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

Ramos Filho A C[1], Santos K T[2], Teixeira S A[2], Mónica F Z[1], Muscará M N[2], Costa S K[2], Grant A D[3], Antunes E[1]

[1]University of Campinas, Brazil; [2]University of São Paulo, Brazil; [3]King's College, London, UK

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 infections1.

✓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 inflammation2.

✓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 responses3.

✓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/6 was obtained and house at University of Campinas, Brazil. Male (22-30g) mice were used in 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 evaluated glucose concentration (as measured with the ACCUCHEK; Roche Diagnostics, Indianapolis, IN). Functional and molecular experiments were made four weeks following STZadministration.

Organ bath study

Mice were euthanased by inhalation of CO2 and the bladder removed. The bladder dome was removed, and cut into 2 longitudinal strips. These were suspended in a 10ml organ bath containing Krebs' solution (pH7.4) at 37ºC and bubbled with a mixture of 95%O_2/5%CO_2. Changes in isometric force were recorded using a Power Lab v.4 system (AD Instruments, UK). 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 pEC_{50} values (stated as mean ± S.E.M) were compared by unpaired t-tests.

Cumulative concentration-response curves to carbachol (3 nM to 100 µM), and KCl (3 –1000 mM) were constructed using one-half log units. The curves were repeated in the presence of Lipopolysaccharides from *E.coli* (Sigma Aldrich, 0111:B4). Nonlinear regression analysis to determine the pEC_{50} 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 homogenised in RIPA buffer with protease inhibitors (Roche, UK). Lysates (20µg of total protein) were 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-Pp38, antiERK1/2, 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 were 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 DNaseI (Invitrogen Corp., Rockville, MD, USA). DNaseI-treated RNA samples obtained were then reverse transcribed with kit Superscript III RT™ (Invitrogen, Life Technologies). The cDNA product was subjected to Real-time reverse transcriptase (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 .

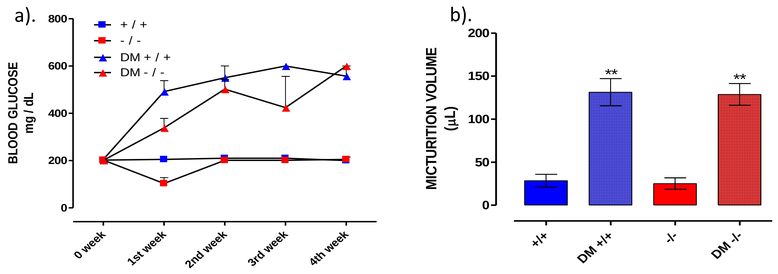

Figure 1. (**A**) Changes in non-fasting serum glucose levels over the weeks in non-diabetic and diabetic TLR4 (+/+) and (-/-). (**B**) Micturition volume (µL) measured over 3h, on the fourth week after STZ injection. n=10 mice. **P<0.01 (One-way ANOVA followed by a Tukey test).

TLR4 knockout mice inhibited carbachol and KCl concentration-response curves hypercontractility in diabetic bladders

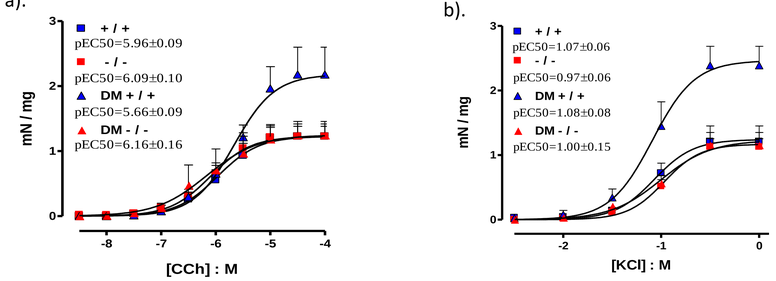

Figure 2. Contraction of isolated bladder strips from TLR4 (+/+) and (-/-) mice, normoglycemic and diabetic, in response to the muscarinic agonist carbachol (**A**; 3nM – 100µM) and KCl (**B**; 3 –1000 mM) . n=5 mice.

TLR4 knockouts inhibited MyD88 and p38MAPKinase overexpression in diabetic bladders

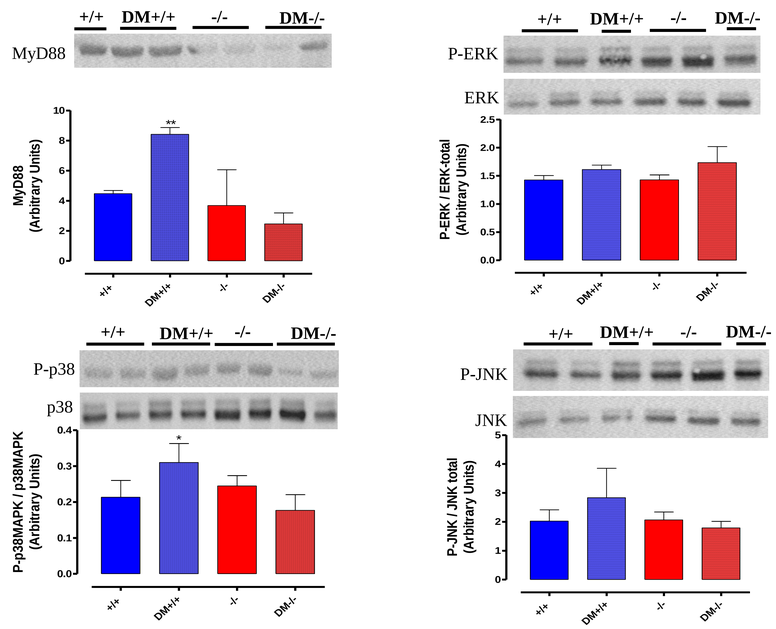

Figure 3. Western blotting analysis of p38MAK phosphorylation (P-p38 MAPK), ERK1/2 phosphorylation (P-ERK1/2), JNK phosphorylation (P-JNK) and MyD88 in isolated bladders from TLR4 (+/+) and (-/-), normoglicemic and diabetic. n= mice. *P<0.05 and **P<0.01 (One-way ANOVA followed by a Tukey test).

TLR4, TLR2 and Trif mRNA expressions were not different between normoglycemic and diabetic mice

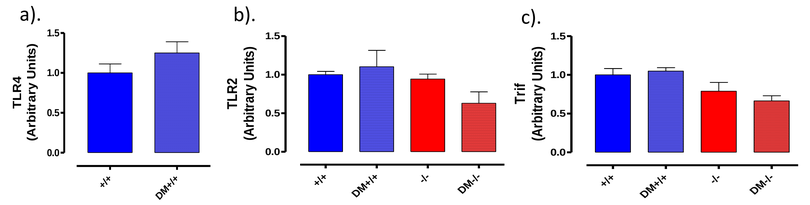

Figure 4. Bladder mRNA expressions for TLR4 (**A**), TLR2 (**B**) and Trif (**C**) in TLR4 +/+ and -/- mice, normoglycemic and diabetic. Values are expressed in arbitrary units. n=4 mice.

Bacterial lipopolysaccharide induced bladder hypercontractility to carbachol in mice through TLR4 activation in urothelium

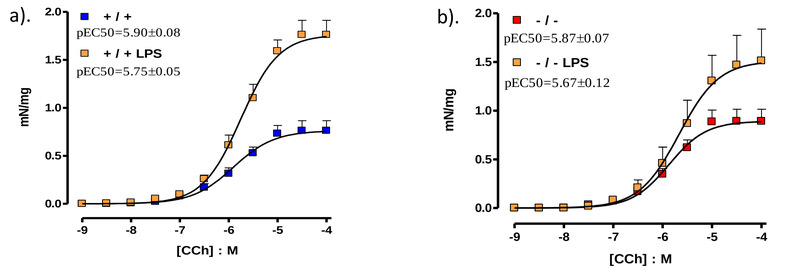

Figure 5. Concentration-response curves to carbachol (1nM – 100µM) in isolated bladder strips from TLR4 (+/+; **A**) and (-/-; **B**) mice, pré-incubated with Lipopolysaccharide (LPS). n=5 mice.

Summary

•TLR4 is activated in urinary bladders from diabetic mice;

•Deletion of TLR4 normalized bladder contractility to carbachol and KCl in diabetic mice;

•TLR4 agonist (LPS) increased muscarinic 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

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