ONLINE SUPPLEMENT

PI3K/Akt Signaling Pathway Activates the WNK-OSR1/SPAK-NCC Phosphorylation Cascade in Hyperinsulinemic db/db Mice

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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.[1] 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). $Spak^{T243A/+}$ and $Osr1^{T185A/+}$ knock-in db/db mice were generated, and genotyping was performed, as previously reported.[2]

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.[3]

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.[4] 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.[5] 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.[6] 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,[7,8] 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,[9] guinea pig anti-NCC antibody, rabbit anti-pOSR1 antibody,[10] rabbit anti-pSPAK antibody,[11] 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

1. O'Brien C, Wallin JJ, Sampath D, GuhaThakurta D, Savage H, Punnoose EA, Guan J, Berry L, Prior WW, Amler LC, Belvin M, Friedman LS, Lackner MR. Predictive biomarkers of sensitivity to the phosphatidylinositol 3' kinase inhibitor GDC-0941 in breast cancer preclinical models. *Clin Cancer Res.* 2010;16:3670-3683.

2. Wang MY, Grayburn P, Chen S, Ravazzola M, Orci L, Unger RH. Adipogenic capacity and the susceptibility to type 2 diabetes and metabolic syndrome. *Proc Natl Acad Sci U S A.* 2008;105:6139-6144.
3. Guo C, Martinez-Vasquez D, Mendez GP, Toniolo MF, Yao TM, Oestreicher EM, Kikuchi T, Lapointe N, Pojoga L, Williams GH, Ricchiuti V, Adler GK. Mineralocorticoid receptor antagonist reduces renal injury in rodent models of types 1 and 2 diabetes mellitus. *Endocrinology.* 2006;147:5363-5373.
4. Sohara E, Rai T, Yang SS, Ohta A, Naito S, Chiga M, Nomura N, Lin SH, Vandewalle A, Ohta E, Sasaki S, Uchida S. Acute insulin stimulation induces phosphorylation of the Na-Cl cotransporter in cultured distal mpkDCT cells and mouse kidney. *PLoS One.* 2011;6:e24277.
5. Garcia-Martinez JM, Wullschleger S, Preston G, Guichard S, Fleming S, Alessi DR, Duce SL. Effect of PI3K- and mTOR-specific inhibitors on spontaneous B-cell follicular lymphomas in PTEN/LKB1-deficient mice. *Br J Cancer.* 2011;104:1116-1125.
6. Nomura N, Tajima M, Sugawara N, Morimoto T, Kondo Y, Ohno M, Uchida K, Mutig K, Bachmann S, Soleimani M, Ohta E, Ohta A, Sohara E, Okado T, Rai T, Jentsch TJ, Sasaki S, Uchida S. Generation and analyses of R8L barttin knockin mouse. *Am J Physiol Renal Physiol.* 2011;301:F297-307.
7. Oi K, Sohara E, Rai T, Misawa M, Chiga M, Alessi DR, Sasaki S, Uchida S. A minor role of WNK3 in regulating phosphorylation of renal NKCC2 and NCC co-transporters in vivo. *Biology Open.* 2011;1:120-127.
8. Susa K, Kita S, Iwamoto T, Yang SS, Lin SH, Ohta A, Sohara E, Rai T, Sasaki S, Alessi DR, Uchida S. Effect of heterozygous deletion of WNK1 on the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 signal cascade in the kidney and blood vessels. *Clin Exp Nephrol.* February 1, 2012. DOI: 10.1007/s10157-012-0590-x. http://www.springerlink.com/content/8212755177t8474q/fulltext.html. Accessed July 20, 2012
9. Yang SS, Morimoto T, Rai T, Chiga M, Sohara E, Ohno M, Uchida K, Lin SH, Moriguchi T, Shibuya H, Kondo Y, Sasaki S, Uchida S. Molecular pathogenesis of pseudohypoaldosteronism type II: generation and analysis of a Wnk4(D561A/+) knockin mouse model. *Cell Metab.* 2007;5:331-344.
10. Ohta A, Rai T, Yui N, Chiga M, Yang SS, Lin SH, Sohara E, Sasaki S, Uchida S. Targeted disruption of the Wnk4 gene decreases phosphorylation of Na-Cl cotransporter, increases Na excretion and lowers blood pressure. *Hum Mol Genet.* 2009;18:3978-3986.
11. Yang SS, Lo YF, Wu CC, Lin SW, Yeh CJ, Chu P, Sytwu HK, Uchida S, Sasaki S, Lin SH. SPAK-knockout mice manifest Gitelman syndrome and impaired vasoconstriction. *J Am Soc Nephrol.* 2010;21:1868-1877.

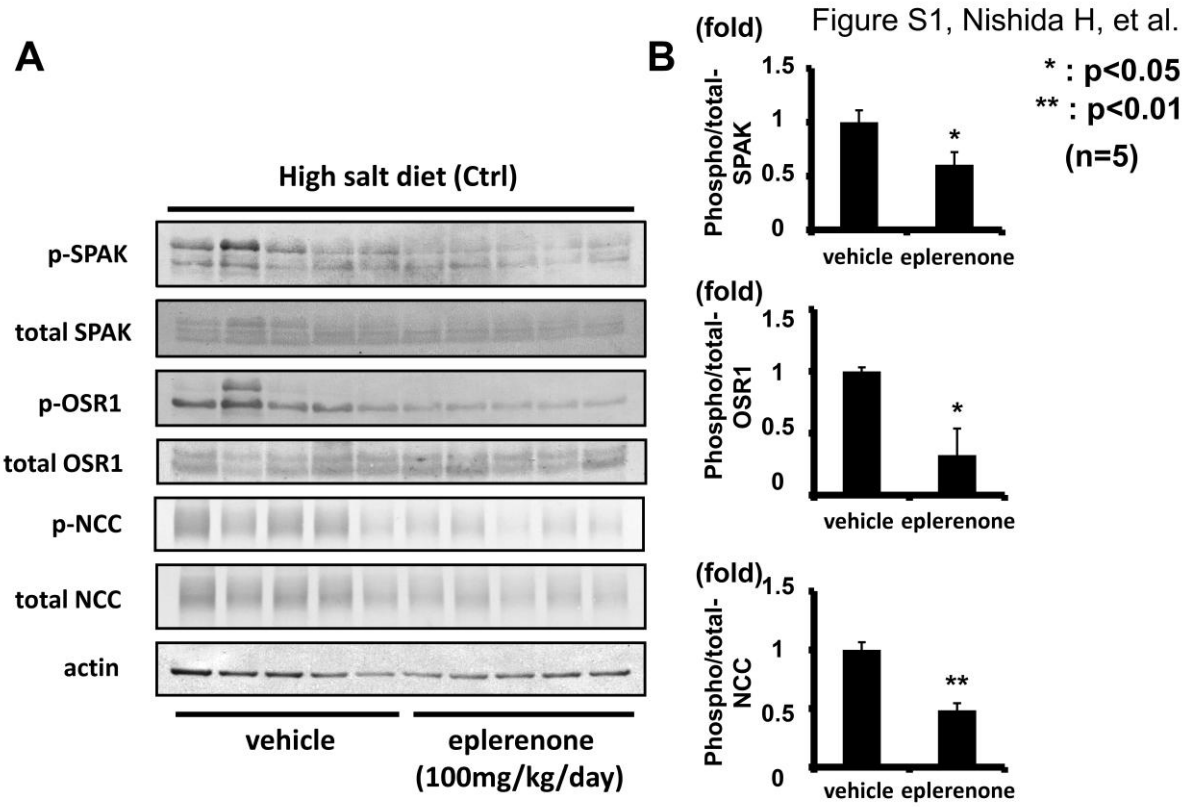

Figure S1. Eplerenone suppressed increased NCC phosphorylation in control mouse kidney

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.

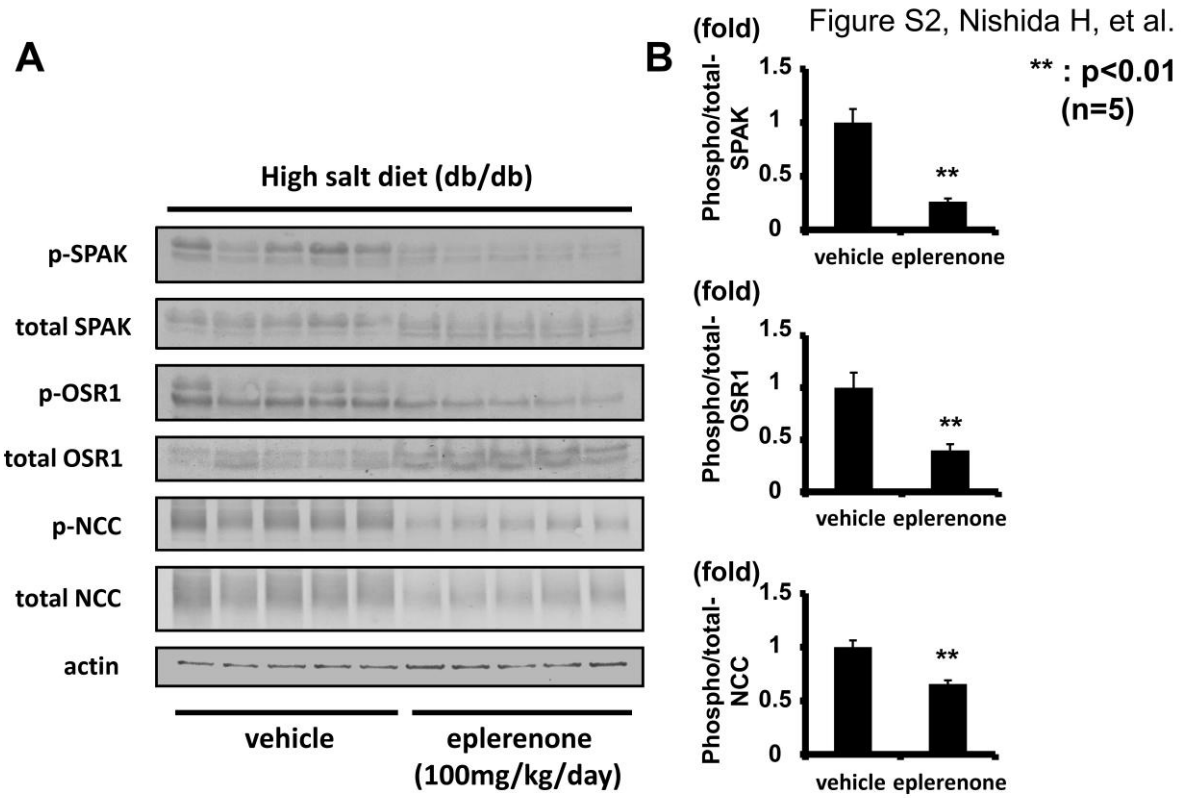

Figure S2. Eplerenone suppressed increased NCC phosphorylation in db/db mouse kidney

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.

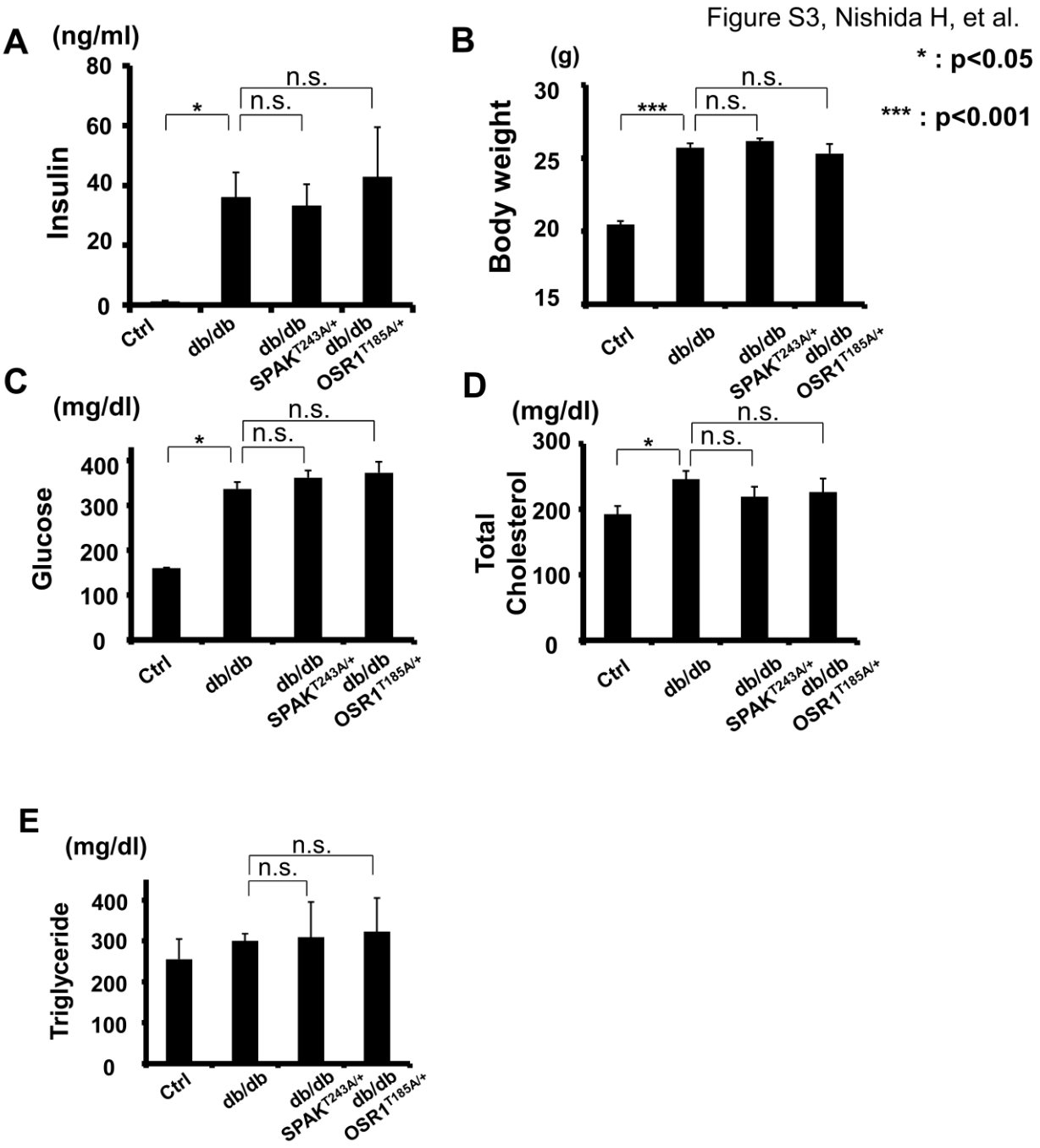

Figure S3. Metabolic characteristics in Spak$^{T243A/+}$ and Osr1$^{T185A/+}$ knock-in db/db mice. Plasma insulin level (A), body weight (B), blood glucose level (C) and lipid profile (D, E) of Spak$^{T243A/+}$ and Osr1$^{T185A/+}$ knock-in db/db mice were not significantly different from those of db/db mice. Mean ± SEM. (n=5). *p<0.05. ***p<0.001.

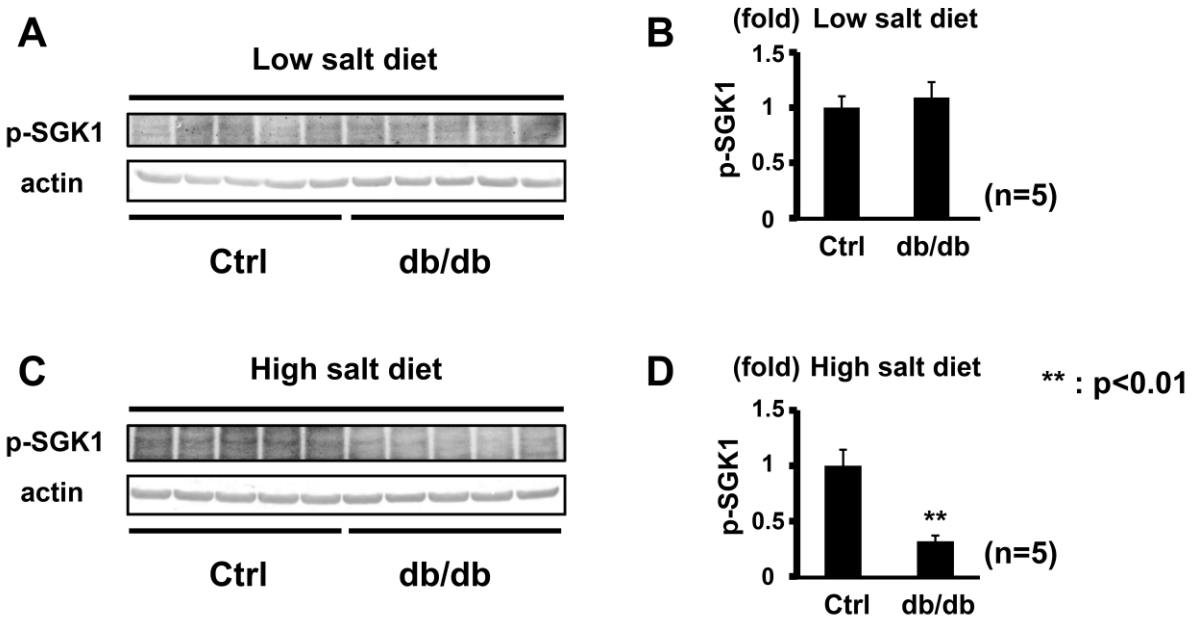

Figure S4. Phosphorylated SGK1 in kidneys of db/db mice

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.

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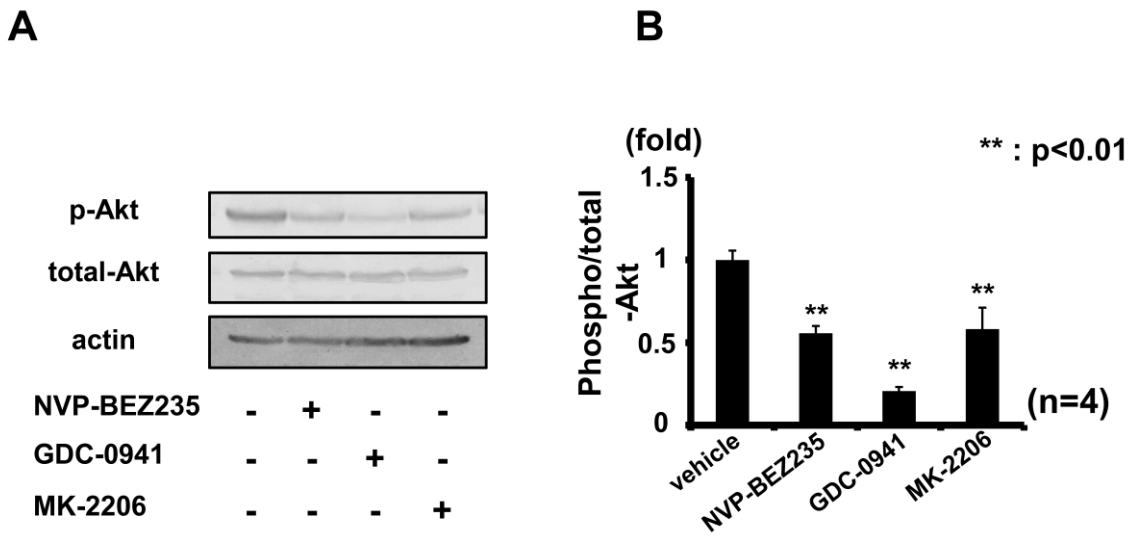

Figure S5. Effect of PI3K inhibitors (NVP-BEZ235 and GDC-0941) and Akt inhibitor (MK-2206) on Akt phosphorylation in mouse kidney

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.

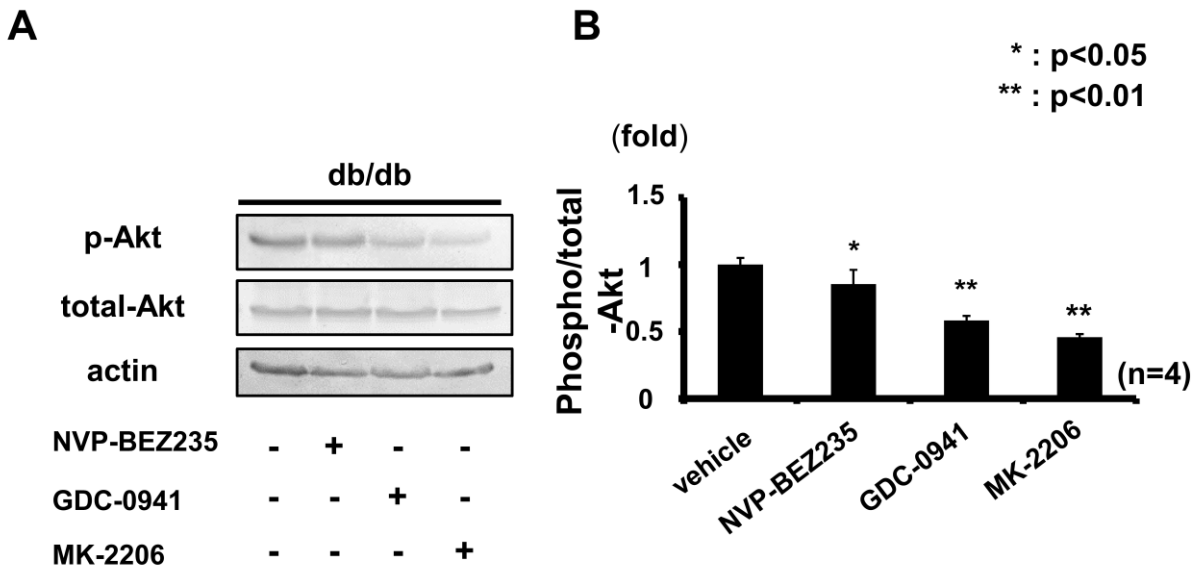

Figure S6. Effect of PI3K inhibitors (NVP-BEZ235 and GDC-0941) and Akt inhibitor (MK-2206) on Akt phosphorylation in db/db mouse kidney

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.

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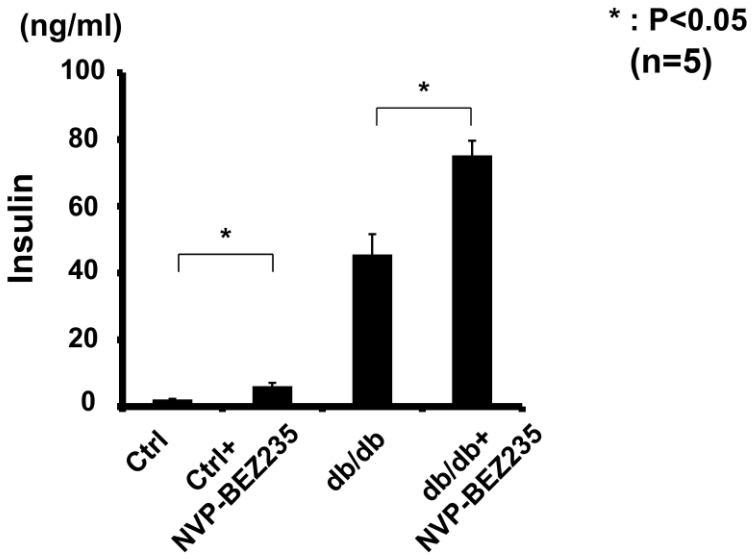

Figure S7. Effect of PI3K inhibitor (NVP-BEZ235) on plasma insulin level in db/db and control mice.

Administration of NVP-BEZ235 increased plasma insulin level in both db/db and control mice. Mean ± SEM. (n=5). *p<0.05.

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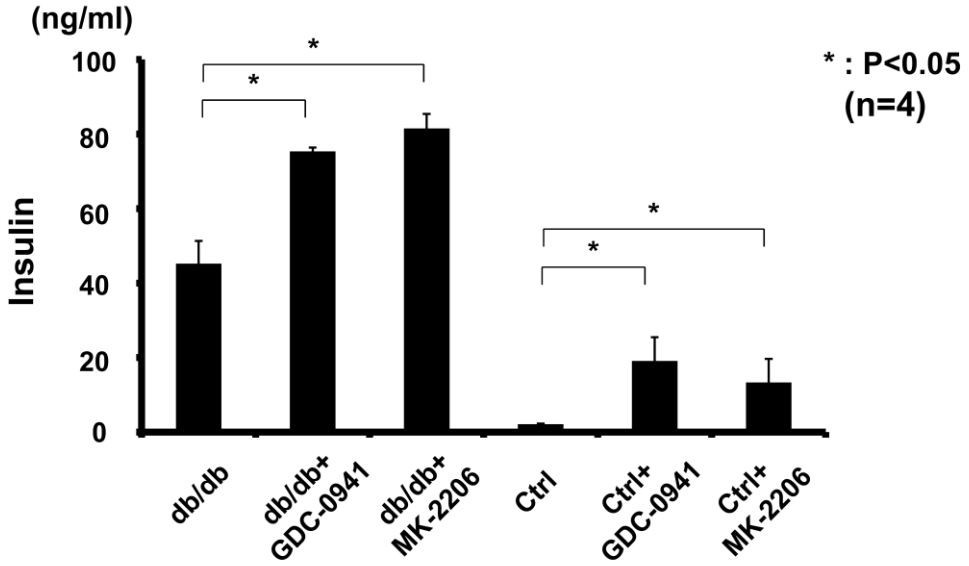

Figure S8. Effect of PI3K (GDC-0941) and Akt (MK-2206) inhibitors on plasma insulin level in db/db and control mice.

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.