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Decreased nitric oxide production is a novel therapeutic target for salt-induced nocturnal polyuria in aging

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Nocturnal polyuria is a major cause of nocturia, which affects quality of life. Aging-related decreases in nitric oxide production have been reported to contribute to salt-induced nocturnal polyuria. We posited that enhanced nitric oxide production from exercise could mitigate salt-induced nocturnal polyuria. To validate, we assessed the impact of 4 weeks of moderate-intensity exercise on intrarenal renin-angiotensin system activity and salt-induced polyuria during inactive periods in 80-week-old mice. The moderate-intensity exercise group (Mod-Ex) had increased nitric oxide production and suppressed intrarenal renin-angiotensin system compared to the sedentary group (Sed) (renal angiotensinogen level: Sed vs. Mod-Ex, 1.101 ± 0.077 vs. 0.630 ± 0.048 arbitrary unit, $P = 0.002$). This exercise also alleviated salt-induced polyuria during inactive periods (diurnal polyuria index: Sed vs. Mod-Ex, 0.292 ± 0.027 vs. 0.193 ± 0.023, $P = 0.018$). These effects were neutralized by suppression of nitric oxide production by oral administration of N^ω-Nitro-L-arginine methyl ester hydrochloride, an nitric oxide synthesis inhibitor. These findings indicate that exercise-induced improvements in salt-induced polyuria during inactive periods are caused by increases in nitric oxide production. In summary, increasing nitric oxide production may serve as a treatment method for salt-induced nocturnal polyuria in older adults.

Nocturia, defined as waking to pass urine during the main sleep period[1], is a common health problem among middle-aged and older adults, affecting approximately 65% of adults over the age of 50[2]. Nocturia is not only a troublesome symptom that affects quality of life[3] but is also a risk factor for death[4]. The pathologies of nocturia include decreased bladder capacity, sleep disturbances, nocturnal polyuria, or a combination of the three[1]. Among these, nocturnal polyuria is the most frequent. Nocturnal polyuria, which is defined as the passage of a large volume of urine during the main sleep period[1], accounts for 50–80% of nocturia[5]. Excessive salt intake has been reported to cause nocturnal polyuria[6,7]. Although nocturnal urine volume increases with aging[8], however, the amount of salt intake does not[9]. These findings suggest that some factors associated with aging are involved in nocturnal polyuria aside from excessive salt intake. Recently, we reported that decreased nitric oxide (NO) production is associated with salt-induced nocturnal polyuria in humans, and that high-salt diet causes nocturnal polyuria in aged mice with decreased NO production but not in young mice. We also reported that high-salt diet causes nocturnal polyuria in young mice when NO production is suppressed by L-NAME administration[10]. These results suggest that decreased NO production plays an important role in the pathogenesis of salt-induced nocturnal polyuria. Moreover, in animal experiments, we found that decreased NO production leads to the activation of the intrarenal renin-angiotensin system (RAS) and sodium chloride co-transporter (NCC), which results in decreased salt excretion and urine volume in the active period and increased salt excretion and urine volume in the inactive period[10]. This mechanism may be responsible for salt-induced nocturnal polyuria in older

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adult humans because decreased NO production is an age-related physiological change[11]. Therefore, increasing NO production may be a novel therapeutic strategy for salt-induced nocturnal polyuria in older adults.

Exercise training has remarkable positive effects on the body and benefits various diseases, such as cardiovascular disease, chronic kidney disease, mental health disorder, cancer, and dementia[12]. One of the positive effects of exercise is to improve endothelial function and increase the release of NO from the endothelium. Increased NO production with exercise decreases vascular resistance, lowers blood pressure in hypertension patients[13], and improves renal function in patients with chronic kidney disease patients[14]. We posited that enhanced NO production from exercise could mitigate salt-induced nocturnal polyuria. To validate, we assessed the impact of exercise on intrarenal RAS activity and salt-induced polyuria during inactive periods in aged mice, finding that four weeks of moderate exercise curbed both by elevating NO levels. These findings indicate that increasing NO production may serve as a novel treatment for elderly patients with this condition.

Results

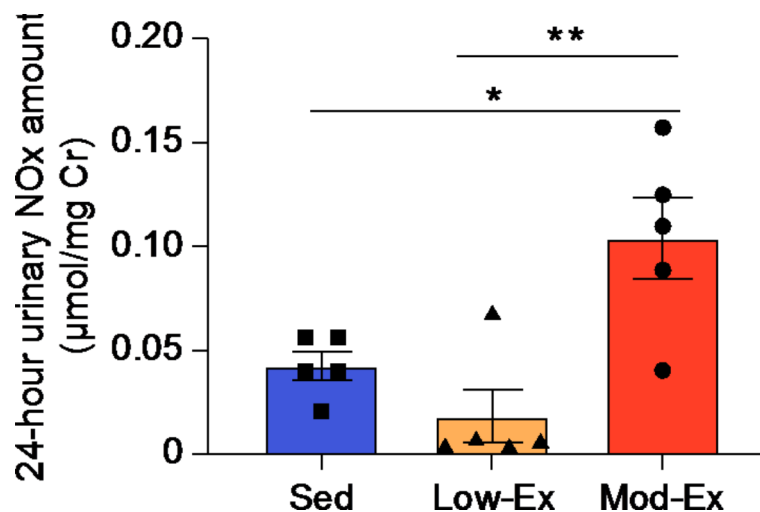### Exercise-induced changes in 24-hour urinary NOx amount

First, we evaluated the effect of exercise on NO production. We used 80-week-old C57/BL6 male mice. Mice were fed 1% high-salt diets (1% HSD) for 2 weeks and were randomized into sedentary (Sed), low-intensity exercise (Low-Ex), and moderate-intensity exercise (Mod-Ex) groups. After 4 weeks, the 24-hour urinary NOx levels were evaluated. The 24-hour urinary NOx amount of the Mod-Ex group was significantly higher than those of the Sed and Low-Ex group (Sed vs. Low-Ex vs. Med-Ex: 0.042 ± 0.005 vs. 0.018 ± 0.001 vs. 0.104 ± 0.015 μmol/Crmg, $P = 0.017$ and 0.006, respectively) (Fig. 1). There was no significant difference in the 24-hour urinary NOx levels between the Sed and Low-Ex groups ($P = 0.122$).

Increasing NO production-induced changes in intrarenal RAS and NCC activity in the active period

Next, we investigated exercise-induced changes in the intrarenal RAS activity in the active period. We performed a quantitative analysis of renal angiotensinogen (AGT) in the Sed and Mod-Ex groups. AGT/β-actin (ACTB) level in the Mod-Ex group was significantly lower than that of the Sed group (1.101 ± 0.077 vs. 0.630 ± 0.048 arbitrary unit, $P = 0.002$) (Fig. 2a). This indicates that moderate-intensity exercise decreases the intrarenal RAS activity in the active period. The intrarenal RAS regulates NCC activity in the distal tubule[15]. Thus, we quantitatively analyzed the phosphorylated NCC (pNCC) in the Sed and Mod-Ex groups to investigate exercise-induced changes in NCC activity. The pNCC/NCC level in the Mod-Ex group was significantly lower than that of the Sed group (0.812 ± 0.045 vs. 0.538 ± 0.030 arbitrary unit, $P = 0.003$) (Fig. 2b). This indicates that moderate-intensity exercise decreases NCC activity.

Increasing NO production-induced changes in sodium excretion kinetics and diurnal polyuria index

NCC activity in the distal tubule is known to control sodium reabsorption[15,16]; thus, we evaluated sodium excretion in the inactive and active periods of the Sed and Mod-Ex groups to investigate exercise-induced changes in sodium excretion kinetics. The urine collection test revealed that there was no significant difference in 24-hour sodium excretion between the two groups (0.219 ± 0.016 vs. 0.204 ± 0.010 mEq, $P = 0.461$). In contrast, the sodium excretion during the active period of the Mod-Ex group was significantly higher than that of the Sed group (0.114 ± 0.017 vs. 0.179 ± 0.012 mEq, $P = 0.022$). The sodium excretion during inactive periods of the Mod-Ex group was significantly lower than that of the Sed group (0.106 ± 0.021 vs. 0.024 ± 0.003 mEq, $P = 0.006$)

Fig. 1. Exercise-induced changes in 24-hour urinary NOx amount. 24-hour urinary NOx levels were compared between Sed, Low-Ex, and Mod-Ex groups. Tukey's honestly significant difference test was performed ($n = 5$ per group). *$P < 0.05$, **$P < 0.01$. Error bars represent s.e.m.
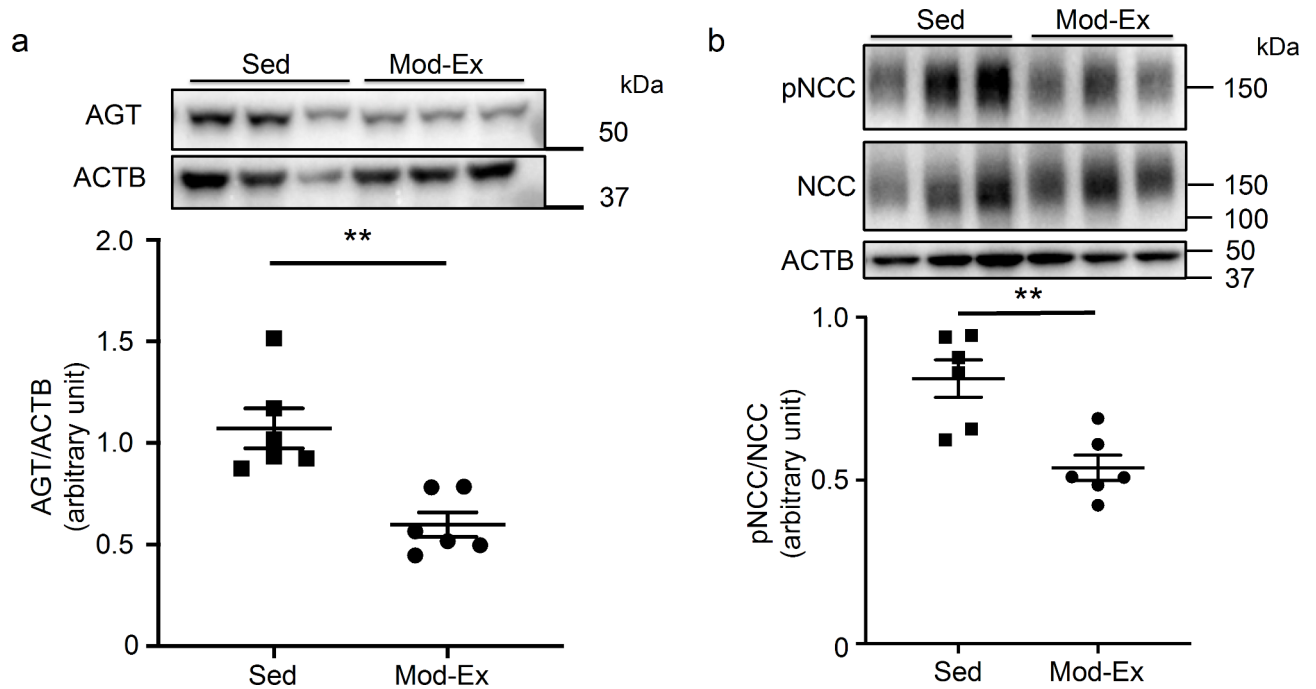
Fig. 2. Increasing NO production-induced changes in intrarenal RAS and NCC activity in the active period. (a) AGT/ACTB levels were compared between Sed and Mod-Ex groups. Representative immunoblotting (above) and quantitative analysis (below) of renal AGT in the active period. Two-tailed Student's *t*-test was performed (*n* = 6, each group), **P < 0.01. Error bars represent s.e.m. (b) pNCC/NCC levels were compared between Sed and Mod-Ex groups. Representative immunoblotting (above and middle) and quantitative analysis (below) of renal phosphorylated NCC (pT53), NCC, and ACTB in the active period. Two-tailed Student's *t*-test was performed (*n* = 6, each group), **P < 0.01. Error bars represent s.e.m.

(Fig. 3a–c). This indicates that moderate-intensity exercise shifts urinary sodium excretion from the inactive to the active period. Next, we evaluated the effect of exercise on the diurnal polyuria index (DPi), the ratio of the diurnal to daily urine volume. Because mice are nocturnal, the DPi in mice is an index corresponding to the nocturnal polyuria index in humans. We analyzed urine volume every 4-hour for 3.5 consecutive days before and after exercise (Fig. 3d and e). In the Mod-Ex group, exercise significantly decreased the DPi (from 0.292 ± 0.027 to 0.193 ± 0.023, *P* = 0.018), while in the Sed, there were no significant changes in the DPi (Sed: from 0.257 ± 0.008 to 0.306 ± 0.025, *P* = 0.236) (Fig. 3f). Notably, the extent of the decrease in DPi was significantly greater in the Mod-Ex group than in the Sed group (*P* = 0.003) (Fig. 3f). These findings suggest that moderate exercise-induced increase in NO production improves salt-induced polyuria during inactive periods in aged mice.

The effect of NO suppression on exercise-induced changes in 24-hour urinary NOx amount, intrarenal RAS activity, and DPi

Moreover, we assessed NO suppression's impact on exercise-induced alterations in 24-hour urinary NOx, intrarenal RAS activity, and DPi using N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), an NO synthase inhibitor. We assigned eighty-week-old C57/BL6 male mice to three groups: Mod-Ex, Mod-Ex with L-NAME, and Sed with L-NAME. The 24-hour urinary NOx levels in the Mod-Ex with L-NAME and Sed with L-NAME groups were markedly lower than in the Mod-Ex group (0.131 ± 0.022 vs. 0.041 ± 0.018 vs. 0.015 ± 0.003 μmol/Crmg, *P* = 0.020 and 0.005, respectively) (Fig. 4a). Subsequently, we investigated the NO suppression's effects on intrarenal RAS activity by quantifying renal AGT in the same groups. AGT/ACTB levels in the Mod-Ex with L-NAME and Sed with L-NAME groups significantly exceeded those in the Mod-Ex group (0.752 ± 0.026 vs. 0.979 ± 0.026 vs. 0.995 ± 0.027 μmol/Crmg, *P* = 0.001 and 0.001, respectively) (Fig. 4b).

Finally, we analyzed the every 4-hour urine volume for 3.5 consecutive days before and after exercise (Fig. 4c–e). The exercise-induced decrease in the DPi in the Mod-Ex group was abolished in the Mod-Ex with L-NAME group (*P* = 0.015) (Fig. 4f). There was no significant difference in the DPi between the Mod-Ex with L-NAME and the Sed with L-NAME group (Mod-Ex with L-NAME: from 0.252 ± 0.015 to 0.286 ± 0.014, *P* = 0.212; Sed with L-NAME: from 0.268 ± 0.010 to 0.305 ± 0.023, *P* = 0.181) (Fig. 4f). These results indicated that exercise-induced changes in salt-induced polyuria during inactive periods in aged mice were mediated by increased NO production and decreased intrarenal RAS activity.

Discussion

In this study, we provide experimental evidence that an increase in NO production by 4 weeks of moderate-intensity exercise can suppress intrarenal RAS and NCC activity, increase sodium excretion during active periods,
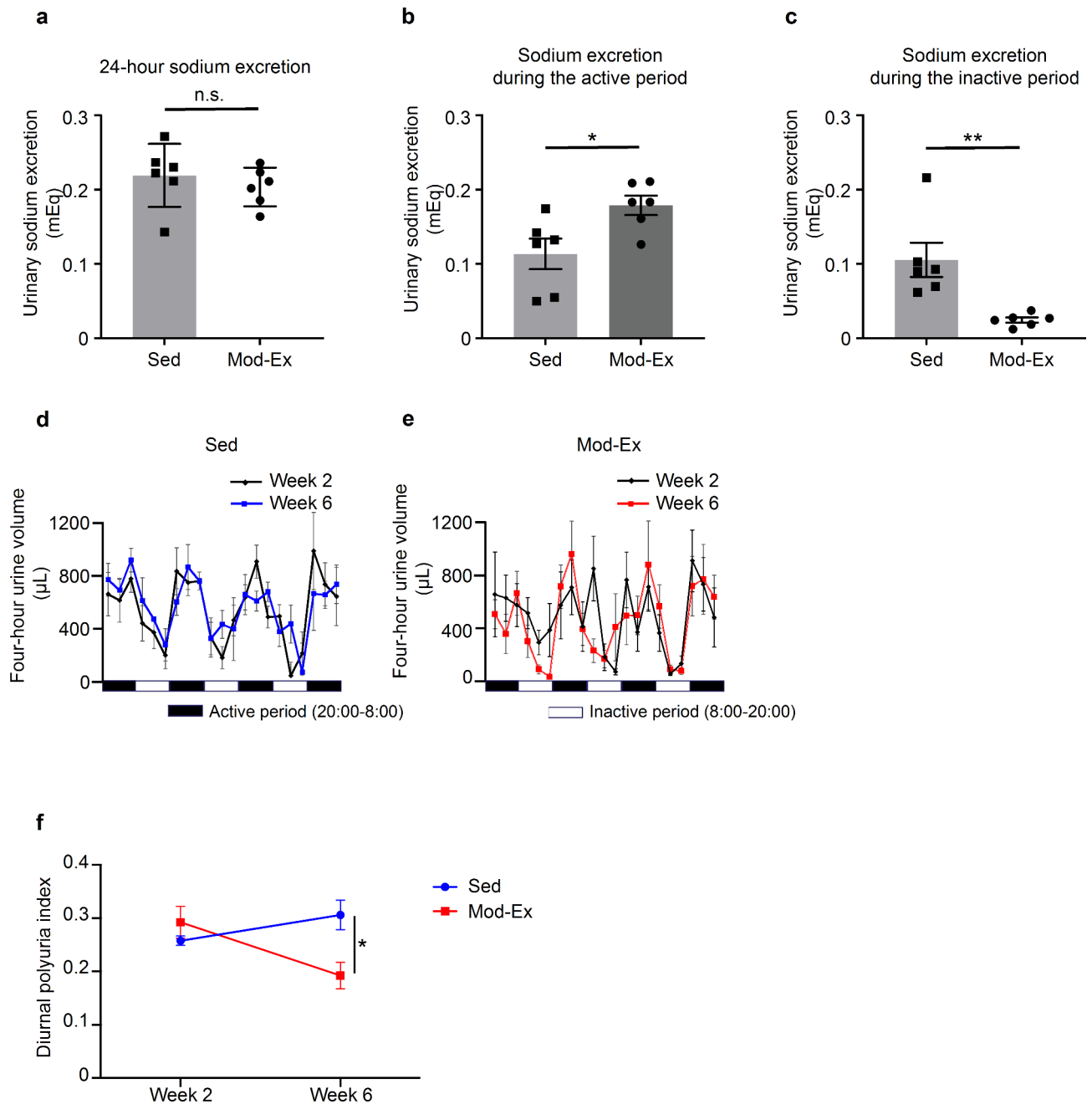
Fig. 3. Increasing NO production-induced changes in urinary sodium excretion kinetics and diurnal polyuria index. 24-hour sodium excretion (a), sodium excretion during the active (b), and sodium excretion during inactive periods (c) were compared between the Sed and Mod-Ex groups. A two-tailed Student's t-test was performed ($n = 6$ per group); *$P < 0.05$, **$P < 0.01$. Error bars represent s.e.m., n.s.: Not significant. The 4-hour urinary volume for 3.5 consecutive days in the Sed (d) and Mod-Ex (e) groups at week 2 (baseline) and week 6 was measured using the automated voiding staining on the paper method (aVSOP). Changes in the DPi were compared between Sed and Ex groups (f). Data were obtained from the same mice. Two-way repeated-measures analysis of variance (ANOVA) was performed ($n = 6$ per group); *$P < 0.05$ (time-by-group interaction). Error bars represent s.e.m.

decrease sodium excretion during inactive periods, and improve salt-induced polyuria during inactive periods in aged mice. Given that mice are nocturnal, polyuria during inactive periods is considered to correspond to nocturnal polyuria in humans. These findings suggest that increased NO production is a potential new treatment for salt-induced nocturnal polyuria in elderly individuals.

Excessive salt intake has been reported to cause nocturnal polyuria[6,7]. However, nocturnal urine volume increases with aging[8], although the amount of salt intake does not increase with aging[9]. These findings suggest that age-related factors are involved in the association between salt intake and nocturnal polyuria. We previously
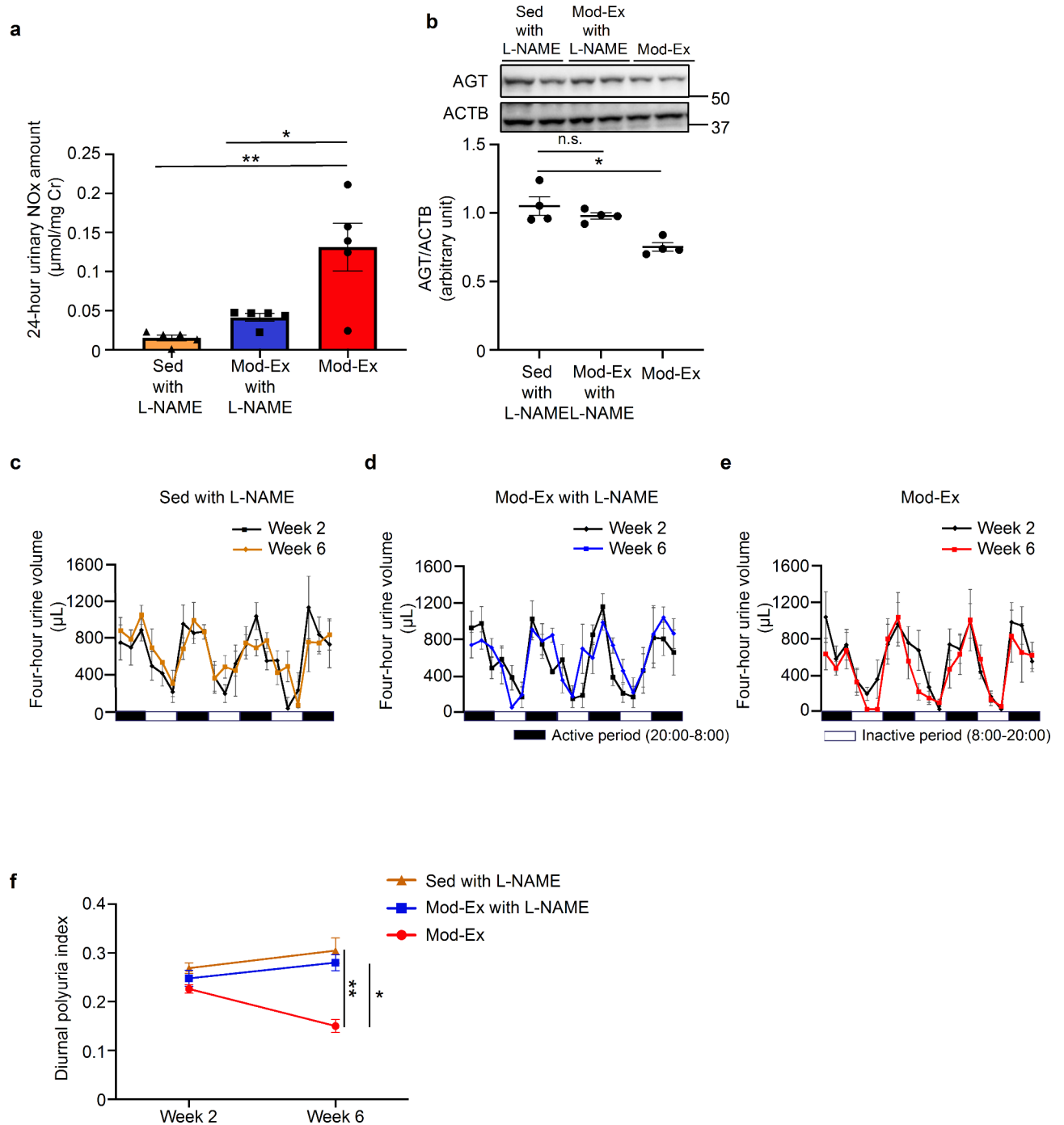
Fig. 4. The effect of NO suppression on exercise-induced changes in 24-hour urinary NOx amount, intrarenal RAS activity, and DPi. (a) 24-hour urinary NOx amounts were compared among Sed with L-NAME, Mod-Ex with L-NAME, and Mod-Ex group. Tukey's honestly significant difference test was performed ($n = 5$, each group). *$P < 0.05$, **$P < 0.01$. Error bars represent s.e.m. (b) AGT/ACTB levels were compared among Sed with L-NAME, Mod-Ex with L-NAME, and Mod-Ex groups. Representative immunoblotting (above) and quantitative analysis (below) of renal AGT in the active period. Tukey's honestly significant difference test was performed ($n = 4$, each group), *$P < 0.05$. Error bars represent s.e.m. The 4-hour urinary volume for 3.5 consecutive days in Sed with L-NAME (c), Mod-Ex with L-NAME (d), and Mod-Ex (e) groups at week 2 (baseline) and week 6 was measured using aVSOP. Changes in the DPi were compared between Sed with L-NAME, Mod-Ex with L-NAME, and Mod-Ex Groups (f). Data were obtained from the same mice. Two-way repeated-measures analysis of variance (ANOVA) was performed ($n = 6$, each group) *$P < 0.05$, **$P < 0.01$ (Time-by-group interaction). Error bars represent s.e.m.

reported that patients with decreased NO production exhibit salt-induced nocturnal polyuria[10]. We also found that decreased NO production leads to activation of the intrarenal RAS, which results in decreased salt excretion and urine volume in the active period and increased salt excretion and urine volume in the inactive period[10]. These findings indicated that decreased NO production is involved in the development of salt-induced nocturnal polyuria and could be a novel therapeutic target for salt-induced nocturnal polyuria. In this study, we hypothesized that exercise-induced increase in NO production would improve salt-induced nocturnal polyuria. To test this hypothesis, we evaluated the effects of exercise on NO production, intrarenal RAS activity, and salt-induced polyuria during inactive periods in aged mice. We found that 4 weeks of moderate-intensity exercise increased systemic NO production, suppressed intrarenal RAS and NCC activity, and improved salt-induced polyuria during inactive periods in aged mice. We also found that the effect of exercise on salt-induced polyuria during inactive periods in aged mice was inhibited by NO suppression. These findings indicate that exercise-induced improvement in salt-induced polyuria during inactive periods is mainly mediated by increased NO production. Therefore, increasing NO production could be a new treatment strategy for salt-induced nocturnal polyuria in older individuals.

Exercise has been reported to be effective in treating nocturnal polyuria[17], but the mechanism by which exercise improves nocturnal polyuria is not well understood. Exercise has been speculated to improve nocturnal polyuria by pumping water from the interstitial space to the blood vessels and expelling it as sweat[18]or urine[19]. However, this mechanism has not been scientifically proven. In this study, we found that moderate-intensity exercise suppressed intrarenal RAS activity and improved salt-induced polyuria during inactive periods by increasing NO production in aged mice. Moderate-intensity exercise may improve salt-induced nocturnal polyuria through a similar mechanism in older adult humans because they have decreased NO production[11]. Exercise is distinguished by its intensity as low-, moderate-, or high-intensity. Low-intensity exercise includes walking and stretching, moderate-intensity exercise includes yoga and golfing, and high-intensity exercise includes marathon running and football[20]. It has been reported that NO production increases with exercise intensity[21]. First, we investigated the effects of high-intensity exercise on NO production in aged mice and found that the high-intensity exercise was too demanding for aged mice to complete the protocol. Therefore, we assessed the effects of low- and moderate-intensity exercises. Low-intensity exercise did not increase NO production, whereas moderate-intensity exercise increased NO production and improved salt-induced polyuria during inactive periods in aged mice. This finding suggests that moderate-intensity exercise is necessary to increase NO production and improve salt-induced nocturnal polyuria in older individuals. The urinary NOx excretion in the Low-Ex group tended to be lower than that in the Sed group, although the difference was not statistically significant. We believe this result may be because four weeks of low-intensity exercise does not increase NO production and the amount of NO production in old mice is highly variable. Several studies have reported that moderate-intensity exercise increases NO production, whereas low-intensity exercise does not[22,23]. These reports suggest that the reasons for the lack of increased NO production with low-intensity exercise may be because exercise-induced stimulus to vascular endothelium is too weak to elicit an increase in NO production during low-intensity exercise.

Exercise is known to improve hypertension. In this study, 4 weeks of moderate-intensity exercise lowered systolic and diastolic blood pressure during both the inactive and active periods (Supplementary Fig. 1a–d). Because non-dipping hypertension is associated with nocturnal polyuria[24], the improvement in salt-induced polyuria during inactive periods by exercise may be related, in part, to the improvement in hypertension during inactive periods. However, lowering blood pressure with the antihypertensive drug hydralazine hydrochloride did not improve DPi (Supplementary Fig. 2a–e), indicating that exercise-induced improvement in polyuria during inactive periods was not due to an improvement in hypertension.

The intrarenal RAS is the pathway by which angiotensinogen produced in the kidney is converted to biologically active angiotensin II by renin and angiotensin-converting enzymes. The intrarenal RAS is distinct from the systemic RAS and plays a significant role in the pathogenesis of hypertension and renal disease[25]. The intrarenal RAS regulates the NCC activity in the distal tubule, which controls sodium reabsorption[16]. Previously, we reported that the suppression of NO production activates intrarenal RAS and NCC activity and increases sodium reabsorption during active periods, which leads to salt-induced polyuria during inactive periods in mice. In this study, we found that increased systemic NO production suppressed intrarenal RAS and NCC activity, decreased sodium reabsorption in active periods, and ameliorated salt-induced polyuria during inactive periods in aged mice. NO plays a crucial role in regulating physiological functions, such as vasodilation, neurotransmission, and immune response[26]. In the kidney, NO increases renal blood flow and glomerular filtration rate and promotes sodium excretion[27]. Moreover, NO suppresses intrarenal RAS activity[13]; however, the mechanism by which increased NO production suppresses intrarenal RAS is unclear. Therefore, it is possible that the increase in NO production associated with moderate-intensity exercise increased renal blood flow and promoted sodium excretion, thereby improving nocturnal polyuria. However, the mechanism by which increased NO production decreases intrarenal RAS activity was not elucidated in this study, and further research is required. Previously, we have reported that decreased renal function decreases daytime sodium excretion and increases nocturnal sodium excretion, resulting in increased nocturnal urine output[28]. We believe that sodium excretion kinetics have a crucial influence on salt-induced nocturnal polyuria. It is known that there are racial differences in sodium excretion kinetics[29], and genetic factors related to sodium excretion kinetics may be involved in salt-induced nocturnal polyuria. It is also possible that NO production affects vasopressin secretion which, in turn, affects urine output. The association between NO production and vasopressin secretion has been reported in several studies. Ota et al. reported an increase in blood vasopressin concentration after intracerebroventricular administration of NO donors[30]. However, in the kidney, NO has been reported to reduce vasopressin responsiveness. Garcia et al. reported that increased NO in the cortical collecting ducts of the kidney inhibits vasopressin-stimulated water reabsorption[31]. Consequently, the impact of NO-induced

changes in vasopressin on urine output remains poorly understood. While increased NO production is believed to enhance vasopressin secretion in the brain, it simultaneously appears to reduce vasopressin responsiveness in the kidneys, creating a complex and unresolved dynamic. However, the possibility that vasopressin is involved in the improvement of salt-induced nocturnal polyuria caused by increased NO production is undeniable.

This study had a limitation. We showed that moderate-intensity exercise is effective in the treatment of salt-induced polyuria during inactive periods in aged mice. Moderate-intensity exercise such as yoga or golfing is also thought to be necessary to improve nocturnal polyuria in humans; however, it may be challenging for some older individuals. Therefore, exercise may not be appropriate for all older adults with salt-induced nocturnal polyuria.

In summary, moderate-intensity exercise improved salt-induced polyuria during inactive periods in aged mice by increasing NO production. This suggests that increasing NO production may be a potential treatment for salt-induced nocturnal polyuria in some elderly individuals who have decreased NO production. Further studies are required to determine its efficacy in humans.

Methods

Experimental animals

All experiments involving animals were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Osaka University and approved by the Animal Care and Use Committee of Osaka University (No. J008231-002). All animal experiments in this study were done in accordance with ARRIVE guidelines. The study complied with all ethical regulations. Eighty-week-old C57BL/6J mice (aged mice were purchased from Charles River Laboratories [Tokyo, Japan]) were kept in a temperature-controlled room with a 12/12-hour light/dark cycle. The active period was from 8:00 p.m. to 8:00 a.m. and the inactive period was from 8:00 a.m. to 8:00 p.m. (both 12 h) and had ad libitum access to food and water.

Experimental protocol

We have adjusted the sodium content of the standard purified product for mouse and rat research published by the National Institute of Nutrition in 1993 to crease a 1% salt-containing diet. The mice were administered a 1% high sodium diet (HSD) for 2 weeks. All the diets were purchased from Oriental Yeast (Tokyo, Japan). We previously reported that a 1% HSD for mice aged 2 weeks increased urine volume during inactive periods without producing differences in daily urine output compared to a normal salt diet[10]. The mice were divided into the low-intensity exercise (Low-Ex), moderate-intensity exercise (Mod-Ex), moderate-intensity with Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Mod-Ex with L-NAME), sedentary (Sed), and sedentary with L-NAME (Sed with L-NAME) groups. Mice in the Low-Ex, Mod-Ex, and Mod-Ex with L-NAME groups underwent forced treadmill exercise, while those in the Sed and Sed with L-NAME groups remained in their cages and listened to the sounds of treadmill running. Mice in the Mod-Ex with L-NAME and Sed with L-NAME groups were administered L-NAME (Sigma-Aldrich, St. Louis, MO, USA, 0.5 g per L in drinking water) for four weeks. After four weeks, urinary behavior was assessed using the automated Voided Stain on Paper (aVSOP) method, and urine samples were collected using metabolic cages. The mice were sacrificed by isoflurane inhalation during the active period, and their kidneys and blood were collected.

Exercise protocol

We conducted adaptive exercises prior to moderate- and low-intensity exercises. Mice were acclimated to the treadmill at 10 m/min and 0° incline for 15 min daily for 1 week. Mice in the Low-Ex, Mod-Ex, and Mod-Ex with L-NAME groups underwent forced treadmill exercises on TMS-6B units (MELQUEST, Toyama, Japan) in individual lanes. The Low-Ex group exercised for four weeks at 10 m/min, 60 min/day, 0° incline, and four days/week. The Mod-Ex group exercised similarly but at a progressive speed: starting at 10 m/min for 5 min, increasing to 12 m/min for 15 min, and reaching 14 m/min for 40 min, with an incline of 15°[32,33]. Mice in the Mod-Ex with L-NAME group were administered L-NAME and subjected to forced treadmill exercise at the same intensity as mice in the Mod-Ex group. All the mice performed the exercise without an electric stimulant.

Urine volume and time measurements

The urine volume was measured using the aVSOP method[34,35]. Briefly, a roll of laminated filter paper pretreated to turn the urine dark purple was rolled under a water-repellent wire grid at a speed of 10 cm/h. The mice were housed for four days in cages with dimensions of 110 mm × 160 mm × 75 mm ($H \times D \times W$). Urine samples were counted, tracked, and converted to volume using ImageJ software (ver. 1.53e, National Institutes of Health, Bethesda, MD, USA). The diurnal polyuria index (DPi) was calculated by dividing the volume of urine produced during inactive periods (8 a.m. to 8 p.m.) by the volume of urine produced per day. We measured urine volume every 4 h for 3.5 consecutive days to calculate DPi. In all experiments, changes in DPi were measured in the same mice to evaluate changes in DPi before and after the intervention.

Blood pressure measurement

Blood pressure was measured using the tail-cuff method (BP-98 A; Softron Corporation, Tokyo, Japan). All mice were first acclimated to the blood pressure measurements for 3 days. Three consecutive measurements were performed, and the average value was recorded.

Mouse urine analysis

Mice were placed individually in metabolic cages (3600M021; Techniplast, Tokyo, Japan), with unrestricted access to food and water for urine collection. Due to reduced urine volume during inactive period, diurnal urine and urinary electrolyte concentrations were calculated using the following formulae: (inactive period urine volume)

= (24 − hour urine volume) − (active period urine volume); (inactive period urinary electrolyte concentration) = {(24 − hour urinary electrolyte amount) − (active period urinary electrolyte amount)} ÷ (inactive period urine volume). Urine electrolytes were assessed via Fujifilm Pet Systems Co. Furthermore, urinary NOx (NO_2/NO_3) levels were quantified using the NK05 NO_2/NO_3 Assay Kit-C II (Colorimetric, Japan), involving protein separation with a column, centrifugation at 7000 ×g for 20 min, and a subsequent centrifugation at 17,000 ×g for 10 min.

Immunoblotting

Proteins extracted from whole kidneys were homogenized in lysis buffer; the lysate and membrane fractions were then collected and prepared in sodium dodecyl sulfate sample buffer (Cosmo Bio Co., Ltd., Tokyo, Japan). Protein concentrations were determined using the Lowry method, and samples were stored at − 80 °C until immunoblotting. A total of 20 μg of protein underwent 8% non-SDS–PAGE or 10% SDS–PAGE, followed by immunoblotting. Membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated overnight at 4 °C in Tris-buffered saline with primary antibodies. Detection used anti-rabbit IgG, horseradish peroxidase-conjugated antibody (Cell Signaling Technology, Danvers, MA, USA), and Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan). A ChemiDoc XRS Plus system (Bio-Rad, Hercules, CA, USA) captured blot images, which were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The primary antibodies were rabbit anti-NCC, anti-phosphorylated NCC (threonine 53), and anti-angiotensinogen, all at 1:1000; the secondary was anti-rabbit IgG HRP at 1:5000.

Statistical analysis

For the analysis of DPi, 24-hour urinary NOx, renal AGT and NCC activity, and sodium excretion kinetics, groups were compared by Student's t-test, paired t-test, two-way repeated measures ANOVA, and Tukey's test when applicable. Bonferroni correction was used for multiple comparison tests. All data are presented as means ± standard errors of the means, and values of $P < 0.05$ were considered statistically significant. All analyses were performed using the JMP version 17.1.0 software (SAS Institute, Cary, NC, USA).

Data availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

TI and KT contributed to conceptualization. TI, GT, and KT contributed to data curation. TI, KT , and NU contributed to the formal analysis. TI, KT, YK, HK, GT, SK, YS, NU, SF, HK, HN and NN contributed to the methodology. TI, KT, and NN contributed to project administration. KT and NN contributed to supervision. TI contributed to Writing – original draft. TI, KT, and NN contributed to Writing – review & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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