Original Article

Arachidonic Acid Metabolites Inhibit the Stimulatory Effect of Angiotensin II in Renal Proximal Tubules

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Angiotensin II (Ang II) regulates renal proximal transport in a biphasic way *via* Ang II type 1 receptor (AT₁). Whereas extracellular signal-regulated kinase (ERK) activation mediates the stimulatory effect, cytosolic phospholipase A₂ (cPLA₂) mediates the inhibitory effect independently of ERK. In this study, we tested the hypothesis that the cPLA₂/P450 epoxygenase pathway might work to suppress the Ang II-mediated ERK activation. In the presence of arachidonic acid or 5,6-epoxyeicosatrienoic acid (EET), Ang II failed to stimulate the Na-HCO₃ cotransporter activity in renal proximal tubules isolated from wild-type, AT_{1A}-deficient, and cPLA₂- α -deficient mice. In addition, Ang II failed to induce a significant ERK phosphorylation in the presence of arachidonic acid or 5,6-EET. Arachidonic acid or 5,6-EET also suppressed the stimulatory effect of Ang II on net proximal tubule bicarbonate absorption without changing cell Ca²⁺ concentrations. These results indicate that the cPLA₂- α /P450/EET pathway blocks the stimulatory effect of Ang II by suppressing the ERK activation. Thus, the cPLA₂- α /P450/EET pathway may operate as a unique negative feedback mechanism to attenuate excessive Ang II activity in the renal proximal tubules, where extremely high concentrations of Ang II are found. (*Hypertens Res* 2008; 31: 2155–2164)

Key Words: angiotensin II, renal proximal tubules, Na-HCO₃ cotransporter, extracellular signal-regulated kinase, epoxyeicosatrienoic acid

Introduction

Angiotensin II (Ang II) is a key hormone in the regulation of blood pressure. In addition to its vascular effects, Ang II has direct effects on renal tubular functions. In particular, Ang II acts on sodium and bicarbonate absorption from the proximal tubules, and this process is thought to have a significant impact on body fluid and sodium homeostasis (1–3). There are two distinct features of the effects of Ang II on renal proximal tubules. First, proximal tubular fluid contains high concentrations of Ang II, which cannot be explained by simple

spillover from the systemic circulation (4). In fact, proximal tubular cells have all the components to locally generate Ang II, which, after being secreted into proximal tubular lumen, may exert paracrine/autocrine effects (5, 6). Second, Ang II regulates renal proximal tubule transport in a biphasic manner that features stimulation by low (picomolar to nanomolar) concentrations of Ang II and inhibition by high (nanomolar to micromolar) concentrations of Ang II (1, 2, 7). Although conflicting data have been reported regarding the identity of the receptor subtype(s) that mediates this unique mode of regulation (7–9), our data from Ang II type 1 receptor (AT₁)-deficient mice have clearly shown that AT₁ mediates both

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stimulatory and inhibitory effects of Ang II (10, 11). Our recent study further revealed that the extracellular signal-regulated kinase (ERK) pathway mediates only the stimulatory effect of Ang II, whereas the group IV cytosolic phospholipase A₂ (cPLA₂- α)/P450 pathway mediates the inhibitory effect of Ang II independently of ERK (12). Arachidonic acid, released by cPLA₂- α , is in turn metabolized by cytochrome P450 epoxygenase in renal proximal tubules, and the final metabolite 5,6-epoxyeicosatrienoic acid (EET) seems to be responsible for the inhibitory effect of Ang II (13).

Unexpectedly, our data also suggested that the cPLA₂- α /P450 pathway might work to prevent ERK activation by Ang II in intact proximal tubules (12). Indeed, high concentrations of Ang II failed to activate ERK in wild-type mice. However, when the cPLA₂- α activity was abrogated by pharmacological means or genetic knockout, high concentrations of Ang II activated ERK. This finding is quite surprising, because P450 metabolites are reported to activate ERK in a number of different types of cells such as endothelial cells, arterial smooth muscle cells, glomerular mesangial cells, and renal tubular epithelial cells (14, 15). In vascular smooth muscle cells, for example, hydroxyeicosatetraenoic acid (HETE) is believed to mediate ERK activation by Ang II (16). These seemingly contradictory results suggest the possibility that P450 metabolites may exert a unique inhibitory effect on Ang II-induced ERK activation in intact proximal tubules. In this study, we directly tested this hypothesis by investigating the effects of Ang II in the presence of arachidonic acid or its metabolite, 5,6-EET. In view of the multiplicity of Ang II-mediated signaling cascades, as well as the diversity of P450 metabolite-mediated biological actions, we used isolated proximal tubules from wild-type, AT_{1A}-deficient, and cPLA₂- α -deficient mice.

Methods

Animals

Male AT_{1A}-deficient (17) and cPLA₂- α -deficient mice (18), 5 to 8 weeks old, were used in the present study. These mice had been backcrossed to C57B6 for more than 9 generations. Moreover, we confirmed that their littermates that expressed normal alleles for AT_{1A} and cPLA₂- α exhibited transport capacities of the renal proximal tubules that were similar to those recorded in wild-type C57B6 mice. Therefore, we used normal C57B6 mice for the wild-type controls instead of the littermates. All the mice were provided with standard food and water *ad libitum*. All animal procedures were in accordance with local institutional guidelines.

Measurements of Na-HCO₃ Cotransporter Activity

Na-HCO₃ cotransporter (NBC) activity was determined as previously described (10, 12). Briefly, the proximal tubule (S2 segment) fragment was microdissected manually and

transferred to a perfusion chamber mounted on an inverted microscope, and both ends of the tubule were sucked into two holding pipettes. To avoid the influence of luminal transporters, the lumenally-collapsed tubule was used. The tubule was incubated with 15 μ mol/L of an acetoxymethyl ester form of a pH sensitive fluorescence dye known as 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Dojindo, Kumamoto, Japan) for 5 min at room temperature. Intracellular pH (pH_i) was monitored with a photometry system (OSP-10; Olympus, Tokyo, Japan). To achieve a rapid exchange of experimental solution, the chamber was perfused at a rate of 10 mL/min with prewarmed (38°C) perfusate equilibrated with 5% CO₂/95% O₂ gas. Dulbecco's modified Eagle's tissue culture medium (DMEM) was used for the peritubular perfusate, which was shown to be essential for the long-term functional preservation of isolated proximal tubules (10, 11, 19). The intracellular buffer capacity was determined by monitoring the pH_i changes that accompanied sudden alterations in the bath CO₂ tension. Bath HCO₃⁻ concentrations were repeatedly reduced from 25 to 12.5 mmol/L in the absence and presence of Ang II. The rate of pH_i decrease to bath HCO₃⁻ reduction and the buffer capacity were used to calculate the NBC activity as described (10, 11, 19). Ang II, arachidonic acid, and ketoconazole were obtained from Sigma (St. Louis, USA). 5,6-EET was from Cayman Chemical (Ann Arbor, USA), and recombinant human epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, USA). 5,6-EET and ketoconazole were dissolved in ethanol, and Ang II and arachidonic acid were dissolved in H₂O.

Measurements of the Rate of Bicarbonate Absorption

To measure the rate of bicarbonate absorption ($J_{\text{HCO}_3^-}$), we used the stop-flow microspectrofluorometric method, which has been shown to be appropriate for both rabbit and mouse proximal tubules (11, 12, 20). Proximal tubules were microperfused according to the method previously described (11, 12, 20), and luminal pH (pH_L) was monitored on the OSP-10 system. The tubular lumen was perfused with Ringer's solution, which contained 25 mmol/L HCO₃⁻, 40 mmol/L raffinose and BCECF, whereas DMEM was used for bath perfusate. To determine $J_{\text{HCO}_3^-}$, the rapid (~80 nL/min) luminal perfusion was abruptly stopped by suddenly reducing the perfusion pressure from ~18 to 0 cmH₂O. The decay in luminal HCO₃⁻ concentration was calculated from the changes in pH_L, and $J_{\text{HCO}_3^-}$ was calculated as described (20). When the perfusion pressure is suddenly reduced to zero upon stop-flow, the tubular lumen tends to partially collapse. A correction for this volume loss into the pipettes was performed using the decaying 440 nm (pH-insensitive) fluorescence signals as a marker of the residual luminal volume as described (20).

Measurements of Intracellular Ca^{2+} Concentrations

Intracellular Ca^{2+} concentrations were measured as previously described (10). Briefly, the tubules were incubated with 30 $\mu\text{mol/L}$ Fura-2/AM (Dojindo) for 60 min in DMEM under 5% CO_2 /95% O_2 gas. Thereafter, the tubule was transferred into the perfusion chamber and sucked into two holding pipettes, and intracellular Ca^{2+} signals were measured on the OSP-10 system. The calibration curves were calculated at the end of each experiment, and intracellular Ca^{2+} concentrations were derived according to the method proposed by Grynkiewicz *et al.* (21).

Analysis of ERK Phosphorylation

ERK phosphorylation was analyzed as previously described (12). Briefly, thin slices of kidney cortex were obtained from mice. They were divided into segments of small bundles, consisting mostly of proximal tubules as described (12). These samples were incubated at 37°C for 40 min in DMEM under 5% CO_2 , which contained an angiotensin converting enzyme inhibitor captoril (0.1 $\mu\text{mol/L}$; Sigma) to suppress the endogenous production of Ang II. After Ang II was added for 2 min, the samples were homogenized in an ice-cold buffer (25 mmol/L Tris-HCl, pH 7.4, 10 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L EGTA, and 1 mmol/L phenylmethylsulfonyl fluoride) and centrifuged. Equal amounts (~20 μg) of protein samples were obtained from the supernatants, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated with anti-ERK or anti-phospho-ERK antibodies (Cell Signaling Technology, Beverly, USA) and then with horseradish peroxidase-conjugated anti-rabbit IgG. The signals were detected by an ECL Plus system (Amersham, Aylesbury, UK), and Scion Image (Scion Corporation, Frederick, USA) was used to perform densitometric analysis.

Statistics

The data were represented as mean values $\pm$ SEM. Significant differences were determined by applying the paired or unpaired *t*-test or ANOVA with Bonferroni's adjustment as appropriate.

Results

Effects of Ang II in Wild-Type Mice

To clarify the roles of arachidonic acid metabolites in Ang II signaling in proximal tubules, we focused on the regulation of NBC, which is one of the main target transporters of Ang II (10, 11). In wild-type mice, Ang II regulates NBC activity in a biphasic manner, which is exclusively mediated by $\text{AT}_{1\text{A}}$

Fig. 1. Effects of Ang II on NBC activity in wild-type mice. *A:* Effects of 10^{-6} mol/L Ang II on NBC activity in the absence and presence of arachidonic acid (A.A., 10^{-7} mol/L) or 5,6-EET (EET, 10^{-7} mol/L). Open bars indicate control data, and closed bars indicate data after 5-min incubation with Ang II. Six or seven observations were made for each experiment. * $p < 0.01$ vs. control. *B:* Effects of 10^{-10} mol/L Ang II on NBC activity in the absence and presence of arachidonic acid or 5,6-EET. Six observations were made for each experiment. * $p < 0.01$ vs. control.

(10, 12). Therefore, we first examined the effects of arachidonic acid or 5,6-EET in inhibiting the effects of Ang II. As shown in Fig. 1A, 10^{-6} mol/L Ang II inhibited NBC activity. Incubation with arachidonic acid or with 5,6-EET for more than 20 min significantly reduced the baseline NBC activity ($p < 0.01$ by ANOVA), as reported (12). Moreover, the inhibitory effect of Ang II was lost in tubules preincubated with arachidonic acid or 5,6-EET. In separate experiments, incubation with 10^{-7} mol/L arachidonic acid for 5 min significantly reduced the NBC activity from 87.1 ± 4.2 to 68.8 ± 3.4 mmol/min ($n = 6$, $p < 0.01$). However, when the tubules were preincubated with 1.5×10^{-5} mol/L ketoconazole, a P450 inhibitor (12), arachidonic acid failed to inhibit the NBC activity (86.2 ± 4.6 vs. 87.7 ± 3.7 mmol/min, $n = 6$, not significant). These results are consistent with data that suggest that P450 metabolites of arachidonic acid such as 5,6-EET mediate the

Fig. 2. Effects of arachidonic acid or 5,6-EET on ERK phosphorylation in wild-type mice. *A:* Kidney cortex samples were incubated with or without 10^{-7} mol/L arachidonic acid or 5,6-EET for 40 min, and 10^{-10} mol/L Ang II was added for 2 min. Western blot analysis was performed with anti-phospho-ERK1/2 (P-ERK1/2) and anti-ERK1/2 (ERK1/2) antibodies. A representative blot from 3 independent experiments is shown. *B:* Densitometric analysis of ERK phosphorylation by 10^{-10} mol/L Ang II. The relative density of P-ERK1/2 to ERK1/2 is shown. Open bars indicate control values, and closed bars indicate values after the addition of Ang II. Three observations were made for each experiment. *C:* EGF-induced ERK phosphorylation. Kidney cortex samples were incubated with or without 10^{-7} mol/L arachidonic acid or 5,6-EET for 40 min, and 10 ng/mL EGF was then added for 5 min. Details are as in *A*. *D:* Densitometric analysis of ERK phosphorylation by 10 ng/mL EGF. Open bars indicate control values, and hatched bars indicate values following addition of EGF. Three observations were recorded for each experiment. * $p < 0.05$ vs. control.

inhibitory effect of Ang II (12, 13). As previously reported (10), 10^{-8} mol/L Ang II did not affect the basal NBC activity. We confirmed that 10^{-8} mol/L Ang II also did not affect the NBC activity in the presence of arachidonic acid or 5,6-EET (data not shown).

We subsequently examined the impact of arachidonic acid or 5,6-EET on the stimulatory effects of Ang II. As shown in Fig. 1B, 10^{-10} mol/L Ang II stimulated the NBC activity, but this stimulation was again lost in tubules preincubated with arachidonic acid or 5,6-EET. Because the stimulatory effect of Ang II was dependent on ERK activation (12), we examined the Ang II-induced ERK phosphorylation. Ang II was applied for 2 min, the protocol of which was shown to induce a maximal ERK phosphorylation in tissues of the renal cortex (12). As shown in Fig. 2A and B, 10^{-10} mol/L Ang II stimulated the ERK phosphorylation as reported (12). However, arachidonic acid or 5,6-EET markedly suppressed Ang II-induced ERK phosphorylation without changing the basal

ERK phosphorylation. These results are consistent with the hypothesis that arachidonic acid and its metabolites suppress the AT_{1A} -mediated stimulation of NBC activity by preventing ERK activation. On the other hand, neither arachidonic acid nor 5,6-EET impacted the EGF-induced ERK phosphorylation, as shown in Fig. 2C and D. This result suggests that arachidonic acid metabolites do not exhibit general inhibitory effects on agonist-mediated ERK activation, but they do have a specific inhibitory effect on Ang II-mediated ERK activation.

Effects of Ang II in AT_{1A} -Deficient Mice

In AT_{1A} -deficient mice, low concentrations of Ang II fail to activate the NBC activity because of a very low level of expression of AT_{1B} . Instead, only 10^{-6} mol/L Ang II stimulates the NBC activity through the AT_{1B} /ERK-pathway (10, 12). Therefore, we examined the effects of 10^{-6} mol/L Ang II

Fig. 3. Effects of Ang II in AT_{1A} -deficient mice. **A:** Effects of 10^{-6} mol/L Ang II on NBC activity in the absence and presence of arachidonic acid or 5,6-EET. Open bars indicate control data, and closed bars indicate data after 5-min incubation with Ang II. Six observations were made for each experiment. * $p < 0.01$ vs. control. **B:** Ang II-induced ERK phosphorylation. Details as in Fig. 2A, but 10^{-6} mol/L Ang II was used. * $p < 0.05$ vs. control.

in these mice. As shown in Fig. 3A, both arachidonic acid and 5,6-EET significantly reduced the baseline NBC activity ($p < 0.05$ by ANOVA). In addition, the stimulatory effect of 10^{-6} mol/L Ang II on NBC activity was lost in the presence of arachidonic acid or 5,6-EET. As shown in Fig. 3B, arachidonic acid and 5,6-EET suppressed the Ang II-induced ERK phosphorylation, without changing the basal ERK phosphorylation. Our results indicate that arachidonic acid and its metabolites also suppress the AT_{1B} -mediated stimulation of NBC activity by preventing the ERK activation.

Effects of Ang II in $cPLA_2\alpha$ -Deficient Mice

The aforementioned results indicate that the inhibitory effect of arachidonic acid and its metabolites on Ang II-mediated ERK activation is independent of AT_1 subtypes. On the other hand, several types of signaling cross-talk exist between $cPLA_2$ and ERK (16, 22). To avoid the influence of such interactions, we examined the effects of Ang II in $cPLA_2\alpha$ -deficient mice. In $cPLA_2\alpha$ -deficient mice, more than 10^{-8}

Fig. 4. Effects of Ang II in $cPLA_2\alpha$ -deficient mice. **A:** Effects of 10^{-6} mol/L Ang II on NBC activity in the absence and presence of arachidonic acid or 5,6-EET. Open bars indicate control data, and closed bars indicate results after 5-min incubation with Ang II. Six observations were made for each experiment. * $p < 0.01$ vs. control. **B:** Ang II-induced ERK phosphorylation. Details as in Fig. 2A, but 10^{-6} mol/L Ang II was used. **C:** Densitometric analysis of ERK phosphorylation by 10^{-10} mol/L Ang II. Three observations were made for each experiment. Details are as in Fig. 2B.

mol/L Ang II is required to induce a spike-like cell Ca^{2+} increase as observed in wild-type mice, indicating that the functional expression of AT_1 in these mice is unaltered (12). Unlike in wild-type mice, however, any concentration of Ang II (from 10^{-10} to 10^{-6} mol/L) stimulates the NBC activity

Fig. 5. Effects of Ang II on the rate of bicarbonate absorption ($JHCO_3^-$) in wild-type mice. Ang II was added to bath perfusate in the absence and presence of 10^{-7} mol/L arachidonic acid or 5,6-EET. Open bars indicate control fluxes, and closed bars indicate fluxes following 5-min incubation events with Ang II. **A:** The effect of 10^{-10} mol/L Ang II is shown. Six observations made used for each experiment. * $p < 0.01$ vs. control. **B:** The effects of 10^{-6} mol/L Ang II is shown. Six observations were made for each experiment. * $p < 0.01$ vs. control.

through the ERK pathway (12). Therefore, cPLA₂- α is considered the cPLA₂ isoform that is responsible for the inhibitory effect of Ang II. We examined the effect of 10^{-6} mol/L Ang II in these mice. As shown in Fig. 4A, arachidonic acid or 5,6-EET significantly reduced the baseline NBC activity ($p < 0.01$ by ANOVA) as reported (12). More importantly, the stimulatory effect of 10^{-6} mol/L Ang II on NBC activity was completely lost in the presence of arachidonic acid or 5,6-EET. As shown in Fig. 4B and C, arachidonic acid and 5,6-EET again suppressed the Ang II-induced ERK phosphorylation without changing basal ERK phosphorylation. These results indicate that arachidonic acid and its metabolites can suppress the Ang II-mediated stimulation of NBC activity by preventing the ERK activation, even in the absence of

cPLA₂- α activity.

Effects of Ang II on $JHCO_3^-$

The results obtained to date with respect to the regulation of NBC activity suggest that arachidonic acid and its metabolites can attenuate the stimulatory effects of Ang II on net proximal tubule transport. To test this hypothesis, we measured $JHCO_3^-$ in wild-type mice using the stop-flow microspectrofluorometric method (20). As shown in Fig. 5A, 10^{-7} mol/L arachidonic acid and 5,6-EET both significantly reduced the control flux ($p < 0.01$ by ANOVA). The addition of 10^{-10} mol/L Ang II into the bath perfusate for 5 min significantly stimulated $JHCO_3^-$ (11). In the presence of 10^{-7} mol/L arachidonic acid or 5,6-EET, however, the stimulatory effect of Ang II was completely lost. Our results confirm that arachidonic acid metabolites prevent the stimulatory effect of Ang II on net proximal tubule bicarbonate absorption. As shown in Fig. 5B, we confirmed that the inhibitory effect of 10^{-6} mol/L Ang II was also lost in the presence of arachidonic acid or 5,6-EET.

Roles of Cell Ca^{2+}

Ang II is generally considered to increase cell Ca^{2+} concentrations through the phospholipase C/inositol-tris-phosphate pathway (23). Douglas and colleagues, however, reported different mechanisms for cell Ca^{2+} increase. On the basis of data obtained from cultured rabbit proximal tubular cells, they proposed that Ang II-induced Ca^{2+} increases in proximal tubules arise secondary to the release of arachidonic acid and the production of 5,6-EET (24). To test whether a similar mechanism operates in intact proximal tubules, we measured cell Ca^{2+} concentrations in wild-type mice. As shown in Fig. 6A, the addition of 10^{-7} mol/L 5,6-EET did not increase cell Ca^{2+} , although the addition of 10^{-6} mol/L Ang II induced a typical spike-like cell Ca^{2+} increase. In 7 tubules, we could not detect a significant cellular Ca^{2+} increase following addition of 5,6-EET. Similarly, we also failed to detect a significant cell Ca^{2+} increase in the presence of 10^{-7} mol/L arachidonic acid ($n=8$). On the other hand, Douglas and colleagues also reported that incubating a cell culture with arachidonic acid significantly enhances the Ang II-induced cell Ca^{2+} increase (24). As shown in Fig. 6B, however, incubating with 10^{-7} mol/L arachidonic acid for up to 10 min did not enhance the cell Ca^{2+} response to Ang II ($n=5$). These results do not support the role of arachidonic acid metabolites as the mediator of Ang II-induced cell Ca^{2+} increase in intact proximal tubules.

Discussion

We previously showed that although the ERK pathway mediates only the stimulatory effect of Ang II, the cPLA₂- α /P450 pathway mediates the inhibitory effect of Ang II independently of ERK in renal proximal tubules (12). In the absence of

Fig. 6. Cell Ca^{2+} measurement in wild-type mice. *A:* Effects of 10^{-7} mol/L EET and 10^{-6} mol/L Ang II on cell Ca^{2+} . Note that a spike-like cellular Ca^{2+} increase was observed only in response to Ang II. *B:* Cell Ca^{2+} response to 10^{-6} mol/L Ang II in the absence and presence of 10^{-7} mol/L arachidonic acid. Note that arachidonic acid did not induce a spike-like Ca^{2+} increase.

cPLA $_2$ - α or P450 activities, however, the ERK activation by high concentrations of Ang II was unmasked, suggesting that P450 metabolites might also work to suppress the Ang II-mediated ERK activation (12). In the present study we directly tested this hypothesis by examining the effects of Ang II on NBC activity or net bicarbonate transport in the presence of arachidonic acid or 5,6-EET. Our results in wild-type mice are consistent with a view that arachidonic acid and its metabolites not only mediate the inhibitory effect of Ang II as previously reported (7, 10–13), but also suppress the Ang II-mediated stimulation of NBC activity by preventing the ERK activation. Furthermore, this inhibitory effect on ERK activation was preserved in both AT $_{1A}$ -deficient and cPLA $_2$ - α -deficient mice. From these observations, we conclude that the inhibitory effect of arachidonic acid and its metabolites on Ang II-induced ERK activation is independent of AT $_1$ subtypes or cPLA $_2$ - α activities and should be attributed to the intrinsic properties of intact proximal tubules.

As in various previous studies (10–12), we did not find significant differences in the basal NBC activity in wild-type, AT $_{1A}$ -deficient, or cPLA $_2$ - α -deficient mice. Our results sug-

gest that the basal NBC activity is independent of AT $_{1A}$ or cPLA $_2$ - α , at least under our experimental conditions. However, proximal tubules from these mice showed different responses to high concentrations of Ang II. As previously reported (10), we confirmed that the repeated addition of Ang II, separated by sufficient wash-out periods, induced similar cell Ca^{2+} increases in individual proximal tubules. This observation suggests that short-term incubation with Ang II may not significantly change the membrane expression level of AT $_{1A}$. We also note that incubation with captoril might not completely suppress the endogenous production of Ang II in renal cortex tissues. Theoretically, such residual Ang II production in renal cortex tissues would significantly shift the concentration dependency of Ang II-mediated ERK activation. However, the concentration dependence of Ang II-induced ERK activation in renal cortex tissues was identical to the concentration dependence of Ang II-induced NBC stimulation in isolated proximal tubules (10–12). The endogenous production of Ang II was negligible under our experimental conditions. These observations strongly suggest that the residual endogenous production of Ang II, if non-negligi-

Fig. 7. Working hypothesis regarding the biphasic regulation of proximal tubule transport by AT_1 . The Ang II-mediated stimulation is dependent on the MEK/ERK pathway; however, in contrast, Ang II-mediated inhibition is dependent on the $cPLA_2\text{-}\alpha$ /P450/EET pathway, but not on the MEK/ERK pathway. The $cPLA_2\text{-}\alpha$ /P450/EET pathway also works to suppress the Ang II-mediated ERK activation.

ble, is not sufficient to modify the responsiveness to Ang II in renal cortex tissues. On the other hand, we do not have any information about intracellular concentrations of arachidonic acid or 5,6-EET in intact proximal tubules treated with Ang II. Our preliminary trial failed to produce reproducible values. We speculate that different experimental methods using radioisotope-labeled arachidonic acid on cultured proximal tubules would be required to precisely measure these concentrations.

5,6-EET is reported to increase cell Ca^{2+} concentrations in several tissues including pituitary or parotid cells (25, 26). Douglas and colleagues, on the basis of observations in cultured proximal tubular cells, even proposed that arachidonic acid metabolites might mediate the Ang II-induced cell Ca^{2+} increase (24). However, our study shows that arachidonic acid metabolites are unlikely to mediate the Ang II-induced cell Ca^{2+} increase in intact proximal tubules. Our conclusion is consistent with our previous observation that the Ang II-induced cell Ca^{2+} response is preserved in proximal tubules isolated from $cPLA_2\text{-}\alpha$ -deficient mice (12).

Several models have been proposed that link G-protein-coupled receptors to the ERK cascade (27). They include Ras-dependent ERK activation *via* transactivation of receptor tyrosine kinases or Ras-independent ERK activation *via* protein kinase C. Depending on cell types, P450 metabolites of arachidonic acid may be also involved in ERK activation. Muthalif *et al.*, for example, identified a key role of HETE in

Ang II-mediated ERK activation in vascular smooth muscle cells (16). They showed that the stimulation of $cPLA_2$ by Ang II or by norepinephrine facilitates arachidonic acid release. P450 metabolites of arachidonic acid, such as 12,15-HETE or 20-HETE, activate ERK by the Ras/Raf/mitogen-activated protein kinase (MEK) pathway. Activation of MEK/ERK in turn amplifies $cPLA_2$ activity and further releases arachidonic acid concentrations.

In sharp contrast to such a positive feedback loop between the $cPLA_2$ /P450/HETE pathway and the ERK cascade in vascular smooth muscle cells, our study indicates that the $cPLA_2\text{-}\alpha$ /P450/EET pathway operates as a negative feedback loop with respect to the ERK cascade in renal proximal tubules. Moreover, the $cPLA_2\text{-}\alpha$ /P450/EET pathway may not exhibit a general inhibitory effect on agonist-mediated ERK activation, but may instead exert a specific inhibitory effect on Ang II-mediated ERK activation. At present the exact molecular mechanism underlying the contrasting effects of $cPLA_2$ /P450 pathway on the ERK cascade in vascular smooth muscle cells and in renal proximal tubules remains unknown. To date, we have not established whether the Ras/Raf family is indeed involved in the Ang II-mediated ERK activation in intact renal proximal tubules. In all likelihood, different approaches using the dominant negative forms of Ras and/or Raf on cultured proximal tubular cells would be necessary to clarify this issue. On the other hand, the P450-eicosanoids are known to activate a variety of intracellular signaling pathways, some of which may be mediated by a putative cell surface receptor or by direct intracellular interactions (15). From a teleological viewpoint, however, it is tempting to speculate that renal proximal tubules may have to develop the unique mechanism to attenuate the excessive activity of Ang II. Indeed, *in situ* proximal tubular fluid is reported to contain high concentrations of Ang II (4), and uncontrolled Ang II actions may result in problematic events such as cell hypertrophy or tissue damage (28, 29). Activation of ERK by EET has been reported in cultured proximal tubular cells (30). However, we previously failed to detect any significant ERK phosphorylation by 5,6-EET in renal cortex tissues of both wild-type and $cPLA_2\text{-}\alpha$ -deficient mice (12). In addition, the negative effects of EET on ERK activation shown in our study are more consistent with a protective role of EET in Ang II-induced renal injury, as previously reported in rats that overexpress the human renin and angiotensinogen genes (28). Interestingly, several EET members are known to exhibit antihypertensive properties and they are therefore potential therapeutic targets (31). It is possible that the negative effect on the ERK cascade in intact proximal tubules may play a role in the antihypertensive properties of EET, because the ERK activation is coupled to the stimulation of proximal transport (12).

Figure 7 summarizes our working hypothesis regarding the biphasic regulation of proximal tubule transport by Ang II. In our model, the Ang II-mediated stimulation is dependent on the MEK/ERK pathway. In contrast, the Ang II-mediated inhibition is dependent on the $cPLA_2\text{-}\alpha$ /P450/EET pathway,

but not on the MEK/ERK pathway. Indeed, other researchers have shown that the pharmacological inhibition of the MEK/ERK pathway does not impact basal NBC activity (12). However, the cPLA₂- α /P450/EET pathway also works to suppress Ang II-mediated ERK activation. At present, the precise mechanism by which the cPLA₂- α /P450/EET pathway exerts negative effects on the Ang II-mediated ERK activation is unknown. However, one possible mechanism would be the involvement of several phosphatases, which are known to negatively regulate ERK pathway activity (32).

In summary, our results show that the cPLA₂- α /P450/EET pathway has a distinct negative effect on Ang II-induced ERK activation in intact proximal tubules. This effect operates not only to suppress the stimulatory effect of Ang II, but it also to explain the antihypertensive properties of EET.

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