TLR4 increases bladder contractility through MyD88/p38MAPK activation in streptozotocin-induced diabetic mice

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Introduction

Urinary tract infections are responsible for nearly 13 million annual doctor visits in the United States. These infections are most commonly caused by the Gram-negative bacterium Escherichia coli, which is responsible for 80-85% of community-acquired urinary tract infections 1.

 Toll-like receptor 4 (TLR4) is expressed in bladder urothelium, and has been suggested to initiate the expulsion of E. coli infected cells from the urinary tract. When activated by lipopolysaccharide (LPS) TLR4 leads to urothelial desquamation and inflammation 2.

 Diabetic patients have greater susceptibility to urinary tract infections than normoglycemic people. Evidence from experimental animal models as well as from humans has indicated that systemic inflammation plays a role in the pathophysiological processes of diabetes that is facilitated by innate immune responses 3.

The present work aimed to study TLR4 activation and function in isolated bladders from streptozotocin-induced diabetic mice.

Methods

Wild type (+/-) and TLR4 knockout (-/-) mice were bred in house at University of São Paulo, Brazil. C57BL/6J was obtained and housed at University of Campinas, Brazil. Male (22-30g) mice were used in all these studies. All procedures were carried out in accordance with Brazilian College for Animal Experimentation (COBEA) guidance. They were approved by the Committee for Ethics in Animal Research (CEEA-UNICAMP, protocol number 3313-1).

Diabetes induction

The animals were divided into four groups, control (TLR4 +/+, TLR4 -/-), and diabetic (DM +/+, DM -/-). Diabetic mice received a single intraperitoneal injection of streptozotocin (STZ) at 125 mg kg^-1 dissolved in citrate buffer (20 mM, pH 4.5). Control mice were treated identically except that a similar volume of buffer was injected instead of STZ Blood samples were taken from the tail weekly to evaluate glucose concentration (as measured with the ACCUCHEK, Roche Diagnostics, Indianapolis, IN). Functional and molecular experiments were made four weeks following STZ administration.

Organ bath study

Mice were euthanized by inhalation of CO2 and the bladder removed. The bladder dome was removed, and cut into 2 longitudinal strips. These were suspended in a 30 ml organ bath containing Krebs’ solution (pH 7.4) at 37°C and bubbled with a mixture of 95% O₂/5% CO₂. Changes in isometric force were recorded using a Power Lab v4.0 system (AD Instruments, USA). Following 60 min equilibration, the resting tension was adjusted to 5 mN at the beginning of the experiments. All drugs were dissolved and administered in Krebs’ solution. Maximal response (E_max) and pEC50 values (stated as mean ± SEM) were compared by unpaired t-tests. Cumulative concentration–response curves to carbacol (3 μM to 100 μM) and KCl (3 – 1000 mM) were constructed using one-half log unit blocks. The curves were repeated in the presence of Lipopolysaccharides from E.coil (Sigma Aldrich, 100 μg/mL). Nonlinear regression analysis to determine the pEC50 and Emax was carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

SDS-PAGE and Western blotting

Bladders were collected as above and homogenized in RIPA buffer with protease inhibitors (Roche, UK). Lysoptic (20% of total protein) was separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membranes and blocked in 5% milk 90 min. Membranes were incubated with rabbit anti-p38, anti-P38, anti-ERK1/2, anti-P-ERK1/2, anti-JNK, anti-P-JNK, anti-MYD88 (Cell Signalling) overnight at 4°C. For densitometric analysis, the ratio of phosphorylated/total signals was calculated.

Real Time RT-PCR

Total RNA was extracted with TRIzol (Gibco-BRL, Gaithersburg, MD) from bladder samples. RNA samples (1 μg) were incubated with DNasel (Invitrogen Corp., Rockville, MD, USA). DNasel-treatment RNA samples obtained were then reverse transcribed with kit Superscript III RT™ (Invitrogen, Life Technologies). The cDNA product was subjected to Real-time reverse transcription (RT-PCR) using SYBR Green Master Mix™ (Invitrogen Corp., Rockville, MD, USA) and selected primers (TLR4, TLR2, Trif). Expression levels of all genes were normalized by HPRT (internal control) levels. The mean expression level in WT non-treated control mice was arbitrarily defined as 1.0. The cycle threshold (Ct) was determined for each sample and normalized to the average Ct of the four housekeeping genes. The comparative ΔCt method (SABiosciences) was used to calculate relative gene expression.

Results

Streptozotocin injection increases serum glucose concentration and micturition volume in TLR4 wild type and knockout animals.

TLR4 knockout mice inhibited carbacol and KCl concentration-response curves in diabetic bladders.

TLR4 knockout mice inhibited MyD88 and p38MAPK mRNA overexpression in diabetic bladders.

Summary

• TLR4 is activated in urinary bladders from diabetic mice;
  • Deletion of TLR4 normalized bladder contractility to carbacol and KCl in diabetic mice;
  • TLR4 agonist (LPS) increased mucuscarinic contractility in isolated bladders from TLR4 wild type. This effect was partially blocked in TLR4 knockout mice.
  • The mechanism that mediates overactive bladder through TLR4 in diabetic mice involves MyD88 activation and p38MAPK phosphorylation.

Concluding message

Inflammation plays an important role in diabetic bladder overactivity. Inhibition of TLR4 decreases the downstream MyD88 and p38MAPK intracellular pathways, thus representing a good pharmacological target to treat urological symptoms associated with diabetes.

References