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Overexpression of HIF Prolyl-Hydroxylase-2 transgene in the renal medulla induced a salt-sensitive hypertension

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Abstract

Renal medullary hypoxia-inducible factor (HIF)-1 α and its target genes, such as heme oxygenase and nitric oxide synthase, have been indicated to play an important role in the regulation of sodium excretion and blood pressure. HIF prolyl hydroxylase domain-containing proteins (PHDs) are major enzymes to promote the degradation of HIF-1 α . We recently reported that high salt intake suppressed the renal medullary PHD2 expression and thereby activated HIF-1 α -mediated gene regulation in the renal medulla in response to high salt. To further define the functional role of renal medullary PHD2 in the regulation of renal adaptation to high salt intake and the longer-term control of blood pressure, we transfected PHD2 expression plasmids into the renal medulla in uninephrectomized rats and determined its effects on pressure natriuresis, sodium excretion after salt overloading and the long-term control of arterial pressure after high salt challenge. It was shown that overexpression of PHD2 transgene increased PHD2 levels and decreased HIF-1 α levels in the renal medulla, which blunted pressure natriuresis, attenuated sodium excretion, promoted sodium retention and produced salt sensitive hypertension after high salt challenge compared with rats treated with control plasmids. There was no blood pressure change in PHD2-treated rats that were maintained in low salt diet. These results suggested that renal medullary PHD2 is an important regulator in renal adaptation to high salt intake and a deficiency in PHD2-mediated molecular adaptation in response to high salt intake in the renal medulla may represent a pathogenic mechanism producing salt sensitive hypertension.

Key words: pressure natriuresis, hypoxia inducible factor-1 α , transcription factor, gene transfection, sodium excretion

Introduction

Salt-sensitive hypertension accounts for 50% of hypertensive population [1-2]. Importantly, the salt sensitivity of blood pressure is closely associated with a much greater propensity to develop organ injuries in hypertension [2-4]. Mechanism for salt-sensitive hypertension is not fully understood. It is well documented that renal medullary function plays an important role in the regulation of renal sodium excretion and arterial blood pressure, and that dysfunction in the renal medulla is involved in salt-sensitive hypertension [5-10]. We have recently demonstrated that the transcription factor hypoxia inducible factor (HIF)-1 α -mediated gene activation in the renal medulla is an important adaptive mechanism in response to high salt intake, which leads to inductions of various protective factors in the renal medulla and promotes extra sodium excretion [11].

HIF-1 α and some of its target genes, such as nitric oxide synthase (NOS), cyclooxygenase-2 (COX-2) and hemeoxygenase-1 (HO-1), are highly expressed in the renal medulla [6,9,12-15]. The products of these HIF-1 α target genes play critical roles in regulating renal medullary blood flow and tubular activity, and thereby maintaining the constancy of body fluid volume and arterial blood pressure. These genes in the renal medulla are up-regulated in response to high salt intake [6,13-16] and inhibition of these genes and/or the enzymes encoded by these genes within the renal medulla reduces sodium excretion and increases salt sensitivity of arterial blood pressure [6,9,13-14,17-19]. We have previously shown that high salt intake increases HIF-1 α levels in the renal medulla [11] and that inhibition of HIF-1 α blocks the activation of its target genes in the renal medulla in response to high salt intake, consequently promoting sodium retention and producing salt-sensitive hypertension [11]. It is suggested that HIF-1 α -mediated gene regulation in the renal medulla represents an important molecular adaptive mechanism in response to high salt intake and plays a crucial role in the maintenance of sodium balance.

HIF prolyl-hydroxylases are the major enzymes to promote the degradation of HIF-1 α [20-22]. HIF prolyl-hydroxylases catalyze site-specific proline hydroxylation of HIF-1 α using oxygen as a cofactor, and prolyl-hydroxylated HIF-1 α is then recognized and targeted for degradation by the ubiquitin-proteasome pathway. Although HIF prolyl-hydroxylases work as oxygen sensor to regulate the destruction of HIF-1 α [20-22], recent evidence has clearly shown that the activities and expressions of HIF prolyl-hydroxylases are also regulated independent of oxygen levels by a variety of factors [23-27].

Three isoforms of HIF prolyl-hydroxylase, including prolyl hydroxylase domain-containing proteins 1, 2, and 3 (PHD1, 2, and 3), have been identified [20-21,28]. It has been demonstrated that PHDs are present in the kidneys with PHD2 as the predominant isoform of PHDs [29-33] and PHD2 is most abundantly expressed in the renal medulla [29,33]. We have shown that inhibition of PHD activity increases the levels of HIF-1 α in the renal medulla and enhances pressure natriuresis, indicating that PHDs participate in the regulation of renal medullary function [29]. We have further demonstrated that high salt intake decreases PHD2 levels in the renal medulla and suggested that the adaptive activation of renal medullary HIF-1 α and its target genes in response to high salt challenge is mediated through reducing PHD2 levels [34]. Given the important role of high salt-induced adaptive response of HIF-1 α and its target genes in the renal medulla, we hypothesize that renal medullary PHD2 importantly participates in the renal adaptation to high salt challenge and sodium handling, and consequently, the long-term control of blood pressure in response to high salt challenge. We transfected PHD2 expression plasmids into the renal medulla to block the decrease of PHD2 levels after high salt challenge and examined its effect on sodium excretion after salt overloading and the effect on pressure natriuresis. Our results demonstrated that overexpression of PHD2 transgenes to disrupt the adaptive activation of

HIF-1 α in response to high salt challenge in the renal medulla inhibited sodium excretion and produced salt-sensitive hypertension.

Materials and Methods

Animal: Experiments were performed in male Sprague-Dawley rats (Harlan, Madison, WI), weighing 250-350 g. Animals were kept on a low salt diet (0.4% NaCl) and some of them were fed with a high salt diet (4% NaCl) during experiments as indicated in the results section. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Transfection of plasmids expressing rat full-length PHD2 cDNA into the renal medulla. Rats were uninephrectomized one week before, and the remaining left kidney was transfected with designated plasmids (50 μ g) into the renal medulla using *in vivo-jetPEI*[™] (Polyplus-transfection, New York, NY), a polyethylenimine derivative, in combination with ultrasound radiation. Previous studies showed that DNA was successfully delivered into the renal medulla using *in vivo-jetPEI*[™] [35] and that combination of ultrasound significantly enhanced DNA transfection with different transfection reagents [11,36-37] including polyethylenimine nanoparticles [38]. Plasmids encoding rat full-length PHD2 cDNA are generous gifts from Dr. Frank S. Lee (University of Pennsylvania). The expression and function of rat PHD2 protein by the plasmids have been validated by Dr. Lee's publications [39-40] and in our previous studies [29,34]. Plasmids expressing luciferase were used in control animals.

To deliver the plasmids into renal medulla, rats were anesthetized with 2.5% isoflurane and 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™* in 600 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 [41] in the middle and at the end of the infusion.

Our previous studies using a similar technique for DNA delivery into cells in the renal medulla showed that >90% of cells were transfected with no cell-type selectivity [11]. We also showed that the expression of transgene in the kidney peaked on around day 5-7 and gradually decreased thereafter, while the mRNA levels in transfected animals remained 4.5 times higher than that in control animals 4 weeks after transfection [42]. The *in vivo* expression time period of transgene in our studies is consistent with reports by others using non-viral vectors and different DNA delivery methods, which have shown that *in vivo* overexpressions of transgenes last for at least 2 or 4 weeks. [43-45].

Measurement of pressure natriuresis in response to the elevations of renal perfusion pressure. Animals were transfected with PHD2 or control plasmids as described above and maintained on low salt diet. Ten days after transfection, pressure natriuresis studies were performed as described previously [6,46]. In brief, after being anesthetized with ketamine (30 mg/kg i.m.) and thiobutabarbital (Inactin; 50 mg/kg

i.p.), the rats were placed on a thermostatically controlled warming table to maintain body temperature at 37°C. Catheters were placed in the left femoral vein for intravenous infusion and in the infrarenal aorta via femoral artery for monitoring renal perfusion pressure (RPP), respectively. A catheter was inserted into the ureter for urine collection.

After surgery, the animals received a continuous infusion of 0.9% NaCl solution containing 2% albumin at a rate of 1 ml/hr/100 g BW throughout the experiment. For pressure natriuresis, vasopressin (52 pg/min), aldosterone (20 ng/min), norepinephrine (100 ng/min), and hydrocortisol (20 µg/min) were included in the intravenous infusion solution to fix the circulating levels of these hormones as previously described [46]. After a 1-h equilibration period, RPP was acutely increased by tying off the celiac and mesenteric arteries and the RPP was set to 140, 110 and 80 mmHg, respectively, by an adjustable clamp placed on the aorta above renal arteries. At each RPP level, after a 10-min equilibration period, urine samples were collected during a 20-min clearance period. The urine flow rate was determined gravimetrically and sodium (Na⁺) concentrations of urine samples were measured using a flame photometer (Buck Scientific, East Norwalk, CT). Urine flow and urinary Na⁺ excretion were factored per gram kidney weight. The animals were euthanized with an excess intravenous dose of pentobarbital sodium (150 mg/kg) after experiments.

Measurement of urinary sodium excretion in response to acute sodium loading. Additional groups of animals transfected with PHD2 or control plasmids as above were surgically prepared similar to that in the pressure natriuresis studies and received a continuous infusion of 0.9% NaCl solution containing 2% albumin at a rate of 1 ml/hr/100g BW throughout the experiment to replace fluid loss. After a 1-hour equilibration and two 10-min control period sample collections, a 5% body weight isotonic saline load

was administered intravenously and three 10-min samples were collected over 30 minutes [11,47], and then three more 10-min post-control samples were taken. Urinary volume and sodium excretion were measured.

Measurement of daily sodium balance. Additional groups of animals the same as above were housed in metabolic cages and daily indexes of sodium balance were computed by subtracting urinary sodium excretion from total sodium intake. After 2 day control measurements, the animals were switched from tap water to 2% NaCl water and experimental measurements were continued for 4 days [48-49].

Chronic monitoring of arterial blood pressure in conscious rats. Mean arterial blood pressure (MAP) were monitored with a telemetry blood pressure measuring system. In brief, after rats were anesthetized with 2.5% isoflurane, a catheter connected with a telemetry transmitter was implanted into the infrarenal aorta via cannulating femoral artery, the transmitter was placed subcutaneously, and the RPP signal from the transmitter was received by a remote receiver, and then recorded by a computer program (Data Sciences International, DSI). Animals were housed with no restriction after recovery from anesthesia. MAP was recorded for 3 hrs each day at the same time period (12-3 pm). After baseline MAP was recorded on 3 consecutive control days while the rats remained on low salt diet, animals were switched to high salt diet (Dyets, Inc) and MAP was recorded for additional 3 weeks. Three groups of animals, including rats treated with control plasmids (Ctrl) + high salt diet (HS), PHD2 plasmids + HS and PHD2 + low salt diet (LS), were examined. At the end of experiment, renal tissues were collected for protein and RNA isolation later.

RNA extraction and quantitative RT-PCR analysis of PHD2 mRNA levels. Total RNA from the renal medulla 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 TaqMan Gene Expression Assays kits (Applied Biosystems). The level of 18S ribosomal RNA was used as an endogenous control. The relative gene expressions were calculated in accordance with the $\Delta\Delta C_t$ method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta C_t}$.

Preparation of tissue homogenate and nuclear extracts and Western blot analyses for protein levels of HIF-1 α . Nuclear protein samples from the renal medulla (50 μ g) were prepared as described previously [29] and subjected to 7% SDS-PAGE gel electrophoresis and electrophoretically transferred onto nitrocellulose membrane. The membranes were probed with antibody against HIF-1 α (monoclonal, 1:300, Novus Biologicals) overnight at 4°C [29,50]. The intensities of the blots were determined using an imaging analysis program (ImageJ, free download from <http://rsbweb.nih.gov/ij/>). The levels of β -actin were used as internal control.

Statistics. Data are presented as means \pm SE. The significance of differences in mean values within and between multiple groups was evaluated using an ANOVA followed by a Duncan's multiple range test. Student's *t*-test was used to evaluate statistical significance of differences between two groups. $P < 0.05$ was considered statistically significant.

Results

Effect of renal medullary transfection of PHD2 plasmids on the levels of PHD2 and HIF-1 α in the renal medulla. The PHD2 mRNA levels are summarized in figure 1A. In consistent with our previous findings [34], high salt challenge inhibited the mRNA levels of PHD2 and overexpression of PHD2 transgenes in the renal medulla blocked the decrease of PHD2 mRNA levels after high salt. As a result, high salt-induced increases in HIF-1 α protein levels were remarkably decreased in PHD2-transfected rats (Fig. 1B&C). These results verified the successful overexpression of PHD2 transgenes that inhibited the high salt-induced activation of HIF-1 α in the renal medulla.

Effects of renal medullary overexpression of PHD2 transgene on pressure natriuresis in response to the elevations of renal perfusion pressure (RPP). Both the urine flow and urinary sodium excretion rates were increased in response to the elevation of RPP. However, these pressure diuretic and natriuretic responses were significantly attenuated in PHD2-transfected rats compared with the control group (Fig. 2).

Effects of renal medullary overexpression of PHD2 transgene on urinary sodium excretion in response to acute sodium loading. Acute sodium loading increased urine volume (U·V) and urinary sodium excretion (U_{Na}·V). These increases in U·V and U_{Na}·V were considerably blunted in rats treated with PHD2 plasmids compared with control (Fig.3).

Effects of renal medullary overexpression of PHD2 transgene on salt balance. High salt intake induced a positive daily and cumulative salt balance. The daily positive salt balances were progressively increased in the first two days and decreased on the third day of high salt intake. The high salt-induced positive salt balance was significantly enhanced in rats treated with PHD2 plasmids compared with control rats (Fig. 4).

Effects of renal medullary overexpression of PHD2 transgenes on long-term arterial blood pressure after high salt challenge. The mean arterial pressures (MAP) changes are presented in figure 5. After the rats were challenged with a high salt diet, the MAP was progressively increased in rats treated with PHD2 plasmids compared with that in control rats. However, there was no change in MAP in rats treated with PHD2 plasmids if the rats were not challenged with high salt diet (Fig. 5).

Discussion

The present study showed that overexpression of PHD2 transgene diminished high salt-induced activation in HIF-1 α in the renal medulla and remarkably impaired the renal medullary function as indicated by reduced sodium excretion, blunted pressure natriuretic responses and increased sodium retention, and consequently, producing salt-sensitive hypertension.

Our results showed that local overexpression of the PHD2 transgene blocked the high salt-induced decrease in PHD2 levels and subsequently inhibited the activation of HIF-1 α in the renal medulla after high salt challenge, which validated the regulatory function of PHD2 on HIF-1 α in the renal medulla.

Given the important role of HIF-1 α in renal adaptive response to high salt intake and kidney sodium handling [11], manipulation of HIF-1 α by PHD2 transgene allowed us to evaluate the role of PHD2 in the regulation of sodium excretion and long-term control of blood pressure in response to high salt intakes.

We first determined the effects of PHD2 transgene on pressure natriuresis. Renal medullary function plays an important role in the regulation of pressure natriuresis [7,51-53] and several HIF-1 α target genes such as HO-1, COX-2 and NOS have been reported to be crucial regulators in renal medullary function and sodium excretion, as well as pressure natriuresis [12,53-56]. Reducing HIF-1 α levels by PHD2 transgene would be expected to impair renal medullary function and blunt pressure natriuresis relationship. Our data showed that a normal PHD2 behavior played a crucial role in the regulation of renal medullary function and pressure natriuresis.

To further evaluate the role of renal medullary PHD2 in the regulation of renal salt handling, we examined sodium excretion after acute sodium loading and salt balance after chronic sodium challenge.

Our data showed that overexpression of the PHD2 transgene remarkably impaired the capability of the kidneys to remove extra sodium load and resulted in sodium retention. The results from these extra sodium loading experiments demonstrated that overexpression of the PHD2 transgene disrupted the renal adaptive response to sodium overloading, additionally suggesting that inhibition of renal medullary PHD expression by high salt intake is an important determinant to promote sodium excretion after high salt challenge.

Since pressure-natriuresis and normal renal medullary function are key determinants to the long-term control of arterial blood pressure [7,10,51-52,57], overexpression of PHD2 transgenes in the renal medulla, which impaired sodium excretions in responses to renal perfusion pressure and extra sodium loading, would lead to an increase in MAP in response to high salt intake. To test this hypothesis, we compared MAPs between animals transfected with PHD2 and control plasmids into the renal medulla. It was found that high salt challenge significantly increased the MAP in PHD2-treated rats, but not in control rats, whereas PHD2-treated rats did not develop hypertension when they were not challenged with high salt. These results suggested that high salt-induced inhibition of PHD2 is an important mechanism in the regulation of salt sensitivity of arterial blood pressure, probably via governing the renal sodium handling through regulating the HIF-1 α pathway.

On the other hand, an impaired response of PHD2 facing high salt challenge, as performed in the present study, could be responsible for the development of salt sensitive hypertension. In this regard, it was interesting that high salt-induced inhibition of PHD2 in the renal medulla was absent in the Dahl salt sensitive hypertensive rat, a widely used genetic model of human salt-sensitive hypertension [34]. Given the significant importance of renal medullary PHD2 in the regulation of sodium excretion and renal adaptation to high salt intake shown in the present study, the impairment in PHD2 response to high salt challenge would be very possibly the pathogenic mechanism in Dahl S rats. Detailed roles of renal medullary PHD2 in the development of hypertension in Dahl S rats require future investigation.

In summary, the present study demonstrated that disruption of high salt-induced inhibition of PHD2 and subsequently adaptive activation of renal medullary HIF-1 α in response to high salt challenge impaired renal medullary function and kidney salt handling, thereby increasing the salt sensitivity of arterial

pressure. It is concluded that PHD2 in the renal medulla is an important regulator in renal adaptation to high salt intake and that a defect in PHD2-associated molecular adaptation in response to high salt intake in the renal medulla may represent a pathogenic mechanism producing salt sensitive hypertension.

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Conflict of Interest

None

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Figure legends

Figure 1. Effect of renal medullary transfection of PHD2 plasmids on the levels of PHD2 and HIF-1 α in the renal medulla. A: PHD2 mRNA levels; B: Representative ECL gel documents of Western blot analyses depicting the protein levels of HIF-1 α ; C: Summarized intensities of the HIF-1 α blots (normalized to LS). * P < 0.05 vs. others (n=7-8). LS = low salt, HS = high salt, Ctrl = control vectors, PHD2 = PHD2 expression vectors, CoCl₂ = sample from cells treated with CoCl₂ as positive control. Renal medullary tissues were obtained at the end of blood pressure recording after 3 week high salt challenge.

Figure 2. Effects of renal medullary overexpression of PHD2 transgene on pressure natriuresis in response to the elevations of renal perfusion pressure (RPP). A: urine flow rates (U·V) in response to the elevations of RPP. B: urinary sodium excretion rates (U_{Na}·V) in response to the elevations of RPP. * P < 0.05 vs. PHD2 (n=6). Ctrl = control vectors, PHD2 = PHD2 expression vectors.

Figure 3. Effects of renal medullary overexpression of PHD2 transgene on U·V (A) and U_{Na}·V (B) in response to acute Na⁺ loading. * P < 0.05 vs. PHD2 (n=6). S=sodium loading; P=post sodium loading. Ctrl = control vectors, PHD2 = PHD2 expression vectors.

Figure 4. Effects of renal medullary overexpression of PHD2 transgene on salt balances. A: daily sodium balance. B: cumulative sodium balance. * P < 0.05 vs. control (n=8). Ctrl = control vectors, PHD2 = PHD2 expression vectors.

Figure 5. Effects of renal medullary overexpression of PHD2 transgene on mean arterial pressure (MAP). * P < 0.05 vs. others. (n=7-8). LS = low salt, HS = high salt, Ctrl = control vectors, PHD2 = PHD2 expression vectors.

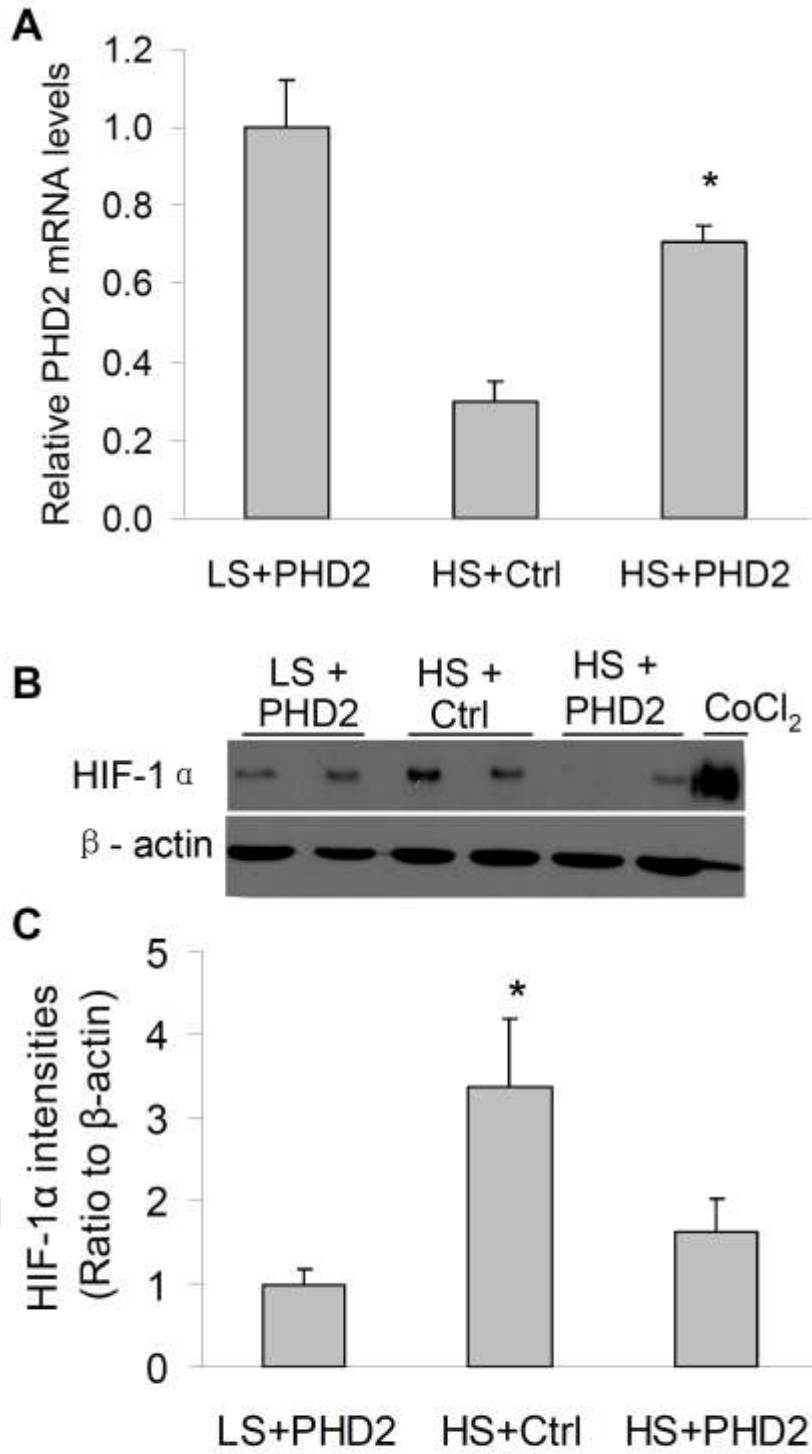


Figure 1

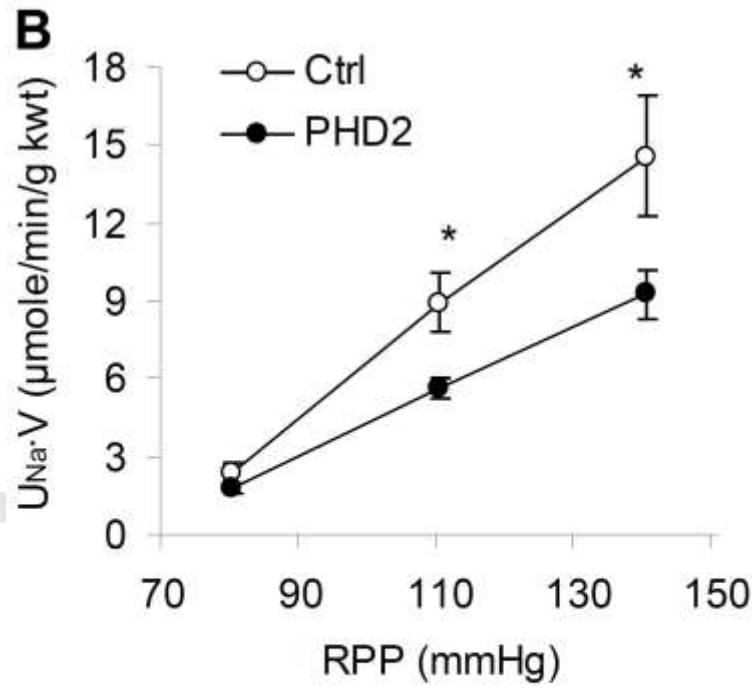
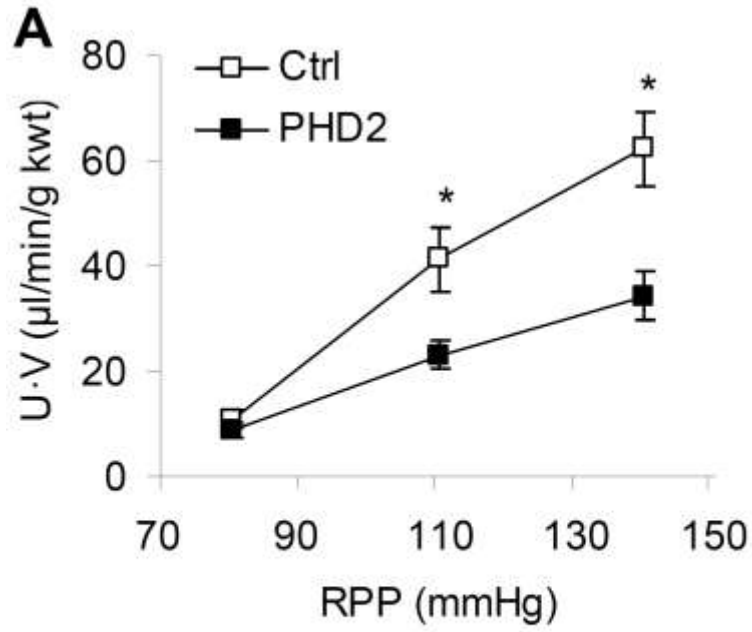


Figure 2

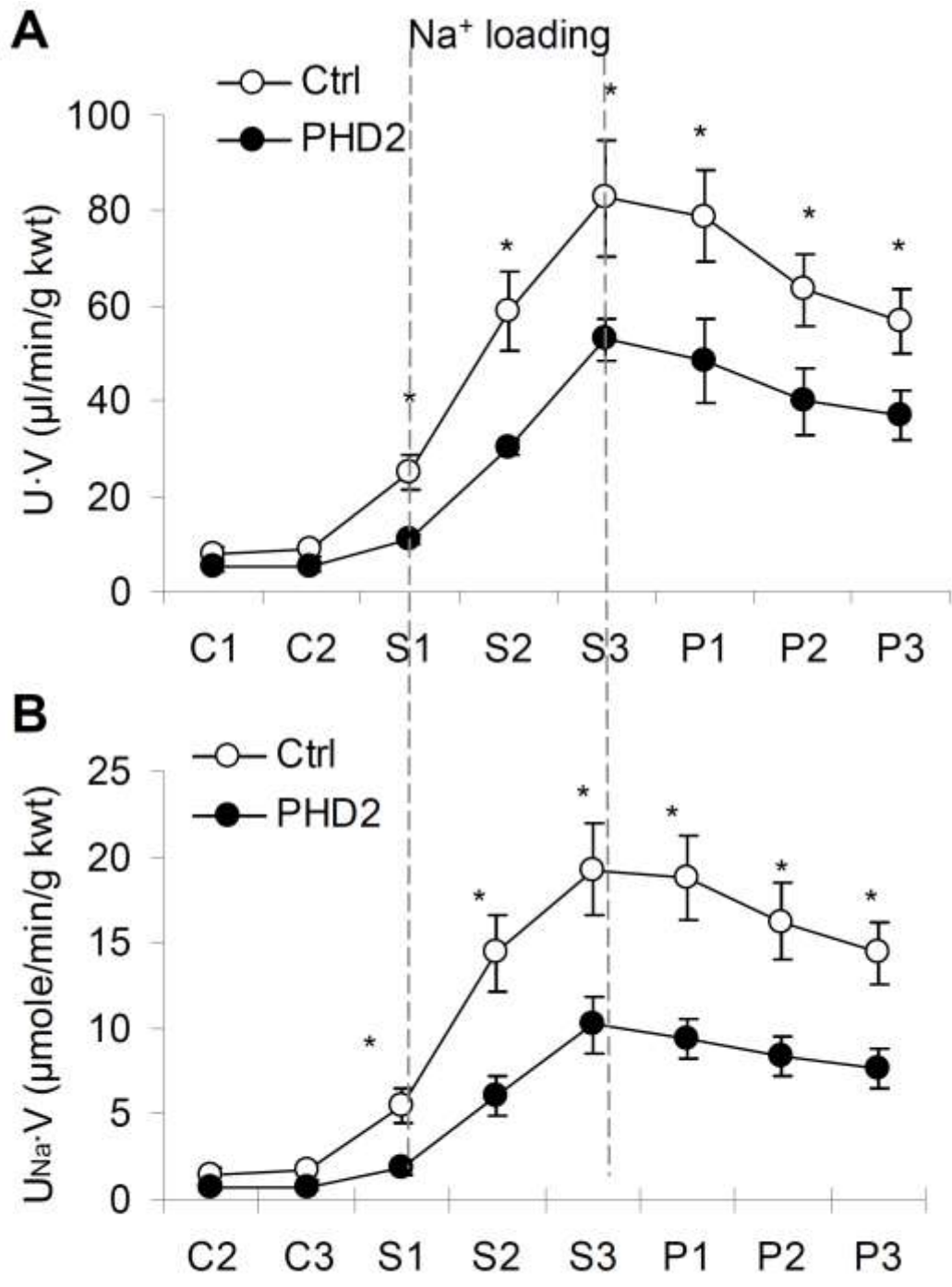


Figure 3

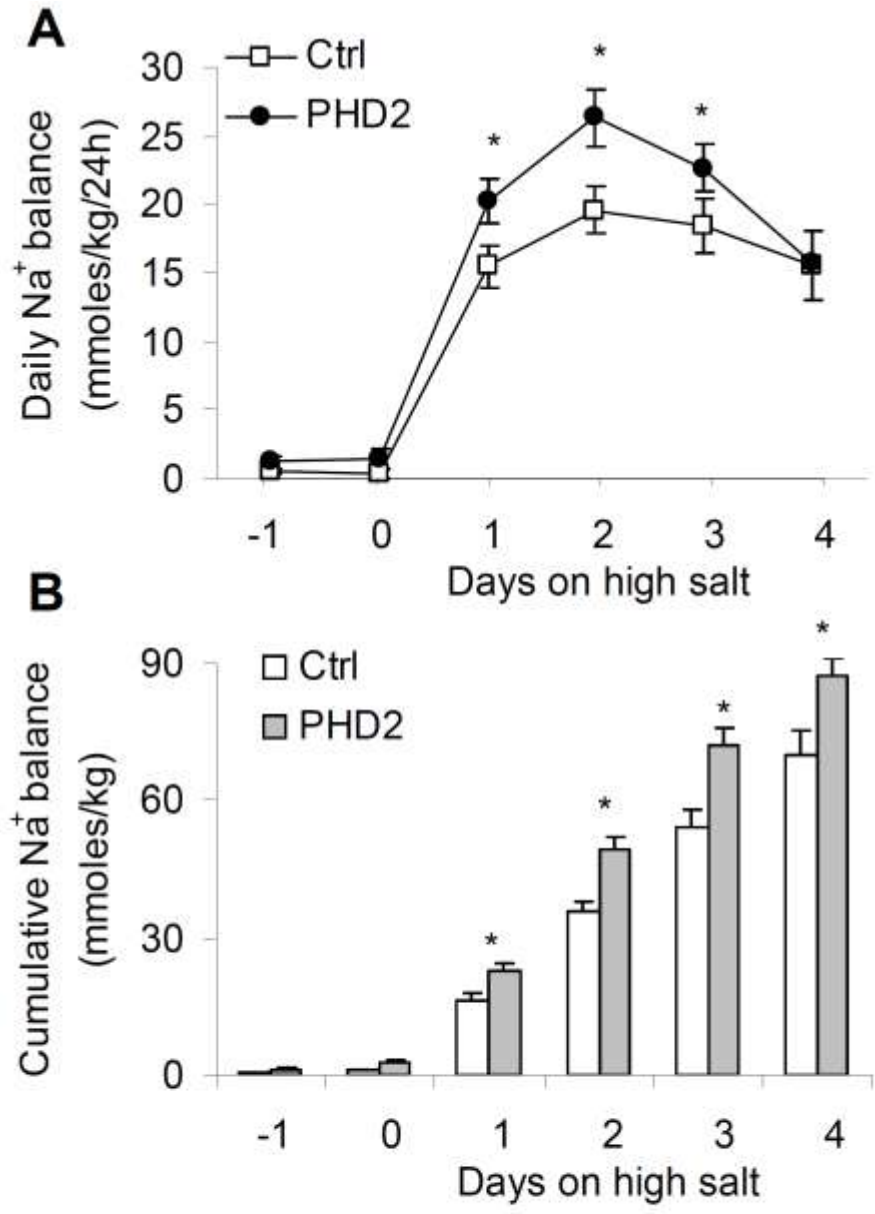


Figure 4

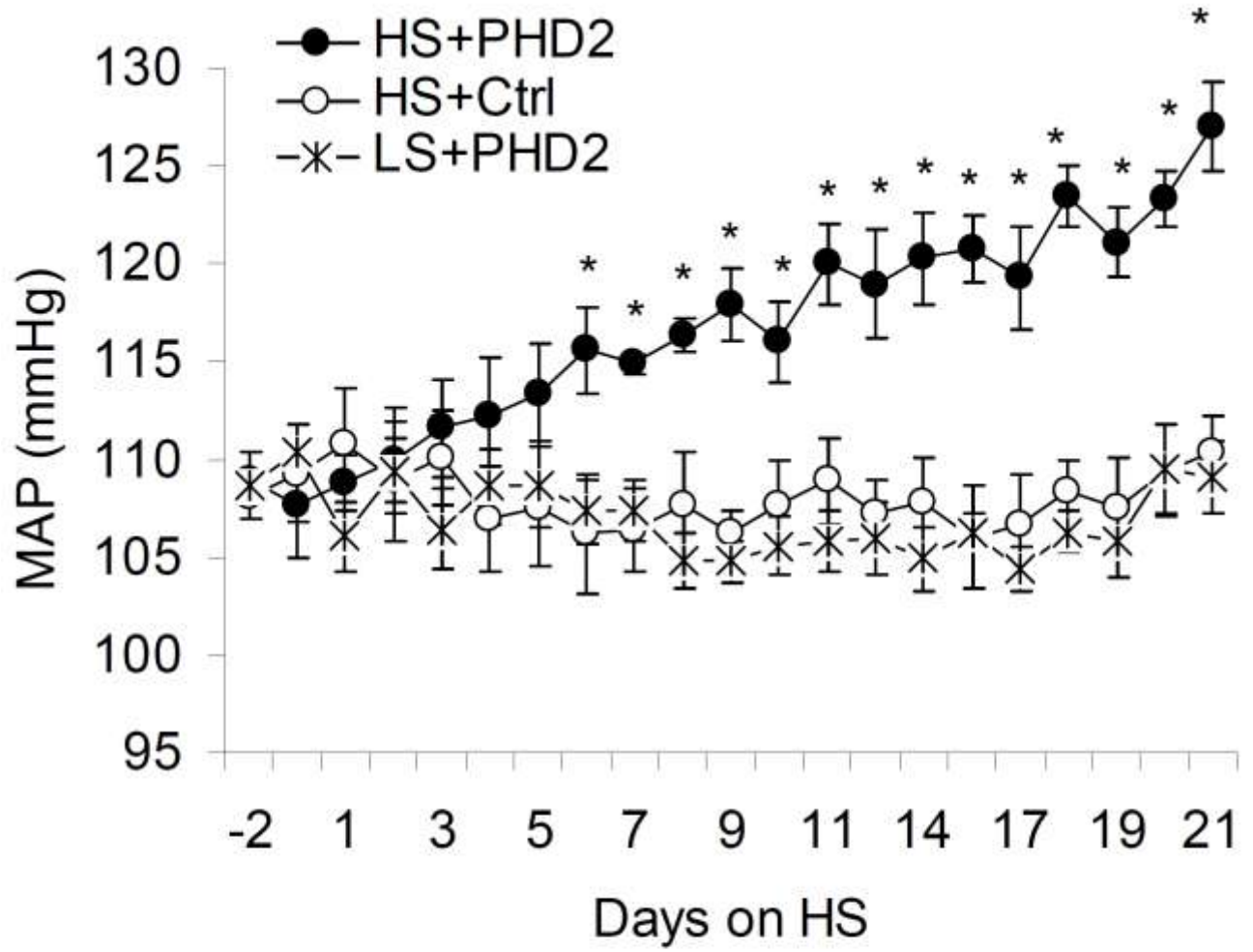


Figure 5