Salt-Sensitive Hypertension Induced by Decoy of Transcription Factor Hypoxia-Inducible Factor-1α in the Renal Medulla

Ningjun Li, Li Chen, Fan Yi, Min Xia, Pin-Lan Li

Abstract—Hypoxia inducible factor (HIF)-1α, a transcription factor, is abundantly expressed in the renal medulla and regulates many oxygen-sensitive genes such as nitric oxide synthase, cyclooxygenase-2, and heme oxygenase-1. Given the important roles of these genes in the control of arterial pressure, the present study was to test the hypothesis that HIF-1α-mediated gene activation serves as an antihypertensive pathway by regulating renal medullary function and sodium excretion. HIF-1α decoy oligodeoxynucleotides (ODNs) or scrambled ODNs were transfected into the renal medulla in uninephrectomized Sprague–Dawley rats. Two weeks after ODN transfection, the HIF-1α binding activities were significantly inhibited by 45%, and high salt–induced increases of nitric oxide synthase-2 and heme oxygenase-1 transcriptions were also inhibited by 70% and 61% in the renal medulla from decoy rats. The natriuretic responses and increases of renal medullary blood flow responding to the elevations of renal perfusion pressure were significantly blunted by 50% and 37% in decoy rats. Intravenously acute sodium loading increased medullary blood flow and urinary sodium excretion, which was remarkably attenuated in decoy rats. In decoy rats, high salt intake caused a greater positive sodium balance. Consequently, arterial pressure was remarkably increased (from 118±1.9 to 154±6.3 mm Hg) in decoy rats but not in control rats when the rats were challenged with a high salt diet. There was no blood pressure change in decoy rats that were maintained in normal salt diet. In conclusion, HIF-1α–mediated gene activation importantly participates in the regulation of renal medullary function and long-term arterial blood pressure. (Circ Res. 2008;102:0-0.)

Key Words: fluid homeostasis ■ urinary sodium excretion ■ pressure natriuresis ■ and renal hemodynamics

It is well documented that renal medullary functions play an important role in the regulation of renal sodium excretion and arterial blood pressure.1–3 Many enzymes producing antihypertensive factors such as nitric oxide synthase (NOS), cyclooxygenase (COX)-2, and heme oxygenase (HO)-1 are highly expressed in this kidney region.4–10 These enzymes in the renal medulla are upregulated in response to high salt intake,5,8–11 and inhibition of these enzymes within the renal medulla reduces sodium excretion and increases salt sensitivity of arterial blood pressure.5–8,10,12–14 In salt-sensitive hypertensive animal models, renal medullary levels of these enzymes are much lower15–17 and their responses to high salt diet and angiotensin II are diminished.8,15,18,19 There is a general agreement that the medullary protective factors produced by these enzymes play critical roles in regulating renal medullary blood flow and tubular activity, which is essential in maintaining the constancy of body fluid volume and arterial blood pressure. However, the mechanisms mediating the activation of these enzymes in the renal medulla are not clear.

Recent studies have indicated that the regulations of the protective factor–producing enzymes described above are associated with transcriptional expression of the genes encoding these enzymes in the renal medulla. These genes are oxygen-sensitive genes and regulated by hypoxia-inducible factor (HIF)-1α,20–22 a transcription factor that is also highly expressed in the renal medulla23–25 because of the low oxygen levels in this kidney region.26–29 HIF-1α has been demonstrated as a master regulator of adaptation to hypoxia and activates gene transcription of many oxygen-sensitive genes, including NOS, COX-2, and HO-1.20,21,30–32 Activation of these genes in the renal medulla leads to vasodilation and inhibition of tubular activity, which maintains the renal medullary function and consequently contributes to the control of sodium excretion and blood pressure.2–4,22,33 Therefore, renal medullary functions are, in fact, associated with the hypoxic gene regulation in this area.

Although it is evident that HIF-1α regulates the transcriptional expressions of these enzymes in the renal medulla, it remains unknown whether this HIF-1α–mediated gene activation is of physiological relevance in the control of renal function, in particular, the renal medullary function. The present study was designed to test the hypothesis that HIF-1α
mediates the activation of the oxygen-sensitive genes such as NOS, COX-2, and HO-1 in the renal medulla and thereby participates in the control of renal medullary functions and consequently regulates blood pressure. We transfected HIF-1α decoy oligodeoxynucleotides (ODNs) into the renal medulla to inhibit the binding activity of HIF-1α and examined the effect of HIF-1α on pressure natriuresis and renal cortical and medullary blood flows in response to the elevations of renal perfusion pressure (RPP) and sodium loading and then determined the chronic effect of this HIF-1α decoy on arterial blood pressure. To our knowledge, the present study provides the first evidence that HIF-1α-mediated gene regulation plays an important role in the regulation of renal medullary function and long-term control of blood pressure.

Materials and Methods

Animals

Experiments were performed on male Sprague–Dawley rats (Harlan Inc) weighing 250 to 300 g. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Renal Medullary Transfection of HIF-1α Decoy Oligodeoxynucleotides

Double-stranded HIF-1α hypoxia-responsive elements (HREs) containing ODNs (decoy ODNs) and scrambled ODNs were prepared based on the sequence reported before.22,24 The ODNs were phosphorothioate-modified, which is the most extensively used modification to significantly enhance nucleases resistance and increase the affinity and potency of the ODNs.33,35 In uninephrecto-
mized rats, 0.6 mL of mixture containing 40 nmol of decoy ODNs or scrambled ODNs and microbubble (Optison) at a ratio of 1:1 was infused into renal medulla at a speed of 10 μL/min. An ultrasound transducer (Ultax UX-301) was directly applied onto the kidneys with a continuous-wave output of 1-MHz ultrasound at 5% power output, for a total of 60 seconds with 30-second intervals in the middle and at the end of the infusion. This ultrasound-microbubble technique has been shown to effectively deliver DNA into cells in the kidneys with a >90% of transfection rate without toxicity to the kidney.37–40 To confirm the delivery of ODNs into the cells, fluorescein isothiocyanate (FITC)-labeled ODNs were transfected into the renal medulla and renal cryostat sections were examined using a fluorescent microscope at days 2 and 10 after transfection. We also performed in vitro experiments to test the transfection and inhibition efficiency of decoy ODNs in cultured renal medullary interstitial cells. For the details of these and the following methods, see the expanded Materials and Methods section in online data supplement, available at http://circres.ahajournals.org.

Chronic Monitoring of Arterial Blood Pressure in Conscious Rats

A telemetry transmitter (Data Sciences International) was implanted for the measurement of mean arterial blood pressure (MAP) as we described previously.10 After baseline MAP was recorded on 3 consecutive control days, while the rats remained on the 1% salt diet, animals were switched to a high salt diet containing 8% NaCl (Dyets Inc), and MAP was recorded for additional 10 days. Three groups of animals, including rats treated with scrambled ODNs plus high salt, decoy ODNs plus high salt HS, and decoy ODNs plus normal salt, were examined. At the end of experiment, renal tissues were collected for protein and RNA isolation later.

Preparation of Renal Tissue Nuclear Extracts and Analyses of HIF Banding Activity

Renal tissue nuclear protein was prepared as we described previously25 and by others.44 HIF-1α banding activities in the nuclear extracts were detected using an ELISA-based HIF binding kit (Panomics). The ELISA-based HIF binding assay kit provides a fast, sensitive, and specific measurement for the HIF-1α binding activities.42

Western Blot Analysis of HIF-1α Protein Levels in Renal Tissue Nuclear Extracts

Nuclear protein samples from renal medulla (50 μg) were subjected to 7% SDS-PAGE gel electrophoresis and electrophoretically transferred onto nitrocellulose membrane. The membranes were probed with antibodies (1:500, Novus Biologicals) against HIF-1α (monoclonal) and HIF-1β (rabbit anti-rat) overnight at 4°C. After washing, the membranes were incubated with IRDye 680 anti-mouse IgG and IRDye 800CW anti-rabbit IgG (Li-Cor Biosciences) as secondary antibodies (1:7500, 60 minutes at room temperature) and then processed with an Odyssey Infrared Imaging System (Li-Cor Biosciences) to obtain fluorescent images and intensities of the blots.

RNA Extraction and Quantitative RT-PCR Analysis of HO-1 and NOS2 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 TagMan Gene Expression Assays kits for rat HO-1 and NOS2 mRNA levels (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.

Measurement of Pressure Natriuresis and Renal Cortical and Medullary Blood Flows in Response to the Elevations of RPP

Animals were transfected with decoy ODNs or scrambled ODNs as described above and maintained on a normal salt diet. Ten days after ODN transfection, pressure natriuresis studies were performed as described previously.43 Optical fiber needle probes (Transonic) were implanted to simultaneously measure cortical (1.5 mm depth) and medullary (5 mm depth) blood flows using a dual-channel laser-Doppler flowmeter (Transonic) as described previously.44 Glomerular filtration rate was measured using FITC-inulin (Sigma) as described previously.45 Glomerular filtration rate, urine flow, and urinary Na⁺ excretion were factored per gram of kidney weight.

Measurement of Urinary Sodium Excretion and Renal Cortical and Medullary Blood Flows in Response to Acute Sodium Loading

Additional groups of animals, the same as above, were surgically prepared as in the pressure natriuresis studies and received a continuous infusion of 0.9% NaCl solution containing 2% albumin at a rate of 1 mL/h per 100 g of body weight throughout the experiment to replace fluid loss. After 1-hour equilibration and two 10-minute control period sample collections, a 5% body weight isotonic saline load was administered intravenously and three 10-minute samples were collected over 30 minutes,46 and then 3 more 10-minute postcontrol samples were taken. Urinary volume, sodium excretion, MAP, and renal cortical and medullary blood flow (CBF and MBF) 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 1 day of control measurements, the animals were switched from tap water to 2% NaCl water, and experimental measurements were continued for 3 days.47,48

Statistics

Data are presented as means±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**

**Effects of Renal Medullary Transfection of HIF-1α Decoy ODNs on HIF Binding Activity and the mRNA Expressions of HO-1 in the Renal Medulla**

On renal cryostat sections from FITC-ODN–transfected animals both at days 2 and 10 after transfection, fluorescent microscopic examinations showed strong fluorescence within the renal medullary cells compared with control and >90% of cells were transected with no cell-type selectivity. Figure 1A is a representative fluorescent image. In animals transfected with decoy ODNs, HIF-1α binding activities in the renal medulla were significantly inhibited compared with that in scrambled ODN–transfected animals (Figure 1B). To verify the inhibitory effect of HIF-1α decoy on the transcription of HIF-1α target genes, mRNA expressions of HO-1 as a prototype of HIF target gene were evaluated. HO-1 mRNA levels were remarkably decreased in decoy ODN–transfected rats compared with control rats (Figure 1C). These results verified the successful delivery and sustenance of ODN transfection in the renal medullary cells and the inhibition of HIF-1α transcriptional activity during the experimental time period. The data from in vitro experiments in cultured cells also confirmed the inhibition of HIF-1α transcription functions by decoy ODNs (see the online data supplement).

**Effects of High Salt Intake and Renal Medullary Transfection of HIF-1α Decoy ODNs on HIF-1α Protein Levels and Transcriptions of HIF-1α Target Genes in the Renal Medulla**

High salt intake significantly increased the protein levels of HIF-1α and the transcriptions of HO-1 and NOS2. HIF1α decoy did not affect HIF-1α protein levels but significantly inhibited the increases of HO-1 and NOS2 mRNA levels induced by high salt intake (Figure 2).

**Effects of Renal Medullary Transfection of HIF-1α Decoy ODNs on Pressure Natriuresis and Renal Cortical and Medullary Blood Flow in Response to the Elevations of RPP**

Both the urine flow and urinary sodium excretion rates were remarkably increased in response to the elevation of RPP. However, these pressure diuretic and natriuretic responses were significantly blunted in HIF-1α decoy group compared with the control group (Figure 3). There was no significant difference in the responses of glomerular filtration rate to RPP between 2 groups of rats when RPP was elevated (data not shown).

The renal CBF and MBF were presented as the percentage of the values at RPP of 80 mm Hg as 100% (Figure 4). The CBF was significantly increased by 24% when RPP was elevated from 80 to 120 mm Hg. However, the CBF was not further increased when RPP was increased from 120 to 160 mm Hg (Figure 4A). There was no difference in the responses of CBF to the elevations of RPP between control animals and decoy ODN–transfected animals. The MBF was also significantly increased by 33% when RPP was elevated from 80 to 120 mm Hg and increased by 60% when the RPP was further elevated to 160 mm Hg in control animals. In contrast, in the animals treated with decoy ODNs the increases in MBF were only 17% and 33%, respectively, when the RPP was elevated from 80 to 120 and 160 mm Hg (Figure 4B). When comparing the RPP-induced increases in CBF and in MBF, elevations of RPP caused a more profound change in MBF than that in CBF in control rats.
Effects of Renal Medullary Transfection of HIF-1α Decoy ODNs on Urinary Sodium Excretion and Renal Cortical and Medullary Blood Flows in Response to Acute Sodium Loading

Acute sodium loading dramatically increased MBF, urine volume (U·V) and urinary sodium excretion (UNa·V). There is no significant change in CBF (data not shown). These increases in MBF, U·V, and UNa·V were considerably attenuated in HIF-1α decoy–treated rats. MAP was also increased during acute sodium loading and returned after sodium loading in control animals. However, in HIF-1α decoy–treated animals, increase of MAP during acute sodium loading was more significant compared with control and sustained after sodium loading (Figure 5).

Effects of Renal Medullary Transfection of HIF-1α Decoy ODNs 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 greater in HIF-1α decoy–treated rats than that in control rats (Figure 6).

Effects of Renal Medullary Transfection of HIF-1α Decoy ODNs on Arterial Blood Pressure

There was no difference in baseline MAP between HIF-1α decoy ODN–treated rats and scrambled ODN–treated rats when the animals were fed with a normal salt diet (1% NaCl). After the rats were challenged with a high salt diet (8% NaCl) for 10 days, the MAPs were progressively increased from 118±1.9 to 154±6.3 mm Hg in decoy ODN–treated rats, whereas no significant increases of MAP was observed in scrambled ODN–treated rats. When decoy ODN–treated rats were maintained on normal salt diet, there was no increase in MAP (Figure 7).
Discussion

The present study demonstrated that renal medullary transfection of HIF-1α decoy ODNs blocked the transcriptional activity of HIF-1α and inhibited the expression of its target genes in the renal medulla and consequently attenuated the increases of renal MBF and urinary sodium excretion in response to the elevations of RPP and sodium loading, which promoted a sodium retention and, as a result, induced a salt-sensitive hypertension in uninephrectomized Sprague–Dawley rats.

Transcription factor decoy ODNs, by blocking transcription factor–chromosomal DNA interaction, have been proven to be a powerful means to manipulate the regulation of gene expression in both in vitro and in vivo studies.35,49 The in vivo decoy effects have been reported to last up to 4 weeks.50 In the present study, locally delivery of HIF-1α decoy ODNs substantially blocked the HIF-1α binding and inhibited the transcription of the target genes of HIF-1α in the renal medulla, which is consistent with previous studies in which HIF-1α decoy blocked HIF-1α–mediated gene activation.22,34 High salt intake significantly upregulated the renal medullary levels of HIF-1α, HO-1, and NOS2, as reported previously.10,51,52 HIF-1α decoy blocked high salt–induced increases of HO-1 and NOS2 transcriptions without effect on HIF-1α expression. These results validated the inhibitory effect of HIF-1α decoy on transcriptions of its target genes in the renal medulla, which allowed us to evaluate the role of HIF-1α-mediated gene activation in the regulation of renal medullary function in response to high salt intakes.

We first determined the effects of inhibition in HIF-1α–mediated gene activation on pressure natriuresis. Because renal medullary function plays an important role in the regulation of pressure natriuresis3,33,53,54 and several HIF-1α target genes such as NOS, COX-2, and HO-1 have been reported as crucial regulators in renal medullary function and sodium excretion, as well as pressure natriuresis,4,54–57 modification of HIF-1α binding activities would be expected to change the levels of these HIF-1α target genes, thereby altering pressure natriuresis relationship. Our data showed that HIF-1α decoy in the renal medulla significantly blunted the pressure natriuresis, suggesting that HIF-1α, possibly through the actions on its target genes, is importantly involved in regulation of renal medullary function. Because the products of enzymes encoded by these HIF-1α target genes have been
shown to dilate the medullary vasculature and inhibit the tubular activities,\textsuperscript{3,33,58} the effect of HIF-1α-mediated pathway on pressure natriuresis may be through both vascular and tubular actions.

Our results showed that inhibition of HIF-1α transcriptional activity significantly impacted the effect of RPP on sodium excretion. On the other hand, there is a question concerning the influence of RPP on HIF-1α activities/levels. HIF-1α may respond to RPP, which could represent a mechanism mediating renal adaptation for RPP alteration. However, in the present study, RPP changes were acutely induced and lasted for a very short period. Therefore, the present study could not address whether high RPP can activate or inhibit HIF-1α functions and/or expression. Nonetheless, the effect of chronic alterations of RPP on HIF-1α is worth further investigation to advance our understanding of the role of HIF-1α-mediated gene activation in the control of renal function.

It has been known that MBF is one of the important determinants to pressure natriuresis\textsuperscript{2,3,33,57} and that products of many enzymes encoded by HIF-1α target genes such as NOS, COX-2, and HO-1 regulate MBF or renal sodium excretion.\textsuperscript{4,54–57} It is, therefore, of interest to investigate the role of HIF-1α-mediated gene activation in the regulation of MBF. In the present study, we compared the changes of CBF and MBF in response to RPP between decoy ODN– and scrambled ODN–treated rats. Consistent with the previous studies, our experiment showed that CBF was correspondingly increased when RPP was elevated during a lower range of RPP, whereas CBF was maintained the same when RPP was increased from 120 to 160 mm Hg, demonstrating an autoregulation of CBF.\textsuperscript{59,60} However, the values of MBF were correlated with the levels of RPP at all ranges, suggesting a poor autoregulation of MBF.\textsuperscript{59,60} It has been shown that increases in RPP are associated with significant increases of many vasodilators, including CO, NO, prostaglandin E2, and kinins, especially in the renal medulla.\textsuperscript{2,3,10,33,53,56} Therefore, inhibition of the enzymes producing these vasodilators by blocking HIF-1α mediated gene activation in the present study would be expected to attenuate the increases of MBF induced by elevation of RPP. Indeed, RPP-induced increases in MBF were significantly lower in HIF-1α decoy rats than in control rats, indicating that HIF-1α–mediated pathway is of importance in determining the RPP-induced elevation of MBF.

To further evaluate the impact of renal medullary HIF-1α decoy on salt handling, we examined the sodium excretion after acute sodium loading and salt balance after chronic sodium challenge. The results from these experiments demonstrated that renal medullary HIF-1α decoy remarkably impaired the capability of the kidneys to remove extra sodium load, which resulted in a sodium retention and sustained increase in MAP. These data additionally suggest that renal medullary HIF-1α is a crucial determinant in the regulation of sodium excretion.

Because pressure-natriuresis and normal renal medullary function are key determinants to the long-term control of arterial blood pressure,\textsuperscript{2,3,33,53,61} the inhibitory effect of HIF-1α decoy on sodium excretions and MBF in response to RPP 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 renal medullary decoy ODN– and scrambled ODN–treated rats. Although the baseline MAPs were not significantly increased in HIF-1α decoy rats, high salt challenge dramatically increased the MAP in these HIF-1α decoyed rats but not in control rats. HIF-1α decoyed rats did not develop hypertension when they were not challenged with high salt. These data indicate that

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**Figure 6.** Effects of renal medullary transfection of HIF-1α decoy ODNs on salt balances. A, Daily sodium balance. B, Cumulative sodium balance. *P*<0.05 vs control. N=6.

**Figure 7.** Effects of renal medullary transfection of HIF-1α decoy ODNs on MAP. *P*<0.05 vs other low groups. N=14.
HIF-1α–mediated pathway importantly participates in high salt adaptation of the renal medulla. High salt intake has been reported to increase the renal medullary tubular activities, which may result in a further decrease of oxygen level in this area. It has been demonstrated that in response to high salt intake HIF-1α51 and its target genes, such as NO-S, HO-1, and COX-2, are upregulated in the renal medulla.5,8–11,52 Activation of these genes by high salt intake would increase the production of corresponding protective factors such as NO, CO, and prostaglandins. These factors could dilate vasa recta, increase renal medullary blood flow, inhibit tubular ion transport activity, and therefore increase sodium excretion to maintain sodium balance. Therefore, high salt–induced activation of HIF-1α–regulated pathways is considered as an adaptive mechanism to high salt intake, which leads to an induction of various protective factors and consequent promotion of extra sodium excretion. Deficiency of HIF-1α–mediated gene transcription in the renal medulla may decrease the production of various protecting factors, impair renal medullary function, prevent excretion of extra salt intake, consequently disrupting salt adaptation, and increase the salt sensitivity of arterial blood pressure. The results from the present study suggested that HIF-1α–mediated gene activation may be a common mechanism regulating the expression of various protecting factors in the renal medulla, thereby exerting an antihypertensive action when animals are exposed to high salt challenge. Further investigation is required to determine which of the many factors downstream of the HIF-1α pathway is the major mediating factor of salt sensitivity of blood pressure and what the relationship is among these downstream factors.

In summary, the present study demonstrated that inhibition of HIF-1α binding activity in the renal medulla by HIF-1α decoy ODNs downregulated the transcription of HIF target gene and induced a resetting of the pressure natriuresis, reduction of RPP-induced elevation of MBF, inhibition of sodium excretion, and promotion of sodium retention. As a result, decoy of HIF-1α in the renal medulla produce a salt-sensitive hypertension. It is concluded that HIF-1α–mediated gene activation importantly regulates the gene expression and production of different renal medullary protective or antihypertensive factors, which tonically control renal medullary function and arterial blood pressure.

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Disclosures
None.

References


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Salt-Sensitive Hypertension Induced by Decoy of Transcription

Factor HIF-1α in the Renal Medulla

**Extended Method**

*Renal medullary transfection of HIF-1α decoy oligodeoxynucleotide (ODN).* HIF-1α HRE containing ODN (decoy ODN) and scrambled ODN were synthesized based on the sequence reported before \(^1,^2\). The ODN were phosphorothioate-modified (Operon), which is the most extensively used modification to significantly enhance nucleases resistance and increase the affinity and potency of the ODN \(^3,^4\). Before use, both sense and antisense ODN (100 µmol/L in TE buffer, pH 8.0) were heated at 95°C for 5 min and then cooled slowly down to room temperature to make double-strand ODN. The rats were first uninephrectomized to remove the right kidney after anesthetized with an intramuscular injection of ketamine (60 mg/kg) and xylazine (5 mg/kg). One week after uninephrectomy, surgeries were performed to transfet decoy ODN or scrambled ODN as control into the renal medulla. For delivery of ODNs, 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 40 nmoles of HIF-1α decoy ODN or scrambled ODN in saline with microbubble (Optison, GE HealthCare) at a ratio of 1:1 \(^5,^8\) in 0.6 ml was infused into renal medulla at a speed of 10 µl/min. After infusion, the catheter was cut and blocked by a piece of fat tissue with Vetbond Tissue Adhesive. An ultrasound transducer (Ultax UX-301; Celcom...
Medico Inc., Japan) was directly applied onto the kidney with a continuous-wave output of 1-MHz ultrasound at 5% power output, for a total of 60 s with 30-s intervals in the middle and at the end of the infusion. This ultrasound-microbubble technique has been shown to effectively deliver plasmid into cells in the kidneys with a >90% of transfection rate without toxicity to the kidney. To confirm the delivery of HIF-1α decoy ODN into the cells, FITC-labeled ODN were transfected into the renal medulla and renal cryostat sections were examined using a fluorescent microscope at day 2 and day 10 after transfection.

Effect of transfection of HIF-1α decoy-ODN on HIF-1α activities in cultured Renal Medullary Interstitial Cells (RMIC). RMIC cells were cultured as we described previously. ODN was transfected using ultrasound-microbubble technique: microbubble (Optison)/ODN mixture was prepared by incubating DNA (10 μg for 35 mm dish) with microbubble (10%, v/v, in 2 ml of medium) for 10min. 80% confluent cells were washed with serum free medium and then switched to the mixture of microbubble/ODN, followed immediately by ultrasound irradiation with 1-MHz ultrasound at 1% power output for 30 s. This ultrasound condition was determined by preliminary experiments, which did not induce cell damages and achieved effective transfection. Cells then were switched to normal medium 30 min after ultrasound and treated with 100 μM of CoCl₂ for 6 hours after 12 hour ODN transfection. Three groups of cells were prepared: scrambled ODN (S)-treated, S+CoCl₂-treated and decoy ODN (D) + CoCl₂–treated cells. Transfection efficiency of ODN was examined by fluorescent microscopy and flow cytometry 18 hours after transfection of FITC-ODN. For flow cytometry analysis, cells were trypsinized and counted for the percentage of FITC-fluorescent positive cells. Effect of decoy-ODN was determined by measurements of HIF-1α binding activity in nuclear extractions.
using an ELISA-based HIF binding kit (Panomics) and HO-1 mRNA levels using quantitative RT-PCR as described below.

**RNA extraction and quantitative RT-PCR analysis of HO-1 and NOS2 mRNA.** Total RNA from renal medullary tissues was extracted using TRIzol solution (Life Technologies, Inc) and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad). The RT products were amplified using TaqMan® Gene Expression Assays kits for rat HO-1 and NOS2 mRNA levels (Applied Biosystems). The levels of 18S ribosomal RNA (rRNA) were used as an endogenous control. The real-time quantitative PCR was performed using an iCycler iQ Real-Time PCR Detection System (Bio-Rad). The cycle threshold (Ct) values were exported into a Microsoft Excel worksheet for calculation of gene expression in accordance with the $\Delta\Delta$Ct method. The Ct values were first normalized with respect to 18S rRNA levels to obtain $\Delta$Ct values. The averaged $\Delta$Ct value from the decoy group was used as a reference to calculate $\Delta\Delta$Ct values for all samples. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta\text{Ct}}$.

**Chronic monitoring of arterial blood pressure in conscious rats.** Mean arterial blood pressure (MAP) was measured using a telemetry blood pressure recording system (DSI, Data Sciences International, Arden Hills, MN). Telemetry transmitter was implanted after the injection of ODN in the same surgery. Briefly, a catheter connected to a telemetry transmitter was inserted into femoral artery. The transmitter was placed subcutaneously and blood pressure signals was recorded through a remote receiver, which allowed rats to be housed unrestrainedly. The animals were allowed to recover from surgery for three days and then daily two hour MAP recording started. After baseline MAP was recorded on 3 consecutive control days while the rats remained
on the 1% salt diet, animals were switched to high salt diet containing 8% NaCl (Dyets, Inc) and MAP was recorded for additional 10 days. Three groups of animals, including rats treated with scrambled ODN + high salt (HS), decoy ODN + HS and decoy ODN + normal salt, were examined. At the end of experiment, the kidneys were removed and dissected into cortical and medullary tissues, which were snap-frozen in liquid nitrogen and stored in -80°C for protein and RNA isolation later.

References


Supplementary data

Salt-Sensitive Hypertension Induced by Decoy of Transcription Factor HIF-1α in the Renal Medulla

Suppl. Fig. 1. Fluorescent microscopic images of cultured Renal Medullary Interstitial Cells (RMIC) transfected with FITC-labeled ODN (A) or non-FITC-labeled ODN (B and C). Images were taken 24 hrs after transfection. (Representative images from 6 sets of experiments, x 400)

Cells transfected with FITC-ODN exhibit fluorescence in almost all cells, while cells transfected with non-FITC-ODN do not exhibit any fluorescence even when exposure time is increased by 100 times.
Suppl. Fig. 2. Histogram plots of flow cytometry analysis of FITC fluorescent positive cells. A: RMIC Cells transfected with non-FITC-labeled ODN. B: RMIC Cells transfected with FITC-labeled ODN. C: Overlay of A and B.

Cells transfected with FITC-ODN or non-FITC-ODN are clearly separated into two populations. Histogram Statistics (M1 in C) demonstrated that 94.6 ± 1.3 % of the cells transfected with FITC-ODN are fluorescence positive, indicating a >90% of transfection efficiency (n=6).
Suppl. Fig. 3. Effects of HIF-1α decoy ODN transfection on HIF-1α binding activity and the mRNA expressions of HO-1 in RMIC cells. 
A: HIF-1α binding assay in the nuclear extracts. B: Realtime RT-PCR analyses of the HO-1 mRNA levels. S=Scrambled ODN, D=decoy ODN, C=CoCl₂ treatment (100 μM, 6 hrs). * P < 0.05 vs. S, # P< 0.05 vs. SC. N=6.

In decoy-ODN-treated cells, both CoCl₂-induced increases in HIF-1α binding and HO-1 mRNA were significantly inhibited.