HIF Prolyl-Hydoxylases Sense High Salt Intake to Increase Hypoxia Inducible Factor-1α Levels in the Renal Medulla

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Supplement methods

Transfection DNA into renal medulla. Rats were uninephrectomized one week before and the remaining left kidney was transfected with designated plasmids into renal medulla. To deliver the plasmids into renal medulla, the anesthetized rats were placed on a thermostatic table to maintain a body temperature of 37°C, and then the left kidney was exposed by a flank incision (1-1.5 cm) and placed in a cup to stabilize the organ for implanting a medullary interstitial catheter (tapped tip, 4-5mm) into the kidney. The catheter was anchored into place on the kidney surface with Vetbond Tissue Adhesive (3M). A mixture containing 50 μg of DNA and 8 μl of *in vivo-jetPEI*™ (Polyplus-transfection, New York, NY), a polyethylenimine derivative, in 500 μl 10% glucose was infused into renal medulla at a speed of 20 μl/min. After infusion, the catheter was cut and blocked by a piece of fat tissue with Vetbond Tissue Adhesive. An ultrasound transducer (Sonitron 2000, Rich-Mar) was directly applied onto the kidneys with a 1-MHz ultrasound at 10% power output, for a total of 60 s with 30-s intervals on each side of the kidney [1] in the middle and at the end of the infusion. A previous study evidenced that shRNA expression plasmids were successfully delivered into renal medulla using *in vivo-jetPEI*™ [2]. It has been shown in our previous studies [3-4] and others [5-7] that combination of ultrasound significantly enhances the DNA transfection with different transfection reagents including polyethylenimine and polystyrene nanoparticles [8-9]. In the present study, combination of ultrasound with *vivo-jetPEI* produced a high efficient gene delivery into the renal medulla as evidenced by a 4-fold increase in overexpression of transgene and 78% decrease in the protein expression of shRNA target gene.

RNA extraction and quantitative RT-PCR analysis of PHD2, heme oxygenase (HO)-1, nitric oxide synthase (NOS)-2 and cyclooxygenase (COX)-2 mRNA. Total RNA from renal medullary tissues was extracted using TRIzol solution and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad). The RT products were amplified using TaqMan® Gene Expression Assays kits (Applied Biosystems) with an iCycler iQ Real-Time PCR Detection System (Bio-Rad). The levels of 18S ribosomal RNA (rRNA) were used as an endogenous control. The relative gene expressions were calculated using cycle threshold (Ct) values in accordance with the ΔΔCt method. The Ct values were first normalized with respect to 18S rRNA levels to obtain ΔCt values. The averaged ΔCt value from the control group was used as a reference to calculate ΔΔCt values for all samples. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Preparation of tissue homogenate and nuclear extracts, and Western blot analyses for protein levels of HIF-1α and PHD2. Nnuclear protein was prepared as we described previously [10]. Briefly, renal medullary tissue was 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 and pellets were collected separately. The supernatants were centrifuged again at 6,000 x g for 10 min and resulting supernatants were used for Western blot analyses of PHD2.

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 [10]. Primary antibodies used in the present study included anti-rat HIF-1α (monoclonal, Novus Biologicals, 1:300 dilution) and PHD2 (rabbit polyclonal, Novus Biologicals, 1:300). The intensity of the blots was determined using an imaging analysis program (ImageJ, free download from http://rsbweb.nih.gov/ij/).

Determination of prolyl hydroxylase activity. Peptide-specific conversion of 2-oxoglutarate into succinate provides a hydroxyl group for HIF-1α to be prolyl hydroxylated. This reaction has been widely used for determination of PHD activity [11-12]. Briefly, dissected renal cortical and medullary tissues were homogenized on ice using Dounce homogenizer in 6 volumes of buffer containing 0.15 mM $MgCl_2$, 10mM KCl, 10 mM Tris-HCl, pH 6.7. Dextrose was added to a final concentration of 0.25 M, and the homogenate was centrifuged at 1500 x g for 15 min at 4 °C to remove nuclei. The mitochondrial fraction was separated by the second centrifugation at 6500 x *g* for 10 min at 4 °C. The hydroxylase reaction was carried out using 30 µg of protein in the reaction buffer containing 40 mM Tris-HCl, pH 7.5, 50 µM $FeSO_4$, 0.1 mM L-[5-^{14}C] α-ketoglutaric acid (Moravek Biochemicals, Brea, CA), 200 µM ODD peptide (amino acids 556–575 of rat HIF-1α) (Advanced ChemTech, Louisville, KY), 0.25 mM ascorbate, 0.4 mg/ml catalase and 0.5 mM dithiothreitol. Samples were incubated for 1 h at 37 °C in a final volume of 50 µl. After incubation, 25 µl of mixture of 20 mM succinate and 2-oxoglutarate was added, followed by the addition of 25 µl of 0.16 M 2,4-dinitrophenylhydrazine in 30% $HClO_4$. The samples were allowed to sit for 30 min at room temperature following the addition of 50 µl of 1 M of 2-oxoglutarate. Supernatants were separated by spinning at 3000 x *g* for 5 min, and radioactivity measured using a liquid scintillation counter. Each set of experiments included control reactions without

addition of peptide of HIF-1α to calculate HIF-1α–dependent conversion of 2-oxoglutarate into succinate, which represents specific HIF prolyl-hydroxylase activity.

References

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