehicle or FEN (1 μmol/L,30-min.) was added to nRPTCa and uRPTCs and PM proteins were isolated. Representative blots are shown above bar graphs for Panels B-D. **A,** a D_1R blot (previously published) above a Ponceau S stained blot to show even loading (β-tubulin blot analysis also previously published). **B**, a 4.77±0.41 fold increase in FEN-induced recruitment of AT_2R (***P<0.001 vs. VEH, N=4). **C**, no effect of FEN on D_5R recruitment, but the basal expression of D_5R is lower in uRPTCs than nRPTCs (44.7±0.07% of control, **P<0.01, N=4). **D**, FEN-induced AT_1R expression is decreased by 36±0.07% and 46.7±0.05% (**P<0.01, N=4) in nRPTCs and uRPTCs, respectively. Basal AT_1R expression is higher in uRPTCs (28.0±0.06% over nRPTC, *P<0.05, N=4).

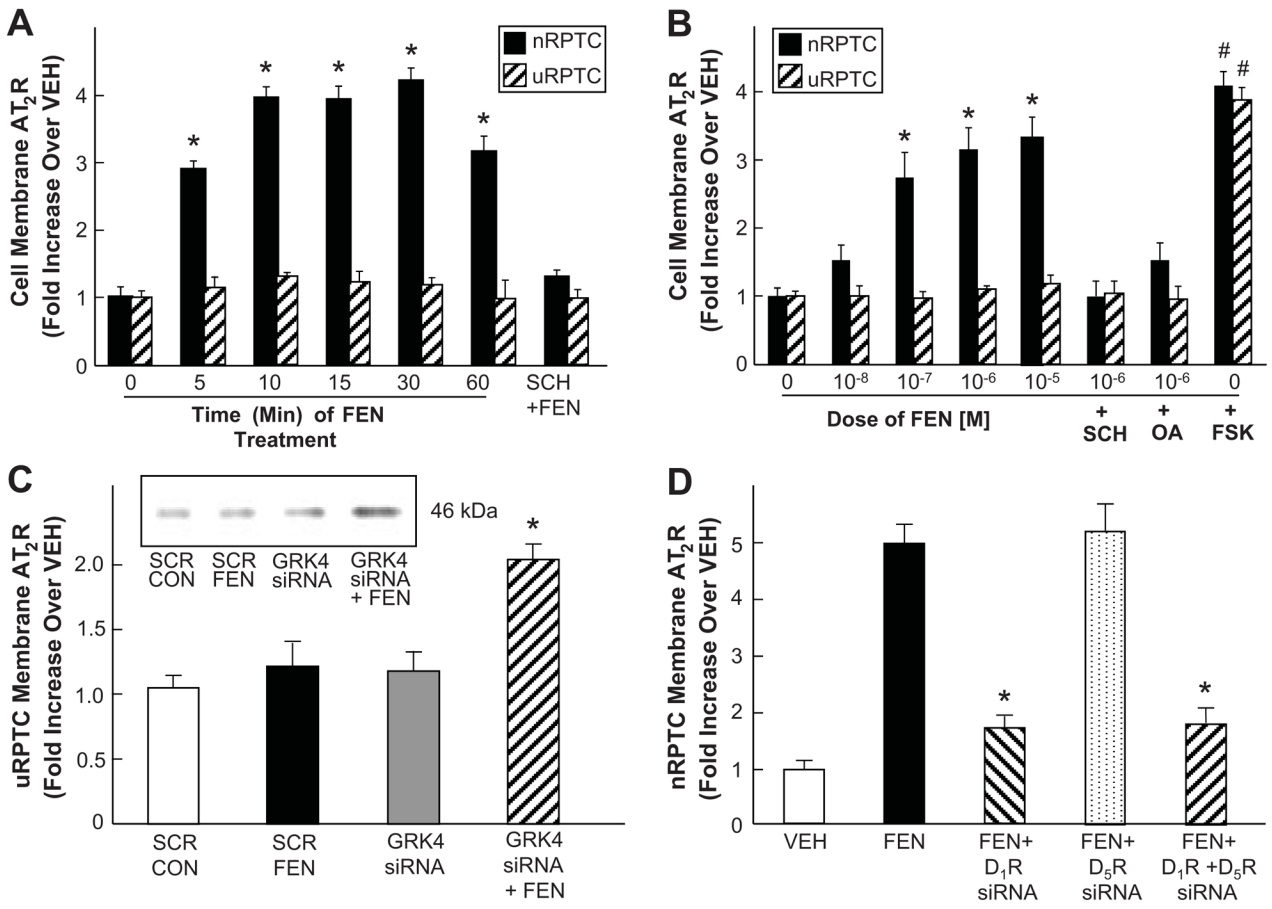

Figure 2. Plasma Membrane AT_2R

A, FEN (1 μmol/L) stimulated a time-dependent recruitment of AT_2R to the plasma membrane (PM) but only in nRPTCs (N =4, *****P<0.05 vs. 0-time). SCH 23390 (5 μmol/L, 30-min) blocked the nRPTC response to FEN. **B**, FEN (30-min) concentration-dependent recruitment of AT_2R to the PM is seen only in nRPTCs (N=4, *P<0.05 vs. VEH). **C,** D_1R is recoupled following silencing of GRK4, allowing FEN-induced AT_2R recruitment in uRPTCs (N=3, *P<0.05 vs. others). One representative immunoblot is displayed. **D,** FEN increased AT_2R PM expression in nRPTCs, which is reversed with siRNA silencing of the D_1R (3 rd bar) gene (not the D_5R, 4 th bar). Silencing the expression of both D_1R and D_5R (5th bar) was the same as D_1R siRNA , indicating that D_1R works without D_5R to recruit PM AT_2R (N=3, *P<0.05 vs. FEN).

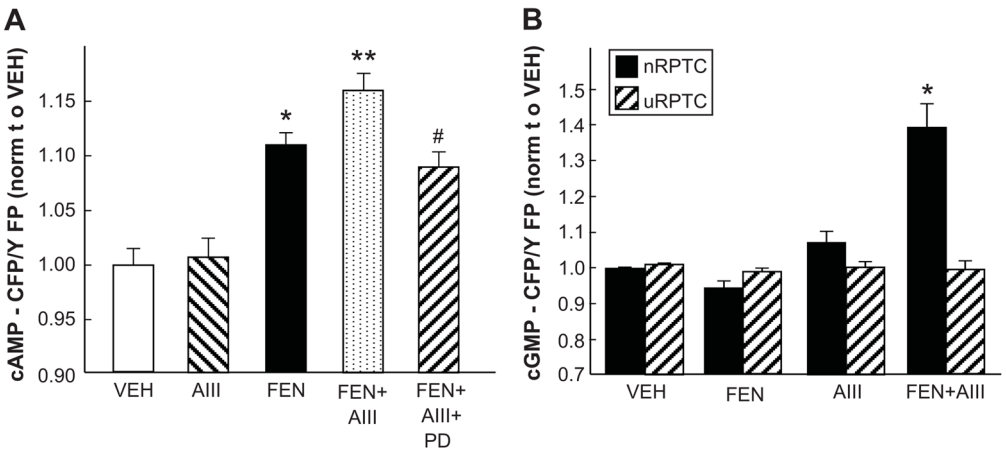

Figure 3. Intracellular cAMP and cGMP accumulation

A, a cAMP FRET-based biosensor was used in nRPTCs (30-min). Angiotensin III (AIII, 10 nmol/L) did not stimulate cAMP accumulation, but FEN (1μmol/L) stimulation of cAMP was enhanced by AIII. This was blocked by PD123319 (PD, AT_2R antagonist, 1 μmol/L, N=6/group, *P<0.05 vs. VEH, **P<0.05 vs. FEN alone, #P<0.05 vs. FEN+AIII). **B,** intracellular cGMP accumulation was measured (20-min) using a cGMP FRET based biosensor. Data is expressed as the ratio of CFP fluorescence intensity divided by the YFP FRET intensity, normalized to T=0 before addition of agonists. Neither FEN nor AIII alone had an effect, but FEN+AIII increased cGMP in nRPTCs only (N=11, *P<0.001 vs. VEH or FEN+AIII in uRPTC).

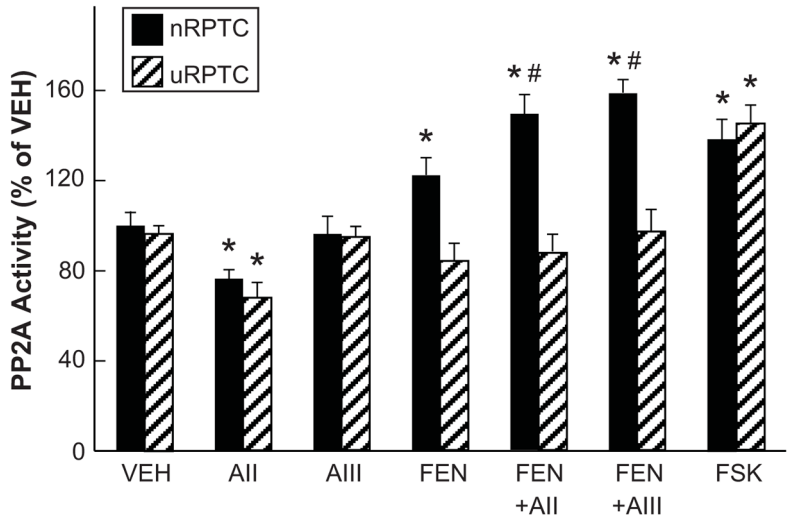

Figure 4. PP2A activity

AII (10 nmol/L) but not AIII (10 nmol/L) decreased PP2A activity in both nRPTCs and uRPTCs. FEN (100 nmol/L) increased PP2A activity only in nRPTCs. Both AII and AIII enhanced FEN stimulation beyond FEN alone. Forskolin (FSK, adenylyl cyclase agonist, 10 μmol/L) served as a positive control for cAMP stimulation (N=4, *P<0.05 vs. VEH, *#P<0.05 vs. FEN nRPTC).

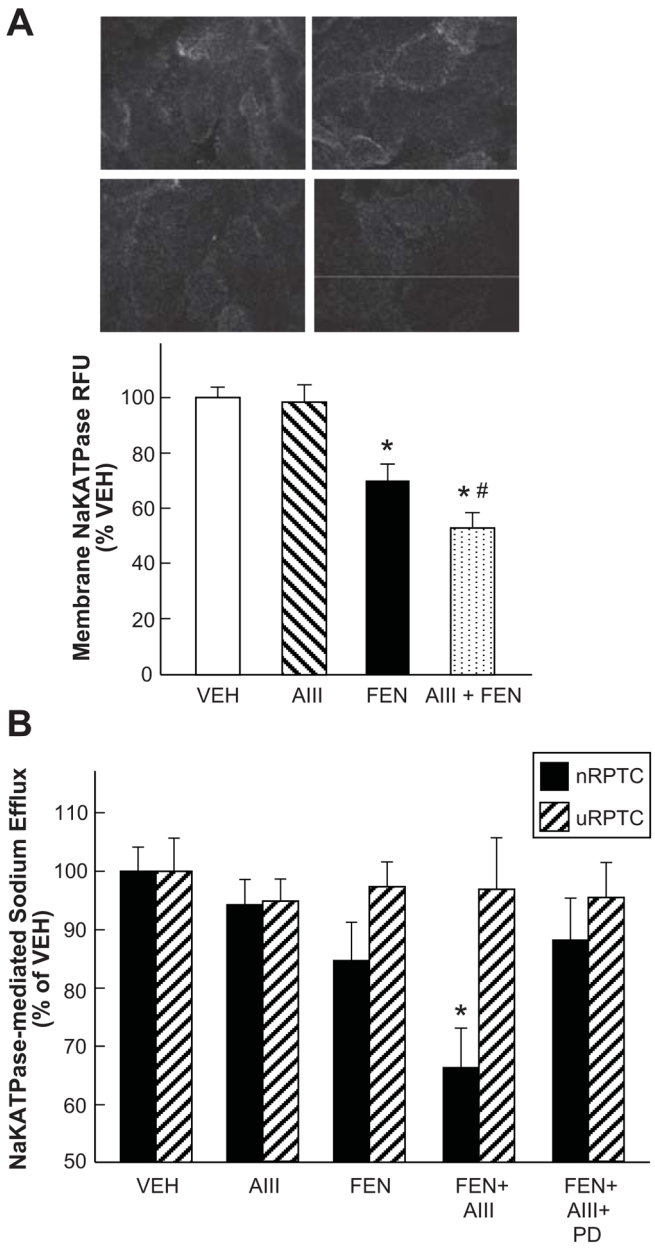

Figure 5. D_1R / AT_2R coupling to NaKATPase

A, The effect of FEN (100 nmol/L, 30-min), angiotensin III (AIII, 10 nmol/L, 30-min) or FEN+AIII on NaKATPase-α expression was measured in nRPTCs using TIRF microscopy. Representative images (taken at the basolateral membrane, 70 nanometers above the coverslip surface) are shown. NaKATPase internalization is seen as a loss of TIRF fluorescence intensity (N=4, *P<0.05 vs. VEH, *#P<0.05 vs. FEN alone). **B**, ouabain-sensitive sodium export was not reduced by FEN (100 nmol/L) or AIII (10 nmol/L), but was inhibited by FEN+AIII in nRPTCs (but not uRPTCs). This was blocked by PD123319 (PD, AT_2R antagonist, 1 μmol/L, N=6 per group, *P<0.01 vs. VEH, *P<0.05 vs. all others).