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Calcineurin and Akt expression in hypertrophied bladder in STZ-induced diabetic rat

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ABSTRACT

Diabetes causes significant increases in bladder weight but the natural history and underlying mechanisms are not known. In this study, we observed the temporal changes of detrusor muscle cells (DMC) and the calcineurin (Cn) and Akt expressions in detrusor muscle in the diabetic rat. Male Sprague–Dawley rats were divided into 3 groups: streptozotocin-induced diabetics, 5% sucrose-induced diuretics, and age-matched controls. The bladders were removed 1, 2, or 9 weeks after disease induction and the extent of hypertrophy was examined by bladder weights and cross sectional area of DMC. Cn and Akt expression were evaluated by immunoblotting. Both diabetes and diuresis caused significant increases in bladder weight. The mean cross sectional areas of DMC were increased in both diabetic and diuretic animals 1, 2, or 9 weeks after disease induction. The expression levels of both the catalytic A (CnA) and regulatory B (CnB) subunits of Cn were increased at 1 and 2 weeks, but not at 9 weeks. Expression of Akt was similar among control, diabetic, and diuretic rat bladder at all time points. In conclusion, diabetes and diuresis induce similar hypertrophy of detrusor muscle during the first 9 weeks, indicating that bladder hypertrophy in the early stage of diabetes is in response to the presence of increased urine output in diabetes. Our results suggest that the Cn, but not the Akt signaling pathway may be involved in the development of bladder hypertrophy.

Introduction

Diabetes mellitus (DM) seriously affects multiple organ systems, including the urinary bladder. In some series, 52% of randomly evaluated diabetic patients were found to have urologic symptoms (Ioanid et al., 1981). Our previous studies showed that diabetes-related polyuria induced time-dependent increase of bladder weight, and the main source was detrusor smooth muscle (DMC) (Liu and Daneshgari, 2006). The increased detrusor smooth muscle may be due to hypertrophy or hyperplasia or both of smooth muscle cells. Hypertrophy refers to an increase in the size of the cell while hyperplasia refers to an increase in the number of cells. However, the temporal changes of DMC and mechanisms of such changes remain unknown, whereas a detailed characterization of hypertrophy in heart diseases has been studied (Heineke and Molkentin, 2006).

Cardiac hypertrophy occurs in response to long-term increases in hemodynamic load (Heineke and Molkentin, 2006). Hemodynamic overloads, including the volume overload (elevated preload) and the pressure overload (elevated afterload), increase ventricular stroke work (Carabello et al., 1992; Gorgulu et al., 2010; Kato et al., 1996; Wojciechowski et al., 2010). The chronically increased ventricular stroke work demands of overloaded states are compensated by the development of ventricular hypertrophy. Pressure overload results in the development of concentric hypertrophy where parallel sarcomere replication produces increased wall thickness; volume overload results in series sarcomere replication and eccentric hypertrophy (Carabello et al., 1992; Gorgulu et al., 2010; Kato et al., 1996; Wojciechowski et al., 2010). Cardiac hypertrophy is at least in part responsible for the symptoms of congestive heart failure that develop in patients with chronic overload hypertrophy (Carabello et al., 1992).

Previous studies indicated the involvement of calcineurin (Cn)-nuclear factor of activated T-cells (NF-AT) signaling in this process of cardiac hypertrophy (Molkentin et al., 1998). In addition, Cn signaling has been shown to be involved in bladder outlet obstruction induced detrusor hypertrophy (Clement et al., 2006; Nozaki et al., 2003). Recently, there were some reports which linked signaling through the phosphoinositide-3-kinase (PI3K)/Akt pathway to induction of cardiac hypertrophy (Naga Prasad et al., 2003). PI3K activates the Akt serine/threonine kinase and enable its activation. Akt mediates a wide variety of cellular responses including growth, proliferation and survival (Lawlor and Alessi, 2001). Mammalian genomes contain three Akt genes, Akt1, Akt2 and Akt3. Akt is phosphorylated at its two regulatory phosphorylation sites, T308/S473 in Akt1, T309/S474 in Akt2 and T305/S472 in Akt3. Akt1-null mice have a 20% reduction in body size, whereas Akt2-null mice do not show any reductions in body growth (Chen et al., 2001; Cho et al., 2001). Akt3-deficient mice present a selective 25% decrease in brain size (Easton et al., 2005). Expression of activated Akt in cardiac muscle

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of mice was found to be sufficient to induce hypertrophy (Shioi et al., 2002).

Similarly to hemodynamic overloads in cardiac hypertrophy, urodynamic overloads in diabetic bladder, including mainly volume overload (increased urine production (Liu and Daneshgari, 2006)) and probably pressure overload (decreased urethral relaxation (Liu et al., 2008; Yang et al., 2007)), are the potential inducers of hypertrophy. We hypothesize that Cn and Akt signaling may play roles in the hypertrophic response of the bladder to diabetes-associated polyuria.

In this study, we aimed to observe the temporal changes of DMC and the Cn and Akt expression in detrusor muscle in the diabetic rat.

Materials and methods

Experimental animals

Male Sprague–Dawley rats matched by date of birth (290 to 310 g, 10 weeks-old, Harlan), were used in this study. The animals were randomly allocated to three groups: diabetics (n = 18), diuretics (n = 18), and age-matched controls (n = 18). Each group was subsequently divided into three subgroups of 6 rats for evaluation at 1 week, 2 weeks or 9 weeks after induction. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, 50 mg/kg dissolved in 0.1 M citrate buffer, pH 4.5), and diuresis was induced by addition of 5% sucrose to their drinking water. Blood glucose levels were measured with the ACCU-CHEK system (Roche Diagnostics Corporation, Indianapolis, IN) 72 h after administration of STZ and at the time of sacrifice to confirm diabetes (blood glucose > 350 mg/dl). At designed time points, animals were sacrificed by a single intraperitoneal injection of pentobarbital (200 mg/kg). The urinary bladder was removed at the level of the bladder neck, weighed and sectioned at the equatorial midline (largest diameter). The bottom half was placed on ice. Tunica adventitia and urothelium were removed under a surgical microscope, and the smooth muscle layer was frozen for immunoblotting. The dome half was allowed to equilibrate for 15 min at room temperature in Krebs' buffer, then embedded in Tissue-Tek® O.C.T. Compound (Torrance, CA), frozen in liquid nitrogen, and stored in −80 °C freezer for immunofluorescence staining. The composition of the Krebs solution was as follows (in mM): 133 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 16.3 $NaHCO_3$, 1.35 NaH_2PO_4, 0.6 $MgSO_4$, and 7.8 Dextrose (Hall et al., 2002). All experimental protocols were approved by our Institutional Animal Care and Use Committee.

Immunofluorescence staining and morphometric analysis

Frozen OCT-embedded bladder tissues were sectioned into 8 μm-thick sections. Sections were treated with 4% paraformaldehyde for 30 min, then placed in 10% normal goat serum with 0.2% Triton X-100 for 30 min, and then incubated with rabbit α-smooth muscle actin antibody (AB 1982, 1:500, Chemicon; Temecula, CA) overnight at 4 °C in a humid chamber. The slides were rinsed with PBS and then incubated with fluorescein-conjugated Alexa 488 sheep anti-rabbit IgG (1:5000; Molecular Probes, Eugene, OR), including 10 μg/ml Rhodamine Wheat Germ Agglutinin (WGA) which can bind to N-acetylglucosamine or chitobiose for glycoprotein staining of membrane. Slides were washed and mounted with medium containing DAPI (Vector Laboratories). Fluorescent images were obtained using a laser confocal microscope.

Immunofluorescence stained sections at equatorial sections of bladder were used to determine mean intracellular cross-sectional area of a single detrusor muscle cell. Fig. 1A is the representative overlaid image of immunofluorescence staining of α-smooth muscle actin (green), membrane (red), and nuclei (blue) in the longitudinal sections of smooth muscle cells in bladder. The immunoreactive α-smooth muscle actin cells were used for analysis. Four regions of longitudinally oriented smooth muscle cells at 3, 6, 9, and 12 o'clock of equatorial sections of bladder were chosen for quantification. The intracellular cross-sectional area of each cell in a region was determined and a mean value was calculated. Then the mean of four regions was calculated to represent the intracellular cross sectional area of a single cell in each animal. The immunofluorescence images were analyzed with Image-Pro Plus 5.1 image analysis software (Media Cybernetics, Silver Spring, MD). The software can automatically distinguish regions stained with different colors and accurately measure the areas by counting the pixels and converting pixels to number of square micrometers. Fig. 1B shows the cell membranes stained in red. The area within the membrane of DMC was measured by tracing the internal edges of the cell. Fig. 1C shows the red circle along the perimeter of the cell produced by the software automatically and used for measuring the intracellular cross-sectional area of each cell. In all cases, the processing of images was performed by the same investigator unaware of treatment group assignments.

Immunoblotting

Frozen detrusor muscle tissues were homogenized in buffer containing 20 mM Tris–HCl, 1% Triton X-100, 100 mM NaCl, 0.5% NP-40 and protease inhibitors. Protein concentration was determined by the detergent-compatible Bio-Rad DC protein assay. Proteins were separated by SDS-PAGE. Equal amounts of protein extract (40 μg) from control, diabetic, and diuretic groups at the same time point were distributed to the same gel (4–15% linear gradient 18 well gel, Bio-Rad Laboratories) to reduce any other non-treatment effects. The proteins then were transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in PBS containing 0.05% Tween-20 and probed with a primary antibody in the blocking buffer, then incubated with the appropriate secondary antibodies (Jackson Immunoresearch, West Grove, PA). The bands were visualized using enhanced chemiluminescence and Kodak Biomax film. Membrane was also incubated with anti β-actin antibodies (A 5441, 1:100000, Sigma-Aldrich, Saint Louis, Missouri). The primary antibodies used were rabbit-anti-CnA (1:100) and rabbit-anti-CnB (1:100) from Biomed Technology (Stoughton, MA), rabbit-anti-Akt (1:1000), rabbit-anti-Akt (Thr308) (1:1000), and mouse-anti-Akt (Ser473) (1:1000) from Cell Signaling Technology (Danvers, MA). Band intensities were evaluated using Image J software. Beta-actin was used as a loading control. The band intensity quantification was normalized to β-actin.

Statistical analysis

All data is expressed as the mean plus or minus standard error of the mean (SEM). Comparisons of measurements among the diabetic, diuretic and control groups at the same time point were performed with the One-way ANOVA test, followed by Newman–Keuls multiple comparison (Graph Pad 4.0 software).

Results

General characteristics

General physical characteristics of the animals are summarized in Table 1. The initial mean body weight was similar for all 3 groups, but the diabetic group weighed less than the corresponding diuretic and control groups 1 week, 2 weeks and 9 weeks after induction ($P<0.01$). The mean blood glucose levels of the diabetic rats were ~4 times higher than those of control and diuretic rats. However, there were no significant differences in body weights and blood glucose levels between control and diuretic animals ($P>0.05$). The bladder weights increased markedly in both the diabetic and diuretic rats compared with controls.