CORRECTION NOTICE


Epigenetic modulation of the renal \(\beta\)-adrenergic–WNK4 pathway in salt-sensitive hypertension

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In the version of this article initially published, the image of the actin bands shown in Supplementary Figure 2b was mistakenly rotated 180 degrees. The image has been replaced with the bands in their correct orientation, and the densitometry shown for this blot has been recalculated, which does not affect the conclusions. The figure legends for Figure 1e and Supplementary Figure 2b have also been edited to indicate that the same kidney samples were used for the blots in Figure 1b,e and Supplementary Figure 2b and that the actin bands shown for these blots are identical.

In the version of this supplementary file originally posted online, there was an error inadvertently made by the authors during the preparation of Supplementary Figure 2a. The bands shown for actin (vehicle) were incorrect. These errors did not affect the quantification of band intensities and did not affect any of the conclusions of the article. The errors have been corrected in this file as of 4 August 2011 and 5 April 2012.
Epigenetic modulation of the renal β-adrenergic-WNK4 pathway in salt-sensitive hypertension

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Supplementary Figure Legends and Table

Figure 1.
β-stimulation elevated blood pressure during high-salt diet, via WNK4 and NCC regulation.
(a) Averages of systolic blood pressure measured by tail-cuff method in sham-operated control (Con), norepinephrine-infused (NE), norepinephrine plus propranolol-treated (NE+PRO), isoproterenol-infused (ISO), and isoproterenol plus propranolol-treated mice (ISO+PRO) fed normal-salt (NS; 0.3%) diet (grey bars) and high-salt (HS; 8%) diet (black bars) for 1 week. (b) The infusion of NE into mice did not change WNK1 (left panel) or kidney specific (KS)-WNK1 (right panel) mRNA in the kidney. The treatment of prazosin (Pra), α-blocker, does not affect NE-induced changes in WNK4 mRNA (c) and NCC protein (d) in the kidneys of mice. The infusion of ISO as well as NE in mice down-regulated renal WNK4 mRNA and NCC protein. n=5~7 mice for each group. *p < 0.01 vs. CON, #p <0.01 vs. NE/ISO. **p<0.05.

Figure 2.
Effect of WNK4 on NCC and ENaC expression.
(a) Effects of WNK4 siRNA on ISO- and dexamethasone (Dex)-induced up-regulation of NCC protein. The transfection of siWNK4 into the charcoal-treated mDCT cells abolished both Dex-induced and Dex-plus-ISO-induced increases in NCC protein (upper panel). The transfection of siWNK4 into mDCT cells down-regulated WNK4 expression by more than 90% (lower panel). n=4~5 experiments of mDCT cells for each group . (b) By Western blotting, both α-ENaC and β-ENaC protein in the kidney were significantly up-regulated in the NE-infused mice, but it was reversed by the additional treatment of propranolol (PRO). However, γ-ENaC was not affected by NE. The same samples were used as in Fig. 1b,e and the actin blot shown is the same as in Fig. 1b,e (note that the lane corresponding to the leftmost control sample is not shown and was not used for the densitometry due to high background staining). n=5~7 mice for each group. *p<0.01 vs. Control.

Figure 3.
Negative control of Fig. 2a.
Acute injection of saline without HCTZ did not change FENa in these groups. n=4~6 rats for each group

Figure 4.
Daily dietary intake in chronic ISO-infused and HCTZ-treated rats fed the high salt diet.
Neither ISO infusion nor HCTZ treatment affected daily dietary intake in rats. Thus, these changes
in UNaV during the ISO infusion and with the subsequent treatment of HCTZ (Fig. 2c left panel) clearly indicated changes in sodium balance (intake-output), followed by the significant increases in PV with isoproterenol and the subsequent decreases with HCTZ (Fig. 2c right panel). n=4–6 rats for each group.

**Figure 5.**

Effects of amiloride, eplerenone and olmesartan on mean arterial pressure and WNK4 expression in isoproterenol-infused mice fed a high-salt diet.

(a) High-salt diet increased arterial pressure moderately in the isoproterenol-infused mice, but the additional treatment of amiloride slightly decreased it. Then there are clear differences of the anti-hypertensive effects between the treatment of thiazide (Fig.2c) and amiloride. (b) Effects of eplerenone and olmesartan on mean arterial pressure and WNK4 expression in isoproterenol-infused mice on a high-salt diet. Neither treatments of eplerenone nor olmesartan affected both salt-induced elevation of blood pressure and WNK4 expression in the isoproterenol infused mice. n=4–6 mice for each group. *p<0.01 vs. Control.

**Figure 6.**

WNK4 expressions in mDCT cells with and without charcoal treatment.

(a) In the charcoal-untreated mDCT cells, isoproterenol (ISO) significantly decreased WNK4 mRNA (upper panel) and protein (lower panel), and this inhibition was augmented by the additional treatment of theophylline (ISO+Theo), but that of H89, a PKA inhibitor (ISO+H89), could reverse it. (b) In charcoal-treated mDCT cells, isoproterenol alone (ISO) did not affect WNK4 protein expression, but dexamethasone (Dex) decreased it, and the co-administration of dexamethasone and isoproterenol (ISO+Dex) further decreased it. n=4–6 experiments of mDCT cells for each group. *p<0.01 vs. Control, **p<0.05.

**Figure 7.**

Immunostaining of NCC in the charcoal-treated mDCT cells

Immunostaining of NCC in the charcoal-treated mDCT cells was up-regulated by dexamethasone (Dex), and further enhanced by the additional treatment of isoproterenol (ISO+Dex) but the treatment of H89, a PKA inhibitor, could inhibit the effects of isoproterenol (ISO+Dex+H89). Of note, isoproterenol alone (ISO) did not affect NCC staining. Nuclei are stained by DAPI. Magnification: x200.

**Figure 8**

Dose-responsive effects of Dex and NE on WNK4 inhibition.
Dose-response curve of dexamethasone (Dex)-induced down-regulation of WNK4 mRNA (open circle), and the additional effect of norepinephrine (NE) (closed circle). n=4~6 experiments of mDCT cells for each group. *p < 0.01 vs. Dex 0 (M), #p <0.05.

**Figure 9.**
*Effects of siGR on GR expression in mDCT cells.*
The transaction of GR siRNA into mDCT cells down-regulated GR expression by more than 90%, but that of control siRNA did not affect it. n=3~4 experiments of mDCT cells for each group.

**Figure 10.**
*WNK4 promoter transcription assay.*
*upper panel* shows WNK4 promoter deletion mutants: -561, -500, -400, -300, -280, -200, and -180 bps of WNK4 promoter. By luciferase reporter assay (*lower panel*), effects of dexamethasone (Dex) and isoproterenol (ISO) on WNK4 transcription with its promoter deletion mutants. Grey bars indicate no treatment, and black bars indicate treatment of Dex plus ISO. Treatment of Dex plus ISO decreases WNK4 transcription in mutant with -400 bps promoter region but not in mutant with -300 bps promoter region, suggesting the presence of nGREs in -400 and -300bps promoter region of WNK4 gene. n=4~6 experiments of mDCT cells for each group. #p <0.05.

**Figure 11.**
*Effect of the GR antagonist on WNK4 expression.*
Effect of RU486 (RU) on renal WNK4 expression in mice with or without norepinephrine (NE) infusion. GR blockade by RU up-regulated WNK4 expression in mice, and NE infusion did not decreased WNK4 expression in RU-treated mice. n=4~5 mice for each group *p<0.01 vs. Con.

**Figure 12**
*Immunofluorescent staining of WNK4.*
WNK4 is apparently stained in the distal nephron in the mouse kidney. Scale bar: 100 μm. Nuclei are stained by DAPI.

**Figure 13.**
*Effects of salt loading on mean arterial pressure in wild-type and GR-KO mice.*
Without isoproterenol infusion, there are little changes in mean arterial pressure with salt loading in both mice. n=4~6 mice for each group
GR-GFP and HSP 90 in mDCT cells.

(a) Time-course of nuclear GR cycling. After transfection of GR-GFP into charcoal-treated mDCT cells, the GRs were mainly distributed in the cytoplasm (upper panel), and exposure to dexamethasone (Dex) immediately targeted GRs into the nucleus. All GRs were clearly observed in the nucleus of the cells 60 minutes after exposure to Dex or Dex plus isoproterenol (ISO) (middle panel), suggesting that ISO has little effect on GR translocation into the nucleus. Consistently, Western blotting showed similar amounts of nuclear GR protein after 60 minutes in both cells exposed to Dex alone and Dex plus ISO (Fig. 4a, upper panel). All GRs remained in the nucleus for 120 minutes, and then the GRs in the nucleus began to be extruded to the cytoplasm. Thereafter, GRs appeared in the cytoplasm of cells exposed to Dex but not in that of the cells exposed to both ISO and Dex (lower panel), suggesting that ISO causes GRs to remain in the nucleus longer. Thus, the Dex-induced increase in nuclear GR protein 180 minutes after the treatment was augmented by ISO (Fig. 4a, middle and lower panels), but exposure to H89 for 180 minutes abolished it (Fig. 4a, lower panel) with the definite appearance of GR-GFP in the cytoplasm of the ISO plus Dex-treated cells (lower panel). (b) Nuclear HSP90 in mDCT cells, by Western blotting. Neither the treatments of isoproterenol (ISO) nor dexamethasone (Dex) affects nuclear HSP90 protein. n=4~7 experiments of mDCT cells for each group

Figure 15.

Effects of acute hydrochlorothiazide (HCTZ) injection on urinary sodium excretion (UNaV) in DOCA-salt rats.

For the clearance study, the bladder was cannulated in the conscious SD rats, and six urine collections were performed at 30 min intervals for 3 hrs to measure urine volume and subsequently calculate UNaV and FENa. Polyethylene catheters were inserted into the left carotid artery for monitoring arterial pressure and the jugular vein for the intravenous infusion of physiological saline (1ml/hr) and the injection of HCTZ (2mg/kg). The degree of renal NCC activity was estimated by scrutinizing changes in UNa in response to HCTZ (ΔUNaV induced by HCTZ). β1AR blocker metoprolol (12mg/kg/day) and β2AR blocker ICI118551 (1mg/kg/day) were subcutaneously administered by the Alzet mini-pump. The administration of HCTZ clearly increased urine volume (a) and UNaV (b) in the untreated rats fed on the high salt diet, HCTZ induced additional diuresis (a) and natriuresis (b) in DOCA-salt rats. However, the treatment of the β2 antagonist, ICI118551, reversed HCTZ-induced diuresis (a) and natriuresis (b), but that of the β1 antagonist, metoprolol, did not affect it in DOCA-salt rats. ΔUNaV with HCTZ was significantly greater in DOCA-salt rats than in the untreated rats and DOCA-salt rats treated with the β2AR antagonist, suggesting that β2AR stimulation induced sodium retention through the increased renal NCC activity in DOCA-salt rats. Consistent with the results of acute injection of HCTZ, chronic administration of HCTZ decreased
daily UNaV and reversed volume retention (Fig. 2b) in the isoproterenol-infused rats. As a result, chronic administration of HCTZ decreased blood pressure moderately in salt-loaded mice who received an isoproterenol infusion, as shown in Figure 2c. These results have strengthened our conclusion that the stimulation of β<sub>2</sub> receptors causes sodium retention and BP elevation in salt-sensitive hypertensive animals. n=6–7 rats for each group *p<0.01 vs. HS, #p<0.01.

**Figure 16.**
Efforts of hydrochlorothiazide (HCTZ) on fractional sodium excretion (FENa) in DOCA-salt rats.
(a), The response of FENa to HCTZ was significantly greater in DOCA-salt rats. However, the treatment of ICI118551 abolished the augmented response of FENa to HCTZ, but metoprolol did not, strongly suggesting that the activation of β<sub>2</sub> receptors increases renal NCC activity in salt-sensitive hypertension animals. (b), The lower panel shows little effects of saline without HCTZ on FENa in these experimental rats. Arterial pressure remained unchanged throughout the study in all groups. n=6–7 rats for each group *p<0.01 vs. HS, #p<0.01.

**Figure 17.**
Effects of renal denervation and amiloride treatment on mean arterial pressure in salt-loaded Dahl-S rats.
Neither amiloride nor renal denervation affected blood pressure, but both treatments clearly decreased it in salt-loaded Dahl-S rats. n=5–6 rats for each group. *p<0.05.

**Figure 18**
Characterization of mDCT cells and controls of ChIP assay.
(a) In mDCT cells, mRNA of β<sub>1</sub>AR, β<sub>2</sub>AR and WNK4 are well expressed by quantitative RT-PCR, and GR protein was also expressed by immunofluorescent staining. (b) Positive and negative control for ChIP assay. Left panel shows input, and right panel shows IgG binding in mDCT cells transfected without (upper panel) and with intact HDAC8 (middle panel) and HDAC8(S39A) mutant (lower panel). n=4–5 experiments of mDCT cells for each group

**Supplementary Table**
Primers for PCR and quantitative PCR.
Mu SY et al: Supplementary Figure 1
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**Graph:**

- Con: Vehicle
- ISO: Vehicle
- Dex: Vehicle
- ISO+Dex: Vehicle

**Bar Chart:**

- Con
- ISO
- Dex
- ISO+Dex

- Relative NCC

### b)

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**Graph:**

- Con
- NE
- NE+PRO

**Bar Chart:**

- αENaC
- βENaC
- γENaC

**Relative ENaC**

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**Mu SY et al: Supplementary Figure 2**

*Nature Medicine doi:10.1038/nm.2337*
Mu SY et al: Supplementary Figure 3
Mu SY et al: Supplementary Figure 4
Mu SY et al: Supplementary Figure 5
Mu SY et al: Supplementary Figure 6

Nature Medicine doi:10.1038/nm.2337
Mu SY et al: Supplementary Figure 7
Mu SY et al: Supplementary Figure 8
Mu SY et al: Supplementary Figure 9
Mu SY et al: Supplementary Figure 10
Mu SY et al: Supplementary Figure 11
Mu SY et al: Supplementary Figure 12
Mu SY et al: Supplementary Figure 13
Mu SY et al: Supplementary Figure 14
Vein catheter
Inject saline 1ml/h
Artery catheter
Inject HCTZ to block NCC
BP
Bladder catheter
Urine collection

HCTZ before after before after before after

HS DOCA+HS DOCA+HS +ICI 118551

ΔUNaV induced by HCTZ

0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
HS HS HS HS

DOCA DOCA Metoprolol DOCA

ICI 118551

Mu SY et al: Supplementary Figure 15

Mu SY et al: Supplementary Figure 15

Nature Medicine doi:10.1038/nm.2337
Mu SY et al: Supplementary Figure 16
Mu SY et al: Supplementary Figure 17
Mu SY et al: Supplementary Figure 18
### Table: Primers for PCR and quantitative PCR

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