Online supplement

Contribution of hypoxia inducible factor-1α to the profibrotic action of angiotensin II in cultured renal medullary interstitial cells

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Running Title: Hypoxia inducible factor-1α and renal fibrosis

Supplement results

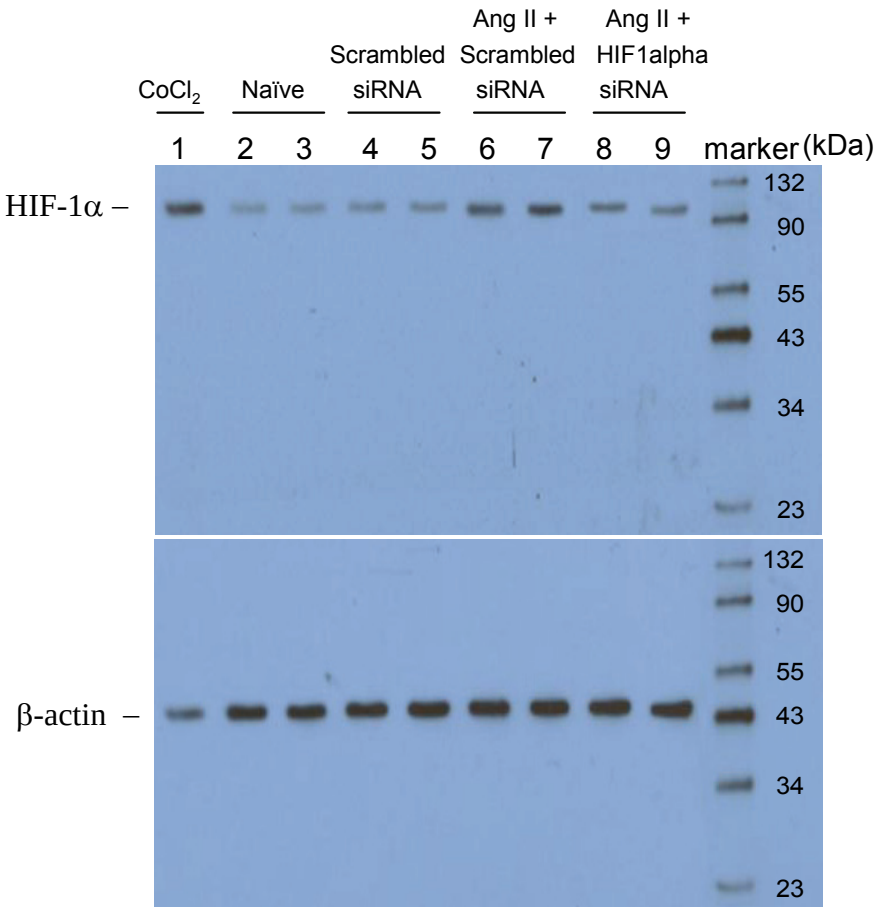

Figure 1. Representative ECL gel documents of Western blot analysis with full size markers depicting the location of HIF-1α blots. The sample from $CoCl_2$-treated cells was used as a positive control to further clarify the size of HIF-1α blots.

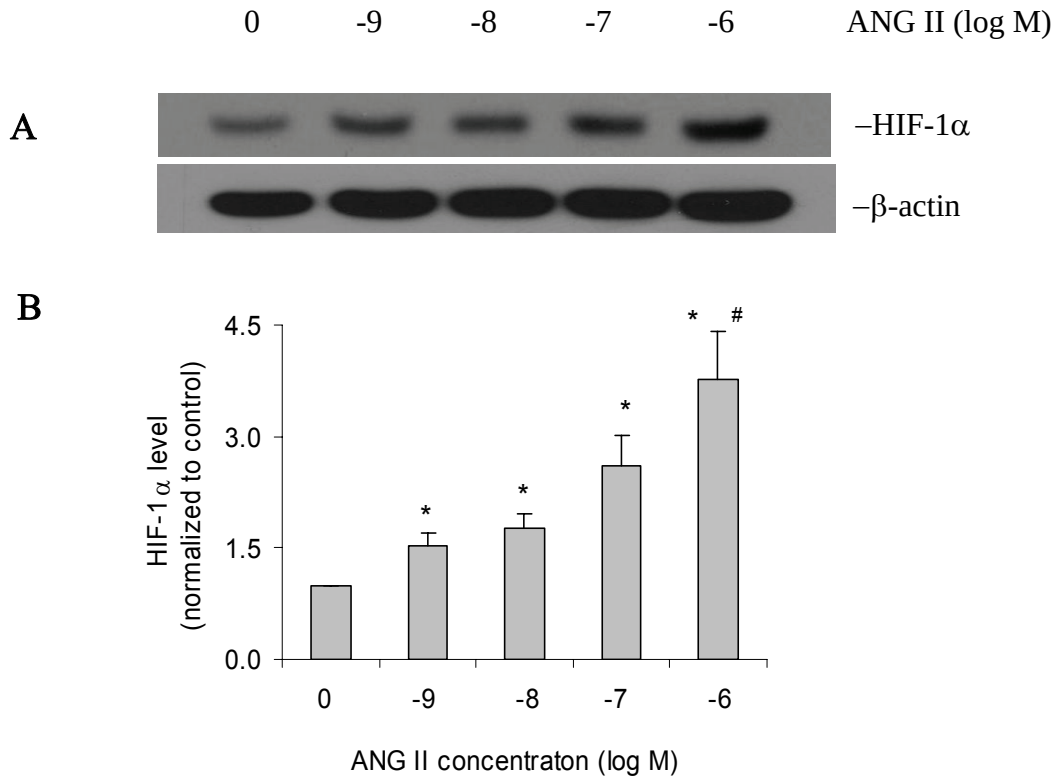

Figure 2. Effect of ANG II on HIF-1α levels at different concentrations in RMICs by Western blot analysis. Panel A: representative ECL gel documents of Western blot analyses depicting the protein levels of HIF-1α. Panel B: summarized intensities of HIF-1α blots normalized to control. * $P < 0.05$ vs. control, # $P < 0.05$ vs. -8 (n=3).

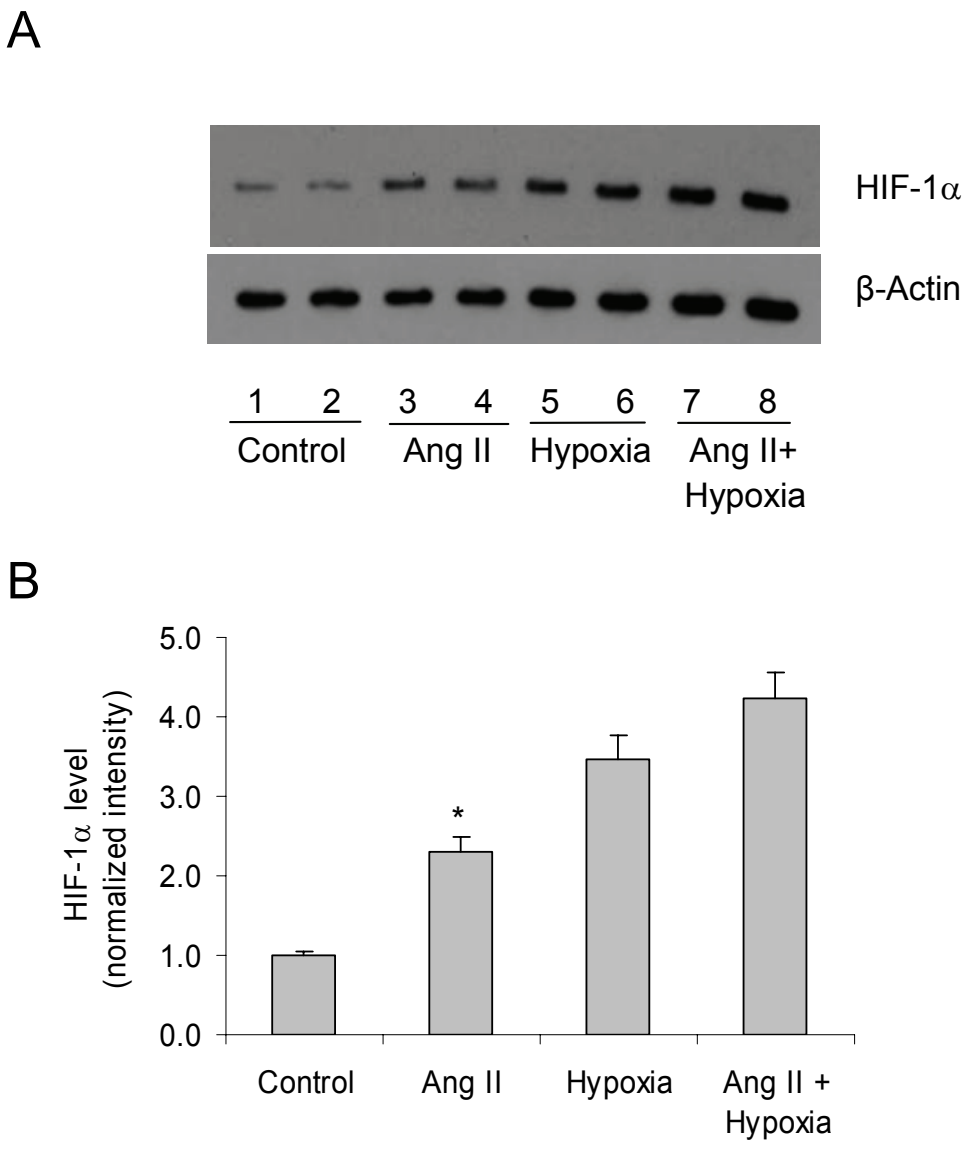

Figure 3. HIF1α protein level in the RMICs treated with Ang II and/or hypoxia (1% O_2 for 4 hours). * P< 0.05 vs. all other groups (n = 6). P = 0.11 Hypoxia vs. ANG II + Hypoxia.

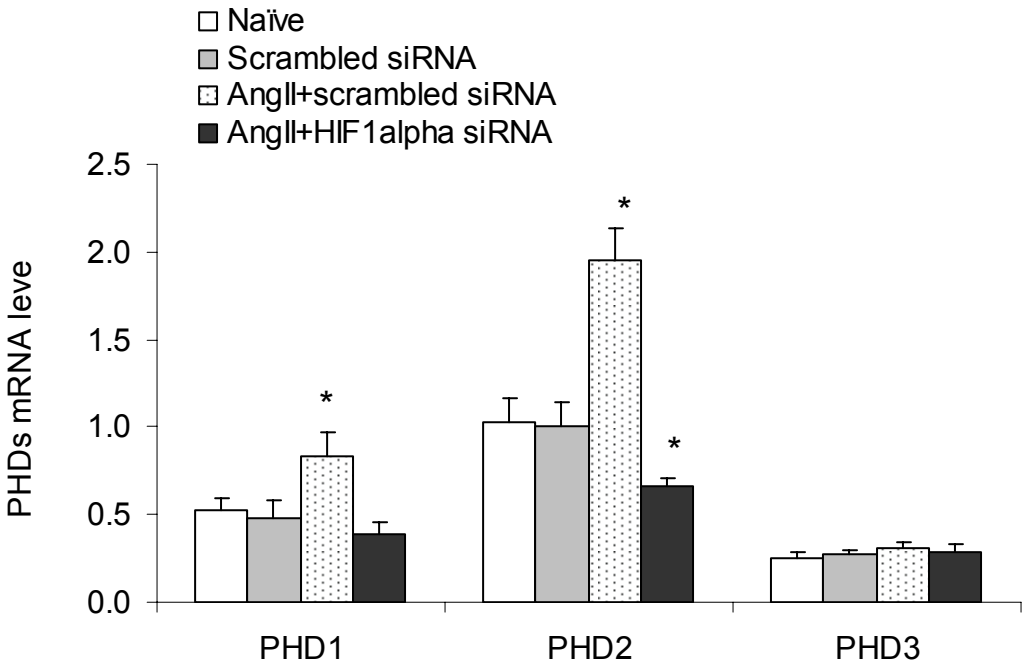

Figure 4. Effect of ANG II and HIF-1α siRNA on the mRNA levels of PHD1, 2 and 3 in RMICs by Real-time RT-PCR analysis. *P < 0.05 vs. all other groups within the same PHD isoform (n=6).

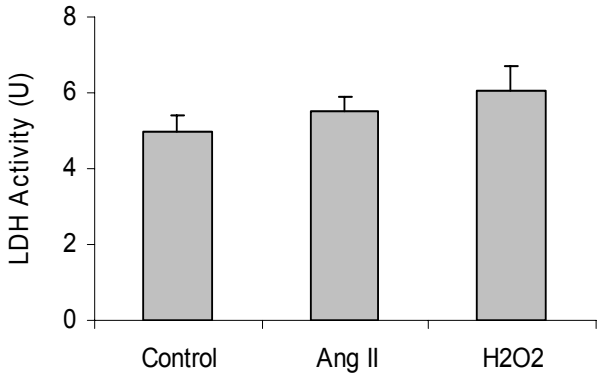

Figure 5. Effect of ANG II and H_2O_2 on lactate dehydrogenase (LDH) activity (assay kit, Cayman). (n=6)

Expanded Materials and Methods

Preparation of nuclear extracts and cytosolic protein, Western blot analyses for protein levels of HIF-1α, TIMP-1, collagen I/III and PHD2. Nnuclear protein was prepared as we described previously (1). Briefly, cells were scraped and washed with PBS by centrifugation at 1,000 x g for 5 min, and then homogenized with a glass homogenizer in ice-cold HEPES buffer (A) containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10% Nonidet P-40. After centrifugation of the homogenate at 1,000 x g for 5 min at 4°C, the supernatants were collected for cytosolic protein preparation and the pellets for nuclear protein isolation. The supernatants were centrifuged again at 6,000 x g for 10 min and resulting supernatants were used as cytosolic proteins for Western blot analyses of TIMP-1, collagen I/III and PHD2. The reason for detecting collagen I/III is that the subtype of collagen is tissue/cell specific and collagen I/III is the one expressed in renal interstitial cells.

For nuclear fraction isolation, the pellets from the first centrifugation, which contains cell nuclei, were washed with buffer A and then incubated with ice-cold HEPES buffer (B) containing 5 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 300 mM NaCl, 400 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 26% glycerol for 30 min to release nuclear proteins. Next, the reaction mixtures were centrifuged at 23,000 rpm for 30 min, and the supernatant was collected and frozen in liquid nitrogen until use as nuclear extracts for Western blot analyses of HIF-1α levels.

Western blot analyses were performed as described previously (1). Briefly, protein samples (20 μg) were subjected to 10% SDS-PAGE gel electrophoresis and electrophoretically transferred onto nitrocellulose membrane. The membranes were then probed with primary antibodies overnight in cold room (4^oC). After washing, the membrane was incubated for 1 h with 1:3,000 horseradish peroxidase-labeled secondary antibodies. After the membrane was washed, enhanced chemiluminescence detection solution (ECL, Pierce) was added directly to the blots on the surface carrying proteins, and the membrane was wrapped in Saran wrap and exposed to Kodak Omat film. The intensity of the blots was determined using an imaging analysis program (ImageJ, free download from http://rsbweb.nih.gov/ij/).

Primary antibodies used in the present study included anti-rat HIF-1α (monoclonal, Novus Biologicals, 1:300 dilution), hydroxylated HIF-1α (HIF-1α-OH, rabbit polyclonal, Novus Biologicals, 1:500), PHD2 (rabbit polyclonal, Novus Biologicals, 1:300), TIMP-1 (monoclonal, R&D systems, 1:1000), and collagen I/III (rabbit polyclonal, Calbiochem, 1:300).

RNA extraction and quantitative RT-PCR analysis of the mRNA levels of PNCA, Vimentin, and PHD. Total RNA was extracted using TRIzol solution (Life Technologies, Inc. Rockville MD) and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad, Hercules, CA). The RT products were amplified using a TaqMan Gene Expression Assays kit (Applied Biosystems). This kit contains target gene primers and FAM™ dye-labeled probes, and has been tested and optimized for the analysis of target gene expression by the manufacturer. A TaqMan Gene Expression Assays kit for detecting the levels of 18S rRNA was used as an endogenous control. The real-time quantitative PCR was performed using an iCycler iQ Real-Time PCR Detection

System (Bio-Rad) according to manufacturer's manual. The cycle threshold (Ct) values were used for calculation of gene expression in accordance with the ΔΔCt method. The Ct values of target genes were first normalized with respect to 18S ribosomal RNA levels from the same samples to obtain ΔCt values. The mean ΔCt value from the control samples was used as a reference to calculate ΔΔCt values for all samples. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Superoxide ($O_2^{\cdot-}$) detection by electronic spin resonance (ESR): The measurement of $O_2^{\cdot-}$ by ESR was performed according to the methods in our previous studies (2-3). Cells were gently collected and suspended in modified Krebs-HEPES buffer containing deferoxamine (100 μM; metal chelator), and then were subsequently mixed with 1 mM of the $O_2^{\cdot-}$-specific spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) in the presence or absence of manganese-dependent SOD (500 U/ml). The cell mixture was then loaded in glass capillaries and immediately kinetically analyzed for $O_2^{\cdot-}$ production for 10 min by an ESR system (Miniscope MS200, Noxygen Science Transfer & Diagnostics GmbH, Denzlingen, Germany). The SOD-inhibitable fraction of the signals were normalized by protein concentration and compared among different experimental groups. The ESR settings were as follows: biofield, 3,350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; 4,096 points of resolution; receiver gain, 100; and kinetic time, 10 min.

Fluorescence spectrometric assay of H_2O_2 concentrations. Amplex red is a fluorogenic substrate with very low background fluorescence; it reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin (4). Fluorescence spectrometric assay of H_2O_2 levels in

culture medium was performed using an Amplex red kit (Molecular Probes, Eugene, OR) as we described previously (5). Briefly, 200 μM Amplex red reagent and 1 U/ml horseradish peroxidase were added to the experimental culture media (50 μl), blank culture media containing a series of H_2O_2 standard and blank culture medium alone as a control for background fluorescence, and the samples were then incubated for 30 min in Falcon 96-well microplates in the dark at room temperature. Fluorescence intensity was measured in an automatic microplate reader (model KC_4, Bio-Tek Instruments, Winooski, VT) at an excitation wavelength of 530 ± 25 nm and an emission wavelength of 590 ± 35 nm. After subtraction of background fluorescence, H_2O_2 concentrations in culture media were calculated on the basis of a H_2O_2 standard curve generated using H_2O_2 and Amplex red. Both the results of $O_2^{\cdot-}$ and H_2O_2 were normalized with total cell numbers in each dish.

Reference

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