Dietary magnesium insufficiency induces salt-sensitive hypertension in mice with genetically low COMT activity

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Abstract:

Catechol-O-methyl transferase (COMT), an enzyme that metabolizes catechol, requires magnesium (Mg) to maintain its activity. Low COMT activity causes insufficient 2-methoxyestradiol (2-ME), a biologically active metabolite from hydroxyestradiol, which leads to hypertensive disorders, including preeclampsia (PE). Hypoestrogenism increases the risk of salt-sensitive hypertension (SSH). SSH and PE are risk factors for each other; however, the molecular mechanism of this interaction is unclear. We focused on the interactive effect of Mg insufficiency and genetic COMT deficiency on SSH using two strains of mice with genetically distinct COMT activity. In male mice, C57/B6 (BL6), a high-activity COMT strain, displayed unaltered blood pressure (BP) regardless of the Mg and salt levels in food; DBA/2J (DBA), a low-activity COMT strain, developed SSH under low Mg and high salt conditions. COMT inhibition in BL6 strain also induced SSH. Treatment with 2-ME cured SSH in both models. The angiotensin II type 1 receptor (ATR1) - STE20-serine-proline alanine-rich kinase (SPAK) - sodium chloride cotransporter (NCC) axis, molecules associated with sodium reabsorption in distal convoluted tubules, was activated in mice that developed SSH. Thiazide, an inhibitor of NCC, also ameliorated SSH. In female DBA mice, ovariectomized mice displayed elevated BP under low Mg and high salt conditions that was ameliorated by 2-ME, whereas the BP of sham mice was unaltered regardless of any intervention. Our findings revealed that Mg insufficiency exaggerated the low COMT activity and induced SSH via the ATR1-SPAK-NCC pathway due to 2-ME insufficiency, suggesting a new pathophysiological role that links COMT/2-ME deficiency with hypertensive syndrome.

Keywords: Magnesium deficiency, Catechol-O-methyl transferase, 2-methoxyestradiol, Sodium chloride, Sodium chloride symporters

Introduction:

Catechol-O-methyl transferase (COMT), a magnesium-dependent enzyme, metabolizes catechols such as catecholamine and hydroxyestradiol. The compound 2-methoxyestradiol (2-ME), an estrogen metabolite of COMT generated from hydroxyestradiol, is a biologically active compound that improves glucose/lipid metabolism and possesses anti-inflammatory, antivasoconstrictive, antiangiogenic, and antitumor effects [1] [2]. COMT activity is determined by various factors, as described in Fig. 1a. Among genetic factors, single nucleotide polymorphisms (SNPs), which determine the stability of the COMT protein, are the best-known factors. In humans, rs4680 (Val158Met) is a common polymorphism in which Val/Val possesses 3-4 times higher COMT activity than Met/Met [3] (Fig. 1a), and low activity of $COMT^{met/met}$ has been linked to an increased risk of hypertension [4] and acute coronary syndrome [5]. Haplotype sequences are also widely investigated, as in patients with preeclampsia (PE) in which haplotypes related to low COMT activity were reported to have a significantly higher risk of recurrence of PE [6] (Fig. 1a). These reports illustrate the global importance of COMT deficiency in the onset of hypertensive syndrome in humans.

Magnesium (Mg) is also an essential factor that induces the correct conformation of the active site of the enzyme [7] (Fig. 1a). Mg is the second most abundant intracellular cation and functions as a cofactor of over 300 enzymes and activator of approximately 200 enzymes [8]. Mg deficiency causes various diseases, such as cardiovascular disease, arrhythmia, hypertension, neurodegenerative disease, migraine, epilepsy, and metabolic disorders, such as type 2 diabetes and lipid metabolism disorder [9]. Magnesium sulfate ($MgSO_4$) reduces the risk of eclampsia and maternal death of patients with PE [10], which is caused by placental dysfunction. $MgSO_4$ reduces oxidative stress and the vasoconstrictive effect of angiotensin II (AII) and endothelin-1 in the human placenta; thus, Mg deficiency is believed to have a

essential role in the development of PE [11] [12].

Mice with 2-ME insufficiency due to genetic COMT defects present a preeclamptic phenotype [13], and COMT activities and plasma 2-ME levels were significantly suppressed in patients with PE compared to healthy pregnant women [6] [13]. With regard to the long-term complications of PE, women with a history of PE are more prone to develop salt-sensitive hypertension (SSH) before menopause [14]. SSH is recognized as an estrogen-related disease based on reports of an increased risk of SSH in postmenopausal women and young women with primary ovarian insufficiency [15] [16] and the positive effect of estrogen replacement therapy on ovariectomized mice and rats that developed SSH [17, 18]. A link between COMT and SSH has been proposed: high salt reduced COMT activity and induced SSH via the accumulation of norepinephrine (NE) [19]; however, the effect of COMT as an estrogen metabolizer has never been discussed.

One of the best-known mechanisms of SSH is hyperactivation of the sodium chloride cotransporter (NCC), a thiazide-sensitive salt absorptive pathway localized at distal convoluted tubules (DCT). NCC is responsible for the reabsorption of 5-10% of filtered sodium chloride (NaCl) and is a fine regulator of Na homeostasis [20]. NCC activation is regulated by with no lysine kinase (WNK)/STE20-serine-proline alanine-rich kinase (SPAK)/NCC phosphorylation cascade, which is activated by AII-induced angiotensin type 1 receptor (ATR1) upregulation [21] [22]. We previously identified that ATR1 upregulation was induced by COMT deficiency in the aorta [23]; therefore, we hypothesized that COMT might regulate NCC via the ATR1-SPAK pathway.

Given the strong association between Mg and COMT activity as well as COMT deficiency and PE/SSH, we hypothesized that COMT deficiency is a shared molecular mechanism of PE and SSH.

Materials and methods:

Data that support the findings of this study are available from the corresponding author upon reasonable request. A detailed section of methods and materials is provided in the supplemental materials.

Summary

All animal experiments were approved by the IACUC of Kanazawa Medical University (protocol numbers 2017-120 & 2019-17) and by the IACUC of Shimane University (protocol number IZ2-52). All experiments are performed according to Japanese guidelines and institutional ethics committee guidelines. For Mg deficient experiment, 7 weeks old male/female DBA/2J (DBA) and male C57/B6 (BL6) were fed either 0.1% Mg or 0.03% Mg which were replaced with 8% NaCl diet or 0.6% NaCl diet with the same Mg concentration after 2 weeks. For COMT inhibitor (COMTi) experiment, male BL6 mice were treated with COMTi Ro41-0960 (25 mg/kg/day) or grape seed oil intraperitoneally a week after 0.1% Mg diet was given. Intraperitoneal injection of either 2-ME (10 ng/day), hydrochlorothiazide (HCTZ) (25 mg/kg/day) or PBS was administered from 10 weeks of age. Ovariectomy was performed at 7 weeks of age before the diet was replaced with 0.1% Mg or 0.03% Mg diet. All mice were sacrificed at 12 weeks of age. Blood pressure (BP) was measured every week by tail-cuff system as previously described [23]. Plasma was collected for analysis of electrolytes, AII, catecholamine, S-adenosyl-L-methionine (SAM) and S-adenosyl-L-homocysteine (SAH) concentrations. Plasma catecholamine, SAM, SAH were analyzed as preciously described [24 25 26 27].Twenty-four-hour urine was collected for analysis of electrolytes and albumin to creatinine ratio. Western blot analysis was performed using whole

kidney extraction. Kidney COMT activity was measured using methyltransferase activity kit. Graph Pad Prism software 8.2.1 was used for statistical analysis.

Results:

Plasma Mg concentration decreased with dietary Mg insufficiency

Similar to SNP-dependent human COMT activity, mouse COMT activity exhibits variation among strains [28]. Different mRNA isoforms exist among mouse strains, in which mice with short proximal 3' untranslated region (3' UTR) isoforms, such as BL6 mice, are known to have greater COMT activity and protein expression than mice with the original length of the 3' UTR, such as DBA mice [29]. This study utilizes these two strains, BL6 and DBA, with high and low COMT activities to evaluate the effect of Mg on COMT activity. The scheme of the study protocol is illustrated in Fig. 1b. Mouse growth was not affected by the concentrations of Mg or NaCl in either strain (Fig. 1c). Under basal conditions, the level of plasma Mg was lower in the DBA strain than in the BL6 strain, and the plasma levels were decreased directly proportionally to the amount of Mg contained in the food in both strains regardless of the NaCl concentration (Fig. 1d).

Mg deficiency induced salt-sensitive hypertension in the DBA mice; 2-ME ameliorated this condition

The sBP values without interventions were comparable between the strains. The sBP of BL6 was not affected by Mg deficiency and/or high salt (Fig. 1e, g). The sBP of DBA was also not affected by Mg deficiency or high salt alone but was significantly increased in the Mg-deficient and high salt conditions. Administration of 2-ME normalized the sBP (Fig. 1e, g) in the DBA mice fed Mg-deficient and high-salt diet. The plasma AII level was relatively higher

in the BL6 mice than in the DBA mice. High salt significantly decreased the plasma AII level in the DBA mice, but only a downward trend was observed in the BL6 mice (Fig. S1).

Percent Na excretion and urinary Mg excretion were higher in the DBA mice than in the BL6 mice under high salt load

The amount of daily water intake and urine increased by high salt loading regardless of the Mg concentration in both strains (Fig. 2a, b). The volume of water that was not excreted as urine (daily water intake – daily urine; $\Delta water^{intake\text{-}urine}$) was stable regardless of any intervention in both strains, although there was a slight increasing trend of Δ $water^{intake\text{-}urine}$ in the DBA mice fed high salt diet (Fig. 2c). Urinary Mg excretion decreased as Mg intake decreased in both strains (Fig. 2d). In the BL6 mice, the amount of urinary Mg excretion was relatively higher than that in the DBA mice and was not affected by a high salt load (Fig. 2d). However, in the DBA mice, urinary Mg excretion increased significantly to a higher level than that of the BL6 mice under high salt conditions regardless of the Mg concentration (Fig. 2d). The amount of Na that was not excreted as urine (daily Na intake- daily urinary Na; $\Delta Na^{intake\text{-}urine}$) was significantly increased by high salt load only in BL6 as described detail in Fig. S2.The % Na excretion (urinary Na/daily Na intake) under a high salt load was much higher in the DBA mice than the BL6 mice (Fig. 2e). To investigate alternative ways to excrete Na, we measured the amount of Na in feces; however, Na excretion in feces was comparable between the strains (Fig. S3).

Kidney COMT activity decreased in the Mg-deficient DBA mice under high salt conditions

While kidney COMT activity was not altered by changes in Mg or salt concentration in the

BL6 mice, COMT activity significantly decreased with high salt only in the Mg-deficient DBA mice. 2-ME did not recover the COMT activity (Fig. S4a-d).

Plasma catecholamine, SAM and SAH in the Mg-deficient mice under high salt conditions

Mg deficiency tended to increase the plasma NE and epinephrine (E) levels compared to those in the normal Mg mice only in DBA mice (Fig. S5a, b). No alternation was found in plasma levels of both SAM and SAH in the high salt loaded mice regardless of the Mg status (Fig. S5c, d).

Chemical inhibition of COMT activity induced salt-sensitive hypertension in the BL6 mice; 2-ME ameliorated

To confirm the relevance of the COMT deficiency in the onset of SSH, we administered a COMTi to high salt-loaded, normal-Mg BL6 mice (Fig. 3a). The body weight (BW) and plasma Mg concentration were not different among all groups (Fig. 3b, c). The sBP did not change by COMTi alone, but coadministration with high salt significantly increased the sBP, which was normalized by 2-ME (Fig. 3d, e). Both Δ water$^{\text{intake-urine}}$ and Δ Na$^{\text{intake-urine}}$ were significantly increased by high salt load, but urinary Mg excretion and % Na excretion were unaltered as described detail in Fig. S6.

Kidney NCC was activated via ATR1-SPAK activation in the high salt-loaded Mg-deficient DBA mice and COMTi-treated BL6 mice

Phosphorylated NCC (p-NCC) forms a complex-glycosylated homodimer with a molecular mass of approximately 310 kDa, and this is the only form that can exist on the plasma

membrane [30]. To identify p-NCC as a homodimer, we prepared protein extracts dissolved in 8 M urea in which protein complexes resist dissolution [31]. In the DBA mice, a high salt load reduced the expression of p-NCC, phosphorylated SPAK (p-SPAK) and ATR1 under normal Mg conditions (Fig. 4a). However, in the Mg-deficient conditions, a high salt load resulted in the highest expression of p-NCC, p-SPAK and ATR1 in all groups (Fig. 4a, b). COMTi treatment of the BL6 mice had a similar effect on increasing p-NCC, p-SPAK and ATR1 protein expression under a high salt load (Fig. 4c, d). The upregulated expression of p-NCC, p-SPAK and ATR1 was suppressed by 2-ME in both SSH models (Fig. 4a-d). To further confirm the activation of the NCC pathway in our model, we administered HCTZ, an inhibitor of NCC, to the Mg-deficient DBA and COMTi-treated BL6 mice under high salt conditions (Fig. 5a, b). A significant decline in sBP was observed in the HCTZ-treated groups to the same extent as 2-ME treatment in both strains (Fig. 5c-f).

Female ovariectomized DBA mice developed SSH under Mg-deficient and high salt conditions

Female DBA mice with or without ovariectomy were fed Mg-deficient and/or high salt diet (Fig. 6a). There was no difference in BW or basal plasma Mg between the sham and ovariectomized (OVX) mice (Fig. 6b, c). While the sham mice maintained a normal sBP regardless of the Mg or salt concentration, the ovariectomized mice developed SSH only in the Mg-deficient and high salt conditions, and 2-ME normalized the sBP of these mice (Fig. 6d, e). Δ water$^{\text{intake-urine}}$ was unaltered by high salt load (Fig. S7a-c). Urinary Mg excretion and % Na excretion were significantly increased by high salt load in OVX mice (Fig. S7d, e). Δ Na$^{\text{intake-urine}}$ was significantly increased by high salt load only in Mg deficient mice (Fig. S7f-h).

The urine albumin to creatinine ratio was significantly increased in the Mg-deficient OVX DBA and COMTi BL6 mice; 2-ME ameliorated this condition

In the male DBA mice, urine alb/cre significantly increased by high salt load (Fig. S8a). In the COMTi BL6 mice, increased urine alb/cre under high salt condition was significantly decreased by either 2-ME or HCTZ (Fig. S8b). In the female DBA mice, only the Mg-deficient OVX mice showed elevated urine alb/cre, which was ameliorated by 2-ME (Fig. S8c).

Discussion:

Elevated salt sensitivity is one of the major causes of hypertension [32] and is also associated with chronic kidney disease, another disease linked with a COMT-deficient genetic background [33]. From previous reports regarding the role of COMT deficiency in the pathogenesis of PE [13] and the link between PE and SSH [14], we hypothesize that COMT deficiency could be a shared molecular mechanism in the onset of PE and SSH. Therefore, this study focused on the effect of COMT deficiency on SSH and identified an interactive effect of dietary Mg insufficiency and genetic COMT deficiency that induced SSH. In brief, our novel findings were as follows: 1) Mg deficiency induced SSH in the genetically low COMT strain DBA under high salt conditions, and 2-ME, a metabolite of COMT, attenuated this condition, 2) upregulation of the SPAK-NCC pathway was involved in COMT deficiency-induced SSH, 3) COMTi-treated BL6, a genetically high COMT strain, expressed similar phenotypes as Mg-deficient DBA and 4) ovariectomy-induced SSH in female DBA mice was observed under low Mg and high salt conditions and was ameliorated by 2-ME.

The recommended daily allowance of Mg is approximately 300-400 mg; studies

from several countries revealed that between 20-80% of the population, depending on gender and age, do not meet their daily requirement of Mg intake [34]. In general, women have a higher risk of inadequate Mg intake than men, especially pregnant women and aged women, because serum Mg gradually decreases during pregnancy due to the significant increase in demand for Mg and aging reduces intestinal permeability to Mg [34 35]. However, Mg deficiency is barely detectable by typical blood tests since 99% of total body Mg is located intracellularly in bone, muscle, and soft tissues, and extracellular Mg accounts for only approximately 1%: 0.3% in serum and 0.5% in red blood cells [8].

In terms of the effect of Mg deficiency on COMT activity and the development of SSH, only DBA, a strain with genetically low COMT activity, was strongly affected by Mg deficiency. Interestingly, high salt load significantly increased urinary Mg excretion only in DBA. Although there was no change in plasma Mg, the high salt-induced upregulation of urinary Mg excretion might lower the intracellular Mg concentration in DBA, which could further reduce the activity of COMT. Indeed, our suggestion is reinforced by the report by Resnick LM et al. describing that although no significant difference was found in plasma Mg between salt-sensitive and insensitive patients under high salt conditions, cytosolic free Mg was significantly decreased only in salt-sensitive patients [36]. Moreover, our results correlate with a previous report of COMT activity suppressed by high salt load in Dahl salt-sensitive rats (DRs), whose COMT expression is known to be lower than that in other rats [19 37].

In the kidneys of the mice with SSH, upregulation of the ATR1-SPAK-NCC pathway was identified and was ameliorated by 2-ME, although plasma AII was suppressed by high salt load. HCTZ treatment mimicking the results of 2-ME confirmed that the ATR1-SPAK-NCC pathway is the target of 2-ME in the kidney. Our results indicated that 2-ME insufficiency under COMT-deficient conditions might lead to the upregulation of ATR1,

which activates SPAK-NCC, resulting in the development of SSH. This assumption agreed with previous reports revealing the link between 2-ME and AII sensitivity in several organs. In the aorta, our group has previously reported that 2-ME insufficiency increased blood pressure by upregulating the expression of ATR1 in the aorta [23], and in the brain, Singh P et al. recently reported that 2-ME attenuated AII-induced upregulation of sympathetic activities and thus revealed a protective effect against hypertension [38].

SSH is an estrogen-related hypertension whose risk increases after menopause for many women [15, 39]. Although endogenous estrogen protects against increasing salt sensitivity, several studies suggest that oral hormone replacement therapy does not affect or can slightly increase BP in postmenopausal women [15 40]. Our study identified that the development of SSH is due to a lack of the estrogen metabolite 2-ME; therefore, we propose that 2-ME administration could be a new treatment option for SSH instead of estrogen for postmenopausal women. The higher possibility of developing SSH in women with a history of PE during the premenopausal period [14 39] might be the consequence of a low COMT genetic background. In addition, a study of DR revealed that DR, but not rats with spontaneous hypertension, developed superimposed PE during pregnancy [41]. Thus, we assume that SSH and PE may share a similar pathogenesis, which we propose is "COMT deficiency" or "lack of 2-ME". Furthermore, among the many factors that could affect COMT activity, Mg is worthy of attention considering its insufficient intake worldwide. Further study with a pregnant model is required to clearly show this relationship.

Limitation and additional discussion are described in supplemental materials.

In conclusion, we revealed that Mg insufficiency exaggerated low COMT activity and subsequently induced SSH, suggesting a new role of COMT as an SPAK-NCC regulator via 2-ME production.

Perspectives:

This study focused on genetical variance of COMT activity as well as the impact of Mg deficiency on COMT activity and identified that Mg deficiency induced COMT deficiency/2-ME insufficiency only in genetically low COMT strain and that COMT deficiency/2-ME insufficiency developed SSH via upregulating ATR1-SPAK-NCC pathway in male. In female, only ovariectomized mice developed SSH under Mg deficiency suggesting the importance of sufficient amount of 2-ME production. Inadequate intake of Mg is a worldwide topic for decades, especially in elderly women who are at increased risk of SSH. This study revealed the importance of adequate Mg intake for prevention of SSH. 2-ME replacement therapy instead of estrogen replacement therapy might be another option for treatment of SSH.

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References

1. Dubey RK. 2-methoxyestradiol: A 17beta-estradiol metabolite with gender-independent therapeutic potential. *Hypertension.* 2017;69:1014-1016

2. Kanasaki M, Srivastava SP, Yang F, Xu L, Kudoh S, Kitada M, Ueki N, Kim H, Li J, Takeda S, Kanasaki K, Koya D. Deficiency in catechol-o-methyltransferase is linked to a disruption of glucose homeostasis in mice. *Sci Rep.* 2017;7:7927

3. Chen J, Lipska BK, Halim N, Ma QD, Matsumoto M, Melhem S, Kolachana BS, Hyde TM, Herman MM, Apud J, Egan MF, Kleinman JE, Weinberger DR. Functional analysis of genetic variation in catechol-o-methyltransferase (comt): Effects on mrna, protein, and enzyme activity in postmortem human brain. *Am J Hum Genet.* 2004;75:807-821

4. Htun NC, Miyaki K, Song Y, Ikeda S, Shimbo T, Muramatsu M. Association of the catechol-o-methyl transferase gene val158met polymorphism with blood pressure and prevalence of hypertension: Interaction with dietary energy intake. *Am J Hypertens.* 2011;24:1022-1026

5. Voutilainen S, Tuomainen TP, Korhonen M, Mursu J, Virtanen JK, Happonen P, Alfthan G, Erlund I, North KE, Mosher MJ, Kauhanen J, Tiihonen J, Kaplan GA, Salonen JT. Functional

comt val158met polymorphism, risk of acute coronary events and serum homocysteine: The kuopio ischaemic heart disease risk factor study. *PLoS One.* 2007;2:e181

6. Roten LT, Fenstad MH, Forsmo S, Johnson MP, Moses EK, Austgulen R, Skorpen F. A low comt activity haplotype is associated with recurrent preeclampsia in a norwegian population cohort (hunt2). *Mol Hum Reprod.* 2011;17:439-446

7. Tsao D, Diatchenko L, Dokholyan NV. Structural mechanism of s-adenosyl methionine binding to catechol o-methyltransferase. *PLoS One.* 2011;6:e24287

8. Jahnen-Dechent W, Ketteler M. Magnesium basics. *Clin Kidney J.* 2012;5:i3-i14

9. Ismail AAA, Ismail Y, Ismail AA. Chronic magnesium deficiency and human disease; time for reappraisal? *QJM.* 2018;111:759-763

10. Duley L, Meher S, Abalos E. Management of pre-eclampsia. *BMJ.* 2006;332:463-468

11. Holcberg G, Sapir O, Hallak M, Alaa A, Shorok HY, David Y, Katz M, Huleihel M. Selective vasodilator effect of magnesium sulfate in human placenta. *Am J Reprod Immunol.* 2004;51:192-197

12. Kawasaki K, Kondoh E, Chigusa Y, Kawamura Y, Mogami H, Takeda S, Horie A, Baba T, Matsumura N, Mandai M, Konishi I. Metabolomic profiles of placenta in preeclampsia. *Hypertension.* 2019;73:671-679

13. Kanasaki K, Palmsten K, Sugimoto H, Ahmad S, Hamano Y, Xie L, Parry S, Augustin HG,

Gattone VH, Folkman J, Strauss JF, Kalluri R. Deficiency in catechol-o-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. *Nature.* 2008;453:1117-1121

14. Martillotti G, Ditisheim A, Burnier M, Wagner G, Boulvain M, Irion O, Pechere-Bertschi A. Increased salt sensitivity of ambulatory blood pressure in women with a history of severe preeclampsia. *Hypertension.* 2013;62:802-808

15. Kim JM, Kim TH, Lee HH, Lee SH, Wang T. Postmenopausal hypertension and sodium sensitivity. *J Menopausal Med.* 2014;20:1-6

16. Schulman IH, Aranda P, Raij L, Veronesi M, Aranda FJ, Martin R. Surgical menopause increases salt sensitivity of blood pressure. *Hypertension.* 2006;47:1168-1174

17. Brinson KN, Rafikova O, Sullivan JC. Female sex hormones protect against salt-sensitive hypertension but not essential hypertension. *Am J Physiol Regul Integr Comp Physiol.* 2014;307:R149-157

18. Izumiyama K, Osanai T, Sagara S, Yamamoto Y, Itoh T, Sukekawa T, Nishizaki F, Magota K, Okumura K. Estrogen attenuates coupling factor 6-induced salt-sensitive hypertension and cardiac systolic dysfunction in mice. *Hypertens Res.* 2012;35:539-546

19. Hirano Y, Tsunoda M, Shimosawa T, Matsui H, Fujita T, Funatsu T. Suppression of catechol-o-methyltransferase activity through blunting of alpha2-adrenoceptor can explain hypertension in dahl salt-sensitive rats. *Hypertens Res.* 2007;30:269-278

20. Frame AA, Puleo F, Kim K, Walsh KR, Faudoa E, Hoover RS, Wainford RD. Sympathetic regulation of ncc in norepinephrine-evoked salt-sensitive hypertension in sprague-dawley rats. *Am J Physiol Renal Physiol.* 2019;317:F1623-F1636

21. Ostrosky-Frid M, Castaneda-Bueno M, Gamba G. Regulation of the renal nacl cotransporter by the wnk/spak pathway: Lessons learned from genetically altered animals. *Am J Physiol Renal Physiol.* 2019;316:F146-F158

22. Murthy M, Kurz T, O'Shaughnessy KM. Wnk signalling pathways in blood pressure regulation. *Cell Mol Life Sci.* 2017;74:1261-1280

23. Ueki N, Kanasaki K, Kanasaki M, Takeda S, Koya D. Catechol-o-methyltransferase deficiency leads to hypersensitivity of the pressor response against angiotensin ii. *Hypertension.* 2017;69:1156-1164

24. Tsunoda M, Takezawa K, Santa T, Imai K. Simultaneous automatic determination of catecholamines and their 3-o-methyl metabolites in rat plasma by high-performance liquid chromatography using peroxyoxalate chemiluminescence reaction. *Anal Biochem.* 1999;269:386-392

25. Takezawa K, Tsunoda M, Watanabe N, Imai K. An automatic analyzer for catecholamines and their 3-o-methyl metabolites using a micro coulometric flow cell as a postcolumn reactor for fluorogenic reaction. *Anal Chem.* 2000;72:4009-4014

26. Iijima H, Okada Y, Tsunoda M, Takamiya T, Imai K. Quantification of norepinephrine and its metabolites in the plasma of renal failure models. *Nephron Physiol.* 2010;116:p9-p16

27. She QB, Nagao I, Hayakawa T, Tsuge H. A simple hplc method for the determination of s-adenosylmethionine and s-adenosylhomocysteine in rat tissues: The effect of vitamin b6 deficiency on these concentrations in rat liver. *Biochem Biophys Res Commun.* 1994;205:1748-1754

28. Parks C, Giorgianni F, Jones BC, Beranova-Giorgianni S, Moore Ii BM, Mulligan MK. Comparison and functional genetic analysis of striatal protein expression among diverse inbred mouse strains. *Front Mol Neurosci.* 2019;12:128

29. Grice DE, Reenila I, Mannisto PT, Brooks AI, Smith GG, Golden GT, Buxbaum JD, Berrettini WH. Transcriptional profiling of c57 and dba strains of mice in the absence and presence of morphine. *BMC Genomics.* 2007;8:76

30. de Jong JC, Willems PH, Mooren FJ, van den Heuvel LP, Knoers NV, Bindels RJ. The structural unit of the thiazide-sensitive nacl cotransporter is a homodimer. *J Biol Chem.* 2003;278:24302-24307

31. McKee JA, Kumar S, Ecelbarger CA, Fernandez-Llama P, Terris J, Knepper MA. Detection of na(+) transporter proteins in urine. *J Am Soc Nephrol.* 2000;11:2128-2132

32. Weinberger MH. Salt sensitivity of blood pressure in humans. *Hypertension.* 1996;27:481-490

33. Yoshida T, Kato K, Yokoi K, Oguri M, Watanabe S, Metoki N, Yoshida H, Satoh K, Aoyagi Y, Nishigaki Y, Nozawa Y, Yamada Y. Association of genetic variants with chronic kidney disease in individuals with different lipid profiles. *Int J Mol Med.* 2009;24:233-246

34. DiNicolantonio JJ, O'Keefe JH, Wilson W. Subclinical magnesium deficiency: A principal driver of cardiovascular disease and a public health crisis. *Open Heart.* 2018;5:e000668

35. Fine KD, Santa Ana CA, Porter JL, Fordtran JS. Intestinal absorption of magnesium from food and supplements. *J Clin Invest.* 1991;88:396-402

36. Resnick LM, Gupta RK, DiFabio B, Barbagallo M, Mann S, Marion R, Laragh JH. Intracellular ionic consequences of dietary salt loading in essential hypertension. Relation to blood pressure and effects of calcium channel blockade. *J Clin Invest.* 1994;94:1269-1276

37. Kajimoto K, Hiura Y, Sumiya T, Yasui N, Okuda T, Iwai N. Exclusion of the catechol-o-methyltransferase gene from genes contributing to salt-sensitive hypertension in dahl salt-sensitive rats. *Hypertens Res.* 2007;30:459-467

38. Singh P, Song CY, Dutta SR, Gonzalez FJ, Malik KU. Central cyp1b1 (cytochrome p450 1b1)-estradiol metabolite 2-methoxyestradiol protects from hypertension and neuroinflammation in female mice. *Hypertension.* 2020;75:1054-1062

39. Pechere-Bertschi A, Burnier M. Female sex hormones, salt, and blood pressure regulation. *Am J Hypertens.* 2004;17:994-1001

40. Chiu CL, Lujic S, Thornton C, O'Loughlin A, Makris A, Hennessy A, Lind JM. Menopausal hormone therapy is associated with having high blood pressure in postmenopausal women: Observational cohort study. *PLoS One.* 2012;7:e40260

41. Gillis EE, Williams JM, Garrett MR, Mooney JN, Sasser JM. The dahl salt-sensitive rat is a spontaneous model of superimposed preeclampsia. *Am J Physiol Regul Integr Comp Physiol.* 2015;309:R62-70

Novelty and Significance:

What is new?

1. Mg deficiency induces SSH via lowering COMT activity and subsequently reducing 2-ME production in the genetically low COMT mice.
2. 2-ME maintains BP homeostasis via regulation of ATR1-SPAK-NCC pathway.

What is relevant?

Elevated salt sensitivity is a major cause of hypertension; however, only a few treatment options such as salt restriction is available. Our findings that the interaction between genetically low COMT activity and Mg deficiency could cause SSH suggest the importance of adequate intake of Mg and 2-ME sufficiency for prevention of SSH.

Summary

Genetic strength of COMT activity affect the incidence of SSH. For low COMT activity individuals, adequate Mg intake and 2-ME replacement prevent development of SSH.

Figure 1

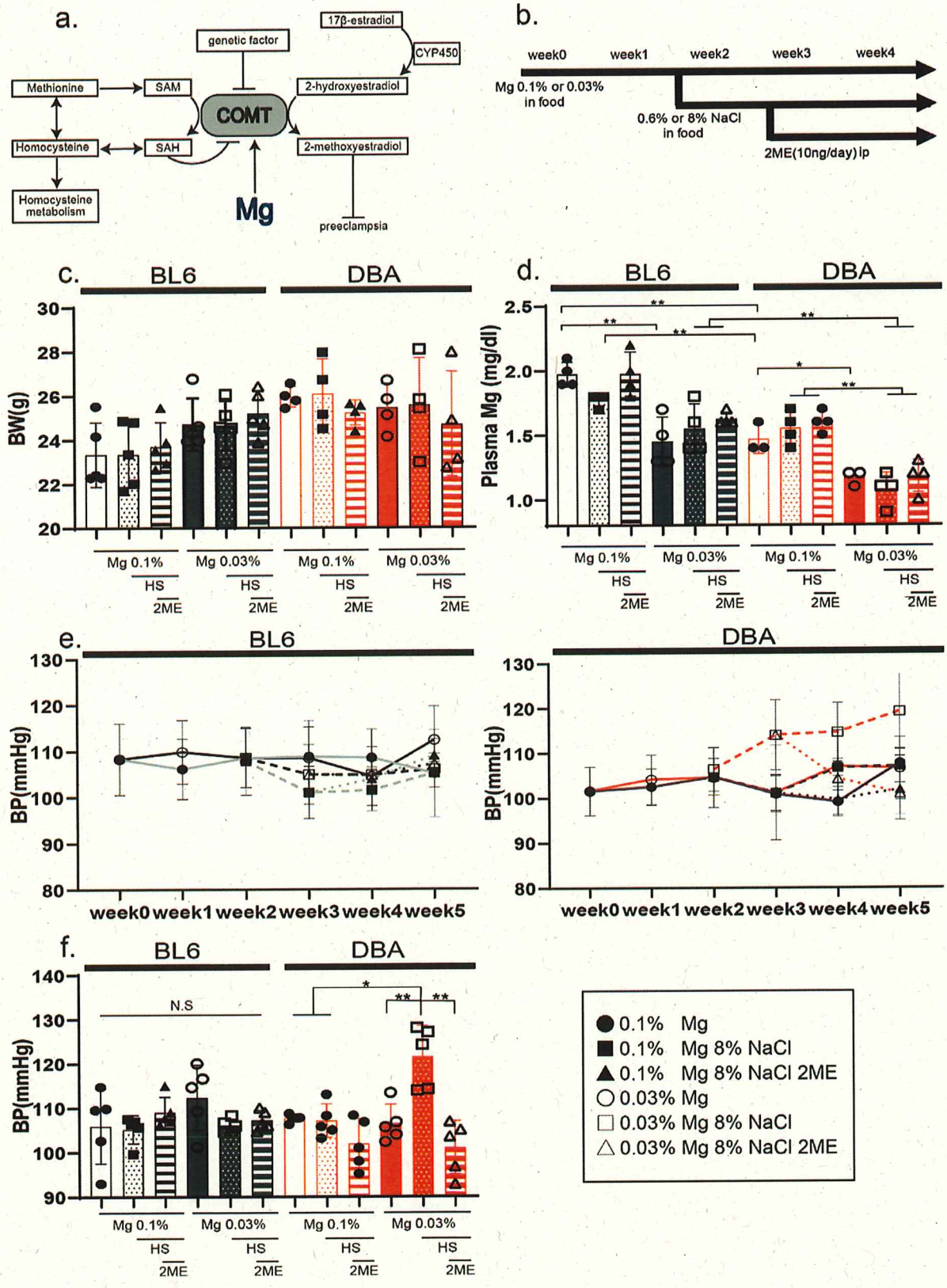

Figure 1. Mg deficiency induced SSH only in the DBA mice under high salt conditions

and 2-ME normalized sBP.

a. COMT is an enzyme that methylates catechol. S-adenosyl-methionine (SAM) is the methyl donor for COMT, and the demethylated compound S-adenosyl-homocysteine (SAH) is known as an endogenous inhibitor of COMT. Genetic factors such as SNPs and haplotypes determine the strength of COMT activity. Mg is a cofactor of COMT and keeps its active site open. An estrogen catechol, 2-hydroxyestradiol, is metabolized by COMT to 2-methozyestradiol (2-ME), which is known for several bioactivities, including preventing the development of preeclamptic phenotypes. **b.** Scheme of the Mg-deficient high salt protocol for the BL6 and DBA mice. A Mg 0.1%/Mg 0.03% + 0.6% NaCl diet was replaced with a Mg 0.1%/Mg 0.03% + 8% NaCl diet at week 2, and 2-ME was injected intraperitoneally from week 3. **c.** BW at week 5. N=3 in the DBA Mg 0.1% and Mg 0.03% groups, N=4 in the other groups. **d.** Plasma Mg concentration at week 5. N=5 for BL6, N=4 for DBA. **e.** Weekly sBP of BL6 and DBA. High salt was administered at week 2, and 2-ME treatment started at week 3. N=5 in each group. **g.** sBP at week 5. N=5 in each group. The graph is expressed as the mean ± SEM. The significance was determined by three-way ANOVA for comparisons within one strain or by t-test for comparison between two strains and is represented as * $P<0.05$ or ** $P<0.01$. High salt is designated 8% NaCl or HS in the figure.

Figure 2

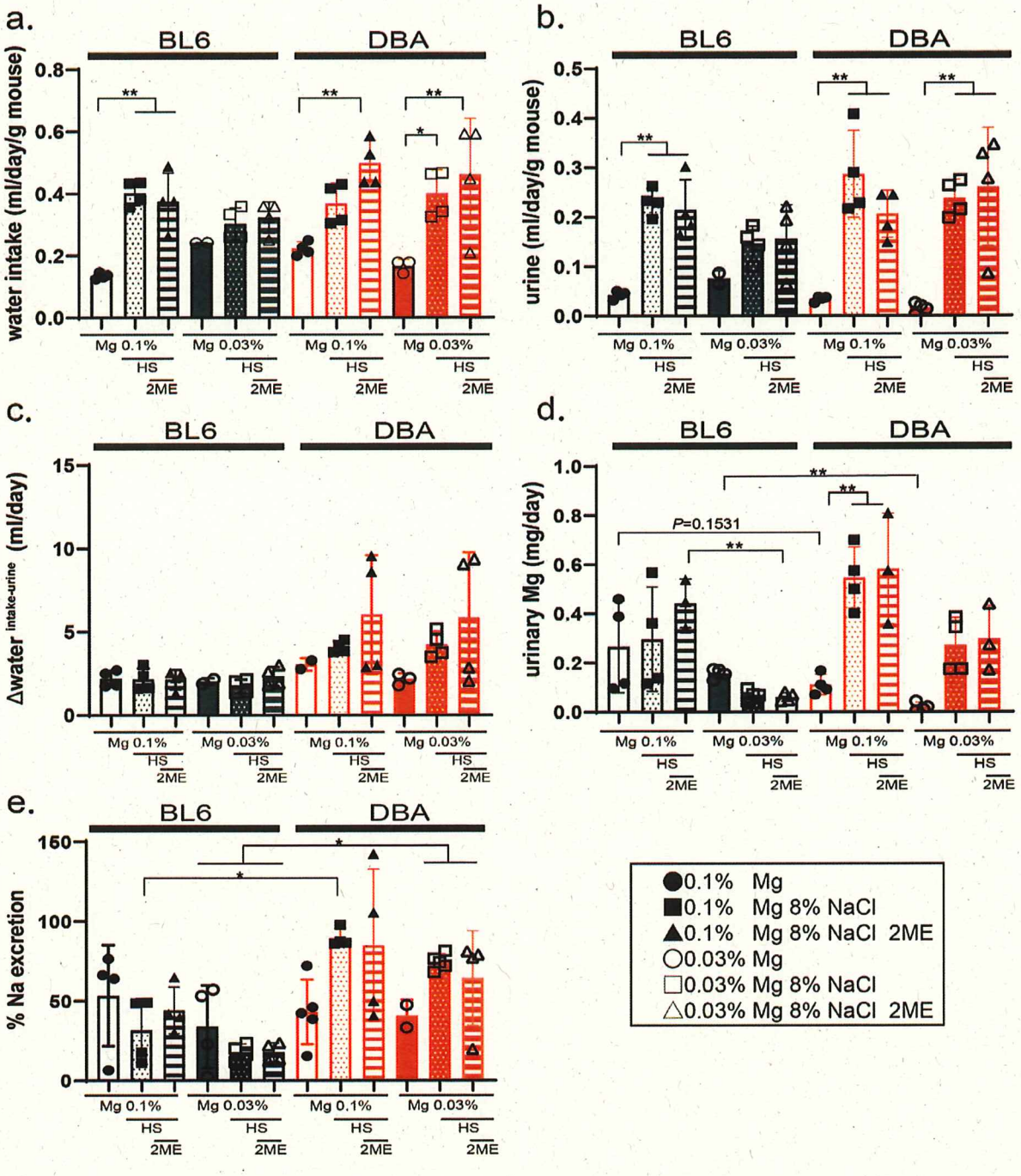

Figure 2. High salt increased urinary Mg excretion and % Na excretion but did not affect water that was not excreted from urine in the DBA mice regardless of the Mg concentration.

a. Volume of 24 hr water intake. N=2 in the BL6 Mg 0.03% group, N=3 in the DBA Mg

0.03% group, N=4 in the other groups. **b.** Volume of 24 hr urine. N=2 in the BL6 Mg 0.03% group, N=4 in the other groups. **c.** Volume of water that was not excreted from urine. The volume of 24 hr urine was subtracted from the volume of 24 hr water intake. N=2 in the BL6 Mg 0.03% and DBA Mg 0.1% groups, N=3 in the DBA Mg 0.03% group, N=4 in the other groups. **d.** Twenty-four-hour urinary Mg excretion. N=3 in the Mg 0.1%/8% NaCl/2-ME, DBA Mg 0.1%/8% NaCl/2-ME, and DBA Mg 0.03%/8% NaCl/2-ME groups, N=4 in the other groups. **e.** % Na excretion; % Na excretion was defined as the ratio of daily urinary sodium excretion and sodium intake. N=4 in each group. The graph shows the mean ± SEM. The significance was determined by three-way ANOVA for comparisons within one strain or by t-test for comparison between two strains and is represented as * $P<0.05$ or ** $P<0.01$. High salt is designated 8% NaCl or HS in the figure.

Figure 3

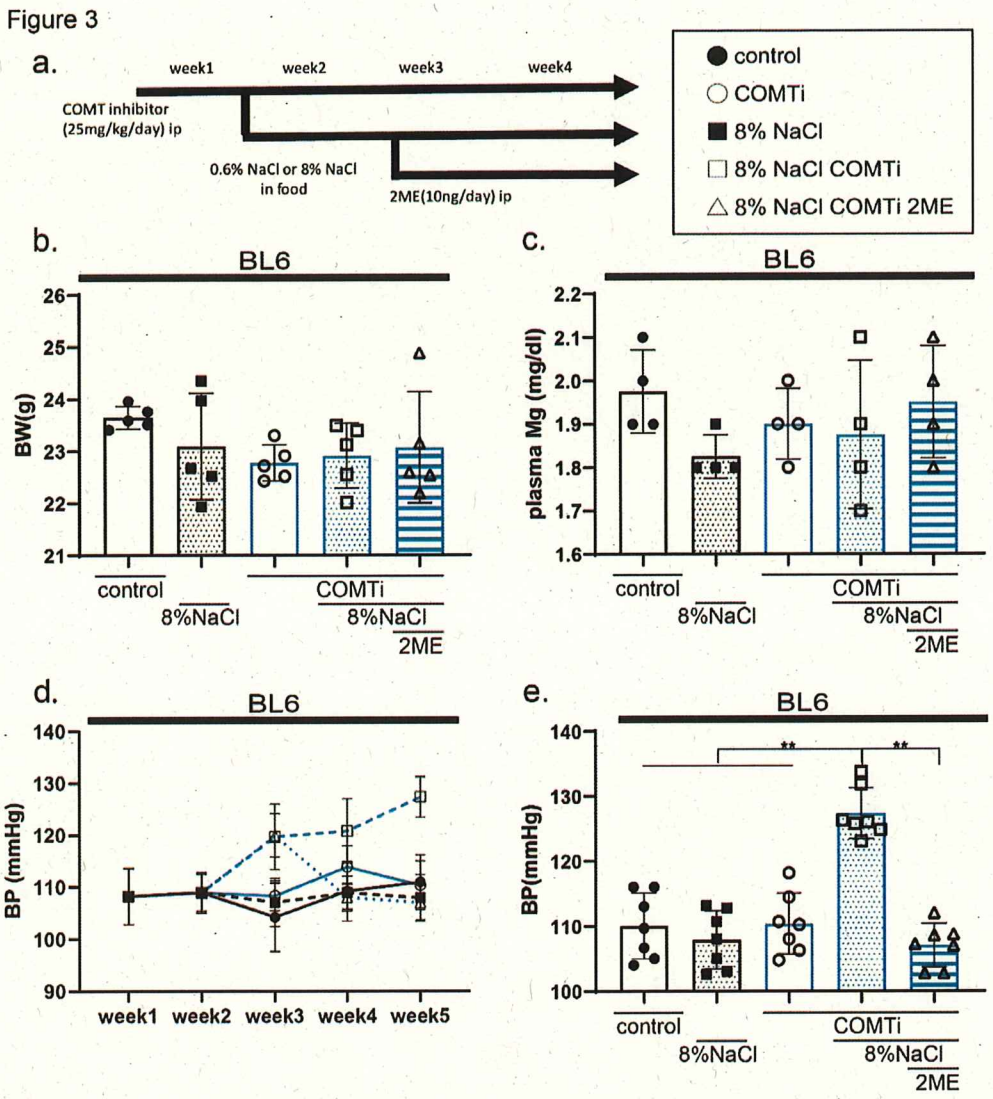

Figure 3. Chemical inhibition of COMT induced and 2-ME ameliorated SSH in normal Mg BL6 mice under high salt conditions.

a. Schemes of the COMTi high salt BL6 protocol. 0.6% NaCl diet was replaced with an 8% NaCl diet at week 2, and 2-ME was injected intraperitoneally from week 3. **b.** BW at week 5. N=5 in each group. **c.** Plasma Mg concentration at week 5. N=4 in each group. **d.** Weekly sBP of BL6. High salt was administered at week 2, and 2-ME treatment started at week 3. N=7 in each group. **e.** sBP at week 5. N=7 in each group. The graph shows the mean ± SEM. The significance was determined by three-way ANOVA and is represented as ** $P<0.01$. High

salt is designated 8% NaCl in the figure.

Figure 4

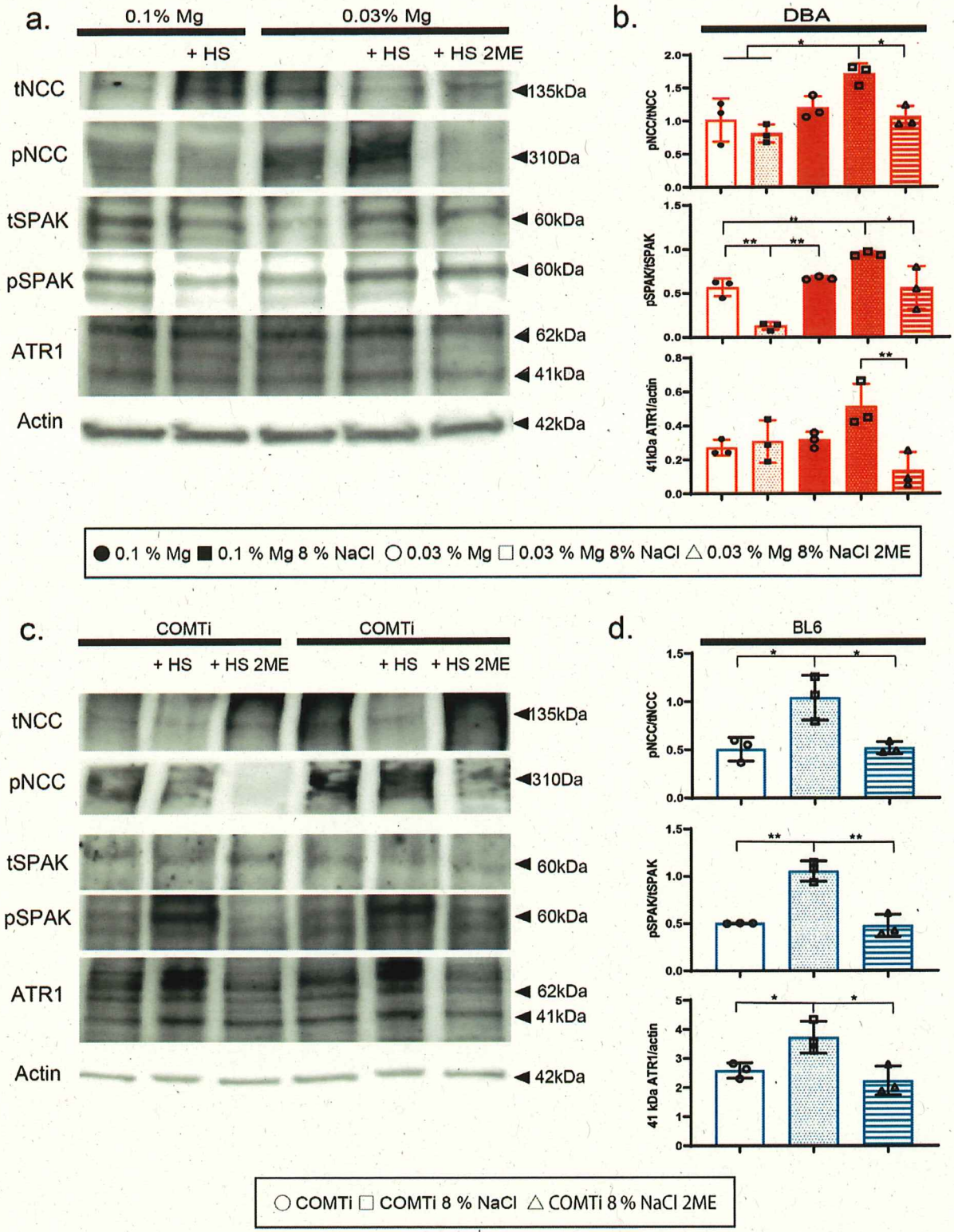

Figure 4. High salt activated the ATR1-SPAK-NCC pathway in the kidneys of the Mg-

deficient DBA- and COMTi-treated BL6 mice.

a. Representative western blotting images of male DBA mice. Actin was used as a loading control. **b.** Densitometric data analysis of ATR1, t/p-SPAK and t/p-NCC in the DBA mice. ATR1 was normalized to actin, and p-SPAK and p-NCC were normalized to t-SPAK and t-NCC, respectively. **c**. Representative western blotting images of the male BL6 mice treated with COMTi. Actin is shown under the corresponding blots as a loading control. **d.** Densitometric data analysis ofATR1, t/p-SPAK and t/p-NCC in the BL6 mice. ATR1 was normalized to actin, and p-SPAK and p-NCC were normalized to t-SPAK and t-NCC, respectively. N=3 in each group. Due to the technical difficulties, we only utilized 3 samples for each group. The graph shows the mean ± SEM. The significance was determined by one-way ANOVA and is represented as * $P<0.05$ or ** $P<0.01$. High salt is designated HS in the figure.

Figure 5

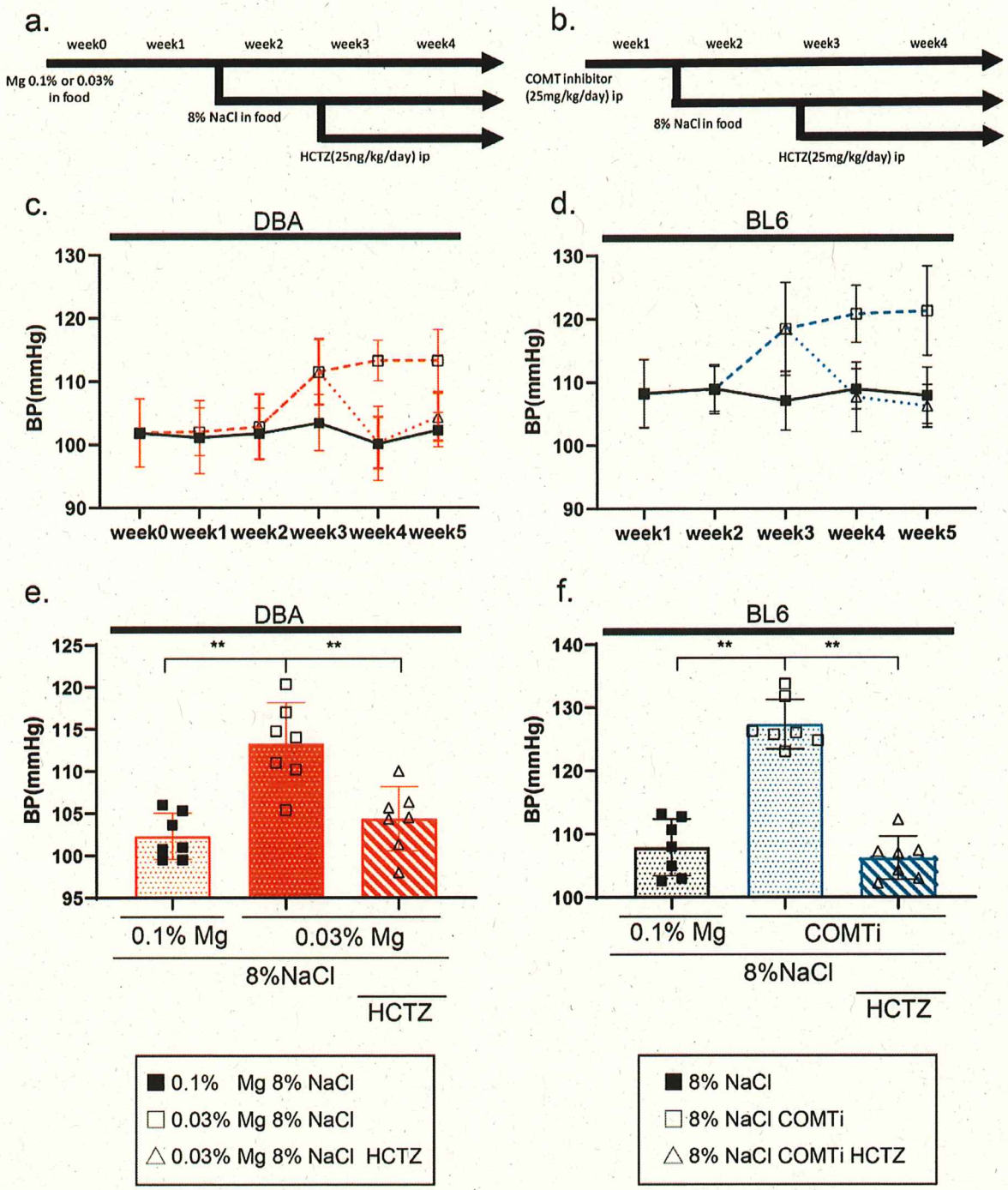

Figure 5 HCTZ ameliorated SSH in the Mg-deficient DBA and COMTi-treated BL6 mice under high salt conditions.

Schemes of the animal protocol with HCTZ treatment. NaCl diet (0.6%) was replaced with 8% NaCl diet at week 2, and HCTZ was injected intraperitoneally from week 3 (**a, b**). **a.**

Protocol for the Mg-deficient DBA mice, **b.** Protocol for the COMTi BL6 mice. Weekly sBP is shown in **c, d**. High salt was administered in week 2, and HCTZ treatment was started at week 3. **c**. Weekly sBP of the Mg-deficient DBA mice. **d.** Weekly sBP of the COMTi BL6 mice. **e.** sBP of the Mg-deficient DBA mice at week 5. **f.** sBP of the COMTi BL6 mice at week 5. For all figures, N=7 in each group. The graph shows the mean ± SEM. The significance was determined by two-way ANOVA and is represented as ** $P<0.01$. High salt is designated 8% NaCl in the figure.

Figure 6

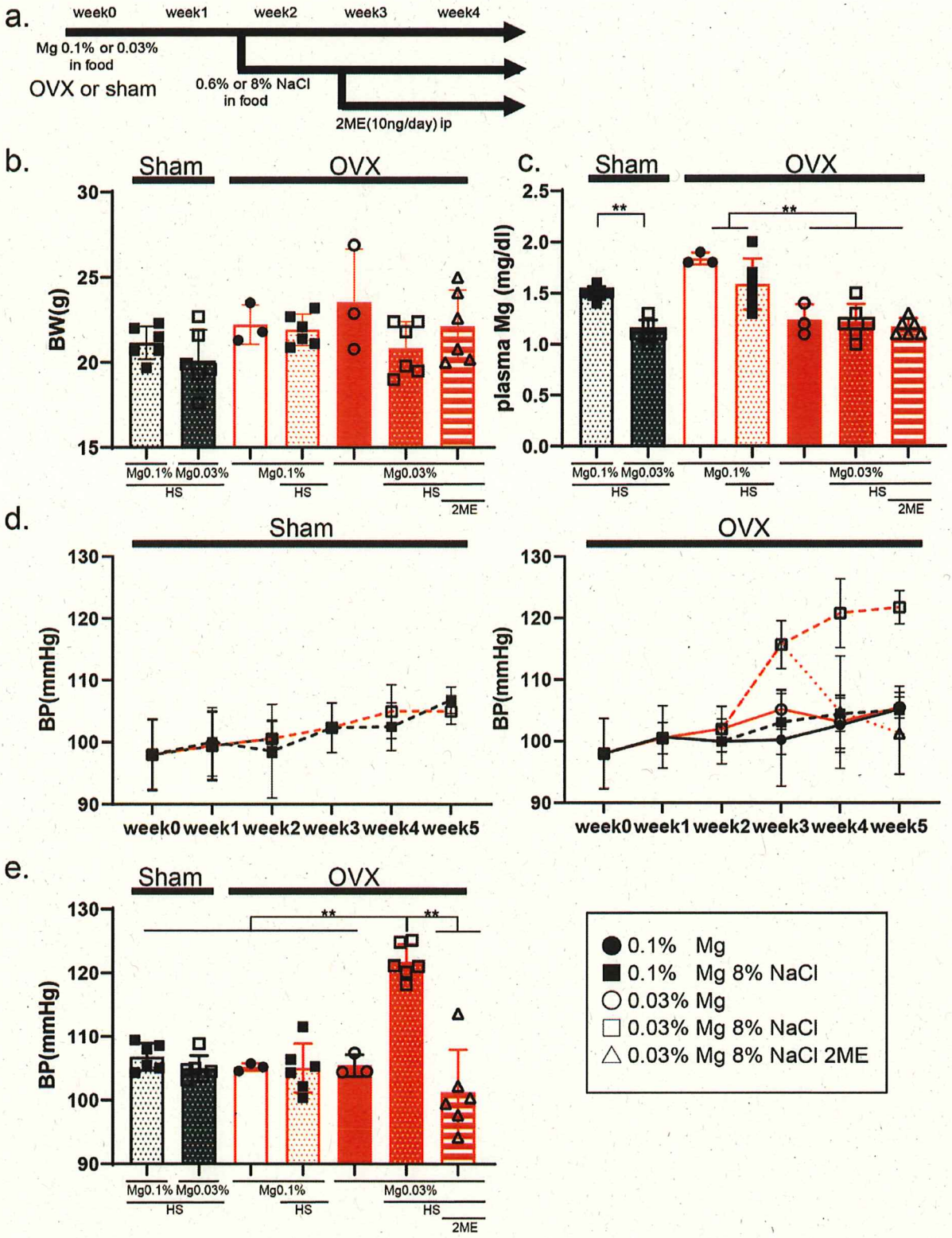

Figure 6. Mg-deficient OVX DBA mice developed SSH under high salt conditions and 2-ME normalized sBP.

a. Scheme of the Mg-deficient high salt protocol for the sham or OVX DBA mice. A Mg 0.1%/Mg 0.03% + 0.6% NaCl diet was replaced with a Mg 0.1%/Mg 0.03% + 8% NaCl diet at week 2, and 2-ME was injected intraperitoneally from week 3. **b.** BW at week 5. **c.** Plasma Mg concentration at week 5. **e.** Weekly sBP of BL6 mice. High salt was administered at week 2, and 2-ME treatment started at week 3. **d.** Weekly sBP of DBA. High salt was administered at week 2, and 2-ME treatment started at week 3. **e.** sBP at week 5. For every figure, N=3 in the OVX Mg 0.1% and OVX Mg 0.03% groups, and N=6 in the other groups. The graph is expressed as the mean ± SEM. The significance was determined by three-way ANOVA for comparisons within one strain or by t-test for comparison between two strains and is represented as * $P<0.05$ or ** $P<0.01$. High salt is designated HS in the figure.