Translocation of NF-κB and Expression of Cyclooxygenase-2 Are Enhanced by Ketamine-Induced Ulcerative Cystitis in Rat Bladder

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The number of ketamine abusers has increased significantly recently. Ketamine abusers exhibit urinary frequency, urgency, and at times urinary incontinence. Our aim was to investigate the role of transcription factor NF-κB and cyclooxygenase (COX)-2 in ketamine-induced cystitis. Sprague-Dawley rats were distributed into three groups, which received saline or treatment with ketamine or ketamine combined with a Cox-2 inhibitor (parecoxib). In addition, the toxic effect of ketamine and its metabolites were examined by primary urothelial cell culture. The ketamine-treated group displayed bladder hyperactivity and decreased bladder capacity. Treatment with ketamine + COX-2 inhibitor prevented these bladder dysfunctions. These bladder dysfunctions were accompanied by increases in the expression of NF-κB and COX-2 at the protein and mRNA levels. Ketamine treatment also enhanced bladder interstitial fibrosis, whereas ketamine + Cox-2 inhibitor decreased the intensity of fibrosis. Treatment of primary urothelial cells in vitro with ketamine or urine obtained from ketamine-treated rats stimulated the expression of NF-κB p65 and COX-2. Ketamine also initiated NF-κB translocation from cell cytoplasm to nucleus. Treatment with NF-κB inhibitor suppressed Cox-2 mRNA expression. Promoter-deletion analysis revealed that NF-κB was a necessary transcription factor for COX-2 gene (Ptgs2) activation. These results demonstrate that the regulation of COX-2 via the NF-κB pathway is involved in the inflammatory signaling of ketamine-induced cystitis in rat urinary bladder. (Am J Pathol 2015, 185: 2269—2285; http://dx.doi.org/10.1016/j.amjpath.2015.04.020)

Ketamine, a noncompetitive N-methyl-D-aspartic acid receptor antagonist, is used as an anesthetic drug and a conscious sedative agent in humans. Recently, ketamine has been used as a recreational and dissociative drug, especially in nightclubs and at dance parties. Previous studies have indicated that ketamine addiction affects lower urinary tract function, resulting in increased urinary frequency, nocturia, urgency, suprapubic discomfort, and at times hematuria. In addition, vesicoureteral reflux, ureteral obstruction, and hydronephrosis have been reported in ketamine-addiction cases. The pathophysiological mechanism of the bladder

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urothelium and voiding dysfunction in ketamine-induced cystitis (KIC) patients is currently unclear. In a mouse ketamine-addiction model, enhanced noncholinergic contractions and P2x1 receptor expression in the bladder indicated that dysregulation of purinergic neurotransmission might underlie detrusor overactivity. Recent studies in KIC patients have shown increases in bladder mast cell and eosinophil cell infiltration, with greater serum IgE levels than in controls and in acute bacterial cystitis patients, revealing that KIC is associated with hypersensitivity and/or allergic reactions.

Cyclooxygenase (COX)-2 is inducible in response to inflammatory stimuli, cytokines, or endotoxins, resulting in exaggerated release of prostanooids. A study in an animal model of chemical-induced hemorrhagic cystitis found that COX-2 expression increases in the bladder, suggesting that it plays an important role in bladder inflammation. Some investigations have suggested that COX-2 expression in renal medullary interstitial cells is induced by driving NF-κB activation with active IκB kinase α. The increased COX-2 expression favors the survival of renal medullary interstitial cells in hypertonic conditions. We have previously shown in a rat model that ketamine treatment enhances bladder interstitial fibrosis; alternates in micturition patterns; accelerates macrophage infiltration; and initiates the up-regulation of COX-2, inducible nitric oxide synthase, and endothelial nitric oxide synthase expression. Moreover, the increased expression of COX-2 in bladder stromal cells may be a potential prognostic biomarker of urothelial cancer, especially of cancer-specific death and recurrence.

Transcription factor NF-κB plays a crucial role in inflammatory response. A variety of extracellular stimuli dissociate NF-κB from cytoplasmic inhibitors, followed by translocation of NF-κB p65 to the nucleus and binding to specific DNA sequences. The COX-2 gene (PTGS2) promoter has several potential response elements for transcription factors, such as cAMP-response element, specificity protein 1, NF-κB, nuclear factor of activated T cells/NF-IL-6, and TATA box. In vascular endothelial cells, lipopolysaccharides may induce COX-2 expression through the activation of nuclear factor of activated T cells/NF-IL-6 binding site. The promoter region of the human COX-2 gene contains two motifs with sequences similar to that of the consensus binding site for NF-κB. These findings suggest that NF-κB mediates COX-2 activation during inflammation.

Despite this progress, little is known about the involvement of the signaling pathway of NF-κB and COX-2 in KIC. Due to the lack of specific biomarkers, most KIC patients are diagnosed late and have irreversible bladder damage. In extension of our previous investigation, the present study elucidated the role of NF-κB and COX-2 and investigated the relationship between ketamine use and the development of KIC symptoms in rat bladder KIC. Elucidation of the potential factors will provide a better understanding of KIC in the bladder and specific biomarkers of KIC.

Materials and Methods

Animals and Ketamine Administration

Experiments were performed in 36 adult female Sprague-Dawley rats (BioLASCO Animal Center, Taipei, Taiwan) weighing 250 g. Our experiments were performed in three groups: i) a saline group (0.9% saline; control), ii) a ketamine group (ketamine 25 mg/kg per day by i.p. injection), and iii) a ketamine + COX-2 inhibitor group [ketamine combined with COX-2 inhibitor (parecoxib sodium; Dynastat [Pfizer, New York, New York]) 10 mg/kg per day by i.p. injection]; each group was treated for 28 days. The study protocol was approved by the Animal Care and Treatment Committee of Kaohsiung Medical University. All experiments were conducted according to the guidelines for laboratory animal care. All efforts were made to minimize animal stress.

Physical Indicators of Bladder Function

As previously described, the rats were placed in individual KDS-TL380 metabolic cages and the MLT0380 transducer (AD Instruments, Colorado Springs, CO). Micturition frequency and the volume of urine output were recorded for 3 days, and a mean value was determined. A 24-hour urine test was conducted for measuring protein and creatinine levels in urine. Blood (serum) creatinine was also determined. The ratio of protein to creatinine in urine and the creatinine clearance rate were calculated.

Cystometrography Study

Cystometrography was performed according to the method previously described. Cystograms were recorded until the bladder pressure was stable, then reproducible micturition cycles were recorded for 1 hour. A voiding contraction was defined as an increase in bladder pressure that resulted in urine loss. Pressure and force signals were amplified (ML866 PowerLab; ADInstruments, Dunedin, NZ) and recorded on a chart recorder (LabChart version 7; ADInstruments). Cystometrography parameters recorded included peak micturition pressure (the maximum pressure during micturition), bladder volume, voiding contractions, and nonvoiding contractions (without urine leakage during bladder infusion).

Ketamine and Metabolites Assay in Urine and Serum by High-Performance Liquid Chromatography

Urine samples were collected for a period of 24 hours after the last ketamine i.p. injection. One milliliter of blood was obtained from each rat’s tail for analysis of serum ketamine and norketamine, and the rats were then sacrificed. The concentrations of ketamine and norketamine in urine and serum were determined by high-performance liquid chromatography. After extraction and purification by liquid—liquid extraction using ethyl ether, urine and serum samples underwent chromatography on a
reversed-phase column, and ketamine and norketamine were detected at 200 nm by UV spectrophotometry.

Histological Study with Masson Trichrome Stain

Deparaffinized sections were stained with Masson trichrome stain (kit HT15; Sigma-Aldrich, St. Louis, MO). The Masson’s trichrome stained the connective tissue blue and detrusor smooth muscle (DSM) red. The color setting and the image-associated quantification were determined using image analysis software (Image-Pro Plus version 7.0; Media Cybernetics, Rockville, MD).

Primary Urothelial Cell and Human J82 Cell Cultures

In parallel to in vivo experiments in rats treated with ketamine or ketamine + Cox-2 inhibitor, in vitro experiments were performed in primary urothelial cells and human bladder J82 cells, using the urine collected from these rats. The urine obtained from the treated rats is referred to as either urine metabolites or urine. The concentrations of ketamine and norketamine in urine are reported in Table 1.

After the rat bladder was excised, a modified Roszell’s procedure was performed.29,30 The bladder mucosa was gently scraped from the muscle tissue. The bladder was immersed in 4 mL of 1% collagenase IV (Sigma-Aldrich) at 37°C on a shaker for 40 minutes. The urothelial cells were plated at a cell density of 5 × 10^6 cells/mL in Gibco keratinocyte serum-free medium (Life Technologies, Grand Island, NY). The culture was supplemented with 5 ng/mL epidermal growth factor, 100 U/mL penicillin, and 1 μg/mL streptomycin (Gibco) for 48 hours. Ultrastructural morphology showed microvilli in stratified urothelium and immunolabeled for cytokeratin 17, which confirmed successful urothelial cell culture. Moreover, human J82 cells were used for examining whether activated NF-κB translocated to nucleus and affected COX-2 mRNA expression. Human J82 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum.

Table 1 Post-Treatment Characteristics, Biochemistry, and Urodynamic Parameters for Different Experimental Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Ketamine (n = 12)</th>
<th>Ketamine + Cox-2 inhibitor (n = 12)</th>
<th>Control (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>267.0 ± 36.8</td>
<td>263.0 ± 31.6</td>
<td>265.0 ± 37.2</td>
</tr>
<tr>
<td>Bladder weight (mg)</td>
<td>200.0 ± 16.0*</td>
<td>157.0 ± 10.0†</td>
<td>150.0 ± 7.0</td>
</tr>
<tr>
<td>Urodynamic parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (no. voids/60 minutes)</td>
<td>12.8 ± 3.2**</td>
<td>3.8 ± 0.8†</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Peak micturition pressure (cm H2O)</td>
<td>48.6 ± 4.5**</td>
<td>34.7 ± 3.8†</td>
<td>30.3 ± 2.4</td>
</tr>
<tr>
<td>Voided volume (mL)</td>
<td>0.66 ± 0.12**</td>
<td>1.78 ± 0.30†</td>
<td>1.96 ± 0.26</td>
</tr>
<tr>
<td>No. nonvoiding contractions between micturition (no./60 minutes)</td>
<td>3.5 ± 0.68**</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Water intake (mL/24 hours)</td>
<td>28.4 ± 5.8</td>
<td>31.0 ± 5.3</td>
<td>30.6 ± 3.5</td>
</tr>
<tr>
<td>Urine output (mL/24 hours)</td>
<td>18.2 ± 3.9*</td>
<td>24.5 ± 4.2†</td>
<td>26.5 ± 3.5</td>
</tr>
<tr>
<td>Serum parameters</td>
<td></td>
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</tr>
<tr>
<td>Ketamine (ng/mL)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Norketamine (ng/mL)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Urine parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketamine conc. (ng/mL)</td>
<td>1468 ± 251.4**</td>
<td>1406 ± 255.9**</td>
<td>ND</td>
</tr>
<tr>
<td>Norketamine conc. (ng/mL)</td>
<td>38,720.2 ± 1762.7**</td>
<td>32,720.0 ± 1485.6**</td>
<td>ND</td>
</tr>
<tr>
<td>Ccr (mL/min per kg)</td>
<td>0.822 ± 0.033*</td>
<td>1.180 ± 0.063†</td>
<td>1.160 ± 0.058</td>
</tr>
<tr>
<td>Urine protein/creatinine ratio</td>
<td>1.08 ± 0.32**</td>
<td>0.860 ± 0.25†</td>
<td>0.51 ± 0.13</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD.

*P < 0.05, **P < 0.01 versus control (saline) group.

1P < 0.05, †P < 0.01 versus ketamine group.

Ccr, clearance rate of creatinine; Cox, cyclooxygenase; ND, not detected.

alamarBlue Reduction Assay for Cell Viability after Ketamine and Urine Treatment

Primary urothelium of bladder was treated with ketamine and urine by alamarBlue assay (Life Technologies) to examine cell viability and in vitro cytotoxicity. Cultured cells were trypsinized from subconfluent cultures, counted with a haemacytometer (Hauser Scientific Company, Horsham, PA), and brought to a concentration of 1 × 10^6 cells/mL and subsequently to serial 1:2 dilutions. The absorbance of test and control wells was read at 540 and 630 nm, respectively, by a standard spectrophotometer at 0, 2, 4, 8, 16, and 24 hours after the addition of alamarBlue. The alamarBlue added to the medium without any cells served as a negative control.

MTT Assay for Cell Viability after Ketamine and Urine Treatment

The viability of rat urothelial cells was also tested by MTT assay after ketamine and urine treatment. Hydrogen peroxide

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was used as a positive control for cell death. The cell viability was measured with the blue formazan that was metabolized from colorless MTT by mitochondrial dehydrogenases, which were active only in the viable cells. The cells were incubated at 37°C for 2 hours after the addition of 0.5 mg/mL MTT. The solubilizing reagent, dimethyl sulfoxide, was then added to extract the blue formazan product, followed by measurement of the absorbance at 550 nm using SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA) at 0, 2, 4, 8, 16, and 24 hours after the addition of MTT. The incubation time and the cell number used for the reaction were optimized for quantification.

Ketamine and Urine Treatment in Cell Cultures

Rat bladder urothelium and human bladder J82 cells were treated with ketamine in the range of 0.5 to 8.0 mmol/L with or without COX-2 inhibitor (parecoxib 10 nmol/L) diluted with Dulbecco’s modified Eagle’s medium. In the urine obtained from rats treated with ketamine and ketamine + COX-2 inhibitor, the urinary concentrations of ketamine were 1468 and 1406 ng/mL, respectively, and those of norketamine were 38,720 and 32,720 ng/mL (Table 1). The cells were treated with urine at different concentrations diluted with Dulbecco’s modified Eagle’s medium (urine dilutions: 2000×, 1000×, 500×, 250×, and 125×). The concentration of urine ketamine used was in the range of 0.7 to 11.2 ng/mL and that of norketamine was 19.0 to 304.0 ng/mL. Concentrations at the different time points after treatment (2, 4, 8, 16, and 24 hours) were also investigated. Urine samples were obtained from all rats as a mixture.

Immunofluorescence Studies of NF-κB and Cox-2 Expression

For in vivo bladder section, immunostaining was performed according to published methods. The sections were then doubly stained with the primary antibody to NF-κB (1:50 approximately 100, mouse IgG3κ; Cell Signaling Technology), Cox-2 (1:50 approximately 100, rabbit IgG; Epitomics, Burlingame, CA), and ED-1 (1:50, mouse IgG1, macrophage biomarker; AbD Serotec, Kidlington, UK) at 4°C overnight, then incubated with secondary antibody (1:800; Invitrogen) conjugated to fluorescein isothiocyanate for NF-κB, conjugated to rhodamine for Cox-2 or Ed-1. The nuclei of the cells were counterstained with DAPI. For in vitro cell culture, the rat urothelial cells and human J82 cells were also treated with ketamine or urine obtained from ketamine-treated rats. The green-stained NF-κB and the red-stained Cox-2/Ed-1 were captured using confocal laser scanning microscopy (MRC 1024 ES; Bio-Rad Laboratories, Segrate, Italy).

Protein Isolation and Western Blot Analysis of Inflammatory and Fibrosis Markers

Thirty micrograms of protein from the bladders was loaded on SDS polyacrylamide electrophoresis gel and transferred to PVDF membranes (Immobilon-P; Millipore, Billerica, MA). Monoclonal antibodies to transforming growth factor β (1:1000, rabbit IgG; R&D Systems, Minneapolis, MN), fibronectin (1:1000, mouse IgG; BD Biosciences, San Jose, CA), type I collagen (1:1000, rabbit IgG; Abcam, Cambridge, UK), and Cox-2 (1:1000, rabbit IgG; Cayman Chemical Company, Ann Arbor, MI) were used for determining the change in protein expression and incubated with the secondary antibody. In each experiment, negative controls without the primary antibody were analyzed. Band intensity was normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and β-actin (1:5000; Upstate Group, Charlottesville, VA).

Preparation of Cytoplasmic and Nuclear Extracts and Western Blot Analysis of NF-κB Translocation

Nuclear and cytoplasmic extracts were prepared by the protocol modified from the method described by NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA). Briefly, primary urothelial cells were harvested with trypsin-EDTA and centrifuged at 500 × g for 5 minutes, pellets were washed with phosphate-buffered saline, and ice-cold cytoplasmic extraction reagent (CER) I was added to the cell pellets. The cell pellets were incubated on ice for 10 minutes after vigorous vortexing, and ice-cold
CER II was added to the tube. The cell pellets were centrifuged for 5 minutes at approximately 16,000 × g, the supernatant (cytoplasmic extract) was transferred to a tube, and nuclear and cytoplasmic extracts were stored at −80°C until use. Primary antibody to NF-κB (1:1000, rabbit IgG; Novus Biologicals, Littleton, CO) was incubated overnight and incubated with anti-rabbit secondary antibody. Band intensity was normalized with respect to laminin A/C (1:5000, mouse IgG; Cell Signaling) for nuclei extract and β-actin (1:5000, mouse IgG; Upstate Group) for cytoplasmic extract.

Real-Time Quantitative PCR Study of COX-2 Gene Expression

For real-time quantitative PCR, National Center for Biotechnology Information primer design was used for making gene-specific primers to Cox-2 gene (Ptgs2) 221 bp (forward primer: 5′-TGAGCATCTACGGTTGGTCTG-3′; reverse primer: 5′-TGCTTGCTGGAAACAATGC-3′), and Gapdh (forward primer: 5′-ATCTCCCTCTGCACTCCGTCGCAAT-3′; reverse primer: 5′-CATGGGACTCCTGCACTCCGAACT-3′). Optimal concentrations were determined with a primer matrix (lowest SD with no change in cycle to threshold). Real-time quantitative PCR was performed with 50-ng cDNA templates in plates using the ABI Prism 7000 sequence-detection system [AB Sciex, Foster City, CA (formerly, Applied Biosystems)]. Analysis of each sample was performed in duplicate. The cDNA templates were combined with optimal concentrations of primers and SYBR Green PCR dye mix (Applied Biosystems) in a total volume of 50 μL and the amplification conducted as recommended by the manufacturer.

The PCR conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of extension at 95°C for 15 seconds and 1 minute at 60°C. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curves. The software automatically recorded the cycle to threshold. *P < 0.05, **P < 0.01 versus saline group; †P < 0.01 versus ketamine group. DSM, detrusor smooth muscle; SL, suburothelial layer; UL, urothelial layer.

RT-PCR

Total RNA was extracted from rat primary urothelial cells and human J82 cells by TRIzol methods (Invitrogen). A
A reverse-transcription reaction was performed on 1 µg of extracted total RNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. For each sample, 1 µg of RNA was treated with DNase I (Invitrogen) to degrade contaminating single- and double-stranded DNA. Treated RNA was converted to single-stranded cDNA using Superscript II Reverse Transcriptase (Invitrogen) as recommended by the manufacturer.

Figure 3 A–C: Immunofluorescence analysis of NF-κB p65 and cyclooxygenase (COX)-2 expression after treatment with saline (A), ketamine (B), or ketamine + COX-2 inhibitor (C) in bladder tissue. Double immunostaining of NF-κB p65 (green, left panels) and COX-2 (red, arrowheads, middle panels) is shown in urothelial layer (UL; areas within the dotted lines). Nuclear DNA was labeled with DAPI (blue). The merged image from left and middle panels (yellow, right panels) is shown. Erythrocytes (arrows) in the UL and suburothelial layer (SL) are identified in ketamine group. The arrowhead (B) indicates NF-κB and COX-2 co-stained cell.

D: Western blots of Cox-2 expression and quantification of the percentage of COX-2 to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) in bladder tissue. E: Real-time quantitative PCR was also applied to assess the COX-2 mRNA expression in the UL of the bladder. Data are expressed as means ± SD. n = 12. **P < 0.01 versus saline group; ††P < 0.01 versus ketamine group.
Single-stranded cDNA was quantified with UV spectrophotometry (Beckman Coulter, Indianapolis, IN) and diluted to 50 ng/mL. For RT-PCR, National Center for Biotechnology Information primer design was used for making gene-specific primers to COX-2 221 bp (sense primer: 5'-ATCAATGCAAGTTCTTCCG-3'; antisense primer: 5'-GACTCTTTTCCGGCAACA-3'), and β-actin 145 bp (sense primer: 5'-ATCTCTTCTG- CATCTGTGTCGGCAAT-3'; antisense primer: 5'-CATGGAGTCTGTCGGCATCCAGAAC-3') were also designed using National Center for Biotechnology Information primer design.

Transfection and Reporter Gene Assay

Human bladder J82 cell lines were seeded in 6-well plates before promoter plasmids were applied. COX-2 promoter plasmids (pXC 918, 250, and 80) were transfected into human bladder J82 cells using Lipofectamine 2000 (Invitrogen). Human COX-2 promoter region (−918/−80 bp) was prepared by PCR amplification of J82 genomic DNA with specific primers (5'-AGAAAGCTTGAGCTTGCA-3' and 5'-AGAAAGCTTGAGCTTGCA-3'). The plasmid sequences were confirmed by DNA sequencing and ligated into luciferase plasmid pXP1. J82 cells were transfected with 0.5 μg of COX-2 promoter plasmid for 24 hours. After transfection, the cells were treated with urine metabolite in Dulbecco’s modified Eagle's medium for 30 minutes. After treatment, cell lysates were tested using a commercial luciferase assay kit (Promega Corp., Heidelberg, Germany). To test the effect of NF-κB inhibitor on human bladder J82 cells in response to urine metabolites, the cells were pretreated with 2 μmol/L BAY 11-7082 for 30 minutes and then stimulated with urine. After treatment, cell lysates were tested using a commercial luciferase assay kit (Promega).

Statistical Analysis

Analysis of variance followed by the Bonferroni test and two-way analysis of variance for individual comparison were performed. The Student’s t-test was used for calculating P values for comparison. Statistical significance was set at P < 0.05.

Results

Effects of Ketamine and COX-2 Inhibitor on Bladder Function

Ketamine-treated rats showed bladder hyperactivity with increases in voiding contraction, nonvoiding contraction, peak micturition pressure, and micturition frequency (Figure 1A and Table 1). However, treatment with ketamine + COX-2 inhibitor significantly reduced peak micturition pressure and micturition frequency and increased bladder capacity compared with ketamine (Table 1). Additionally, tracing analysis of voiding behavior by metabolic cage revealed that ketamine treatment decreased voiding volume and increased micturition frequency compared with saline (Figure 1B and Table 1). Similarly, ketamine + COX-2 inhibitor treatment improved bladder storage function compared with ketamine. These results demonstrated that ketamine treatment caused significant bladder storage dysfunction, whereas ketamine + COX-2 inhibitor prevented these changes.

Concentrations of Ketamine and Its Metabolites in Urine and Serum

Ketamine is metabolized by liver to norketamine (primary metabolite) and is excreted in urine. After treatment of rats with ketamine with or without COX-2 inhibitor, significant amounts of ketamine and norketamine were found in urine (Table 1). However, ketamine and norketamine were
Primary urothelial cells after 4hrs ketamine treatment

A  Saline

B  Ketamine

C  Ketamine + Cox-2 inhibitor

Primary urothelial cells after 4hrs urine treatment

D  Saline

E  Ketamine

F  Ketamine + Cox-2 inhibitor

G

- % of NF-κB⁺/Cox-2⁺/DAPI⁺
- % of NF-κB⁺/Ed-1⁺/DAPI⁺
undetectable in the serum of all three groups. The urinary concentrations of ketamine and norketamine were very high after ketamine treatment. However, there was no significant difference in the urinary concentrations of ketamine and norketamine between the ketamine group and the ketamine + COX-2 inhibitor group. These results indicated that the toxic effect of ketamine on the bladder might come from both ketamine and its metabolites. Moreover, COX-2 inhibitor exhibited no effect on ketamine metabolism.

**Histological Features of Ketamine-Associated Bladder Damage**

In the control group (Figure 2A), there was only sparse collagen distributed between DSM, and urothelium was three to five layers in thickness. In the ketamine group (Figure 2B), the bladder tissues were characterized by ulcerated urothelium, erythrocyte accumulation, monocellular infiltration, and increased interstitial fibrosis. The observed ketamine-induced inflammatory changes in bladder mucosa were similar to ulcerative cystitis. However, in the ketamine + COX-2 inhibitor group (Figure 2C), the thickness of urothelium restored to three to four layers; interstitial fibrosis and mononuclear cell infiltration were reduced.

Transforming growth factor β, fibronectin, and type I collagen expression were significantly increased (5.5-, 3.7-, and 10.5-fold, respectively) at 28 days after ketamine administration compared with those in the control group (Figure 2, D–F). In contrast, in the ketamine + Cox-2 inhibitor treatment group, transforming growth factor β, fibronectin, and type I collagen expression were significantly decreased (0.41-, 0.52-, and 0.18-fold, respectively) compared with those in the ketamine group. In addition, the ratio of collagen to DSM was significantly increased after ketamine treatment, whereas in the ketamine + COX-2 inhibitor group, the collagen-to-DSM ratio was lessened, indicating decreased interstitial fibrosis. These findings revealed that COX-2 inhibitor reduced fibrotic biosynthesis and protected bladders from ketamine-induced interstitial fibrosis changes.

**Increased COX-2 Expression after Ketamine Treatment at the Protein and mRNA Levels**

Immunofluorescence study demonstrated no staining in both the control and ketamine + COX-2 inhibitor groups (Figure 3, A and C). After ketamine treatment, NF-κB p65 and COX-2 proteins were co-labeled in urothelial layer (Figure 3B). Moreover, the COX-2+ cells were co-stained with Ed-1 and were mainly distributed in the suburothelial layer. The COX-2 expression coincided with NF-κB p65 expression after ketamine induction, indicating that COX-2 and NF-κB p65 were synthesized during the inflammatory process of KIC.

Western blots showed that COX-2 expression increased by 1.8-fold after ketamine treatment in bladder tissue (Figure 3D). COX-2 expression in the ketamine + COX-2 inhibitor group was not significantly increased compared with that in the control group. Real-time quantitative PCR was also applied to assess the COX-2 mRNA expression in the urothelial layer of the bladder (Figure 3E). Treatment with ketamine resulted in a 10.1-fold increase in COX-2 mRNA expression compared with saline (Figure 3E). In contrast, rats treated with ketamine + COX-2 inhibitor had a significant decline in COX-2 mRNA expression compared with ketamine-treated rats (Figure 3E).

**alarBlue and MTT Analysis of Bladder Urothelial Cell Viability**

To determine the optimal working concentrations of ketamine or urine and optimal incubation time, rat primary urothelial cells were treated with ketamine at a concentration range of 0.5 to 8.0 mmol/L (Figure 4A) by alamarBlue assay and MTT. Urinary ketamine and norketamine concentrations are listed in Table 1. The urothelial cells were also treated with urine (Figure 4B) by alamarBlue assay and MTT. A significant dose-dependent reduction in cell viability was found after ketamine and urine treatment. Cell viability of primary urothelial cell was confirmed by MTT test. Similar results for cell viability after treatment with ketamine (Figure 4, C and E) and urine (Figure 4, D and F) were obtained. Quantitative RT-PCR of primary urothelial cells after ketamine (Figure 4G) and urine (Figure 4 H) treatment was applied to the transcriptional level of Cox-2 mRNA expression. These data revealed that the optimal working concentration of ketamine was 1 to 2 mmol/L, and that optimal incubation time was 4 to 8 hours. In addition, the concentration of ketamine in urine after 1000× dilution was 1.4 to 2.8 ng/mL, and that of norketamine in urine was 38 to 76 ng/mL. Therefore, the working concentration of 1 mmol/L of ketamine and the incubation time of 4 hours were used for exploring the role of NF-κB and Cox-2 in the inflammatory process of KIC.
Primary urothelial cells after 4hrs ketamine treatment

A

Saline  NF-κB  Cox-2  DAPI  Merge

B  Ketamine

C  Ketamine + Cox-2 inhibitor

Primary urothelial cells after 4hrs urine treatment

D  Saline

E  Ketamine

F  Ketamine + Cox-2 inhibitor

G

Cytoplasmic NF-κB
cytoplasmic actin
Nuclear NF-κB
Lamin A/C

H  Optical density of NF-κB

I  Optical density of nuclear NF-κB with lamin A/C

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Immunofluorescence Analysis of NF-κB and Cox-2 Expression after Ketamine and Urine Stimulation in Urothelial Cell Culture

To elucidate the role of NF-κB and COX-2 in KIC, primary cell culture from urothelium was treated with saline (Figure 5A), 1 mmol/L ketamine (Figure 5B), and 1 mmol/L ketamine combined with 10 mmol/L COX-2 inhibitor (Figure 5C) for 4 hours. Double-labeled immunofluorescence of NF-κB p65, COX-2, or ED-1 protein was performed. No staining was found in the primary urothelial cells in the saline group (Figure 5, A and G). However, after 4 hours of 1 mmol/L ketamine treatment, all NF-κB p65+/DAPI+ cells were co-labeled with Cox-2, and the percentage of NF-κB p65 with Cox-2 co-staining was 15.0% ± 2.5% of total DAPI+ cells (Figure 5, B and G). In contrast, some NF-κB p65+/DAPI+ cells were co-labeled with ED-1, and the percentage of NF-κB p65 with ED-1 co-staining was 9.6% ± 2.1% (Figure 5G). In addition, in the ketamine + COX-2 inhibitor group, NF-κB p65+ and COX-2+ co-staining was weak, but all NF-κB p65+/DAPI+ cells were co-labeled with COX-2. The percentage of NF-κB p65 co-stained with COX-2 was 1.40% ± 0.03%. However, only a few NF-κB p65+/DAPI+ cells were co-labeled with ED-1, and the percentage of NF-κB p65 co-stained with Ed-1 was 2.2% ± 0.3% (Figure 5, C and G).

In parallel to the ketamine experiments, in vitro experiments were also performed using the urine obtained from rats treated with ketamine or ketamine + COX-2 inhibitor. No staining was found in primary urothelial cells from bladder in the saline group (Figure 5D). On the contrary, treatment with urine from ketamine-injected rats showed significant NF-κB and COX-2 staining, and all NF-κB p65+/DAPI+ cells were co-labeled with COX-2 at 30.6% ± 4.5% (Figure 5, E and G). However, some NF-κB p65+/DAPI+ cells were co-labeled with Ed-1 at 9.88% ± 2.5% (Figure 5G). Moreover, after treatment of urine from rats treated with ketamine + COX-2 inhibitor, all NF-κB p65+/DAPI+ cells were co-labeled with Cox-2 at 4.8% ± 1.1%. Only a few NF-κB p65+/DAPI+ cells were co-labeled with Ed-1 at 5.0% ± 0.4% (Figure 5, F and G).

Effects of Ketamine and Its Metabolites on NF-κB p65 Translocation and COX-2 Expression

Confocal microscopy was performed to examine the nuclear and cytoplasmic morphology of urothelium as well as the expression of NF-κB p65 and COX-2 after treatment with ketamine (Figure 6, A–C) or urine (Figure 6, D–F). Double-labeled immunofluorescence study of primary urothelial cells with NF-κB p65 and COX-2 proteins was performed. No immunofluorescence staining was found in either of the saline groups (Figure 6, A and D). In the ketamine-treated group (Figure 6B) and urine-treated group of cells from ketamine-treated rats (Figure 6E), NF-κB p65+ cells were strongly co-labeled with Cox-2 in nucleus and cytoplasm. In contrast, in the ketamine + COX-2 inhibitor group (Figure 6C) and in urine-treated cells from rats treated with ketamine + COX-2 inhibitor (Figure 6F), only weak NF-κB p65 and Cox-2 staining was observed in the cytoplasm of urothelial cells (Figure 6, C and F). Double-labeled analysis revealed that treatment with ketamine and urine induced the translocation of NF-κB p65 to nucleus and a strong expression of COX-2 in nucleus and cytoplasm. In contrast, COX-2 inhibitor suppressed the translocation of NF-κB p65 and COX-2 expression in urothelial cells.

Protein levels of NF-κB p65 in nuclear and cytoplasmic extracts were also investigated (Figure 6, G–I). Treatment with urine obtained from rats treated with ketamine or ketamine + COX-2 inhibitor significantly increased nuclear NF-κB p65 expression, by about 10.1- and 5.2-fold, respectively, compared with saline (Figure 6, G and I). In addition, treatment with urine obtained from these two groups significantly increased cytoplasmic NF-κB p65 expression, by about 4.6- and 1.5-fold, respectively (Figure 6, G and H). In comparison, ketamine + COX-2 inhibitor treatment resulted in a significant decline in NF-κB p65 expression in nucleus and cytoplasm compared with those with ketamine treatment (Figure 6, G–I). These findings demonstrated that ketamine and urine treatment induced the translocation of NF-κB p65 to nucleus and activated COX-2 expression in primary urothelium, whereas COX-2 inhibitor suppressed NF-κB p65 translocation and decreased COX-2 expression.

Ketamine and Its Metabolites Induce NF-κB Translocation and Regulate Inflammatory Gene COX-2 in Human Bladder J82 Cells

Immunofluorescence study was performed to examine the morphology as well as the expression of NF-κB p65 in human J82 cells stimulated with ketamine and urine (Figure 7, A–C). Double-labeled immunofluorescence with NF-κB p65 and COX-2 revealed almost no immunostaining in the saline group (Figure 7A). However, all NF-κB p65+ cells were co-labeled with COX-2 after treatment with urine obtained from ketamine-treated rats, and the percentage of NF-κB p65 with COX-2 co-staining was 17.0% ± 4.2% (Figure 7, B and D). After treatment with urine obtained from rats treated with ketamine + COX-2 inhibitor, although all NF-κB p65+/DAPI+ cells were co-labeled with COX-2, the percentage decreased to 6.40% ± 2.3% (Figure 7, C and D).
These results suggest that ketamine and its primary metabolite, norketamine, evoked the nuclear translocation of NF-κB p65 and COX-2 activation in J82 cells.

Analysis of NF-κB—Responsive Regions in the Promoter Area of COX-2 Gene by Ketamine and its Metabolites

To determine the molecular effect of NF-κB binding on the induction of COX-2 promoter in urine stimulation, the human J82 cell line was treated with diluted urine. Therefore, to identify promoter sites responding to NF-κB, we focused on the COX-2 promoter region ranging from −918 to −80 bp (Figure 8A). The J82 cells were transfected with COX-2 promoter plasmid (pXC 918) (Figure 8B), COX-2 promoter mutant pXC 250 (Figure 8C), or pXC 80 (Figure 8D), and then were treated with urine. The urine from ketamine-treated rats evoked COX-2 promoter (pXC 918) activation (Figure 8B). When the region to 250 bp including intact cAMP-response element and nuclear factor
of activated T cells/NF-κB and IL-6 binding sites was deleted, this response to urine treatment was abolished (Figure 8C). A similar result was obtained using the mutant pXC 80 of COX-2 promoter (Figure 8D). These findings suggest that the cAMP-response element and nuclear factor of activated T cells/NF-κB IL-6 regions were not necessary for ketamine- and norketamine-mediated activation of COX-2 transcription. Promoter deletion analysis did not provide a clear conclusion with respect to which binding sites (activator protein 2, specificity protein 1, and NF-κB) ranging from /C0 918 to /C0 250 bp were involved in the COX-2 response to urine metabolites (ketamine and norketamine). Additionally, pretreatment of the J82 cells with NF-κB inhibitor (BAY 11-7082) inhibited COX-2 promoter activity and COX-2 mRNA after stimulated urine from ketamine-treated rat (Figure 8E). Quantitative RT-PCR of human J82 cells showed that COX-2 mRNA expression increased by three-fold after urine treatment, whereas ketamine + COX-2 inhibitor treatment suppressed COX-2 mRNA expression compared with saline (Figure 8F), demonstrating that ketamine and its primary metabolites induced an increase in the promoter activities of COX-2 gene in J82 cells.

The present results revealed that ketamine and its metabolites evoked COX-2 promoter activation through NF-κB. These findings suggest that stimulation of cells by urine metabolites induced translocation of NF-κB, and that NF-κB activation afterward regulated COX-2 gene activity. A possible model for illustrating urinary metabolite (ketamine and norketamine)-mediated nuclear translocation of NF-κB and activation of COX-2 genes in KIC is proposed in Figure 9.

**Discussion**

Ketamine treatment caused bladder hyperactivity by altering peak micturition pressure, rising micturition frequency, increasing nonvoiding contraction, and decreasing bladder capacity. Moreover, ketamine enhanced bladder interstitial fibrosis, decreased urothelium thickness, augmented submucosal hemorrhage, and accelerated macrophage infiltration. Ketamine administration induced overexpression of NF-κB and Cox-2 protein in bladder urothelium and enhanced NF-κB translocation from cytoplasm to nucleus. Treatment with Cox-2 inhibitor lessened NF-κB p65 translocation and eliminated Cox-2 overexpression. In addition, our previous study found that ketamine injection induced the up-regulation of Cox-2 expression, and most Cox-2+ cells in bladder suburothelium were co-stained with Ed-1+ macrophages in KIC. The
The present investigation demonstrated that NF-κB cells were co-labeled with COX-2 in vivo and in vitro. Taken together, our observations revealed that treatment with ketamine or urine stimulated the expression of NF-κB p65a and COX-2 in rat primary urothelium and activated macrophage at both the mRNA and protein levels.

**Role of COX-2 in Bladder Overactivity and Altered Micturition Patterns in KIC**

COX-2 enzyme is activated in response to the presence of pro-inflammatory cytokines and growth factors. It has been suggested that COX-2 is the key mediator after the induction of cyclophosphamide-induced cystitis.\(^3\) The product of COX-2, prostaglandins (PGs), has strong inflammatory effects. A number of studies have demonstrated the role of COX-2 in bladder overactivity in association with inflammation and/or hypertrophy, where the participation of PG produced by the COX-2 enzyme has been well studied. In sensory neuron culture and chemical-induced cystitis, PGE\(_2\) was shown to induce sensitization of sensory neurons, revealing an important role of COX-2 and PGs in altered micturition pattern.\(^3\) Another study showed that water deprivation and hypertonicity activate NF-κB and consequently increase COX-2 expression, which favors the survival of renal medullary interstitial cells in hypertonic conditions.\(^1\) Inhibition of renal medullary interstitial cell COX-2 could contribute to nonsteroidal anti-inflammatory drug-induced papillary injury.\(^3\) In the present results, ketamine treatment showed significant COX-2 overexpression, whereas treatment with ketamine + COX-2 inhibitor significantly decreased COX-2 expression. Ketamine + COX-2 inhibitor also ameliorated micturition overactivity as well as lessened bladder urothelial damage and interstitial fibrosis caused by direct toxic effects of ketamine or its metabolites on bladder DSM and interstitial cells. This prominent overexpression of COX-2 after ketamine treatment suggests that COX-2 or its downstream product (PG) might play a crucial role in altering micturition pattern and bladder interstitial fibrosis in KIC.

**NF-κB Is the Key Transcription Factor for Ketamine- and Norketamine-Mediated COX-2 Promoter Activation**

NF-κB signaling pathways have been linked with the inflammation process. Many NF-κB target genes are involved in inflammatory reaction and regulation of immunity.\(^4\) Our results indicated that NF-κB was the key transcription factor that responded to ketamine-mediated Cox-2 activation. Inhibition of NF-κB (BAY 11-7082) resulted in a reduction in COX-2 expression. The promoter region of the COX-2 gene contains a binding site for the p65 subunit of NF-κB, and activation of NF-κB can regulate downstream COX-2 expression. Our promoter-deletion analysis also confirmed that NF-κB plays a crucial role in ketamine-regulated signaling. There are at least two potential NF-κB binding sites that have been observed in the COX-2 promoter region. It remains unclear which binding site is specifically involved in the development of KIC.

To determine the molecular effect of NF-κB binding on the induction of COX-2 promoter due to ketamine and urine stimulation, in vitro models can provide important insight into the potential factors regulating bladder function and pathogenesis. The present study used cell cultures of rat primary urothelial cell and human J82 cells to identify COX-2 promoter sites that responded to NF-κB activation. Only in human J82 cells, the binding sites involving COX-2 response to ketamine and norketamine could be identified by promoter-deletion analysis. The reported protein activity within transfected human J82 cells was proportional to the steady-state COX-2 mRNA level. Moreover, translocation of NF-κB p65 to the nucleus induced by ketamine and its metabolites in human J82 cells was found to coincide with a steady increase in COX-2 mRNA expression in primary urothelial cells and animal models.

Treatment of ketamine combined with COX-2 inhibitor reduced the protein expression of NF-κB and COX-2 in bladder tissue. In primary urothelial cell culture, treatment with urine obtained from rats treated with ketamine + COX-2 inhibitor also reduced COX-2 transcriptional level. The in vitro gene transfection and luciferase reporter gene assay elucidated the regulatory role of NF-κB in COX-2 activation. Our results...
demonstrated that the regulation of COX-2 via NF-κB pathway was involved in the inflammatory signaling of KIC in rat urinary bladder. Findings from a previous study in gastric cancer suggested that COX-2 activates NF-κB, thus regulating the transcription and expression of E-cadherin through the SNAI1 signaling pathway in gastric cancer, and that COX-2-mediated down-regulation of E-cadherin was dependent on the NF-κB pathway. Blockade of COX-2 activity, either by COX-2 siRNA or celecoxib, restored the expression of E-cadherin. COX-2 appeared to function upstream of NF-κB and regulated the expression of E-cadherin via the NF-κB/SNAI1 signaling pathway. In human lung cancer, the COX-2 inhibitor celecoxib abrogates the activation of cigarette smoke-induced NF-κB by suppressing the activation of IκB kinase. Taken together, whether COX-2 is a downstream or an upstream product of NF-κB is still inconclusive.

Toxic Effects of Ketamine and Its Metabolites on Bladder Urothelium

Chu et al previously suggested that ketamine or its metabolite norketamine might exert either a direct effect or microvascular damage or an autoimmune reaction. Ketamine is metabolized by the liver to norketamine (primary metabolite) and is excreted in urine. After ingestion, ketamine can be found in urine for up to 5 days and norketamine for up to 6 days. The present study demonstrated that direct interaction of both ketamine and norketamine significantly decreased cell viability in the primary urothelial cells and human J82 cells. Ketamine and norketamine were found to stimulate the expression of NF-κB p65 and COX-2 in rat primary urothelial cells, activate macrophage, as well as induce the translocation of NF-κB to nucleus and activate COX-2 expression. Therefore, the observed toxic effects of ketamine were a result of both ketamine and norketamine acting on bladder urothelium, which resulted in damaging the urothelial barrier function and the consequent development of KIC. An indirect effect might also occur by causing the macrophage activation against bladder urothelium and submucosa due to circulation of ketamine or its metabolites. Such information is valuable for any future research aiming at blocking the urinary effects of ketamine. It remains unclear what kind of receptor of urothelium is stimulated by urinary ketamine and norketamine, resulting in NF-κB p65 translocation and expression of COX-2 in the present KIC animal model. This issue will be explored in future investigations.

The present in vitro studies of urothelial cells revealed that 1 to 2 mmol/L ketamine after 4 to 8 hours of incubation initiated NF-κB translocation and induced the up-regulation of COX-2 expression. Bai et al found that 100 μmol/L ketamine increases proliferation in human neural stem cells after 6 hours of ketamine treatment. Moreover, after 24 hours of treatment, ketamine induced neuronal apoptosis, decreased mitochondrial membrane potential, and increased cytochrome c release as well as production of reactive oxygen species. In addition, Braun et al indicated that ketamine at 2 mmol/L concentration induces apoptosis of human lymphocytes and neuronal cells via the mitochondrial pathway, but that necrosis is the predominant mechanism at higher concentrations (6–8 mmol/L). These investigations have demonstrated that the cell-associated inflammatory system might provide a promising in vitro model for studying the dose–response relationship between ketamine and cell damage. The present study also used cell cultures of human J82 cells to identify COX-2 promoter sites that responded to NF-κB. The plasmids with different mutants of COX-2 promoter (pXC 918, 250, 80) were transfected into cultured cells. The reported protein activity or fluorescence within transfected J82 cells was approxi- mately proportional to the steady-state COX-2 mRNA level. Moreover, translocation of NF-κB p65 to the nucleus induced by ketamine and its metabolite in human J82 cells was found to coincide with a steady increase in COX-2 mRNA expression in primary urothelium and animal models.

Ketamine Initiates NF-κB Translocation and Induces Up-Regulation of COX-2 Expression in KIC

Currently, there is no general consensus on a ketamine concentration range that will cause urinary tract damage. Generally, the anesthetic dose of ketamine in humans is 0.5 to 2 mg/kg. To establish an in vivo animal model of ketamine abuse and to identify potential factors underlying KIC, the present rats received 25 mg/kg per day ketamine i.p. injection for 28 days, which resulted in bladder overactivity, increased micturition frequency, and decreased micturition volume. In addition, the bladder tissues were characterized by ulcerated urothelium, mononuclear cell infiltration, and increased interstitial fibrosis between DSM bundles. These symptoms and histological images are similar to the characteristics of ketamine abuse in humans. Meng et al found that after 8 weeks of daily 100-mg/kg ketamine injection in mice, there were increased voiding frequency and decreasing bladder capacity, similar to the symptoms that developed in human ketamine abusers. Moreover, Yeung et al demonstrated that the administration of 30 mg/kg per day of ketamine for 6 months resulted in infiltration of inflammatory mononuclear cells around and between glomeruli or tubules in kidney. These findings revealed a relationship between clinical symptoms not only with ketamine dosage but also duration of abuse.

The present findings support the hypothesis that ketamine injection incites the inflammation of bladder by inducing COX-2 gene expression via the NF-κB signal transduction pathway. The present investigation provides valuable insight into the understanding of KIC in bladder and specific biomarkers of KIC, which have relevance to clinical significance in treating KIC. Future studies will employ other inhibitors of COX-2 and NF-κB to further investigate NF-κB translocation and COX-2 gene activation, to examine the presently proposed model, and to consider an experiment focused on the COX-2 variation tendency in NF-κB blockade. Such studies might...
provide valuable insight into the understanding of KIC in rat bladder and specific biomarkers of KIC.

Conclusion

Ketamine treatment induces bladder overactivity, increases micturition frequency, and decreases micturition volume. Ketamine treatment also enhances bladder interstitial fibrosis, whereas COX-2 inhibitor prevents these changes and reduces the intensity of interstitial fibrosis. Moreover, ketamine and norketamine initiate the up-regulation of COX-2 expression and accelerate NF-κB translocation in urothelial cells. These results demonstrate that the regulation of COX-2 expression via the NF-κB pathway is involved in the inflammatory signaling in KIC. These findings may help us to design treatment strategies for KIC patients.

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