Impaired phosphorylation of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter by oxidative stress-responsive kinase-1 deficiency manifests hypotension and Bartter-like syndrome

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Na$^+\text{-K}^+\text{-2Cl}^-$ cotransporters (NKCCs), including NKCC1 and renal-specific NKCC2, and the Na$^+\text{-Cl}^-$ cotransporter (NCC) play pivotal roles in the regulation of blood pressure (BP) and renal NaCl reabsorption. Oxidative stress-responsive kinase-1 (OSR1) is a known upstream regulator of N(K)CCs. We generated and analyzed global and kidney tubule-specific (KSP) OSR1 KO mice to elucidate the physiological role of OSR1 in vivo, particularly on BP and kidney function. Although global OSR1$^{-/-}$ mice were embryonically lethal, OSR1$^{+/+}$ mice had low BP associated with reduced phosphorylated (p) STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase (SPAK) and p-NKCC1 abundance in aortic tissue and attenuated p-NKCC2 abundance and Gi protein expression in the kidneys. KSP-OSR1$^{-/-}$ mice had normal BP and hypercalcuria and maintained significant hypokalemia on a low-K$^+$ diet. KSP-OSR1$^{-/-}$ mice exhibited impaired Na$^+$ reabsorption in the thick ascending loop on a low-Na$^+$ diet accompanied by remarkably decreased expression of p-NKCC2 and a blunted response to furosemide, an NKCC2 inhibitor. The expression of total SPAK and p-SPAK was significantly increased in parallel to that of total NCC and p-NCC despite unchanged total NKCC2 expression. These results suggest that, globally, OSR1 is involved in the regulation of BP and renal tubular Na$^+$ reabsorption mainly via the activation of NKCC1 and NKCC2. In the kidneys, NKCC2 but not NCC is the main target of OSR1 and the reduced p-NKCC2 in KSP-OSR1$^{-/-}$ mice may lead to a Bartter-like syndrome.

Bartter syndrome | electrolytes | hormone | knockout mice | volume

Recent studies have shown that Na$^+\text{-K}^+\text{-2Cl}^-$ cotransporters (NKCCs) and the Na$^+\text{-Cl}^-$ cotransporter (NCC) play very important roles in the regulation of blood pressure (BP) and extracellular volume. NKCCs consist of ubiquitous NKCC1 and renal-specific NKCC2. NKCC1 can modulate BP through vascular and renal effects (1–4). NKCC2 and NCC are two renal Na$^+$ cotransporters expressed in the thick ascending limbs (TALs) and distal convoluted tubules (DCTs) of the kidney, respectively, accounting for 20% and 10% of filtered Na$^+$ reabsorption (5). In human essential hypertension and salt-sensitive or spontaneously hypertensive animal models, activation of NKCC1 and NKCC2 has also been reported to play a pivotal role in the pathogenesis of hypertension (6, 7). In addition, activation of NCC by gene mutations in WNK1 and WNK4 leads to an autosomal dominant salt-sensitive hypertension known as pseudohypoaldosteronism type II (PAHII) (8). On the other hand, loss-of-function mutations in the $\text{S}L\text{C}1\text{Z}2\text{A}4$ and $\text{S}L\text{C}1\text{Z}2\text{A}3$ genes encoding NKCC2 and NCC can lead to renal salt-wasting hypertension with hypokalemic metabolic alkalosis, known as Bartter syndrome (BS) (9) and Gitelman syndrome (GS) (10), respectively.

In vitro studies have shown that posttranscriptional phosphorylation of NKCC1/2 and NCC plays a crucial role in the regulation of normal transport activity. Oxidative stress-responsive kinase-1 (OSR1) (11) and STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase (SPAK) (12), two downstream substrates of With-No-Lysine kinase (WNK) (1/4), are the upstream phosphorylators of NKCC1/2 and NCC. Threonine or serine residues in their N-terminal conserved domains (T206/96, T211/101, and T224/114 in mouse NKCC1/2, T53, T58 and S71 in mouse NCC) are the phosphorylation sites of OSR1 and SPAK. The docking site on the conserved C-terminal domains of OSR1/SPAK interacts with the c-Jun amino-terminal kinase (JNK) (13). Together, these interactions can lead to renal salt wasting and hypocalcemia with hypokalemia and increased total and p-NCC expression and function (13–16). We have also reported that increased phosphorylated (p) OSR1/SPAK abundance can enhance p-NCC expression in the PHAI-I-causing Wnk4 D561A knock-in mice (17), whereas the reverse is true in the Wnk4 hypomorphic knockout (KO) mice (18). These findings support that OSR1 and SPAK are important regulators of NKCC and NCC in vivo.

Because OSR1 and SPAK share high homology in their catalytic and regulatory domains and their expression in tissues often overlaps, the creation and analysis of distinct OSR1 or SPAK KO mice is warranted to tease apart the role of each kinase in vivo. For this purpose, we first generated SPAK KO mice and found that SPAK$^{-/-}$ mice exhibited hypotension with decreased p-NKCC1 abundance in aortic tissues and SPAK$^{-/-}$ mice presented a GS phenotype caused by reduced total and p-NCC expression (19). In the present study, we generated global and kidney tubule-specific (KSP) OSR1 KO mice to elucidate the physiological role of OSR1 in vivo (SI Text and Figs. S1 and S2). Results to be reported indicate that global OSR1$^{-/-}$ mice were embryonically lethal and OSR1$^{+/+}$ mice had lower BP associated with reduced p-SPAK expression and p-NKCC1 abundance in aortic tissue and attenuated p-NKCC2 abundance with increased total and p-NCC expression in the kidney. KSP-OSR1$^{-/-}$ mice manifested Bartter-like syndrome because of impaired NKCC2 phosphorylation and function in the TAL with a compensatory increase in NCC phosphorylation and expression. This study provides in vivo evidence that OSR1 is primarily involved in the regulation of BP and renal tubular Na$^+$ reabsorption via the phosphorylation of NKCC1 and NKCC2 but not NCC.

Results

Phenotype in Global OSR1$^{-/-}$ and KSP-OSR1$^{-/-}$ Mice. First, we examined BP and electrolyte homeostasis in the global OSR1$^{-/-}$ and KSP-OSR1$^{-/-}$ mice on a normal diet (0.4% Na$^+$ wt/wt, 1% K$^+$ wt/wt). Compared with WT littermates, the global OSR1$^{-/-}$ mice had relative hypotension ($P < 0.05$) without serum and urine

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electrolyte abnormalities (Table 1). The KSP-OSR1−/− mice had normal BP; however, unlike the global OSR1−/− mice, they showed significant hypokalemia with an increased fractional excretion of K+ (FEK+) (P < 0.05) and hypercalcuria (P < 0.05) (Table 2). In addition, the ambient osmolarity of spot urine was significantly reduced in KSP-OSR1−/− mice (1.805 ± 0.389 vs. 2.414 ± 0.525 mOsm/L in WT, n = 10; P < 0.05). When the global OSR1−/− and KSP-OSR1−/− mice were fed a low-Na+ diet (0.05% Na+ wt/wt), the

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**Table 1. BP and blood and urine biochemistry values in global OSR1−/− mice**

| Diet       | Normal | Low Na+ |
|------------|--------|---------|
| Genotype (n) | WT (n = 12) | OSR1−/− (n = 12) | WT (n = 8) | OSR1−/− (n = 8) |
| **BP, mmHg** |        |         |        |         |
| Systolic    | 111 ± 6 | 99 ± 5 | 108 ± 7 | 95 ± 6 |
| Diastolic    | 58 ± 4 | 49 ± 4 | 54 ± 4 | 51 ± 9 |
| Mean        | 74 ± 4 | 64 ± 7 | 71 ± 5 | 62 ± 7 |
| **Weight, g** | 22.8 ± 2.2 | 21.6 ± 2.3 | 23.6 ± 3.5 | 22.1 ± 4.3 |
| **Plasma** | | | | |
| Na+, mmol/L | 157 ± 3 | 156 ± 2 | 153 ± 6 | 154 ± 5 |
| K+, mmol/L | 4.3 ± 0.3 | 4.1 ± 0.3 | 4.2 ± 0.4 | 4.3 ± 0.4 |
| Mg2+, mg/dl | 9.5 ± 0.2 | 9.6 ± 0.4 | 9.3 ± 0.5 | 9.4 ± 0.6 |
| Ca2+, mg/dl | 2.9 ± 0.1 | 3.1 ± 0.2 | 3.0 ± 0.3 | 3.1 ± 0.4 |
| Cr, mg/dl | 0.13 ± 0.05 | 0.12 ± 0.04 | 0.11 ± 0.08 | 0.11 ± 0.06 |
| Urine, ml/d | 1.77 ± 0.92 | 1.86 ± 0.73 | 1.25 ± 0.63 | 1.23 ± 0.65 |
| Na+, mmol/d | 220 ± 56 | 262 ± 65 | 53 ± 8 | 50 ± 10 |
| K+, mmol/d | 435 ± 133 | 520 ± 108 | 514 ± 155 | 539 ± 178 |
| Cl−, mmol/d | 256 ± 85 | 306 ± 85 | 80 ± 22 | 84 ± 14 |
| Mg2+, mg/d | 0.47 ± 0.11 | 0.50 ± 0.13 | 0.53 ± 0.21 | 0.48 ± 0.22 |
| FEK+, % | 0.65 ± 0.24 | 0.68 ± 0.37 | 0.25 ± 0.09 | 0.23 ± 0.11 |
| FEK+, % | 18.3 ± 6.1 | 20.2 ± 8.6 | 20.6 ± 8.2 | 22.1 ± 9.4 |
| FEK+, % | 0.57 ± 0.12 | 0.61 ± 0.19 | 0.21 ± 0.04 | 0.16 ± 0.05 |
| CaCr, mg/mg | 0.17 ± 0.05 | 0.19 ± 0.08 | 0.19 ± 0.10 | 0.18 ± 0.07 |

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**Table 2. BP and blood and urine biochemistry values in KSP-OSR1−/− mice**

| Diet       | Normal | Low Na+ |
|------------|--------|---------|
| Genotype (n) | WT (n = 10) | KSP-OSR1−/− (n = 10) | WT (n = 8) | KSP-OSR1−/− (n = 8) |
| **BP, mmHg** |        |         |        |         |
| Systolic    | 110 ± 3 | 107 ± 4 | 109 ± 2 | 101 ± 6 |
| Diastolic    | 54 ± 7 | 60 ± 7 | 51 ± 4 | 46 ± 9 |
| Mean        | 75 ± 2.3 | 74 ± 6 | 69 ± 3 | 62 ± 7 |
| **Weight, g** | 23.6 ± 3.5 | 22.8 ± 4.3 | 22.1 ± 4.5 | 23.2 ± 5.3 |
| **Plasma** | | | | |
| Na+, mmol/L | 154 ± 3 | 153 ± 2 | 152 ± 6 | 150 ± 5 |
| K+, mmol/L | 4.2 ± 0.2 | 3.7 ± 0.2 | 4.3 ± 0.3 | 3.6 ± 0.3 |
| Mg2+, mg/dl | 9.8 ± 0.3 | 9.7 ± 0.2 | 9.8 ± 0.3 | 9.7 ± 0.2 |
| Ca2+, mg/dl | 2.9 ± 0.2 | 3.0 ± 0.1 | 2.8 ± 0.3 | 3.0 ± 0.3 |
| Cr, mg/dl | 0.14 ± 0.06 | 0.13 ± 0.05 | 0.13 ± 0.05 | 0.12 ± 0.08 |
| Urine, ml/d | 1.92 ± 0.80 | 2.68 ± 0.21 | 1.54 ± 0.80 | 2.13 ± 0.68 |
| Na+, mmol/d | 232 ± 72 | 250 ± 55 | 93 ± 17 | 103 ± 26 |
| K+, mmol/d | 311 ± 122 | 375 ± 110 | 489 ± 222 | 518 ± 110 |
| Cl−, mmol/d | 203 ± 84 | 247 ± 78 | 65 ± 34 | 72 ± 24 |
| Mg2+, mg/d | 0.60 ± 0.29 | 0.56 ± 0.14 | 0.58 ± 0.21 | 0.55 ± 0.15 |
| FEK+, % | 0.55 ± 0.14 | 0.58 ± 0.27 | 0.22 ± 0.06 | 0.24 ± 0.12 |
| FEK+, % | 20.1 ± 4.1 | 29.5 ± 4.5 | 22.1 ± 6.2 | 32.8 ± 6.5 |
| FEK+, % | 0.52 ± 0.14 | 0.54 ± 0.16 | 0.20 ± 0.04 | 0.22 ± 0.05 |
| FEK+, % | 11.4 ± 2.1 | 10.1 ± 1.1 | 10.4 ± 3.1 | 9.1 ± 2.5 |
| CaCr, mg/mg | 0.16 ± 0.03 | 0.21 ± 0.04* | 0.18 ± 0.02 | 0.23 ± 0.05* |

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**Fig. 1**. Na+ balance and renal K+ handling in OSR1−/− and KSP-OSR1−/− mice. The daily urinary Na+ excretion rate in WT and global OSR1−/− (A) or KSP-OSR1−/− (B) mice on a normal-Na+ diet for 3 d and then on a low-Na+ diet for 6 d was determined. (Inset) Cumulative Na+ excretion from day 1 to day 5 of the low-Na+ diet between WT and KSP-OSR1−/− mice. *P < 0.05 vs. WT (n = 6 per group). Serum K+ concentration and urinary FEK+ in WT, OSR1−/− (C), and KSP-OSR1−/− (D) mice on a normal-K+ (NK) diet and a low-K+ (LK) diet. *P < 0.05 vs. WT (n = 8 per group).
mice, whereas mice. Com-
in the kidneys, we had previ-
excretion rates ± 22%; P <
and KSP-OSR1 and K
Mice. (%) handling in the mice on a low-K
diet in the KSP-
represent the fractional excretion of Na
< P < 0.05) was signi-
< 2.2% in KSP-OSR1
Handling in Global OSR1
diet. ( (%)
Effect of furosemide (FURO) and HCTZ administration on Na
< excretion quickly
0.4 mmol/L in WT ( < 0.05 vs. WT
0.3 mmol/L in KSP-OSR1
< P < 0.05) expression was reduced in OSR1
handling in the global
0.05 for systolic BP only). A low-Na
= 8) vs. 3.8
Fig. S3 between WT and KSP-OSR1
mice, which
< mice after HCTZ
6%;
< mice, we
excretion between the global OSR1
15%;
< mice, which
< mice, we
< mice, we
30.0
0.0
1.0
3.0
50.0
20.0
10.0
0.0
1.0
3.0
4.0
11%
< mice after FURO (Fig. 2C) but a normal response to
HCTZ (Fig. 2D), indicating that their NKCC2 function was re-
duced and NCC function was well preserved (Fig. 2D).

Diuretic Response in Global OSR1+/− and KSP-OSR1+/− Mice. To
determine the function of NKCC2 and NCC, two substrates of
OSR1, in these global OSR1+/− and KSP-OSR1+/− mice, we
administered the NKCC2 inhibitor furosemide and NCC
inhibitor hydrochlorothiazide (HCTZ), respectively. Like WT
mice, global OSR1−/− mice showed a dramatic and similar in-
fraction in the FE, Na, FE, and FEi, in response to furosemide (Fig.
2C and HCTZ (Fig. 2D), suggesting that the NKCC2 and
NCC functions were not obviously affected. Compared with WT
and global OSR1+/− mice, KSP-OSR1+/− mice exhibited a blun-
ted response to furosemide (Fig. 2C) but a normal response to
HCTZ (Fig. 2D), indicating that their NKCC2 function was re-
duced and NCC function was well preserved (Fig. 2D).

Expression of OSR1, SPAK, and NKCC1 in Aortic Tissues of Global
OSR1+/− Mice. Because OSR1, SPAK, and NKCC1 are co-
expressed in vascular smooth muscle and NKCC1 activity is known
to play an important role in the regulation of aortic contractility and
BP (1, 2), we examined whether the OSR1/SPAK-NKCC1 pathway
could be involved in the hypotension of global OSR1+/−
mice. Relative protein expression of total OSR1 (59 ± 22%; P <
0.01), p-OSR1 (44 ± 12%; P < 0.01), and p-SPAK (54.6 ± 2.6%;
P < 0.01) was significantly reduced, along with dramatically re-
duced p-NKCC1 expression (T206) (53 ± 22%; P < 0.01) despite
unchanged total SPAK and NKCC1 abundance in aortic tissue
(Fig. 3). These findings suggested that defective phosphorylation
of OSR1 may cause decreased NKCC1 phosphorylation in blood
vessels, which led to the lower BP.

Renal OSR1, SPAK, NKCC2, and NCC in Global OSR1+/− and KSP-
OSR1+/− Mice. In the kidneys, we had previously reported that OSR1
is mainly distributed in the TAL to downstream renal tubules and
is dominantly expressed in the medulla, colocalizing with NKCC2
(19). Because KSP-OSR1−/− mice displayed a BS-like phenotype
with hypercalcemia and normal magnesium and a blunted re-
sponse to furosemide corresponding to a TAL lesion rather than
GS physiology (a DCT lesion with hypocalciuria and hypomagne-
semia) (20), the expression of total and p-OSR1, SPAK, NKCC2,
and NCC in the kidney of both OSR1−/− and KSP-OSR1−/− mice
was further evaluated by semiquantitative immunoblotting (IB).
Although renal total OSR1 (75 ± 11%; P < 0.05) and p-OSR1
(69 ± 21%; P < 0.05) expression was reduced in OSR1−/− mice
(Fig. 4A), p-SPAK (128 ± 11%; P < 0.05) was mildly increased
(Fig. 4B). The expression of total NKCC2 was not affected, but p-
NKCC2 (T96) (72 ± 15%; P < 0.05) was significantly reduced (Fig.
4C). Total NCC (135 ± 7%; P < 0.05), p-NCC (T58) 121 ± 6%;
< 0.05, and p-NCC (S71) (156 ± 8%; P < 0.05) were also signifi-
cantly increased (Fig. 4D and Fig. S3A). As expected, total OSR1
and p-OSR1 were virtually absent from the kidney tissue of KSP-
OSR1−/− mice (Fig. 5A). However, total SPAK (130 ± 13%; P <
0.05) and p-SPAK (138 ± 13%; P < 0.05) (Fig. 5B) were markedly
increased. As in OSR1−/− mice, expression of total NKCC2 was not
affected but p-NKCC2 (T96) was more dramatically reduced
(32 ± 14%; P < 0.01) (Fig. 5C). Total NCC (158 ± 9%; P < 0.01),

severity of hypotension did not increase in OSR1+/− mice, whereas
the previously normotensive KSP-OSR1−/− mice developed rela-
tive systolic hypertension (P < 0.05 for systolic BP only). A low-Na+
diet also caused significant increases in the plasma aldosterone
concentration (PAC) and plasma renin activity (PRA) in both
global OSR1+/− and KSP-OSR1+/− mice, which were not signifi-
cantly different from their WT littermates (Tables 1 and 2).

Renal Na+ and K+ Handling in Global OSR1+/− and KSP-OSR1+/− Mice.
We further evaluated renal Na+ and K+ handling in the global
OSR1+/− and KSP-OSR1+/− mice. On normal Na+ diets, all these
different groups of mice had similar urine Na+ excretion rates and
fractional excretion of Na+ (FE,Na+) (Tables 1 and 2). On
paired-fed low-Na+ diets, the urinary Na+ excretion quickly
dropped in all three groups (WT, global OSR1+/−, and KSP-
OSR1+/−) of mice. There was no significant difference in the de-
gree of reduced urine Na+ excretion between the global OSR1+/−
and WT littermates (Fig. 1A). However, KSP-OSR1+/− mice
clearly demonstrated more urine Na+ excretion than WT controls

Fig. 2. Effect of furosemide (FURO) and HCTZ administration on Na+, K+, and
Cl− excretion. FE, Na, FE, K, and FE, Cl represent the fractional excretion of Na+, K+,
and Cl−, respectively. Preserved response of FE, Na, FE, K, and FE, Cl in OSR1+/−
mice after FURO treatment (C) but a similar response of FE, Na, FE, K, and FE, Cl in WT and
KSP-OSR1−/− mice after HCTZ (D) treatment. *P < 0.05 vs. WT (n = 6 per group).
p-NCC (T53) (138 ± 11%; P < 0.05), p-NCC (T58) (127 ± 9%; P < 0.05), and p-NCC (S71) (145 ± 15%; P < 0.05) (Fig. S3B) were significantly increased in KSP-OSR1−/− mice.

We also examined the cellular localization of NKCC2 and NCC in the kidney tissue of OSR1+/− and KSP-OSR1+/− mice. The cellular distribution of total NKCC2 and p-NKCC2 (T96) was still luminally condensed, albeit less so for p-NKCC2 (T96) (Fig. S4 A and B). Likewise, total NCC and p-NCC were still luminally condensed (Fig. S4 C and D).

Discussion

In this study, we generated and analyzed global and KSP-OSR1 KO mice to elucidate the physiological role of OSR1 in vivo in the regulation of the NKCC1 and kidney-specific NKCC2 and NCC, focusing on BP and renal tubular Na+ reabsorption. As previously reported in OSR1 gene-trapped or kinase-dead knock-in mice (21, 22), global homozygous OSR1−/− mice were embryonically lethal. Heterozygous OSR1+/− mice exhibited hypotension, markedly reduced p-NKCC1 abundance in aortic tissue, and attenuated p-NKCC2 in kidney. KSP-OSR1−/− mice recapitulated the reduced renal Na+ reabsorption on low-Na+ diets and markedly decreased expression of p-NKCC2. They also had a blunted response to furosemide and a parallel increase in NCC expression and phosphorylation, supporting the notion that TAL function was defective. These results indicate that OSR1 is crucial not only in the regulation of BP but in renal tubular Na+ reabsorption, primarily in the TAL rather than the DCT.

In the global OSR1+/− mice, markedly decreased p-NKCC1 in aortic tissue and kidneys may contribute to obvious hypotension. NKCC1, as a downstream target of OSR1, has been known to play a pivotal role in BP control through vascular and renal effects, as shown in NKCC1 KO mice (1–4). On the one hand, inactivation of NKCC1 in blood vessels causes reduced intracellular Cl− concentration and, consequently, decreased Ca2+ influx through L-type Ca2+ channels, which may lead to vessel relaxation and hypotension (23). On the other hand, defective NKCC1 expression in the basolateral membrane of inner medullary collecting ducts and renin-producing juxtaglomerular (JG) cells (24) may cause the impairment of renal Na+ reabsorption. However, hyperreninemia and hyperaldosteronism with increased renal Na+ transporters, including NKCC2 and NCC observed in NKCC1 KO mice, could help minimize hypotension (3, 4).

Reminiscent of the reduced NKCC1 phosphorylation with reduced aortic contractility in SPAK+/− mice featuring hypotension but normal serum and urine electrolytes (19), the hypotension observed in global OSR1+/− mice reiterates the importance of OSR1/SPAK-NKCC1 phosphorylation signaling in the vascular tissue on BP control. Because both OSR1+/− and SPAK+/− mice had normal total NKCC1 but reduced p-NKCC1, it appeared that intact expression of both OSR1 and SPAK was required for adequate NKCC1 phosphorylation in the aortic tissue. Based on the attenuated rather than increased p-SPAK expression in the aortic tissue of OSR1+/− mice, SPAK phosphorylation may be dependent on the OSR1 activity in the vessels.

In addition to vascular NKCC1, kidney-specific NKCC2 and NCC; two other OSR1 substrates in the TAL and DCT, respectively (5), were also examined in global OSR1+/− mice. A significant decrease in p-NKCC2 and a parallel increase in both p-SPAK and p-NCC in the OSR1+/− mice strongly suggested that a salt-wasting phenotype was present. Based on the Guyton type renal function curve (a plot between mean arterial pressure and urinary Na+ intake and excretion) (25), OSR1+/− mice showed a shift to the left, supporting a defect in renal Na+ transport. Their renal tubule Na+ defect was mild, however, because they did not exhibit negative renal Na+ balance even on low-salt diets. Furthermore, their responses to furosemide and thiazide challenges were also normal. Nevertheless, hypotension in OSR1+/− mice might help dampen the tendency of impaired renal Na+ reabsorption. In response to vascular hypotension or renal Na+ wasting, one should expect an increase in PRA and PAC. However, PRA and PAC were similar between the WT and OSR1+/− mice, suggesting that PRA and PAC were inappropriately low in OSR1+/− mice. Perhaps the reduced p-NKCC1 observed in OSR1+/− mice may have blunted the tendency of impaired renal Na+ reabsorption. In response to vascular hypotension or renal Na+ wasting, one should expect an increase in PRA and PAC. However, PRA and PAC were similar between the WT and OSR1+/− mice, suggesting that PRA and PAC were inappropriately low in OSR1+/− mice. Perhaps the reduced p-NKCC1 observed in OSR1+/− mice may have blunted tubuloglomerular feedback, leading to the impaired release of renin from JG cells as shown in NKCC2 isofrom KO mice (26, 27). However, we could not exclude the direct regulation of aldosterone secretion by OSR1, which was also abundantly expressed in adrenal tissues.

To focus on the specific role of OSR1 in the regulation of NKCC2 and NCC in the kidney, we further created the KSP-OSR1 KO mice. NKCC2 has three different full-length splice variants including NKCC2 and NCC observed in NKCC1 KO mice, could help minimize hypotension (3, 4).

Fig. 3. Expression of ORS1, SPAK, and NKCC1 in aortic tissues of ORS1+/− mice. Semiquantitative IB (Upper) and densitometry (Lower) of total and p-ORS1 (A), total and p-SPAK (B), and total and p-NKCC1 (T206) (C) in aortic tissues of WT and ORS1+/− mice (n = 4 per group). ∗P < 0.05 vs. WT.
mice had marked diminution in p-NKCC2 (T96) in kidney tissues of WT and OSR1−/− mice (n = 4 per group). *P < 0.05 vs. WT.

**Fig. 4.** Expression of OSR1, SPAK, NKCC2, and NCC in kidney tissues of OSR1−/− mice. Semiquantitative IB (Upper) and densitometry (Lower) of total and p-OSR1 (A), total and p-SPAK (B), total and p-NKCC2 (T96) (C), and total and p-NCC (T58) (D) in kidney tissues of WT and OSR1−/− mice (n = 4 per group). *P < 0.05 vs. WT.

KSP-OSR1 wasting. The TAL also accounts for 20% of Na+ excretion. When these mice may be used further to explore the mechanisms of NKCC2 and NCC phosphorylation on their protein trafficking.

In addition to Na+ wasting, KSP-OSR1−/− mice had other electrolyte disturbances, such as hypercalcemia and hypokalemia with renal K+ wasting. The TAL also accounts for 20–25% of filtered K+ and Ca2+ reabsorption in parallel with Na+ reabsorption. Although K+ is directly reabsorbed by NKCC2, the reabsorption of Na+ provides the main driving force for Ca2+ reabsorption via paracellular routes (5). Reduced NKCC2 function would thus impede normal K+ and Ca2+ reabsorption in the TAL, leading to increased urine K+ and Ca2+ excretion. When these...
mice were fed low-K+ diets, significant hypokalemia was maintained, supporting that KSP-OSR1+/− mice had renal K+ wasting.

In conclusion, the analysis of global OSR1+/− and KSP-OSR1−/− mice sheds some light on the physiological role of OSR1 in BP regulation and renal Na+ handling. OSR1−/− mice exhibit hypernatraemia associated with reduced p-SPAK and p-NKCC1 abundance in aortic tissue and decreased p-NKCC2 with an increase in both p-SPAK and p-NCC in the kidney, which is indicative of a salt-wasting phenotype. KSP-OSR1−/− mice show markedly decreased p-NKCC2 expression in the TAL with a blunted response to furosemide and enhanced p-NCC expression in the DCT, supporting the notion that NKCC2 is the main target of OSR1 and accounts for the BS-like phenotype. These results show that OSR1 plays a dual role in arterial tonicity and renal Na+ reabsorption, primarily through NKCC1 and NKCC2, respectively. The development of OSR1 inhibitors suppressing vascular NKCC1 and renal NKCC2 may be a promising direction for antihypertensive therapy in the future.

Materials and Methods

Blood and Urine Analysis and BP Measurement. The phenotype of male mice was evaluated at the age of 12–14 wk. Mice were kept in metabolic cages for 24-h urine collection. Urine osmolalities under ambient conditions were determined using spot urine samples. Blood pressure, plasma, and urine electrolytes, and hormone were obtained and measured as previously described (17, 33).

Na+ and K+ Balance Study. The mice were raised on a 12-h day/night cycle, fed a normal rodent Chow diet, and given plain drinking water ad libitum. For the evaluation of renal Na+ and K+ handling, a low-Na− diet or low-K+ diet was fed for 6 d (34).

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