

hypotonic solution the cells from AQP7^{+/+} animals swelled reaching a maximum relative cell volume of 1.23 ± 0.01 ($n = 7$) within 3 to 4 min (Fig. 5A, squares). The cells then exhibited a regulatory volume decrease (RVD) to 1.12 ± 0.01 by the end of the hypotonic period. The response of the β -cells from AQP7^{-/-} mice (Fig. 5A, circles) was almost identical to that of cells the AQP7^{+/+} animals. Thus cell volume increased to a maximum of 1.24 ± 0.01 ($n=4$; $p > 0.1$), and exhibited a RVD which was similar to the $+/+$ cells, i.e. the initial rate of decrease was -0.016 ± 0.001 RCV.min⁻¹ in both populations ($p > 0.1$), and the final volume was 1.14 ± 0.01 in the AQP7^{-/-} cells which is not different to AQP7^{+/+} ($p > 0.1$). Fig. 5B shows the rate of the initial volume changes in response to the hypotonic solution with a resolution of 15 seconds. There is no difference in the rate of volume change between six cells from AQP7^{+/+} mice and six cells from $-/-$ animals (0.092 ± 0.011 versus 0.075 ± 0.010 , $p > 0.1$).

In six AQP7^{+/+} cells exposed to an increase in D-glucose concentration from 4 to 20 mM, the volume increased to a maximum of 1.06 ± 0.01 during a 10 min exposure to 20 mM D-glucose (Fig. 6A, squares). Similar increases in cell volume were observed in β -cells from $-/-$ mice ($n = 6$). Thus the maximum volume was 1.06 ± 0.01 (not different to wild-type, $p > 0.1$). Furthermore the volume changes (measured as the area under the curve different to 1.0), were not different, being 0.35 ± 0.10 RCV.min in $+/+$ and 0.32 ± 0.09 RCV.min in $-/-$ ($p > 0.1$). β -cell volume also increased when cells were exposed to 50 mM glycerol (Fig. 6B). However, the volume change in AQP7^{-/-} cells (0.49 ± 0.11 RCV.min, $n = 6$) was significantly lower than that observed in AQP7^{+/+} cells (volume change = 1.02 ± 0.16 RCV.min; $p < 0.02$). Incidentally, at variance with the response to hypotonicity (Fig. 5), that to either a rise in D-glucose concentration or the substitution of mannitol by glycerol (Fig. 6) failed to display a regulatory volume decrease.

Electrophysiological data

Electrical responses of β -cells to various stimuli are shown in Fig. 7 and 8. In the AQP7^{+/+} β -cells, a rise in D-glucose concentration from 2.8 mM to 8.3, 11.1 or 16.7 mM provoked a depolarization of the plasma membrane leading to the generation of action potentials (Fig. 7). Exposure of the β -cells from AQP7^{+/+} mice to a 33% hypotonic medium, to 50 μ M tolbutamide or to 50 mM glycerol (substituted for an equal concentration of mannitol) also caused plasma membrane depolarization and electrical activity (Fig. 8). Essentially comparable

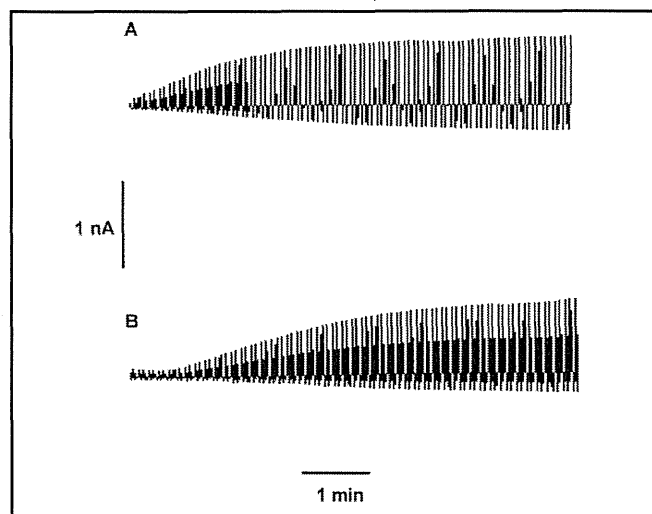


Fig. 9. Conventional whole-cell recordings of VRAC activity in AQP7^{+/+} (A) and AQP7^{-/-} (B) mouse pancreatic β -cells induced by an increase in the osmolarity of the intracellular pipette solution. Recordings are representative of 9-10 cells.

results were recorded in β -cells from AQP7^{-/-} mice when exposed to a rise in D-glucose concentration up to 8.3, 11.1 or 16.7 mM (Fig. 7), to the 33% hypotonic medium or to 50 μ M tolbutamide (Fig. 8, upper traces). However, in sharp contrast to the β -cells from AQP7^{+/+} mice, cells from AQP7^{-/-} mice failed to display any significant change in plasma membrane potential when exposed to 50 mM glycerol (Fig. 8, lower traces). Thus, in the experiments concerning the response to glycerol, the resting membrane potential in the presence of 2.8 mM D-glucose was virtually identical in β -cells from AQP7^{+/+} (-65.5 ± 2.3 mV; $n = 6$) and AQP7^{-/-} (-67.0 ± 1.2 mV; $n = 4$). In cells from AQP7^{+/+} mice, addition of glycerol caused a significant ($p < 0.05$) change in membrane potential to -53.8 ± 3.7 mV ($n = 6$). However, in AQP7^{-/-} cells, the corresponding value during exposure to glycerol was -66.8 ± 0.8 mV ($n = 4$, $p > 0.05$).

The last series of experiments investigated activity of the volume-regulated anion channel (VRAC) in β -cells from AQP7^{+/+} and -AQP7^{-/-} mice (Fig. 9). In both cases, channel activation was induced by swelling the cells using a hypertonic intracellular solution under conventional whole-cell recording conditions. In AQP7^{+/+} β -cells, maximal outward (I_o) and inward (I_i) currents averaged, respectively, $+99.5 \pm 6.8$ and -26.0 ± 3.2 pA/pF ($n = 10$). Comparable values were recorded from AQP7^{-/-} cells, i.e. respectively $+112.2 \pm 13.9$ and -32.2 ± 4.3 pA/pF ($n = 9$). The rate of activation of the VRAC current was also comparable in the AQP7^{+/+} and AQP7^{-/-} cells, half-maximal activation ($T_{0.5}$) being achieved, respectively, 120 ± 25 ($n = 9$) and 173 ± 43 ($n = 9$) seconds following

rupture of the membrane patch. The reversal potential of the VRAC current (V_{rev}) was -13.0 ± 0.4 mV ($n = 5$) and -10.3 ± 0.8 mV ($n = 5$) in the +/+ and -/- cells respectively. Thus, no significant differences in VRAC kinetics were apparent between AQP7^{+/+} and AQP7^{-/-} β -cells.

Discussion

The present study reveals that the release of insulin evoked by a rise in D-glucose concentration, by extracellular hypotonicity or by the isosmotic addition of glycerol in isolated pancreatic islets was always lower in AQP7^{-/-} than in AQP7^{+/+} mice. These findings contrast with those reported by Matsumura et al. [5] who observed, at both low (5.6 mM) and high (25.0 mM) D-glucose concentrations, a higher insulin output from islets isolated from AQP7^{-/-}, as distinct from AQP7^{+/+}, mice, despite a lower islet insulin content in the former AQP7^{-/-} mice than in the latter AQP7^{+/+} mice. In considering such conflicting findings, it should be emphasized that all secretory data in the present study were analyzed by paired comparison with the basal insulin output recorded over 30 min preincubation in an isotonic medium containing 2.8 mM D-glucose. Pooling together all available data, the basal insulin output averaged 17.6 ± 1.6 ($n = 112$) and 16.7 ± 1.1 μ U/islet ($n = 131$) in AQP7^{+/+} and AQP7^{-/-} mice respectively, leaving little doubt as to the lack of any statistically significant difference ($p > 0.66$) between these two mean values.

In terms of volume regulation, isolated β -cells from AQP7^{+/+} and AQP7^{-/-} mice both showed comparable rates and degrees of cell swelling when exposed to a hypotonic solution. This finding implies the existence of at least one water transport pathway in mouse β -cells in addition to AQP7. In this regard, Matsumura and colleagues [5] noted the expression of AQP8 in mouse β -cells, whilst the Na/K/2Cl transporter NKCC1, also expressed in β -cells [20], has been reported to act as a water co-transporter [21]. The present study confirms the expression of AQP8 and reveals that of AQP5 in mouse β -cells. β -cells from AQP7^{+/+} and AQP7^{-/-} cells also showed similar increases in volume in response to a rise in D-glucose concentration, an effect previously attributed to the intracellular accumulation of D-glucose metabolites [17]. This finding suggests that D-glucose metabolism is not significantly impaired in AQP7^{-/-} β -cells. In contrast, a significant difference was observed in the volume responses of AQP7^{+/+} and AQP7^{-/-} cells to glycerol. Thus, the isosmotic addition of glycerol (substituting for mannitol) caused a

significantly smaller increase in cell volume in the AQP7^{-/-} cells compared to those from wild type mice, presumably reflecting the activity of AQP7 as a glycerol transport pathway. This finding is consistent with the previous suggestion that AQP7 mediates both the influx [4] and efflux [5] of glycerol from insulin-producing cells. It should, however, be noted that the residual degree of swelling in AQP7^{-/-} cells in response to glycerol implies the existence of an additional, as yet unidentified, pathway of glycerol transport.

The recordings of β -cell electrical activity in the present study were consistent with cell volume changes. Thus, a rise in glucose to stimulatory concentrations was routinely found to cause depolarization and generate electrical activity in cells from both AQP7^{+/+} and AQP7^{-/-} mice. Similarly, exposure to hypotonic solutions was found to cause depolarization in wild type and knockout β -cells, presumably reflecting swelling-induced VRAC activation generating an inward current. Virtually identical responses were also seen in these cells to the application of the hypoglycemic sulfonylurea tolbutamide. Whilst sulfonylureas are known to inhibit K(ATP) channel activity [22], there is evidence that these drugs can also cause (beta)-cell swelling and increase VRAC activity [23]. However, whilst the isotonic addition of glycerol caused a marked depolarization and electrical activity in AQP7^{+/+} cells, little or no response was observed in β -cells from AQP7^{-/-} mice. Again, this finding underlines the proposed role of AQP7 as a glycerol transport pathway.

In view of the apparently normal volume and electrical responses of AQP7^{-/-} β -cells to various stimuli (with the notable exception of glycerol), it is interesting to note that secretory activity in these cells was impaired compared with those from wild type mice. In this regard, we should emphasise that the secretory data were obtained using intact islets, whereas cell volume and electrophysiological experiments required the use of isolated β -cells. Nevertheless, it is conceivable that, whilst 'proximal' events in β -cell function (including glucose metabolism, cell volume changes and the regulation of electrical activity) are unaffected by the absence of AQP7, this glyceroaquaporin could play a role at a 'distal' site of the exocytotic pathway. Such a proposal is compatible with the finding that the release of insulin by β -cells from AQP7^{-/-} mice was affected to a comparable relative extent in response to either nutrient or non-nutrient secretagogues. For example, it might imply the perturbed participation of some cytosolic protein otherwise tightly coupled in functional terms to AQP7. Alternatively, the

impaired secretory activity of AQP7^{-/-} β -cells could be related to a secondary consequence of AQP7 absence, such as the accumulation of triglyceride previously reported in these cells [5]. Consistent with this latter observation, it was noted during the course of the present study that islet cells from AQP7^{-/-} mice frequently appeared to contain large vacuoles, possibly representing lipid deposits.

In conclusion, the findings of the present investigations strongly suggest a dual role for AQP7 in the regulation of insulin release. On one hand, they are consistent with a key role for AQP7 in allowing both entry [4] and exit [5] of glycerol across the β -cell plasma membrane. Second, the alteration of the secretory response to D-glucose or extracellular hypoosmolarity in the β -cells from AQP7^{-/-} mice suggests a direct or indirect

role for AQP7 at a distal or downstream site in the stimulus-secretion pathway in pancreatic β -cells. The precise determinant(s) of such a second role remain(s), however, to be elucidated.

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ARTICLE

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ASK3 responds to osmotic stress and regulates blood pressure by suppressing WNK1-SPAK/OSR1 signaling in the kidney

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Changes in the osmolality of body fluids pose a serious danger to cells and living organisms, which have developed cellular systems to sense and respond to osmotic stress and to maintain homeostasis of body fluid. However, these processes are incompletely understood in mammals. Here we show that apoptosis signal-regulating kinase 3 (ASK3) is predominantly expressed in the kidney and alters its kinase activity bidirectionally in response to osmotic stress. We further demonstrate that ASK3 interacts with WNK1, mutation in which causes an inherited form of hypertension in humans. Knockdown of Ask3 by short interfering RNA enhances the activation of the WNK1-SPAK/OSR1 signalling pathway. Moreover, Ask3 knockout mice exhibit a hypertensive phenotype, in addition to hyperactivation of SPAK/OSR1 in renal tubules. Our results suggest that ASK3 is a unique bidirectional responder to osmotic stress and that it has a role in the control of blood pressure as an upstream suppressor of the WNK1-SPAK/OSR1 signalling pathway.

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The maintenance of the fluid balance is one of the most important issues for organisms. Thus, all living organisms are equipped with systems that sense and respond to osmolality at the cellular level^{1,2}. Both hypotonic and hypertonic situations are serious stress for cells, resulting in the perturbation of various cell functions, such as cell adhesion, gene expression, proliferation and apoptosis^{1–4}. Cells must transduce not only the ‘quantity’ but also the ‘direction’ of the osmotic changes to maintain osmotic homeostasis. Several signal transduction pathways have been proposed to be involved in either the hypertonic or hypotonic stress response^{1,2,4}. However, because there have been few studies that deal with osmolality as a graded amount of stress throughout a range from hypotonic to hypertonic situations, it is largely unknown whether and, if so, how a common machinery can manage both osmotic stresses.

The mitogen-activated protein kinase (MAPK) cascades are one of the major pathways regulating osmotic stress response and are well conserved from yeast to humans^{1,5,6}. Activated MAPK induces various stress responses, such as gene expression and apoptosis^{7,8}. Several MAP kinase kinase kinases (MAP3Ks), including MEKK2, MEKK3 and TAK1, have been reported to become activated in hypertonic stress to initiate the MAPK cascade in mammalian cells^{9,10}. However, little is known about the activation mechanisms and physiological roles of these MAP3Ks in osmotic stress responses. Furthermore, the involvement of other MAP3Ks in osmotic stress response is still elusive in mammals.

Recently, with-no-lysine [K] 1 (WNK1), which belongs to a unique kinase family, has been proposed to regulate the osmotic stress response^{11,12}. Activated by osmotic stress, WNK1 phosphorylates and activates STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1)^{13,14}. These two downstream kinases regulate ion transporters that control the cell volume^{15–17}. The WNK1-SPAK/OSR1 pathway is also proposed to function in renal salt reabsorption and blood pressure control^{12,14,17}. The *WNK1* gene mutation, which causes overexpression of the WNK1 protein

and misregulation of its splicing, results in an inherited hypertension termed pseudohypoaldosteronism type II (PHA II) in humans^{18–20}, presumably due to excess NaCl reabsorption in the renal tubules^{12,20}. Thus, the WNK1-SPAK/OSR1 pathway, which is activated by osmotic stress, appears to be involved in the pathogenesis of inherited hypertension and might be a cue to integrate the cellular osmotic response into the control of systemic blood pressure. However, it is still unclear how the activity of the WNK1-SPAK/OSR1 pathway is regulated by osmotic stress and whether the hypertensive phenotype induced by *WNK1* mutation is attributable to the misregulation of the WNK1-SPAK/OSR1 pathway.

In the present study, we demonstrate that a newly characterized MAP3K, apoptosis signal-regulating kinase 3 (ASK3)²¹, is activated and inactivated by hypotonic and hypertonic stress, respectively. We also found that ASK3 interacted with WNK1 and inhibited the WNK1-SPAK/OSR1 pathway in a kinase activity-dependent manner. Consistent with these results, ASK3 knockout mice exhibited hyperactivation of SPAK/OSR1 and Na⁺-Cl⁻ cotransporter (NCC) in the renal tubules, in addition to suffering from mild hypertension. Our results suggest that ASK3 is a unique bidirectional responder to both hypo- and hyper-tonic stress and that it functions as an upstream suppressor of the WNK1-SPAK/OSR1 pathway in the kidney to control the systemic blood pressure.

Results

ASK3 is predominantly expressed in the kidney. To find new kinases involved in stress response, we performed a database search based on homology with ASK1 (MAP3K5)²². We determined that MAP3K15 is highly homologous to ASK1 and ASK2 (MAP3K6), and thus, we have named MAP3K15 as ‘ASK3’ (Fig. 1a)²¹. Because ASK1 and ASK2 have been reported to activate the JNK and p38 MAPK pathways^{22,23}, we examined whether ASK3 also functions as a MAP3K. Similar to ASK1, overexpression of ASK3 WT but not a kinase-negative mutant of ASK3 (ASK3 KM) activated both JNK and p38 but not ERK

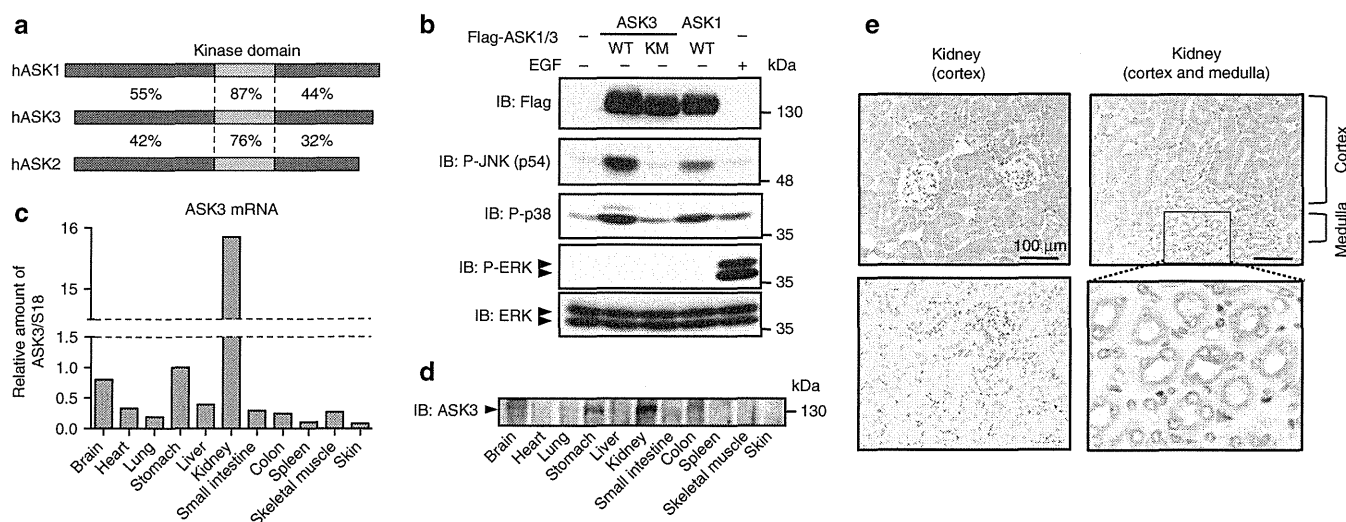


Figure 1 | The properties of ASK3 as an ASK family protein with a unique tissue distribution. (a) Schematic drawings of the ASK family kinases. The identity of the amino-acid sequences of human ASK3 (hASK3) with hASK1 and hASK2 are indicated in the N-terminus, the kinase domain and the C-terminus. (b) The activation of MAPKs due to the overexpression of ASK constructs was analysed by immunoblotting (IB). Epidermal growth factor (EGF) was applied as a positive control for ERK activation. (c) A real-time quantitative PCR for ASK3 mRNA in various tissues. The amount was normalized to the mRNA of S18 ribosomal protein as an internal control, and the relative amount to that in stomach are shown. (d) A tissue immunoblot analysis of ASK3. Tissue lysates containing equal amount of total protein (150 μ g protein per lane) were immunoprecipitated with ASK3 antibody, and then detected by IB. (e) Immunohistochemical staining of ASK3 in the kidney. The ASK3 signals were visualized by DAB staining (brown). The lower left panel shows a negative control treated with control IgG. The lower right panel represents a magnified image of the square in the upper panel. Scale bar, 100 μ m.

(Fig. 1b), suggesting that ASK3 serves as a MAP3K of the JNK and p38 pathways.

Different from the expression pattern of ASK1 or ASK2 (ref. 24), ASK3 was predominantly detected in the kidney by real-time quantitative PCR analysis (Fig. 1c) and immunoblot analysis probed with an ASK3-specific antibody (Fig. 1d and Supplementary Fig. S1a). In the mouse kidney, ASK3 was expressed in the epithelium of the renal tubules in both the cortex and the medulla but not in the glomeruli (Fig. 1e). Interestingly, the ASK3 signal was localized around the apical lumen of renal epithelial cells in the medulla. We also confirmed the presence of the ASK3 transcript in the renal tubules by *in situ* hybridization (Supplementary Fig. S2). These findings suggested that ASK3 is expressed in renal epithelial cells that are directly exposed to the massive osmotic changes in the kidney.

ASK3 responds bidirectionally to osmotic stress. According to the tissue distribution, we examined whether ASK3 responds to the osmotic stress. We monitored the kinase activity of endogenous ASK3 in HEK293A cells using a P-ASK antibody²⁵ (see Supplementary Figs S1c and S3 for detailed information about the P-ASK antibody) after changing the culture medium to hypo- (200 mOsm), iso- (300 mOsm) or hyper- (500 mOsm) osmotic solutions (Fig. 2a). Intriguingly, ASK3 exhibited moderate basal activity in isotonic solution and was activated and inactivated in the hypotonic and hypertonic stress, respectively. The osmolality dependency of the kinase activity was further confirmed by the *in vitro* kinase assay (Supplementary Figs S3d and S4). We also investigated the osmotic response of other ASK isoforms using the P-ASK antibody. ASK2 but not ASK1 showed similar bidirectional response to osmotic stress (Supplementary Fig. S5). To investigate how sensitively ASK3 responds to osmotic changes, we gradually altered the concentration of NaCl by 10-mM steps (Fig. 2b) or mannitol by 20-mM steps (Supplementary Fig. S6) in the extracellular solutions. The kinase activity of ASK3 was affected in both directions by a 10–20 mOsm change around the isotonic condition (130 mM NaCl). Both the activation in hypotonic stress and the inactivation in hypertonic stress occurred as early as 2 min after the osmolality changes (Fig. 2c). Furthermore, the activity of ASK3 was reversed within 10 min by the inversion of the osmotic stress (Fig. 2d). These results suggest that the activity of ASK3 is tightly regulated by osmolality in a sensitive, rapid and reversible manner.

We suspected that the progressive change of the P-ASK signal in the immunoblot analysis might be merely due to the change in the number of cells that exhibit a certain activity of ASK3 in the population. To examine whether a gradual change in the phosphorylation of ASK3 occurs at a single-cell level, we performed an immunocytochemical analysis using a newly generated P-ASK antibody (PA41) (Supplementary Fig. S1c). The P-ASK signal was gradually altered depending on the osmotic stress in each cell transfected with Flag-ASK3 WT (Fig. 2e). We could not detect any P-ASK signal in the cells transfected with Flag-ASK3 KM even under a hypotonic stress condition, suggesting that the phosphorylation of ASK3 relies on its own kinase activity. Furthermore, to assess the degree of ASK3 activity more quantitatively, we calculated the ratio of phospho-signal to total protein signal (P-ASK/Flag) in each cell using an image analyser (ArrayScan; Thermo Scientific Cellomics). A comparison of the frequency distribution of the ratio under the different osmotic conditions showed that the phosphorylation of ASK3 was gradually changed in each cell (Fig. 2f), confirming that ASK3 is a bidirectional responder to osmotic stress.

ASK3 regulates the WNK1-SPAK/OSR1 signalling pathway. To explore the role of ASK3 in the kidney, we performed a yeast two-hybrid screen of a human kidney cDNA library and identified WNK1 as an ASK3-binding protein. WNK1 is a Ser/Thr kinase with a unique kinase domain¹¹ that has recently been reported to regulate diverse ion cotransporters and channels that are involved in the control of cell volume and blood pressure. Moreover, it has also been reported that the kinase activity of WNK1 is altered by osmotic stress^{12,14,26}. Therefore, we investigated the relationship between ASK3 and WNK1 in human cells. Endogenous WNK1 was coimmunoprecipitated with ASK3 in HEK293F cells, confirming the interaction between ASK3 and WNK1 (Fig. 3a). The interaction was not affected by the osmotic conditions (Fig. 3b). The kinase activity of coimmunoprecipitated ASK3 showed a bidirectional response as is described in Fig. 2a, suggesting that ASK3 and WNK1 form a stable complex independently from the ASK3 activity. Other ASK isoforms were also coimmunoprecipitated with WNK1 (Supplementary Fig. S7).

We next examined the mutual regulation of kinase activities between ASK3 and WNK1. The kinase activity of WNK1 was monitored by immunoblotting with a phospho-specific antibody (P-Ser³⁸² in WNK1) as reported previously²⁷. Consistent with previous reports^{12,27,28}, WNK1 was activated by hypertonic stress in control siRNA-treated cells (Fig. 3c and Supplementary Fig. S8), indicating that the kinase activity of WNK1 is oppositely regulated to that of ASK3 in response to hypertonic stress. When endogenous ASK3 was depleted by siRNA, the kinase activity of WNK1 was enhanced. In contrast, WNK1 depletion did not yield any detectable effect on the ASK3 activity (Fig. 3c). These results suggest that ASK3 functions as an upstream regulator of WNK1 to suppress its kinase activity.

WNK1 phosphorylates and activates SPAK and OSR1¹⁴. These two downstream kinases are known to regulate ion transporters in the osmotic stress response^{15–17}. We generated a phospho-specific antibody to a conserved WNK1-mediated phosphorylation site of SPAK and OSR1 (P-SPAK/OSR1) (Supplementary Fig. S1d,e) (Thr²³³ and Thr¹⁸⁵ in SPAK and OSR1, respectively)^{14,27} and examined the response of SPAK/OSR1 to osmotic stress. In HEK293A cells, SPAK and OSR1 were activated by hypertonic stress, and SPAK was inactivated by hypotonic stress, which was the reciprocal pattern of the activity of ASK3 (Fig. 3d), implying that ASK3 may function as a negative regulator of the WNK1-SPAK/OSR1 pathway in a kinase activity-dependent manner.

We further examined the relationship between ASK3 and the WNK1-SPAK/OSR1 pathway in HeLa cells, in which a tight regulation of OSR1 activity by WNK1 had been reported^{15,27}. We found that the overexpression of ASK3 WT, but not of ASK3 KM, strongly suppressed the activity of OSR1 in hypotonic stress (Fig. 3e), suggesting that ASK3 inhibits OSR1 in a kinase activity-dependent manner. ASK3 depletion by two independent siRNAs resulted in the acceleration of OSR1 activity, particularly in hypotonic stress rather than in hypertonic stress (Fig. 3f), which is consistent with the hypothesis that ASK3 suppresses OSR1 with its kinase activity. The P-ASK signal was reduced but still observed under the ASK3 depletion, which appeared to be derived from other ASK isoforms (Supplementary Fig. S9a). However, either depletion of ASK1 or ASK2 had marginal effect on OSR1 activity (Supplementary Fig. S9b). Moreover, an additional depletion of ASK1 and ASK2 together with ASK3 (ASK triple knock down) did not lead to further activation of OSR1 than ASK3 single depletion (Supplementary Fig. S9a). These results suggested that ASK3 may have a pivotal role in the regulation of OSR1 activity among ASK isoforms. The downstream MAPKs, JNK and p38 (Fig. 1b), did not seem to

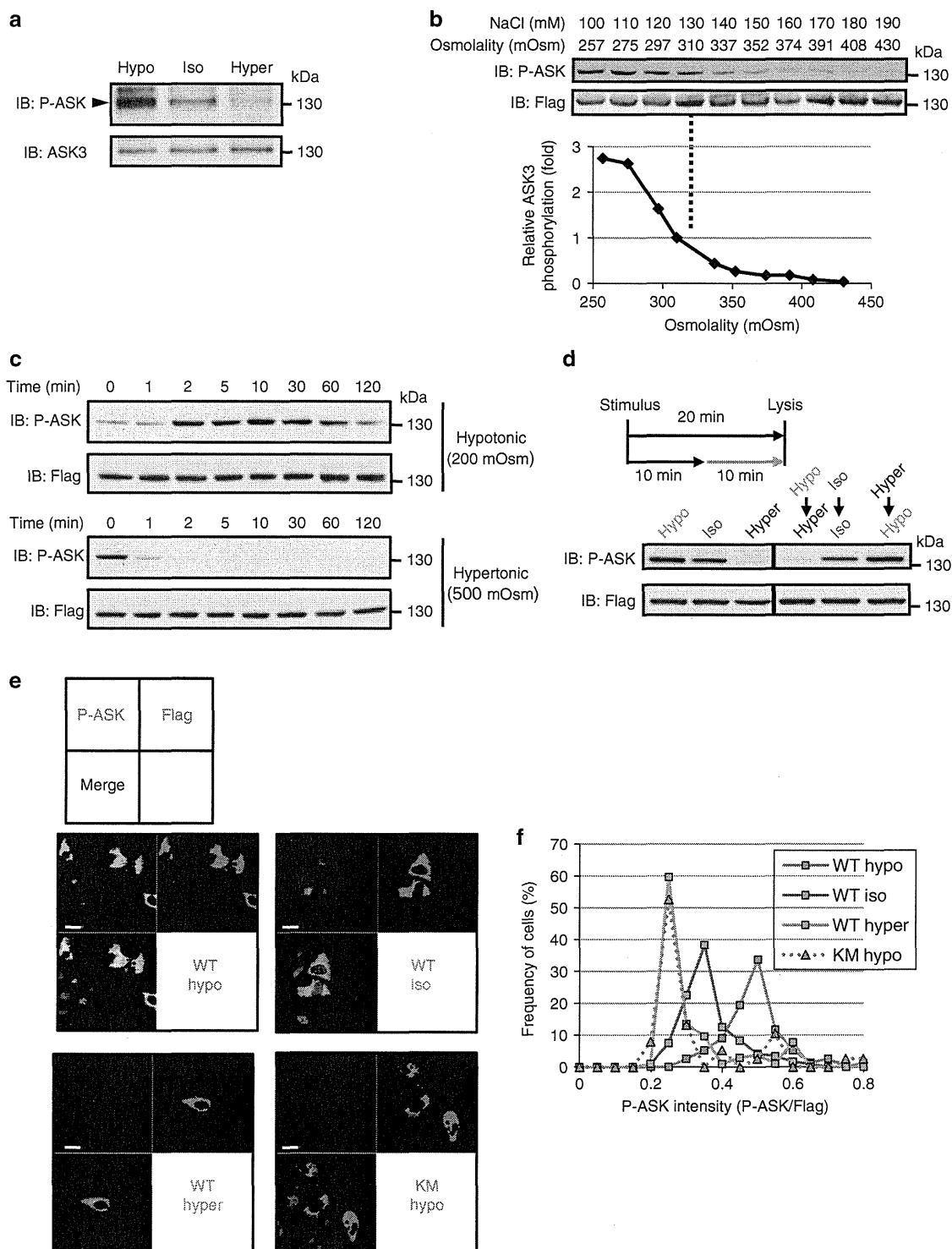


Figure 2 | ASK3 responds bidirectionally to osmotic stress. (a) The osmotic response of endogenous ASK3. ASK3 was immunoprecipitated from HEK293A cells and its kinase activity was evaluated by immunoblotting (IB) using a P-ASK antibody. (b) The NaCl concentration of the extracellular solutions was gradually altered in 10-mM steps. The actual osmolality of the solutions measured by osmometer are shown under the NaCl concentrations. The relative ASK3 activity was normalized to the value at 130 mM NaCl (isotonic). (c) The time course of ASK3 activity in response to osmotic stress. (d) The reversibility of ASK3 activity. HEK293A cells were exposed to the indicated osmotic solution for 20 min in the three lanes on the left. In the three lanes on the right, the solution was exchanged at the halfway point (10 min). (e) The confocal images of double immunofluorescence with P-ASK (green) and Flag (red) in HEK293A cells transfected with Flag-ASK3 WT or Flag-ASK3 KM. Cells were exposed to the indicated osmotic solutions for 20 min before the fixation. Scale bar, 20 μ m. (f) The frequency distribution of the P-ASK/Flag ratio derived from the immunofluorescence images under conditions similar to those of panel e. The Flag signal-positive cells were analysed by an image analyser (ArrayScan) to calculate the P-ASK/Flag signal ratio in each cell ($n = 38-120$).

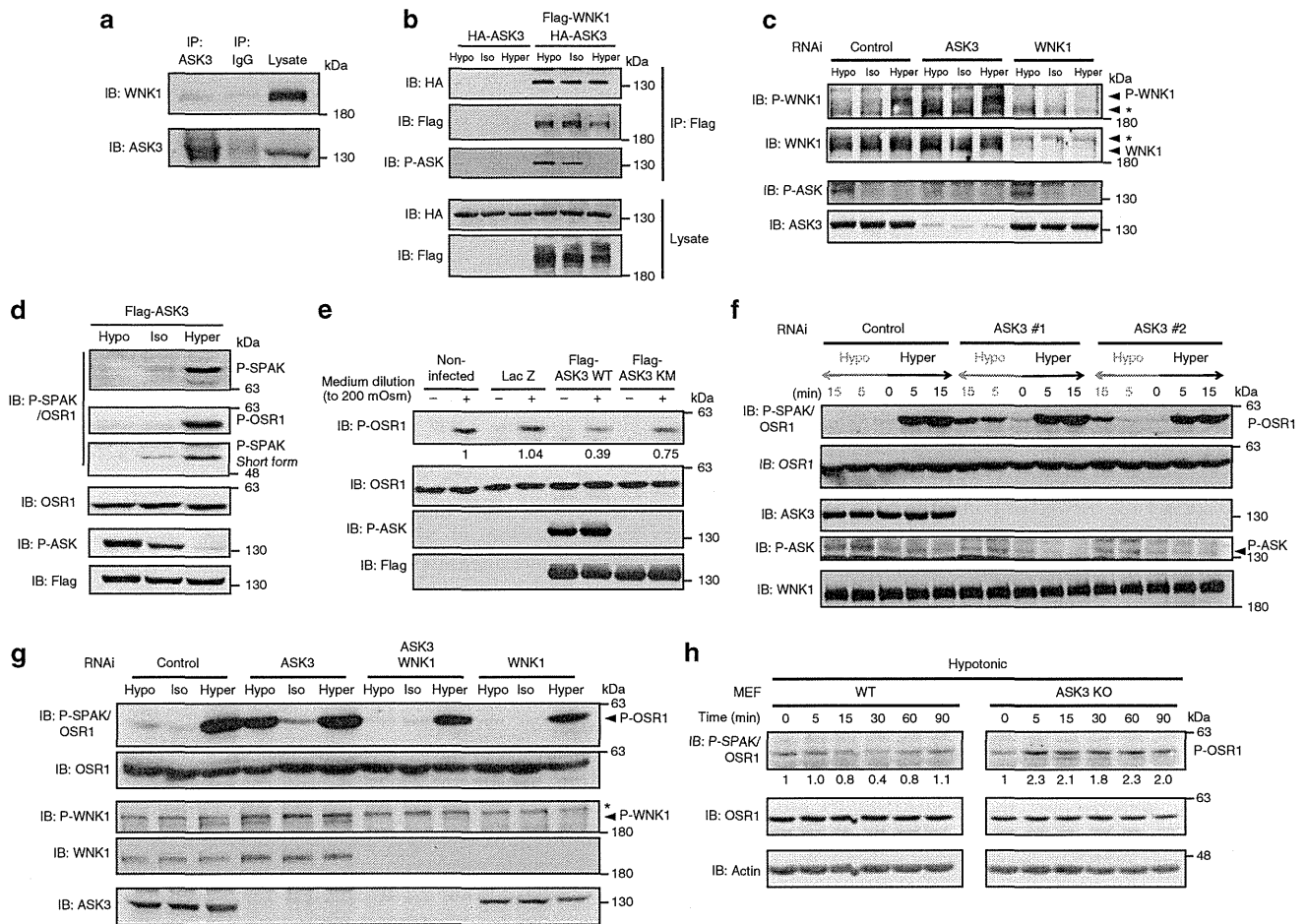


Figure 3 | ASK3 suppresses the WNK1-SPAK/OSR1 pathway through its kinase activity. (a) Coimmunoprecipitation of endogenous WNK1 with ASK3 in HEK293F cells. (b) Coimmunoprecipitation of HA-ASK3 with Flag-WNK1 under the hypo-, iso- and hyper-tonic conditions. (c) The indicated siRNAs were transfected into HEK293A cells, and cells were exposed to each osmotic stress for 10 min. The cell lysates were analysed by immunoblotting (IB) using phospho-specific antibodies to monitor the kinase activities of WNK1 and ASK3. The asterisks (*) indicate nonspecific bands. (d) HEK293A cells were transfected with Flag-ASK3 and exposed to solutions of varying osmolality. The activities of endogenously expressed SPAK and OSR1 were analysed by IB using a P-SPAK/OSR1 antibody. (e) HeLa cells were infected with adenovirus (100 MOI) encoding Lac Z, Flag-ASK3 WT or Flag-ASK3 KM to overexpress these proteins. The cells were exposed to diluted medium (200 mOsm) for 10 min. The quantified relative amounts of P-OSR1 are indicated below the bands. (f) HeLa cells were transfected with a control or two independent ASK3 siRNAs, followed by the treatment with both hypo- and hypertonic solutions for the indicated durations. (g) HeLa cells were transfected with a single siRNA or a combination of siRNAs (ASK3 + WNK1). The cells were treated with both hypo- and hyper-tonic solutions for 15 min. * indicates nonspecific bands. (h) MEFs derived from WT or ASK3 KO mice were exposed to hypotonic stress for the indicated durations. The intensity of P-OSR1 signal was quantified by densitometry and shown under the bands (normalized to each intensity at time 0).

be involved in the regulation of the WNK1-SPAK/OSR1 pathway, because the inhibitors of JNK and p38 did not enhance the OSR1 activity in response to hypotonic stress as ASK3 depletion did (Supplementary Fig. S10).

To examine whether ASK3 inhibits OSR1 activity through the suppression of WNK1, we performed a combinatorial knockdown of ASK3 and WNK1 (Fig. 3g). In the control knockdown cells, OSR1 was activated slightly and strongly by hypotonic and hypertonic stress, respectively. The activation of OSR1 was suppressed by the depletion of WNK1 alone, confirming the previous report that WNK1 is a major activator of OSR1 in osmotic stress²⁷. In ASK3-depleted cells, OSR1 was strongly activated not only by hypertonic stress but also by hypotonic stress, which was consistent with the WNK1 activity monitored by P-WNK1 signal (Fig. 3g). The hypotonic stress-induced activation of OSR1 was completely abolished by the additional depletion of WNK1 with ASK3 knockdown. These results suggest that the hypotonicity-induced OSR1

hyperactivation in the absence of ASK3 was mediated by the misregulation of WNK1. The hyperactivation of endogenous WNK1 under ASK3 depletion was confirmed by an *in vitro* kinase assay (Supplementary Fig. S11). On the other hand, we found that other WNK family kinases (WNK2-4), among which WNK3 and WNK4 have been reported to activate SPAK/OSR1 (ref. 29), were also coimmunoprecipitated with ASK3 (Supplementary Fig. S12a). However, the depletion of WNK1 showed more dominant effect than those of other WNK isoforms on the OSR1 activity (Supplementary Fig. S12b). The result endorses the notion that the hyperactivation of OSR1 upon ASK3 depletion is mainly regulated through the WNK1 misregulation.

We further examined the hypotonic stress response of OSR1 in mouse embryonic fibroblasts (MEFs) derived from WT and ASK3 KO mice (Supplementary Fig. S13). Consistent with the result of ASK3-knockdown HeLa cells, the activation of OSR1 was accelerated in ASK3 KO MEFs (Fig. 3h). The hyperactivation of OSR1 in ASK3 KO MEFs was suppressed by the exogenous ASK3

expressed via the adenovirus infection (Supplementary Fig. S14). These results further supported the hypothesis that ASK3 functions as a suppressor of the WNK1-OSR1 pathway in hypotonic stress.

ASK3 knockout mice exhibit a hypertensive phenotype. A mutation of *WNK1* gene, which leads to the overexpression of the WNK1 protein and the misregulation of its splicing, has been reported to be a cause of a hereditary hypertension termed PHA II^{18,30}. This pathogenic mutation has been considered to eventually cause hypertension through the hyperactivation of NCC and Na⁺-K⁺-2Cl⁻ cotransporter (NKCC), the substrates of SPAK/OSR1 (refs 12,16,18–20,31). Furthermore, it has recently been demonstrated *in vivo* that the SPAK activity that is regulated by WNK kinases is physiologically important for controlling the blood pressure through the regulation of the renal ion transporters NCC and NKCC³². Considering the suppressive function of ASK3 on the WNK1-SPAK/OSR1 pathway together with the fact that ASK3 is also expressed in the kidney (Fig. 1c–e), we speculated that ASK3 might regulate the WNK1-SPAK/OSR1-NCC pathway in renal tubules to control the systemic blood pressure. Thus, we next examined the activity of SPAK/OSR1 and of its substrate NCC in the renal tubules of ASK3 KO mice.

The immunostaining of serial cryosections of the renal cortex with P-SPAK/OSR1, total NCC (T-NCC) and phospho-NCC (P-NCC)³³ antibodies suggested that the serial renal tubules labelled by the T-NCC antibody (the distal convoluted tubules, or DCT) were properly detected by the P-SPAK/OSR1 and P-NCC antibodies (Fig. 4a). Additionally, the P-SPAK/OSR1 and P-NCC but not T-NCC signals disappeared after treatment with λ -phosphatase (Supplementary Fig. S15), confirming the phospho-specificity of these antibodies. In the renal cortex of ASK3 KO mice, the P-SPAK/OSR1 signals were stronger and the number of signal-positive tubules was increased compared with those of WT mice (Fig. 4b). We objectively quantified the confocal images using the image analyser ArrayScan (Supplementary Fig. S16). The area of the signal-positive region was significantly increased in the ASK3 KO mice (Fig. 4c). These results suggest that the genetic depletion of ASK3 enhanced the SPAK/OSR1 activity and increased the area of SPAK/OSR1-active renal tubules in the kidney. We performed a similar analysis of the immunofluorescence images probed with T-NCC and P-NCC. In the ASK3 KO mice, the expression area of NCC (labelled with the T-NCC antibody) was not altered (Fig. 4d), and the P-NCC-positive area was slightly, but not significantly, increased (Fig. 4e). To totally evaluate the NCC activity at the whole-kidney level, we performed an immunoblot analysis in the kidney lysate with the P-NCC antibody. The P-NCC intensity was modestly but significantly increased in the kidneys of ASK3 KO mice compared with those of WT mice without any alteration in the protein levels of WNK1 or OSR1 (Fig. 4f). These results suggest that ASK3 functions as a suppressor of SPAK/OSR1 and thus NCC in the DCT of the mouse kidney.

It has been demonstrated that the hyperactivation of the SPAK/OSR1-NCC pathway leads to systemic hypertension through the excess reabsorption of NaCl in the DCT^{17,32,34}. Therefore, we measured the blood pressure of ASK3 KO mice by a tail-cuff method. The systolic blood pressure (SBP) of the ASK3 KO littermates (SBP: 105.9 \pm 1.8 mm Hg) was similar to that of WT (105.9 \pm 2.1 mm Hg) at 14 weeks of age. However, the SBP of the ASK3 KO mice increased significantly along with aging, reaching 115.9 \pm 3.6 mm Hg at 30 weeks, whereas that of the WT mice was only slightly increased (109.3 \pm 1.5 mm Hg) at the same age (Fig. 5a).

We next performed the NaCl loading on the ASK3 KO mice, as excess salt intake is well known to be involved in the pathogenesis of hypertension^{35,36}. The application of a high-NaCl diet for 21 days significantly increased the SBP of the ASK3 KO mice at the age of 13 weeks (earlier than the spontaneous hypertension, see Fig. 5a), whereas the SBP of the WT mice was slightly decreased by the same treatment (Fig. 5b). This phenotype was unique to ASK3 among ASK isoforms, because neither ASK1 KO nor ASK2 KO mice exhibited hypertension upon the NaCl loading (Supplementary Fig. S17). We further performed telemetry measurement of the blood pressure to more accurately evaluate the salt susceptibility of ASK3 KO mice (Fig. 5c–e). Consistent with the result by the tail-cuff method, the SBP of the ASK3 KO mice became significantly higher than that of the WT after NaCl loading in the dark period of 15th day (Fig. 5e). The diastolic blood pressure after the NaCl loading was modestly, but not significantly ($P=0.10$), higher in the ASK3 KO mice (Supplementary Fig. S18).

Together with the result that the ASK3 KO mice showed hyperactivation of SPAK/OSR1 and NCC in the kidney, the hypertensive phenotype of the ASK3 KO mice suggests that ASK3 is involved in blood pressure control, at least in part, through the suppression of the WNK1-SPAK/OSR1 pathway in the renal tubules.

Discussion

Recent studies have revealed that the WNK1-SPAK/OSR1 pathway activates NCC¹⁶ and NKCC¹⁵ and that WNK1 inhibits K⁺-Cl⁻ cotransporter independent of both SPAK and OSR1 (ref. 26) (Fig. 6). Because these transporters have important roles in cell volume regulation and renal salt reabsorption^{2,17}, WNK1 has received substantial attention in the regulation of the osmotic stress response and systemic blood pressure^{12,14}. However, little is known about upstream regulators of WNK1 activity. In the present study, we have demonstrated that ASK3 bidirectionally responds to osmotic stress and suppresses the WNK1-SPAK/OSR1 pathway in a kinase activity-dependent manner. The bidirectional alteration of ASK3 activity in a single cell (Fig. 2e,f) may provide a plausible regulation of cellular osmotic response, as cells must respond inversely to oppositely directed stresses (for example, the uptake and excretion of ions) (Fig. 6). Moreover, ASK3 showed a quick response and a reversible flexibility to osmotic stress (Fig. 2c,d), which also appear helpful for the immediate and tight cellular osmotic response. With these properties, we consider that ASK3 may integrate hyper- and hypo-tonic stress responses into a single signal system.

The graduated response of ASK3 to osmotic stress could be observed in response to the change of either NaCl or mannitol in the solution (Fig. 2b and Supplementary Fig. S6), suggesting that ASK3 responds to changes not in the NaCl concentration *per se*, but in the osmolality. The hypotonicity-induced phosphorylation of Thr⁸⁰⁸ could not be observed in ASK3 KM (Fig. 2e,f), suggesting that the activation of ASK3 is achieved by autophosphorylation, which may correspond to the autophosphorylation of ASK1 in response to reactive oxygen species²⁵. However, it is still unclear what mechanism is involved in the inactivation of ASK3 in hypertonic stress. We are speculating that there should be ASK3 phosphatase(s) activated and/or recruited in response to hypertonic stress. We examined *in vitro* whether purified ASK3 could directly alter its activity in osmotic solution (Supplementary Fig. S4). Neither the activation in hypotonic solution nor the inactivation in hypertonic solution could be observed for the isolated ASK3. This result suggested that certain cellular components are required to alter the activity of ASK3. The elucidation of the mechanism responsible for the

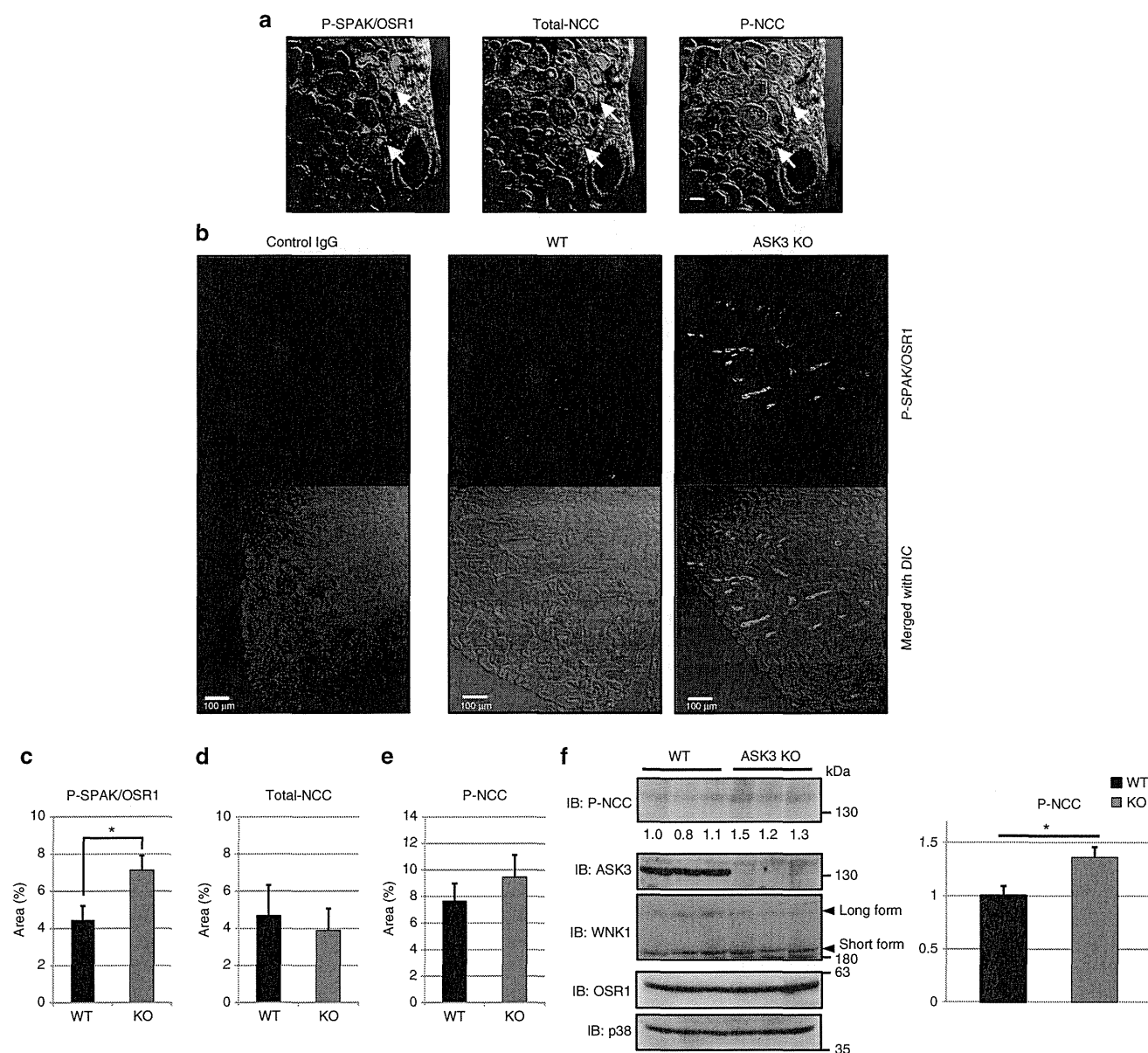


Figure 4 | SPAK/OSR1 and NCC is hyperactivated in the kidney of ASK3 knockout mice. (a) Immunofluorescence images of serial cryosections of the mouse kidney probed with P-SPAK/OSR1, total-NCC and P-NCC antibodies. The representative signals (shown in red) in serial renal tubules are indicated by white arrows. The differential interference contrast (DIC) images are merged. Scale bar, 20 μm . (b) Low-power immunofluorescence images of the kidney probed by control IgG (left) and P-SPAK/OSR1 (middle: WT, right: ASK3 KO). The lower panels show the images merged with the DIC (the fluorescence signals are shown in red). Scale bar, 100 μm . (c–e) The fractions of the areas that are immunostained by each antibody were quantified by an image analyser (black bar, WT; $n=3$; red bar, ASK3 KO; $n=3$, $P=0.026$ in Fig. 3c); Data are means \pm s.e.m. (details are described in Supplementary Fig. S16). * P is for a two-sided t -test comparing WT with ASK3 KO. (f) The kidney lysate of WT and ASK3 KO mice were analysed by immunoblotting with the described antibodies. The quantified relative amounts of P-NCC are indicated below the bands and summarized in a bar graph (black bar, WT; $n=3$; red bar, ASK3 KO; $n=3$, $P=0.048$); The data are represented as the means \pm s.e.m. * P is for a two-sided t -test comparing WT with ASK3 KO.

bidirectional regulation of ASK3 is one of the most interesting issues awaiting future studies.

We also found that ASK3 functions as a negative regulator of the WNK1-SPAK/OSR1 pathway in the osmotic stress response (Fig. 3). ASK3 knockdown by siRNA resulted in the acceleration of the activity of WNK1 in both HEK293A and HeLa cells (Fig. 3c,g). Additionally, our results suggested that ASK3 suppresses the WNK1-OSR1 pathway through its kinase activity (Fig. 3e). The fact that ASK3 depletion affected OSR1 activity in hypotonic stress more than in hypertonic stress further supported the importance of kinase activity in the suppression (Fig. 3f). It is

conceivable that ASK3-dependent inhibitory regulation counteracts and overrides the activation mechanism of WNK1 in hypotonic stress, whereas WNK1 may be freely activated in hypertonic stress, as ASK3 is inactivated in the hypertonic condition (Fig. 6). The balance between ASK3-independent activation and ASK3-dependent inhibition might determine whether the WNK1-SPAK/OSR1 pathway is activated or inhibited in hypotonic stress. Such hypotonicity-specific inhibition by ASK3 may provide an opportunity for the WNK1-SPAK/OSR1 pathway to be oppositely regulated in hypo- and hypertonic conditions (Fig. 6).

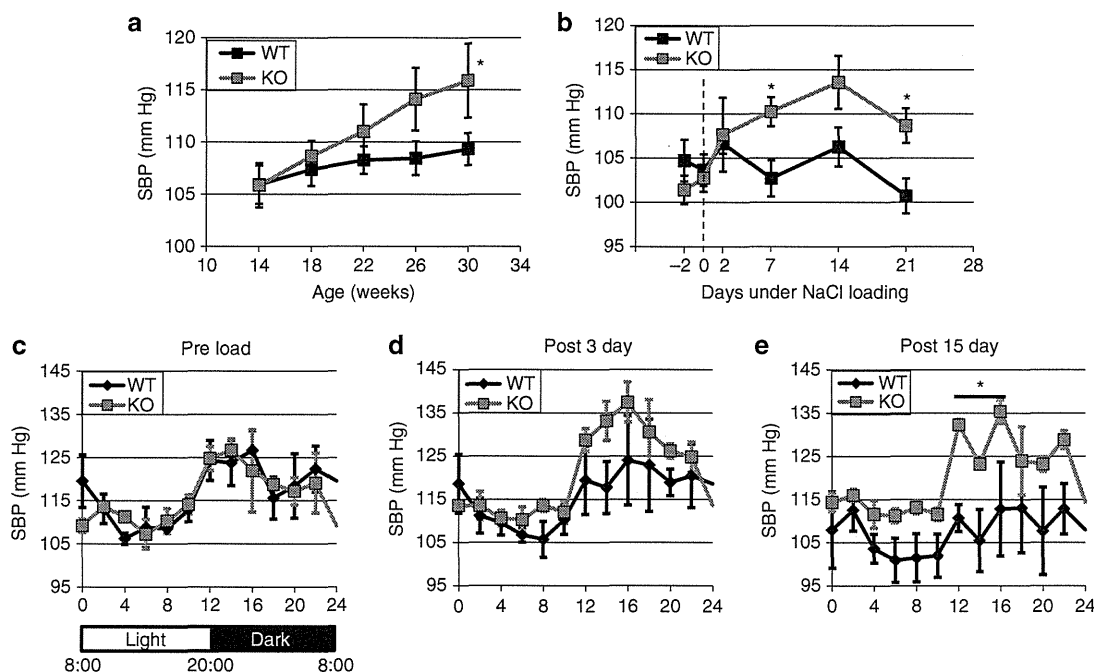


Figure 5 | ASK3 knockout mice exhibit a hypertensive phenotype. (a) The SBP of WT and ASK3 KO littermates (black square, WT: $n=14$; red square, ASK3 KO: $n=9-10$, $P=0.018$) at the indicated ages. The data are represented as the means \pm s.e.m. * P -value is for a two-sided t -test comparing the SBP at 30 weeks with that at 14 weeks. (b) The changes in the SBP after dietary NaCl loading. The SBP was measured on the indicated days of NaCl loading (black square, WT: $n=6$; red square, ASK3 KO: $n=7-8$, $P=0.010$ for 7th day and $P=0.010$ for 21st day); The data are represented as the means \pm s.e.m. * P -value is for a two-sided t -test comparing the SBP of the ASK3 KO with that of the WT at the same day. (c–e) The telemetry measurement of the SBP before loading (Pre load) and 3 and 15 days after the start of NaCl loading (black square, WT: $n=3$; red square, ASK3 KO: $n=3$). Data are obtained every minute and averaged every 2 h. The light and dark periods are indicated by the white and black bars, respectively. * $P=0.045$ for a two-sided t -test comparing the SBP of the ASK3 KO with that of the WT at the first half of the dark period of 15th day; The data are represented as the means \pm s.e.m.

Although we have demonstrated the endogenous interaction between ASK3 and WNK1 (Fig. 3a) and the importance of the kinase activity of ASK3 (Fig. 3e), it is still unknown so far whether ASK3 suppresses WNK1 through the direct phosphorylation of WNK1 or through the phosphorylation of other unidentified protein(s). At least, WNK1 appears to be the pivotal player among the WNK isoforms in the OSR1 hyperactivation induced by ASK3 depletion, because the combinatorial knockdown of WNK1, rather than those of other WNK isoforms, together with ASK3 resulted in the complete abolishment of the effect of ASK3 knockdown (Fig. 3g and Supplementary Fig. S12). Thus, we consider that ASK3 depletion resulted in the OSR1 hyperactivation through the WNK1 misregulation. Therefore, our results propose ASK3 as a novel regulator of the WNK1-SPAK/OSR1 pathway in the osmotic stress response (Fig. 6).

Consistent with the cell-based study, we found that the genetic depletion of ASK3 leads to the hyperactivation of SPAK/OSR1 in the kidney (Fig. 4). It is suggested that PHA II patients harbouring genetic mutations of *WNK1* or *WNK4* and the mouse model of PHA II are affected with hypertension at least in part through the hyperactivation of NCC in the kidney^{18,30,33,37}. In the case of *WNK1* gene mutation, it is suggested that the mutation causes the overexpression of the WNK1 protein and the misregulation of the splicing of the WNK1 transcript, presumably leading to the hyperactivation of NCC via the abnormally enhanced WNK1-SPAK/OSR1 signaling^{18–20}. We considered that the hypertensive phenotype of ASK3 KO mice might be interpretable in a similar context, as hyperphosphorylation of NCC was observed in the kidneys of ASK3 KO mice (Fig. 4f). The susceptibility to acute NaCl loading in the maintenance of blood pressure also endorsed the defects in salt homeostasis in the

ASK3 KO mice (Fig. 5b,e). Although it is well established that the patients and mouse model of PHA II are affected not only with hypertension but also with hyperkalemia due to impaired renal K^+ secretion^{18,30,33,37}, ASK3 KO mice exhibit no alteration in the plasma K^+ concentration and a slight decrease in urinary K^+ secretion ($P=0.14$) (Supplementary Table S1). We suspected that the impact of ASK3 depletion on the renal salt regulation would be milder than that of WNK1 mutation itself, because ASK3 suppresses WNK1 primarily in situations where ASK3 is active, such as in a hypotonic circumstance. The late onset of the mild hypertension in ASK3 KO mice (Fig. 5a) may be attributable to the same reason. Alternatively, it is also possible that there might be some 'back-up' systems that would maintain plasma K^+ level under the disruption of ASK3, or ASK3 might exert positive effects on the K^+ reabsorption independent of its negative effect on the WNK1-SPAK/OSR1 pathway, as ASK3 is also expressed in other renal tubules than the DCT, such as the collecting duct (Fig. 1e and Supplementary Fig. S2).

The results presented in our study demonstrate that ASK3 is a unique osmotic stress-responsive kinase that suppresses the WNK1-SPAK/OSR1 pathway in cells and is involved in the control of blood pressure, at least in part through the regulation of SPAK/OSR1 activity in the kidney. Our results also endorse the notion that the hyperactivation of the WNK1-SPAK/OSR1 pathway leads to hypertension from the viewpoint of a novel upstream regulator. Recently, it is suggested by using *WNK1*^{+/-} mice that the WNK1-SPAK/OSR1 pathway is involved in the vasoconstriction in the regulation of the systemic blood pressure^{31,38}. Therefore, it is an interesting issue to investigate whether ASK3 regulates the WNK1-SPAK/OSR1 pathway also in the blood vessels. Further studies on ASK3, including the

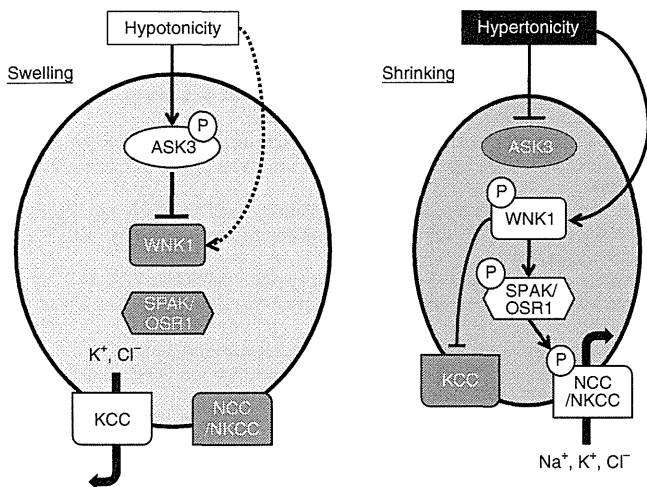


Figure 6 | A schematic model of ASK3's role in the cellular osmotic stress response. When a cell is exposed to hypotonic or hypertonic stress, the cell volume is increased (swelling) or decreased (shrinking), respectively. It is broadly understood that various types of ion channels and transporters are regulated by phosphorylation during the restitution of cell volume. K^+Cl^- cotransporter (KCC) is activated by the dephosphorylation in response to cell swelling and releases ions to the outside from the cell to decrease the cell volume. In contrast, NKCC is activated by phosphorylation in response to hypertonic stress and imports ions into the cell to increase the cell volume. Our results suggest that hypotonic stress activates ASK3, leading to the inhibition of the WNK1-SPAK/OSR1 pathway. In contrast, hypertonic stress inhibits ASK3, which relieves WNK1 to activate the downstream SPAK/OSR1, resulting in the activation of NCC and NKCC. The activities of NCC and NKCC are also involved in salt reabsorption in the renal tubules, which has a substantial impact on the regulation of the systemic blood pressure.

relationship with other WNK kinases (Supplementary Fig. S12a), will provide a novel understanding of the physiological and pathophysiological signal transduction pathways related to body fluid homeostasis in humans.

Methods

Antibodies. Monoclonal antibodies to human ASK3, mouse ASK3 and human SPAK were established as described previously²³. GST-tagged C-terminal fragments of human ASK3 (corresponding to amino acids 909–1,313), mouse ASK3 (amino acids 1,085–1,331) and human SPAK (amino acids 340–547) were produced and purified from *Escherichia coli* strain BL21 as antigens. All of them were used in a 1,000-fold dilution for the immunoblot. The antigen of the monoclonal antibody to phospho-specific ASK (PA41) was constructed by conjugating the synthesized phospho-peptide (amino acids in human ASK3 805–815: CTE[pT]FTGTLQY) with keyhole-limpet hyemecyanin²³. WYK/Izm rats were immunized via the hind footpads with the recombinant proteins using Freund's complete adjuvant. The enlarged medial iliac lymph nodes from the rats were used for cell fusion with mouse myeloma cells, SP2, and the hybridoma cells were cultured in GIT medium (Wako Pure Chemicals) supplemented with 100 μ M hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine, 10% FBS and 2% BM Condensed H1 (Roche Applied Science). The antibody-secreting hybridoma cells were selected by limiting dilution, screening with enzyme-linked immunosorbent assay and immunoblotting (Supplementary Fig. S1). PA41 was used only in the immunocytochemistry shown in Fig. 2e,f in a 100-fold dilution.

Rabbit polyclonal antibodies to mouse ASK3 (amino acids 201–218: CESDAQRRASEYMQPNWD), P-WNK1 (amino acids 376–388: RASFAK[pS]VIGTPE) and P-SPAK/OSR1 (amino acids 226–239: TRNKVRK[pT]FVGTGTPC) were constructed as described previously²⁵ and used in 1,000- (ASK3 and P-WNK1) and 300-fold (P-SPAK/OSR1) dilutions for the immunoblot. P-SPAK/OSR1 antibody was also used in a 500-fold dilution for the immunohistochemistry. The indicated synthetic peptides were conjugated with KLH and used as immunogens. The antisera obtained from immunized rabbits were applied to affinity column for positive selection. The polyclonal antibody to phospho-ASK1 (pT845) was described previously²⁵, which was used in all immunoblot experiments as P-ASK antibody in a 2,000-fold dilution. The

monoclonal antibody to ASK2 was described previously²³ and used in a 1,000-fold dilution for the immunoblot. The antibody to P-NCC was described and characterized in the previous report²³, which was used in a 5,000-fold dilution for immunoblot and a 500-fold dilution for immunohistochemistry.

The antibody to Flag-tag (M2) and actin (AC-40) were purchased from Sigma and used 500- and 10,000-fold dilutions for the immunoblot, respectively. The antibody to HA-tag (3F10) was purchased from the Roche Applied Science and used in a 500-fold dilution for the immunoblot. Phospho-specific antibodies to JNK (P-JNK: Thr¹⁸³/Tyr¹⁸⁵), p38 MAPK (P-p38: Thr¹⁸⁰/Tyr¹⁸²) and ERK (P-ERK: Thr²⁰²/Tyr²⁰⁴) were purchased from Cell Signaling and used in 1,000- or 2,000-fold dilutions for the immunoblot. The antibody to ERK was also purchased from Cell Signaling and used in a 1,000-fold dilution for the immunoblot. The antibodies to JNK (JNK-FL) and p38 (C-20-G) were purchased from Santa Cruz Biotechnology and used in 5,000- and 2,000-fold dilutions for the immunoblot, respectively. The antibody to WNK1 was purchased from Bethyl and used in a 5,000-fold dilution for the immunoblot. The antibodies to OSR1 (M06 and M01) were purchased from Abnova and used in a 10,000-fold dilution for the immunoblot. The antibody to total-NCC was purchased from Millipore and used in a 500-fold dilution for the immunohistochemistry.

Cell culture and osmotic stress treatments. HEK293A cells, HeLa cells and MEFs were cultured as described previously²³. Cells were exposed to osmotic stress by changing the extracellular solutions having three kinds of osmolality (hypotonic, isotonic and hypertonic buffer). Isotonic buffer (300 mOsm per kg H₂O) contained 130 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM glucose and 20 mM mannitol. Hypertonic buffer (500 mOsm per kg H₂O) contained additional 200 mM mannitol compared with isotonic buffer. In hypotonic buffer (200 mOsm per kg H₂O), mannitol was excluded and NaCl was reduced to 90 mM from isotonic buffer. The absolute osmolality was verified by a cryoscopic osmometer (OSMOMAT 030; Gonotec). We also used distilled water-diluted culture medium (200 mOsm per kg H₂O) for hypotonic stimulus.

Immunoblotting analysis. Cell extracts and immunoprecipitates were resolved on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 8.0), the membranes were probed with appropriate antibodies. The antibody-antigen complexes were detected using the ECL system (GE Healthcare) as described previously²³. In some experiments, the amount of signals was measured by an image densitometer (ImageJ 1.37v).

Immunostaining of the cell and image analyser. HEK293A cells were transfected with Flag-tagged ASK3 WT or KM and exposed to osmotic buffer for 20 min. After stimulation, cells were fixed in 4% formaldehyde in PBS, permeabilized in 1% Triton X-100. Blocking was performed with 5% skim milk in TBS-T for 1 h, and then incubated with anti-Flag (M2) and anti-phospho ASK (PA41) antibodies overnight. Then, cells were incubated with secondary antibody (Alexa Fluor 488 anti-rat IgG and Alexa Fluor 555 anti-mouse IgG; Molecular Probes) overnight. Cells were counterstained with Hoechst 33258 (Dojindo) for 3 min before mounting with GEL/MOUNT (Biomedica). Images were collected by a confocal microscope (LSM 510 META; Carl Zeiss).

In imaging analysis by ArrayScan VTI (Thermo Scientific Cellomics), sample preparation was identical as described above. Cells were identified by the Hoechst signal in nuclei and ASK3-transfected cells were selected by the Flag signals. The region of interest (ROI) was defined by Flag signal and the fluorescence intensity ratio of P-ASK and Flag (P-ASK/Flag) in the ROI was calculated in each cell to define an ASK3 activity (38–120 cells in each preparation) using Cellomics Colocalization V3 software.

Coimmunoprecipitation analysis. Coimmunoprecipitation analysis was performed as described previously²³. For endogenous proteins, HEK293F cells were lysed with the IP lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 1 μ M Leupeptin). The cell extracts were clarified by centrifugation and the supernatants were immunoprecipitated with anti-ASK3 antibody or control rat IgG using Protein G sepharose beads (GE Healthcare). For exogenous tagged protein, HEK293A cells were lysed with the IP lysis buffer. The cell extracts were clarified by centrifugation and the supernatants were immunoprecipitated with Flag antibody gel (M2 gel; Sigma). The beads were washed with the IP lysis buffer before supplied to SDS-PAGE.

Immunohistochemistry. C57BL/6J strain mice (male, 12-week old) were dissected and the kidney was fixed and embedded in paraffin. Tissue sections were de-paraffined and heat activated in citrate buffer (pH 6.0) for 15 min at 95 °C. After the blocking with ChemMate Antibody Diluent (Dako), samples were incubated with the monoclonal antibody to mouse ASK3 overnight at 4 °C, and then reacted with Simple Stain MAX-PO (NICHIREI), an amino-acid polymer reagent combined with secondary antibody and peroxidase. Antigen-antibody reaction was visualized with diaminobenzidine tetrahydrochloride and hydrogen peroxide. The

samples were counterstained with Mayer's hematoxylin. The experimental protocol was approved by the Animal Research Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo Japan).

Cryosection, immunofluorescence and image analysis. For the immunofluorescence, the kidneys of age-matched WT and ASK3 KO mice (male, 19–26 weeks old; WT: $n = 3$, KO: $n = 3$) were fixed by perfusion (through the left ventricle) with paraformaldehyde (4%) in PBS as previously described³³. The tissue samples were soaked for several hours in 20% sucrose in PBS, embedded in Cryo Mount I (Muto Pure Chemicals Co., Ltd.) and frozen in liquid nitrogen. P-SPAK/OSR1 antibody (Supplementary Fig. S1d), T-NCC antibody (Millipore) and P-NCC antibody³³ were used as the primary antibodies. When appropriate, λ -phosphatase was administered for 1 h before the primary antibodies. Dylight 546-conjugated anti-rabbit IgG (Jackson ImmunoResearch) was used as the secondary antibody for the immunofluorescence. The immunofluorescence images were obtained with an LSM 510 META (Carl Zeiss) and then analysed using ArrayScan VTI (Thermo Scientific Cellomics) as follows, using Cellomics Colocalization V3 software (Supplementary Fig. S16). The total area of an individual specimen of an image was measured based on the intrinsic fluorescence of the preparation. We carefully obtained images containing the periphery of the kidney to confirm that the objects were in the cortical region. The regions exhibiting stronger fluorescence intensity than a threshold were defined as positive renal tubules, and the summation of these areas was divided by the total area in each specimen to calculate the ratio of positive/total area (%) (Fig. 4c–e). We completely analysed 38 images for P-SPAK/OSR1, 18 images for T-NCC and 27 images for P-NCC in each genotype, using three pairs of WT and ASK3 KO mice.

Blood pressure measurements. In the tail-cuff method, the blood pressure measurements were performed from 20:00 to 00:00, around the start of the dark period of the animal room. The normal blood pressure of unanesthetized mice (ASK3 KO mice or WT littermates; male, 14–30 weeks old) was measured by indirect tail-cuff measurements in every 4 weeks using a BP-98A (Softron). The mice were placed in a cloth holder warmed to 38 °C, and 3–5 consistent measurements (± 5 mm Hg between each measurement) were obtained and averaged for each mouse. In the NaCl-loading experiments, the NaCl concentration in the diet was increased from 0.4 to 4% at 0 day under free-feeding conditions (male, 13-weeks-old). The blood pressure was measured at days of -2, 0, 2, 7, 14 and 21 after changing the diet.

For the radiotelemetric blood pressure measurements, blood pressure transducers (Data Sciences International) were inserted into the left common carotid artery of age-matched WT and ASK3 KO mice (male, 19–23 weeks old; WT: $n = 3$, KO: $n = 3$). After 1 week of recovery, the values of the systolic and diastolic blood pressure were recorded every minute by telemetry. The mice showed alternating periods of low blood pressure (8:00 to 20:00; the light period) and high blood pressure (20:00 to 8:00; the dark period). For each recording, the blood pressure values were represented by the average for 2 h. At 15 days after the surgery, the mice were fed a high-salt diet (4% NaCl) as a NaCl loading, and the blood pressure values were recorded for 16 more days under free-feeding conditions.

Statistical analysis. Data are presented as means \pm s.e.m. Paired means were compared using *t*-tests. Other methods are presented in the Supplementary information.

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Author contributions

I.N. and H.I. drafted the manuscript. I.N., T.U., Y.K., J.M., K.H. and Y.S. performed the experiments. K.K. performed the telemetry measurement of the blood pressure. All authors contributed to discussion and the design of the research and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper on <http://www.nature.com/naturecommunications>

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Phosphatidylinositol 3-Kinase/Akt Signaling Pathway Activates the WNK-OSR1/SPAK-NCC Phosphorylation Cascade in Hyperinsulinemic db/db Mice

Hidenori Nishida, Eisei Sohara, Naohiro Nomura, Motoko Chiga, Dario R. Alessi, Tatemitsu Rai, Sei Sasaki, Shinichi Uchida

Abstract—Metabolic syndrome patients have insulin resistance, which causes hyperinsulinemia, which in turn causes aberrant increased renal sodium reabsorption. The precise mechanisms underlying this greater salt sensitivity of hyperinsulinemic patients remain unclear. Abnormal activation of the recently identified with-no-lysine kinase (WNK)-oxidative stress-responsive kinase 1 (OSR1)/STE20/SPS1-related proline/alanine-rich kinase (SPAK)-NaCl cotransporter (NCC) phosphorylation cascade results in the salt-sensitive hypertension of pseudohypoaldosteronism type II. Here, we report a study of renal WNK-OSR1/SPAK-NCC cascade activation in the db/db mouse model of hyperinsulinemic metabolic syndrome. Thiazide sensitivity was increased, suggesting greater activity of NCC in db/db mice. In fact, increased phosphorylation of OSR1/SPAK and NCC was observed. In both Spak^{T243A/+} and Osr1^{T185A/+} knock-in db/db mice, which carry mutations that disrupt the signal from WNK kinases, increased phosphorylation of NCC and elevated blood pressure were completely corrected, indicating that phosphorylation of SPAK and OSR1 by WNK kinases is required for the increased activation and phosphorylation of NCC in this model. Renal phosphorylated Akt was increased in db/db mice, suggesting that increased NCC phosphorylation is regulated by the phosphatidylinositol 3-kinase/Akt signaling cascade in the kidney in response to hyperinsulinemia. A phosphatidylinositol 3-kinase inhibitor (NVP-BEZ235) corrected the increased OSR1/SPAK-NCC phosphorylation. Another more specific phosphatidylinositol 3-kinase inhibitor (GDC-0941) and an Akt inhibitor (MK-2206) also inhibited increased NCC phosphorylation. These results indicate that the phosphatidylinositol 3-kinase/Akt signaling pathway activates the WNK-OSR1/SPAK-NCC phosphorylation cascade in db/db mice. This mechanism may play a role in the pathogenesis of salt-sensitive hypertension in human hyperinsulinemic conditions, such as the metabolic syndrome. (*Hypertension*. 2012;60:981-990.) • [Online Data Supplement](#)

Key Words: WNK ■ PI3K ■ Akt ■ NaCl cotransporter ■ insulin ■ obesity ■ sodium-dependent hypertension

Pseudohypoaldosteronism type II (PHAII) is an autosomal dominant disease characterized by salt-sensitive hypertension attributed to increased renal salt reabsorption.¹⁻³ Mutations in with-no-lysine kinases 1 and 4 (WNK1 and WNK4) have been reported to cause PHAII.⁴ Previously, WNK4^{DS61A/+} knock-in mice, an ideal mouse model of PHAII, were analyzed; the pathogenesis of PHAII was shown to involve abnormal constitutive activation of the WNK kinase-oxidative stress-responsive kinase 1 (OSR1), STE20/SPS1-related proline/alanine-rich kinase (SPAK)-NaCl cotransporter (NCC) phosphorylation cascade, resulting in increased NCC function.⁵

Recently, several physiological regulators of NCC phosphorylation have been reported. We have reported that NCC phosphorylation was increased by a low-salt diet and decreased

by a high-salt diet through aldosterone, which is a strong regulator of NCC phosphorylation.⁶ Angiotensin II was also found to regulate NCC phosphorylation.⁷⁻⁹ In addition, extracellular potassium ions are reported to regulate the WNK-OSR1/SPAK-NCC phosphorylation cascade.¹⁰ Moreover, Vallon et al¹¹ reported that serum and glucocorticoid-inducible kinase 1 is involved in the regulation of NCC phosphorylation by potassium intake. These findings indicate that the WNK-OSR1/SPAK-NCC phosphorylation cascade is important for NaCl homeostasis and blood pressure regulation under physiological conditions, as well as in PHAII.

There has been a striking worldwide increase in the prevalence of the metabolic syndrome, which is characterized by hypertension, glucose intolerance, obesity, and dyslipidemia.¹²

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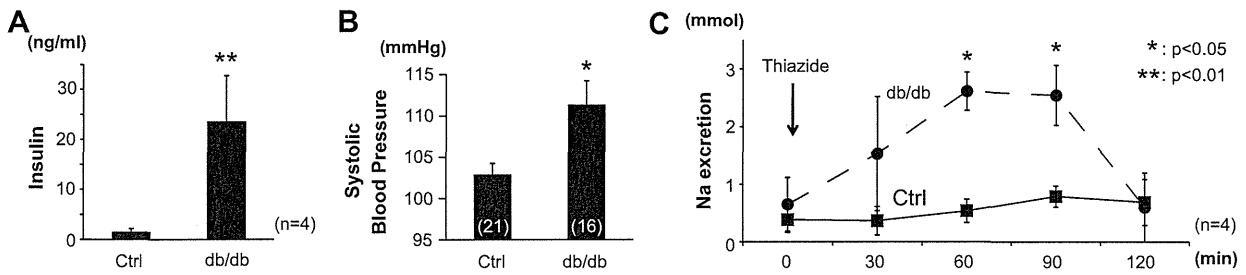


Figure 1. Increased thiazide sensitivity and high blood pressure in db/db mice. **A**, Hyperinsulinemia in db/db mice. Plasma insulin was increased in db/db mice vs control db/m mice. Mean±SEM (n=5). ***P*<0.01. **B**, Systolic blood pressure is higher in db/db mice vs control db/m mice. Mean±SEM (n as indicated). **P*<0.05. **C**, Thiazide infusion test. Response to hydrochlorothiazide (25 mg/kg IP), an inhibitor of NaCl cotransporter (NCC), was significantly greater in db/db mice fed a low-salt diet vs control db/m mice. Mean±SEM (n=4). **P*<0.05.

It has been reported that the metabolic syndrome enhances salt sensitivity, leading to salt-sensitive hypertension.^{13–15} The metabolic syndrome causes hyperinsulinemia as a result of insulin resistance,¹² and hyperinsulinemia causes an aberrant increase in sodium reabsorption by the kidney.^{16–19} However, the precise mechanisms responsible for the increased salt sensitivity of hyperinsulinemic patients have not been clarified. Recently, it was demonstrated that acute insulin stimulation increases OSR1/SPAK and NCC phosphorylation in vivo.²⁰ However, it remains unclear whether insulin increases NCC phosphorylation under physiological and chronic hyperinsulinemic conditions. It is also uncertain what type of signaling pathway links NCC phosphorylation with insulin.

The current study was an investigation of whether and how NCC phosphorylation is increased in the db/db mouse model of the metabolic syndrome. Results demonstrated that the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays a key role in this process, thereby revealing a mechanism that links hyperinsulinemia with salt-sensitive hypertension.

Materials and Methods

See the online-only Data Supplement.

Results

Hyperinsulinemic db/db Mice Have Elevated Systolic Blood Pressure and Increased Phosphorylation of OSR1/SPAK and NCC

To investigate whether chronic hyperinsulinemia can activate the WNK-OSR1/SPAK-NCC signaling cascade (as can acute insulin administration²⁰), hyperinsulinemic db/db mice were used (Figure 1A). The db/db mice lack leptin receptors, and therefore develop features of metabolic syndrome.²¹ It has been reported previously that db/db mice have salt-sensitive hypertension.^{22–26} This study confirmed presence of higher blood pressure in these animals as compared with control mice (db/db, 111.3±2.93 versus control, 102.9±1.36 mm Hg; *P*<0.05; Figure 1B). The blood pressure of db/db mice at the same age was comparable with the previous report.²⁴ To confirm the contribution of NCC to the enhanced salt sensitivity, responsiveness to thiazide, an inhibitor of NCC, was investigated. For 7 days before the thiazide

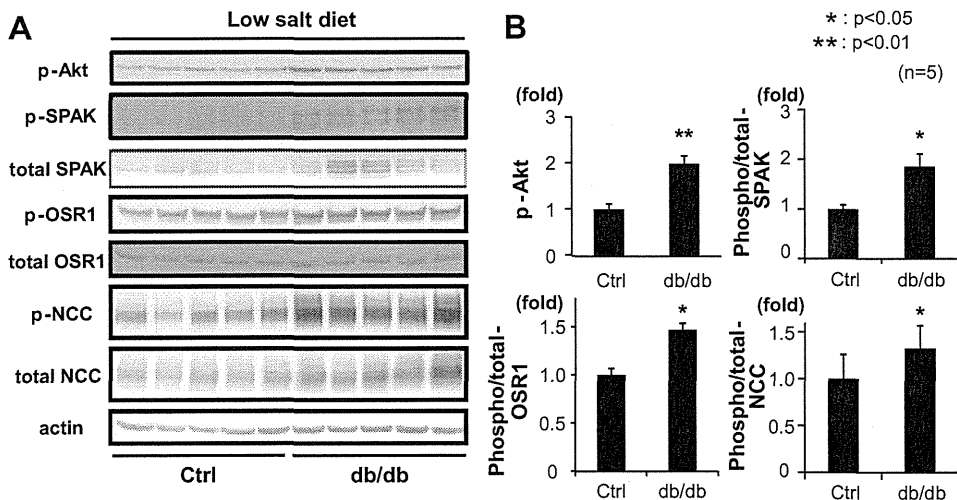


Figure 2. Increased oxidative stress-responsive kinase-1 (OSR1)/STE20/SPS1-related proline/alanine-rich kinase (SPAK) and NaCl cotransporter (NCC) phosphorylation in kidneys of db/db mice fed a low-salt diet. **A**, Immunoblots of OSR1/SPAK and NCC phosphorylation in kidneys of hyperinsulinemic db/db mice fed a low-salt diet. OSR1/SPAK and NCC phosphorylation increased in db/db mice vs controls. Renal Akt phosphorylation was increased. **B**, Densitometry analyses of phosphorylation of Akt, OSR1/SPAK and NCC in the kidney. Values expressed as the ratio to the average of signals in the vehicle group. ***P*<0.01.

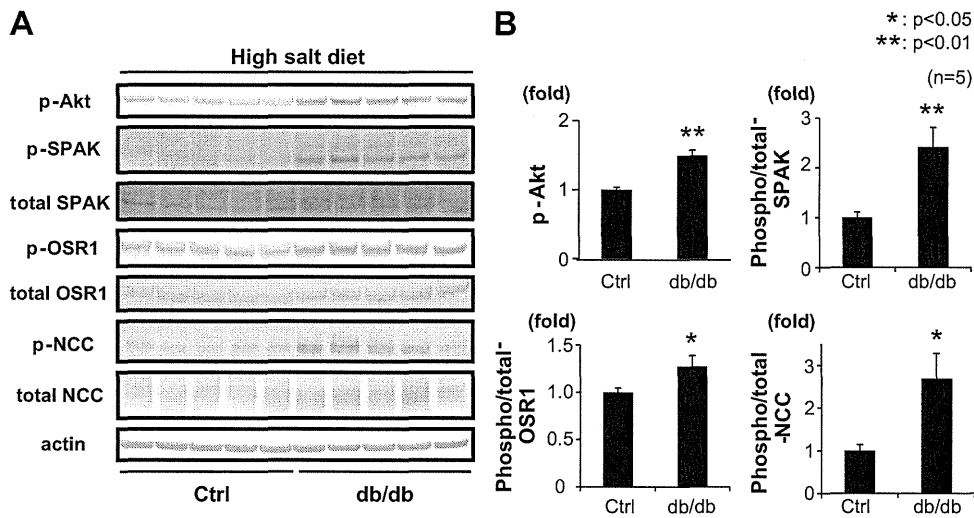


Figure 3. Increased oxidative stress-responsive kinase-1 (OSR1)/STE20/SPS1-related proline/alanine-rich kinase (SPAK) and NaCl cotransporter (NCC) phosphorylation in kidneys of db/db mice fed a high-salt diet. **A**, Immunoblots of OSR1/SPAK and NCC phosphorylation in kidneys of hyperinsulinemic db/db mice fed a high-salt diet. Despite lower plasma aldosterone, OSR1/SPAK and NCC phosphorylation increased in db/db mice fed high- and low-salt diets. Akt phosphorylation increased. **B**, Densitometry analyses of phosphorylation of Akt, OSR1/SPAK, and NCC in the kidney. Values expressed as the ratio to the average of signals in the vehicle group. *P<0.05, **P<0.01.

infusion test, mice were fed a low-salt diet to allow the salt intake of db/db and control mice to equilibrate. As a result, although plasma aldosterone levels in the db/db mice were not significantly lower compared with controls (db/db, 632±461.7 versus control, 822.4±309.6 pg/mL; P=0.11), thiazide sensitivity was higher in the hyperinsulinemic mice (Figure 1C), suggesting greater NCC activity. Therefore, whether renal phosphorylation of OSR1/SPAK and NCC was increased in the db/db mice was ascertained, and, as expected from the results of thiazide infusion test, increased phosphorylation of OSR1/SPAK and NCC was observed in db/db mice fed a low-salt diet (Figure 2).

To minimize the effect of aldosterone, after a week of a high-salt diet, the OSR1/SPAK-NCC phosphorylation cascade was

assayed in the db/db mouse kidney. Plasma aldosterone levels in db/db mice were significantly lower than in control mice on a high-salt diet (db/db 15.2±14.8 versus control 58.6±32.5 pg/mL; P<0.05). As reported previously, a low plasma aldosterone level downregulates the WNK-OSR1/SPAK-NCC phosphorylation cascade in the kidney.⁶ However, despite lower plasma aldosterone levels, db/db mice on a high-salt diet had increased phosphorylation of OSR1/SPAK and NCC (Figure 3). We also used eplerenone to investigate further the contribution of aldosterone to the enhanced WNK-OSR1/SPAK-NCC phosphorylation cascade in db/db mice. Eplerenone (100 mg/kg per day) suppressed WNK-OSR1/SPAK-NCC phosphorylation cascade in both db/db and control mice (please see Figures S1 and S2 in the

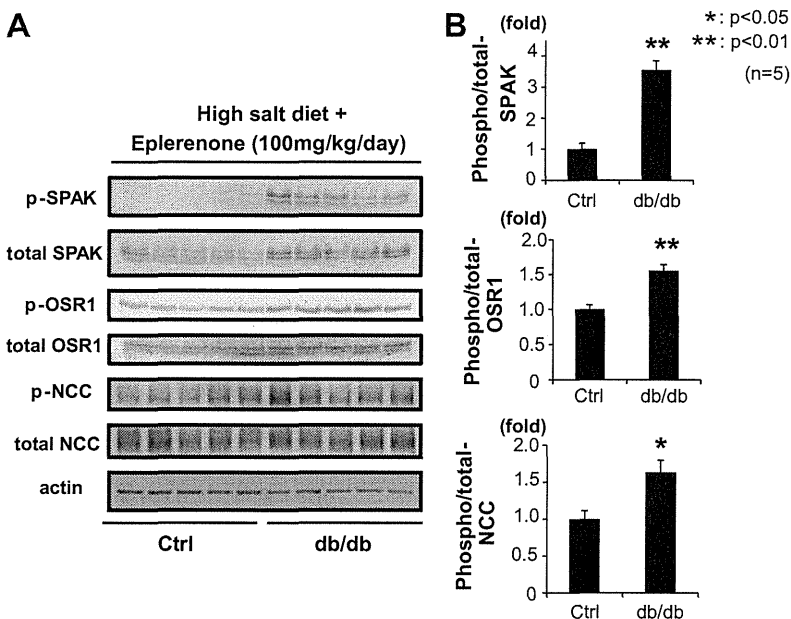


Figure 4. Increased oxidative stress-responsive kinase-1 (OSR1)/STE20/SPS1-related proline/alanine-rich kinase (SPAK) and NaCl cotransporter (NCC) phosphorylation in kidneys of db/db mice fed a high-salt diet with eplerenone. **A**, Immunoblots of OSR1/SPAK and NCC phosphorylation in kidneys of hyperinsulinemic db/db mice fed a high-salt diet with eplerenone (100 mg/kg per day). Phosphorylation of OSR1/SPAK and NCC was still increased in db/db mice, even with eplerenone treatment. **B**, Densitometry analyses of phosphorylation of OSR1/SPAK and NCC in the kidney. Values expressed as the ratio to the average of signals in the vehicle group. *P<0.05, **P<0.01.

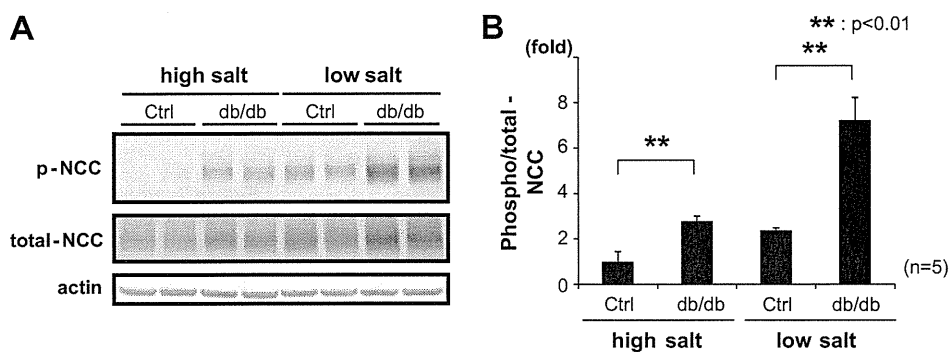


Figure 5. Increased NaCl cotransporter (NCC) phosphorylation in db/db mice is independent of aldosterone. **A**, Representative immunoblot of the renal crude membrane fraction of db/db mice fed high- and low-salt diets, probed with phosphorylated NCC antibody. **B**, Densitometry analysis; values expressed as ratio to average of signals in control mice fed a high-salt diet. In both high- and low-salt diet groups, db/db mice had increased phosphorylated NCC. (n=5). **P<0.01.

online-only Data Supplement). However, as shown in Figure 4, even after administration of eplerenone, the phosphorylation of OSR1/SPAK and NCC was still increased in db/db mice compared with control mice. These results indicated that aldosterone-independent mechanism(s) might be involved in the increased NCC phosphorylation in db/db mice. Moreover, as shown in Figure 5, NCC phosphorylation in db/db mice on a high-salt diet was similar to the increase seen in control mice on a low-salt diet, suggesting that the magnitude of increased NCC phosphorylation in db/db mice reached a level sufficient to explain their increased salt sensitivity.

OSR1/SPAK Kinases Are Involved in the Mechanism of Increased NCC Phosphorylation in Hyperinsulinemic db/db Mice

To confirm the contribution of WNK kinases and OSR1/SPAK to NCC phosphorylation in hyperinsulinemic db/db mice, they were mated with Spak^{T243A/+} and Osr1^{T185A/+} knock-in mice, which have reduced NCC phosphorylation and decreased blood pressure, as described previously.²⁷ These Spak^{T243A/+} and Osr1^{T185A/+} knock-in mice in which the T-loop threonine residue in SPAK and OSR1 (243 and 185, respectively) were mutated to alanine to prevent activation by WNK kinases.²⁷ Plasma insulin level, weight gain, blood glucose, and lipid profile of these double knock-in db/db mice were not different from those of db/db mice, suggesting

that Spak^{T243A/+} and Osr1^{T185A/+} knock-in did not affect metabolic characteristics of db/db mice (please see Figure S3). However, as shown in Figure 6, increased blood pressure and NCC phosphorylation were completely corrected in these double knock-in mice, indicating that phosphorylation of SPAK and OSR1 by WNK kinases is required for generating these effects in db/db mice.

PI3K and Akt Inhibitors Prevent NCC Phosphorylation by Acute Insulin Administration

To investigate the mechanisms of action underlying increased NCC phosphorylation in db/db mice, attention was focused on PI3K and Akt, because renal phosphorylation of Akt at 473S, which accompanies Akt activation,^{28,29} was clearly increased in these mice when fed both low- and high-salt diets (Figures 2 and 3) and because it is well known that Akt, a key downstream substrate of insulin signaling, is phosphorylated by insulin through PI3K.³⁰ Moreover, Akt reportedly contributes to sodium reabsorption in the kidney,³¹ although the precise mechanisms involved have not been determined. In contrast, phosphorylation of serum and glucocorticoid-inducible kinase 1 was not increased in db/db mice fed either low- or high-salt diets (please see Figure S4), suggesting that serum and glucocorticoid-inducible kinase 1 might not be involved in activating the WNK-OSR1/SPAK-NCC signaling cascade in this model. Therefore, the working hypothesis was

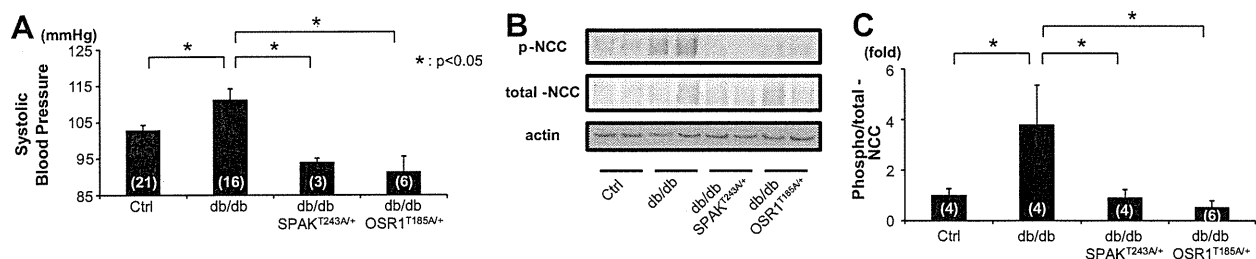


Figure 6. Phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase-1 (OSR1) by with-no-lysine kinase (WNK) is required for the high blood pressure and increased NaCl cotransporter (NCC) phosphorylation of db/db mice. **A**, Systolic blood pressure of Spak^{T243A/+} and Osr1^{T185A/+} knock-in db/db mice. Increased systolic blood pressure of db/db mice corrected in SPAK^{T243A/+} or OSR1^{T185A/+} knock-in mice (threonines in T-loop mutated to alanine to prevent activation by WNK kinases). Mean±SEM (n as indicated). *P<0.05. **B**, Representative immunoblot of renal NCC phosphorylation in SPAK^{T243A/+} and OSR1^{T185A/+} knock-in db/db mice. Compared with db/db controls, NCC phosphorylation was decreased. **C**, Densitometry analyses of renal NCC phosphorylation of NCC in SPAK^{T243A/+} and OSR1^{T185A/+} knock-in db/db mice. Values are expressed as the ratio to the average of signals in vehicle group. Mean±SEM (n as indicated). *P<0.05.

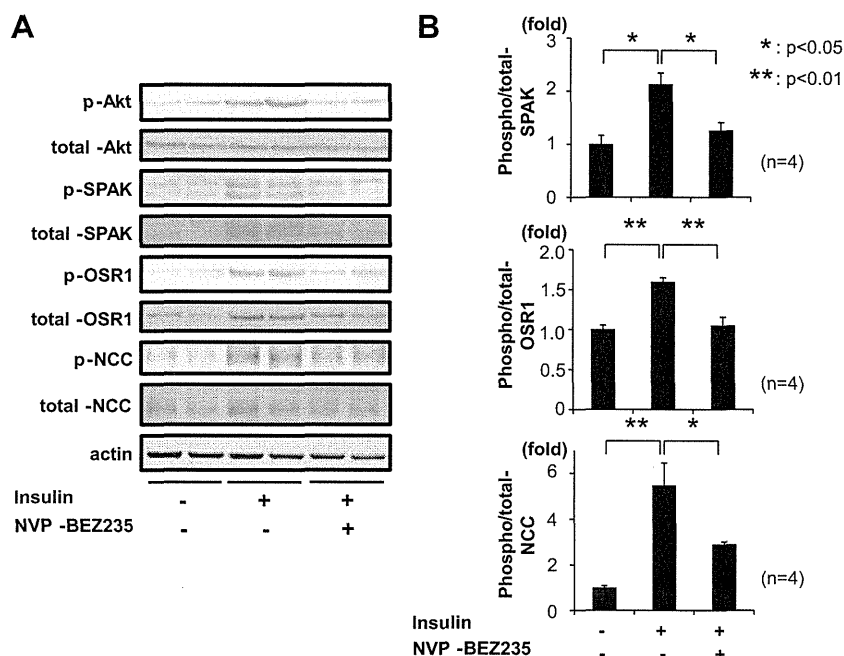


Figure 7. Phosphatidylinositol 3-kinase (PI3K) inhibitor (NVP-BEZ235) suppressed NaCl cotransporter (NCC) phosphorylation by acute insulin administration. **A**, Representative immunoblots of renal Akt, oxidative stress-responsive kinase 1 (OSR1)/STE20/SPS1-related proline/alanine-rich kinase (SPAK) and NCC phosphorylation in insulin-injected mice with or without administration of a PI3K inhibitor (NVP-BEZ235). **B**, Densitometry analysis; NVP-BEZ235 inhibited acute insulin-induced phosphorylation of OSR1/SPAK and NCC. Mean±SEM (n=4). *P<0.05, **P<0.01.

that increased NCC phosphorylation in hyperinsulinemic db/db mice is regulated by the PI3K/Akt signaling cascade in the kidney. To verify this, the first investigation was into whether PI3K and Akt inhibitors could inhibit acute insulin-induced NCC phosphorylation by insulin injection. Because the classic PI3K inhibitors LY294002 and wortmannin were not tolerated for in vivo use because of their toxicities, NVP-BEZ235, a novel orally bioavailable imidazoquinoline derivative that inhibits PI3K activity by binding to the ATP binding cleft of these enzymes, was used.³²⁻³⁴ In addition, to increase specificity and reliability of inhibitor assays, GDC-0941 was used as a PI3K inhibitor and MK-2206 as an Akt inhibitor. GDC-0941 is a selective oral inhibitor of class I PI3K with promising pharmaceutical properties but without significant activity with regard to any other kinases.^{35,36} MK-2206 is an orally active and highly selective allosteric non-ATP competitive Akt inhibitor.^{37,38} Drugs were administered to mice by oral gavage, as reported previously.^{32,35} Because these PI3K and Akt inhibitors had not been tested in the kidney previously, renal phosphorylation of Akt was ascertained. Oral administration of these inhibitors was confirmed to suppress phosphorylation of Akt in mouse kidney (please see Figure S5). Next, to see whether the acute insulin effect on NCC phosphorylation could be suppressed, insulin

was injected to mice intraperitoneally with concurrent oral administration of the inhibitors. As shown in Figure 7, NVP-BEZ235 inhibited insulin-induced OSR1/SPAK and NCC phosphorylation in mouse kidney 30 minutes after insulin stimulation.

To confirm the involvement of PI3K/Akt in the mechanism of increased NCC phosphorylation by acute insulin stimulation, the effect of GDC-0941 and MK-2206 administration was determined. As shown in Figure 8, these inhibitors, like NVP-BEZ235, also inhibited insulin-induced NCC phosphorylation in mouse kidney. Considered together, these results indicate that PI3K and Akt are involved in insulin-induced NCC phosphorylation in vivo. In particular, the Akt inhibitor MK-2206 suppressed NCC phosphorylation to the same level as that seen in controls, suggesting that insulin-induced NCC phosphorylation might be mainly activated by Akt.

PI3K and Akt Are Involved in Mechanisms Leading to Increased NCC Phosphorylation in db/db Mice

To investigate whether the PI3K/Akt signaling pathway regulates NCC phosphorylation in chronic hyperinsulinemic db/db mice, NVP-BEZ235, GDC-0941, or MK-2206 was

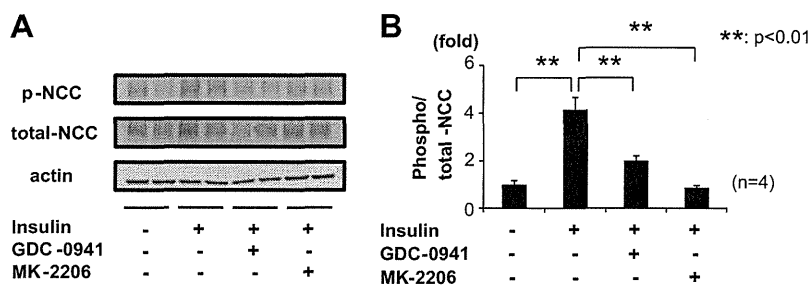


Figure 8. Inhibition of insulin-induced NaCl cotransporter (NCC) phosphorylation by phosphatidylinositol 3-kinase (PI3K) inhibitor (GDC-0941) and Akt inhibitor (MK-2206). **A**, Representative immunoblots of renal NCC phosphorylation in insulin-injected mice, with or without PI3K inhibitor (GDC-0941) and Akt inhibitor (MK-2206) administration. **B**, Densitometry analysis; GDC-0941 and MK-2206 inhibited acute insulin-induced NCC phosphorylation. Mean±SEM (n=4). *P<0.05, **P<0.01.

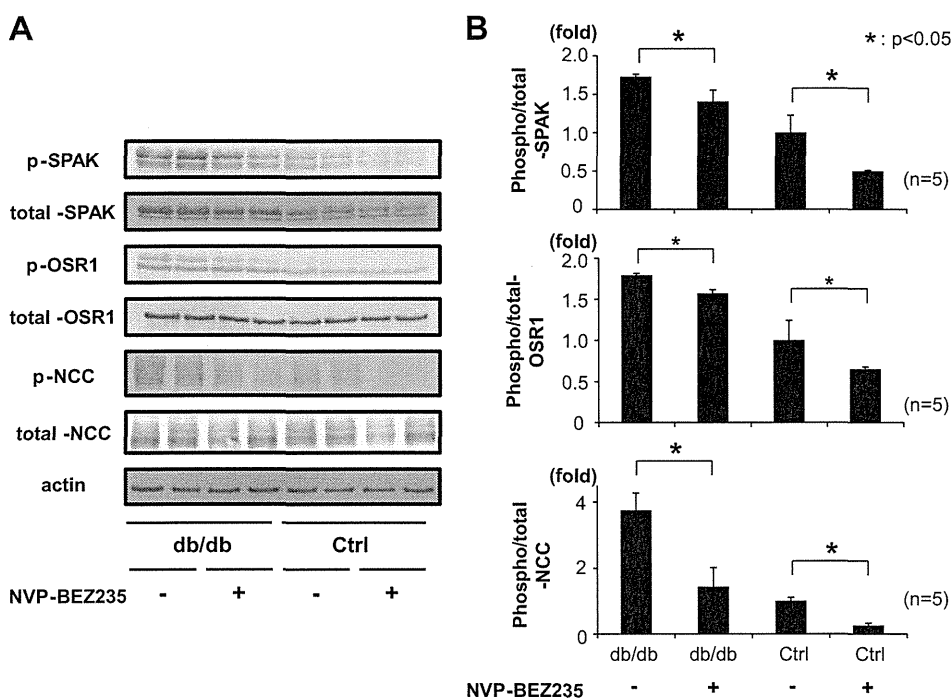


Figure 9. Phosphatidylinositol 3-kinase (PI3K) inhibitor (NVP-BE2235) suppressed increased NaCl cotransporter (NCC) phosphorylation in db/db mouse kidney. **A**, Representative immunoblots of phosphorylation of Akt, oxidative stress-responsive kinase 1 (OSR1)/STE20/SPS1-related proline/alanine-rich kinase (SPAK) and NCC from db/db and control mouse kidney, with or without administration of a PI3K inhibitor (NVP-BE2235). **B**, Densitometry analysis; NVP-BE2235 suppressed increased OSR1/SPAK and NCC phosphorylation in db/db mouse kidney. Mean±SEM (n=4). *P<0.05, **P<0.01.

administered. All of these inhibitors decreased Akt phosphorylation in db/db mouse kidney, indicating that NVP-BE2235 and GDC-0941 inhibited PI3K and MK-2206 inhibited Akt in db/db mouse kidney, as expected (please see Figure S6). Consistent with the previous reports,³⁹ plasma insulin levels were increased in both control and db/db mice by these inhibitors because of feedback mechanisms, indicating that the PI3K and Akt signaling pathway was properly blocked with these drugs (please see Figures S7 and S8). As shown in Figure 9, NVP-BE2235 suppressed NCC phosphorylation, suggesting that increased phosphorylation of NCC in db/db mice is regulated by PI3K. In addition, phosphorylation of OSR1/SPAK was suppressed by NVP-BE2235 at 30 minutes after NVP-BE2235 administration. As shown in Figure 10, the inhibitors GDC-0941 and MK-2206 also

suppressed NCC phosphorylation in db/db and control mice, as well as NVP-BE2235, suggesting that regulation by the PI3K/Akt signaling cascade occurs. Similar to acute insulin administration, MK-2206 suppressed NCC phosphorylation in db/db mice to the same level as that seen in controls, suggesting that increased NCC phosphorylation in db/db mice is mainly activated by Akt.

To see a chronic effect of the PI3K inhibitor on WNK-OSR1/SPAK-NCC phosphorylation cascade in db/db mice, we also performed chronic administration of NVP-BE2235 to mice. As expected, chronic treatment with NVP-BE2235 decreased phosphorylation of OSR1/SPAK and NCC in the db/db mice kidney (Figure 11A and 11B). In addition, chronic treatment with NVP-BE2235 decreased blood pressure only in db/db mice but not in control mice

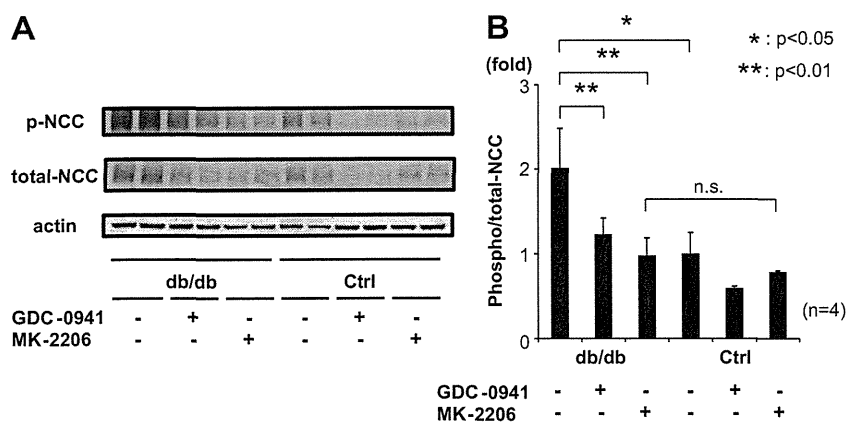


Figure 10. Inhibition of increased NaCl cotransporter (NCC) phosphorylation in db/db mice by phosphatidylinositol 3-kinase (PI3K) inhibitor (GDC-0941) and Akt inhibitor (MK-2206). **A**, Representative immunoblots of phosphorylation of NCC in db/db mice kidney, with or without administration of PI3K inhibitor (GDC-0941) and Akt inhibitor (MK-2206). **B**, Densitometry analysis; GDC-0941 and MK-2206 inhibited increased phosphorylation of NCC in db/db mouse kidney. Mean±SEM (n=4). *P<0.05, **P<0.01.