Biphasic Regulation of Renal Proximal Bicarbonate Absorption by Luminal AT$_{1A}$ Receptor

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Abstract. Angiotensin II (AngII) regulates renal proximal transport in a biphasic way. It has been recently shown that the basolateral type 1A receptor (AT$_{1A}$) mediates the biphasic regulation of Na$^+$/HCO$_3^-$ cotransporter (NBC) by AngII. However, the receptor subtype(s) responsible for the luminal AngII actions remained to be established. To clarify this issue, the luminal AngII effects in isolated proximal tubules from wild-type (WT) and AT$_{1A}$-deficient mice (AT$_{1A}$ KO) were compared. In WT, the rate of bicarbonate absorption ([JHCO$_3^-$]), analyzed with a stop-flow microspectrofluorometric method, was stimulated by 10$^{-10}$ mol/L luminal AngII but was inhibited by 10$^{-6}$ mol/L luminal AngII. Both stimulatory and inhibitory effects of AngII were completely blocked by valsartan (AT$_1$ antagonist) but unaffected by PD 123,319 (AT$_2$ antagonist). In AT$_{1A}$ KO, in contrast, luminal AngII (10$^{-10}$ – 10$^{-6}$ mol/L) did not change JHCO$_3^-$ in WT, 10$^{-6}$ mol/L luminal AngII increased cell Ca$^{2+}$ concentrations ([Ca$^{2+}$])$_i$, which was again blocked by valsartan but not by PD 123,319. However, luminal AngII did not increase [Ca$^{2+}$]$_i$ in AT$_{1A}$ KO. On the other hand, the addition of arachidonic acid similarly inhibited JHCO$_3^-$ in WT and AT$_{1A}$ KO. Furthermore, the acute activation of protein kinase C by phorbol 12-myristate 13-acetate similarly stimulated JHCO$_3^-$ in WT and AT1A KO, indicating that the inhibitory and stimulatory pathways necessary for the AngII actions were preserved in AT$_{1A}$ KO. These results indicate that the luminal AT$_{1A}$ mediates the biphasic regulation of bicarbonate absorption by luminal AngII, while no evidence was obtained for a role of AT$_2$.
which we have recently developed for the determination of \( \text{J}_2 \) in isolated rabbit proximal tubules (14,15).

Materials and Methods

Animals

Male AT1A KO mice (16) and WT mice (Discovery Research Laboratory, Tanabe Seiyaku), 5- to 8-wk-old, from the same genetic background were used in the present study. They were provided with standard food and water ad libitum. All animal procedures were in accordance with local institutional guidelines.

Microperfusion Technique

Mice were anesthetized with pentobarbital sodium, and the thin sections from the left kidney were obtained and stored in ice-cold, gas-equilibrated (5% CO\(_2\)/95% O\(_2\)) Ringer solution containing: 144 mmol/L Na\(^+\), 5 mmol/L K\(^+\), 1.5 mmol/L Ca\(^{2+}\), 1 mmol/L Mg\(^{2+}\), 125 mmol/L Cl\(^-\), 25 mmol/L HCO\(_3\)\(^-\), 2 mmol/L H\(_2\)PO\(_4\)\(^-\), 1 mmol/L SO\(_4\)\(^2-\), and 5.5 mmol/L D-glucose. Proximal tubules (S2 segment) were microdissected without collagenase treatment and then micropерфused according to the method described by Burg et al. (17) with a modified version of the perfusion and sampling capillary system (14,15). The tubular lumen was perfused with the same gas-equilibrated Ringer solution; however, D-glucose was omitted and 20 mmol/L NaCl was replaced by 40 mmol/L raffinose. We have previously shown that tissue culture media such as Dulbecco modified Eagle medium (DMEM) are essential to maintain a better functional state of isolated proximal tubules (18–20). In pilot experiments, we indeed confirmed that HCO\(_3\)\(^-\) in isolated mouse proximal tubules was kept at high rates for more than 60 min in DMEM solution, but it deteriorated within 60 min in Ringer solution. In the present study, DMEM was therefore used as the bath perfusate. The experimental chamber was continuously perfused at a rate of approximately 10 nl/min with prewarmed (38°C) and gas-equilibrated (5% CO\(_2\)/95% O\(_2\)) DMEM solution for 30 min, and the JHCO\(_3\)\(^-\) measurements were started.

Determination of JHCO\(_3\)\(^-\)

We used the recently described stop-flow microperfusion technique (14,15). In brief, isolated tubules were mounted on the stage of an inverted epifluorescence microscope (IMT-2, Olympus). After background fluorescence was measured, a pH-sensitive fluorescence dye 2',7'-bis(carboxyethyl)-5 (6)-carboxyfluorescein (BCECF) was added to the luminal perfusate. Luminal pH (pH\(_L\)) was monitored by a laser scanning confocal microscope (LSM 510, Zeiss) and an inverted epifluorescence microscopy (IMT-2, Olympus). After back-correction, the luminescent images were analyzed using Confocal Assistant software. Luminal pH was measured every 100 ms during the measurement period.

Measurements of Cell Ca\(^{2+}\) Concentrations ([Ca\(^{2+}\)]\(_i\))

[BECF] was measured as described previously (11). Briefly, after autolysis, the tubules were incubated with 20–40 mmol/L Fura-2/AM for 60 min in DMEM under 5% CO\(_2\)/95% O\(_2\) gas at 37°C. Thereafter, the tubule was transferred into the perfusion chamber, microperfused as described above, and [Ca\(^{2+}\)]\(_i\) was recorded with the OSP-10 system. The calibration curves were obtained at the end of each experiment, and [Ca\(^{2+}\)]\(_i\) was calculated according to the method by Grynkiewicz et al. (21).

Results

The Validity of Stop-Flow Microspectrofluorometric Method

To test whether the stop-flow microspectrofluorometric method, which we have recently developed to measure bicarbonate absorption from rabbit proximal tubules (14,15), can also apply to mouse proximal tubules, we performed a series of experiments on isolated proximal tubules from WT. Bicarbonate absorption from proximal tubules has been shown to largely depend on the activities of carbonic anhydrases as well as on the Na\(^+\) gradient (22). To test for the role of carbonic anhydrases, we examined the effect of acetazolamide. The addition of acetazolamide (1 mmol/L) to both luminal and basolateral sides for 10 min substantially reduced JHCO\(_3\)\(^-\) from 13.0 ± 1.1 to 4.1 ± 0.5 pmol/cm s (68 ± 3% inhibition; n = 6; P < 0.005). To test for Na\(^+\)-dependency, we examined the effect of ouabain. The addition of ouabain (1 mmol/L) to the basolateral side for 10 min markedly reduced JHCO\(_3\)\(^-\) from 12.3 ± 0.8 to 3.0 ± 0.3 pmol/cm s (76 ± 2% inhibition; n = 7; P < 0.005). We also tested for Na\(^+\)-dependency more directly by removing Na\(^+\) from the ambient solutions. Similarly to all the other experiments, tubules were first perfused for 30 min using Ringer solution and DMEM as the luminal and the basolateral perfusate, respectively. After the control JHCO\(_3\)\(^-\) value was obtained, however, both perfusates were switched to Na\(^+\)-free solution, in which the Na\(^+\) in Ringer solution was replaced by N-methyl-D-glucamine. The bilateral Na\(^+\)-removal by this procedure for 10 min also markedly reduced JHCO\(_3\)\(^-\) from 11.7.
In the presence of valsartan, JHCO₃⁻ did not change (n = 5) when Ringer solution, instead of Na⁺-free solution, was used for the bilateral perfusates, indicating that the reduction in JHCO₃⁻ after the Na⁺-removal was not due to the omission of DMEM. These results are consistent with the characteristics of bicarbonate absorption from proximal tubules (22), indicating that the stop-flow microspectrofluorometric method can successfully apply to measure bicarbonate absorption from isolated mouse proximal tubules.

**Effects of Luminal AngII in WT**

To examine the roles of luminal AngII receptors in the regulation of proximal bicarbonate absorption, we first examined the effects of luminal AngII in isolated proximal tubules from WT. As can be seen in Figure 1, 10-min perfusion with the luminal solution containing 10⁻¹⁰ mol/L AngII increased JHCO₃⁻ by 42 ± 7% (n = 8; P < 0.005). On the other hand, 10⁻⁸ mol/L AngII had no effects (n = 8), and 10⁻⁶ mol/L AngII rather decreased JHCO₃⁻ by 34 ± 4% (n = 7; P < 0.05). Time control experiments without AngII confirmed that JHCO₃⁻ did not change during repeated stop-flow (n = 6). These results indicate that luminal AngII has biphasic effects on JHCO₃⁻ in isolated mouse proximal tubules as previously shown in isolated rabbit proximal tubules (12).

To examine the receptor subtype(s) mediating these AngII effects, we tested an AT₁ antagonist, valsartan. The addition of 10⁻⁶ mol/L valsartan into the luminal perfusate did not change JHCO₃⁻ (11.9 ± 1.8 versus 11.6 ± 2.2 pmol/cm s; n = 6; NS) and did not modify the effects of luminal AngII. Thus, in the presence of PD 123,319, 10⁻¹⁰ mol/L AngII increased JHCO₃⁻ by 39 ± 8% (n = 7; P < 0.005), and 10⁻⁶ mol/L AngII decreased JHCO₃⁻ by 32 ± 3% (n = 7; P < 0.005). These results indicate that the luminal AT₁ mediates both stimulation and inhibition of JHCO₃⁻ by AngII.

A previous study on isolated rabbit proximal tubules (12) suggested that the presence of physiologic concentrations of AngII in the basolateral side could counteract the inhibition by high concentrations of luminal AngII. We therefore examined the effect of luminal 10⁻⁶ mol/L AngII in the presence of basolateral 10⁻¹⁰ mol/L AngII. The addition of 10⁻¹⁰ mol/L AngII to the basolateral side alone for 10 min increased JHCO₃⁻ from 10.7 ± 0.7 to 14.1 ± 0.8 pmol/cm s (33 ± 5% stimulation; n = 6; P < 0.01), as expected. On the other hand, the simultaneous addition of basolateral 10⁻¹⁰ mol/L AngII and luminal 10⁻⁶ mol/L AngII for 10 min significantly reduced JHCO₃⁻ from 11.7 ± 0.6 to 7.8 ± 0.5 pmol/cm s (34 ± 2% inhibition; n = 7; P < 0.005). The degree of inhibition by luminal 10⁻⁶ mol/L AngII in the presence of basolateral 10⁻¹⁰ mol/L AngII was not statistically different from that by luminal 10⁻⁶ mol/L AngII alone.

**Effects of AngII in AT₁A KO**

We next examined the effects of luminal AngII in AT₁A KO. The control JHCO₃⁻ in AT₁A KO (11.9 ± 0.8 pmol/cm s; n = 24) was very similar to that in WT (11.7 ± 0.5 pmol/cm s; n = 26). As shown in Figure 2, the addition of 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ mol/L AngII all failed to change JHCO₃⁻ in AT₁A KO. Time control experiments without AngII confirmed that JHCO₃⁻ did not change during repeated stop-flow (n = 5). These results are consistent with a view that the biphasic regulation of JHCO₃⁻ in WT was mediated by the luminal AT₁A.

**Effects of AngII on [Ca²⁺]ᵢ**

The increase in [Ca²⁺]ᵢ could be an important factor in AngII-mediated signaling pathways (8,9,23,24); we therefore

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![Figure 1. Effects of luminal angiotensin II (AngII) on rate of bicarbonate absorption (JHCO₃⁻) in wild-type mice (WT). Open bars indicate control values; closed bars indicate values after AngII addition. n = 8, 8, and 7 for 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ mol/L AngII, respectively. *P < 0.005 versus control responses.](image-url)

![Figure 2. Effects of luminal AngII on JHCO₃⁻ in type 1A receptor (AT₁A)−deficient mice (AT₁A KO). Open bars indicate control values; closed bars indicate values after AngII addition. n = 8 for each concentration of AngII.](image-url)
also examined the \([Ca^{2+}]_i\) responses to luminal AngII. In WT, the addition of \(10^{-10}\) mol/L AngII did not induce a significant increase in \([Ca^{2+}]_i\) \((n = 6)\), and \(10^{-8}\) mol/L induced only a marginal increase in three of seven tubules. However, \(10^{-6}\) mol/L AngII consistently induced a spikelike increase in \([Ca^{2+}]_i\) as shown in Figure 3. To examine the receptor subtype mediating the \([Ca^{2+}]_i\) response to AngII, we tested valsartan and PD 123,319. In three tubules, sequential additions of \(10^{-6}\) mol/L AngII in the same tubule, separated for more than 4 min, induced comparable \([Ca^{2+}]_i\) responses. As also shown in Figure 3, however, the addition of \(2 \times 10^{-7}\) mol/L valsartan into the luminal perfusate almost completely inhibited the \([Ca^{2+}]_i\) increase by \(10^{-6}\) mol/L AngII \((n = 4)\). On the other hand, the addition of \(10^{-5}\) mol/L PD 123,319 into the luminal perfusate did not inhibit the \([Ca^{2+}]_i\) increase by \(10^{-6}\) mol/L AngII \((n = 4)\). To test whether the luminal AngII actions are mediated solely by the apical receptor(s), we examined the effect of basolateral valsartan on the \([Ca^{2+}]_i\) response to luminal AngII. In three tubules, the \([Ca^{2+}]_i\) increase by the luminal addition of \(10^{-6}\) mol/L AngII was not inhibited by valsartan added into the basolateral perfusate. These results indicate that the \([Ca^{2+}]_i\) response to luminal AngII is solely mediated by the luminal AT1.

We also examined the effects of luminal AngII in AT1A KO. However, up to \(10^{-5}\) mol/L AngII did not induce a significant \([Ca^{2+}]_i\) increase in AT1A KO.

**Effects of Arachidonic Acid and PMA on JHCO3−**

Previous studies have shown that the activation of phospholipase A2 (PLA2) and the subsequent release of arachidonic acid could be responsible for the inhibition by high concentrations of AngII \((9,25,26)\). We therefore compared the effects of arachidonic acid on JHCO3− in WT and AT1A KO. As shown in Figure 4, the addition of \(10^{-5}\) mol/L arachidonic acid to the bath perfusate similarly reduced JHCO3− in WT \((−27 ± 2\%; n = 7)\) and AT1A KO \((−32 ± 1\%; n = 7)\).

On the other hand, activation of protein kinase C (PKC) could be involved in the stimulation by low concentrations of AngII \((24,27)\). Therefore we finally compared the effects of acute PKC activation in WT and AT1A KO. As shown in Figure 5, the addition of \(5 \times 10^{-7}\) mol/L PMA similarly increased JHCO3− in WT \((33 ± 4\%; n = 5)\) and AT1A KO \((36 ± 6\%; n = 5)\).

**Discussion**

In 1977, Harris and Young \((4)\) reported that AngII added into the peritubular fluid regulates volume absorption from rat proximal tubules in the biphasic way. This finding has been confirmed in various experimental conditions \((8,9,23,28,29)\), and other investigators have shown that AngII added into the luminal fluid can also exert the similar biphasic effects \((12,13)\). Regarding the signal transduction pathways, the stimulation by AngII is generally thought to be mediated by the activation of PKC and/or the decrease in the level of cAMP in the cell \((7,23,24,27)\), though a recent study suggests that the activation
of tyrosine kinases could be also involved (30). On the other hand, the activation of PLA₂ and the subsequent release of arachidonic acid seem to be involved in the inhibition by AngII. In particular, a P₄₅₀–dependent arachidonic acid metabolite, 5,6-epoxyeicosatrienoic acid, could be the final mediator of the inhibition by AngII (9,25,26). Although the stimulation by AngII is certainly mediated by AT₁, conflicting results have been reported as to the receptor subtype responsible for the inhibition by AngII (8,9,10). By comparing the effects of AngII in WT and AT₁A KO, we have recently shown that the biphasic regulation of NBC by AngII is mediated by AT₁A in the basolateral membranes (11). The study was, however, limited on the basolateral receptors; we could not therefore exclude a possibility that the luminal AT₂ may mediate the inhibition by AngII. To clarify this issue, we compared the effects of luminal AngII in WT and AT₁A KO in the present study. To measure bicarbonate absorption, we used the stop-flow microspectrofluorometric method, which we have recently developed for isolated rabbit proximal tubules (14,15). The validity of this method was confirmed by series of experiments with acetazolamide, ouabain, and Na⁺–free solution on isolated proximal tubules from WT, which reproduced the previously characterized properties of proximal bicarbonate transport (22).

In WT, the biphasic regulation of JHCO₃⁻ by AngII was completely inhibited by the highly selective AT₁ antagonist, valsartan (31). However, the AT₂ antagonist PD 123,319 did not modify the effects of AngII. In AT₁A KO, the effects of luminal AngII on JHCO₃⁻ were completely lost. On the other hand, the stimulation by PMA as well as the inhibition by arachidonic acid was very similar in WT and AT₁A KO, indicating that the stimulatory and inhibitory signaling pathways required for AngII actions are intact in AT₁A KO as we previously reported (11). These results are consistent with a view that the luminal AT₁A mediates the biphasic regulation of bicarbonate absorption from proximal tubules.

Because of remarkably high proximal intratubular concentrations of AngII and the presence of angiotensinogen and its mRNA in proximal tubules, AngII is thought be directly secreted into proximal tubular lumen by the epithelial cells (13). Baum et al. (32) reported that this endogenously secreted AngII was able to modify bicarbonate absorption from isolated proximal tubules. In the present study, however, the addition of sufficient concentrations of valsartan did not change JHCO₃⁻ in WT, which did not support a role of endogenous AngII. One possible explanation for these apparently conflicting results could be that we used the very high luminal perfusion rates (>80 nl/min) to perform stop-flow measurements, whereas Baum et al. (32) used the much lower (approximately 10 nl/min) perfusion rates to measure JHCO₃⁻ by the conventional picapnotherm method. On the other hand, a previous study on isolated rabbit proximal tubules (12) provided data suggesting that the inhibition by high concentration of luminal AngII could be blunted by the presence of physiologic concentrations of basolateral AngII. In contrast, we showed that the inhibition by 10⁻⁶ mol/L luminal AngII in WT was preserved even in the presence of basolateral 10⁻¹⁰ mol/L AngII. The reason for these discrepant results is not apparent, but species difference could be involved.

In the previous study, we observed that 10⁻⁶ mol/L basolateral AngII slightly stimulated the NBC activity in AT₁A KO, which was completely inhibited by valsartan (11). In the present study, however, 10⁻⁶ mol/L luminal AngII had no effects on JHCO₃⁻ in AT₁A KO. The pharmacologic properties of AT₁A and AT₁B are known to be very similar (1,2); therefore, the most likely explanation for these observations would be that AT₁B is expressed at a low level in the basolateral membranes but not in the apical membranes of proximal tubules. A previous study has confirmed the low-level expression of AT₁B in proximal tubules (33), but whether it is expressed in the basolateral or the apical membranes has not been determined. The future studies on AT₁B-deficient mice would help clarify this issue.

When high concentrations of AngII were added into the luminal fluid, some of the peptide could diffuse out of the lumen through the paracellular pathways and might act also on the basolateral receptors. However, the [Ca²⁺], increase by 10⁻⁶ mol/L luminal AngII was completely inhibited by the luminal valsartan but unaffected at all by the basolateral valsartan in WT. In addition, up to 10⁻⁵ mol/L luminal AngII did not increase [Ca²⁺], in AT₁A KO. These observations indicate that the effects of luminal AngII are really mediated by the luminal AT₁A, but not by the basolateral receptor. Regarding the concentration dependency of [Ca²⁺], responses to AngII in WT, 10⁻¹⁰ mol/L luminal AngII did not increase [Ca²⁺], and only 10⁻⁶ mol/L luminal AngII induced a typical spike-like [Ca²⁺] increase. Previously Nagami (34) reported, however, that much lower concentrations of luminal AngII can increase [Ca²⁺] in isolated mouse proximal tubules. At present the reason for these discrepant results is unknown, but the difference in metabolic status of isolated tubules could be responsible.

Taken together with the results of our previous study about the NBC regulation by AngII (11), we can now conclude that AT₁A, whether expressed in the basolateral or the apical membranes, mediates the biphasic regulation of proximal transport. In contrast to our conclusion, Haithcock et al. (10), using cultured proximal tubular cells, presented the evidence that AT₂ mediates the inhibition by high concentrations of AngII. Other studies on cultured proximal tubular cells also suggested a role of AT₂ in the acute inhibitory effect of AngII (35,36). While the reason for the discrepant results is not apparent, it has been shown that the expression of AT₂ in kidney is very high in the developing fetus but declines soon after birth (37,38). It could be possible that cultured proximal tubular cells, depending on the culture conditions, might express a significant amount of AT₂. On the other hand, we cannot exclude a possibility that AT₂ may play some physiologic roles in adult kidney. For example, AngII has been shown to activate NF-κB in proximal tubules through both AT₁ and AT₂ (39), and AT₂ could mediate long-term effects of AngII in vivo through such a mechanism. Nevertheless, the results from our studies on isolated proximal tubules strongly argue against a
significant role of AT₂ in the acute regulation of proximal transport by AngII.

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References


