AT₁ receptor-mediated uptake of angiotensin II and NHE-3 expression in proximal tubule cells through a microtubule-dependent endocytic pathway

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IT IS WELL RECOGNIZED THAT G protein-coupled ANG II type 1 (AT₁) receptor (GPCR)-mediated endocytosis, or internalization, of extracellular ANG II plays a major role in the desensitization and subsequent desensitization of acute responses to ANG II in various target cells (2, 14, 19, 41). Circulating or extracellular ANG II is thought to be primarily internalized

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and phospholipase C activation. These studies suggest that AT₁ receptor-mediated endocytosis or uptake of extracellular ANG II by PT cells may be mediated by the cytoskeleton microtubule-dependent mechanisms.

In addition to the endocytic pathways that may mediate the uptake of extracellular ANG II by PT cells, whether AT₁ (AT₁a) receptor-mediated uptake of extracellular ANG II may induce intracellular signaling in PT cells of the kidney is still not fully understood. The sodium and hydrogen exchanger-3 (NHE-3) is the major Na⁺/H⁺ antipporter that is mainly expressed in polarized PT cells and mediates the bulk of sodium and bicarbonate transport in PTs of the kidney (3, 7, 15). The changes in NHE-3 expression or activity have been widely used as an index of cellular responses to ANG II by PT cells (3, 7, 15, 28). The present study tested the hypotheses that the microtubule-associated endocytic pathway, rather than the clathrin-dependent endocytic pathway, regulates AT₁ receptor-mediated uptake of extracellular ANG II and ANG II-induced NHE-3 expression in cultured rabbit PT cells.

METHODS

Cell culture. Immortalized rabbit PT cells were purchased from American Type Culture Collection (v-EPT, ATCC), whereas AT₁a receptor-deficient mouse PT cells (mPT) were provided by Dr. Ulrich Hopfer of Case Western Reserve University School of Medicine. The morphological, biochemical, and transport characteristics of these cells have been described (25, 28, 47). PT cells of passages 8–12 were subcultured to 90% confluence in six-well plates or glass coverslips in complete DMEM/F-12 growth medium at 37°C and 95% O₂-5% CO₂, which was supplemented with 50 μM hydrocortisone, 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (25, 28). AT₁a-deficient mPT cells were maintained as described by Woost et al. (47) and used as an AT₁ receptor knockout control, or transfected with a mouse AT₁a receptor full-length cDNA (4 μg/well, Origene) as a knockin control to determine the specific role of AT₁ (AT₁a) receptors. PT cells were first transfected with or without specific small interfering RNAs (siRNAs) or their respective control (or scrambled) siRNAs for 24 or 48 h before they were used for live cell fluorescent imaging of FITC-ANG II and/or Alexa Fluor 594-transferin (Molecular Probes) or Western blot analysis of NHE-3 protein expression, as described (25, 28, 29, 50). Lipofectamine 2000 was used in all transfection experiments.

siRNA knockdown of AT₁ receptor expression. To inhibit AT₁ receptor-mediated uptake of FITC-ANG II in PT cells grown on glass coverslips or ANG II-induced NHE-3 expression in PT cells grown in six-well plates, 20–25 nucleotide human AT₁ receptor siRNAs (AT₁R siRNAs) were used to knock down AT₁ receptor expression, as described (25, 28, 44, 50). Humans and rabbits share a highly conserved homology of AT₁ receptor expression (28), PT cells were transfected with or without non-AT₁ receptor-targeting, control siRNAs as described previously (25, 28, 44, 50). Untransfected and AT₁R siRNA- or control siRNA-transfected PT cells were used for live cell fluorescent imaging or Western blot studies.

siRNA knockdown of clathrin LC and HC subunit expression. The expression of LC or HC proteins in PT cells was knocked down by transfection of PT cells with specific 20–25 nucleotide siRNAs targeting either clathrin LC or clathrin HC subunits (Santa Cruz Biotechnology), as described above for AT₁R siRNAs. To determine the peak inhibitory response, the time course of clathrin LC protein knockdown by its siRNAs was first evaluated at 24, 48, and 72 h, respectively. Additional groups of PT cells were transfected with control siRNAs (a scrambled sequence of clathrin LC or HC siRNAs) for comparisons before live cell fluorescent imaging of FITC-ANG II and/or Alexa Fluor 594-transferin, or Western blot studies of NHE-3 or total or phosphorylated MAP kinases ERK1/2 were performed, as described (25, 28).

siRNA knockdown of cytoskeleton microtubule-associated proteins. The expression of cytoskeleton microtubule-associated proteins, MAP-1A or MAP-1B, in PT cells was knocked down by transfection of PT cells with specific 20–25 nucleotide siRNAs targeting either MAP-1A or MAP-1B, as described above for AT₁R siRNAs. To determine the peak inhibitory response, the time course of MAP-1A protein knockdown by its siRNAs was first evaluated at 24, 48, and 72 h, respectively. Again, additional groups of PT cells were transfected with control siRNAs (a scrambled sequence of MAP-1A or MAP-1B siRNAs) for control before live cell fluorescent imaging of FITC-ANG II and/or Alexa Fluor 594-transferin, or Western blot studies were performed, as described (23, 28, 50).

Live cell fluorescent imaging of AT₁ receptor-mediated uptake of FITC-ANG II in PT cells. To determine whether knockdown of AT₁ receptor, clathrin LC and HC, and MAP-1A or MAP-1B protein expression inhibits AT₁ receptor-mediated uptake of FITC-ANG II, all control untransfected and specific siRNA- or control siRNA-transfected PT cells grown on glass coverslips were first incubated with FITC-ANG II (1 nM) at 4°C for 3 h without exposure to light. Under this condition, all FITC-ANG II binds to cell surface AT₁ receptors, but AT₁ receptor-mediated uptake of FITC-ANG II is inactive (25, 28). Some of these PT cells were then transferred to a cell culture incubator at 37°C for 1 h, which initiates AT₁ receptor-mediated uptake of FITC-ANG II (25, 28). At the end of incubation, the medium was removed, PT cells were washed twice with warm PBS and counterstained with the nuclear acid marker 4,6-diamidino-2-phenylindole (DAPI; 300 nM) for 5 min (25, 28). Glass coverslips were mounted on a perfusion chamber maintained at 37°C, which in turn was mounted on a Nikon-Eclipse TE2000-U inverted fluorescence microscope. Green (FITC-ANG II) and blue (DAPI) fluorescence images were captured sequentially, and the relative fluorescence levels of internalized or intracellular FITC-ANG II (cell surface AT₁ receptor-bound FITC-ANG II excluded) in PT cells were analyzed using MetaMorph Imaging analysis (Molecular Devices), as described (25, 28, 29). For better visualization of colocalization of green and blue fluorescence, DAPI-stained nuclei were converted into red to ensure that the colocalization could be viewed as yellow or orange in the merged fluorescent images.

Fig. 1. Effects of specific AT₁ receptor small interfering (si) RNAs or control siRNAs on AT₁ receptor expression in rabbit proximal tubule (PT) cells. The peak inhibition of AT₁ receptor expression by AT₁ receptor siRNAs occurred at 48 h after transfection. **P < 0.01 vs. control PT cells.
Live cell fluorescent imaging of the uptake of Alexa Fluor 594-transferrin in PT cells. To determine whether knockdown of clathrin LC or MAP-1A proteins inhibits the clathrin-dependent uptake of transferrin by PT cells, untransfected, clathrin LC siRNA-, or MAP-1A siRNA-transfected PT cells were subcultured on glass coverslips as described above. PT cells were first incubated with FITC-ANG II (1 nM) and Alexa Fluor 594-transferrin (25 μg/ml) at 4°C for 3 h in the dark to stop receptor-mediated endocytosis (20), which was followed by incubation of the cells at 37°C for 1 h to initiate the uptake of FITC-ANG II or Alexa Fluor 594-transferrin. At the end of incubation, PT cells were counterstained with DAPI (blue, 300 nM) for 5 min before live cell fluorescent imaging of FITC-ANG II (green) and/or Alexa Fluor 594-transferrin (red) was performed, as described above (25, 28, 29).

Western blot analysis of AT1 receptor, clathrin LC or HC, MAP-1A, and NHE-3 protein expression and MAP kinases ERK1/2. For Western blot analysis of AT1 receptor, clathrin LC or HC, MAP-1A or MAP-1B, and phosphorylated (p-ERK1/2) and total ERK1/2 (t-ERK1/2) proteins, untransfected and specific siRNA- or control siRNA-transfected PT cells were incubated with ANG II (1 nM) in six-well plates at 37°C for 1 h. To determine AT1 (AT1a) receptor-specific mechanisms of ANG II-induced ERK1/2 activation, the AT1 receptor antagonist losartan (10 μM), AT1a receptor-deficient mPTs (knockout) and AT1a-deficient mPTs retransfected with a mouse full-length AT1a receptor (Knockin) were used. At the end of treatment, the medium was removed and PT cells were washed twice with ice-cold PBS and lysed with a modified RIPA buffer as described previously (25, 28). Proteins were extracted and

![Fig. 2. Effects of AT1 siRNAs on AT1-mediated uptake of FITC-ANG II in rabbit PT cells.](image-url)
determined using a BCA protein assay kit (Pierce), and Prism 4.0. PT protein samples (10 μg each) were electrophoretically separated on 8–16% Tris-glycine gels at 120 V for 1.5–2 h. After SDS separation, proteins were transferred to Millipore Immobilon-P membranes using a Bio-Rad Trans-Blot Semi-Dry system powered by a Bio-Rad PowerPac HC (25 V, 0.12 A, 1.5 h). The membranes were blotted overnight at 4°C with 5% nonfat dry milk and incubated for 3 h at room temperature with primary antibodies against the human AT₁ receptor (1:200), clathrin LC or HC (1:200), MAP-1A or MAP-1B (1:200, Santa Cruz Biotechnology), NHE-3 proteins (1:200, Chemicon), and t-ERK1/2 and p-ERK1/2 (1:200, Cell Signaling) (7, 25, 28). To ensure equal protein loading, the same membranes were treated with stripping buffer (Pierce) for 20 min, blotted with 5% nonfat dry milk, and reprobed with a mouse anti-β-actin monoclonal antibody at 1:2,000 (Sigma-Aldrich). Western blot signals were detected using enhanced chemiluminescence (Amersham) and analyzed using a microcomputer imaging device with a digital camera (MCID, Imaging Research) (25, 28, 29). The change in each protein expression was calculated as the ratio of each respective protein to actin or as the ratio of p-ERK1/2 to t-ERK1/2 for MAP kinases ERK1/2, respectively.

Results

Effects of AT₁R siRNAs on AT₁ receptor expression, FITC-ANG II uptake, and ANG II-stimulated NHE-3 protein expression in PT cells. Western blot analysis showed that transfection of PT cells with specific AT₁R siRNAs resulted in a marked inhibition of AT₁ receptor expression by >80% 48 h after transfection (P < 0.01) (Fig. 1). The specificity of AT₁R siRNAs for AT₁ receptor knockdown was confirmed in PT cells transfected with control siRNAs, which did not significantly inhibit AT₁ receptor expression [not significant (NS) vs. control]. In untransfected PT cells incubated with FITC-ANG II for 3 h at 4°C, FITC-labeled ANG II (green) was mainly restricted to the cell surface bound to AT₁ receptors with little FITC-ANG II seen inside the cells (Fig. 2, A and C). In PT cells transfected with control siRNAs and incubated with FITC-ANG II at 37°C for 1 h, FITC-ANG II was internalized and clearly seen in the cytoplasm, perinuclear locations, and over the nuclei (Fig. 2, D and F). Colocalization of FITC-ANG II (Fig. 2D) and the DAPI-stained nuclei (Fig. 2E) is shown as yellow and orange fluorescence in the nuclei (Fig. 2F). This indicates that control siRNAs had no effect on AT₁ receptor-mediated uptake of FITC-ANG II in PT cells. By contrast, the AT₁ receptor-mediated uptake of FITC-ANG II was completely inhibited in PT cells pretransfected with AT₁R siRNAs, so that FITC-ANG II was not seen in the cytoplasm and the nuclei (Fig. 2, G and I). Cell surface-bound FITC-ANG II was also markedly reduced in AT₁R siRNA-transfected cells due to the inhibition of AT₁ expression (Fig. 2G). Figure 2J shows the semiquantitative levels of internalized or intracellular FITC-ANG II levels as analyzed using fluorescence imaging analysis.

The effects of AT₁R siRNAs on ANG II-stimulated NHE-3 expression in PT cells are shown in Fig. 3A. ANG II significantly increased NHE-3 protein expression in PT cells by >60% (Fig. 3A, top, P < 0.01). The ANG II-stimulated NHE-3 expression was blocked in PT cells pretransfected with AT₁R siRNAs, but not in PT cells pretransfected with control siRNAs. In further experiments in which PT cells were treated with AT₁R siRNAs or control siRNAs alone without the addition of ANG II, NHE-3 expression was not changed by control siRNAs, but was decreased by AT₁R siRNAs to well below control levels, likely due to a tonic effect of endogenous ANG II on NHE-3 expression (Fig. 3B).

Effects of clathrin LC or HC siRNAs on clathrin LC or HC protein expression, uptake of Alexa Fluor 594-transferrin or FITC-ANG II, and ANG II-stimulated NHE-3 protein expression. Compared with PT cells transfected with control siRNAs, Western blot analysis shows that transfection of PT cells with specific clathrin LC subunit siRNAs induced time-dependent inhibition of clathrin LC protein expression with a peak response at 24 h after transfection (>90% knockdown, P < 0.01) (Fig. 4). Similar levels of clathrin HC expression knockdown were observed for clathrin HC siRNAs. Thus 24 h after transfection was chosen as the time point for live cell fluorescence imaging and Western blot analysis. The effects of clathrin LC siRNA on intracellular uptake of Alexa Fluor 594-transferrin by PT cells are shown in Fig. 5. Under control condition at 37°C for 1 h, both FITC-ANG II and Alexa Fluor 594-transferrin were internalized, with FITC-ANG II seen in the cell surface, cytoplasm, and also in the nucleus (Fig. 5A), whereas transferrin was observed primarily in the cytoplasm (Fig. 5B). Clathrin LC siRNA had no significant effect on FITC-ANG II uptake (Fig. 5E), but largely blocked the uptake of Alexa Fluor 594-transferrin (Fig. 5F). Thus internalized FITC-ANG II was observed in the cytoplasm and around the
nucleus (green), whereas Alexa Fluor 594-transferrin was confined to the cell surface outlining the cells (Fig. 5H). Figure 5I summarizes the semiquantitated results of internalized Alexa Fluor 594-transferrin in control or clathrin LC siRNA-transfected PT cells, which shows that clathrin LC siRNA blocked ~90% of transferrin uptake.

The effects of clathrin LC or HC siRNAs on FITC-ANG II uptake in PT cells are further shown in Fig. 6. As expected, control siRNAs did not inhibit AT1 receptor-mediated FITC-ANG II uptake in PT cells (Fig. 6, A and C). Clathrin LC siRNAs had no significant effects on AT1 receptor-mediated FITC-ANG II uptake in PT cells (Fig. 6, D and F). Thus internalized FITC-ANG II was observed in the cytoplasm and over the cell nuclei (Fig. 6F). Similarly, clathrin HC siRNAs also had no effects on AT1 receptor-mediated FITC-ANG II uptake in PT cells (Fig. 6, G and I). The semiquantitated effects of clathrin LC and control siRNAs on the uptake of FITC-ANG II are summarized in Fig. 6J. Pretransfection of PT cells with clathrin LC siRNAs, HC siRNAs (not shown), or control siRNAs failed to inhibit ANG II-induced NHE-3 protein expression (Fig. 7A). By contrast, ANG II-stimulated NHE-3 expression was completely blocked by losartan (not shown) and in AT1a-deficient mPT cells with or without clathrin LC knockdown, suggesting an AT1 (AT1a)-mediated effect (Fig. 7B).

**Fig. 5.** Effects of clathrin LC siRNAs on the classic clathrin-dependent uptake of Alexa Fluor 594-transferrin (red) in rabbit PT cells compared with the uptake of FITC-ANG II (green). In control PT cells not transfected with clathrin LC siRNAs (A–D), both FITC-ANG II (A, white arrows) and Alexa Fluor 594-transferrin (E, green arrows) were internalized, so they could be seen in the cytoplasm and perinuclear regions of the cells (D, yellow arrows). In PT cells transfected with clathrin LC siRNAs for 24 h (E–H), internalization of FITC-ANG II persisted (E, white arrows) whereas that of Alexa Fluor 594-transferrin was largely inhibited (F, green arrows). Thus the merged image (H) shows that Alexa Fluor 594-transferrin was seen outlining the cells (yellow arrows) whereas internalized FITC-ANG II was observed in the cytoplasm and perinuclear regions of the cells. I: semiquantitated results of the inhibitory effect of clathrin LC siRNAs on the uptake of Alexa Fluor 594-transferrin. **P < 0.01 vs. control. The nuclei of the cells were stained by the nuclear marker DAPI. Magnification ×40.
Effects of MAP-1A or MAP-1B siRNAs on MAP-1A or MAP-1B protein expression, uptake of FITC-ANG II or Alexa Fluor-transferrin, and ANG II-stimulated NHE-3 protein expression.

MAP-1A (and MAP-1B) siRNAs produced time-dependent inhibitions of MAP-1A (or MAP-1B) protein expression in PT cells, with peak responses observed at 48 h after transfection (Fig. 8A). Figure 8B shows that control siRNAs had no effect on FITC-ANG II uptake. D–F: AT1 receptor-mediated uptake of FITC-ANG II was not blocked in PT cells pretransfected with clathrin LC siRNAs for 24 h. G–I: clathrin HC siRNAs also had no effect. FITC-labeled ANG II was clearly seen in the supranuclear regions as well as over the nuclei (yellow or orange staining over the red nuclei: yellow arrows (F and I)). J: semiquantitated results of clathrin LC siRNAs or control siRNAs on FITC-ANG II uptake. **P < 0.01 vs. control PT cells.

Effects of MAP-1A or MAP-1B siRNAs on MAP-1A or MAP-1B protein expression, uptake of FITC-ANG II or Alexa Fluor-transferrin, and ANG II-stimulated NHE-3 protein expression. MAP-1A (and MAP-1B) siRNAs produced time-dependent inhibitions of MAP-1A (or MAP-1B) protein expression in PT cells, with peak responses observed at 48 h after transfection (Fig. 8A). Figure 8B shows that control siRNAs had no effect on FITC-ANG II expression, whereas MAP-1A siRNAs decreased MAP-1A protein expression in PT cells by 86% 48 h after transfection (P < 0.01). As shown in Fig. 9, control siRNAs had no effect on FITC-ANG II uptake, as expected (Fig 9, A and C), but knockdown of MAP-1A protein by its siRNAs almost completely inhibited AT1 receptor-mediated FITC-ANG II uptake in PT cells (Fig. 9, D and F). Thus FITC-ANG II largely remained at the cell surface bound to AT1 receptors (Fig. 9, D and F). Similar inhibitory effects of MAP-1B siRNAs on FITC-ANG II uptake are shown in Fig. 9, G and I. The semiquantitated levels of internalized FITC-ANG II are summarized in Fig. 9J. In contrast to the effects of clathrin LC or HC siRNAs, MAP-1A siRNAs had significant effects on ANG II-stimulated NHE-3 expression. Figure 10 shows that ANG II significantly increased NHE-3 expression...
in PT cells, which was blocked by MAP-1A siRNAs, but not by control siRNAs.

In contrast to its inhibitory effect on FITC-ANG II uptake, knockdown of MAP-1A protein expression by MAP-1A siRNA had no effect on the uptake of Alexa Fluor 594-transferrin in PT cells (Fig. 11). Thus, in addition to cell surface receptor-bound transferrin, internalized Alexa Fluor 594-transferrin was observed in the cytoplasm (Fig. 11B), whereas FITC-ANG II was still bound to cell surface ANG II receptors (Fig. 11A). Neither control siRNA nor MAP-1A siRNA alone have significant effects on AT1 receptor or NHE-3 protein expression (Fig. 11E).

MAP-1A siRNA exerted its effects on ANG II-induced NHE-3 expression in part through the inhibition of ANG II-evoked MAP kinases ERK1/2 activation (Fig. 12). ANG II increased p-ERK1/2 proteins by more than threefold in PT cells (Fig. 12, band 1; *P < 0.01). The stimulatory effect of ANG II on p-ERK1/2 was largely blocked by pretreatment of the cells with losartan and MAP-1A siRNAs, respectively (*P < 0.01). MAP-1A siRNA alone as a specific control had no effect on p-ERK1/2 activation. Deletion of AT1a receptors completely blocked ANG II-induced p-ERK1/2 activation in AT1a-deficient mPT cells (band 2). Conversely, transfection of AT1a receptor-deficient mPT cells with a mouse full-length AT1a receptor (band 3) completely rescued the response of p-ERK1/2 to ANG II stimulation, suggesting a specific AT1 (AT1a) receptor-mediated effect.

DISCUSSION

The major focus of the present study was to determine whether AT1 (AT1a) receptor-mediated endocytosis and uptake of extracellular ANG II in PT cells are mediated by the canonical clathrin-dependent pathway via clathrin LC and HC subunits or the clathrin-independent pathway via microtubule-associated proteins MAP-1A and MAP-1B. The current results suggest that the canonical clathrin-mediated pathway does not play an important role in AT1 receptor endocytosis and ANG II uptake in PT cells. By contrast, a cytoskeleton microtubule-dependent vesicle-trafficking pathway appears to be the major route for AT1 receptor-mediated ANG II uptake in this particular cell type. This conclusion is based on the findings that AT1 receptor-mediated ANG II uptake in PT cells was insensitive to the knockdown of either the HC or LC subunit of clathrin expression, but was markedly attenuated by the inhibition of MAP-1A or MAP-1B expression. These contrasting responses were unlikely caused by the off-target effects of siRNAs employed, since the specificity of the siRNAs targeting the clathrin- or MAPs was established by inclusion of appropriate positive and negative controls. For example, the knockdown of clathrin LC subunits markedly inhibited transferrin uptake, an effect known to be mediated primarily by clathrin in many cell types, including PT cells (24, 25, 35, 46, 50), while the downregulation of either of the MAPs did not affect transferrin uptake. Our results are consistent with earlier observations that depletion of clathrin-coated pits by hyperosmotic sucrose in rabbit PT cells failed to inhibit AT1-mediated ANG II uptake,
whereas AT1 receptor endocytosis and ANG II-stimulated sodium transport in PT cells were inhibited by colchicine, a microtubule-depolymerizing agent (13, 25, 38).

The prevalent view is that GPCRs including AT1 (AT1a) are primarily endocytosed with the agonist(s) via clathrin-coated vesicles in various cells (4, 14, 19, 41). However, recent reviews of membrane protein and GPCR trafficking in epithelia suggest that the generalizations from one GPCR to others, or from one cell type to other cell types, are probably invalid due to the differences in membrane protein trafficking, lack of differentiated cells as in vivo models for trafficking studies, and the diversity of GPCR trafficking associated with or without specialized adaptor proteins (12, 18). For example, a specific, non-PDZ adaptor protein has been identified in the carboxyl terminal region of AT1a receptor, which may determine specific trafficking and/or recycling patterns of AT1a receptors in HEK-293 cells (17). The current study supports a potential role of microtubules or MAPs in regulating AT1 (AT1a) receptor-mediated ANG II uptake in PT cells. Although it is not well understood how the microtubule-dependent endocytic pathway regulates these processes, two different mechanisms or models may likely be involved. The first mechanism

Fig. 9. Effects of MAP-1A or MAP-1B siRNAs on AT1 receptor-mediated uptake of FITC-labeled ANG II in rabbit PT cells. A–C: AT1 receptor-mediated uptake of FITC-ANG II (white arrows) in PT cells pretransfected with control siRNAs for 48 h and incubated at 37°C for 1 h. FITC-ANG II was seen in the supranuclear regions and nuclei (C, yellow arrows), suggesting that control siRNAs had no effect on FITC-ANG II uptake. D–F: AT1 receptor-mediated uptake of FITC-ANG II was completely blocked in PT cells pretransfected with MAP-1A siRNAs for 48 h. G–I: MAP-1B siRNAs also inhibited FITC-ANG II uptake. FITC-labeled ANG II was absent from the supranuclear regions or in the nuclei (F). J: semiquantitated effects of MAP-1A siRNAs vs. control siRNAs on FITC-ANG II uptake. Magnification ×40. **P < 0.01 vs. control PT cells. + +P < 0.01, comparisons between ANG II-treated PT cells transfected with or without specific siRNAs.
may rely on microtubule-dependent recycling of AT₁ (AT₁a) receptors between apical membranes and recycling endosomes and the trafficking of internalized membrane vesicles, AT₁ receptors, and agonists via microtubules to perinuclear locations. Alternatively, the second mechanism may rely on microtubule-dependent endocytosis of ciliary membrane proteins, which is a characteristic of polarized PT cells in the kidney.

Microtubules play an important role in trafficking membrane vesicles with their associated intrinsic proteins, which includes GPCRs (5, 8, 13, 21, 22, 32). In polarized epithelial cells, many apical membrane GPCRs are moved from apical early endosomes to recycling endosomes along microtubules (5, 31). Furthermore, exocytosis of AT₁ receptors in mouse PT cells has been shown to be microtubule dependent (22). Thus a potential mechanism (model 1) for AT₁-mediated ANG II uptake by PT cells may involve a continuous recycling of AT₁ receptors between the surface membrane and intracellular endosomal compartments, with ANG II binding to the receptor at
the surface and hitching a ride to endosomal compartments where this peptide is dissociated from internalized receptors (2, 4, 14, 19, 41). In this model, agonist-induced AT1 endocytosis may act as a catalyst for ANG II uptake by PT cells, but internalized ANG II may well exceed the number of intracellular AT1 receptors, because ANG II levels are so high in the luminal fluid compartment and the receptors would be expected to recycle back to the surface membrane, leaving internalized ANG II inside the cells (33, 43, 49, 51). It is unclear whether apical recycling endosomes have a perinuclear location in PT cells or whether additional endosomal compartments exist in this cell type. Microtubule dependence of perinuclear repositioning has been shown for other recycling surface membrane proteins in polarized cells, such as aquaporin-2 in principal cells of the collecting ducts (45). However, the mechanisms underlying internalized ANG II dissociation from AT1 receptors within endosomes and its trafficking to perinuclear regions are unknown. In this model, depletion of microtubule-associated proteins by their respective siRNAs could affect the microtubule assembly and the gross cytoskeletal structure, including formation of a cilium (8, 13, 21, 32) and the rates with which motors can travel with their cargo along microtubules (5, 31, 32). Either of these effects could greatly alter the recycling rate of AT1 receptors and hence the uptake rate of ANG II. However, this model may not completely exclude a possible involvement of clathrin-coated pits in the initial step of membrane endocytosis but not in later AT1 receptor trafficking and recycling. A mechanism similar to this model has been proposed for V-ATPase recycling in proximal tubules and renal intercalated cells (9).

An alternative mechanism (model 2) may rely on an initial process targeting AT1 (AT1a) receptors to the ciliary membrane, analogous to the GPCR Smo (Smoothened). Smo interacts with the adaptor protein β-arrestin after ligand activation, similar to AT1 (14, 34, 48), and then moves to the ciliary membrane through interactions with motors travelling on the microtubules of the solitary cilium (23). The cilium represents a site where microtubules come in very close contact with the plasma membrane without any intervening actin filaments. While the endocytosis rate of Smo or AT1 receptors through the ciliary membrane has not been investigated, it could follow the general turnover of this membrane and thus depend on the “intraflagellar transport machinery” that uses microtubular motors and tracks (36). Endocytosis of AT1 (AT1a) receptors and uptake of ANG II via this route are possible in principle but remain to be confirmed in future studies. In this model, AT1 receptors could recycle between the surface membrane and endosomal compartments, and the stoichiometric uptake of ANG II and its trafficking to perinuclear locations may still be dependent on the cytoskeleton microtubule system.

The present study shows that inhibition of AT1 (AT1a) receptor-mediated ANG II uptake by MAP-1A siRNAs blocked ANG II-induced NHE-3 expression in PT cells in part by inhibiting ANG II-induced MAP kinases ERK1/2 activation. Activation of MAP kinases ERK1/2 by ANG II or other hormones plays an important role in NHE-3 or AQP2 protein expression and trafficking to apical membranes of polarized PT or collecting duct cells (6, 15, 28, 42, 30). Whether ANG II-evoked AT1 receptor endocytosis directly induces MAP kinases ERK1/2 signaling and NHE-3 expression in PT cells is not well understood. Inhibition of AT1 receptor endocytosis in VSMCs by concanavalin A blocked ANG II-induced ERK1/2 activation via concanavalin A-mediated proteolysis of the EGF receptor (40). Concanavalin A blocked AT1 receptor endocytosis without interfering with agonist binding to the receptor or inhibiting ANG II-induced classic intracellular signaling. However, ANG II-induced MAP kinases ERK1/2 activation in Clone 9 hepatocytes was independent of AT1 receptor internalization (39). Since the endocytic inhibitors commonly used primarily target the clathrin-dependent pathway, the same mechanisms may not apply to AT1 receptor endocytosis and ANG II uptake in PT cells, which is microtubule dependent. Schelling et al. (38) previously showed that colchicine blocked ANG II-stimulated phospholipase C activation and sodium uptake in rat PT cells. ANG II-induced transactivation of the EGF receptor and ERK1/2 activation appear to be calcium dependent (1, 11, 16). Since not all internalized AT1 (AT1a) receptors are sorted to lysosomes and recycle back to the cell surface, some are likely to be retained within the endosomal compartments and traffic to perinuclear regions with internalized ANG II (10, 14). These receptors may mediate internalized ANG II-induced ERK1/2 activation, analogous to intracellular or nuclear effects of ANG II in PT cells (29, 50).

In summary, the present study demonstrates that the alternative microtubule-associated endocytotic pathway via type 1 MAPs, rather than the canonical clathrin-dependent pathway via clathrin LC or HC subunits, regulates AT1 receptor-mediated uptake of ANG II and ANG II-induced NHE-3 expression in rabbit PT cells. This novel endocytic pathway may play an important physiological role in mediating intraretinal uptake of circulating and intracellular ANG II and ANG II-stimulated NHE-3 expression and sodium transport in proximal tubules of the kidney.

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