Clinical significance of interleukin-6 and inducible nitric oxide synthase in ketamine-induced cystitis

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Abstract. Ketamine is an ionotropic glutamatergic N-methyl-D-aspartate receptor antagonist, which is widely used among recreational drug abusers. Ketamine abusers exhibit substantially reduced bladder capacity, which can lead to urinary frequency. The molecular pathogenesis of ketamine-induced cystitis has been scarcely reported. Given previous clinical findings, it may be hypothesized that pathological alterations in smooth muscle cells (SMCs) of the urinary bladder serve a crucial role in the mechanism underlying cystitis. In the present study, two lineages of SMCs, one from differentiated foreskin-derived fibroblast-like stromal cells and the other from cultured normal aortic SMCs, were used to study ketamine-induced molecular alterations. Polymerase chain reaction was used to study the effects of ketamine on oxidative stress. The effects of adjuvant chemotherapy with cyclophosphamide (CTX) were also investigated. The results indicated that the expression levels of interleukin-6 and inducible nitric oxide synthase (iNOS) were decreased, whereas collagen expression and deposition were increased in ketamine-treated SMCs. Conversely, treatment with CTX restored the expression of iNOS, which may prevent or limit oxidative damage. In conclusion, the present study demonstrated that ketamine may induce several molecular alterations in SMCs and these changes may be associated with the clinical symptoms observed in ketamine abusers. In addition, the specific chemotherapeutic agent CTX may reverse these ketamine-induced aberrations.

Introduction

Ketamine, which is an ionotropic glutamatergic N-methyl-D-aspartate (NMDA) receptor antagonist, can produce a fast-acting antidepressant response in patients with major depressive disorder (1-3). Ketamine is used clinically for pediatric anesthesia and sedation, as an anesthetic in the treatment of asthma, and in pain management (4,5). In addition, ketamine, in liquid or powdered form, is a widely used recreational drug that can be injected, ingested or added to materials for smoking (6,7). The Drug Enforcement Administration of Taiwan categorizes ketamine as a Schedule III substance due to its illegal abuse.

Although ketamine rarely produces serious withdrawal symptoms, it does exert dose-dependent psychedelic and psychotic effects (8,9). In addition to its neurotoxic effects, ketamine affects other cell lineages due to its role as an NMDA receptor antagonist (10). For example, ketamine abuse can induce cystitis and cause serious damage to the urinary tract (11,12). Ketamine abusers may experience urinary frequency, urgency, dysuria, urge incontinence, hemorrhagic cystitis and hydronephrosis (13-15). In long-term ketamine abusers, bladder capacity may decrease substantially to one-tenth of the primary capacity (13,16,17). Major surgical operations, including augmentation ileocystoplasty, have been reported to efficaciously relieve refractory ketamine-associated bladder pain and to reduce urinary tract symptoms (7,18). In addition to surgery, chondroitin sulfate therapy can relieve symptoms of severe ketamine-induced contracted bladder (19).

In addition, transformation of the bladder wall and increased interstitial fibrosis in the bladder tissue have been observed in rats following long-term ketamine administration (7,20,21).

The mechanism underlying bladder contracture, a serious ketamine-induced side effect, remains unclear. Previous studies have suggested that clinical manifestations induced by ketamine may be mediated through a specific neurogenic mechanism or possible hypersensitivity (18,22). Furthermore, clinical data have suggested the involvement of chronic inflammation and epithelial repair in this lower urinary tract symptom (22-24). The side effects of ketamine abuse have emerged as a novel challenge for urologists (16).

Given the previous clinical findings, it may be hypothesized that pathological alterations in smooth muscle cells (SMCs) of the urinary bladder serve a crucial role in the mechanism underlying ketamine-induced cystitis. The present study used two lineages of SMCs, one from differentiated foreskin-derived fibroblast-like stromal cells (FDSCs) and the other from cultured normal aortic SMCs, to investigate ketamine-induced molecular alterations. Immunocytochemical staining was used to examine the effects of ketamine on differentiated FDSCs and cell viability. To determine the effects of ketamine on oxidative stress (25), polymerase chain reaction (PCR) array-based expression profiling was conducted. The ketamine-induced molecular alterations were subsequently validated using quantitative (q)PCR or western blot analysis. An understanding of the molecular alterations involved in ketamine-associated bladder dysfunction may contribute to the development of adjuvant therapy for ketamine abusers.

Materials and methods

Differentiation of human FDSCs to SMCs and cultivation of normal aortic SMCs. SMCs from two different lineages, FDSC-derived SMCs and cultured human normal aortic SMCs, were used to evaluate the effects of ketamine. Initially, FDSCs were harvested from foreskin tissues. This protocol was approved by the institutional review board of Cathay General Hospital (CGH-P100084). To differentiate isolated stromal cells into SMCs, FDSCs were cultivated in complete Dulbecco's modified Eagle's medium (#12100046; Thermo Fisher Scientific, Inc., Waltham, MA, USA) on fibronectin-coated culture plates, supplemented with two growth factors, transforming growth factor (TGF)-β1 (2.5 ng/ml) (#240-B; R&D Systems, Inc., Minneapolis, MN, USA) and platelet-derived growth factor-BB (PDGF-BB, 5 ng/ml) (#220-BB; R&D Systems, Inc.) in a humidified incubator at 37˚C and 5% CO_2, for 5-10 passages within 4 weeks (26-28). In addition, human normal aortic SMCs [American Type Culture Collection (ATCC) CRL-1999] were cultured in ATCC-formulated F-12K medium (ATCC #30-2004; both ATCC, Manassas, VA, USA) at 37˚C in an incubator containing 5% CO_2 with extra components [0.05 mg/ml ascorbic acid; 0.01 mg/ml insulin; 0.01 mg/ml transferrin; 10 ng/ml sodium selenite; 0.03 mg/ml endothelial cell growth supplement; 10% fetal bovine serum; 10 mM(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); 10 mM TES] suggested by the manufacturer.

Treatment of SMCs with ketamine and cyclophosphamide (CTX). To determine the effects of ketamine on normal SMCs, the concentration of residual ketamine (7.744 μg/ml) in the urinary bladder was used as a reference concentration in the direct treatment of FDSC-derived SMCs and human normal aortic SMCs (7). FDSC-derived SMCs were subjected to cyclic ketamine treatment. The prescription license for the use of ketamine (Ketalar Injection; Pfizer Ltd., Surrey, UK) in the present study was permitted by the Food and Drug Administration of the Ministry of Health and Welfare in Taiwan (announcement no. 1000088118; Taipei, Taiwan, R.O.C.) and by Cathay General Hospital (announcement no. CRM09500000101; Taipei, Taiwan, R.O.C.). One cycle of ketamine treatment comprised incubation of SMCs in a growth factor-containing medium with various doses of ketamine (0, 1 and 10 μg/ml) for 1 day, followed by a 1 day culture without ketamine, both at 37˚C. In an additional experiment, cultured human normal aortic SMCs were incubated with the chemotherapeutic agent CTX (10 μM) (Endoxan Injection; Baxter Deutschland GmbH, Unterschleißheim, Germany) for 5 h following 1-day ketamine (10 μg/ml) treatment at 37˚C (29).

Immunodetection of smooth muscle actin (SMA), collagens, interleukin (IL)-6 and inducible nitric oxide synthase (iNOS). For a comprehensive study, immunocytochemical and immunofluorescent (IF) staining, and western blot analysis were performed on FDSC-derived SMCs and normal aortic SMCs treated with various concentrations of ketamine. Briefly, cells on glass slides were fixed with 4% paraformaldehyde in phosphate-buffered saline for 5 min and were then permeabilized with 0.1% Triton X-100 for 20 min at room temperature. To ensure the differentiation efficacy of the FDSCs, SMA was detected by incubating the slides with undiluted anti-SMA antibody (#IR611) for 30 min at room temperature, and staining was developed using a Dako REAL detection system (#K4065) (both Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocols. IF staining was used to detect type II collagen (COL2), IL-6 and iNOS expression. Briefly, COL2 was detected by incubating the FDSC-derived ketamine-treated SMCs with anti-COL2 (MAB1330, 1:5) for 12 h at 4˚C and fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Ig)G (AP192F, 1:200) for 1 h at 25˚C (both EMD Millipore, Billerica, MA, USA).

To identify alterations in IL-6 and iNOS expression following CTX (0 or 10 μM) and ketamine (0 or 10 μg/ml) treatment, normal aortic SMCs were subjected to IF immunostaining. Briefly, SMA was detected using anti-SMA (sc-1616, 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and FITC-conjugated anti-goat IgG (#02-13-06, 1:200; KPL, Inc., Gaithersburg, MD, USA). Anti-IL-6 (ab6672, 1:500) and anti-iNOS (ab3523, 1:20) (both Abcam, Cambridge, UK) were used to colocalize SMA with IL-6 and iNOS, respectively, and Cy3-conjugated anti-rabbit IgG was used as a secondary antibody (AP182C, 1:200; EMD Millipore). The incubation time for all primary antibodies was 12 h at 4˚C and for secondary antibodies was 1 h at 25˚C. Finally, the nuclei were identified by counterstaining with 4',6-diamidino-2-phenylindole. The stained samples were dehydrated, mounted and analyzed using a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands).

Western blot analysis was performed to detect COL1 expression in FDSC-derived SMCs that underwent various

Table I. Probes and primer sequences for quantification of *IL-6*, *TGF-β1* and *COX-2*.

Gene name	Accession no.	Sequence (5'-3')	Probe no.
IL-6	NM_000600	F: gatgagtacaaaagtcctgatcca	40
		R: ctgcagccactggttctgt	
TGF-β1	NM_000660	F: tggacacgcagtacagcaa	11
		R: cttgcggcccacgtagta	
COX-2	AY462100	F: gctttatgctgaagccctatga	2
		R: tccaactctgcagacatttcc	
GAPDH	NM_002046	F: ctctgctcctcctgttcgac	60
		R: acgaccaaatccgttgactc	

COX-2, cyclooxygenase-2; F, forward; *IL-6*, interleukin-6 ; R, reverse; *TGF-β1*, transforming growth factor-β1.

numbers of ketamine treatment cycles according to the standard protocol. Briefly, cell lysates were prepared using a radioimmunoprecipitation assay buffer (Intron Biotechnology, Inc., Seongnam, South Korea) and quantified using Bio-Rad Protein Assay kit II (#5000002; Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturers' protocols. The protein aliquot (30 μg) of each lysate was separated by 12% SDS-PAGE and was transferred to a polyvinylidene fluoride membrane using a TE70 semidry transfer unit (GE Healthcare Life Sciences, Little Chalfont, UK). The membrane was blocked in 3% bovine serum albumin (#A3803; Merck KGaA, Darmstadt, Germany) for 1 h at 25°C, and COL1 was immunoblotted using anti-COL1 (ab292, 1:5,000; Abcam) for 1 h at 25°C. Anti-GAPDH (AM4300, 1:4,000; Thermo Fisher Scientific, Inc.) was used as a protein-loading control to ensure equal protein levels in all samples (incubation time, 1 h at 25°C). The secondary antibodies used were as follows: Biotinylated anti-rabbit IgG (BA-1000, 1:1,000; Vector Laboratories, Inc., Burlingame, CA, USA) for COL1 (30 min at 25°C) and horseradish peroxidase-conjugated anti-mouse IgG for GAPDH (1 h at 25°C) (AP192P, 1:5,000; EMD Millipore). The bands were visualized using a VECTASTAIN ABC-AmP Chemiluminescence Detection kit (AK-6601; Vector Laboratories, Inc.) for COL1 (10 min at 25°C) and Western Lightning Plus-ECL (#NEL103001EA; PerkinElmer, Inc., Waltham, MA, USA) for GAPDH, according to the manufacturers' protocols. Finally, images were captured using an Alpha Innotech FluorChem FC2 Imager (ProteinSimple, San Jose, CA, USA). Each protein band was recorded using UVP Bioimaging systems (UVP, LLC, Upland, CA, USA). The relative protein expression levels of COL1 normalized to GAPDH were identified by densitometry using ImageQuant, version 5.2 (Molecular Dynamics Inc, Chatsworth, CA, USA) and are expressed as the mean ± standard deviation.

Molecular alterations induced by ketamine in FDSC-derived SMCs. Total RNA was isolated from FDSC-derived SMCs treated with or without ketamine, using TRI Reagent (T9424; Sigma-Aldrich; Merck KGaA). RNA was reverse transcribed to cDNA using oligo(dT) primer and a PowerScript® Reverse

Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) following appropriate DNA digestion (AM2222; Thermo Fisher Scientific, Inc.), according to the manufacturers' protocols.

The inflammatory response and oxidative stress are believed to be involved in ketamine-associated cystitis. The present study examined the effects of ketamine on the differential expression of inflammation and oxidative stress markers in FDSC-derived SMCs with or without ketamine treatment (10 μg/ml) by comparing their expression levels. The relative mRNA expression levels of specific inflammatory markers were quantified using a gene expression assay with universal TaqMan probes in a LightCycler 1.5 system (both Roche Diagnostics, Basel, Switzerland). Primer sequences are listed in Table I. The thermocycling conditions were as follows: IL-6, TGF-β1, COX-2, and GAPDH were pre-incubated (95°C for 5 min) and amplified (95°C for 10sec, 60°C for 30 sec) for 55 cycles. To detect ketamine-induced oxidative stress, an RT^2 Profiler™ PCR array (Human Oxidative Stress Plus, PAHS-065Y; Qiagen N.V., Venlo, The Netherlands) was used with an Applied Biosystems 7300 Real-Time PCR system (Thermo Fisher Scientific, Inc.), according to the manufacturers' protocols.

The data were analyzed using software supplied by Qiagen (RT^2 Profiler™ PCR Array Data Analysis, version 3.5) and were normalized to the expression of five internal housekeeping genes: β-actin, NM_001101; β-2-microglobulin, NM_004048; *GAPDH*, NM_002046 and the comparative $2^{-\Delta\Delta Cq}$ method was used to analyze the relative changes in gene expression; hypoxanthine phosphoribosyltransferase 1, NM_000194; and ribosomal protein lateral stalk subunit P0, NM_001002. The significant targets [code numbers for titin (*TTN*): PPH06931F and *iNOS*: PPH00173F] were validated using a specific RT^2 qPCR primer assay (Qiagen N.V.) with the Applied Biosystems 7300 Real-Time PCR system. Dissociation curves were analyzed after each reaction to assess the quantification specificity in the RT^2 qPCR primer assay. Each quantification was calculated relative to the level of an endogenous control, *GAPDH* (NM_002046). Those showing changes in specific genes >2-fold were considered significant candidates.

Statistical analysis. The Student's t-test was used to compare the significant differences in gene expression between different groups. All statistical analyses were performed using SPSS software (v. 22.0; IBM Corp., Armonk, NY, USA). The data shown here are representative of at least three experiments with similar results, and P-values <0.05 are considered significant.

Results

Deformation of FCSC-derived SMCs by ketamine. The effects of ketamine on human SMCs were studied using FDSC-derived SMCs and normal aortic SMCs. Differentiation of FDSCs into SMCs was confirmed by the positive signals observed by immunostaining with an anti-SMA antibody (Fig. 1A). The FDSC-derived SMCs shrank and deformed into a spindle shape when treated with 10^2 μg/ml ketamine. Based on our observations of the molecular effects of ketamine on the urinary bladder (7) and the marginal concentra-
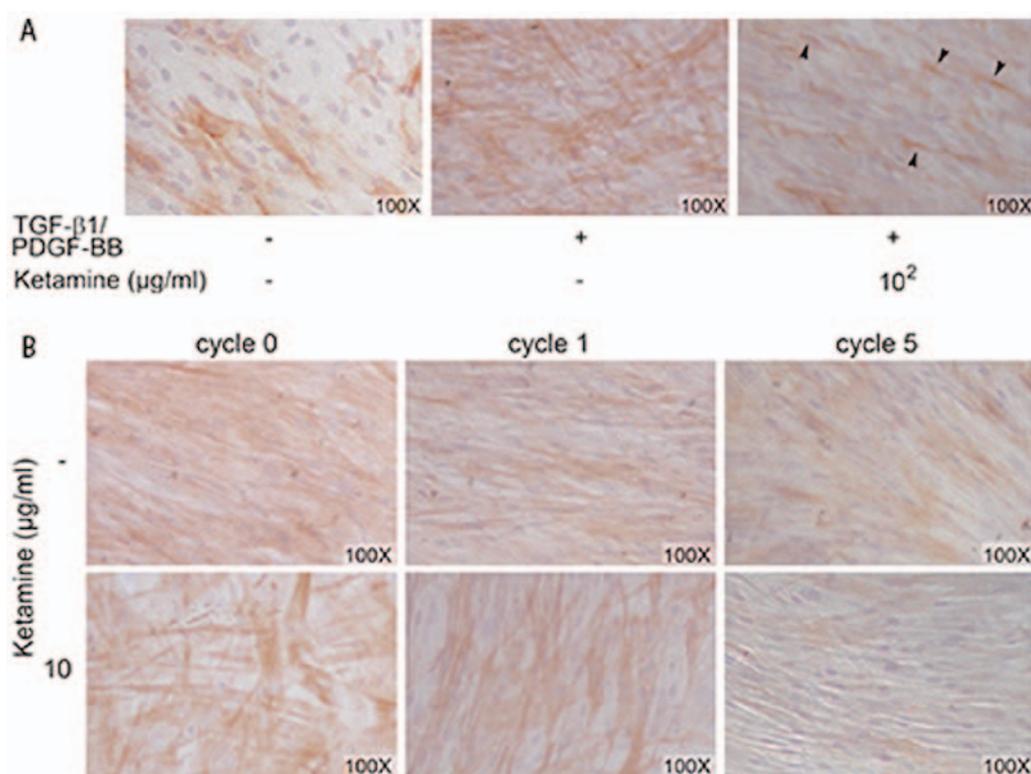
Figure 1. Immunodetection of SMA from human FDSC-derived SMCs treated with ketamine. (A) SMA expression in human FDSC-derived SMCs treated with ketamine. Isolated foreskin cells were harvested, seeded on fibronectin-coated culture plates and were then treated with (+) or without (-) the myogenic differentiation agent, containing TGF-β1 (2.5 ng/ml) and PDGF-BB (5 ng/ml). After a 14-day incubation for differentiation, cells were treated with ($10^2 \mu$g/ml) or without (-) ketamine for 5 days. Arrow heads represent cells with spindle shape. (B) Morphological alterations of human FDSC-derived SMCs following cyclic ketamine treatment. All isolated cells were treated with the myogenic differentiation agent followed by treatment with (10 μg/ml) or without (-) ketamine for various cycles (0, 1 and 5 cycles). Each cycle composed 1 day ketamine treatment and 1 day of culturing in medium alone. The cells were fixed and incubated with anti-SMA. FDSC, foreskin-derived fibroblast-like stromal cells; PDGF-BB, platelet-derived growth factor-BB; SMA, smooth muscle actin; TGF-β1, transforming growth factor-β1.

tion of ketamine detected in the urine of ketamine abusers, 10 μg/ml was selected as the concentration for cyclic treatment of SMCs (30,31). Compared with untreated FDSC-derived SMCs, cells grew to achieve a spindle-like morphology with additional cycles of ketamine treatment (Fig. 1B).

Collagen expression and deposition is increased in FDSC-derived SMCs following ketamine treatment. To investigate whether fibrosis is induced in FDSC-derived ketamine-treated SMCs, the protein expression levels of frequently studied collagens, COL1 and COL2, were detected. Western blot analysis demonstrated that COL1, which is associated with fibrosis, was decreased in FDSC-derived SMCs (Fig. 2A). This decrease was prevented in cells exposed to cyclic ketamine treatment. Densitometric analysis was used to semi-quantify the relative expression levels of COL1; the protein expression levels of COL1 were increased with increasing cycles (Fig. 2B). COL2 deposition around cell nuclei in the cytosol of FDSC-derived SMCs was identified after 5 cycles of ketamine treatment. Obvious COL2 deposition appears increased in ketamine-treated cells (Fig. 2C), not in cells treated with TGF-β1 and PDGF-BB only (Fig. 2D).

Detection of altered mRNA expression in FDSC-derived SMCs following ketamine treatment. Molecular alterations were detected in cells following cyclic ketamine treatment.

Cyclooxygenase-2 levels were increased and *IL-6* levels were decreased, whereas *TGF-β1* levels were not markedly altered after 5 cycles of ketamine treatment in FDSC-derived SMCs (Fig. 3A). The mRNA expression levels of *IL-6* were increased when the concentration of ketamine was lowered from 10 to 1 μg/ml during cyclic ketamine treatment (Fig. 3B). A pathway-focused PCR array was used to examine oxidative stress; the results indicated that the expression levels of two genes associated with oxidative stress, *TTN* (NM_003319) and *iNOS* (NM_000625), were significantly reduced, with fold-changes of 0.41 and 0.29, respectively, in FDSC-derived SMCs following 8 cycles of ketamine treatment (Fig. 4A). All other genes exhibited fold-changes within the range of 0.5 and 2.0. However, only *iNOS* was downregulated (0.25-fold) after repeated ketamine treatment, as determined by specific qPCR validation in FDSC-derived SMCs (Fig. 4B). The mRNA expression levels of *iNOS* were restored when the concentration of ketamine was decreased from 10 to 1 μg/ml during cyclic ketamine treatment (Fig. 4C).

Restoration of protein levels of IL-6 and iNOS by CTX in ketamine-treated normal aortic SMCs. Similar to the results presented in Fig. 1B, normal aortic SMCs also became deformed and exhibited a spindle-like morphology following treatment with 10 μg/ml ketamine for 24 h (Fig. 5). All SMCs expressed consistent levels of SMA (labeled green with FITC
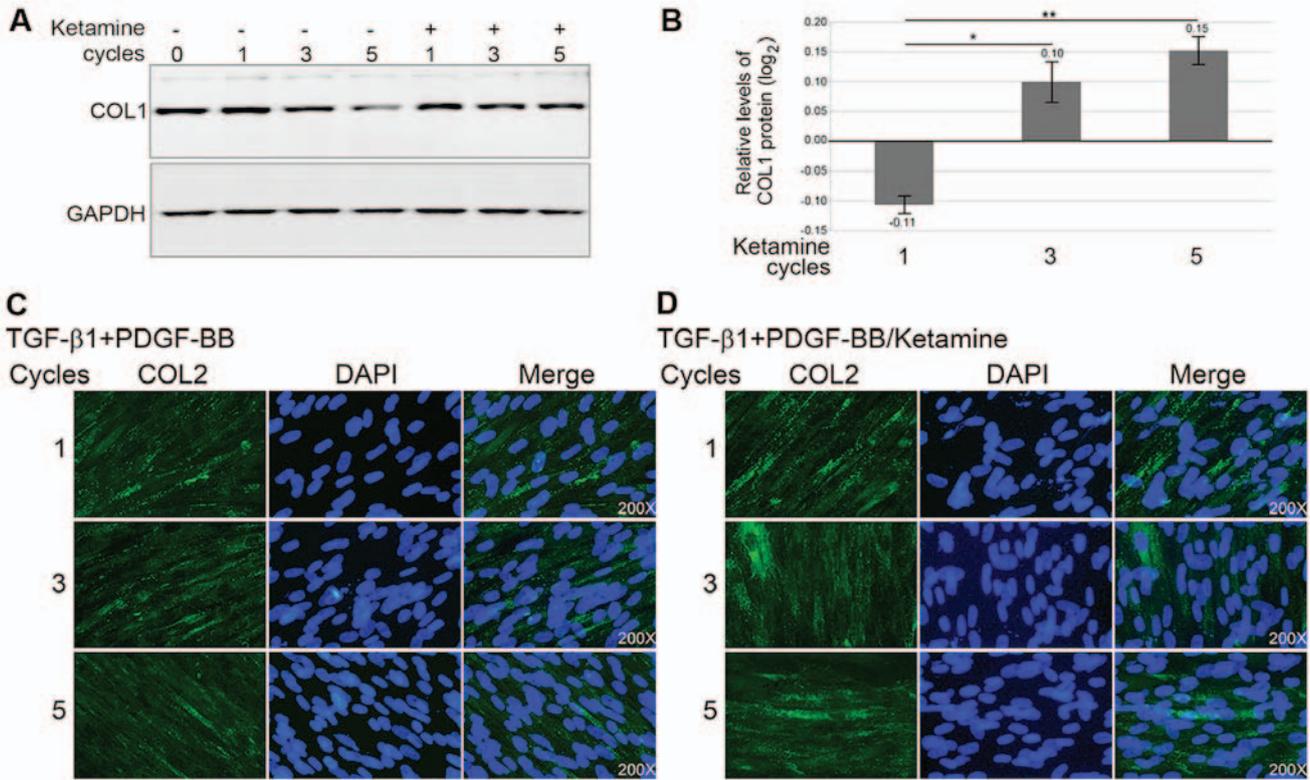
Figure 2. Immunodetection of collagens from human foreskin-derived stromal cells following cyclic ketamine treatment. (A) Detection of COL1. (B) Semi-quantification of immunoblotting. Expression was semi-quantified to GAPDH and $\log_2$ values were calculated. *P<0.05 and **P<0.01. (C) Fluorescent detection of COL2 from cells without ketamine treatment. (D) Fluorescent detection of COL2 from cells following ketamine treatment. Isolated cells were harvested and seeded on fibronectin-coated culture plates and were then treated with the myogenic differentiation agent, containing TGF-β1 (2.5 ng/ml) and PDGF-BB (5 ng/ml). After a 14-day incubation for differentiation, cells were treated with (+) or without (-) ketamine (10 μg/ml) for various cycles (1, 3 and 5 cycles). Each cycle composed 1 day ketamine treatment followed by 1 day culturing in medium alone. COL1, type I collagen; COL2, type II collagen; DAPI, 4',6-diamidino-2-phenylindole; PDGF-BB, platelet-derived growth factor-BB; TGF-β1, transforming growth factor-β1.
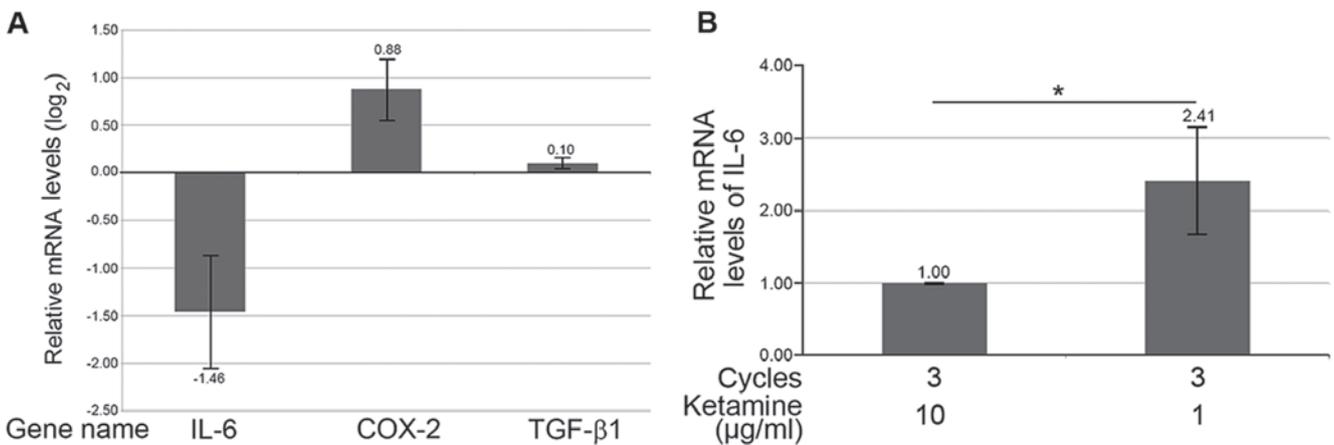
Figure 3. Detection of molecular alterations in cells following cyclic ketamine treatment. (A) Relative mRNA expression levels of *IL-6*, *COX-2* and *TGF-β1* in cells following 5 cycles of ketamine treatment. (B) Alterations in the mRNA expression levels of *IL-6* from cells following 3 cycles of ketamine treatment. All mRNA levels were quantified by quantitative polymerase chain reaction and were relatively obtained by dividing the mRNA levels of cells with cyclic ketamine treatment by those of cells without any treatment. *P<0.05. *COX-2*, cyclooxygenase-2; *IL-6*, interleukin-6; *TGF-β1*, transforming growth factor-β1.

in the figure); however, reduced levels of IL-6 and iNOS (both labeled red with Cy3) were detected following ketamine treatment (Fig. 5). Conversely, the levels of IL-6 and iNOS were restored following 10 μM CTX treatment (Fig. 5).

Discussion

Ketamine abusers exhibit numerous clinical symptoms (32). Typically, long-term (>2 years) ketamine abuse results in
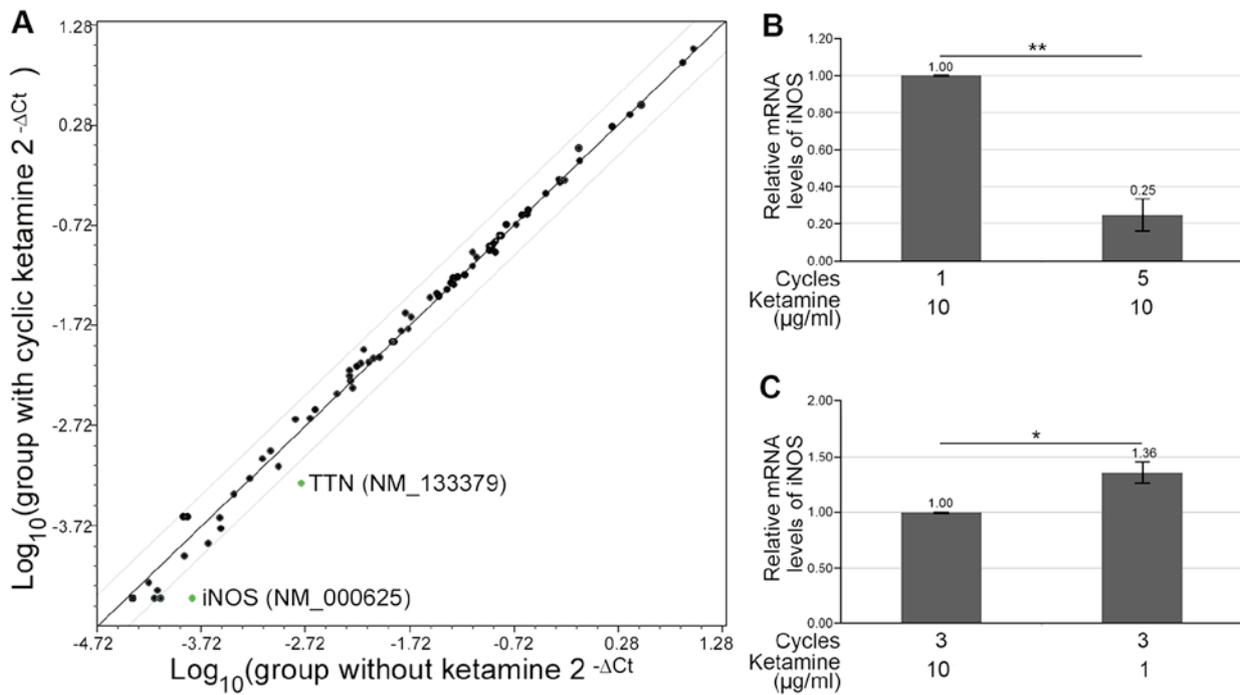
Figure 4. Genes correlated with ketamine-induced oxidative stress. (A) Scatter plot of the expression of 84 candidate genes associated with oxidative stress. Green dots indicate downregulated genes in cells with cyclic ketamine treatment compared with the group without ketamine treatment. The central line indicates unaltered gene expression; boundaries represent the 2-fold regulation cut-off. (B) Relative mRNA expression levels of *iNOS* in cells following 5 cycles of ketamine treatment. (C) Alterations in iNOS mRNA expression from cells following 3 cycles of ketamine treatment. mRNA expression levels were quantified by quantitative polymerase chain reaction and were relatively obtained by dividing the mRNA levels of cells with cyclic ketamine treatment by those of cells without any treatment. *P<0.05 and **P<0.01. *TTN*, titin (NM_003319); *iNOS*, inducible nitric oxide (NM_000625).

bilateral lower ureteral stricture and a contracted bladder with severely reduced bladder capacity (<55 ml) (33,34). Clinical observations include urinary frequency (average, >31 times/day) and nocturia (12.5 times/day) in ketamine abusers due to their reduced bladder capacity (7). In addition, genetic alterations have been observed in ketamine-induced cystitis (35,36). However, to the best of our knowledge, no translational study has indicated any potential therapy for these pathological and molecular alterations in the ketamine-injured human bladder.

Although ketamine-induced cystitis can cause bladder pain, which is mediated through a specific neurogenic mechanism on the urothelium, we proposed that the main muscle component of the urinary bladder wall, the detrusor muscle, is also damaged by ketamine (22,37,38). However, dynamic genetic alterations involving irreversible bladder contracture in the human detrusor muscle are difficult to analyze in ketamine abusers. Therefore, two types of cultured normal human SMCs, FDSC-derived SMCs and aortic SMCs, were used in the present study to evaluate *in vitro* ketamine-induced molecular alterations.

Ketamine and its active metabolites (e.g., norketamine) are believed to exert a direct toxic effect on the bladder mucosa (17,35). In the cultured normal SMCs used in the present study, ketamine, but not norketamine, induced a cytotoxic effect on cell growth (data not shown). Similarly, ketamine has been reported to exert toxicity in chondrocyte cell cultures and induce cytoskeleton interruption (39,40). Collectively, these results suggested that ketamine may dose-dependently damage the urinary bladder and affect SMC

stability. This is consistent with the findings of Shahani *et al* who reported that severe ulcerative cystitis results from chronic ketamine use (13). Since 1998, mouse models have been used to demonstrate that accelerated fibrosis and collagen deposition coincide in the renal interstitium during ureteral obstruction (41). Furthermore, a rat model has been used to indicate that ketamine increases interstitial fibrosis in urinary bladder tissues (20,42,43). Increased fibrosis can be examined by measuring the deposition of various types of collagen (44), and excessive synthesis of COL1 and COL2 indicates fibrosis in various organs (45,46). The excessive COL1 and COL2 levels detected in response to ketamine in the present study suggested that fibrosis occurs in ketamine-treated normal human SMCs.

The expression of IL-6, which is an IL with pleiotropic functions as a proinflammatory cytokine and an anti-inflammatory myokine (47), was reduced following cyclic ketamine treatment in the present study. However, some inflammatory markers were upregulated in our previous model of ketamine-induced cystitis (35,42). Given the present data and those of previous studies, it may be concluded that inflammatory markers are expressed differently between various types of ketamine-treated cells (48). Numerous studies regarding antidepressants and neural pathways have indicated that ketamine is associated with the downregulation of proinflammatory cytokines, including IL-6 (36,49). In addition, the expression of IL-6 was positively correlated with the migration of vascular SMCs (50). Therefore, reduced IL-6 expression in ketamine-treated SMCs may be associated with the function of IL-6 as an anti-inflammatory myokine,
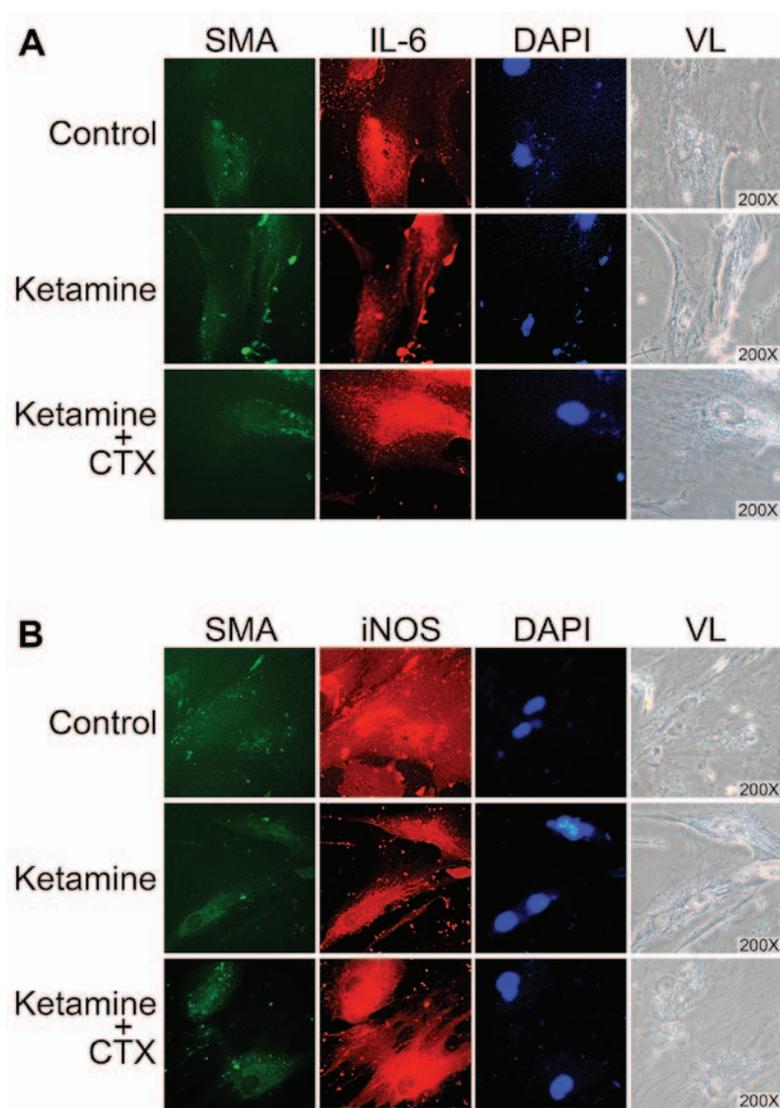
Figure 5. Ketamine-induced molecular alterations in normal aortic SMCs, and the effects of CTX. (A) IL-6 and (B) iNOS expression was restored with CTX treatment. Normal aortic SMCs were treated with 10 μg/ml ketamine alone or in combination with 10 μM cyclophophamide. Nuclei were stained with DAPI. CTX, cyclophosphamide; DAPI, 4',6-diamidino-2-phenylindole; IL-6, interleukin-6; iNOS, nitric oxide synthase 2; SMA, smooth muscle actin; SMCs, smooth muscle cells; VL, visible light.
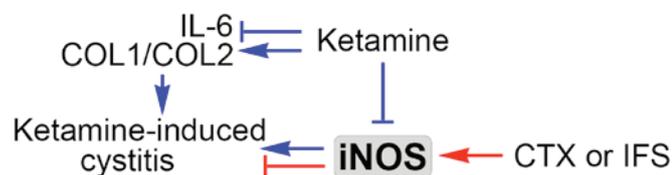
Figure 6. Molecular pathogenesis of ketamine-induced cystitis. Red lines or arrows indicate effects of CTX or IFS; blue lines or arrows indicate effects of ketamine. COL1, type I collagen; COL2, type II collagen; CTX, cyclophosphamide; IFS, ifosfamide; IL-6, interleukin-6; iNOS, nitric oxide synthase 2.

thus suggesting that reduced IL-6 levels may impair muscle contraction (1). Conversely, the proinflammatory effects of IL-6 may increase the migratory rate of SMCs (51). This indicates that the ketamine-caused decreasing IL-6 is negatively correlated with the bladder stretching. Clinical observations have indicated that patients feel better and that their symptoms improve when they stop or reduce ketamine use. The observation that IL-6 expression was restored in the present study when ketamine treatment was removed or its concentration was reduced is consistent with these clinical observations. Therefore, ketamine abusers are strongly advised to terminate ketamine use. In addition, ketamine induced COL1 and COL2 deposition, and reduced IL-6 expression in SMCs; these effects may be associated with muscle contraction in the urinary bladder. In other words, the increased fibrosis and decreased myokine additionally cause the bladder dysfunction.

The ketamine-induced molecular alterations detected in SMCs may also include the inhibition of iNOS (48). There is evidence to suggest that reactive oxygen species are crucial to the pathogenesis of various diseases (52,53). In a rat model, subanesthetic doses of ketamine increased oxidative stress and induced a schizophrenia-like condition (25). The effects of iNOS in response to oxidative stress were observed in epithelial cells (54). The findings of the present study, and those of other studies, suggested that the ketamine-induced

cystitis may also result from a reduction in iNOS levels following treatment of SMCs with cyclic ketamine. However, in a previous study, selective inhibition of NOS could increase muscle thickness (55). The reduction in nitric oxide levels due to reduced iNOS may also increase smooth muscle contraction (e.g., frequency or strength) (56). Therefore, in ketamine abusers, cyclic ketamine treatment may lead to continuous muscle contraction and to the development of small bladder capacity due to iNOS downregulation. These findings indicated that reversing the effects of oxidative stress is a potential therapeutic strategy in addition to surgery.

CTX, which belongs to the group of oxazaphosphorines, exhibits strong activity against several experimental tumors and is widely used in the treatment of various clinical conditions, including lymphoproliferative disorders, small cell lung cancer, breast cancer and other severe forms of rheumatic diseases, such as lupus erythematosus, systemic sclerosis and vasculitis (57,58). However, CTX and its isomeric analogue ifosfamide (IFS) can dose-dependently induce hemorrhagic cystitis, a urological disorder (59,60). Following administration of CTX, iNOS bladder activity increases, which is associated with histopathological alterations (61). Ozguven et al reported that appropriate ketamine pretreatment attenuated experimental IFS-induced hemorrhagic cystitis in a rat model (62). This result suggested that IFS (or CTX) and ketamine have opposing effects on the urinary bladder, probably due to their different effects on iNOS activities. In the present study, the results observed in the ketamine-treated SMCs are consistent with the results of previous studies, which suggested that the production of proinflammatory mediators, such as IL-6 and free oxygen radicals (nitric oxide), are reduced in SMCs following ketamine treatment (60,62).

It seems likely that ketamine-induced cystitis is propagated via an accumulation of molecular alterations in the urinary bladder, particularly in SMCs. These alterations may result in the clinical symptoms observed in ketamine abusers. A specific therapeutic agent, CTX, may counteract oxidative damage and reverse ketamine-induced aberrations (Fig. 6).

In conclusion, the findings of the present study provided a novel insight into the molecular mechanisms underlying ketamine-induced cystitis and may suggest novel clinical treatments.

Acknowledgments

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