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Original Research Article

Involvement of the Renal Kallikrein–Kinin System in the Diuretic Action of a Thiazide Using a Crossover Design in Rats: An Exploratory Study

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Abstract

Background: We have reported that the renal kallikrein–kinin system is involved in the diuretic action of potassium or furosemide in rats.

Objectives: The aim of this study was to investigate whether the renal kallikrein–kinin system is involved in the diuretic action of thiazides in rats.

Materials and Methods: In the first study, indapamide (INDP) was administered at 0.1 or 0.3 mg/kg/day, or a solvent, and urine was collected using metabolic cages at 0–6, 6–12, and 12–24 h. Urine output, urinary excretion of sodium, chloride, and potassium, and urine kallikrein activity were measured. In the second study, a bradykinin B_2 receptor antagonist, icatibant, or a solvent was administered before INDP administration in a crossover design with a washout period, which was conducted under conditions with and without saline loading. Urine output and urinary excretion of sodium, chloride, and potassium were measured at 0–6 h after INDP administration.

Results: The results showed that the diuretic actions of INDP increased with high-dose INDP and reached a maximum at 0–6 h: urine outputs were 5.0 (median, interquartile range 4.7–5.9) versus 1.8 (1.4–2.1, $p < .05$) mL/100 g body weight (b.w.), and urinary excretions of sodium, chloride, and potassium were 0.21 (0.18–0.23) versus 0.06 (0.05–0.07, $p < .01$), 0.18 (0.16–0.19) versus 0.05 (0.05–0.05, $p < .01$), 0.11 (0.08–0.13) versus 0.04 (0.03–0.05, $p < .05$) mmol/mg urinary creatinine (Cr) in the INDP versus control groups. Urinary kallikrein activity did not change with either dose of INDP. Icatibant did not inhibit the diuretic action of INDP under either condition.

Conclusion: The conclusions were that although the crossover design was used to evaluate the urine data collected by a metabolic cage in rats, the study could not determine whether the renal kallikrein–kinin system is involved in the diuretic actions of thiazides. Future studies should improve regarding fluid loading and long-term sodium loading.

Keywords

Bradykinin B_2 receptor antagonist, crossover design, renal kallikrein–kinin system, thiazides

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Introduction

Kallikreins are a group of serine proteases, and there are two types of kallikreins: plasma and tissue kallikreins. Plasma kallikrein is a protein of about 88,000 Da, and it is activated with the initiation of a surface-dependent activation of endogenous blood clotting, and participates in inflammation, fibrinolysis, and regulation of vascular tone.[1] Tissue kallikreins are derived from a multigene family, and they are smaller proteins than plasma kallikrein. They are expressed in a number of tissues, including epithelial cells, salivary glands, pancreas, prostate, distal nephron, and neutrophil, and they act at

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these tissues specifically.[1] Renal kallikrein is localized along the distal tubules.[2,3] When renal kallikrein is secreted into the lumen of the distal tubules, it produces bradykinins from its substrate, kininogen.[1]

We have reported that the renal kallikrein–kinin system suppresses the salt-sensitive hypertension, which is caused by the diuretic and natriuretic effects of bradykinin.[4–6] In addition, increasing the urinary bradykinin levels has been shown to cause diuresis and natriuresis through the bradykinin B_2 receptors (B_2 receptors) in rats.[7,8] The sites in the kidney where bradykinin acts have been thought of as the cortical collecting duct and medullary collecting duct in rats.[9,10] Although molecular mechanisms of the diuretic and natriuretic effects of bradykinin remain unclear, Tomita et al. have reported that bradykinin inhibits sodium and chloride transport electroneutrally in the rat cortical collecting duct.[11]

Thiazides are known to inhibit the electroneutral transport of sodium and chloride via a sodium-chloride cotransporter (NCC) in the distal convoluted tubule. However, Leviel et al. have reported that thiazides may inhibit sodium transport electroneutrally in the rat cortical collecting tubules via the sodium-coupled chloride-bicarbonate exchanger.[12]

We have reported effects of other diuretic-like agents and diuretics, a high potassium and furosemide, are also involved in the renal kallikrein–kinin system in rats.[13,14] Taken together, we hypothesized that the effects of thiazides are also involved in the renal kallikrein–kinin.

It has been known that there is a large interindividual variability in the excretory patterns of urine output and electrolytes in rats when assessing the effects of diuretics.[15] In order to reduce the variability of data, several efforts have been made, including a large volume of saline loading given before the administration of diuretics, and paired rats were placed in one metabolic cage as one dataset.[15,16] It is known that a crossover design can reduce the variability of data, as it compares the data inter-individually. It has been used in clinical studies as well as in animal studies.[17,18] Therefore, we decided to use a crossover design to reduce the variability of the urine data.

Thus, the study aimed to examine whether the renal kallikrein–kinin system is involved in the diuretic actions of a thiazide using a crossover design, and to probe a complex interaction of the renal kallikrein–kinin system and thiazides to inform future, more definitive research.

Materials and Methods

Animals

Five-week-old, male Wistar rats (Oriental Yeast Co., Ltd.) were housed with food and tap water *ad libitum* at a constant temperature (22°C ± 1°C) and 12-h light/dark-controlled conditions in the animal facility. After 1 week of acclimatization, rats were used for the experiment.

Study Design

Indapamide (INDP, CAS Registry Number 26807-65-8, FUJIFILM Wako Pure Chemical Corporation), a thiazide, and icatibant (D-Arg-Arg-Pro-Hyp-Thi-Ser-Tic-Oic-Arg, CAS Registry Number 130308-48-4, 138614-30-9, Peptide Institute, Inc.), an antagonist of the B_2 receptors, were used. INDP was chosen as it has the advantages of a lower effective dose, higher oral availability, and longer half-life than other thiazides.[19]

In the first study, INDP or a vehicle, a methylcellulose 400 solution (MC, CAS Registry Number 9004-67-5, 0.5 w/v%, FUJIFILM Wako Pure Chemical Corporation), was administered by gastric gavage using a feeding needle (SN-6201, Shinano Seisakusho Corporation) once daily for 4 days. Doses of INDP were set at 0.1 and 0.3 mg/kg body weight (b.w.). They were determined based on a clinical dose in adults (2 mg once per day), which was converted to the dose in rats by a body surface area,[20] and the effective doses in rats.[21] INDP was not dissolved in water; thus, it was suspended in MC. The INDP suspension or vehicle was administered at a volume of 1 mL/kg. A repeated administration was conducted in order to obtain a maximal and stable effect of INDP. Rats were fasted for 15 h before the last administration of INDP or vehicle, and each rat was placed in an individual metabolic cage (SN-781, Shinano Seisakusho Corporation) after the administration. Subsequently, urine was collected over 0–6, 6–12, and 12–24 h periods.

In the second study, a crossover design was used. Rats were randomly divided into two subgroups and were subcutaneously administered vicariant, or a vehicle, 1 h before administration of INDP. After a 2-day washout, the rats of each subgroup received an opposite treatment in order to reduce the sequential effect of the treatment. The 2-day washout was determined by the duration of action of icatibant, which was about 5 h after subcutaneous injection, and the half-life of INDP was 1.3 h after gastric-gavage administration in rats.[22,23] INDP was administered in two conditions, without and with a large volume of saline (saline loading). The large volume of saline was administered in order to place the rats in a state of sodium retention, based on the previous study, where urinary excretion of sodium was decreased in rats genetically devoid of kinin substrates compared to normal rats on high-sodium diets.[24] A dose of icatibant was set at 1.2 mg/kg according to the previous study, where icatibant at 300 μg/kg four times a day decreased urine output and the urinary excretion of sodium in the sodium-retained rats.[25] Saline was used as the vehicle of icatibant. Icatibant or its vehicle was injected at a volume of 1 mL/kg. Doses of INDP were set at 0.1 and 0.3 mg/kg for the study without saline loading, and at 0.3 mg/kg for the study with saline loading. In the saline loading, saline was simultaneously administered with INDP at a volume of 4% b.w. Rats were fasted for 15 h before administration of INDP or vehicle, and each rat was placed in an individual metabolic cage after administration. Subsequently, urine was collected over a 0–6 h period.

Measurement of Urine Output and Urinary Excretion of Sodium, Chloride, Potassium, and Creatinine (Cr)

Urine output was measured by the volume after removal of the deposits by centrifugation of the urine sample. Concentrations of urinary sodium, chloride, and potassium were measured by an ion-selective electrode, and those of Cr by the enzymatic assay (SRL Inc., Hachioji Laboratory, Tokyo). Urine output was expressed as mL/100 g b.w. Urinary excretion of sodium, chloride, and potassium was expressed as millimoles per milligrams of Cr in order to correct a difference in the glomerular filtration rate.

Measurement of Urinary Kallikrein Activity

Enzymatic activity of urinary kallikrein was determined using the fluorogenic synthetic substrate L-Pro-Phe-Arg 4-methylcoumaryl-7-amide (Peptide Institute, Inc., Osaka, Japan) according to our previous study.[26] The diluted urine was incubated with the synthetic substrate at 37°C for 20 min in the presence of soybean trypsin inhibitor (Warthington Biochem Corp., Lakewood, NJ, USA) or aprotinin (FUJIFILM Wako Pure Chemical Corp., Osaka, Tokyo), which inhibits a kallikrein-like activity in plasma or that in plasma and urine. Thus, a difference between the values of these incubation mixtures indicates urinary kallikrein activity. The fluorescence intensity was measured by a fluorescence spectrophotometer (F-7000: Hitachi High-Tech Science Corporation, Tokyo, Japan). Kallikrein activity was quantified by a calibration curve of the reference, 7-amino-4-methylcoumarin (Peptide Institute, Inc.). Values were expressed as nanomoles per milligram of urinary Cr to correct for differences in the glomerular filtration rate.

Statistical Analyzes

The International Business Machines (IBM) Statistical Package for the Social Sciences (SPSS) Statistics (version 29.0.1.0) was used (IBM Japan, Tokyo, Japan). The normality of the data distribution regarding the first study was tested by the Shapiro–Wilk test. Most data showed a lack of normality except for the 6–12 h period urine output and urinary excretion of sodium in the control group, 12–24 h period urinary excretion of sodium in the 0.3 mg/kg INDP group, and 12–24 h period urinary kallikrein activities in the 0.1 and 0.3 mg/kg INDP groups (Figure 1). Therefore, all data in the first and second studies were analyzed by a nonparametric test, such as the Kruskal–Wallis test and the one-sample Wilcoxon test, and the data were expressed as boxplots including the minimum, first quartile, median, third quartile, and maximum.

In the first study, the Kruskal–Wallis test with the Bonferroni correction, multiple comparisons, and *post hoc* tests was used for a comparison among groups of the control and two doses of INDP. In the second study regarding a comparison between the groups without and with icatibant, the one-sample Wilcoxon test was used as the study was conducted in the same individuals by the crossover design. In the second study regarding a comparison among the two doses of INDP and the control, the Kruskal–Wallis test with the Bonferroni correction was used for each study without or with icatibant. The difference with a probability of 5% or less was considered to be significant.

Results

Effects of INDP on urine output and urinary excretion of sodium, chloride, and potassium, and urinary kallikrein activity for the 0–6, 6–12, and 12–24 h urines.

Urine output was increased in the 0–6 and 6–12 h urines of the 0.3 mg/kg INDP group compared with those of the control groups, respectively (Figure 1a, 1b). Urinary excretion of sodium and chloride was also increased in the 0–6 h urine of the 0.3 mg/kg INDP group compared with that of the control group (Figure 1d, 1e, 1g, 1h). Urinary excretion of potassium was only increased in the 0–6 h urine of the 0.3 mg/kg INDP group compared with that of the control group (Figure 1j). Urinary kallikrein activities were not changed in any of the urine periods for either dose of the INDP group compared with those of the control groups (Figure 1m–1o).

Effects of icatibant on urine output and urinary excretion of sodium, chloride, and potassium after administration of INDP without saline loading.

The 0–6 h urine was examined in these studies, as the effect of INDP appeared most strongly in this period (Figure 1a, 1d, 1g, 1j, 1m). Urine output showed a dose-dependent increasing trend, but was not significant in the INDP groups compared with the control group, without icatibant (Figure 2a). A reason why the increase in urine output was not significant in the INDP groups was probably due to insufficient volume loading. The INDP-induced increasing trends of urine output were not suppressed significantly in the groups with icatibant compared with the groups without icatibant (Figure 2a). Urinary excretion of sodium and chloride increased in the 0.3 mg/kg INDP group compared with those in the control group, and dose-dependent increases were shown in either group without or with icatibant. The INDP-induced increases in urinary excretion of sodium and chloride were not suppressed significantly in the groups with icatibant compared with the groups without icatibant (Figure 2b, 2c). Urinary excretion of potassium increased in the 0.3 mg/kg INDP group compared with that in the control group, and a dose-dependent increase was shown in the group without icatibant. A dose-dependent increasing trend of urinary excretion of potassium was shown in the group with icatibant. The INDP-induced increase and its increasing trend of urinary excretion of potassium were not suppressed significantly in

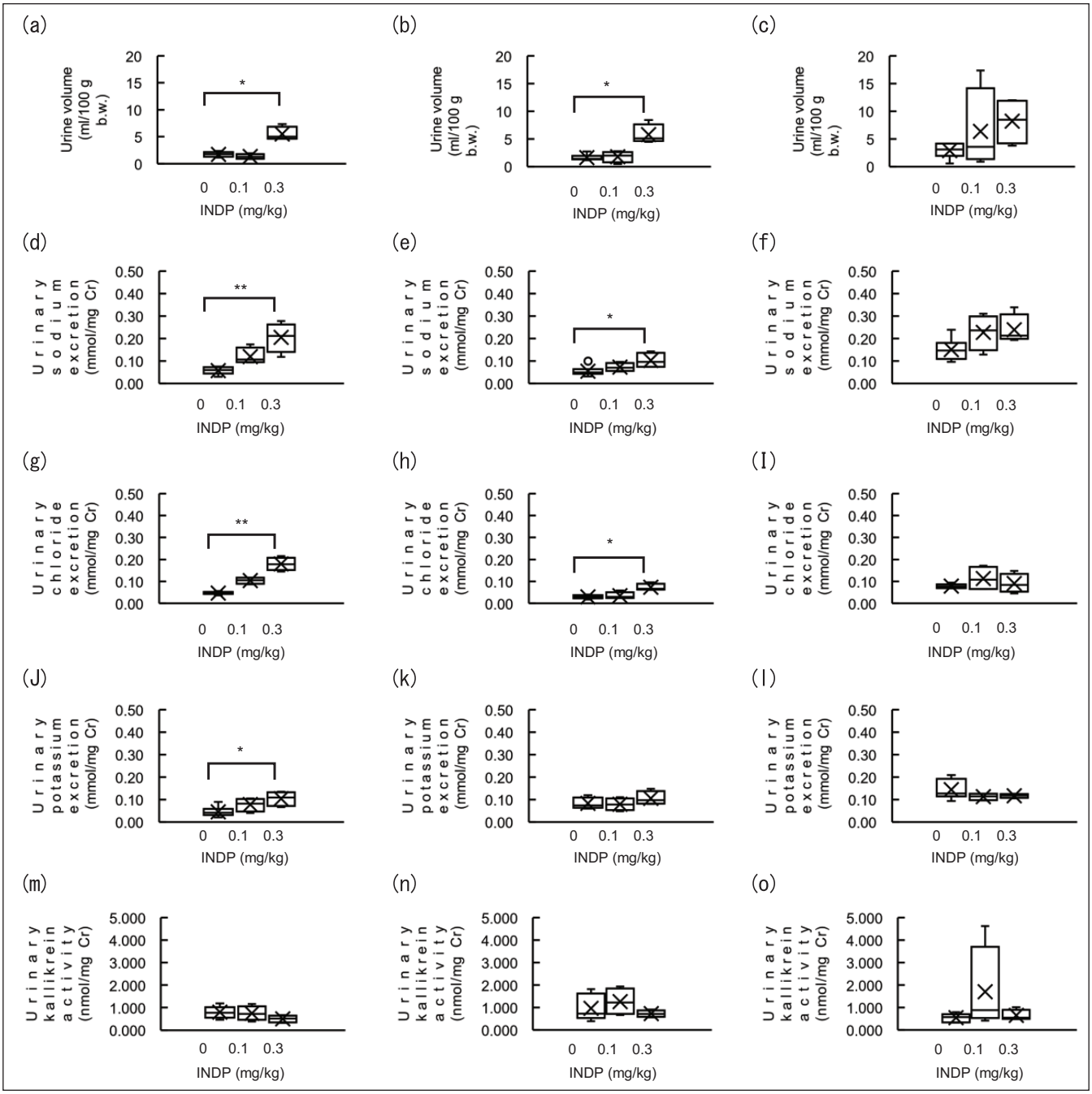


Figure 1. Effects of Indapamide (INDP) on Urine Output (a–c), Urinary Excretion of Sodium (d–f), Chloride (g–i), and Potassium (j–l), and Urinary Kallikrein Activity (m–o) in the 0–6 (a, d, g, j, m), 6–12 (b, e, h, k, n), and 12–24 (c, f, I, l, o) h Urines. Rats Were Orally Administered with INDP (0.1, 0.3 mg/kg) or 0.5% Methylcellulose (MC) Once a Day for 4 Days. Urine Was Collected for 0–6, 6–12, and 12–24 h After the Last Administration. Graphs Show the Boxplots Including the Minimum, First Quartile, Median, Third Quartile, and Maximum (n = 4 for the INDP Groups at 0.1 and 0.3 mg/kg, Respectively, n = 6 for the Control Group).

Note: $^{*}p < .05$, $^{**}p < .01$ compared between the indicated groups in the same time. The significance of the difference was calculated using the Kruskal–Wallis test and Bonferroni correction.

the groups with icatibant compared with the groups without icatibant (Figure 2d).

Effects of icatibant on the urine output and urinary excretion of sodium, chloride, and potassium after the administration of INDP with saline loading.

The 0–6 h urine was also examined in these studies. These studies compared urine output and urinary excretion of sodium, chloride, and potassium between the 0.3 mg/kg INDP groups without and with icatibant in the state with saline loading. The results showed that the urine

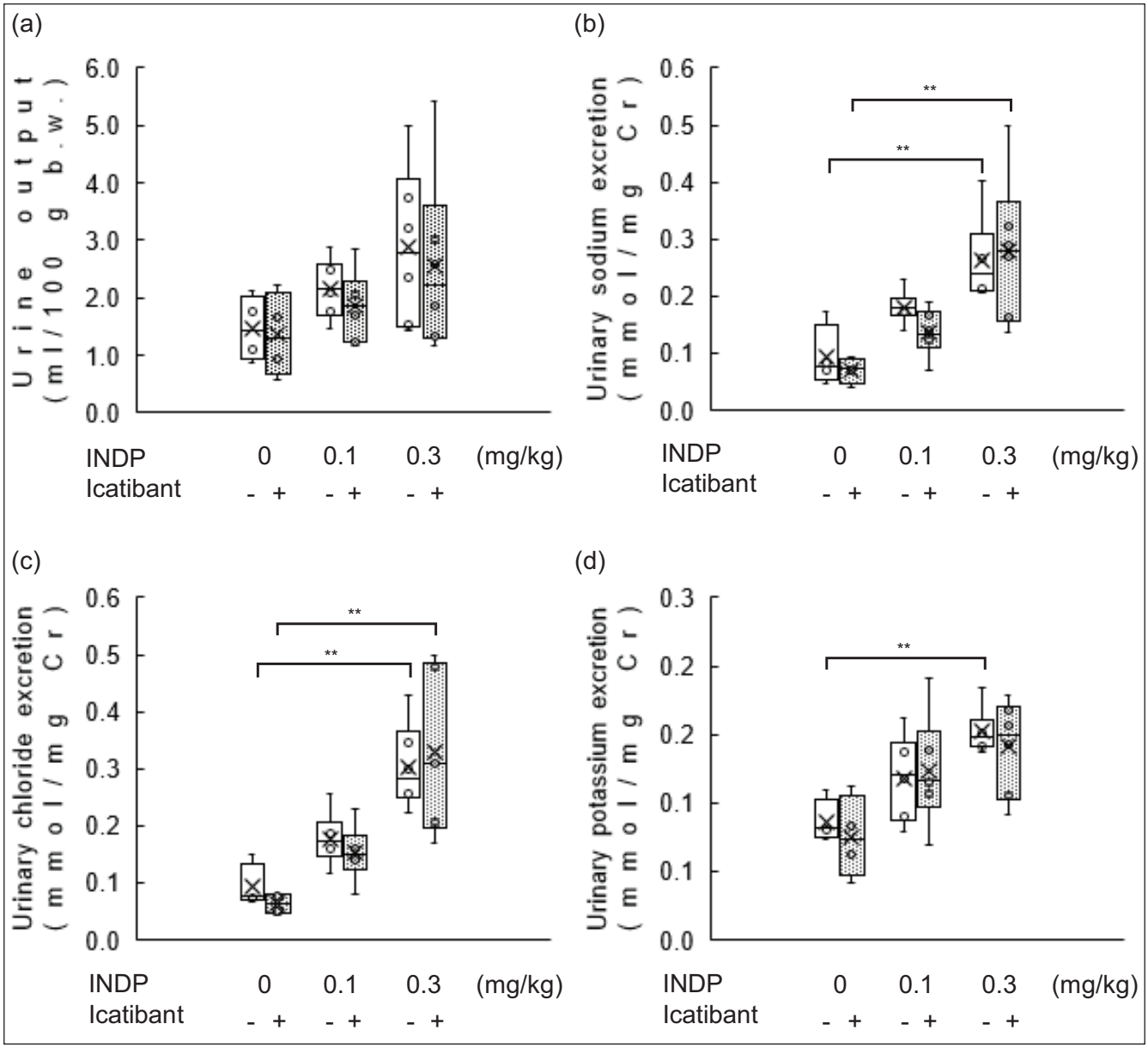


Figure 2. Effects of Icatibant on Urine Output (a) and Urinary Excretion of Sodium (b) Chloride (c) and Potassium (d) After Administration of Indapamide (INDP) Without Saline Loading. The Study Was Conducted in a Crossover Design. In Brief, Rats Received Two Treatments, Icatibant (1.2 mg/kg) and a Saline Solution, Respectively, with a 2-day Washout. One Hour After the Subcutaneous Administration of Icatibant or Saline INDP (0.1, 0.3 mg/kg) or 0.5% Methylcellulose (MC) Was Orally Administered to Rats. Urine Was Collected for 0–6 h After Administration of INDP or 0.5% MC. Graphs Show the Boxplots Including the Minimum, First Quartile, Median, Third Quartile, and Maximum (n = 6 for the INDP Groups at 0.1 and 0.3 mg/kg, Respectively, n = 4 for the Control Group).

Note: $^{**}p < .01$ compared between the indicated groups. The significance of the difference between the groups without and with administration of icatibant was calculated using the one-sample Wilcoxon test. The significance of the difference among the control and INDP groups was calculated using the Kruskal–Wallis test and Bonferroni correction.

output and urinary excretion of sodium, chloride, and potassium were not suppressed in the groups with icatibant compared with the groups without icatibant (Figure 3a–3d).

Discussion

In the first study, increases in urine output were shown in the 0–6 h and 6–12 h urines by administration of high-dose INDP,

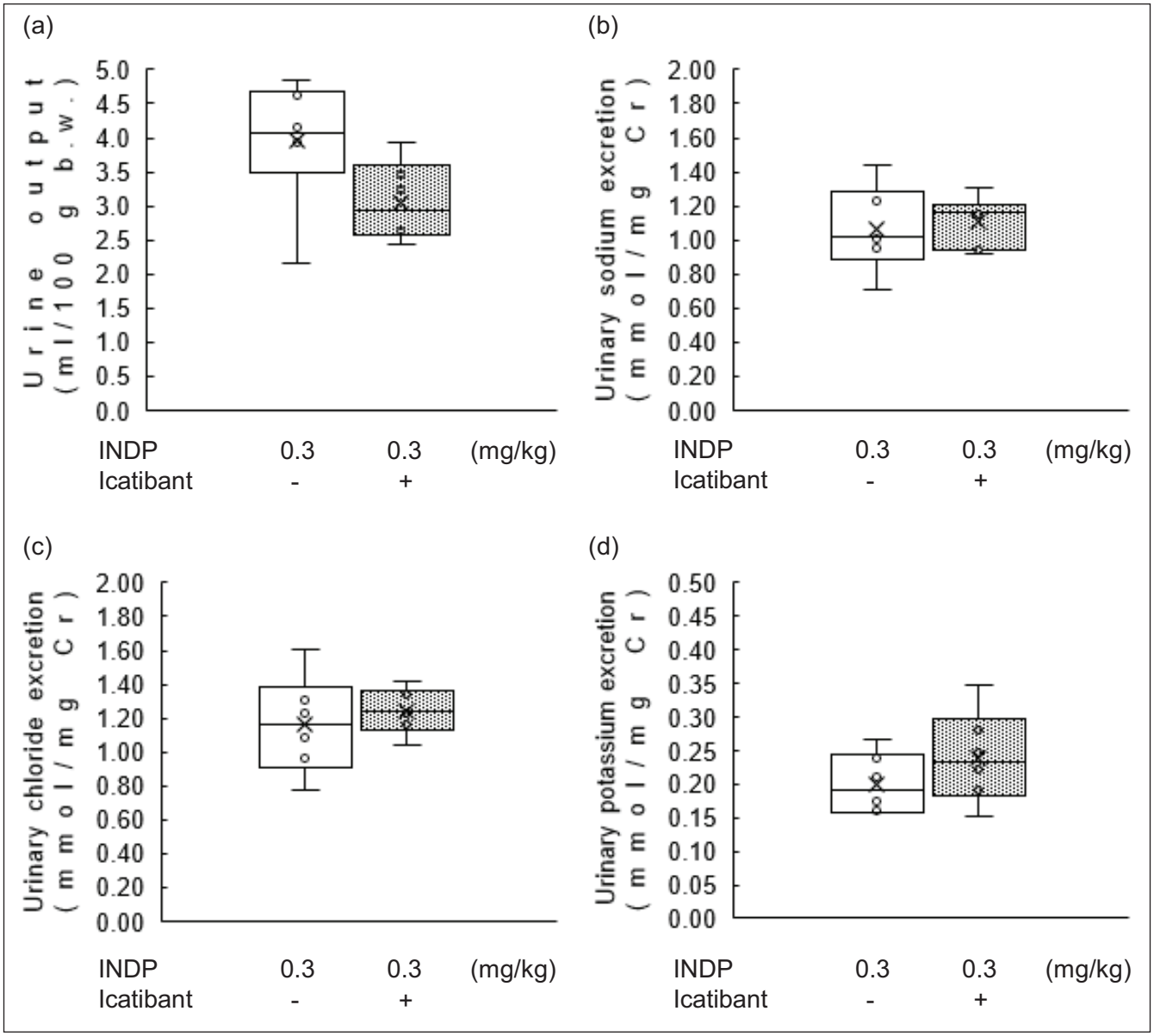


Figure 3. Effects of Icatibant on Urine Output (a) and Urinary Excretion of Sodium (b) Chloride (c) and Potassium (d) After Administration of Indapamide (INDP) with Saline Loading. The Study Was Conducted in a Crossover Design. In Brief, Rats Received Two Treatments: Icatibant (1.2 mg/kg) and Saline, Respectively, with a 2-day Washout. One Hour After the Subcutaneous Administration of Icatibant or Saline INDP (0.3 mg/kg), Was Orally Administered to Rats with Saline at a Volume of 4% b.w. Urine Was Collected for 0–6 h After Administration of INDP. Graphs Show the Boxplots Including the Minimum, First Quartile, Median, Third Quartile, and Maximum (n = 6 for Each Group). The Significance of the Difference Between the Groups Without and With Administration of Icatibant Was Calculated Using the One-sample Wilcoxon Test.

but not by that of low-dose INDP (Figure 1a, 1b). Dose-dependent increases in urinary excretion of sodium and chloride were shown in the 0–6 h (Figure 1d, 1g) and 6–12 h (Figure 1e, 1h) urines by INDP administration. A dose-dependent increase in urinary excretion of potassium was shown in the 0–6 h urine, but not in the 6–12 h urine. Increases in urinary kallikrein activities were not shown in any of the urine periods (Figure 1m–1o).

A reason why the increase in urine output was not shown by low-dose INDP may be due to insufficient water intake before administration, as rats had been fasted for 15 h before. A smaller increase in the urinary excretion of potassium compared to increases in the urinary excretion of sodium and chloride suggests the increase in potassium excretion is secondarily caused by an increased influx of sodium into a downstream of the NCC where sodium and

potassium are exchanged. A previous report supports these results, that the slope of the dose-response curve was small for the potassium excretion compared to those for the sodium and chloride excretion in the first 5-h urine after INDP administration at doses from 0.05 to 10 mg/kg in rats.[27] The result that urinary kallikrein activity did not increase suggests that INDP does not have a direct effect on the increase in renal kallikrein secretion, which was consistent with our previous study. Renal kallikrein secretion was not increased by trichlormethiazide, another thiazide, when it was administered in the incubated kidney cortex sections.[26] This result also suggests that the increasing effect of INDP on the urinary excretion of potassium is not enough to cause an increase in the renal kallikrein secretion, as we have reported that the administration of a high potassium concentration increases the renal kallikrein secretion in the incubated kidney cortex sections.[26] Thus, it is unlikely that thiazides increase urinary excretion of sodium by increasing the renal kallikrein secretion, followed by activation of the renal kallikrein–kinin system.

Therefore, in the second study, we examined whether icatibant, a B_2 receptor antagonist, inhibits the INDP-induced increases in urine output and urinary excretion of sodium, potassium, and chloride from the perspective of the involvement of the renal kallikrein–kinin system in the diuretic actions of a thiazide via a possible common mechanism, the inhibition of the sodium-driven chloride-bicarbonate exchanger. In the results, we could not detect differences in the diuretic actions of INDP between the groups with and without icatibant despite the use of a crossover design (Figures 2 and 3). We consider the following reasons for our failure to detect these differences. One is that the diuretic actions of INDP were not large enough, as indicated by the results that the increase in urine output was not significant in the INDP groups compared with the control group without icatibant (Figure 2a). These results were probably due to insufficient volume loading; in previous studies, diuretics were administered with a large volume of saline, a volume of 2.5%–4% b.w., in rats.[15,16,28] The other is that sodium retention was not large enough for bradykinin to exert its action, as the saline loading was conducted in a short time. In a previous study, a high-sodium diet for more than 1 week was shown to decrease the urinary excretion of sodium and to increase systolic blood pressure in rats genetically devoid of kinin substrates compared to normal rats.[24]

We used a crossover design in order to reduce variability in the urine data. As far as we have searched, two studies used the crossover design to examine differences in the urine data collected by a new device and a metabolic cage in rats.[29,30] Thus, the crossover design can be reliably used to assess the urine data by urine collection. It may also be advantageous when the number of animals is limited, for example, genetically-engineered animals, animals subjected to special instruments or treatments.[31]

Conclusion

The study could not determine whether the renal kallikrein–kinin system is involved in the diuretic actions of a thiazide using the crossover design in rats. We need to improve the study in the future, for example, fluid loading before administration of the diuretic and long-term sodium loading before administration of the B_2 receptor antagonist in combination with the crossover design.

Abbreviations

Am J: American Journal; **ATP:** Adenosine triphosphate; **Biol Pharm Bull:** Biological and Pharmaceutical Bulletin; **Br J:** British Journal; **b.w.:** Body weight; **B2 receptors:** Bradykinin B_2 receptors; **CAS:** Chemical abstracts service; **CDER:** Center for Drug Evaluation and Research; **Circ:** Circulation; **Clin:** Clinical; **Co.:** Company; **Corp.:** Corporation; **Cr:** Creatinine; **°C:** Degrees celsius; **et al.:** Et alii (and others); **Eur J:** European Journal; **Exp:** Experimental; **FDA:** Food and Drug Administration; **g:** Grams; **h:** Hours; **Hypertens:** Hypertension; **IBM SPSS:** International Business Machines Statistical Package for the Social Sciences; **Inc.:** Incorporated; **INDP:** Indapamide; **Int:** International; **Invest:** Investigation; **J Am Assoc Lab Anim Sci:** Journal of the American Association for Laboratory Animal Science; **J Clin Invest:** Journal of Clinical Investigation; **Jpn J:** Japanese Journal; **J Pharmacol Methods:** Journal of Pharmacological Methods; **J Pharm Pharmacol:** Journal of Pharmacy and Pharmacology; **J Pharm Sci:** Journal of Pharmacy and Pharmaceutical Sciences; **KCl:** Potassium chloride; **KW-3902:** 8-(Noradamantan-3-yl)-1,3-dipropylxanthine; **Lab:** Laboratory; **Ltd.:** Limited; **MC:** Methyl cellulose 400 solution; **mg/kg:** Milligrams per kilogram; **min:** Minutes; **mL:** Milliliters; **NaCl:** Sodium chloride; **NCC:** Sodium-chloride cotransporter; **NJ:** New Jersey; **nm:** Nanometers; **Osaka:** Osaka (location abbreviation); **pH:** Potential of hydrogen; **Pharmacol:** Pharmacology; **Physiol:** Physiology; **Res:** Research; **Rev:** Review; **Sci:** Science; **SE-1520:** Chemical compound designation; **SGLT2:** Sodium-glucose cotransporter-2; **SN:** Serial number; **SRL:** Laboratory service company; **Tokyo:** Tokyo (location abbreviation); **µg/kg:** Micrograms per kilogram; **USA:** United States of America; **vs.:** Versus; **w/v%:** Weight per volume percentage.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The experimental protocol for this study was approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine (approval number 2015 122) and performed in accordance with the rules of the animal experiments by Kitasato University.

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Informed Consent

Not applicable.

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