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CORRESPONDENCE

Does a β_2-adrenergic receptor–WNK4–Na-Cl co-transporter signal cascade exist in the *in vivo* kidney?

To the Editor:

We read with great interest the article by Mu *et al.*[1] reporting that sympathetic overactivity leads to glucocorticoid receptor stimulation, WNK4 suppression and Na-Cl co-transporter (NCC) activation. The authors of this report suggested that this signal cascade may be involved in the development of salt-sensitive hypertension induced by norepinephrine with dietary salt excess.

We attempted to reproduce the fundamental findings of this study: suppression of WNK4 expression, a regulator of sodium reabsorption, and activation of NCC by treatment with norepinephrine in mice (as shown in Fig. 1b,e of Mu *et al.*[1]). However, we could not reproduce their results, as shown in **Figure 1**. We performed norepinephrine infusion experiments as described by Mu *et al.*[1] and confirmed that norepinephrine did induce salt-sensitive hypertension (**Fig. 1a**). High salt increased blood pressure only in NE-infused mice as Mu *et al.*[1] reported (**Fig. 1a**). We also confirmed an increase in serum norepinephrine concentration in the norepinephrine-infused mice (**Fig. 1b**). However, we did not observe any change in WNK4 mRNA levels in the kidney between vehicle- and NE-infused C57BL6/J mice (**Fig. 1c**). RT-PCR was performed as described by Mu *et al.*[1]. However, in our hands, the PCR primers used by Mu *et al.*[1] for WNK4 (ABIMm00841400_m1) were much less sensitive than the primers reported by O'Reilly *et al.*[4], and we therefore used the primer set from ref. 4 in this experiment. The representative immunoblots and the results of quantification of the bands clearly show no significant changes in WNK4 protein, total NCC or phosphorylated NCC (pSer71) between vehicle- and NE-infused C57BL6/J mouse kidneys (**Fig. 1d**). We speculate that the major reason for the discrepant results regarding WNK4 protein levels as measured by immunoblots is the use of different WNK4-specific antibodies. The

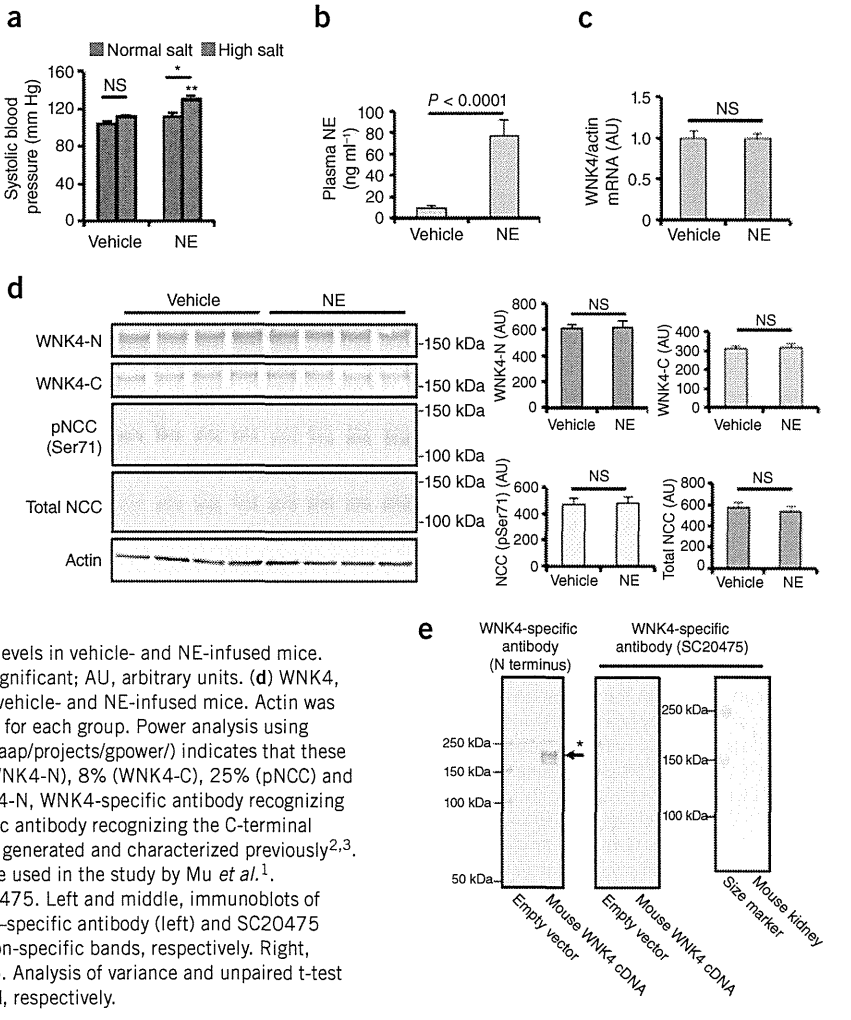

Figure 1 Effects of norepinephrine (NE) infusion on WNK4 and NCC in the mouse kidney. (**a**) Systolic blood pressure measured by the tail-cuff method in vehicle (67 mM HCl in 0.9% NaCl)- and NE-infused C57BL6/J mice fed normal salt (0.3%) or high salt (8%) diet for 1 week. Data are means ± s.e.m.; $n = 8$ for each group. $^{**}P < 0.01$ versus vehicle, $^{*}P < 0.05$, NS, not significant. (**b**) Plasma NE concentrations in vehicle- and NE-infused mice (data are means ± s.e.m.; $n = 5$ for each group). (**c**) WNK4 mRNA levels in vehicle- and NE-infused mice. Data are means ± s.e.m.; $n = 5$ for each group. NS, not significant; AU, arbitrary units. (**d**) WNK4, NCC and pNCC (phosphorylated at Ser71) abundance in vehicle- and NE-infused mice. Actin was used as a loading control. Data are means ± s.e.m.; $n = 9$ for each group. Power analysis using G*power software (http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/) indicates that these immunoblot data would have been able to detect 10% (WNK4-N), 8% (WNK4-C), 25% (pNCC) and 24% (total NCC) differences with 80% confidence. WNK4-N, WNK4-specific antibody recognizing the N-terminal portion of WNK4; WNK4-C, WNK4-specific antibody recognizing the C-terminal portion of WNK4. WNK4-N and WNK4-C antibodies were generated and characterized previously[2,3]. Antibodies against pNCC and NCC were the same as those used in the study by Mu *et al.*[1]. (**e**) Characterization of the WNK4-specific antibody SC20475. Left and middle, immunoblots of mouse WNK4 overexpressed in COS7 cells with WNK4-N–specific antibody (left) and SC20475 (middle). An arrow and an asterisk indicate WNK4 and non-specific bands, respectively. Right, immunoblot of normal kidney homogenate with SC20475. Analysis of variance and unpaired t-test were used to assess the statistical difference in **a** and **b–d**, respectively.

identity of the WNK4-specific antibody used was not provided in Mu *et al.*[1], but in further communication with the authors they indicated that the antibody used was SC-20475 (Santa Cruz Biotechnology). We also discussed and confirmed with the authors of Mu *et al.*[1] experimental conditions and protocols (for example, the dosage of norepinephrine and how norepinephrine was dissolved and prepared for administration). We recently published a detailed characterization of one of our own WNK4-specific antibodies and reported that most commercially available antibodies against WNK4 are not suitable for detecting WNK4 in mouse kidney[2,3]. Full-length mouse WNK4 transiently expressed in COS7 cells was clearly detected by a WNK4-specific antibody recognizing the N terminus (**Fig. 1e**). However, in the case of the antibody used in Mu *et al.*[1], we were unable to detect mouse WNK4 protein overexpressed in COS7 cells (**Fig. 1e**). We also could not detect any bands using SC20475 in normal C57BL6/J kidney samples (**Fig. 1e**).

Given these observations, we are concerned about the fundamental finding of Mu *et al.*[1] that norepinephrine regulates WNK4 and NCC abundance in the kidney. However, the discovery that norepinephrine may be involved in the development of salt-sensitive hypertension is noteworthy. Further studies clarifying the role of WNK4 in this context may be necessary. A *Wnk4*-knockout mouse would be a valuable tool in clarifying this issue.

The Animal Care and Use Committee of Tokyo Medical and Dental University approved these experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Mu *et al.* reply:

In their correspondence[1], Uchida *et al.* respond to our paper[2] in which we showed that renal β_2-adrenergic receptor stimulation downregulates *WNK4* expression and upregulates Na-Cl co-transporter (NCC) expression, hence contributing to the development of salt sensitive hypertension. We appreciate the study of Uchida *et al.*[1] and their findings, which confirm our original data that norepinephrine treatment contributes to the development of salt-sensitive hypertension, with a similar efficacy as in our study[2]. However, they did not detect any changes in WNK4 or NCC mRNA or protein levels in the kidney upon norepinephrine stimulation[1].

To address the concerns raised by Uchida *et al.*[1], we first reexamined whether WNK4 mRNA levels are decreased under norepinephrine stimulation. An obvious methodological difference between the two studies is that Uchida *et al.*[1] performed their salt diet experiments for 1 week in contrast to our 3-d regimen; we therefore performed all additional experiments after 1 week of salt diet. We collected RNA samples and analyzed them by both quantitative RT-PCR and northern blotting (**Fig. 1a,b**). In their study, Uchida *et al.*[1] chose a different primer set from the one used in our study[2] (the ABI Mm00841400_m1 primer set) and claimed that our primer set was "less sensitive." We evaluated both primer sets and found the presence of nonspecific bands and pronounced primer-dimer formation when using the primer set recommended by Uchida *et al.*[1] in comparison with the ABI primer set (**Fig. 1c**). This suggested that the ABI primer set, in fact, has high specificity and gives a low background signal and can therefore be used to assess the effect of norepinephrine treatment on *WNK4* expression. Various factors can influence the results of quantitative RT-PCR reactions and the subsequent interpretation of

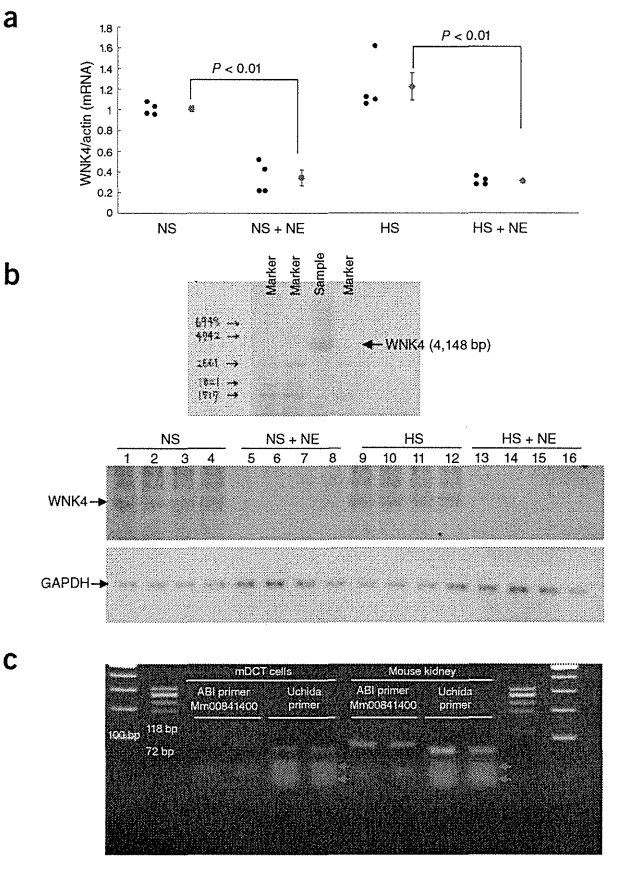

Figure 1 Effects of norepinephrine (NE) infusion on renal WNK4 mRNA expression in mice. (**a**) WNK4 mRNA expression in mice on normal-salt (NS) or high-salt (HS) diet with or without NE infusion was evaluated by quantitative RT-PCR using the ABI primer set Mm00841400_m1. Each dot refers to the results of one mouse. Data are means ± s.e.m., and data were analyzed by analysis of variance with Dunnett's *post hoc* test. β-actin served as a loading control (ABI Mm00607939_s1). Mice were fed indicated salt diets (normal-salt (NS; 0.3% NaCl) or high-salt (HS; 8% NaCl)) for 1 week as in Uchida *et al.*[1]. Quantitative data are presented as means ± s.e.m.; $n = 4$ for each group. (**b**) Top, to confirm the specificity of the WNK4 probe, we performed electrophoresis of mRNA together with molecular-weight markers. Bottom, WNK4 mRNA expression measured by northern blotting with a cDNA probe (3,258–3,754 base pairs (bp) of NM_175638.3) labeled with DIG-11-dUTP (PCR DIG Probe Synthesis Kit, Roche 1636090). GAPDH probe (570–1,021 bp of NM_008084.2) served as the loading control. The same RNA samples were used for quantitative RT-PCR and for northern blotting. (**c**) Comparison of quantitative RT-PCR products obtained using the ABI primer set and the primer set used by Uchida *et al.*[1]. The same mDCT cell and mouse kidney cDNA templates and matching primer concentrations were used to compare the two primer sets. Red arrows indicate possible nonspecific bands and primer dimers formed during the PCR step. The two lanes on both the left and right sides of the gel are molecular weight ladders.