Inhibition of NMDAR Reduces Bladder Hypertrophy and Improves Bladder Function in Cyclophosphamide Induced Cystitis

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Abbreviations and Acronyms

Akt = protein kinase B
BOO = bladder outlet obstruction
CCh = carbachol
CYP = cyclophosphamide
D-AP5 = D-2-amino-5-phosphonopentanoate
MK-801 = dizocilpine
NGF = nerve growth factor
NMDAR = N-methyl-d-aspartate receptor
phospho = phosphorylated
PI3K = phosphoinositide 3-kinase

Accepted for publication December 19, 2014.

Study received Virginia Commonwealth University institutional animal care and use committee approval.

Supported by NIH Grant DK077917 (LYQ).

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Purpose: We examined the role of NMDAR in the regulation of bladder hypertrophy and function in a rat model of cyclophosphamide induced cystitis.

Materials and Methods: Cystitis was induced by intraperitoneal injection of cyclophosphamide (150 mg/kg body weight). NMDAR phosphorylation (activity) and signal transduction pathways were examined by direct measurement and by specific inhibitors in vivo. Bladder hypertrophy was measured by bladder weight/body weight and type I collagen expression. Bladder function was examined by metabolic recording, conscious cystometry and detrusor muscle strip contractility in response to carbachol.

Results: NMDAR activity measured by the phosphorylation level of the NMDAR1 (NR1) subunit was expressed in the spinal cord but not in the bladder at 48 hours of cystitis. NMDAR inhibition with dizocilpine (MK-801) reduced the cystitis induced increment of bladder weight and type I collagen up-regulation in the bladder. NMDAR regulated type I collagen up-regulation was mediated by the PI3K/Akt pathway. NMDAR inhibition also attenuated cystitis induced urinary frequency measured by metabolic cage and cystometry. Cystitis decreased the responsiveness of detrusor muscle strips to carbachol, which was reversed by MK-801 in vivo. Unlike MK-801 the NMDAR antagonist D-AP5, which could not block central NMDAR activity, had no effect on bladder hypertrophy, type I collagen up-regulation or Akt activation caused by cystitis in the bladder.

Conclusions: Findings suggest that NMDAR activity has a role in cystitis induced bladder hypertrophy and overactivity. NMDAR mediated Akt activation may underlie the mechanism of bladder dysfunction.

Key Words: urinary bladder, overactive; receptors, N-methyl-D-aspartate; collagen; cystitis, interstitial; hypertrophy

THE role of NMDAR in synaptic plasticity is well established. Emerging evidence shows that NMDAR also has an irreplaceable role in the transmission of visceral and inflammatory pain.[1–3] In irritable bowel syndrome and overactive bladder NMDAR activity was found in the central nervous system and it mediates visceral hyperactivity.[3,4] Blockading NMDAR activity with the noncompetitive antagonist MK-801 inhibits

0022-5347/15/1935-1676/0
THE JOURNAL OF UROLOGY®

http://dx.doi.org/10.1016/j.juro.2014.12.092
Vol. 193, 1676-1683, May 2015
Printed in U.S.A.

bladder overactivity caused by cerebral infarction[5] and attenuates hyperreflexia in the micturition reflex caused by crystalluria, partial BOO and nerve injury.[6–9] The common pathogenesis of irritable bowel syndrome and overactive bladder also involves smooth muscle overactivity.[10,11] However, to our knowledge the role of NMDAR in regulating cytological changes in the bladder, thereby regulating bladder function, has not been investigated.

NMDAR has an ionotropic property that regulates Ca^{2+} influx and Ca^{2+} dependent physiological effects such as presynaptic neurotransmitter and neuropeptide release.[1] Our recent study showed that according to the phosphorylation level of the NR1 subunit the activity level of NMDAR was increased in the lumbosacral spinal cord in CYP induced cystitis.[12] NMDAR mediated neurotransmitter release to the bladder may possibly facilitate molecular and physiological changes in the viscera.

In CYP induced cystitis the expression level of type I collagen is increased in the inflamed bladder, acting as one of the major factors contributing to bladder hypertrophy.[13] It was suggested that collagen production and extracellular matrix remodeling in peripheral organs may be facilitated by neurotransmitter release from the central and peripheral nervous systems.[14,15] In addition to neuronal expression of NMDAR, recent studies showed that NMDAR activity is also present in peripheral tissues, including macrophages, bone marrow and the cardiovascular system.[16,17] The effects of central and peripheral NMDAR are often differentiated using the specific antagonists MK-801 and D-AP5. MK-801 is used to block overall NMDAR activity for its ability to cross the blood-brain barrier[18,19] and D-AP5 is used to study the role of peripheral NMDAR.[16]

In the current study we used these tools to investigate whether NMDAR has a role in the development of bladder hypertrophy. At the functional level the increase in detrusor wall thickness can reduce bladder capacity, thereby decreasing urine volume per void and increasing urinary frequency. In addition, bladder wall thickening causes poor detrusor smooth muscle compliance and affects smooth muscle contractility.[20] Thus, the role of NMDAR in regulating bladder function during cystitis was also investigated.

MATERIALS AND METHODS

Experimental Animals and Reagents

We used adult male Sprague Dawley® rats at ages 6 to 8 weeks. All experimental procedures were approved by the Virginia Commonwealth University institutional animal care and use committee. Animal care was done in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) and NIH (National Institutes of Health) guidelines. CYP, β-actin antibody and other chemicals used in this experiment were obtained from Sigma-Aldrich®. Primary antibodies against type I collagen (sc-28657, 1:1,000), phospho-NR1 (sc-31669, Santa Cruz Biotechnology, Houston, Texas, 1:1,000), phospho-Akt (4060, 1:1,000) and Akt (9272, Cell Signaling Technology®, 1:1,000) were used for Western blot. Secondary antibodies for Western blot were obtained from Pierce®. MK-801 and D-AP5 were obtained from Tocris Bioscience, Bristol, United Kingdom.

CYP Induced Cystitis

Cystitis was induced in rats by intraperitoneal injection of CYP at a single dose of 150 mg/kg body weight. Rats were survived for 48 hours and then sacrificed by isoflurane overdose. Control rats received volume matched saline injection. All injections were performed using isoflurane (2%) anesthesia.

Drug Treatment

Immediately after CYP injection the MK-801 treated cystitis group received a single administration of MK-801 intravenously at a dose of 3 mg/kg body weight. This dose was tested in our previous study and attenuated cystitis induced spinal plasticity in vivo.[12] D-AP5 was injected in rats with cystitis at a single dose of 5 mg/kg body weight in the same manner as MK-801. This dose was chosen based on a recent study showing that it inhibits peripheral NMDAR activity with no effect on central NMDAR activity.[16] The PI3K inhibitor LY294002 was injected at a single dose of 50 μg/kg body weight. This dose and treatment regimen inhibited Akt activity in our previous study.[12] All inhibitor stocks were stored in dimethyl sulfoxide solution and diluted with saline before injection. Control and cystitis rats received vehicle, that is the same amount of dimethyl sulfoxide diluted in saline.

Western Blot

The bladder was freshly dissected out and homogenized in T-PER buffer (Pierce®) supplemented with protease and phosphatase inhibitor cocktails (Sigma). The homogenate was centrifuged at 20,200 × gravity for 10 minutes at 4C. The protein concentration in the supernatant was determined using the DC™ Protein Assay Kit. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred to nitrocellulose membrane by a semidry transfer technique. The membrane was blocked with 5% milk in tris-buffered saline for 1 hour and incubated with a specific primary antibody followed by horseradish peroxidase conjugated secondary antibody. Bands were identified by echochemiluminescence and x-ray film exposure. Band density was digitized with FluorChem™ and normalized to nonphospho-protein or β-actin as the internal control. The L6 spinal cord was extracted from rats with cystitis to compare with phospho-NR1 in the bladder.

Immunohistochemistry

The bladder was freshly dissected out and postfixed with 4% paraformaldehyde. After dehydration 7 μm transverse sections were obtained and immunostained with type I collagen antibody (1:200) followed by Cy3 conjugated