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Roles of Na⁺/H⁺ Exchanger Type 1 and Intracellular pH in Angiotensin II-Induced Reactive Oxygen Species Generation and Podocyte Apoptosis

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Abstract

A growing body of evidence suggests that podocyte apoptosis is a major cause of decreased podocyte number, which leads to albuminuria and glomerular injury. The aim of this study was to clarify the molecular mechanisms of angiotensin II (Ang II)-induced apoptosis in cultured mouse podocytes. We examined the effects of Ang II (100 nmol/L) on apoptosis, superoxide anions, and cytosol pH in podocytes. For intracellular pH measurements, image analysis was conducted using confocal laser microscopy after incubation with carboxy-seminaphthorhodafluor-1. Superoxide anions and intracellular pH were elevated with Ang II treatment. Apoptotic cell numbers, as measured by TUNEL staining and caspase 3 activity, were also augmented in the Ang II-treated group. Pre-treatment with olmesartan (100 nmol/L, an Ang II type 1-receptor blocker), apocynin (50 μmol/L, NADPH oxidase inhibitor), or 5-*N*, *N*-hexamethylene amiloride [30 μmol/L, Na⁺/H⁺ exchanger type 1 (NHE-1) inhibitor] abolished Ang II-induced podocyte apoptosis, whereas *NHE-1* mRNA and protein expression was not affected by Ang II treatment. Moreover, Ang II increased NHE-1 phosphorylation. These results suggest that superoxide production, NHE-1 activation, and intracellular alkalization were early features prior to apoptosis in Ang II-treated mouse podocytes, and may offer new insights into the mechanisms responsible for Ang II-induced podocyte injury.

Keywords

angiotensin II; Na⁺/H⁺ exchanger type 1 (NHE-1); intracellular pH; apoptosis; podocyte

Introduction

Podocytes are highly specialized, terminally differentiated, epithelial cells that play a key role in both maintenance of the glomerular filtration barrier and structural integrity (1). There is an increasing body of experimental and clinical evidence indicating the contribution of decreased podocyte number to the progression of chronic kidney disease (2, 3). The consequences of reduced podocyte number include albuminuria and glomerulosclerosis. Conversely, angiotensin II (Ang II) is an important risk factor in the progression of chronic kidney disease (4, 5). We recently reported that Ang II blockade prevents the onset of

albuminuria via its protective effects on podocytes (6). Additionally, in vitro experiments revealed that Ang II induces a large number of non-hemodynamic effects, including induction of oxygen radicals and stimulation of cell apoptosis (7, 8). Ang II induces podocyte apoptosis both in vivo and in vitro (9, 10); however, the molecular mechanisms responsible for Ang II-induced podocyte apoptosis are not entirely clear.

Reactive oxygen species (ROS), especially the superoxide anion, also play an important role in cell survival and apoptosis (11, 12). We and others reported that Ang II mediates NADPH-derived superoxide production via Ang II type 1 (AT₁) receptor in various types of cells and subsequently induces renal injury (13–15). Recent reports revealed that apoptosis contributed to the downregulation of antioxidant protein in podocytes, indicating the involvement of ROS production in podocyte apoptosis (16).

Na⁺/H⁺ exchanger type 1 (NHE-1) is a member of the solute carrier family 9A, and it is a ubiquitously distributed protein with central roles in the control of cell volume and intracellular pH (pHi). The activation of NHE-1 and subsequent alkalinization of the cytosol generally precedes the activation of many cellular functions, including intracellular signaling pathways associated with apoptosis (17). It has been also reported that Ang II activates NHE-1 in various tissues (18, 19). NHE-1 is predominately expressed in differentiated podocytes (20), although its functions, including in the involvement of podocyte apoptosis, are still unclear.

In the present study, we hypothesized that Ang II induces podocyte apoptosis via NHE-1-induced pHi changes and NADPH oxidase-derived ROS production. To test this hypothesis, we examined the effects of the NHE-1 inhibitor and an antioxidant on Ang II-induced apoptotic changes in cultured mouse podocytes.

Materials and Methods

Cell culture

Cell culture of conditionally immortalized mouse podocytes was performed, as previously reported (21). To propagate podocytes, cells were grown on type I collagen-coated dishes at 33°C in the presence of 10 U/mL of mouse recombinant interferon- γ (Sigma-Aldrich, St. Louis, MO, USA), which enhances the expression of a thermosensitive T-antigen, in RPMI 1640 (Sigma-Aldrich). To induce differentiation, cells were maintained at 37°C without interferon- γ for 10–14 days. All experiments were performed with differentiated podocytes.

Superoxide anion measurement

Superoxide anion production was determined with dihydroethidium (DHE) staining, as described previously (22). Briefly, cells were plated onto a glass-bottom dish (Asahi Glass, Tokyo). At the appropriate time after stimulation, DHE (10 μ mol/L) was added into the medium, and the incubation was continued for 30 min. Then, cells were washed with PBS, and images were obtained with a laser scanning confocal microscope system (LSM 700; Carl Zeiss, Oberkochen, Germany). The averages of fluorescence intensity values were calculated using NIH ImageJ software.

pHi measurement

For pHi imaging, podocytes were grown on 2-well glass chamber slides (BD Bioscience, Franklin Lakes, NJ, USA). Cells were incubated with the acetoxymethyl ester of the pH-reporter dye carboxy-seminaphthorhodafluor (SNARF)-1 (Life Technologies, Carlsbad, CA, USA) at 10 μ mol/L for 30 min as previously reported (23). Excess extracellular dye was

washed away with medium. The pHi dye was excited using a 488 nm argon laser, and fluorescence was detected with confocal microscopy at 580 and 640 nm. The 580/640 nm fluorescence ratio was then converted into pHi using a calibration curve. The standard curve was obtained by measuring the ratio signals of carboxy-SNARF-1-loading cells in high-potassium buffers (25 mmol/L HEPES, 145 mmol/L KCl, 0.8 mmol/L CaCl₂, 5.5 mmol/L glucose) at different pHs (pH 6.7, 7.0, 7.3, 7.6, or 7.9) in the presence of 10 µg/mL of nigericin, an electroneutral H⁺/K⁺ ionophore.

Real-time RT-PCR

For real-time RT-PCR analysis, total RNAs were extracted using ISOGEN (Nippon Gene, Tokyo). cDNA (from 1 µg RNA) was synthesized, as described previously (24). The expression of mRNA was analyzed with a Light Cycler Fast Start DNA Master SYBR Green I kit (Life Technologies). RT-PCR was performed using pre-designed primers for mouse *NHE-1* (TaqMan Gene expression Assays: Life Technologies) and mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (F: 5'-TGAACGGGAAGCTCACTGG-3' and R: 5'-TCCACCACCCTGTTGCTGTA-3') (25). All data were normalized to the expression of *GAPDH*.

Western blot analysis

Cells at 80% – 90% confluence were made quiescent by incubation with medium containing 0.1% FBS for 24 h. Cells were lysed as described previously (26); solubilized proteins were isolated by centrifugation (12,500 rpm, 10 min) and quantified by the Bradford assay. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies against NHE-1 (Cell Signaling Technology, Beverly, MA, USA). The membranes were then embedded with Infrared Dye were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). To confirm equal protein loading, each membrane was re-probed with an anti- β -actin antibody (Sigma-Aldrich). Band intensities were quantified by immunoblot densitometry using NIH ImageJ software.

Immunoprecipitation

We determined NHE-1 phosphorylation by co-immunoprecipitation and western blotting, as reported previously (26, 27). Protein samples (130 µg) from podocytes were immunoprecipitated by overnight incubation with anti-phospho-Ser 14- 3-3 antibody (Cell Signaling Technology), followed by western blot analysis with the antibody against NHE-1.

TUNEL method

TUNEL staining was performed using a MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya), as previously reported (28). This method detects nucleosome-sized DNA fragments by tailing their 3'-OH ends with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT). After treatment, the cells were fixed with 4% paraformaldehyde and incubated with TdT buffer. Cells were double-labeled with 1 µg/mL of DAPI for 15 min at 37°C. The numbers of TUNEL-positive cells were counted using fluorescence microscopy.

Caspase 3 activity

An APOPCYTO caspase 3 colorimetric assay kit (MBL) was used for the measurement of caspase activities, as previously reported (28). Briefly, podocytes were plated in 100-mm dishes and cultured in medium. After treatment, the cells were lysed with 150 µL of ice-cold lysis buffer. After centrifugation at 10,000 *g* at 4°C for 5 min, protein concentrations in the supernatants were quantified using the Bradford assay. A 4-amino acid sequence was

labeled with *p*-nitroanilide (pNA) at the C-terminal side. Free pNA was released from the labeled synthetic substrate following cleavage with active caspase. The lysates were incubated with the reaction buffer and then incubated with DEVD-pNA substrate for 2 h at 37°C. Absorbance was monitored at a wavelength of 405 nm.

Statistical analyses

Values are presented as means \pm S.E.M. Multiple-group comparisons were made using one-way analyses of variance (ANOVA), followed by a Bonferroni's test. Student's *t*-tests were performed to compare the means when the experimental design comprised of two individual groups. $P < 0.05$ was considered statistically significant.

Results

Effects of Ang II on superoxide production in podocytes

An incubation of podocytes with 100 nmol/L of Ang II resulted in a significant increase in DHE-positive areas compared with the control group (Fig. 1A). Pre-incubation with the appropriate inhibitors [i.e., olmesartan (RNH-6270, an active form of olmesartan medoxomil, 100 nmol/L), an AT₁-receptor blocker; apocynin (50 μ mol/L), an NADPH oxidase inhibitor; or 5-*N*, *N*-hexamethylene amiloride (HMA, 30 μ mol/L), an NHE-1 inhibitor], inhibited superoxide production induced by Ang II, indicating the involvement of AT₁ receptors, NADPH oxidase, and NHE-1 in the Ang II-mediated increases in ROS production.

Effects of Ang II on pHi in podocytes

We used SNARF-1 staining to obtain a calibration line to measure pHi (Fig. 2A). Treatment of podocytes with 100 nmol/L Ang II caused an increase in pHi (Fig. 2B). Ang II-induced intracellular alkalinization in podocytes was inhibited by HMA (Fig. 2C). Olmesartan and apocynin also prevented Ang II-induced intracellular alkalinization, indicating that Ang II activates NHE-1 via the AT₁ receptor and NADPH oxidase.

Furthermore, to clarify the mechanisms behind the pHi changes induced via NHE-1, we measured *NHE-1* mRNA expression by RT-PCR. We found that *NHE-1* was expressed in cultured podocytes under basal condition. However, Ang II failed to increase *NHE-1* mRNA expression (Fig. 3A) or affect NHE-1 protein expression (Fig. 3B). By contrast, Ang II augmented NHE-1 phosphorylation, which was abolished by pretreatment with inhibitors (Fig. 3C). These results suggest that intracellular alkalinization was primarily the result of NHE-1 activation and not due to an increase in NHE-1 expression.

Effects of Ang II on apoptosis in podocytes

The incubation of podocytes with 100 nmol/L Ang II for 18 h resulted in a significant increase in apoptosis compared with the control, as determined via TUNEL staining (Fig. 4A). Pre-treatment with olmesartan, apocynin, or HMA inhibited Ang II-induced podocyte apoptosis (Fig. 4B). The findings with respect to caspase 3 activity were similar to those obtained with TUNEL staining, where Ang II was found to augment caspase 3 activity, and this was completely inhibited by olmesartan, apocynin, and HMA (Fig. 5). These results suggest that both NHE-1 and ROS promote apoptosis in mouse podocytes.

Discussion

Ang II is directly involved in podocyte injury (6, 29). Studies have indicated that the major cause of Ang II-induced podocyte injury is due to apoptosis in podocytes (9, 10, 16). A reduced podocyte number due to apoptosis induces proteinuria and subsequent

glomerulosclerosis (2, 30). Although Ang II induces podocyte apoptosis, its molecular mechanisms are not fully elucidated. In the present study, we revealed that Ang II-induced podocyte apoptosis is associated with NHE-1 activation, intracellular alkalization, and an accumulation of intracellular ROS. These findings reveal the possible mechanisms of Ang II-induced podocyte injury via pHi-mediated apoptosis.

NHE-1, which participates in the regulation of pHi by mediating the exchange of Na⁺ for H⁺ across the plasma membrane (31), is activated by a wide range of stimuli, including many growth factors and hormones, cell adhesion, and stress stimuli (32, 33). Ang II also activates NHE-1 in various tissues. We demonstrated that 100 nmol/L Ang II induces intracellular alkalization via NHE-1 activation in cultured mouse podocytes. Furthermore, Ang II-induced intracellular alkalization was reduced with a selective NHE-1 inhibitor, indicating that NHE-1 stimulation was primarily involved. We also demonstrated that there was a remarkable increase of pHi in Ang II-treated cells in conjunction with ROS production and apoptosis. Several reports suggested that NHE-1 promotes apoptosis via actively inducing intracellular alkalization, activation of Bax (34), the inhibition of mitochondrial ADP transport (35), and endonucleases (36). However, the mechanisms behind alkalization-related apoptosis still need to be evaluated. Conversely, although our findings clearly reveal the involvement of pHi in podocyte apoptosis, it is difficult to evaluate pH changes of podocytes in *in vivo* experiments. Several studies have reported on organ injury via pH changes in animal model (37, 38); however, measurements of pHi in podocytes have not yet been evaluated. Therefore, podocyte-specific NHE-1 inhibition may be useful in revealing the effects of NHE-1-mediated pHi in glomerular injury, instead of *in vivo* pH measurements.

Ang II is known to induce oxidative stress in a variety of renal cells, including podocytes (39). Ang II augments ROS production via the activation of NADPH oxidase through the AT₁ receptor (13). Moreover, increased ROS production has been implicated in the pathogenesis of Ang II-induced apoptosis in cardiovascular and renal tissues (40, 41). In the present study, Ang II augmented ROS generation in podocytes. We also found that the AT₁-receptor antagonist and NADPH-oxidase inhibitor blocked Ang II-induced apoptosis. These results indicate that Ang II-induced podocyte apoptosis is mediated via NADPH-dependent ROS production through the AT₁ receptor. Our results also demonstrated that the inhibition of NADPH oxidase in the presence of Ang II caused an increase in pHi, indicating that ROS production may be upstream from NHE-1 activation, as previously reported in cardiomyocytes (42).

In conclusion, our results highlight the role of NHE-1 stimulation, intracellular alkalization, and accumulation of intracellular ROS in the Ang II-induced, caspase-3-dependent apoptosis of podocytes. The inhibition of NHE-1 and NADPH oxidase may have protective effects against podocyte apoptosis, thereby improving renal injury. Although further studies are necessary to fully elucidate the mechanisms of Ang II-induced podocyte injury, the inhibition of these pathways may potentially serve as clinical therapeutic targets.

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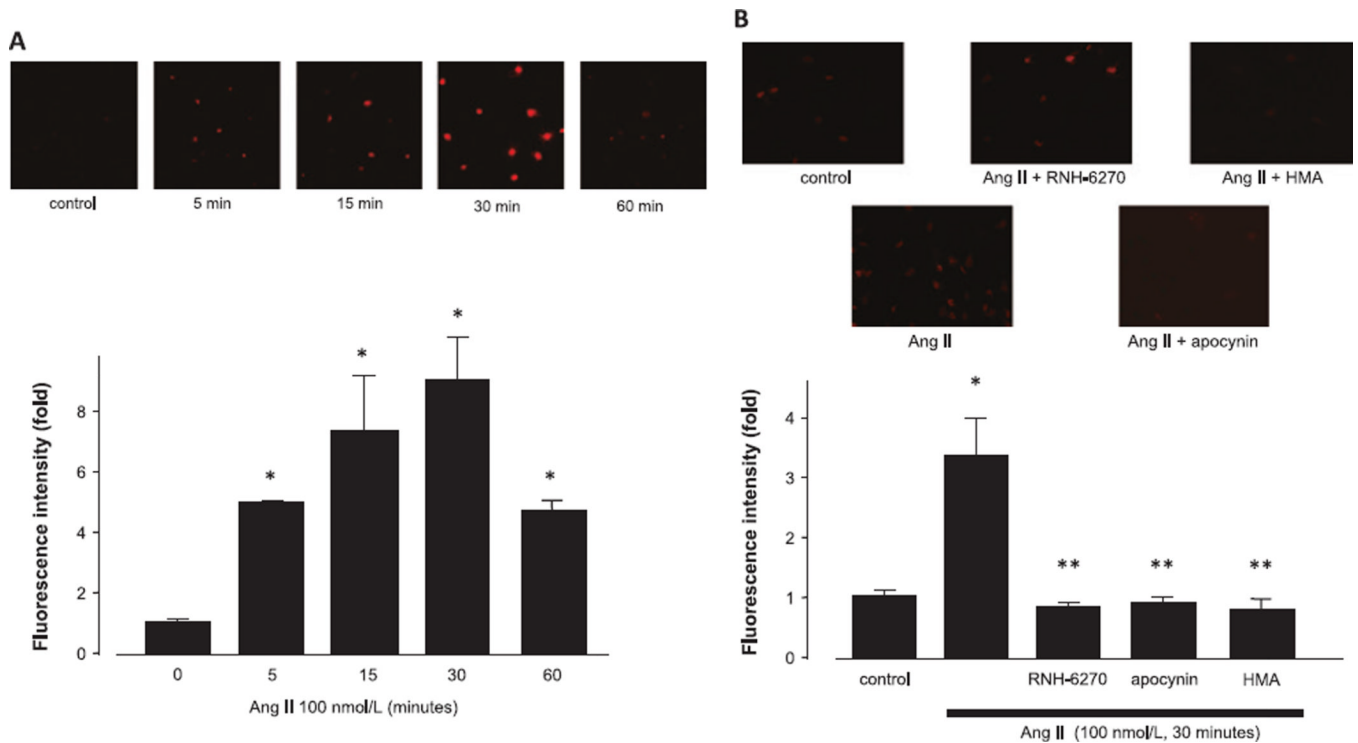


Fig. 1. Effects of Ang II on superoxide production. A: Podocytes were incubated with 100 nmol/L Ang II for the indicated times. B: Podocytes were pre-treated with olmesartan (100 nmol/L), apocynin (50 μ mol/L), or HMA (30 μ mol/L) for 1 h, and then stimulated with 100 nmol/L of Ang II for 30 min prior to DHE staining. DHE fluorescence was used to evaluate superoxide production in podocytes. Representative photographs of DHE-stained podocytes are shown. Data are reported as the mean \pm S.E.M. (n = 6), expressed as fold change compared with unstimulated cells. * P < 0.05 vs. control podocytes, ** P < 0.05 vs. Ang II alone.

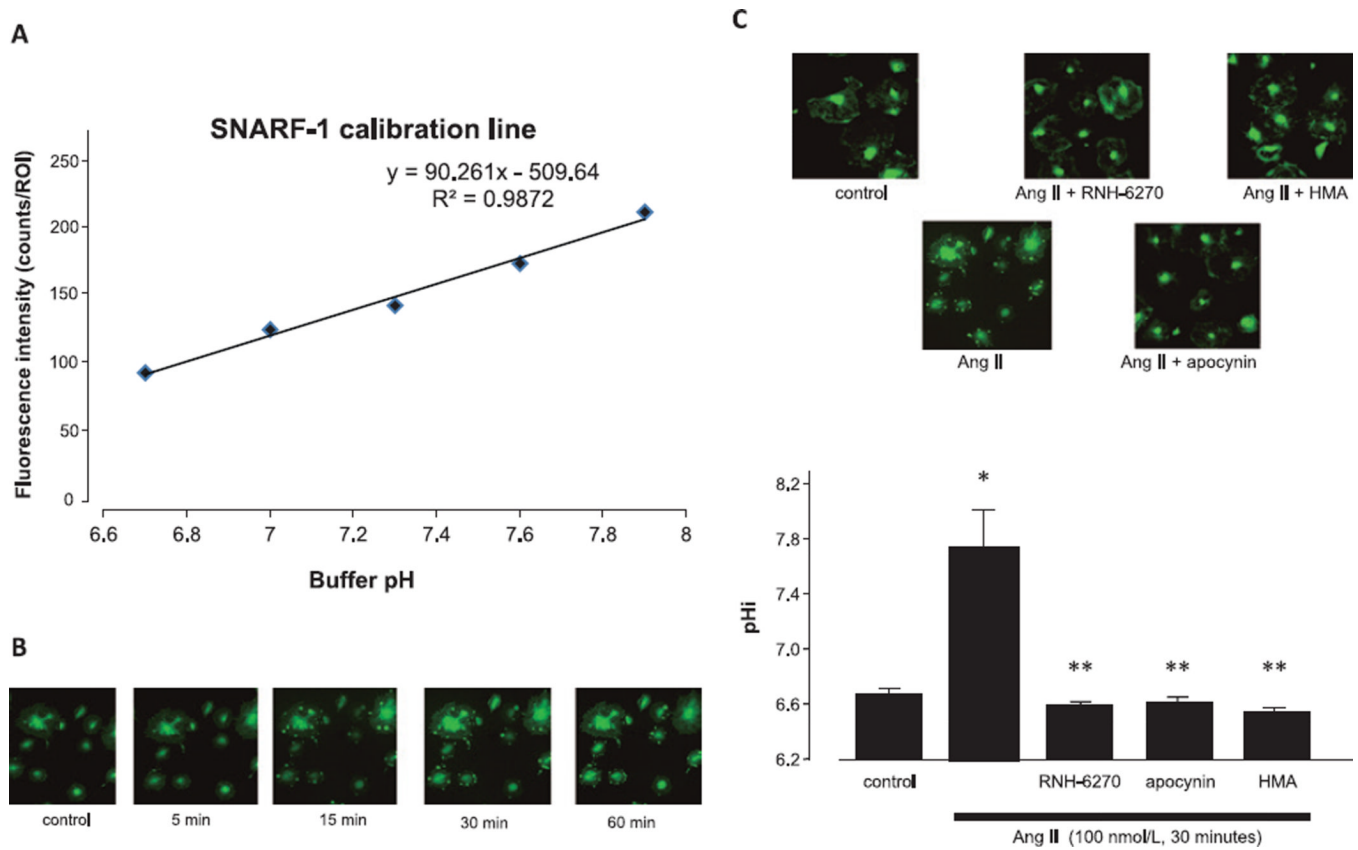


Fig. 2. Effects of Ang II on intracellular pH. **A:** The calibration line was obtained by measuring the signals of carboxy-SNARF-1-loading cells at different pHs in the presence of 10 $\mu\text{g}/\text{mL}$ of nigericin. **B:** Podocytes were incubated with 100 nmol/L Ang II for the indicated times. **C:** Podocytes were pre-treated with olmesartan (100 nmol/L), apocynin (50 $\mu\text{mol}/\text{L}$), or HMA (30 $\mu\text{mol}/\text{L}$) for 1 h and then stimulated with 100 nmol/L Ang II for 30 min before SNARF-1 staining. Representative photographs of SNARF-1-stained podocytes are shown. Data are reported as the mean \pm S.E.M. ($n = 6$). * $P < 0.05$ vs. control podocytes, ** $P < 0.05$ vs. Ang II alone.

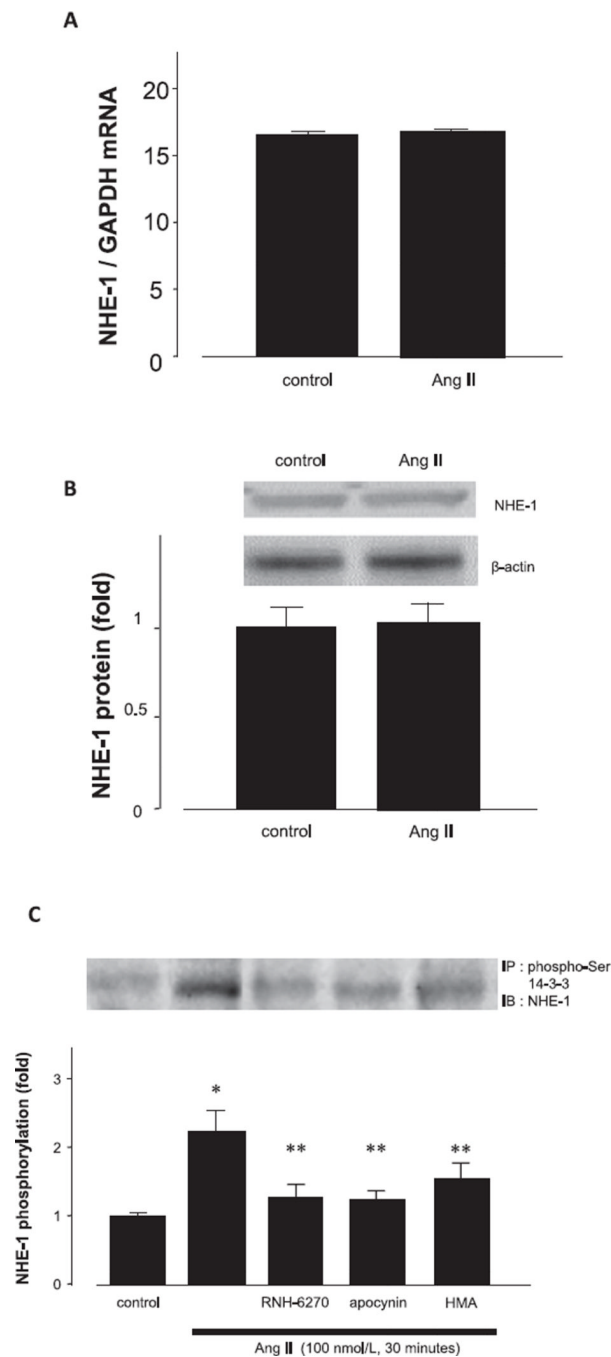


Fig. 3. Effects of Ang II on NHE-1 mRNA, protein, and phosphorylation. Podocytes were treated with 100 nmol/L Ang II for 24 h (A and B) or 30 min (C). A: *NHE-1* mRNA expression was measured by RT-PCR. B: NHE-1 protein expression was measured by western blot analysis. C: Protein samples of podocytes were immunoprecipitated with anti-phospho-Ser 14-3 antibody, followed by western blot analysis with the antibody against NHE-1. Data are reported as the mean \pm S.E.M. (n = 6). * P < 0.05 vs. control podocytes, ** P < 0.05 vs. Ang II alone.

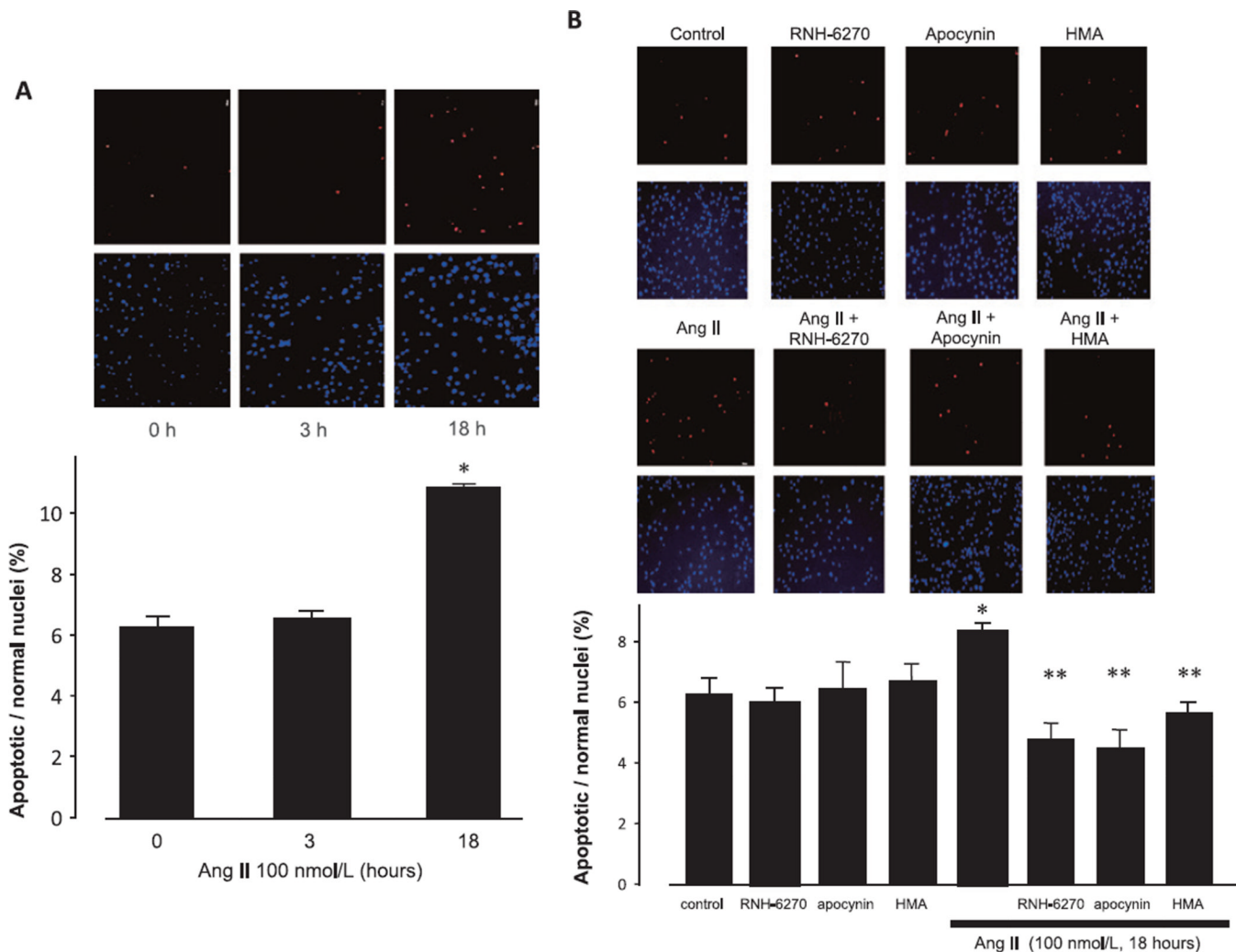


Fig. 4. Effects of Ang II on TUNEL staining. A: Podocytes were incubated with 100 nmol/L Ang II for the indicated times. B: Podocytes were treated with 100 nmol/L Ang II for 18 h prior to TUNEL staining. Blue: DAPI stained normal nuclei and Red: TUNEL-positive nuclei. Representative photographs of TUNEL staining are shown. Data are reported as the mean \pm S.E.M. (n = 6), expressed as apoptotic/normal nuclei. * P < 0.05 vs. control podocytes. ** P < 0.05 vs. Ang II alone.

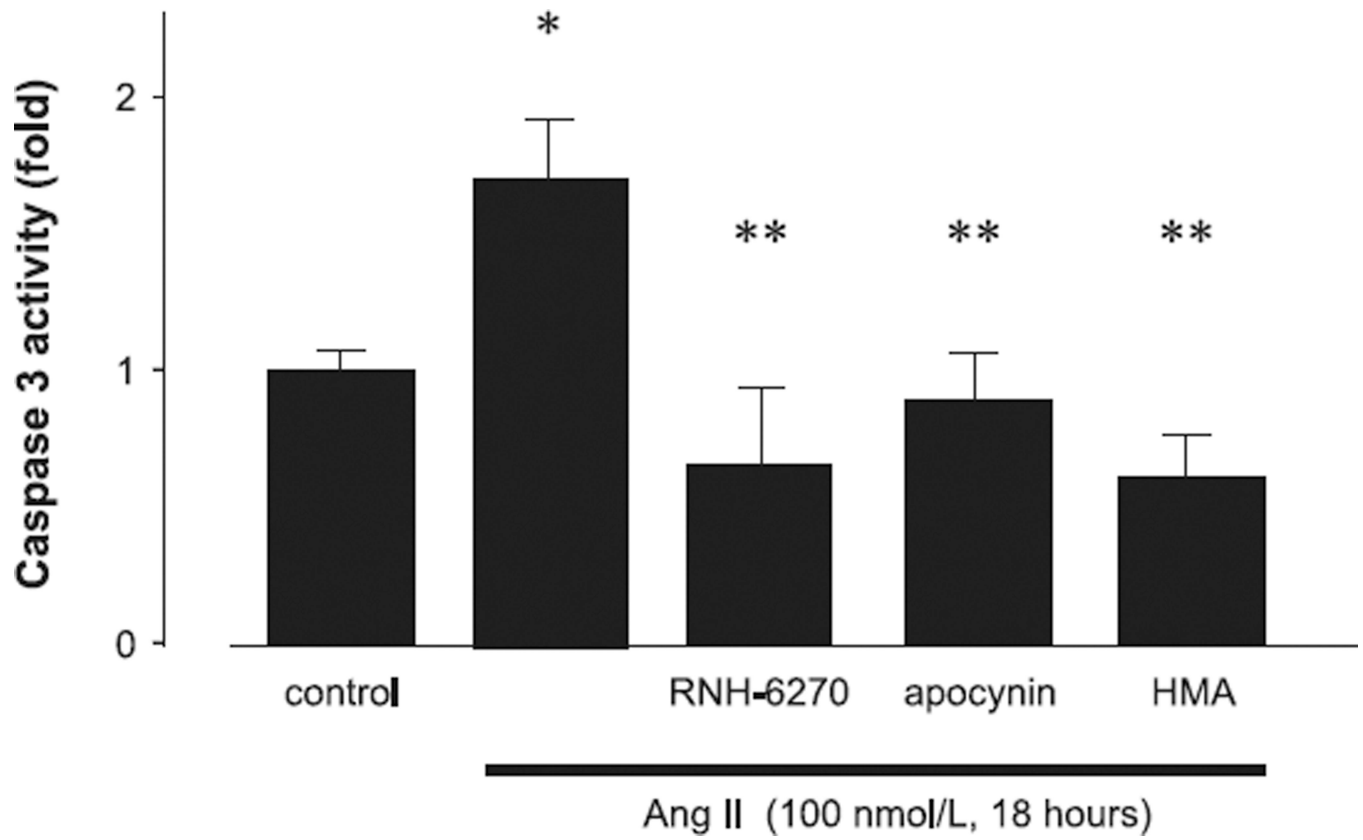


Fig. 5. Effects of Ang II on caspase 3 activity. Podocytes were treated with 100 nmol/L of Ang II for 18 h prior to caspase 3 assay. Data are reported as the mean \pm S.E.M. (n = 6), expressed as fold change compared with unstimulated cells. * P < 0.05 vs. control podocytes, ** P < 0.05 vs. Ang II alone.