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Metformin increases urinary sodium excretion by reducing phosphorylation of the sodium-chloride cotransporter

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Abstract

Objective. Metformin is an antidiabetic drug that is widely used to treat patients with diabetes mellitus. Recent studies have reported that treatment with metformin not only improved blood glucose levels but also reduced blood pressure. However, it remains unclear how metformin reduces blood pressure. We hypothesized that metformin affects sodium reabsorption in the kidneys.

Methods. Urinary sodium excretion and expression of renal sodium transporters were examined

in 8-week-old male C57BL/6 mice with acute and chronic treatment of metformin. In addition, we examined metformin effects using *ex vivo* preparations of mice kidney slices.

Results. In this study, we demonstrated that metformin increased urinary sodium excretion by reducing phosphorylation of the thiazide-sensitive Na-Cl cotransporter (NCC) in acute and chronic metformin administration. We also confirmed reduction of phosphorylated NCC in an *ex vivo* study. The activity of other renal sodium transporters, such as NKCC2, ENaC, and NHE3 did not show significant changes. WNK-OSR1/SPAK kinase signals were not involved in this inactivation effect of metformin on NCC.

Conclusion. Metformin increased urinary sodium excretion by reducing phosphorylation of NCC, suggesting its role in improving hypertension.

Keywords: metformin, sodium-chloride cotransporter, sodium excretion, hypertension

1. Introduction

Metformin, a biguanide derivate, is an anti-hyperglycemic drug that has been widely used to treat patients with type 2 diabetes mellitus for over 50 years [1]. The conventional signal pathway of metformin is to partially inhibit the mitochondrial respiratory chain complex I by accumulating in mitochondria through organic cation transporters (OCTs) [2-4], which results in an increase in the

AMP:ATP ratio. This increase activates AMP-activated protein kinase (AMPK) in the liver and adipocytes [2,5,6], followed by inhibition of gluconeogenic gene transcription and lipogenesis, which improves insulin sensitivity [7-11]. Many researchers have shown that metformin has multiple beneficial effects such as anti-aging, anti-cancer, and anti-hypertension effects, in addition to its antidiabetic effect [8,9,12]. In addition, a recent meta-analysis of randomized trials reported that metformin decreased blood pressure in nondiabetic patients [13], which indicated that metformin also has an anti-hypertensive effect. However, the mechanism underlying the blood pressure-lowering effect of metformin remains unclear.

The kidneys regulate total body sodium balance and blood pressure by controlling sodium excretion. Sodium transporters and channels on the apical membrane of renal tubules are responsible for transepithelial sodium reabsorption [14]. The major apical transporters for sodium reabsorption are sodium-hydrogen exchanger 3 (NHE3) in the proximal tubule, sodium–potassium chloride cotransporter 2 (NKCC2) in the thick ascending limb of the loop of Henle, sodium-chloride (Na-Cl) cotransporter (NCC) in the distal convoluted tubule, and epithelial sodium channel (ENaC) in the collecting duct [15]. Analysis of mutant phenotypes in these sodium transporter proteins has revealed the role of each transporter in control of fluid homeostasis and blood pressure [16]. We and others have clarified that NCC has an important role in blood pressure regulation and was mainly regulated by the with-no-lysine kinase (WNK)-oxidative stress-responsive 1 (OSR1)/Ste-like proline/alanine rich kinase (SPAK)-NCC phosphorylation cascade [17-19]. WNK1 and WNK4 were found as

responsible for pseudohypoaldosteronism type II (PHAII) [19-21]. WNKs phosphorylate OSR1 and SPAK [22-24], and phosphorylated OSR1/SPAK activate NCC by phosphorylation and concurrently increase its membrane localization in the distal convoluted tubule [25-31]. Constitutive activation of WNK signals in the kidneys causes increased sodium reabsorption through NCC, leading to salt-sensitive hypertension in PHAII [32].

In the present study, to investigate the mechanisms underlying the blood pressure-lowering effect of metformin, we investigated whether and how metformin affects renal sodium absorption through sodium transporters *in vivo* and *ex vivo*. We found that metformin increased sodium excretion and decreased the abundance of phosphorylated NCC in the distal convoluted tubule independently from the WNK4-OSR1/SPAK cascade. These results demonstrated one of the mechanisms underlying the blood pressure-lowering effect of metformin.

2. Methods

2.1 Animals and treatments

8-week-old male C57BL/6 mice (Sankyo Laboratory Service Corporation, Inc., Tokyo, Japan), weighing 23 to 25 g, were housed on a 12-hr/12-hr light/dark cycle, with free access to food and water. To examine the effect of metformin on renal sodium transport, the mice were administered

metformin (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) at a dose of 300 mg/kg once daily for five days by oral gavage since metformin at the dose of 300 mg/kg/day has been used in previous studies reporting metformin effects using mice [7,33-35] and mouse metformin dose of 300 mg/kg/day was equivalent to the dose for clinical use for human patients (1200-2400 mg/day for 50-100 kg human patient), normalized by body surface area [36]. To evaluate the effect of acute metformin, the mice were administered metformin at a dose of 300 mg/kg by oral gavage and were euthanized 1 h after treatment. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.2 Urinary analysis

To evaluate urinary sodium excretion, acute sodium loading was performed, as previously described [37]. Mice were administered 1 ml of 0.9% saline orally after five days of metformin treatment (300 mg/kg). The mice were placed in metabolic cages for 8 h to collect urine samples. To evaluate the short-term effects of metformin treatment, the mice were administered metformin (300 mg/kg) and placed in metabolic cages for 4 h to collect urine samples. The urinary sodium concentration (U_{Na}) was analyzed by DRI-CHEM (FUJIFILM Medical Co., Ltd, Tokyo, Japan).

2.3 *Ex vivo* kidney slice experiment

Kidney slices were prepared, as previously described [38]. Kidneys were harvested and cut into <500-μm slices using a microslicer (Natsume Seisakusho Co., Ltd, Tokyo, Japan) and then placed in ice-cold Hank's buffer medium (pH 7.4, 110 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 4 mM Na acetate, 1 mM Na citrate, 6 mM D-glucose, 6 mM L-alanine, 1 mM NaH_2PO_4, 3 mM $Na2HPO_4$, 25 mM $NaHCO_3$). The slices were incubated for equivalation in Hank's buffer medium for 20 min at room temperature. Then, slices from the same kidney were separated in normal or metformin (5 mM) medium and then incubated for 30 min at 28°C. During the experiments, all solutions were continuously bubbled with 95% O_2 and 5% CO_2. After the incubation, the slices were snap frozen in liquid nitrogen and prepared for immunoblotting.

2.4 Immunoblotting

Extraction of protein samples and semiquantitative immunoblotting were performed, as previously described [39]. For immunoblotting, kidneys were lysed in detergent-free buffer {250 mM sucrose, 10 mM Triethanolamine, 1 mM EGTA, 1 mM EDTA, 1 mM Na orthovanadate, 50 mM Na fluoride, complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd., Basel, Switzerland.)} with a homogenizer, and then the homogenates were centrifuged to separate to entire kidney samples without the nuclear fraction, as either whole kidney lysates (600 g, supernatant) and crude membrane

fraction (17000 g, pellet). The crude membrane fractions were used to measure the expression levels of NCC, NKCC2, ENaC and NHE3. The relative intensities of immunoblot bands were analyzed and quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Primary antibodies used in this study were as follows: rabbit anti-WNK4 [31], rabbit anti-phosphorylated SPAK (S383) [40], rabbit anti–total SPAK (Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-phosphorylated OSR1 (S325) [41], mouse anti–total OSR1 (M10; Abnova, Taipei, Taiwan) rabbit anti-phosphorylated NCC (S71) [42], rabbit anti-NCC [43], rabbit anti-phosphorylated NKCC2 [44], rabbit anti-NKCC2 (Alpha Diagnostic Intel. Inc., San Antonio, TX), rabbit anti-ENaC α subunit (Stressmarq Biosciences, Inc., Victoria, BC), anti-ENaC β subunit (kindly provided by Kumamoto university), anti-ENaC γ subunit (kindly provided by Kumamoto university), rabbit anti-NHE3 (Alpha Diagnostic Intel. Inc., San Antonio, TX), rabbit anti-phosphorylated AMPK (T172) (Cell Signaling Technology, Inc., Danvers, MA), anti-pan-α AMPK (Cell Signaling Technology, Inc.), rabbit anti-phosphorylated serine/threonine protein kinase Akt [PKB (protein kinase B: also called Akt); Cell Signaling Technology, Inc.], rabbit anti-Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-phosphorylated PP1α (T320) (Cell Signaling Technology, Inc.), mouse anti-PP1α (E-9) (Santa Cruz Biotechnology, Inc.), rabbit anti-I-1 (Abcam, Inc., Cambridge, UK), rabbit anti-pan-calcineurin A (Cell Signaling Technology, Inc.), rabbit anti-PP4c (Abcam, Inc.), and rabbit anti–β-actin antibody (Cytoskeleton, Inc., Denver, CO). Alkaline phosphatase-conjugated anti-IgG antibodies (Promega Corporation, Fitchburg, WI) were used as

secondary antibodies, and Western Blue (Promega Corporation) was used to detect the signal.

2.5 Immunofluorescence

Immunofluorescence was performed as previously reported [45]. The kidneys were fixed by perfusion with periodate lysine (0.2 M) and paraformaldehyde (2%) in phosphate-buffered saline (PBS). Tissue samples were soaked for several hours in 20% sucrose in PBS, embedded in Tissue-Tek OCT compound (Sakura Finetek Japan Co., Ltd. Tokyo, Japan) and snap frozen in liquid nitrogen. The primary antibodies used were rabbit anti-phosphorylated NCC (S71) and guinea pig anti-total NCC. Alexa Fluor 488 or 546 dye-labeled (Molecular Probes, Inc., Engene, OR) were used as secondary antibodies. Immunofluorescence images were obtained using the LSM510 Meta Confocal Microscope (Carl Zeiss, Oberkochen, Germany).

2.6 Laser capture microdissection (LCM) and reverse transcription-PCR (RT-PCR)

The mouse kidneys were cut and embedded in OCT compound and frozen in liquid nitrogen. Frozen tissue blocks were cut into 10 μm sections, and were mounted on uncoated, uncharged glass slides. Sections were stained for Carrazzi's Hematoxylin Solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). LCM was performed using Leica LMD 7000 microscope system (Leica

Microsystems, Wetlzlar, Germany). Total RNA from LCM samples was extracted from captured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) and total RNA from mouse kidneys was extracted using Sepasol®-RNA Ⅰ Super G (Nakalai tesque Inc., Kyoto, Japan), according to the manufacturer's instructions. cDNA was synthesized using ReverTra Ace® (Toyobo Co., Ltd., Osaka, Japan). The primers used for RT-PCR were the following; OCT1 (sense; 5'-GCGGTGTGGCTGGAGCCCGGCAGAGGA-3' and antisense; 5'-GGCCACCCCAGCAAGCCCCACTAGCC-3'), OCT2 (sense; 5'-CTACTCTGCCCTGGTGGAATTCCCAGC-3' and antisense; 5' - CCAGCAAAGCAATGGGCATTCCCCAGG-3') [46] and GAPDH (sense; 5'-CCATCACGCCACAGCTTTCC-3' and antisense; 5' - CCTGGCCAAGGTCATCCATG-3').

2.7 Statistical analysis

The two groups were compared using unpaired t tests. Data are presented as means ± SEM. P values <0.05 were considered statistically significant.

3. Results

3.1 Chronic administration of metformin increased urinary sodium excretion via decreased NCC

phosphorylation.

We first investigated whether metformin treatment affects sodium reabsorption in the kidneys. We treated mice with metformin at a dose of 300 mg/kg or vehicle once daily by oral gavage for five days as previously used [7,33-35]. After five days of metformin treatment, we measured sodium excretion in urine after acute sodium loading to test whether sodium is properly excreted, as described previously [37]. Urinary sodium excretion in the metformin-treated mice was significantly higher than that in the vehicle-treated mice (Fig. 1). To clarify which part of the nephron and sodium transporters were responsible for this sodium loss from the kidneys, we examined the activity of these transporters and channel in metformin-treated mice by immunoblotting. Treatment with metformin for five days reduced the abundance of phosphorylated NCC in mice, whereas the abundance of ENaC isoforms and phosphorylated NKCC2 were not significantly changed (Fig. 2A and B). In addition, we also performed immunofluorescence of NCC. Consistent with the immunoblots, we confirmed that the signals of both total and phosphorylated NCC were reduced on the apical membrane of the distal convoluted tubules 5 days after metformin treatment (Fig. 2C and D). Metformin is known to activate AMPK by phosphorylation at T172 of the α-subunit in hepatocytes [5]. However, the phosphorylation levels at T172 of the AMPK α-subunit in the kidneys were not significantly changed in the metformin-treated mice relative to that in the vehicle-treated mice (Fig. 2E). These results indicated that urinary sodium excretion increased with metformin

treatment because NCC phosphorylation reduced independently from AMPK activation.

3.2 WNK4-OSR1/SPAK cascade was not involved with decreased phosphorylated NCC by metformin

WNK4 regulates NCC activity in the distal convoluted tubule of the kidney through phosphorylation of OSR1 and SPAK. Furthermore, the WNK-OSR1/SPAK-NCC cascade is activated by activated Akt [40,42]. Therefore, we examined the abundance of WNK4 and phosphorylation levels of OSR1, SPAK, and Akt in mice kidneys after metformin treatment. However, there were no significant changes in the abundance of WNK4, phosphorylated OSR1, and phosphorylated SPAK by metformin treatment (Fig. 3A). The Akt expression level also did not change with metformin treatment (Fig. 3B). These results demonstrated that the decrease in NCC phosphorylation with metformin treatment was independent of the WNK-OSR1/SPAK cascade.

3.3 A single dose of metformin increased urinary sodium excretion by decreasing NCC phosphorylation

As shown above, NCC phosphorylation decreased independently from the WNK-OSR1/SPAK cascades. This result suggested that metformin directly regulates NCC phosphorylation. To

investigate the acute effects of metformin on sodium transporters, we examined whether the metformin effect on decreasing NCC phosphorylation occurs in the short term. We treated mice with metformin at a dose of 300 mg/kg or vehicle by oral gavage for 1 h. Subsequently, we collected urine 2 and 4 hours after metformin treatment for measurement of urinary sodium excretion levels and examined the amounts of sodium transporters 1 h after metformin treatment. We found that sodium excretion in urine increased after short-term treatment with metformin as well as after five days of treatment (Fig. 4). Immunoblotting analysis showed that the abundance of phosphorylated NCC was reduced in metformin-treated mice, and there was no significant change in the abundance of AMPK, NKCC2, ENaC, and NHE3 relative to those in the control mice (Fig. 5A-C). Akt, WNK4, OSR1, and SPAK also did not change significantly after a single dose administration of metformin (Fig. 6A and B), similar to the results of the chronic administration experiment described above.

3.4 Metformin directly dephosphorylated NCC *ex vivo*

Short-term experimental results suggested that metformin could directly modulate NCC. However, it is possible that other biological factors mediate the effect of metformin on NCC. To eliminate systemic physiological effects, such as that of endocrine and nervous systems, we further investigated the mechanisms underlying the decreased NCC phosphorylation effect of metformin in *ex vivo* preparations of mice kidney slices, as previously described [47]. Kidney slices were

incubated with metformin (5 mM) *ex vivo* for 30 min. Then, we analyzed NCC phosphorylation levels by immunoblotting. Phosphorylated NCC was significantly reduced and phosphorylated SPAK was significantly increased (Fig. 7). Our *ex vivo* study results clearly demonstrated that metformin regulated NCC, but not by systemic effects of metformin, and that the WNK-SPAK signal was not involved in this mechanism.

4. Discussion

The study results provided new insights into the effect of metformin on increasing urinary sodium excretion by decreasing NCC phosphorylation. To the best of our knowledge, it has not been previously shown that metformin directly regulates NCC activity. Our results demonstrated that metformin reduced phosphorylated NCC, whereas other sodium transporters and channels, which are known to be related to insulin signaling or AMPK (Fig. 2A and B, Fig. 5A and B), were not significantly changed. In addition, the *ex vivo* experimental results demonstrated that metformin directly regulated NCC independently from the known hormonal regulators of NCC, such as insulin signaling or Ang II. Although we examined the WNK-OSR1/SPAK cascade, which has an important role in NCC regulation, this cascade was not involved in the effect of metformin on NCC.

It remains controversial whether metformin increases AMPK activity in the kidneys *in vivo*. Although it is well known that metformin is an activator of AMPK, several previous studies have

reported that metformin did not increase phosphorylated AMPK α at Thr172 *in vivo* in the kidneys, a result that differed from those in other tissues [35,48]. In the present study, the activity of AMPK in the kidneys did not significantly change after long-term and short-term metformin treatment, although NCC phosphorylation decreased significantly (Fig. 2C, Fig. 5C). Therefore, our data indicated that metformin did not change the AMPK activity in the kidneys, which indicated that the effect of metformin on NCC regulation was independent of AMPK activity. The fact that AMPK α subunits knockout mice showed a salt-wasting phenotype [49] also supports this indication because AMPK activation is expected to promote salt retention. Considering that metformin did not activate AMPK at all even in the *ex vivo* study (Supplemental Figure 1), although NCC phosphorylation decreased at the same time, it is natural to think that metformin directly decreased NCC phosphorylation. Organic cation transporter 1 (OCT1), which is reported to transport metformin [50], is expressed in DCT (Supplemental Figure 2), also supporting this "direct" effect of metformin in DCT.

Our data showed that the abundance of phosphorylated NCC significantly decreased after metformin treatment, whereas the other major sodium transporters were not significantly changed. We previously demonstrated that NCC phosphorylation inhibited its ubiquitination, which is a major mechanism in regulating NCC abundance following changes in NCC phosphorylation [24]. That is, dephosphorylation of NCC results in endocytosis from the apical membrane of the distal convoluted tubule and is subject to ubiquitination. Consequently, decreased phosphorylated NCC restricts

NCC-dependent sodium reabsorption. Further, our data showed that renal sodium excretion in mice significantly increased after metformin treatment.

We demonstrated that metformin reduced phosphorylated NCC in a single-dose treatment *ex vivo* and in a five-day treatment. The rapid onset of decreasing phosphorylated NCC suggested that metformin directly regulates NCC. In this study, the expression levels of WNK4 and SPAK did not change significantly *in vivo* with metformin treatment (Fig. 3A, Fig. 6A). Moreover, in the *ex vivo* experiment, elevated levels of phosphorylated SPAK were observed (Fig. 7). We considered that upregulation of phosphorylated SPAK in the *ex vivo* study could have been caused by feedback from reduced phosphorylated NCC. These data strongly indicated that there are direct effects of metformin on NCC that do not depend on the WNK-OSR1/SPAK cascade.

Metformin affects insulin concentration and downstream PI3K/Akt signaling. We also confirmed the abundance of phosphorylated Akt because we previously demonstrated that the WNK-OSR1/SPAK-NCC cascade was activated through phosphorylation of the activation site of Akt [40,42]. There was no significant change in the phosphorylated Akt expression level *in vivo* (Fig. 3B, Fig. 6B). The *ex vivo* study clearly demonstrated that the insulin/PI3K/Akt signal was not involved in NCC regulation by metformin. Therefore, we concluded that the WNK-OSR1/SPAK cascade was not involved in the decreased NCC phosphorylation caused by metformin.

SPAK phosphorylation was not decreased by metformin *in vivo* and *ex vivo*; therefore, we considered that protein phosphatases (PP) could be involved in the mechanism of decreased NCC

phosphorylation by metformin. It has been reported that some protein phosphatases, PP1 [51], PP4, and calcineurin (known as PP2B) [52–55], are involved in the regulation of NCC dephosphorylation. However, the expression levels of the catalytic subunit of PP1, PP4, calcineurin did not change significantly and phosphorylated PP1 (T320) and I-1, which is an endogenous inhibitor against PP1 [56], did not change significantly either (Supplemental Figure 3). In addition, although we investigated whether metformin dephosphorylates NCC through PP1 and calcineurin using specific inhibitors *in vivo* and *ex vivo*, the involvement of these protein phosphatases was not evident in our experiments (data not shown). However, it is still possible that the mechanism of metformin regulation of NCC phosphorylation could be due to activation of specific phosphatases, inhibition of kinases yet to be identified, or involvement of unknown pathways. Further studies will be necessary to elucidate the mechanism of action of metformin in regulating NCC phosphorylation and activity. With regards to the effects on blood pressure reduction, we could not observe the decrease of blood pressure by 14 days of metformin administration (500 mg/kg/day, in drinking water) to C57BL/6 mice (data not shown). In the case of mouse, it was reported that even $NCC^{-/-}$ mice did not show decreased blood pressure [57]. On the contrary, human homozygous mutation of NCC is well known to cause Gitelman syndrome, which is a hypotensive disease. Therefore, this could be due to the difference between human and mouse.

A major strength of our study is that we discovered that metformin is a potent antihypertension drug by decreasing phosphorylation of NCC and increasing sodium excretion, for the first time. In

contrast, the weakness of this study is that we confirmed the effects only in mouse models. Therefore, whether this effect of metformin is significantly involved in the mechanisms of lowering blood pressure in human remains unclear. In any case, our study implied translational potential of metformin. As mentioned above, recent meta-analysis of randomized trials reported that metformin decreased blood pressure in nondiabetic patients [13], which indicated that metformin also has an anti-hypertensive effect. Considering that metformin could have natriuresis effect, metformin could be an ideal drug for treatment of excessive salt intake, as well as excessive calorie intake. From the point of view of treatment for chronic kidney disease (CKD) patients, metformin has long been avoided considering risk of lactic acidosis. However, recent systematic reviews reported that metformin could be prescribed to type 2 DM patients with mild to moderate CKD with no greater risk of lactic acidosis compared with the overall population with diabetes [58] and that metformin reduced all-cause mortality and heart failure readmissions in type 2 DM with CKD [59]. As it becomes evident that metformin may not necessarily be unsafe in CKD patients, our results suggest that metformin can be beneficial even in CKD patients by increasing urinary sodium excretion. Further investigation will be required to establish the evidences between metformin and NCC in human.

In conclusion, metformin reduced NCC phosphorylation and increased sodium excretion in the kidneys. These results provide novel insights into the anti-hypertensive effects of metformin.

Author Contributions

H.H. performed experiments and wrote the manuscripts. E.S. designed and directed the project. N.N. and S.W. helped with *ex vivo* study. K.I. revised the manuscripts. H.K. helped with design the experiments. K.Y. and T.F. performed and helped with laser capture microdissection. S.U., T.R., T.O., T.M., and F.A. contributed to the data discussion.

Figure legends

Fig. 1 - Metformin treatment for five days increased urinary sodium excretion. 1 ml of 0.9% saline was orally administered to mice after five days of metformin (300 mg/kg/day, by oral gavage) or vehicle treatment. Urine was collected for 8 hours, and the amount of sodium for 8 hours was evaluated. Values are expressed as mean ± SEM. n = 9. *p < 0.05.

Fig. 2 - Effects of metformin treatment for five days on renal sodium transporters and channels. Representative immunoblots of (A) NCC, (B) NKCC2, ENaC, NHE3 in the kidneys of metformin-treated mice (300 mg/kg/day, by oral gavage, for five days). The arrowhead indicates the full length of ENaC a. Decreased NCC was observed in the metformin-treated group relative to that in the vehicle-treated group. (Right) Densitometric analysis. (C) Double immunofluorescence of pNCC (green) and tNCC (red) in the kidneys after metformin treatment. Both pNCC and tNCC were reduced on apical membrane of the distal convoluted tubule in metformin treated-mice. Scale bars =

10 μm. (D) High power view of double immunofluorescence of pNCC and tNCC in the kidneys after metformin treatment. Scale bars = 10 μm. (E) Representative immunoblots of AMPK in the kidneys of metformin-treated mice. Values are expressed as mean ± SEM. n = 6. **p < 0.01. n.s., not significant; p, phosphorylated; t, total; Ctrl, control; Met, metformin.

Fig. 3 - NCC dephosphorylation induced by metformin treatment for five days was independent of the WNK-OSR1/SPAK cascade. Representative immunoblots of (A) WNK4, SPAK, OSR1 and (B) Akt in the kidneys of metformin-treated mice (300 mg/kg/day, by oral gavage, for five days). Densitometric analysis of WNK4, SPAK, OSR1, and Akt blots. Values are expressed as mean ± SEM. n = 6. n.s., not significant; p, phosphorylated; t, total.

Fig. 4 - A single dose of metformin treatment increased urine sodium excretion. Metformin (300 mg/kg) or vehicle were administered to mice by oral gavage. The urine was collected for 2 hours and 4 hours. The total amount of sodium in urine collected for 2 and 4 hours were evaluated, respectively. Values were expressed as mean ± SEM. n = 7. **p < 0.01.

Fig. 5 - Effects of a single dose of metformin on renal sodium transporters and channels. Representative immunoblots of (A) NCC, (B) NKCC2, ENaC, NHE3, and (C) AMPK in the kidneys 1 h after metformin administration by oral gavage (300 mg/kg). Decreased NCC was observed in the metformin-treated group relative to that in the vehicle-treated group. Densitometric analysis; metformin treatment decreased NCC phosphorylation. Values are expressed as mean ± SEM. n = 6. **p < 0.01. n.s., not significant; p, phosphorylated; t, total.

Fig. 6 - NCC dephosphorylation induced by a single dose of metformin treatment was independent of the WNK-OSR1/SPAK cascade. Representative immunoblots of (A) WNK4, SPAK, OSR1, and (B) Akt in the kidneys 1 h after metformin administration by oral gavage (300 mg/kg). Densitometric analysis of WNK4, SPAK, OSR1, and Akt blots. Values are expressed as mean ± SEM. n = 6. n.s., not significant; p, phosphorylated; t, total.

Fig. 7 - Effects of metformin treatment using *ex vivo* preparations of mouse kidney slices. Representative immunoblots of NCC and SPAK in *ex vivo* preparations of mouse kidneys from two different mice (#1 and #2) incubated with metformin (5 mM) for 30 min. Decreased NCC and increased SPAK levels were observed in the metformin-treated group relative to those in the control group. Densitometric analysis; metformin treatment decreased NCC phosphorylation and increased SPAK. Values are expressed as mean ± SEM. n = 6. *$p < 0.05$. n.s., not significant; p, phosphorylated; t, total; MF, membrane fraction; WL, whole lysate.

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Conflict of Interest

All the authors declared no competing interests.

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Highlights

- Metformin administration increased urinary sodium excretion *in vivo*.
- Metformin reduced NCC phosphorylation at the activation site in kidneys.
- *Ex vivo* experiments confirmed direct effect of metformin on NCC.

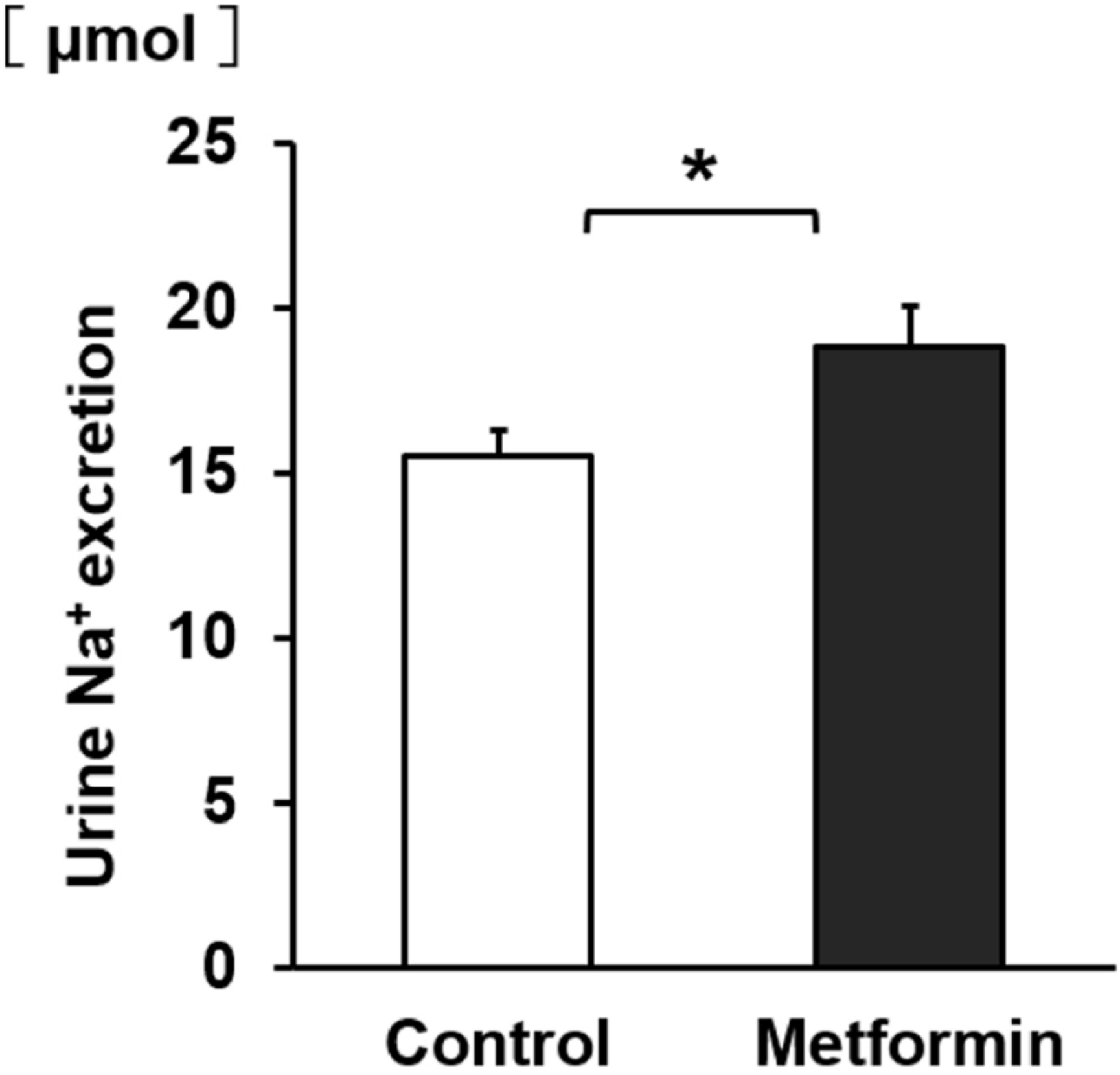

Figure 1

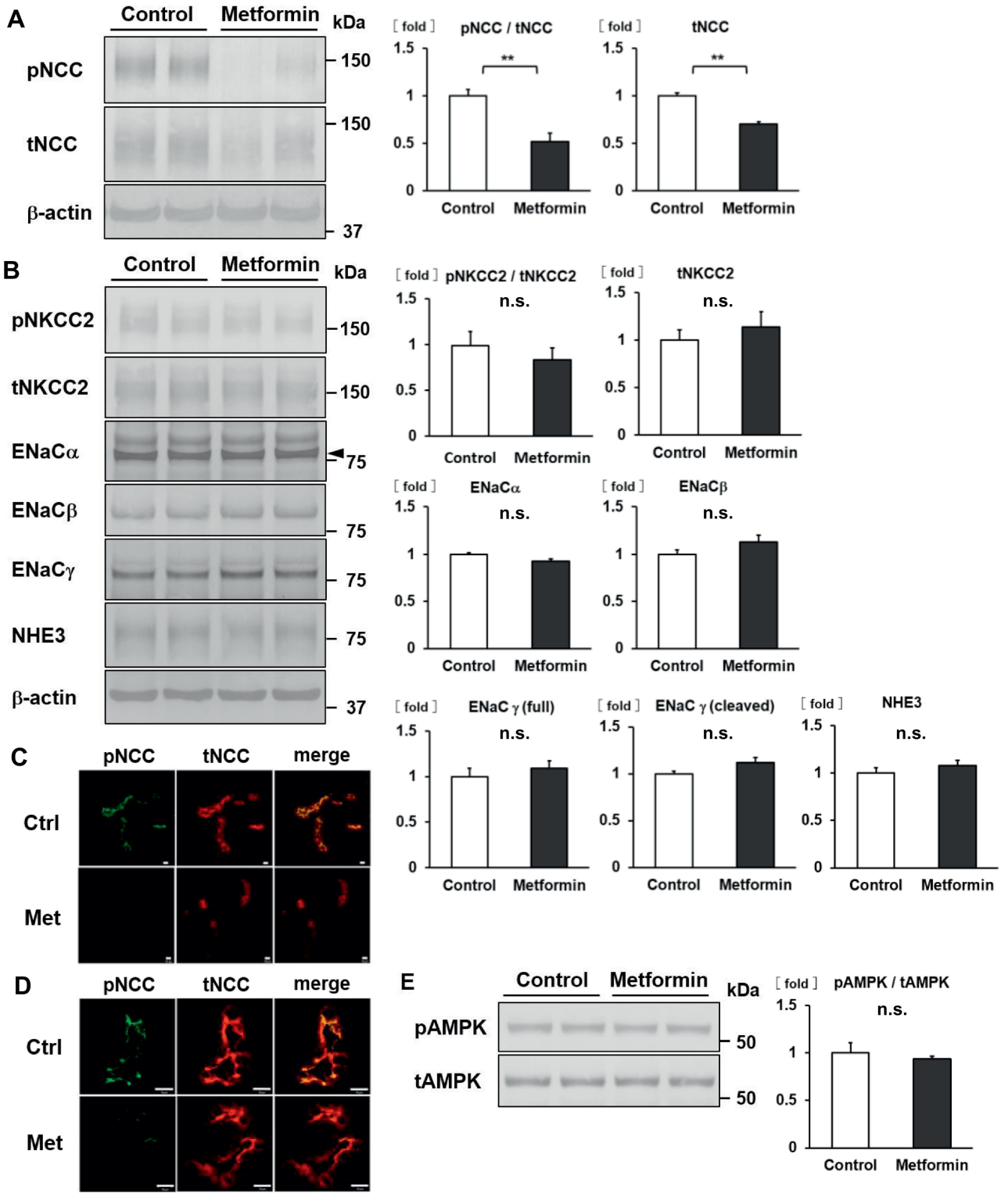
A
Control
Metformin
kDa
pNCC
tNCC
β-actin
150
150
37
[fold] pNCC / tNCC
**
[fold] tNCC
**
Control
Metformin
B
pNKCC2
tNKCC2
ENaCα
ENaCβ
ENaCγ
NHE3
β-actin
150
150
75
75
75
75
37
[fold] pNKCC2 / tNKCC2
n.s.
[fold] tNKCC2
n.s.
[fold] ENaCα
n.s.
[fold] ENaCβ
n.s.
[fold] ENaC γ (full)
n.s.
[fold] ENaC γ (cleaved)
n.s.
[fold] NHE3
n.s.
C
pNCC
tNCC
merge
Ctrl
Met
D
pNCC
tNCC
merge
Ctrl
Met
E
Control
Metformin
kDa
pAMPK
tAMPK
50
50
[fold] pAMPK / tAMPK
n.s.

Figure 2

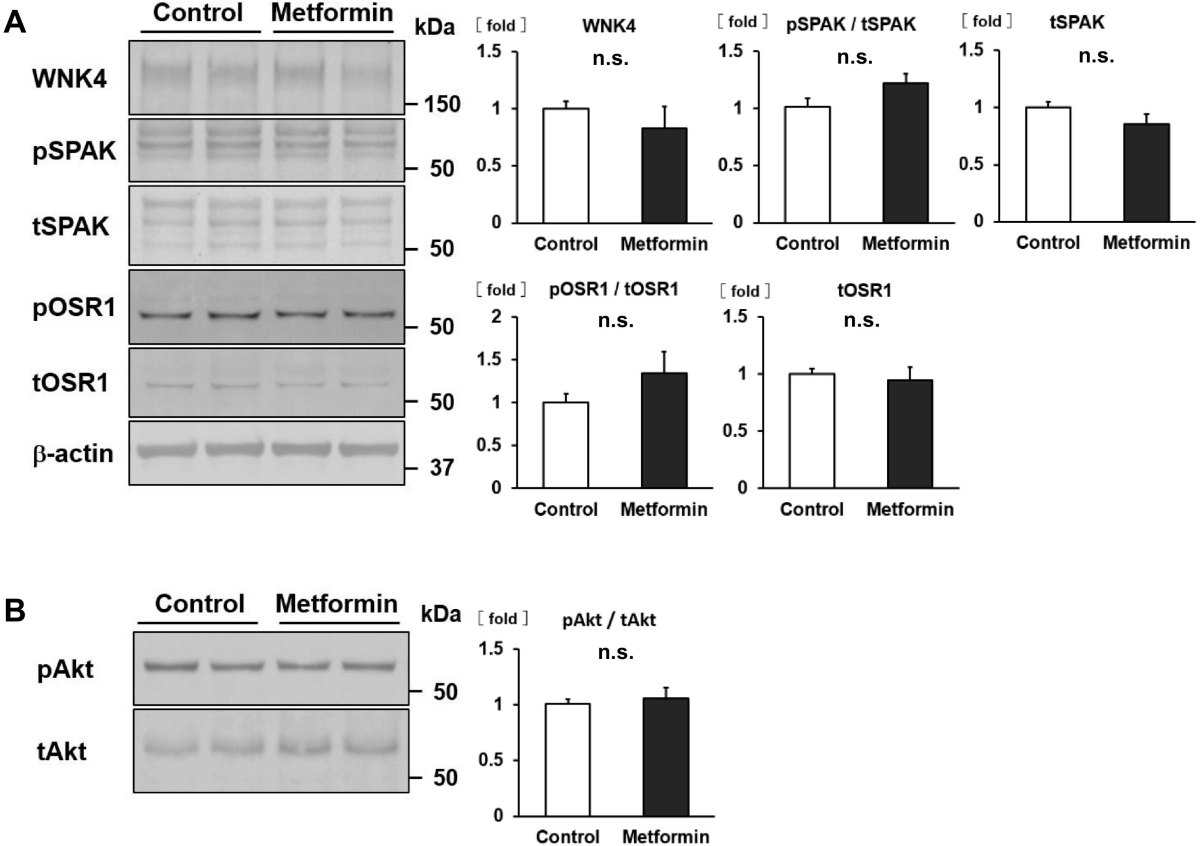
A
Control
Metformin
kDa
WNK4
pSPAK
tSPAK
pOSR1
tOSR1
β-actin
150
50
50
50
50
37
[fold]
WNK4
n.s.
[fold]
pSPAK / tSPAK
n.s.
[fold]
tSPAK
n.s.
[fold]
pOSR1 / tOSR1
n.s.
[fold]
tOSR1
n.s.
Control
Metformin
B
Control
Metformin
kDa
pAkt
tAkt
50
50
[fold]
pAkt / tAkt
n.s.
Control
Metformin

Figure 3

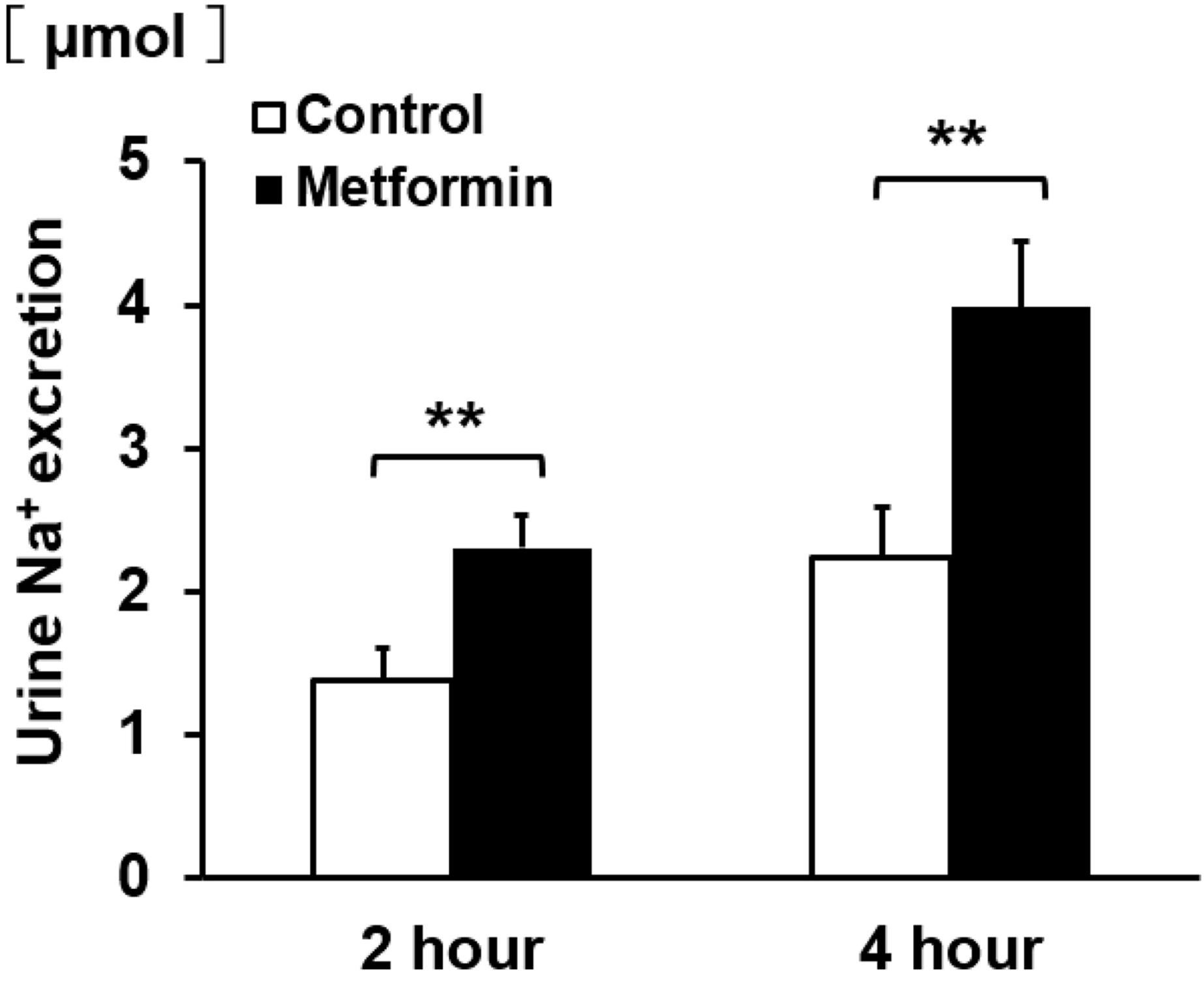
[µmol]
Control
Metformin
Urine Na+ excretion
5
4
3
2
1
0
**
**
2 hour
4 hour

Figure 4

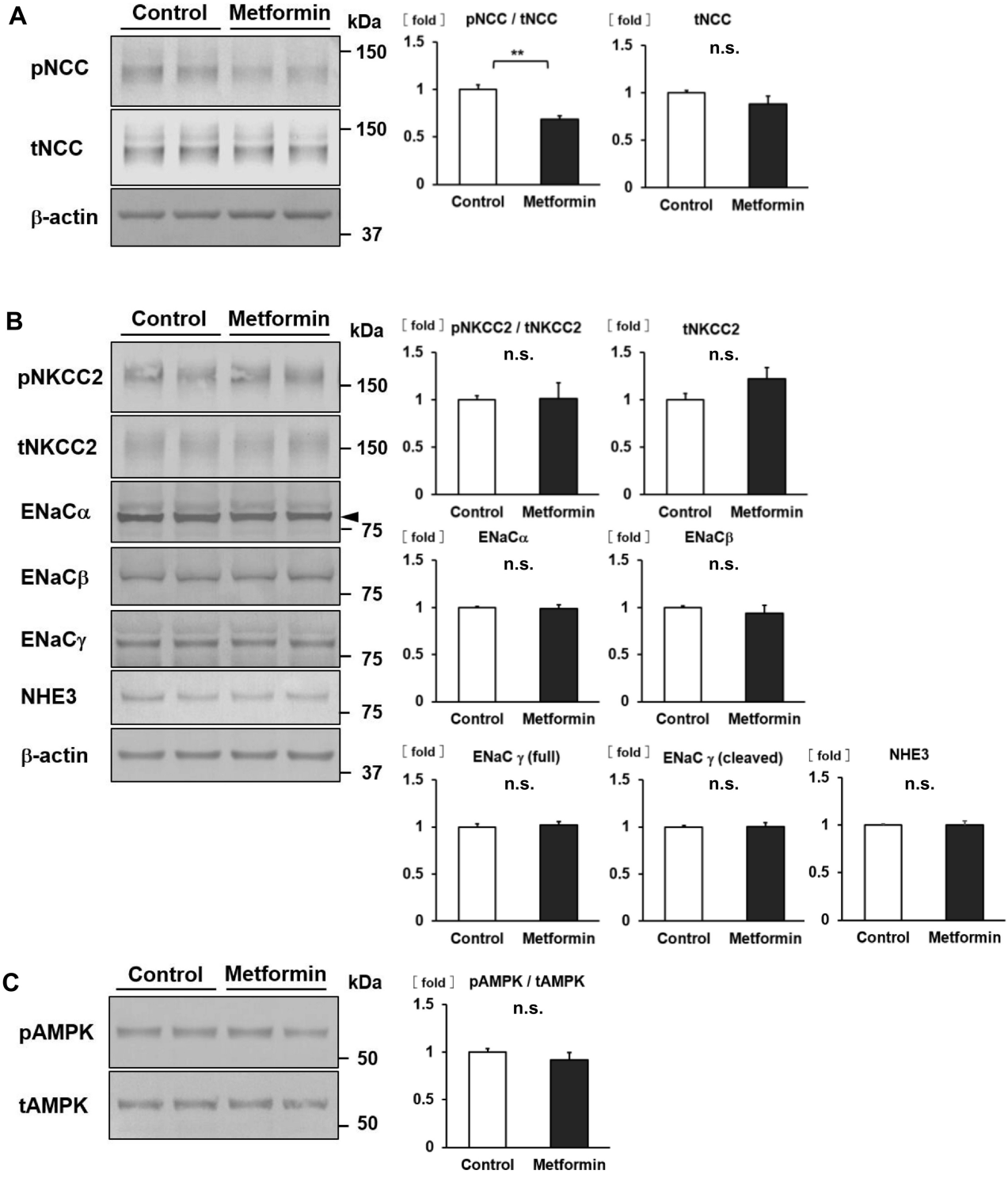
A
Control
Metformin
kDa
pNCC
tNCC
β-actin
150
150
37
[fold]
pNCC / tNCC
**
Control
Metformin
[fold]
tNCC
n.s.
Control
Metformin
B
Control
Metformin
kDa
pNKCC2
tNKCC2
ENaCα
ENaCβ
ENaCγ
NHE3
β-actin
150
150
75
75
75
75
37
[fold]
pNKCC2 / tNKCC2
n.s.
[fold]
tNKCC2
n.s.
[fold]
ENaCα
n.s.
[fold]
ENaCβ
n.s.
[fold]
ENaC γ (full)
n.s.
[fold]
ENaC γ (cleaved)
n.s.
[fold]
NHE3
n.s.
C
Control
Metformin
kDa
pAMPK
tAMPK
50
50
[fold]
pAMPK / tAMPK
n.s.
Control
Metformin

Figure 5

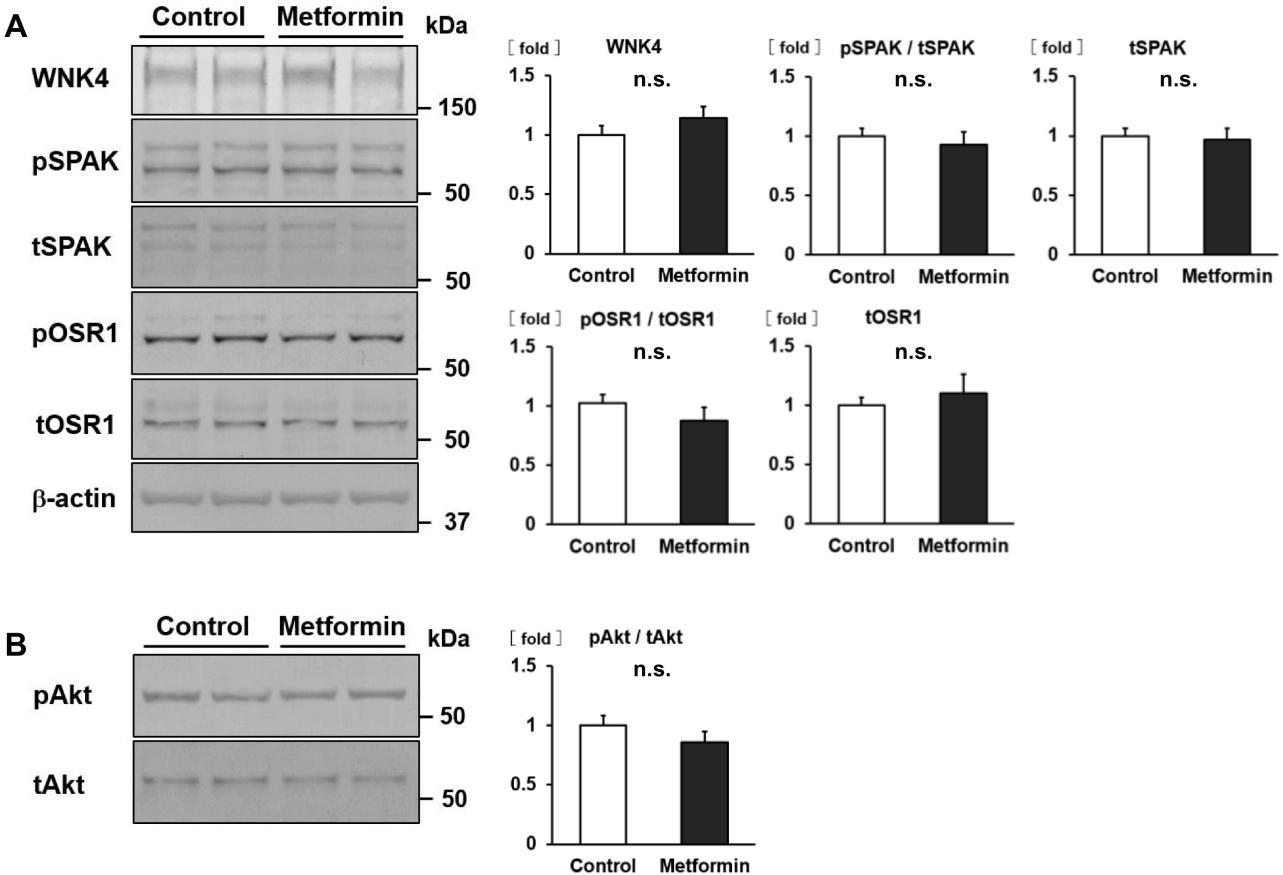
A
Control
Metformin
kDa
WNK4
pSPAK
tSPAK
pOSR1
tOSR1
β-actin
150
50
50
50
50
37
[fold]
WNK4
n.s.
[fold]
pSPAK / tSPAK
n.s.
[fold]
tSPAK
n.s.
[fold]
pOSR1 / tOSR1
n.s.
[fold]
tOSR1
n.s.
1.5
1
0.5
0
Control
Metformin
B
Control
Metformin
kDa
pAkt
tAkt
50
50
[fold]
pAkt / tAkt
n.s.
1.5
1
0.5
0
Control
Metformin

Figure 6

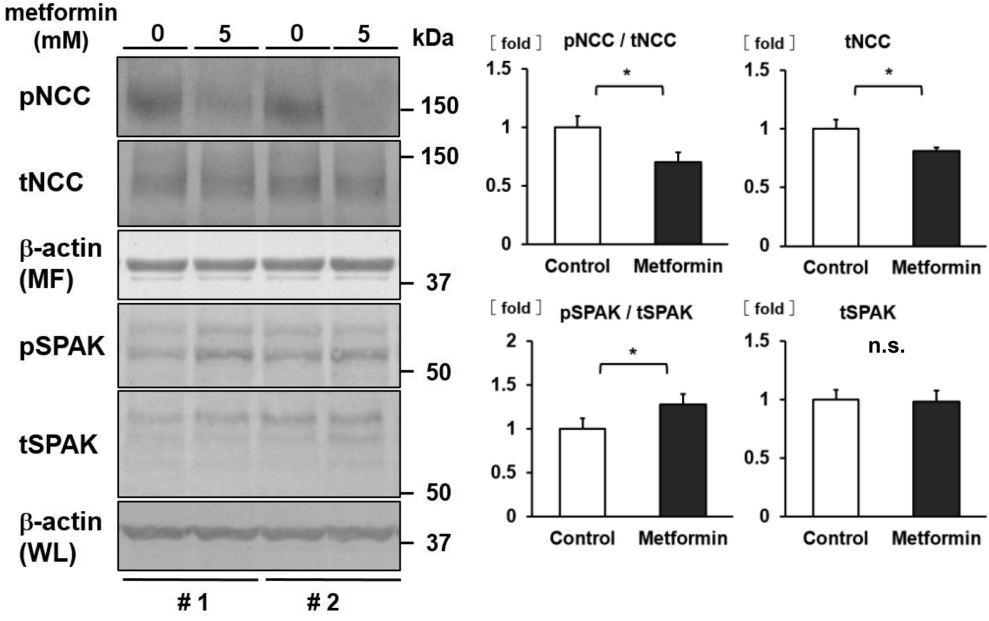
metformin
(mM)
0
5
0
5
kDa
pNCC
tNCC
β-actin
(MF)
pSPAK
tSPAK
β-actin
(WL)
150
150
37
50
50
37
1
2
[fold]
pNCC / tNCC
1.5
1
0.5
0
*
Control
Metformin
[fold]
tNCC
1.5
1
0.5
0
*
Control
Metformin
[fold]
pSPAK / tSPAK
2
1.5
1
0.5
0
*
Control
Metformin
[fold]
tSPAK
n.s.
1.5
1
0.5
0
Control
Metformin

Figure 7