Age-dependent activation of PKC isoforms by angiotensin II in the proximal nephron

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Age-dependent activation of PKC isoforms by angiotensin II in the proximal nephron. Am J Physiol Regulatory Integrative Comp Physiol 281: R861–R867, 2001.—ANG II increases fluid absorption in proximal tubules from young rats more than those from adult rats. ANG II increases fluid absorption in the proximal nephron, in part, via activation of protein kinase C (PKC). However, it is unclear how age-related changes in ANG II-induced stimulation of the PKC cascade differ as animals mature. We hypothesized that the response of the proximal nephron to ANG II decreases as rats mature due to a reduction in the amount and activation of PKC rather than a decrease in the number or affinity of ANG II receptors. Because PKC translocates from the cytosol to the membrane when activated, we first measured PKC activity in the soluble and particulate fractions of proximal tubule homogenates exposed to vehicle or 10⁻¹⁰ M ANG II from young (26 ± 1 days old) and adult rats (54 ± 1 days old). ANG II increased PKC activity to the same extent in homogenates from young rats (from 0.119 ± 0.017 to 0.146 ± 0.015 U/mg protein) (P < 0.01) and adult rats (from 0.123 ± 0.020 to 0.156 ± 0.023 U/mg protein) (P < 0.01). Total PKC activity did not differ between groups (0.166 ± 0.018 vs. 0.181 ± 0.023). We next investigated whether activation of the α-, β-, and γ-PKC isoforms differed by Western blot. In homogenates from young rats, ANG II significantly increased activated PKC-α from 40.2 ± 6.5 to 60.2 ± 9.5 arbitrary units (AU) (P < 0.01) but had no effect in adult rats (46.1 ± 5.1 vs. 48.5 ± 8.2 AU). Similarly, ANG II increased activated PKC-γ in proximal tubules from young rats from 47.9 ± 13.2 to 65.6 ± 16.7 AU (P < 0.01) but caused no change in adult rats. Activated PKC-β, however, increased significantly in homogenates from both age groups. Specifically, activated PKC-β increased from 8.6 ± 1.4 to 12.2 ± 2.1 AU (P < 0.01) in homogenates from nine young rats and from 19.0 ± 5.5 to 25.1 ± 7.1 AU (P < 0.01) in homogenates from 12 adult rats. ANG II did not alter the amount of soluble PKC-α, -β, and -γ significantly. The total amount of PKC-α and -γ did not differ between homogenates from young and adult rats, whereas the total amount of PKC-β was 59.7 ± 10.7 and 144.9 ± 41.8 AU taken from young and adult rats, respectively (P < 0.05). Maximum specific binding and affinity of ANG II receptors were not significantly different between young and adult rats. We concluded that the primary PKC isoform activated by ANG II changes during maturation.

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tigated age-related changes in ANG II-induced stimulation of the PKC cascade, or whether the major PKC isoform activated by ANG II in the proximal nephron changes as rats mature due to a reduction in the amount and activation of PKC rather than a decrease in the number or affinity of ANG II receptors.

**METHODS**

Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were fed a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 4 days before experimental use. Two groups of rats were used in each protocol: young (26 ± 1 days old) and adult (54 ± 1 days old). The Institutional Animal Care and Use Committee approved all procedures, and rats were cared for according to institutional guidelines.

**Isolation of proximal tubule suspensions.** Rats were anesthetized with xylazine (20 mg/kg body wt ip) and ketamine (100 mg/kg body wt ip) and given 0.1 ml heparin (1,000 USP/ml ip). The abdominal cavity was exposed, and the kidney perfused at 37°C with type I collagenase (0.75 mg/ml; Sigma, St. Louis, MO) containing 25 mmol mannitol in perfusion solution as described previously [7]. The perfusion solution contained (in mM): 114 NaCl, 4.0 KCl, 25 NaHCO3, 2.5 NaH2PO4, 1.0 Na lactate, 5.5 glucose, 6.0 alanine, and 1.0 NaH2O as measured by freezing-point depression. Each kidney was perfused with 40 ml of solution for 10 min, and then 150 mM cold NaCl was poured over it. The kidney was removed and placed in cold perfusion solution. The cortex was cleaved, minced, and stirred on ice in perfusion solution for 10 min, and the suspension passed through a nylon mesh (250 μm). The suspension was then stirred for 1 min and allowed to sit for 5 min to allow glomeruli to settle. In preliminary experiments, aliquots taken from the upper portion of the suspension were more than 99% proximal tubules by number.

For determination of ANG II-induced PKC activity, the protocol was modified. After perfusion with collagenase, the kidney was perfused with 20 ml cold perfusion solution containing a protease inhibitor cocktail to inhibit enzymatic digestion of PKC, and protease inhibitors were added to the final suspension. The cocktail included (in μg/ml): 5 antipain, 10 aprotinin, 5 leupeptin, 5 chymostatin, 2.5 pepstatin A, and 25 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Sigma).

For determination of ANG II-stimulated activation of PKC isoforms, the same protocol was followed except that the protease inhibitor cocktail also included 2 mM benzamidine.

**PKC activity assay.** Two 10-ml aliquots were taken from the upper portion of the suspension and warmed to 37°C for 10 min with agitation. The tubules were then exposed to either 10−10 M ANG II (Sigma) or the vehicle (perfusion solution) for 5 min. Previously, 10−10 M ANG II was shown to induce maximum fluid absorption in proximal tubules [8]. After exposure to ANG II, 10 ml cold perfusion solution were added to the samples, and the tubules were centrifuged at 100 g for 4 min at 4°C. The tubules were resuspended in 250 μl homogenization buffer containing the protease inhibitor cocktail. The buffer was composed of 50 mM Tris·HCl and 150 mM NaCl. The tubules were sonicated on ice for 3 min at output 2 of an ultrasonic processor, W-385 sonicator (Ultrasonics, Farmingdale, NY). The sonicate was centrifuged at 1,140 g for 10 min at 4°C to pellet cell debris and nuclei, and the supernatant was subjected to ultracentrifugation at 100,000 g for 60 min at 4°C. The supernatant was considered to be the soluble fraction, and Triton X-100 was added to obtain a final concentration of 0.1%. The pellet was then resuspended in homogenization buffer containing 0.1% Triton X-100 and the protease inhibitor cocktail. The suspension was subjected to ultracentrifugation at 100,000 g for 60 min at 4°C, with the resulting supernatant considered to be the particulate fraction. Protein concentrations were determined and the protein was immediately used to measure PKC activity using a Pierce Colorimetric PKC Assay Kit, Spin-Zyme Format (Pierce, Rockford, IL). Results were expressed in units per milligram protein, where 1 U equals the amount that will transfer 1 nmol of phosphate per minute at 30°C. PKC from rat brain (Calbiochem-Novabiochem, La Jolla, CA) was used as a standard.

**Western blot of PKC-α, -β, and -γ isoforms.** Tubules were treated with either ANG II or vehicle as described above, except that benzamidine was added to all solutions. Soluble and particulate fractions of cell homogenates were separated as described in PKC activity assay, except that benzamidine was again added to all solutions. Protein concentrations were determined, and the protein was stored at −80°C. Samples were then thawed, and 10–50 μg were aliquoted for Western blot. The protein aliquots were diluted with lysis buffer to give a total volume of 40 μL. The lysis buffer contained 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1.0% NP-40, 1.0% SDS, and 2 mM EDTA. Ten microliters of sample buffer were added to each aliquot for a total volume of 50 μL. The sample buffer contained 0.3 M Tris·HCl (pH 6.8), 5 mM EDTA, 10% SDS, 25% β-mercaptoethanol, 50% glycerol, and 0.05% bromophenol blue. PKC from rat brain (Calbiochem-Novabiochem) was used as a standard. All samples were heated in near-boiling water for 5 min and loaded onto a 8.0% polyacrylamide gel. The gel was run at 15 mA for ~2 h. After electrophoresis, the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) overnight at 1 A/h. Nonspecific binding was blocked by incubating the membrane for 1 h in Tris-buffered saline-Tween 20 (TBS-T) with 5% nonfat dry milk. TBS-T contained 50 mM Tris (pH 7.5), 500 mM NaCl, and 0.1% Tween 20. For detection of PKC-α, -β, and -γ, the membrane was then incubated for 1 h in TBS-T with 5% nonfat dry milk and an anti-PKC-α, -β, or -γ monoclonal IgG antibody isolated from mice (Transduction Laboratories, Lexington, KY). The PKC-α, -β, and -γ antibodies were diluted 1:1,000, 1:2,500, and 1:5,000, respectively. The membrane was washed with TBS-T four times for 15 min, then incubated in TBS-T with 5% nonfat dry milk and an anti-mouse IgG-horse-radish peroxidase-linked antibody (Amersham, Arlington Heights, IL), diluted 1:1,000 for 1 h, and finally washed again. Protein was detected using enhanced chemiluminescence Western reagents (Amersham). The films were scanned using a densitometer equipped with the Molecular Analyst Program (Bio-Rad Laboratories, Hercules, CA).

**ANG II receptor binding.** Twelve 0.5-ml aliquots were taken from the upper portion of the suspension and placed in reaction tubes coated with 0.1% BSA to prevent nonspecific binding. Two aliquots were used to conduct a protein assay (Pierce) to normalize data. The aliquots were spun at 4°C for 2 min at ~1,140 g to pellet tubules. The perfusion solution was removed, and tubules were resuspended in 50 μl binding buffer with 0.05−2 nM 35S-ANG II (specific activity, 3,761 cpm/fmol; NEN Life Science Products, Boston, MA). One-half
of the aliquots simultaneously received $10^{-8}$ M unlabeled ANG II to measure nonspecific binding. All dilutions were done in binding buffer containing (in mM): 140 NaCl, 4.0 KCl, 1.2 MgSO$_4$, 2.5 NaH$_2$PO$_4$, 1.0 Ca lactate, 5.5 glucose, 6 alanine, 1.0 Na$_3$ citrate, and 10 HEPES (pH 7.4). The samples were allowed to incubate at room temperature for 30 min. The tubules were then washed four times with 500 ml binding buffer and counted. Specific binding was taken to be the difference between nonspecific and total binding. Specific binding was 70% at 2 nM 125I-ANG II.

Analysis. All values are expressed as means ± SE. Results concerning PKC were analyzed using ANOVA for repeated measures. Paired or unpaired t-tests were used for post hoc testing. Results concerning ANG II binding data were analyzed using paired t-tests.

RESULTS

First, we examined whether ANG II-stimulated PKC activity differed between young and adult rats. Because PKC translocates from the cytosol to the membrane when activated, we measured PKC activity in the soluble and particulate fractions of proximal tubule homogenates exposed to vehicle or $10^{-10}$ M ANG II from young and adult rats. In proximal tubule homogenates from young rats, PKC activity increased from 0.119 ± 0.017 to 0.146 ± 0.015 U/mg protein ($P < 0.01$) in six of seven experiments. In one experiment, the value was extremely high, but it also increased from 0.227 to 0.729 U/mg protein. ANG II had a similar effect on proximal tubule homogenates from seven adult rats (0.123 ± 0.020 to 0.156 ± 0.023 U/mg) ($P < 0.01$) (Fig. 1). Total PKC activity did not differ significantly between young and adult groups (0.166 ± 0.018 vs. 0.181 ± 0.023 U/mg).

Because we did not find a significant difference in ANG II-induced activation of total PKC activity in young vs. adult rats, we next investigated whether activation of the α-, β-, and γ-PKC isoforms differed. Figure 2 shows a representative Western blot of PKC-α from a young rat. ANG II exposure increased activated PKC-α from 36.6 to 77.5 arbitrary units (AU) but had no effect on soluble PKC-α. In homogenates from 10 young rats, ANG II significantly increased activated PKC-α from 40.2 ± 6.5 to 60.2 ± 9.5 AU ($P < 0.01$). In contrast, ANG II had no effect on activated PKC-α in homogenates from nine adult rats (46.1 ± 5.1 vs. 48.5 ± 8.2 AU) (Fig. 3). Figure 4 shows a representative Western blot of PKC-γ from a young rat. ANG II exposure increased activated PKC-γ from 18.7 ± 13.2 to 65.6 ± 16.7 AU ($P < 0.01$) in homogenates from nine young rats but failed to change in homogenates from nine adult rats (57.8 ± 16.3 vs. 60.6 ± 17.1 AU) (Fig. 5). Figure 6 shows a representative Western blot of PKC-β from an adult rat. ANG II exposure increased activated PKC-β from 18.0 to 33.0 AU but had no effect on soluble PKC-β. In contrast to the data for PKC-α and -γ, activated PKC-β increased significantly in ho-

![Fig. 1. Comparison of protein kinase C (PKC) activity in proximal tubule homogenates from young and adult rats before and after addition of $10^{-10}$ M ANG II (hatched bars). *$P < 0.01$ vs. appropriate control value. Open bars, control.](image1)

![Fig. 2. Representative Western blot of PKC-α in proximal tubule homogenates from a young rat before and after addition of $10^{-10}$ M ANG II. The homogenate was separated into soluble (Sol) and particulate (Part) fractions. Right: molecular masses of marker proteins (in kDa).](image2)

![Fig. 3. Comparison of activated PKC-α in proximal tubule homogenates of particulate fractions from young and adult rats before and after addition of $10^{-10}$ M ANG II (hatched bars). *$P < 0.01$ vs. appropriate control value. Open bars, control.](image3)
Specifically, ANG II-stimulated activation of PKC-β increased from 8.6 ± 1.4 to 12.2 ± 2.1 AU (P < 0.01) in homogenates from nine young rats and from 19.0 ± 5.5 to 25.1 ± 7.1 AU (P < 0.01) in homogenates from 12 adult rats (Fig. 7). ANG II did not alter the amount of soluble PKC-α, -β, and -γ significantly. The total amount of PKC-α and -γ did not differ between young and adult rats (209.2 ± 38 vs. 174.3 ± 39.4 AU and 177 ± 50.3 vs. 228.8 ± 57.1 AU, respectively). In contrast, the total amount of PKC-β was 59.7 ± 10.7 AU for young rats and 144.9 ± 41.8 AU for adult rats (P < 0.05).

Finally, we investigated whether the increased response to ANG II in young rats was due to a difference in ANG II receptor number or affinity. Maximum specific binding was 12.7 ± 2.4 fmol/mg protein for the young rats (n = 13), not significantly different from the adult rats (12.5 ± 1.4 fmol/mg protein) (n = 8). Similarly, the ANG II receptor affinity (Kd) for ANG II did not differ between the two age groups (0.80 ± 0.16 vs. 0.70 ± 0.13 nM) (Table 1).

**DISCUSSION**

We found that the ability of ANG II to activate PKC isoforms differed between proximal tubule homogenates from young and adult rats, although it increased total PKC activity to the same extent in both groups. Specifically, ANG II-stimulated activation of PKC-α and -γ was greater in proximal tubule homogenates from young rather than adult rats, whereas with PKC-β activation was significant in both age groups. Although the amounts of PKC-α and -γ did not differ between young and adult animals, PKC-β levels increased with age. Additionally, ANG II receptor number and affinity did not differ significantly in homogenates from young and adult rats.

Although there appears to be a discrepancy between the amount of activated PKC in the particulate fraction from the Western blot data and the total PKC activity, it is important to note that different antibodies were used to recognize each isoform. Therefore, one cannot simply assume that a 10-AU increase in α is equal to a 10-AU increase in β. Consequently, although there appears to be an increase in the total particulate fraction from young proximal tubule homogenates by Western blot, without a mirroring increase in total PKC activity assayed by measuring PO₄⁻, it is important to note that one cannot simply sum the data from Western blots and get an accurate indication of activated PKC due to three individual isoforms.

Previously, we reported that proximal tubules from young rats display a greater response to ANG II than those from adult rats. When proximal tubules were exposed to 10⁻¹⁰ M ANG II, fluid absorption increased 50% more in young rats (8). ANG II works to increase fluid absorption in the proximal nephron via activation of two second messenger cascades; one involves inhibition of adenylate cyclase, which leads to a decrease in cAMP, and the other involves activation of PKC (9, 18, 22, 23). We found that this exaggerated response to ANG II is partly due to changes in cAMP metabolism, as ANG II decreased cAMP by 30% in young rats but had no effect in adult rats (8). Our present data show that this age-dependent response to ANG II is not due to an increase in ANG II’s ability to stimulate total PKC activity in the proximal nephrons of young rats, as no difference was found between young and adult proximal tubules. However, ANG II’s ability to stimulate the activation of different PKC isoforms changed as rats matured. A change in the predominant PKC isoform activated by ANG II in the proximal nephron during maturation suggests a functional difference between individual PKC isoforms. To our knowledge, no one has yet studied the influence of individual PKC isoforms on transport in freshly isolated proximal tubule cells.

The effects of individual PKC isoforms on Na⁺-K⁺-ATPase have been studied in cultured cells. Efendiev
et al. (5) reported that in opossum kidney (OK) cells, phorbol ester-dependent stimulation of Na\(^+\)-K\(^+\)-ATPase is mediated by activation of PKC-\(\beta\), whereas dopamine-dependent inhibition of Na\(^+\)-K\(^+\)-ATPase requires PKC-\(\zeta\). However, these studies were based on the use of selective inhibitors rather than direct demonstration that a single isoform can alter pump activity. Liang and Knox (21) reported that PKC-\(\alpha\) inhibits Na\(^+\)-K\(^+\)-ATPase in OK cells, but these authors did not study other PKC isoforms. Consequently, it is not clear whether their results were due to PKC-\(\alpha\) or another isoform.

Other investigators (2, 24, 36) have also demonstrated age-related alterations in the expression of PKC isoforms in renal and nonrenal tissues. Serlachius et al. (36) reported a decrease in PKC-\(\alpha\) mRNA in the rat renal cortex during development. In addition, Busquets et al. (2) reported an age-dependent increase in the immunoreactive material recognized by an antibody directed against the \(\alpha\)- and \(\beta\)-isoforms in the frontal cortex of the human brain, and an increase in the \(\beta\)-isoform has also been reported in human platelets during aging (24). The differential activation of PKC isoforms during development further supports the idea that each isoform may have distinct cellular functions.

It is interesting to note that unlike our study, other investigators have measured the soluble fraction only to determine PKC activity in the developing kidney and reported an age-related decrease in PKC activity in proximal tubule cells (26). However, these data are not necessarily contradictory, because the previous study compared proximal tubule cells isolated from 10- and 40-day-old rats, whereas in our study we compared 26- and 54-day-old rats. It could be that the decline in PKC activity occurs between 10 and 25 days following parturition and therefore was not seen in our experiments. The different results could also be due to an age-related decrease in renin levels. Furthermore, these investigators also noted a difference in the apparent molecular weight of the immunoreactive material recognized by an antibody directed against a conserved region in the \(\alpha\)-, \(\beta\)-, and \(\gamma\)-isoforms of PKC in immature and mature cells. As the molecular weights of the \(\alpha\)- and \(\beta\)-isoforms differ slightly, this variation is consistent with our findings regarding a change in the predominant PKC isoform active during development.

Karim et al. (20) have examined the effect of pharmacological concentrations of ANG II on PKC isoforms in rat proximal tubules. They found a large increase in PKC-\(\alpha\) (100%) and only a 15% change in PKC-\(\varepsilon\) in proximal tubule suspensions exposed to 10\(^{-7}\) M ANG II. Consequently, we chose to examine only the effect of ANG II on the classical isoforms. Unlike our study, these researchers were unable to detect PKC-\(\beta\) or -\(\gamma\) in their proximal tubule suspensions. One possible explanation for this discrepancy could be the method used to isolate the proximal tubule suspensions. Although we perfused the kidney with protease inhibitors before extraction, and kept the tissue in a solution containing protease inhibitors for the duration of the experiment, the other investigators did not use protease inhibitors until after the suspension was prepared. As we discovered, significant protein degradation occurs if protease inhibitors are not infused into the kidney. Thus it is possible that the amount of PKC-\(\beta\) and -\(\gamma\) in their samples degraded to an undetectable level during preparation.

Table 1. ANG II receptor number and \(K_d\) in proximal tubule homogenates from young and adult rats

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<tr>
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<th>Young rats</th>
<th>Adult rats</th>
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<tr>
<td>Receptor No., fmol/mg</td>
<td>12.7 ± 2.4</td>
<td>12.5 ± 1.4</td>
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<tr>
<td>(K_d), nM</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.2</td>
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Values are means ± SE.
Karim et al. (19) also reported that in luminal membrane vesicles isolated from rat kidney cortical tubule suspensions, only PKC-ζ increased significantly, whereas PKC-α did not change. Given that we used proximal tubule suspensions, it is difficult to compare data from luminal membranes with our studies.

Although the causes for these changes in PKC and cAMP are unclear, stimulation of phospholipase C and adenylate cyclase is mediated through G proteins. Other researchers have reported a decrease in G proteins associated with aging (14, 27, 37). Michel et al. (27) found an age-related reduction in renal Gαi and Gαq proteins. Similarly, Hanai et al. (14) reported an age-related decrease in G proteins in renal cortical cells. These researchers also reported finding attenuation of parathyroid hormone-stimulated cAMP production and adenylate cyclase activity in senescent rats. Furthermore, Young et al. (37) described an age-related decrease in stimulatory G protein-coupled adenylate cyclase activity in rat osteoblasts. However, we did not investigate whether a similar age-related decrease in G proteins was present in the proximal nephron.

In summary, we report that ANG II-stimulated activation of PKC and ANG II receptor number and affinity do not differ between proximal tubule homogenates from young and adult rats. However, the primary PKC isoforms activated by ANG II in the proximal tubule differ as rats mature. This difference may partially account for the diminished increase in urinary volume and sodium excretion following volume expansion in young rats compared with adult rats.

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