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 Osr1T185A/+ 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, WNK4V501M/V501M 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 high-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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From the Department of Nephrology (H.N., E.S., N.N., M.C., T.R., S.S., S.U.), Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan; Medical Research Council Protein Phosphorylation Unit (D.R.A.), College of Life Sciences, University of Dundee, Dundee, Scotland, United Kingdom.
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Correspondence to Eisei Sohara, Department of Nephrology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1–5–45 Yushima, Bunkyo, Tokyo 113–8519. E-mail esohara.kid@tmmd.ac.jp
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It has been reported that the metabolic syndrome enhances salt sensitivity, leading to salt-sensitive hypertension. It has been reported that the metabolic syndrome causes hyperinsulinemia as a result of insulin resistance, and hyperinsulinemia causes an aberrant increase in sodium reabsorption by the kidney. 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. 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 administration), hyperinsulinemic db/db mice were used (Figure 1A). The db/db mice lack leptin receptors, and therefore develop features of metabolic syndrome. It has been reported previously that db/db mice have salt-sensitive hypertension. 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. To confirm the contribution of NCC to the enhanced salt sensitivity, responsiveness to thiazide, an inhibitor of NaCl cotransporter (NCC), was investigated. For 7 days before the 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.

**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.

**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/SPAKm and NCC in the kidney. Values expressed as the ratio to the average of signals in the vehicle group. **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, 632.4±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 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.

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 are 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.

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,21 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 that SpakT243A/+ and Osr1T185A/+ 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.

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 SpakT243A/+ and Osr1T185A/+ knock-in db/db mice. Increased systolic blood pressure of db/db mice corrected in SpakT243A/+ or Osr1T185A/+ knock-in mice (threonines in T-loop mutated to alanine to prevent activation by WNK kinases). Means±SEM (n as indicated). *P<0.05. B, Representative immunoblot of renal NCC phosphorylation in SpakT243A/+ and Osr1T185A/+ knock-in db/db mice. Compared with db/db controls, NCC phosphorylation was decreased. C, Densitometry analyses of renal NCC phosphorylation in SpakT243A/+ and Osr1T185A/+ knock-in db/db mice. Values are expressed as the ratio to the average of signals in vehicle group. Means±SEM (n as indicated). *P<0.05.
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
administered. All of these inhibitors decreased Akt phosphorylation in db/db mouse kidney, indicating that NVP-BEZ235 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-BEZ235 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-BEZ235 at 30 minutes after NVP-BEZ235 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-BEZ235, 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-BEZ235 to mice. As expected, chronic treatment with NVP-BEZ235 decreased phosphorylation of OSR1/SPAK and NCC in the db/db mice kidney (Figure 11A and 11B). In addition, chronic treatment with NVP-BEZ235 decreased blood pressure only in db/db mice but not in control mice.
Akt Regulates NCC Phosphorylation

(Figure 11C), indicating that PI3K signaling cascade significantly contributed to the mechanisms of hypertension in db/db mice.

**Discussion**

The metabolic syndrome is related to an increased risk for cardiovascular disease, chronic kidney disease, and mortality. Furthermore, salt sensitivity is associated with an increased risk of cardiovascular disease and premature death. Therefore, the mechanisms underlying the salt sensitivity seen in hyperinsulinemic patients are potentially important therapeutic targets. It has been reported that hyperinsulinemia enhances salt sensitivity, which in turn leads to salt-sensitive hypertension. However, to the best of our knowledge, the molecular mechanisms involved have not been well established. The results of this study demonstrate that phosphorylation of OSR1/SPAK and NCC is increased in a mouse model of the metabolic syndrome. Moreover, use of kinase-dead Spak T243A/+ and Osr1T185A/+ knock-in db/db mice clarified that phosphorylation of OSR1 and SPAK by WNK kinase(s) is required to effect increased NCC phosphorylation. The current results clearly indicate that the WNK-OSR1/SPAK-NCC phosphorylation cascade plays an important role in the development of salt-sensitive hypertension in hyperinsulinemic conditions.

Although it is widely accepted that metabolic syndrome pathophysiology involves insulin resistance, renal insulin...
resistance does not develop in the same manner as in glucogenic tissues like muscle and adipose tissue.\textsuperscript{43-45} Consistent with a previous report,\textsuperscript{41} the current study demonstrated that phosphorylated Akt is increased in kidneys from db/db mice, indicating that, although their activities were decreased in muscle and adipose tissue,\textsuperscript{50,51} PI3K and Akt were activated in the kidney compared with control mice. It was shown that PI3K and Akt inhibitors suppressed increased phosphorylation of NCC in db/db mice. Therefore, as suggested previously,\textsuperscript{12,13,49} direct insulin stimulation appears to activate PI3K/Akt signaling and to cause inappropriate sodium reabsorption and salt-sensitive hypertension through activation of the WNK-OSR1/SPAK-NCC signaling cascade.

As shown in Figure 5, db/db mice fed a high-salt diet had increased phosphorylated NCC equal to the level seen in control mice fed a low-salt diet. Considering that phosphorylated NCC in mice fed a low-salt diet is highly increased by the physiological response to salt depletio, this increased level of renal phosphorylated NCC could explain the salt sensitivity occurring in the hyperinsulinemic state. Moreover, these data indicate that the insulin effect on NCC phosphorylation is independent of aldosterone. In normal mice, as reported previously,\textsuperscript{6} a high-salt diet results in downregulation of the WNK-OSR1/SPAK-NCC phosphorylation cascade through a low plasma aldosterone level. Also reported previously is the fact that constitutive activation of this cascade, even with a high-salt diet (ie, lack of downregulation), causes hypertension in PHAII.\textsuperscript{5} Because the insulin signal appears to be independent of aldosterone, hyperinsulinemia could bring about a situation similar to PHAII via the PI3K/Akt signaling pathway even during a high-salt diet. Hyperinsulinemia prevents proper downregulation of NCC phosphorylation by high-salt intake and thus causes salt-sensitive hypertension (Figure 12).

The detailed mechanisms underlying WNK activation in db/db mice remain to be determined. Considering that PI3K/Akt inhibitors suppressed increased phosphorylation of NCC in the kidney, it is clear that the PI3K/Akt signaling pathway plays a key role. One possible mechanism is that Akt activated by insulin/PI3K signaling modifies the function of WNK4, because WNK4 has several Akt phosphorylation motifs, and phosphorylation at one of these sites has been reported to alter WNK4 kinase activity.\textsuperscript{50,51} It is also possible that WNK1 might be involved in the mechanism of increased NCC phosphorylation in db/db mice, because Thr-60 of WNK1 is phosphorylated by insulin and Akt.\textsuperscript{52} Activation of WNK1 could result in OSR1, SPAK, and NCC phosphorylation by insulin as well.

Finally, we briefly mention the limitation of our study. We only used db/db mice in this study as a model animal showing hyperinsulinemia. Because the phenotypes of db/db mice essentially come from the defect of leptin signaling,\textsuperscript{43,44} it is possible that activation of the WNK-OSR1/SPAK-NCC cascade in the kidney might be caused not only by hyperinsulinemia but also by inactivation of leptin signaling. Therefore, analyses on other model animals showing hyperinsulinemia may be necessary. Very recently, activation of NCC in Zucker obese rats was reported,\textsuperscript{53} although the detailed mechanism in the kidney was not investigated in vivo. We also have to be cautious about the interpretation of data from conventional knockout and knock-in mice because significant compensatory mechanisms might occur in those mice. In this respect, our data obtained from OSR1 and SPAK knock-in mice should be confirmed by other genetically engineered mice, such as conditional knockout mice of OSR1 and SPAK.

**Perspectives**

In summary, it was determined that increased NCC phosphorylation in hyperinsulinemic db/db mice is regulated by the PI3K/Akt signaling pathway, indicating that the PI3K-Akt-WNK-OSR1/SPAK-NCC signaling cascade plays an essential physiological role in this phenomenon. Because the same mechanism has been proven to be the cause of human hypertension, this cascade may be one factor operating in the development of salt-sensitive hypertension in human hyperinsulinemic conditions. Future investigations are warranted to further elucidate details of the underlying mechanisms involved.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is New?**
- It was determined that increased NCC phosphorylation in db/db mice is regulated by the PI3K/Akt signaling pathway. Moreover, the PI3K-Akt-WNK-OSR1/SPAK-NCC signaling cascade plays an essential physiological role in salt-sensitive hypertension under hyperinsulinemic conditions.

**What Is Relevant?**
- It has been reported that the metabolic syndrome enhances salt sensitivity, leading to salt-sensitive hypertension. The metabolic syndrome causes hyperinsulinemia as a result of insulin resistance, and hyperinsulinemia causes an aberrant increase in sodium reabsorption by the kidney. We discovered that hyperinsulinemia in db/db mice can activate NCC through the PI3K-Akt-WNK-OSR1-SPAK-NCC signaling cascade. Because the same mechanism has been proven to be the cause of human hypertension (PHAII), this cascade may be one factor operating in the development of salt-sensitive hypertension in human hyperinsulinemic conditions.

**Summary**

In this article, we report that the PI3K/Akt signaling pathway activates the WNK-OSR1/SPAK-NCC phosphorylation cascade in hyperinsulinemic 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.
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PI3K/Akt Signaling Pathway Activates the WNK-OSR1/SPAK-NCC Phosphorylation Cascade in Hyperinsulinemic db/db Mice

Hidenori Nishida¹, Eisei Sohara¹, Naohiro Nomura¹, Motoko Chiga¹, Dario R Alessi², Tatemitsu Rai¹, Sei Sasaki¹ and Shinichi Uchida¹

¹Department of Nephrology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
²MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland, UK

Correspondence: Eisei Sohara, M.D., Ph.D.
1-5-45 Yushima Bunkyo Tokyo, 113-8519, Japan
Phone: +81-3-5803-5214
Fax: +81-3-5803-5215
e-mail: esohara.kid@tmd.ac.jp
Materials and Methods

Chemicals
NVP-BEZ235 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in N-methyl-2-pyrrolidone (Tokyo Chemical Industry, Tokyo, Japan) and then diluted 10 times with PEG300 (Tokyo Chemical Industry, Tokyo, Japan) to make a working solution, as previously reported. GDC-0941 (synthesized by Dr. Natalia Shpiro at the University of Dundee, Scotland, UK) and MK-2206 (provided by AstraZeneca Alderley Park, Cheshire, UK) were formulated in 0.5% methocellulose - 0.2% Tween20, as previously reported. All drug solutions were prepared within 1 h before use.

Animal Studies
Animals were maintained under specific pathogen-free conditions, and all procedures and experiments were approved by the Animal Care and Use Committee of Tokyo Medical and Dental University. For 7 days before experiments, 5-week-old db/db male mice were fed a high- or low-NaCl diet (4.0% or 0.01% NaCl [w/w], respectively). Same-aged db/m mice were used as controls. All foods were obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). Spak<sup>T243A/+</sup> and Osr1<sup>T185A/+</sup> knock-in db/db mice were generated, and genotyping was performed, as previously reported. For eplerenone administration, male mice were fed a high-NaCl diet (4.0% NaCl [w/w]), and eplerenone was administered for 7 days via chow (0.6 mg eplerenone per gram chow) to achieve an eplerenone dose of approximately 100 mg/kg/day, as previously reported. For an acute insulin injection model, male mice were fed a high-NaCl diet (4.0% NaCl [w/w]) for 7 days before insulin administration. All foods were obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). Insulin was administered intraperitoneally at a dose of 5 U/kg, as previously reported. Control mice received vehicle instead. Mice were sacrificed 60 min after injections. For in vivo inhibitor experiments, 5-week-old male mice were fed a high-NaCl diet (4.0% NaCl [w/w]) for 7 days before administration of PI3K and Akt inhibitors (NVP-BEZ235, GDC-0941 and MK-2206). Inhibitors were administered by oral gavage at a dose of 50 mg/kg (NVP-BEZ235) and 75 mg/kg (GDC-0941 and MK-2206), as previously reported. Control mice received vehicle instead. Mice were sacrificed 30 min after NVP-BEZ235 administration and 60 min after GDC-0941 and MK-2206 administration. For chronic treatment, NVP-BEZ235 was administered once daily by oral gavage at the same dose (50 mg/kg/day). Mice were sacrificed after 7 days of treatment.

Thiazide Infusion Test
Mice received 70 microliters/gram body weight 5% glucose solution, injected intraperitoneally, to facilitate spontaneous voiding, as previously reported. Hydrochlorothiazide (25 mg/kg body weight) was injected intraperitoneally 1 h later. Urine was collected every 30 min by spontaneous voiding or bladder massage, and sodium excretion was measured by DRI-CHEM (Fujifilm).

Blood and Blood Pressure Measurement
Blood was drawn from the venous plexus near the mandible just before sacrifice. Plasma aldosterone levels were measured by the SRL clinical laboratory service (Tokyo, Japan). Plasma insulin levels were measured by mouse insulin enzyme-linked immunosorbent assay (AKRIN-011 and AKRIN-011H, Shibayagi, Japan). Plasma cholesterol and triglyceride were measured by Cholesterol E-Test and TG E-Test (Wako Pure Chemical, Osaka, Japan), respectively. Blood pressure of restrained conscious mice was measured by a programmable tail-cuff sphygmomanometer (MK-2000A, Muromachi-Kikai CO. LTD, Tokyo, Japan).

Immunoblotting
Semiquantitative immunoblotting was performed, as described previously, to assess relative expression levels of proteins in whole kidney homogenates without the nuclear fraction (600 g) or the crude membrane fraction (17000 g). The intensity of bands was analyzed using Image J (NIH, USA). Rabbit anti-pNCC (Ser71) antibody, guinea pig anti-NCC antibody, rabbit anti-pOSR1 antibody, rabbit anti-pSPAK antibody, mouse anti-OSR1 antibody (Abnova, Taipei, Taiwan), rabbit anti-SPAK antibody (Cell Signaling, Beverly, MA), rabbit anti-Akt antibody (Santa Cruz, CA, USA), rabbit anti-pAkt antibody (Cell Signaling), rabbit anti-pSGK1 antibody (Cell Signaling), and rabbit anti-actin antibody (Cell Signaling) were used, as previously reported. Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI, USA) were used as secondary antibodies for immunoblotting.

Statistical Analysis
Statistical significance was evaluated using an un-paired t-test. All data are expressed as mean ± SEM. When more than three groups were compared, one-way ANOVA with Fischer’s post-hoc test was used. P<0.05 was considered statistically significant.

References
Figure S1. Eplerenone suppressed increased NCC phosphorylation in control mouse kidney
A. Representative immunoblots of phosphorylation of OSR1/SPAK and NCC in control mouse kidney, with or without administration of eplerenone (100 mg/kg/day).
B. Densitometry analysis; eplerenone suppressed increased OSR1/SPAK and NCC phosphorylation in control mouse kidney. Mean ± SEM. (n=5). *p<0.05, **p<0.01.
Figure S2. Eplerenone suppressed increased NCC phosphorylation in db/db mouse kidney
A. Representative immunoblots of phosphorylation of OSR1/SPAK and NCC in db/db mouse kidney, with or without administration of eplerenone (100 mg/kg/day).
B. Densitometry analysis; eplerenone suppressed increased OSR1/SPAK and NCC phosphorylation in db/db mouse kidney. Mean ± SEM. (n=5). **p<0.01.
Figure S3. Metabolic characteristics in Spak$^{T243A/+}$ and Osr1$^{T185A/+}$ knock-in db/db mice. Plasma insulin level (A), body weight (B), blood glucose level (C) and lipid profile (D, E) of Spak$^{T243A/+}$ and Osr1$^{T185A/+}$ knock-in db/db mice were not significantly different from those of db/db mice. Mean ± SEM. (n=5). *$p<0.05$, ***$p<0.001$. 
**Figure S4. Phosphorylated SGK1 in kidneys of db/db mice**

A. Immunoblots of SGK1 phosphorylation in kidneys of hyperinsulinemic db/db mice fed a low-salt diet. SGK1 phosphorylation was not significantly decreased in db/db mice fed a low-salt diet compared to controls.

B. Densitometry analyses of phosphorylated SGK1 in the kidney. Values expressed as the ratio to the average of signals in the vehicle group.

C. Immunoblots of SGK1 phosphorylation in kidneys of hyperinsulinemic db/db mice fed a high-salt diet. SGK1 phosphorylation was decreased in db/db mice fed a high-salt diet compared to controls.

D. Densitometry analyses of phosphorylated SGK1 in the kidney. Values expressed as the ratio to the average of signals in the vehicle group. **p<0.01.
Figure S5. Effect of PI3K inhibitors (NVP-BEZ235 and GDC-0941) and Akt inhibitor (MK-2206) on Akt phosphorylation in mouse kidney
A. Representative immunoblots of phosphorylated and total Akt in mouse kidney with and without inhibitors.
B. Densitometry analyses of phosphorylation of Akt in mouse kidney. Phosphorylation of Akt in kidney was significantly suppressed by these PI3K and Akt inhibitors. Mean ±SEM. (n=4).
*p<0.05, **p<0.01.
Figure S6. Effect of PI3K inhibitors (NVP-BEZ235 and GDC-0941) and Akt inhibitor (MK-2206) on Akt phosphorylation in db/db mouse kidney
A. Representative immunoblots of phosphorylated and total Akt in db/db mouse kidney with and without inhibitors.
B. Densitometry analyses of phosphorylation of Akt in db/db mouse kidney. Phosphorylation was significantly suppressed by these PI3K and Akt inhibitors. Mean ± SEM. (n=4). *p<0.05, **p<0.01.
Figure S7. Effect of PI3K inhibitor (NVP-BEZ235) on plasma insulin level in db/db and control mice.
Administration of NVP-BEZ235 increased plasma insulin level in both db/db and control mice. Mean ± SEM. (n=5). *p<0.05.
Figure S8. Effect of PI3K (GDC-0941) and Akt (MK-2206) inhibitors on plasma insulin level in db/db and control mice.

Administration of GDC-0941 or MK-2206 increased plasma insulin level in both db/db and control mice. Mean ± SEM. (n=5). *p<0.05.