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Title page

Title: Glucocorticoids Improve Renal Responsiveness to ANP by Upregulating NPR-A Expression in the Renal Inner Medullary Collecting Duct in Decompensated Heart Failure

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Running title page

Running title: glucocorticoids increase renal NPR-A expression

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Abbreviations:

ANP: atrial natriuretic peptide

NPR-A: Natriuretic peptide receptor-A

IMCD: inner medullary collecting duct

Dex: dexamethasone

GR: glucocorticoid receptor

NP: natriuretic peptide

CHF: congestive heart failure

cGMP: cyclic guanosine monophosphate

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GFR: glomerular filtration rate

LVEDP: left ventricular end diastolic pressure

NEP: neutral endopeptidase

PDE: phosphodiesterase

Recommended section assignment:

Cardiovascular

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Abstract

In heart failure, the renal responsiveness to exogenous and endogenous atrial natriuretic peptide (ANP) is blunted. The mechanisms of renal hyporesponsiveness to ANP are complex, but one potential mechanism is decreased expression of natriuretic peptide receptor-A (NPR-A) in the inner medullary collecting duct (IMCD) cells. Newly emerging evidence shows that glucocorticoids could produce potent diuresis and natriuresis in patients with heart failure, but the precise mechanism is unclear. In the present study, we found dexamethasone (Dex) dramatically increased the expression of NPR-A in the IMCD cells in vitro. The NPR-A overexpression induced by Dex presented in a time- and dose-dependent manner, which emerged after 12 hours and peaked after 48 hours. The cultured IMCD cells were, then, stimulated with exogenous rat ANP. Consistent with the findings with NPR-A expression, Dex greatly increased cyclic guanosine monophosphate (cGMP, the second messenger for the ANP) generation in the IMCD cells, which presented in a time- and dose-dependent manner as well. In rats with decompensated heart failure, Dex dramatically increased NPR-A expression in inner renal medulla which was accompanied by a remarkable increase in renal cGMP generation, urine flow rate, and renal sodium excretion. Of note, Dex dramatically lowered plasma ANP, cGMP levels and left ventricular end diastolic pressure. These favourable effects induced by Dex were glucocorticoid receptor (GR) mediated and were abolished by the GR antagonist RU486. Collectively, glucocorticoids could improve renal responsiveness to ANP by upregulating NPR-A expression in the IMCD and induce a potent diuretic action in decompensated heart failure rats.

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Introduction

Atrial natriuretic peptide (ANP), a peptide hormone secreted by the atrial myocytes in response to volume expansion, provides a potent mechanism counterbalancing the salt- and water-retaining actions of the renin–angiotensin–aldosterone system in the body fluid control. The ANP and its primary receptor, natriuretic peptide receptor A (NPR-A), were found in the kidney and hypothalamus. Systemic ANP administration activates renal and hypothalamic NPR-A. Renal NPR-A activation produces a potent diuresis and natriuresis while hypothalamic NPR-A activation inhibits water drinking and salt intake (Antunes-Rodrigues et al., 2004; Potter et al., 2006). Volume depletion ensues. It was predicted that ANP and its analogues would be useful in the treatment of congestive heart failure. However, their favorable diuretic and natriuretic effects were blunted in heart failure (Cody et al., 1986; Munzel et al., 1991; Schrier and Abraham, 1999). The failure of natriuretic peptides (NPs) to reverse salt and water retention in heart failure is very likely due to the tonic effect of counterregulatory systems (Tsutamoto et al., 1993; Schrier and Abraham, 1999; Potter et al., 2006). The discovery of ANP-sensitizing reagents is anticipated and will be of extraordinary importance in treatment of heart failure (Potter et al., 2006).

Traditional teaching indicates that glucocorticoids cause hypertension by sodium and water retention. However, newly emerging evidence overthrew this notion and demonstrated that the rise in blood pressure is very likely due to the increased pressor responsiveness induced by glucocorticoids (Turner et al., 1996; Wallerath et al., 1999; Whitworth et al., 2000). Contrary to the notion that glucocorticoids cause renal water and sodium retention, recent evidence showed glucocorticoid administration could produce potent diuresis and natriuresis in rats (Thunhorst et al., 2007; Liu et al., 2010). It has also been found, in clinical observations, chronic glucocorticoid administration could produce potent diuresis and restore body fluid homeostasis in heart failure patients with severe fluid overload (Liu et al., 2006; Liu et al., 2007; Zhang et al., 2008; Massari et al., 2011). Previous evidence showed that glucocorticoids could upregulate ANP mRNA expression in the atrial myocytes and increase the ANP levels in the circulation (Gardner et al., 1988;

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Dananberg and Grekin, 1992). But it cannot account for the potent diuretic effect induced by glucocorticoids in decompensated congestive heart failure (CHF), because ANP loses its favorable effects in this situation (Cody et al., 1986; Schrier and Abraham, 1999; Cotter et al., 2008). Recently, we demonstrated that glucocorticoids not only produced a potent diuretic effect, but inhibited dehydration-induced water intake and sodium depletion-induced sodium intake by upregulating the expression of NPR-A in the hypothalamus, resulted in an overt volume depletion (Liu et al., 2010). It is reasonable to assume that the natriuretic action of glucocorticoids in the kidney is also mediated by NPR-A activation, which is matched physiologically by hypothalamic NPR-A activation to produce the volume depleting effect. The inner medullary collecting duct (IMCD), as the terminal nephron segment of the kidney, is the target of natriuretic peptide system that promotes sodium excretion (Koseki et al., 1986; Light et al., 1990). We raised a hypothesis that glucocorticoids may upregulate the NPR-A expression in the IMCD cells, thus producing a potent diuresis and natriuresis. To elucidate the precise mechanism underlying this protective renal effect, we designed this study.

Methods and materials

Chemicals.

Dexamethasone, mifepristone/RU486, ANP 127-150 rat (Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH), collagenase used to digest inner medullary tissue, 3-isobutyl(1-methyl)-xanthine used in ANP stimulation test to inhibit phosphodiesterase activity, and inulin used to calculated glomerular filtration rate (GFR) were provided by Sigma-Aldrich (Sigma-Aldrich Co, China.). Ethylenediaminetetraacetic acid and aprotinin was kindly provided by Beijing North Institute of Biological Technology. Dexamethasone sodium phosphate used in rats with decompensated CHF was kindly provided by CSPC Pharma (CSPC Pharma, China).

Animals

Male Wistar rats weighing 220-250g (provided by Hebei Medical University) were housed in a

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temperature-controlled environment, exposed to a 12:12-h light-dark cycle, and given a commercial standard diet of rat chow and water ad libitum. All animals were managed in accordance with the guidelines of the Hebei Medical University, and the position of the American Heart Association on Research Animal Use. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University.

Isolation and culture of IMCD cells

The IMCD cells from Wistar rats were isolated and cultured using the method previously described (Ye et al., 2003). In brief, Adult Wistar male rats were killed by cervical dislocation after CO_2 narcosis. The inner medullary tissue from each kidney was dissected free from the outer medulla, minced, and digested with 1 mg/mL collagenase at 37°C with gentle agitation during each 30 minutes cycle. These IMCD cells were enriched in the preparation using hypotonic lysis as described previously (Grenier et al., 1981). The cells were re-suspended in Dulbecco's Modified Eagle Medium and seeded on to culture plates. After 24 hours, the cells were placed in Dulbecco's Modified Eagle Medium and cultured for 5 to 7 days.

ANP stimulation test

Natriuretic peptides (NPs) elicit their physiological responses through synthesis of cyclic guanosine monophosphate (cGMP) when binding to their receptors. Therefore, cGMP levels in the renal cells serves as a useful biological marker for the renal activity of ANP in vitro (Ballermann et al., 1985; Cole et al., 1989). The ANP stimulation test was performed by the method previously prescribed (Kanda et al., 1989). Briefly, the IMCD cells were cultured with 96 Well Cell Culture Plates. The number of cells ranged between 8×10^4 and 10×10^4 cell/well. The cells were preincubated with 2.0 mmol/L 3-isobutyl(1-methyl)-xanthine (Sigma-Aldrich) for 10 min to inhibit phosphodiesterase activity, and then stimulated with increasing concentrations of rat ANP (10^{-10} to 10^{-7}mol/L) for 10 min at 37°C under constant shaking. The incubations were terminated by removal of medium and addition of lysis buffer (cGMP HTS Immunoassay Kit, Millipore Corporation). The plates were kept at room temperature for 10 min to allow intracellular cyclic nucleotide to be extracted. Cyclic

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GMP levels in supernatant that was obtained after centrifugation at 4°C were then assayed.

Western blotting analysis

Western blotting measurements were performed using the method previously described (Santos-Araujo et al., 2009)**.** The NPR-A antibody (ab70848) was provided by Abcam Inc. Protein bands were digitally analyzed with Quantity One 1-D Analysis Software (Bio-Rad). The densities were expressed as a relative value compared with the average density measured in the vehicle treated rats. β-Actin was used as a loading control.

Surgical procedure:

The post-infarction rat model of heart failure used in the study was prepared by the standard method previously prescribed (Trueblood et al., 2005). Briefly, rats were anesthetized, incubated, and ventilated with a rodent ventilator. A left thoracotomy was performed in the fourth intercostal space to expose the heart and the pericardial sac was gently removed. The left coronary artery was visualized and ligated between the left atrial appendage and the right ventricular outflow tract with a 6-0 silk suture. Ligation of the coronary artery was confirmed by noting the pallor of the left ventricular wall. The chest wall was then closed and sutured in layers, and the air was evacuated by slight lateral pressure of the thorax. The survived rats were raised for 12 weeks to have decompensated CHF (Supplemental table 1) .

Analysis of blood and urine samples.

Plasma ANP and cGMP were measured by radioimmunoassay. Blood samples were collected into chilled plastic tubes containing ethylenediaminetetraacetic acid (1.5 mg/ml) and aprotinin (500 KIU/ml). Blood samples were centrifuged at 4°C, and the plasma samples were separated and stored at -70°C until assayed for these peptides using radioimmunoassay. One milliliter of plasma was extracted on a C_{18} SEP Pak column for measurement for ANP. The extracts were dried down and reconstituted in 1.0 ml buffer and measured by radioimmunoassay with use of ANP radioimmunoassay kits (North Institute of Biological Technology, Beijing).

Plasma cGMP was measured with a radioimmunoassay kit (Shanghai University of T.C.M.) after

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ethanol extraction. Urinary cGMP was determined directly using the radioimmunoassay kit after 1:3000 dilution.

Hemodynamic study

Rats were anesthetized and left carotid artery was exposed, and cannulated with a fluid-filled polyethylene catheter that was connected to a pressure transducer. The fluid-filled catheter was advanced through the carotid artery across the aortic valve into the left ventricular chamber. Hemodynamic data were analyzed off-line with PowerLab (Model ML 870, ADInstruments, Australia).

Measurement of GFR

The right femoral artery was cannulated for infusion of 8% inulin in isotonic saline solution, at a rate of 2 ml/h, as described previously (Zhang et al., 1994). Urine samples were analyzed by spectrophotometry for calculation of GFR. Renal cGMP production was calculated as follows (Stevens et al., 1994): Renal cGMP generation = (Urinary flow×Urinary cGMP concentration) - (GFR×Plasma cGMP concentration).

Statistical analyses

All the data were express as mean ± standard error of mean (s.e.m). Differences between groups were evaluated with the standard *t*-test, the Wilcoxon rank sum test, as indicated in the figure legends. Data in ANP stimulation test were analyzed by two-way repeated measures ANOVA, followed by a Student-Newman-Keuls test for multiple comparisons.

Results

The effect of glucocorticoids on NPR-A expression in IMCD cells.

Treatment with dexamethasone (Dex, Sigma-Aldrich) for 48 hours dramatically upregulated NPR-A expression in IMCD cells in a concentration-dependent manner (Fig. 1A). The NPR-A expression in IMCD cells was dramatically increased by Dex after 24-hour Dex treatment (Dex, 10^{-7} mol/L) and peaked at 48 hours (Fig 1B). To test whether the effect of Dex on NPR-A was glucocorticoid

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receptor (GR) mediated, IMCD cells were treated with mifepristone/RU486 (Sigma-Aldrich) alone or coadministration with Dex. RU486 alone had no effect on NPR-A expression in the IMCD cells (Fig 1C). The effect of Dex (10^{-7} mol/L) on NPR-A expression in IMCD cells was completely inhibited by GR antagonist, RU486 (10^{-6} mol/L), indicating it was GR-mediated (Fig 1C).

The effect of glucocorticoids on cGMP content in IMCD cells.

To test whether the upregulation in NPR-A expression induced by Dex was associated with an increase in its action, we conducted the ANP stimulation test. After 48-hour treatment, IMCD cells were stimulated by rat ANP (Sigma-Aldrich), and cGMP levels in IMCD cells were measured. It showed that cGMP levels were well correlated with the NPR-A expression induced by Dex in IMCD cells, presenting in a dose- and time-dependent manner as well.

After stimulation with increasing concentration of ANP, the IMCD cells treated with higher concentration of Dex had much higher levels of cGMP than did the IMCD cells treated with lower concentration of Dex or vehicle (n = 5 in each point, $P<0.01$; Fig 2A). Then, the IMCD cells with increasing time period of Dex (10^{-7} mol/L) treatment were stimulated with ANP (10^{-7} mol/L). The data showed that cGMP contents in IMCD cells dramatically increased with Dex-treated time and peaked after 48-hour Dex treatment (n = 8 in each group, $P<0.01$; Fig 2B). Finally, RU486 (10^{-6} mol/L) completely abolished the effect of 48-hour Dex (10^{-7} mol/L) treatment on cGMP contents in IMCD cells (n = 8 in each group; Fig 2C).

The effect of glucocorticoids on NPR-A expression in inner renal medulla, renal cGMP generation, plasma ANP and plasma cGMP.

To determine whether glucocorticoids can potentiate ANP's action in the kidney in rats with heart failure by upregulation the NPR-A expression in the kidney, Wistar rats with decompensated CHF were randomized to receive dexamethasone sodium phosphate (Dex, 1.0mg/kg), vehicle, RU486 (100mg/kg) + Dex or RU486 (100mg/kg) alone.

Consistent with the findings in vitro, western blotting analysis showed Dex dramatically increased NPR-A expression in inner renal medulla after 24-hour treatment (Fig 3A). Consequently, Dex

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greatly increased renal cGMP generation (n = 6-7 in each group, P<0.01; Fig 3B); whereas Dex remarkably reduced plasma ANP levels and plasma cGMP levels (n = 6-7 in each group, P<0.01; Fig 3C, D). The effect of Dex on renal cGMP generation, plasma cGMP, and plasma ANP levels were abolished by RU486 (100mg/kg) (Fig 3B, C, D).

The effect of glucocorticoids on urine flow rate, renal sodium excretion and GFR.

After 24-hour treatment, Dex greatly increased urine flow rate (n = 6-7 in each group, P<0.001; Fig 4A) and renal sodium excretion (n = 6-7 in each group, P<0.01; Fig 4B), whereas it had no significant effect on GFR (n = 6-7 in each group, P = 0.88; Fig 4C).

The effect of glucocorticoids on the diuretic effect and left ventricular end diastolic pressure (LVEDP).

To test whether the effect of glucocorticoids on renal NPR-A expression could be translated to a real diuretic action in vivo. Rats with decompensated CHF were randomized to receive Dex (1mg/kg) or vehicle. During 24-hour treatment period, Dex doubled urinary volume and dramatically increased renal sodium excretion in rats with heart failure (n = 10 in each group, P<0.01; Fig 5A, B). Of note, Dex-treated animals did not drink more water than vehicle-treated animals despite having much higher urine volumes (n = 10 in each group, P = 0.73; Fig 5C). Dexamethasone, therefore, produced potent volume depleting effect in rats with heart failure. As a result, LVEDP, a known index of left ventricular end-diastolic filling status, was dramatically lowered (n = 7-8 in each group, P<0.01; Fig 5D).

Discussion

In summary, glucocorticoids could upregulate the NPR-A expression in IMCD cells both in vitro and vivo. Consequently, glucocorticoids greatly increased renal cGMP generation, urine flow rate, and renal sodium excretion in rats with decompensated CHF; whereas dramatically reduced plasma ANP levels and plasma cGMP levels.

The inner medullary collecting duct is the terminal nephron segment of the kidney, and the target of ANP that promotes sodium excretion (Koseki et al., 1986; Light et al., 1990). The ANP, acting

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through cGMP, reduces Na^+ absorption across the IMCD (Light et al., 1990). In chronic situation such as CHF, patients are resistant to the natriuretic effect of ANP. Increased ANP level is proportional to the severity of heart failure. The increased ANP levels cannot induce potent diuretic effects to excrete the excessively retained sodium and water, and decompensated CHF occurs. The mechanisms of renal hyporesponsiveness to the ANP in CHF are complex, including diminished NPR-A expression in the IMCD segment (Yechieli et al., 1993); decreased ANP delivery to the IMCD segment by increased enzymatic degradation of ANP by neutral endopeptidase (NEP) in the proximal tubule (Margulies et al., 1995); decreased sodium delivery to the collecting duct (Abraham et al., 1995); or increased activity of cGMP phosphodiesterases (PDEs) (Supaporn et al., 1996). Theoretically, any agents targeting above mechanisms could partly reverse ANP resistance and produce a potent diuresis in heart failure. It has been reported that either NEP inhibition (Wilkins et al., 1990), or increasing distal tubular sodium delivery (Abraham et al., 1995), or PDE inhibition (Chen et al., 2006) could reverse ANP resistance and produce potent diuresis and natriuresis in heart failure. But, there was no report that any reagent could increase NPR-A expression in the IMCD segment, and produce a potent diuretic effect. Dexamethasone could upregulate NPR-A expression in IMCD, improve renal responsiveness to endogenous ANP, result in a potent diuresis in decompensated CHF, and finally restore the homeostasis of body fluid. This is why Dex could decrease plasma ANP and cGMP levels in decompensated CHF. It is without doubt that our finding may provide a new therapeutic option for heart failure.

The role of glucocorticoids in body fluid metabolism is provocative. Traditionally, it is assumed that glucocorticoids may cause hypertension by water and sodium retention. But, large amount of data demonstrated that the rise in blood pressure is due to the increased pressor responsiveness induced by glucocorticoids (Handa et al., 1984; Turner et al., 1996; Wallerath et al., 1999; Whitworth et al., 2000). There is no relation between sodium retention and the rise in blood pressure (Whitworth et al., 2000). Moreover, studies from animals and humans have documented the potent diuretic effect induced by glucocorticoids presented. It was reported that glucocorticoids could potentiate renal

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responsiveness to acute volume expansion and restore body fluid hemostasis more quickly than did untreated rats by producing a potent diuresis and natriuresis (Nevskaia et al., 1977). In heart patients with severe fluid retention, glucocorticoids could induced potent diuretic effect and restore fluid hemostasis (Liu et al., 2006; Liu et al., 2007; Zhang et al., 2008; Massari et al., 2011). Recently, we demonstrated that glucocorticoids even produced potent diuresis in rats with overnight water deprivation (Liu et al., 2010). The data presented here coupled with our previous findings suggested glucocorticoids could potentiate ANP's action in body fluid control by upregulating NPR-A expression in the hypothalamus and kidney (Liu et al., 2010). The overall effect of glucocorticoids on body fluid is to have the body under systemic volume depletion (Thunhorst et al., 2007; Liu et al., 2010), which is consistent with the physiological effects of NPR-A activation on body fluid control. Our findings were also supported by data from humans. Damjancic reported glucocorticoids could potentiate renal responsiveness to ANP in humans, and therefore leading to a dramatic increase in natriuresis and diuresis (Damjancic and Vierhapper, 1990). Nevskaia reported that methylprednisolone induced potent diuretic effect in a patient with severe heart failure but lowered natriuretic peptide levels in the circulation at the same time, suggesting glucocorticoid might have a role in natriuretic peptide system (Massari et al., 2011).

Previous evidence showed that glucocorticoids increased blood pressure and increase GFR. There effects are also time-dependent, which begin to appear after one-day Dex treatment (Handa et al., 1984Handa et al., 1984; Aguirre et al., 1999). Our study did show that there was a trend that Dex may slightly raise blood pressure and GFR, but there were no statistically significant differences between Dex-treated rats and vehicle-treated rats due to short treatment period and small sample size (supplemental data).

There are several limitations to this study. First, the focus of the study was on the IMCD that is thought to be the primary site for ANP's action. However, the role of glucocorticoids on other parts of the nephron remains to be determined by future studies. Second, even though the glucocorticoids' effect on collecting duct was confirmed by western blotting analysis and renal cGMP generation

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study, the inner medulla in heart failure model used to perform western blotting analysis mainly (but not only) contains collecting duct, which may compromise our findings. Third, our findings were based on the rat model and should not be extrapolated to humans.

Collectively, the data suggested that glucocorticoids could induce a potent diuretic action in the rats with decompensated CHF, which was associated with a dramatic increase in NPR-A expression in IMCD cells. Future studies are warranted to confirm whether glucocorticoids have the same effect on the renal NPR-A expression in humans.

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Authorship Contributions.

Participated in research design: C. Liu and K. Liu

Conducted experiments: C. Liu, Y. Chen, Y. Kang, Z. Ni, H. Xiu and J. Guan

Performed data analysis: C. Liu and K. Liu

Wrote or contributed to the writing of the manuscript: C. Liu and K. Liu

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Legends for Figures

Figure 1. The effect of glucocorticoids on NPR-A expression in IMCD cells. (A) Concentration dependent effect of Dex on NPR-A expression after 48-hour treatment; (n = 4 in each group); * $P < 0.05$. **(B)** Time dependent effect of Dex (10^{-7} mol/L) on NPR-A expression; (n = 4 in each group); * $P < 0.05$. **(C)** Effect of Dex (10^{-7} mol/L) on NPR-A expression with or without RU486 (10^{-6} mol/L); (n = 4 in each group); * $P < 0.05$.

Figure 2. The effect of glucocorticoids on cGMP content in IMCD cells. (A) Concentration dependent effect of Dex on cGMP production; each point represents means±s.e.m of 5 wells; **P<0.01 compared with cells treated with Dex (-); **P <0.01 compared with cells treated with Dex 10^{-8}mol/L. **(B)** Time dependent effect of Dex (10^{-7} mol/L) on cGMP production in IMCD cells (stimulated by ANP [10^{-7} mol/L]); n = 8 wells in each group; **P<0.01 compared with 0 h. **(C)** Effect of Dex (10^{-7} mol/L) on cGMP production with or without RU486 (10^{-6} mol/L) in IMCD cells (stimulated by ANP [10^{-7} mol/L]); n = 8 wells in each group; **P<0.01 compared with vehicle.

Figure 3. ***The effect of glucocorticoids on NPR-A expression in inner renal medulla, renal cGMP generation, plasma ANP and plasma cGMP.*** **(A)** Effect of glucocorticoids on NPR-A expression in IMCD cells in renal medulla (visualized by western blotting); **P<0.01 compared with rats treated with vehicle. **(B)** Effect of glucocorticoids on renal cGMP generation; n = 6-7 in each group; data were analyzed by the Wilcoxon rank sum test; **P<0.01 compared with rats treated with vehicle. **(C)** Effect of glucocorticoids on plasma ANP; n = 6-7 in each group; data were analyzed by the Wilcoxon rank sum test, **P<0.01 compared with rats treated with vehicle. **(D)** Effect of glucocorticoids on plasma cGMP; n = 6-7 in each group; data were analyzed by the Wilcoxon rank sum test, **P<0.01 compared with rats treated with vehicle.

Figure 4. The effect of glucocorticoids on urine flow rate, renal sodium excretion and GFR. (A)

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Effect of glucocorticoids on urine flow rate; n = 6-7 in each group; data were analyzed by the standard *t*-test, **P<0.01 compared with rats treated with vehicle. **(B)** Effect of glucocorticoids on renal sodium excretion; n = 6-7 in each group; data were analyzed by the standard *t*-test, **P<0.01 compared with rats treated with vehicle. **(C)** Effect of glucocorticoids on GFR; n = 6-7 in each group; data were analyzed by Wilcoxon rank sum test.

Figure 5. ***The effect of glucocorticoids on the diuretic effect and LVEDP.*** **(A)** Effect of glucocorticoids on urinary volume; n = 10 in each group; data were analyzed by the standard *t*-test, **P<0.01 compared with rats treated with vehicle. **(B)** Effect of glucocorticoids on urinary sodium excretion; n = 10 in each group; data were analyzed by the standard *t*-test, **P<0.01 compared with rats treated with vehicle. **(C)** Effect of glucocorticoids on water intake; n = 10 in each group; data were analyzed by the standard *t*-test. **(D)** Effect of glucocorticoids on LVEDP; n = 7-8 in each group; data were analyzed by the standard *t*-test; **P<0.01 compared with rats treated with vehicle.

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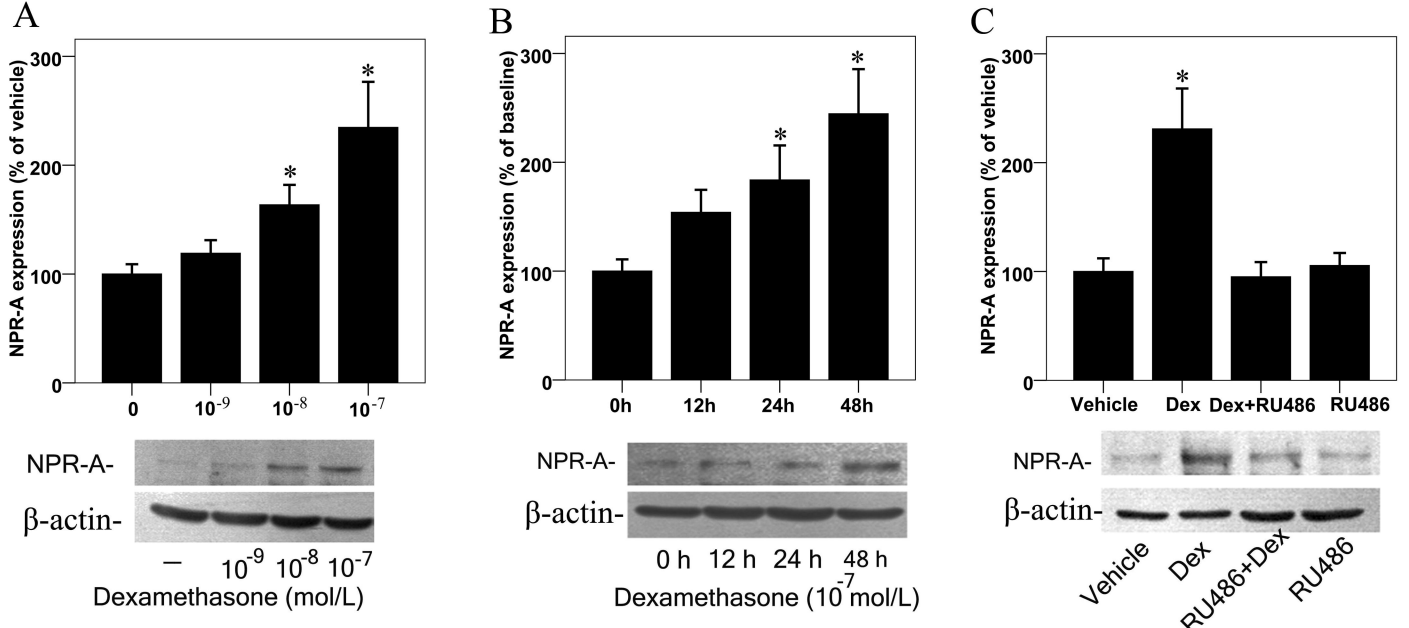
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Figure 1

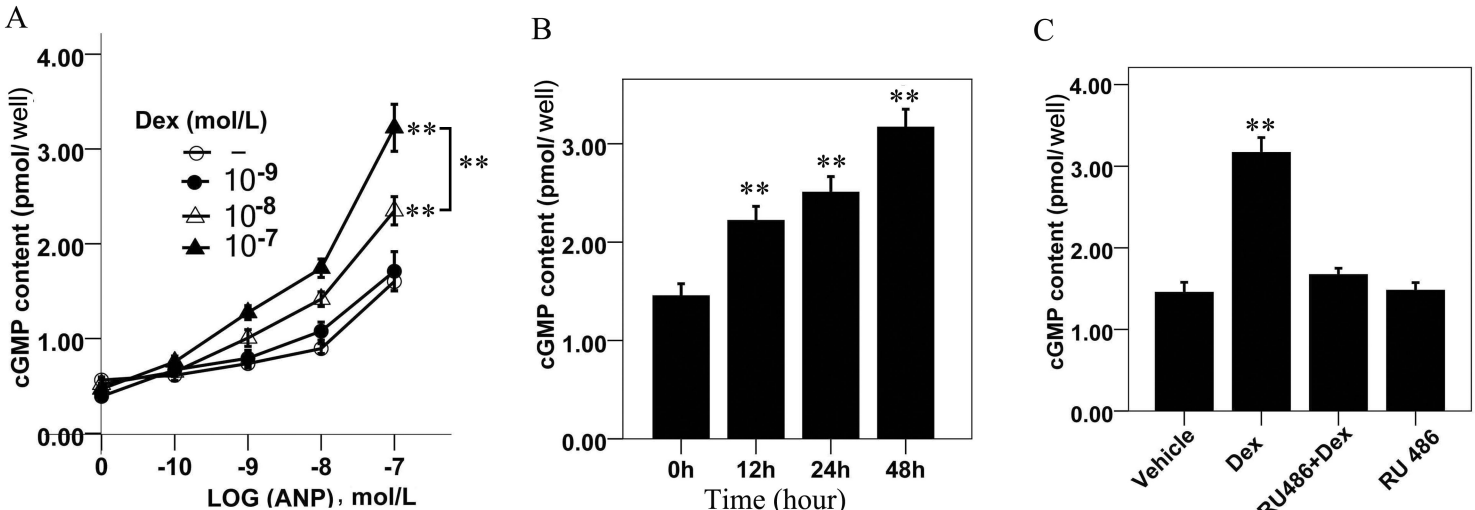
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Figure 2

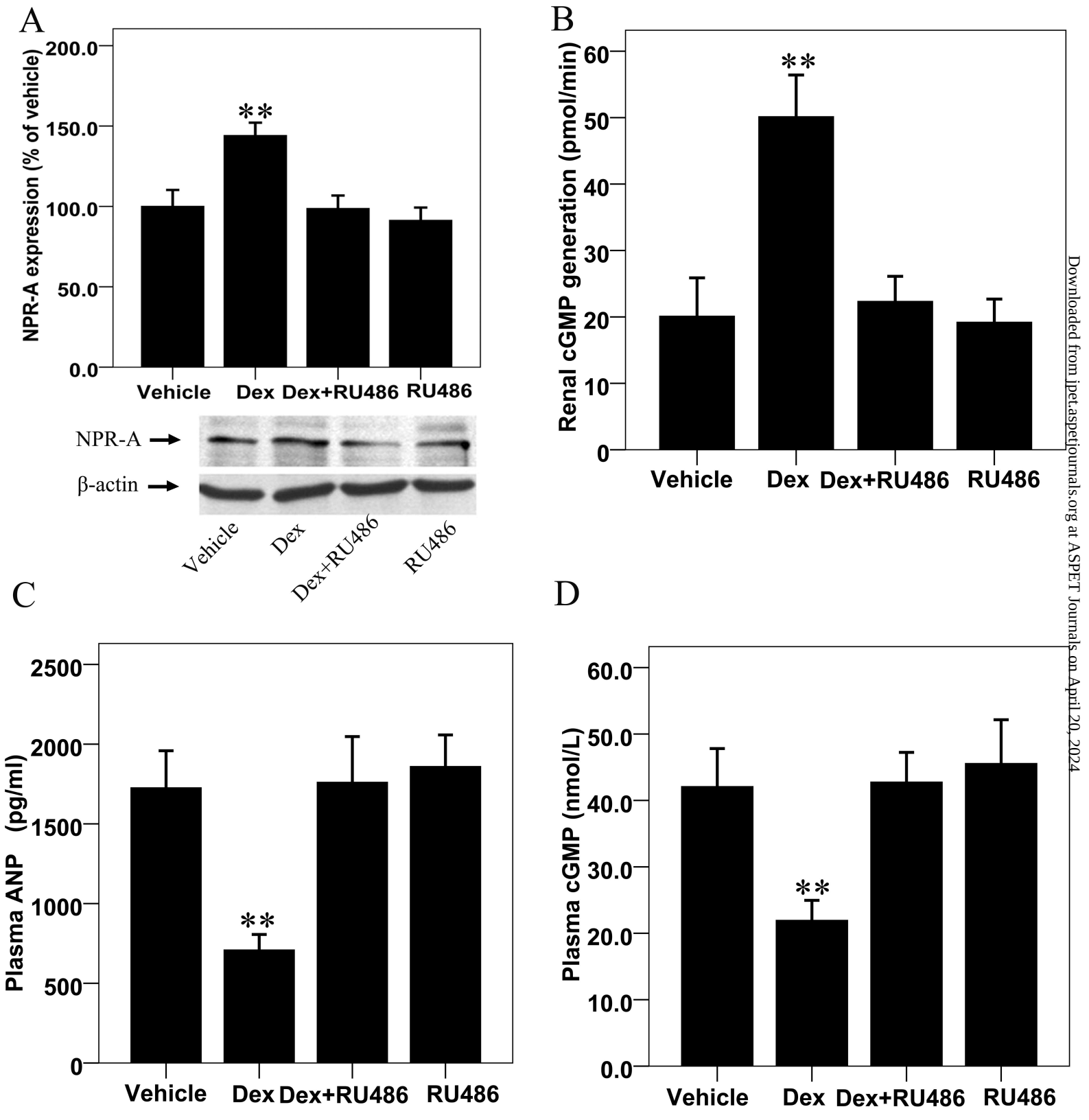

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Figure 3

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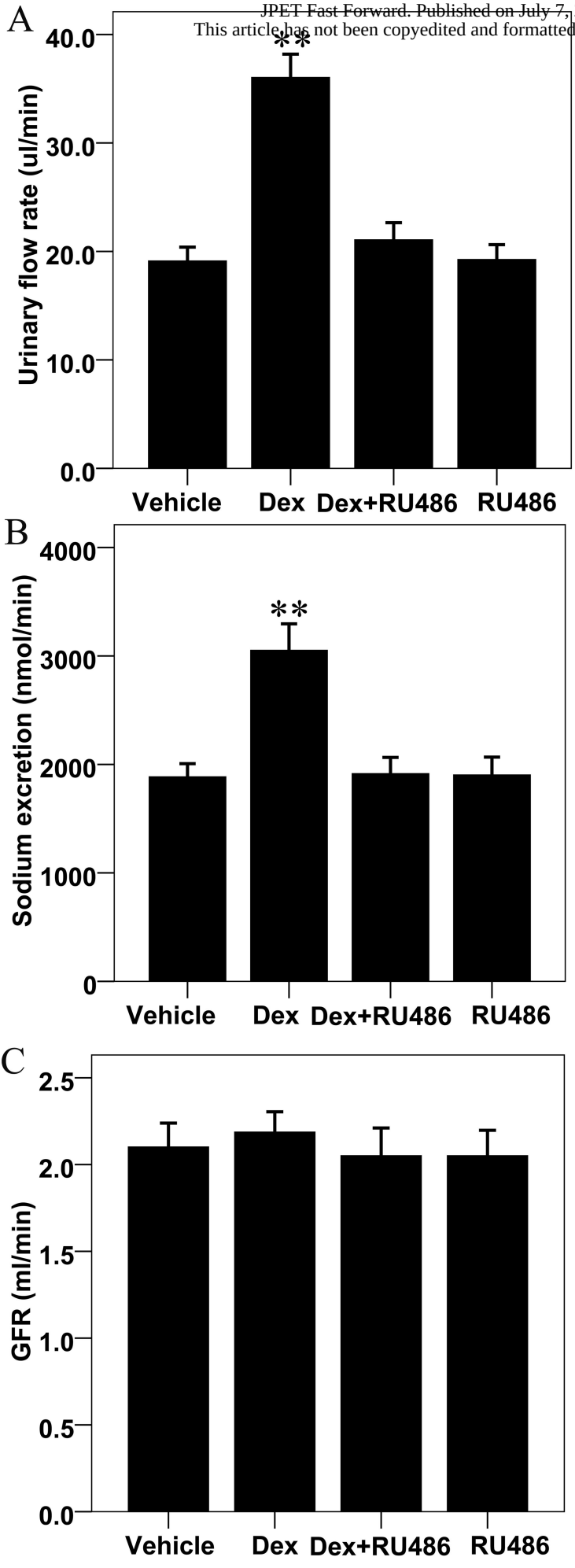

Figure 4

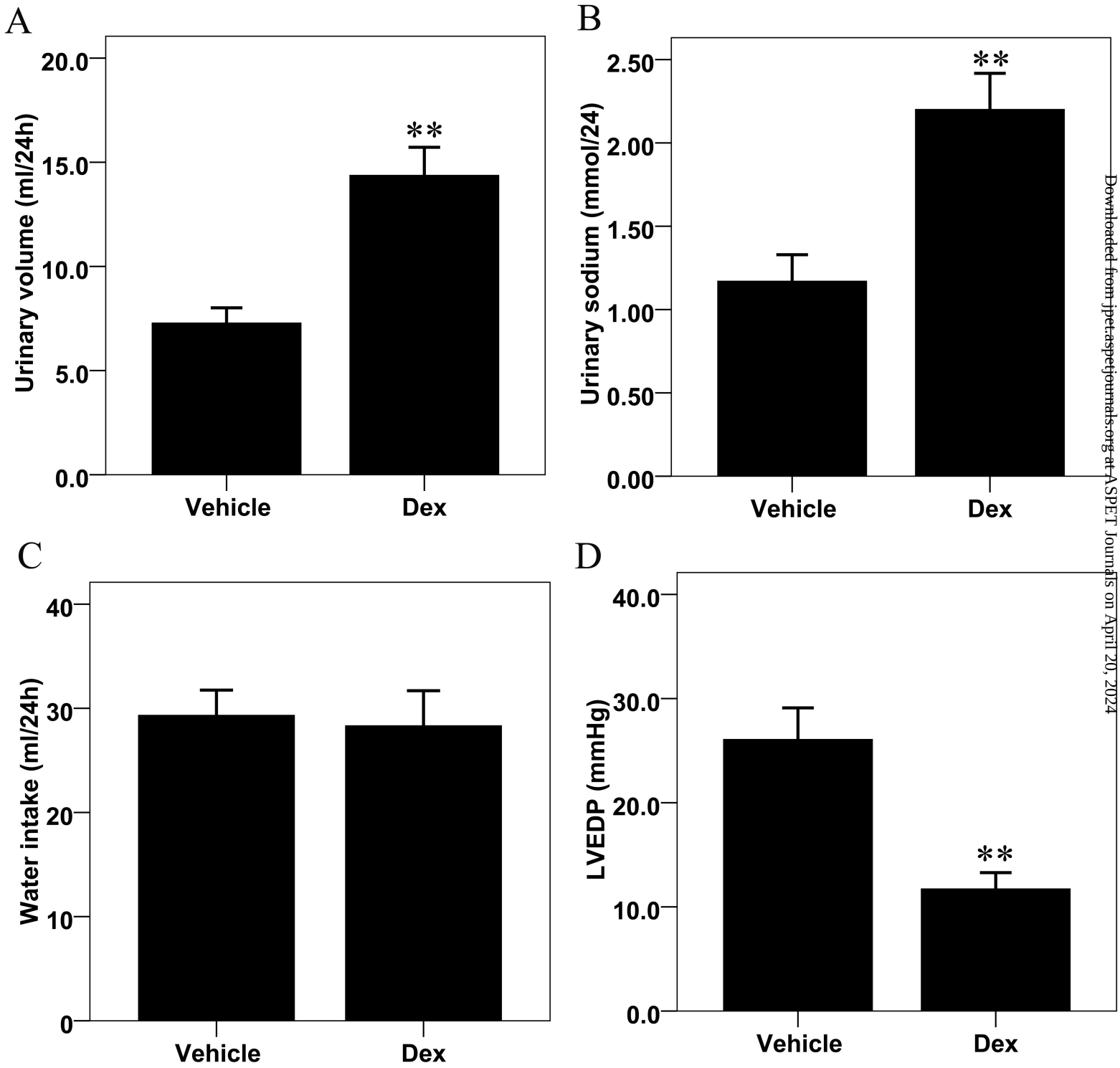

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Figure 5