Cardiotonic Steroids Induce Vascular Fibrosis Via Pressure-Independent Mechanism in NaCl-Loaded Diabetic Rats

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Abstract

Endogenous cardiotonic steroid, marinobufagenin (MBG), induces Fli1-dependent tissue fibrosis. We hypothesized that an increase in MBG initiates the development of aortic fibrosis in salt-loaded rats with type 2 diabetes mellitus (DM2) via pressure-independent mechanism. DM2 was induced by a single intraperitoneal administration of 65 mg/kg streptozotocin to neonatal (4–5 days) male Wistar rats. Eight-week-old DM2 rats received water or 1.8% NaCl (DM-NaCl) solution for 4 weeks (n = 16); half of DM-NaCl rats were treated with anti-MBG monoclonal antibody (mAb) (DM-NaCl-AB) during week 4 of salt loading; control intact rats received water (n = 8/group). Blood pressure, MBG, erythrocyte Na/K-ATPase activity, aortic weights, levels of fibrosis markers (Fli1, protein kinase Cδ, transforming growth factor-β1, receptors of the transforming growth factor beta5, fibronectin, collagen-1), and sensitivity of the aortic explants to the vasorelaxant effect of sodium nitroprusside were assessed. No changes in systolic blood pressure were observed while erythrocyte Na/K-ATPase was inhibited by 30%, plasma MBG was doubled, and aortic markers of fibrosis became elevated in DM-NaCl rats versus control. Treatment of DM-NaCl rats with anti-MBG mAb activated Na/K-ATPase, prevented increases in aortic weights, and the levels of fibrosis markers returned to the control levels. The responsiveness of the aortic rings from DM-NaCl rats to the relaxant effect of sodium nitroprusside was reduced (half maximal effective concentration (EC₅₀) = 29 nmol/L) versus control rings (EC₅₀ = 7 nmol/L) and was restored by anti-MBG mAb (EC₅₀ = 9 nmol/L). Our results suggest that in salt-loaded diabetic rats, MBG stimulates aortic collagen synthesis in a pressure-independent fashion and that 2 profibrotic mechanisms, Fli1 dependent and transforming growth factor-β dependent, underlie its effects.

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The authors report no conflicts of interest.
INTRODUCTION

Marinobufagenin (MBG) is an endogenous ligand of the Na/K-ATPase and a natriuretic hormone that belongs to the class of cardiotonic steroids.\(^1,^2\) MBG is an inhibitor of \(\alpha_1\) Na/K-ATPase that is produced primarily by the adrenal cortex in response to high NaCl intake.\(^3,^4\) MBG causes renal sodium reabsorption via the inhibition of Na/K-ATPase in the proximal tubuli and thick ascending limb of Henle’s loop.\(^3,^5,^6\) Vascular smooth muscle Na/K-ATPase is another target for MBG that promotes increased intracellular sodium and subsequently calcium via Na/Ca exchange.\(^3,^5\) Increased MBG levels were shown to accompany elevated arterial pressure in essential hypertension,\(^7\) myocardial infarction,\(^8\) heart failure,\(^9\) preeclampsia,\(^10\) and chronic renal failure.\(^11\) Recently, we demonstrated that in rats with renal failure\(^11\) and in patients with preeclampsia,\(^10\) MBG, acting via Na/K-ATPase, stimulates synthesis of collagen by protein kinase C\(\delta\)-dependent mechanism involving downregulation of Fli1. Fli1 is a nuclear transcription factor that inhibits the promoter of Col1 gene and acts as a negative regulator of collagen synthesis.\(^12\) In preeclampsia, elevated plasma MBG levels are associated with impairment of ex vivo vasorelaxation of the umbilical arteries.\(^10\) Culturing of the explants of the human umbilical arteries from the healthy subjects with MBG in nanomolar concentration resulted in the reduction of Fli1 level and the increase of collagen-1 abundance.\(^10,^12\) This is accompanied by the loss of sensitivity of arterial rings to the vasorelaxant effect of sodium nitroprusside (SNP) following endothelin-1-induced contracture.\(^10,^13\) In rats, following subtotal nephrectomy, elevated MBG led to a reduction in the level of Fli1 and an increase in collagen-1 level in myocardium.\(^11\) A single administration of monoclonal (clone 3E9) anti-MBG antibody (mAb) to uremic rats produced an anti-fibrotic effect, that is, restored Fli1 levels and significantly reduced collagen-1 abundance in the myocardium.\(^11\)

Dysregulation of many cellular regulatory systems, including Na/K-ATPase, is implicated in the pathogenesis of diabetes mellitus (DM).\(^14,^15\) DM is associated with renal sodium retention, and the diabetic subjects are prone to NaCl-sensitive hypertension.\(^16–18\) Altered expression of the Na/K-ATPase and enhanced levels of MBG have been reported in the patients with type 1 and type 2 DM, as well as in the rats with streptozotocin (STZ)-induced DM.\(^19\) Previously, it has been demonstrated that blood pressure (BP) did not increase in patients with type 2 DM (DM2) in the presence of the elevated level of digoxin-like immunoreactive factor.\(^20,^21\)

Antifibrotic effect of anti-MBG mAb in uremic rats,\(^11\) in rats with pregnancy-induced hypertension,\(^22\) and in hypertensive Dahl rats\(^23\) was associated with a simultaneous decrease in arterial pressure. However, in other models, for example in the Wistar\(^23\) and Spargue-Dawley\(^24\) normotensive rats, this association was pressure independent. Two signaling pathways were shown to modulate profibrotic effects of MBG. Profibrotic effects initiated
by MBG may be receptors of the transforming growth factor beta (SMAD) and transforming growth factor (TGF)-β1 dependent and underlie vascular fibrosis in salt-induced normotensive and hypertensive rats.23–25 Another system, the inhibition of Fli1, a nuclear transcription factor and a member of E-twenty-six transcription factors family family is implicated in MBG-induced fibrosis in preeclampsia and renal failure.11,26,27 The rationale for the present study was to explore (1) whether heightened MBG level underlies profibrotic signaling in the vascular wall in DM2 and (2) whether this mechanism is pressure independent in DM2 NaCl-loaded rats.

METHODS

Study Design

The experimental design of the rat DM2 model was approved by the Institutional Animal Care and Use Committee of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences.

Experiment 1: Effects of High Salt Intake, DM2, and Their Combination

Four groups (n = 8 per group) of 8-week-old Wistar male rats were included in this experiment. The DM2 was induced by a single intraperitoneal injection of 65 mg/kg STZ to sixteen 4–5 days old rats.26 The control animals consumed tap water (Ctrl group; n = 8) or hypertonic 1.8% saline (Ctrl + NaCl group; n = 8) for 4 weeks, and DM2 rats consumed tap water (STZ group; n = 8) or 1.8% saline (STZ + NaCl; n = 8) for 4 weeks. Glucose oral tolerance test was used as described previously at 8 weeks of age.20 Rats from all 4 groups were fasted overnight, in the morning they were given glucose solution orally (5% glucose, 2 g/kg per body weight), and the blood glucose levels were estimated before and 15, 30, 60, and 120 minutes after glucose administration. All animals in STZ groups have developed DM2. Salt loading was started in 8-week-old animals by replacing the drinking water with 1.8% NaCl saline as reported previously27 and continued for 4 weeks.

Experiment 2: Effect of Anti-MBG Monoclonal Antibody and MBG Signaling

Three groups of 8-week-old Wistar male rats were included in this experiment. Rats without DM2 on tap water intake were used as controls (Ctrl group, n = 8). DM2 was developed as in experiment 1. Rats were placed on a high NaCl intake by drinking 1.8% saline instead of water for 4 weeks (NaCl group). DM2 animals were intraperitoneally administered vehicle (STZ + NaCl group, n = 8) or anti-MBG mAb (STZ + NaCl + AB group; n = 8) 3 times during the last week (week 4) of the experiment; the total anti-MBG mAb dose was 50 μg/kg of body weight.27

Blood Pressure and Urinary Electrolytes

BP was recorded by tail-cuff plethysmography (IITC Inc, Life Science, Woodland Hills, CA) in conscious animals at week 4 of both experiments 1 and 2. All animals were placed in metabolic cages for 24 hours for urine collection and water or 1.8% saline intake estimation at the end of week 4, after which the rats were euthanized by abdominal exsanguination under deep anesthesia with ketamine 100 mg/kg and xylasine 10 mg/kg. Erythrocytes were used for the measurement of Na/K-ATPase activity.27 Plasma was collected for insulin
(Cayman Chemical, Ann Arbor, MI), MBG, and creatinine measurements. MBG was estimated in 24-hour urine using the competitive immunoassay based on 4G4 monoclonal anti-MBG antibody. Concentration of urinary Na\(^+\) was measured with Roche-Hitachi 917 flame photometry (Roche, Vienna, Austria). Urinary creatinine was measured with a creatinine assay kit (Cayman Chemical). Plasma electrolytes and creatinine were measured by an i-Stat analyzer (Abbott Laboratories, Abbott Park, IL). Fractional Na\(^+\) excretion FENa was calculated as follows: FENa = uNa × pCr × 100/(pNa × uCr), where uNa and pNa are urine Na\(^+\) and plasma Na\(^+\) concentrations (mmol/L), uCr and pCr are urine creatinine and plasma creatinine concentrations (mmol/L), and expressed as percent.

**Tissue and Plasma Collection**

The rats were euthanized by exsanguination from the abdominal aorta under deep anesthesia by ketamine (100 mg/kg) and xylazine (5 mg/kg). Blood was collected in heparinized tubes (15.8 units of heparin per 1 mL of blood). Thoracic aortae were collected, and weights were expressed in milligrams per 100 g of body weight per millimeter of the total length. Two-millimeter aortic rings were used for contractile studies (below), the remains of the aortae were collected for Western blotting analysis (below).

**Isolated Rat Aorta Contractile Studies**

Vasorelaxation of the thoracic aorta was measured as described previously. In details, endothelium-denuded rings of rat thoracic aortae (2.0-mm wide) were suspended at a resting tension of 1.0 mN in a 15-mL organ bath (Isolated Organ Isometric Force 7003 Transducer; Ugo Basile, Gemonio, Italy) at 37°C in a solution containing in mmol/L of NaCl 130, KCl 4.0, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, NaH\(_2\)PO\(_4\) 0.4, NaHCO\(_3\) 19, and glucose 5.4 and gassed with a mixture of 95% O\(_2\) and 5% CO\(_2\) (pH 7.45); isometric contractions were recorded. The aortic rings were constricted twice with 80 mmol/L KCl, and after the washout, we studied the effect of increasing concentrations of a nitric oxide donor SNP (1 nmol/L−10 μmol/L) following constriction of vascular rings with 100 nmol/L endothelin-1 to investigate the role of anti-MBG mAb treatment, administered in vivo 1 week before the ex vivo studies, on the relaxation of aortae. The force of contractions was expressed as the percent of the vasoconstrictor response to 80 mmol/L KCl (Ugo Basile, Isolated Organ Baths system with 7006 Isometric Transducers and DataCapsule-Digital Recorder). The percent relaxation was calculated relative to the plateau of contractile force that was achieved in response to 100 nmol/L endothelin-1.

**Western Blotting**

The thoracic aortae were homogenized in cell lysis and protein extraction buffer buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Solubilized proteins were separated by 4%–12% Tris-glycine polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Health Care/Life Sciences, Pittsburgh, PA). The proteins were visualized using rabbit polyclonal anti-TGF-β–1 (1:500), anti-SMAD5 (1:250) antibodies (Cell Signaling Technology, Danvers, MA), goat polyclonal anti-collagen-1 (1:500) and anti-collagen-5 (1:200) antibodies (Southern Biotechnology, Birmingham, AL), goat polyclonal antifibronectin antibody (Santa Cruz Biotechnology, Inc), rabbit polyclonal anti-PKCδ(1:1000), anti-phospho-PKCδ-Ser643/676 (1:1000), and anti-Fli1 (1:100) antibodies.
(Santa Cruz Biotechnology, Inc) followed by the incubation with peroxidase-conjugated antimouse, antirabbit antiserum (GE Health Care/Life Sciences, 1:1000) or antigoat antiserum (Santa Cruz Biotechnology, Inc, 1:1000). Bands were visualized by 1–20 minutes of exposure of nitrocellulose membrane on Premium blue x-ray film (Fenix Research Products, Candler, NC), and optical density was quantified by the laser densitometry (version 5.0; Kodak Molecular Imaging Software, Rochester, NY). To normalize levels of proteins against levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), membranes were stripped and reprobed with a rabbit monoclonal anti-GAPDH antibody (Cell Signaling Technology, 1:1000).

**Immunoassays**

Heparinized plasma and urine samples (500 μL) were extracted on Sep-Pak C-18 cartridges (Waters, Milford, MA). MBG concentration was measured by competitive fluoroimmunoassay, based on a monoclonal murine anti-MBG 4G4 antibody, performed as described recently in detail.11 The cross-reactivity of 4G4 anti-MBG antibody was as follows: MBG, 100%; ouabain, 0.005%; digoxin, 0.03%; digitoxin, <0.001%; bufalin, 0.08%; cinobufagin, 0.07%; cinobufotalin, 40%; prednisone, <0.001%; spironolactone, <0.001%; proscillaridin, <0.001%; and progesterone, <0.001%. Excretion of MBG during 24 hours was calculated as MBG concentration (pmol/L) × 24-hour volume of urine (in liters) and expressed as pmol/24-hour.

**Statistical Analysis**

Data are presented as mean ± SEM. For the statistical analysis, the Graph Pad Prism 7 software (La Jolla, CA) was used. The data were analyzed using the 2-way analysis of variance, followed by Bonferroni’s post tests. A 2-sided P value of less than 0.05 is considered to be statistically significant.

**RESULTS**

**Experiment 1: Effect of High Salt Intake, DM2, and Their Combination on MBG and Fibrosis**

We studied 4 groups of rats, normal animals drinking tap water (Ctrl group) or 1.8% saline (Ctrl + NaCl group) and DM2 rats consuming water or 1.8% saline (n = 8 per each group). Data on glucose oral tolerance test, systolic BP, excretion of MBG, and activity of Na/K-ATPase in the erythrocytes are presented in Figure 1. High salt intake led to increased diuresis and water excretion, reduced creatinine clearance, and increased sodium excretion (Table 1). At 8 weeks, STZ animals developed mild diabetes, which was confirmed by an impaired glucose tolerance test (Fig. 1A). Plasma insulin was elevated in this group, and administration of high salt further increased plasma insulin (Table 1).

BP was not affected by a high salt intake, nor by STZ treatment or by a combination of both versus that in the control group (Fig. 1B). Renal MBG became elevated in rats after 4 weeks of 1.8% saline intake and in DM2 animals (STZ group), and it further increased in DM2 rats on a high salt intake in STZ + NaCl group (Fig. 1C). Erythrocyte Na/K-ATPase activity was negatively associated with the changes in MBG levels (Fig. 2D).
As presented in Table 1, left ventricle (LV) and aortic weights, as well as body weights, increased in DM2 rats on a high salt intake versus that in DM2 and in control animals. Levels of collagen 1 and Fli1 in the thoracic aorta, assessed by Western blotting, are presented in Figure 2. Collagen-1 exhibited a progressive increase in all groups of treated rats (NaCl, STZ, STZ + NaCl), reaching maximum in DM2 rats on a high salt intake (STZ + NaCl) (Fig. 2A). Fli1 was inhibited in DM2 animals (STZ) and was surprisingly higher in rats with a combination of both treatments (STZ + NaCl) versus that in STZ group but significantly lower than in both Ctrl and Ctrl + NaCl groups (Fig. 2B).

**Experiment 2: Effect of Anti-MBG Monoclonal Antibody and MBG Signaling and Fibrosis Markers**

Eight-week-old DM2 rats were placed on a high NaCl intake by drinking 1.8% saline instead of water for 4 weeks; during the last week, animals were 3 times administered anti-MBG mAb or vehicle. No changes were observed in systolic BP, whereas erythrocyte Na/K-ATPase was inhibited in salt-loaded DM rats. Anti-MBG mAb did not affect BP, although restored inhibited Na/K-ATPase activity (STZ + NaCl + AB group; Figures 3A, B). As presented in Figures 3D–F, plasma MBG concentration, as well as LV and aortic weights in this group, became elevated compared with the parameters in control animals. Pretreatment of DM and salt-loaded rats with 3E9 anti-MBG mAb prevented increases in LV and aortic weights (Figs. 3E, F).

As shown in Figure 3C, aortic rings from control rats, pretreated with 10 nmol/L SNP, exhibited 50% relaxation following submaximal (100 nmol/L) concentration of endothelin-1. The responsiveness of the aortic rings obtained from salt-loaded DM2 rats (STZ + NaCl group) to the relaxant effect of SNP was markedly reduced (half maximal effective concentration = 29 nmol/L) as compared with the control rings. In the presence of anti-MBG mAb, a combination of diabetes and salt load failed to reduce the sensitivity of aortic rings to SNP (STZ + NaCl + AB group) (half maximal effective concentration = 9 nmol/L) (Fig. 3C).

Figure 4 demonstrates changes in the proteins, participated in the possible signaling pathway underlying vascular fibrosis and sensitive to MBG immunoneutralization. Aortic levels of Fli1, PKC, TGF-β1, and SMAD 5 proteins became elevated, which was accompanied by increases in fibrosis markers collagen 1 and fibronectin. When salt-loaded DM rats were treated with anti-MBG mAb, the aortic levels of signaling molecules and markers of fibrosis returned to control levels (Figs. 4A–H).

**DISCUSSION**

The main novel finding of the present study is that elevation of an endogenous inhibitor of Na/K-ATPase, MBG, in DM2 rats on a high salt intake is accompanied by the aortic wall remodeling in the absence of BP changes. Fli1-dependent and TGF-β-dependent profibrotic pathways were activated in the aorta of diabetic NaCl-loaded rats. Immunoneutralization of MBG in STZ + NaCl rats by a monoclonal antibody against MBG prevented aortic remodeling and restored the abundance of the proteins related to Fli1 and TGF-β signaling.
DM is associated with a positive sodium balance and with an activation of natriuretic factors like MBG.\textsuperscript{28,29} STZ + NaCl rats from the present study exhibited increased production of collagen, that is, initiated tissue fibrosis. This profibrotic mechanism is initiated by the inhibition of Na/K-ATPase by MBG and involves PKC$\delta$/Fli1-dependent signaling, which is in agreement with our previous findings.\textsuperscript{7,10,11,13,23–25} We demonstrated that in addition to Fli1-dependent signaling, TGF-\(\beta\) profibrotic pathway was also activated in the present study because the abundance of the proteins related to this pathway, that is, TGF-\(\beta\)1, SMAD5, collagen-1, collagen-5 and fibronectin, was also increased.

In the present study, salt-loaded DM2 rats exhibited higher MBG levels, inhibited Na/K-ATPase, and higher aortic weight in the absence of BP changes versus control. Against our expectations, Fli1 abundance did not further decrease after the addition of NaCl to DM2 (STZ + NaCl group), although remained substantially downregulated (40\%) versus SALT group and versus control in both experiments 1 and 2. This observation indicates that another growth-promoting pathway, that is, SMAD-dependent TGF-\(\beta\) pathway, is activated in addition to Fli1 in salt-loaded DM2. Recently, we studied the effects of administration of a dietary high NaCl (4\%) and its reduction on the production of MBG and on the activity of TGF-\(\beta\) profibrotic signaling in the aortae of the young male Sprague-Dawley rats.\textsuperscript{25} High NaCl diet was associated with an increased MBG, activation of TGF-\(\beta\) profibrotic signaling, and higher collagen abundance in aortae but no changes in Fli1.\textsuperscript{25} Reduction in salt intake was associated with the lowering of MBG and TGF-\(\beta\) and decrease of aortic collagen level.\textsuperscript{25} Another pathway, that is, inhibition of Fli1, a nuclear transcription factor, is implicated in MBG-induced fibrosis in preeclampsia\textsuperscript{22} and chronic renal failure.\textsuperscript{11} In the present study, immunoneutralization of MBG by 3E9 anti-MBG antibody in STZ + NaCl group (STZ + NaCl + AB group) led to the increase of Fli1 protein, that is, downregulation of Fli1-controlled profibrotic signaling and down-regulation of TGF-\(\beta\)-dependent signaling, which is in agreement with the results of the previous publications.\textsuperscript{11,22–24} Thus, it is very likely that MBG regulates both Fli1- and TGF-\(\beta\)-dependent pathways in salt-loaded rats with type 2 DM. The mechanisms involved in the modulation of Fli1 signaling by a dietary high salt remain to be investigated.

In the present study, DM2 rats after 4 weeks of a high salt intake were associated with a marked reduction in the sensitivity of vascular rings to the vasorelaxant effect of SNP. Our results demonstrated blunted vasorelaxation of aortic rings from STZ + NaCl rats, which was reduced by immunoneutralization of MBG, a profibrotic factor.\textsuperscript{12,13} Thus, the improvement of the elastic properties of the aortic wall by reducing collagen levels due to blockade of MBG was associated with a restoration of the aortic vasorelaxation in response to SNP. Our observation of the pressure-independent effect of MBG is in accordance with the previous data, which showed that BP does not increase even in the presence of high salt in DM2 rats\textsuperscript{30,31} and becomes elevated only when salt consumption is very high (8\% of NaCl).\textsuperscript{32}

**CONCLUSIONS**

We have demonstrated, for the first time, that the young normotensive rats with DM2 in response to a high salt intake exhibit elevated MBG levels. In vivo immunoneutralization of
MBG reduced vascular fibrosis and improved vascular relaxation in these animals. Thus, in the rat DM2 model, high dietary NaCl intake can induce vascular fibrosis via pressure-independent and MBG-dependent mechanisms, which can be counterbalanced by immunoneutralization of MBG.

ACKNOWLEDGMENTS

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REFERENCES


FIGURE 1.
Oral glucose tolerance test (A), SBP (B), renal MBG excretion (C), and activity of Na/K-ATPase (NKA) in erythrocytes (D) in control group (Ctrl), in rats on a high salt intake (Ctrl NaCl), in those with DM (STZ), and in those with DM on a high NaCl intake (STZ NaCl). Data presented as mean ± SEM of 8 individual measurements. By repeated-measures analysis of variance and Bonferroni’s test (A): *P < 0.05, **P < 0.01 versus baseline (0 minutes). By 2-way analysis of variance (B–E): *P < 0.05, **P < 0.01 versus Ctrl.
FIGURE 2.
Levels of collagen-1 (A) and Fli1 (B) in the thoracic aortae of rats on a high salt intake (Ctrl NaCl), in the rats with DM (STZ), and in those with DM on a high NaCl intake (STZ NaCl) in Comparison to the control group (Ctrl). Top of each panel: Western blotting representative bands; bottom of each panel, statistical analysis of band density standardized for GAPDH. Each bar represents individual samples pooled and averaged to give means and SEM of 5–7 measurements. STZ, streptozotocin-treated rats developed DM; Ctrl, control; Ctrl NaCl, salt-loaded control rats; STZ NaCl, salt-loaded rats with DM. By 2-way analysis of variance followed by Bonferroni’s test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus Ctrl; #$P < 0.05$ versus STZ.
FIGURE 3. Effect of the vehicle and anti-MBG antibody (3E9 mAb) on erythrocyte Na/K-ATPase (A), systolic BP (B), and responsiveness of rat aortic rings to SNP-induced vasorelaxation following contractions induced by 100 nmol/L endothelin-1 (C) in NaCl-loaded DM rats versus control. Effect of the combination of DM2, NaCl, and vehicle and DM2, NaCl, and anti-MBG mAb treatment on plasma MBG (D), LV weight (E), and aortic weight (F). Data presented as mean ± SEM from 8 measurements, except (C), below. By 2-way analysis of variance followed by Bonferroni’s test: *P < 0.05, **P < 0.01 versus controls, #P < 0.05 versus vehicle (A). Concentration-response curves were determined 5–7 times on individual samples pooled and averaged to give means and SEM for IC_{50} (C).
FIGURE 4.
Effect of combination of DM, NaCl, and vehicle and DM, NaCl, and anti-MBG mAb treatment on aortic level of Fli1 (A), ratio of phosphorylated to total PKCδ (B), TGF-β1 (C), SMAD-5 (D), collagen-1 (E), collagen-5 (F), fibronectin (G), and GAPDH (H). Top of each panel: Western blotting representative bands; bottom of each panel: statistical analysis of the band density standardized for GAPDH. Each bar represents individual samples pooled and averaged to give means and SEM of 5–7 measurements. Ctrl, control; STZ + NaCl, salt-loaded rats with DM; Veh, vehicle-treated rats; mAb, 3E9 anti-MBG antibody treatment. By 2-way analysis of variance followed by Bonferroni’s test: *P < 0.05 versus control (Ctrl); #P < 0.05 versus vehicle.
### TABLE 1.

Physiological Parameters of Control and Rats With Diabetes Mellitus Type 2

<table>
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<tr>
<th></th>
<th>Control (n = 8)</th>
<th>Control + NaCl (n = 8)</th>
<th>STZ (n = 8)</th>
<th>STZ + NaCl (n = 8)</th>
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<tr>
<td><strong>BW (g)</strong></td>
<td>304 ± 6</td>
<td>280 ± 11</td>
<td>272 ± 14</td>
<td>256 ± 9*</td>
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<td><strong>LV weight (g/kg BW)</strong></td>
<td>1.32 ± 0.05</td>
<td>1.43 ± 0.02</td>
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<td>1.58 ± 0.06**,** ***</td>
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<td><strong>Aortic weight (g/kg BW)</strong></td>
<td>0.36 ± 0.02</td>
<td>0.39 ± 0.04</td>
<td>0.38 ± 0.02</td>
<td>0.51 ± 0.03**,** ***</td>
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<td><strong>Volume of urine (mL/24 h)</strong></td>
<td>8.5 ± 1.3</td>
<td>65.0 ± 7.3**</td>
<td>21.7 ± 5.4*</td>
<td>69.1 ± 9.0**,** ***</td>
</tr>
<tr>
<td><strong>Plasma insulin (pg/mL)</strong></td>
<td>463 ± 16</td>
<td>520 ± 13</td>
<td>648 ± 14**</td>
<td>852 ± 19**,** ***</td>
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<tr>
<td><strong>Volume of water or 2x saline (NaCl 1.8%) (mL/24 h)</strong></td>
<td>18.8 ± 2.3</td>
<td>80.8 ± 7.0**</td>
<td>31.7 ± 2.6*</td>
<td>84.9 ± 8.4**,** ***</td>
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<td><strong>Urine Na</strong> (mmol/24h)</td>
<td>0.7 ± 0.2</td>
<td>29.0 ± 2.5**</td>
<td>1.7 ± 0.5</td>
<td>38.2 ± 3.9**,** ***</td>
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<tr>
<td><strong>Urine creatinine (mmol/L)</strong></td>
<td>8.00 ± 0.17</td>
<td>1.24 ± 0.25*</td>
<td>6.84 ± 1.14</td>
<td>1.29 ± 0.21**,** ***</td>
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<td><strong>Creatinine clearance (mL/min)</strong></td>
<td>3.38 ± 0.17</td>
<td>2.55 ± 0.34*</td>
<td>1.98 ± 0.30**</td>
<td>2.12 ± 0.21**</td>
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<td><strong>FENa</strong></td>
<td>0.25 ± 0.10</td>
<td>5.42 ± 1.01**</td>
<td>0.41 ± 0.11</td>
<td>8.46 ± 1.88**,** ***</td>
</tr>
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Data presented are means ± SEM. By 2-way analysis of variance:

*  
P < 0.05,

**  
P < 0.01 versus control;

***  
P < 0.01 versus STZ.

STZ, animals, treated with streptozotocin to induce diabetes type 2.

BW, body weight; FENa, fractional excretion of sodium.