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Baker, Simon C., Shabir, Saqib, Georgopoulos, Nikolaos T. and Southgate, Jennifer

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Ketamine-induced apoptosis in normal human urothelial cells: a direct, N-methyl-D-aspartate receptor-independent pathway characterised by mitochondrial stress.

Simon Charles Baker[1], Saqib Shabir[1], Nikolaos Theodoros Georgopoulos[2] and Jennifer Southgate[1,*]

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Affiliation

[1]Jack Birch Unit of Molecular Carcinogenesis, Department of Biology, University of York, United Kingdom

[2]Department of Biological Sciences, School of Applied Sciences, University of Huddersfield, United Kingdom

Contact Information for Corresponding Author*

Prof Jennifer Southgate, Jack Birch Unit of Molecular Carcinogenesis, Department of Biology, University of York, York YO10 5DD, United Kingdom

Tel: +44 (0)190 432 8705

Fax +44 (0)190 432 8704

E-mail: jennifer.southgate@york.ac.uk

Abstract

Recreational abuse of ketamine has been associated with the emergence of a new bladder pain syndrome, ketamine-induced cystitis, characterised by chronic inflammation and urothelial ulceration. This study investigated the direct effects of ketamine on normal human urothelium maintained in organ culture or as finite cell lines *in vitro*.

Exposure of urothelium to ketamine resulted in apoptosis, with cytochrome c release from mitochondria and significant subsequent caspase 9 and 3/7 activation. The anaesthetic mode-of-action for ketamine is mediated primarily through N-methyl D-aspartate receptor (NMDAR) antagonism; however, NHU cells were unresponsive to NMDAR agonists or antagonists and no expression of NMDAR transcript was detected. Exposure to non-cytotoxic concentrations of ketamine (≤1 mM) induced rapid release of ATP, which activated purinergic P2Y receptors and stimulated the inositol trisphosphate receptor to provoke transient release of calcium from the endoplasmic reticulum into the cytosol. Ketamine concentrations >1 mM were cytotoxic and provoked a larger-amplitude increase in cytosolic $[Ca^{2+}]$ that was unresolved. The sustained elevation in cytosolic $[Ca^{2+}]$ was associated with pathological mitochondrial oxygen consumption and ATP deficiency.

Damage to the urinary barrier initiates bladder pain and in ketamine-induced cystitis, loss of urothelium from large areas of the bladder wall is a reported feature. This study offers first evidence for a mechanism of direct toxicity of ketamine to urothelial cells by activating the intrinsic apoptotic pathway.

Introduction

The phencyclidine-derivative ketamine is an N-methyl-D-aspartate (NMDA) receptor antagonist,[1] which is used as a rapid onset, short duration anaesthetic and analgesic in clinical and veterinary practice. In the clinical setting, ketamine is particularly used as an anaesthetic in paediatric and asthmatic cases, and for palliative care. Recreational use of ketamine, which has been increasing since the 1980s, is based around its phencyclidine-like effects whereby it induces hallucinations, stimulates out-of-body experiences and increases empathy and insight (reviewed[2]).

The emergence of upper and lower urinary tract damage resulting from ketamine abuse was reported originally as case studies in 2007, where symptoms of urinary frequency/urgency, nocturia, haematuria and suprapubic pain were accompanied by a thickened, contracted and inflamed bladder.[3, 4]

The sloughing of the bladder epithelium (urothelium) observed by histology in ketamine cystitis patients is suggestive of a direct toxicity against the urothelium,[5] but no mechanism has yet been established. Ketamine has been classified as an N-methyl D-aspartate receptor (NMDAR) antagonist[1] and *in vitro* studies with cortical neurons from rats[6] and monkeys[7] have identified the NMDAR as a mediator of ketamine-induced apoptosis. NMDAR subunits have been reported to be expressed in the urinary tract of rats,[8] which suggests this as a potential mechanism in human urothelium. The aim of this study was to identify a mechanism for the direct toxicity of ketamine on normal (non-immortalised) human urothelial (NHU) cells in order to enhance understanding of the clinical pathology.

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Materials and Methods

Chemicals and Reagents

Racemic ketamine hydrochloride salt (without preservatives) was soluble in cell culture medium at 10 mM and was 0.2 μm filter-sterilised prior to use. Unless specified otherwise, all chemicals were of analytical or tissue culture grade, as appropriate, and were obtained from Sigma-Aldrich Company Ltd. (Gillingham, UK).

Due to the high concentrations of ketamine used in this study (and recreationally), it was important to assess the effects of ketamine (0.1mM to 10mM) on the osmolality of culture medium. No change in osmolality of complete keratinocyte serum-free medium (KSFMc) was observed at ketamine concentrations <5 mM and whilst slight increases were recorded at 5 mM and above, the concentrations used in this study did not exceed the normal osmolality range of serum (275-299 mOsmol.kg^{-1}).

Urothelial Cell Isolation and Culture

Urothelium was collected with NHS Research Ethics Committee approval and required informed consent from urological procedures that excluded urothelial neoplasia. Finite (non-immortal, serially-passaged) normal human urothelial (NHU) cell lines were established and maintained in KSFMc (Invitrogen, UK), as described in detail elsewhere, [9] but without the addition of cholera toxin. For this study, cell lines derived from eight individuals were used up to passage 5.

Cell counts were performed using Trypan Blue (Sigma, UK) exclusion to identify viable cells using an improved Neubauer Haemocytometer (SLS, UK).

Organ cultures were established from human ureteric tissue and maintained in DMEM:RPMI 1640 (50:50 mix) supplemented with 5% fetal bovine serum. Following 72 hours exposure to 3mM ketamine, ureteric organ cultures from 6 donors were fixed in 10% formalin for 24 hours, dehydrated through graded alcohols and embedded in paraffin wax for histological evaluation.

Histological Evaluation of Organ Cultures

Dewaxed, 5 µm tissue sections were either stained with haematoxylin and eosin (following standard methods) or immunoperoxidase-labelled using the M30 Cytodeath antibody to cleaved cytokeratin 18 (Roche).

For immunoperoxidase labelling, blocking steps were included to neutralise endogeneous peroxidase and avidin-binding activities. Heat-mediated antigen retrieval was performed by microwave boiling for 10 min in either 10 mM citric acid buffer (pH 6) or 1 mM EDTA buffer (pH 8). After overnight incubation in primary antibody (diluted 1:100) at 4 °C, slides were washed, incubated in biotinylated secondary antibodies and a streptavidin-biotin horseradish peroxidase complex (Dako Cytomation) and visualised using a diaminobenzidine substrate reaction (Sigma-Aldrich). Sections were counterstained with haematoxylin, dehydrated and mounted in DPX (CellPath).

Quantification of cell number by Alamar Blue assay

Alamar Blue (AbD Serotec) diluted 1:10 with KSFMc was added to cells grown in 96-well plates. After 4 hours incubation at 37 °C, the absorbance was measured at 570 nm and

630 nm. The reduction of the Alamar Blue dye is proportional to mitochondrial enzyme activity and can be used as a proxy for viable cell number.[10]

Calcium Imaging

NHU cells were seeded at 5×10^4 cells/cm^2 onto collagen-coated (0.1 mg/ml rat-tail collagen, Becton Dickinson) glass coverslips and maintained for 24 hours. Prior to imaging, cultures were washed with HEPES-buffered saline solution (HBSS; 138 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.3 mM $NaHPO_4$, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM HEPES pH 7.4) and then loaded with 5 µM fluo-4(AM) and 5 µM fura-red(AM) in HBS with 0.02 % pluronic acid for 25 minutes. Cultures were washed twice in HBS and the coverslips placed in a perfusion chamber (Warner Instruments) on the stage of a Revolution XD spinning disc confocal microscope (Andor). The chamber was perfused with an automated pump (Scientifica) at a flow rate of 1.5 ml/min and images recorded at a rate of 1/sec.

In some experiments, to differentiate exogenous from intrinsic calcium stores, cells were pre-incubated for 10 minutes in Ca^{2+}-free HBSS (138 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.3 mM $NaHPO_4$, 1 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES pH 7.4) and experiments were performed in the calcium-free buffer.

Changes in the intensity of the fura-red and fluo-4 fluorescent signal were measured in all the cells in the field of view using Andor iQ software, which was used to record and export raw data over time. The data was imported in Origin 8.6 (Origin labs, Microcal) and the ratio of fluorescence calculated to indicate the change in intracellular $[Ca^{2+}]$.

Reverse-Transcribed Polymerase Chain Reaction (RT-PCR)

RNA was extracted in Trizol reagent (Fisher), any contaminating DNA was digested using DNA-free (Ambion) and cDNA was synthesised using Oligo(dT)12-18 primers (Invitrogen). RT-PCR was performed using SureStart Taq polymerase (Stratagene) and a thermal cycler (PCR Express, Hybaid). With the following annealing temperatures, primers were designed to amplify GRIN1 (5'-GCATCCTCGGGCTGCAGCTC-3' and 5'-AGCGGCCCGGTCTTCCAGAT-3', 61 °C), GRIN2A (5'-GTGGTCTATCAACGGGCAGT-3' and 5'-AGGTGAGACGGTGCCATTAC-3', 51 °C), GRIN2B (5'-GATGGGAGCCCCTACGCCCA-3' and 5'-CCACCGTGGGCTGCCTGAAG-3', 60 °C), GRIN2C (5'-CGACGCCAGCCACGTGAGTT-3' and 5'-AGAGCACCTCGGCCTCCTCG-3', 54 °C with 5 % DMSO added to the reaction), GRIN2D (5'-CCACCTTCCTGCAGCTGGGC-3' and 5'-GAGCTGGGCACTGAGCACGG-3', 61 °C), GAPDH (5'-ACCCAGAAGACTGTGGATGG-3' and 5'-TTCTAGACGGCAGGTCAGGT-3', 60 °C) and UPK2 (5'-CTCCCGCAAGTAAGGAGGT-3' and 5'-GAAGGATGGGGGAATTGTTA-3', 58 °C) transcripts. Reverse transcriptase negative controls were included in all experiments.

Mitochondrial Oxygen Consumption Rate (OCR) Monitoring

The mitochondrial OCR of NHU cells in the presence and absence of 3 mM ketamine was assessed using an XFp Analyzer to perform an XF Cell Mitochondrial Stress Test according to the manufacturer's instructions (Seahorse Bioscience, UK), as detailed [11]. Briefly, NHU cells were seeded at $1x10^4$ cells/well and cultured for 48 hours to near confluence, when cultures were exposed to medium containing 3 mM ketamine and

maintained for a further 48 hours. Prior to the assay, cultures were washed with, and then incubated in, XF Assay modified DMEM (unbuffered, Seahorse Bioscience) containing 5 mM L-glutamine, 5 mM sodium pyruvate and 6mM glucose. The XFp Analyzer performed fluorimetric detection of O_2 consumption by sequentially adding and mixing 1 μM oligomycin, 1 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 0.5 μM Antimycin A with 0.5 μM Rotenone at defined time-points. Values were normalised to total cellular protein derived from a bicinchoninic acid (BCA) assay (Pierce, UK) and mitochondrial respiration was quantified by normalisation to OCR values in the presence of Antimycin A and Rotenone, as described elsewhere.[11]

Tetramethylrhodamine (TMRM) Staining and Flow Cytometry

NHU cultures were treated for 48 hours with either control KSFM or 3 mM ketamine in KSFM, washed twice with KSFM and labelled with 200 nM TMRM methyl ester (Biotium, US) in KSFM for 30 min at 37 °C. Live cultures were imaged immediately at fixed exposures using an Olympus B60 epifluorescent microscope.

For flow cytometry, the same procedure was followed, but cells were first harvested with trypsin in versene before TMRM staining was performed in suspension. Staining was quantified using a CyAn ADP analyser (Beckman Coulter) and data were summarised for three donor cell lines as the mean of median fluorescence from each donor, normalised to the no ketamine control.

Cellular ATP Assay

Cellular ATP was quantified using the ENLITEN ATP assay according to the manufacturer's instructions (Promega, UK) and normalised using a BCA protein assay. Briefly, NHU cells were cultured to 80 % confluence in 10 cm dishes before exposure to 3 mM ketamine or medium only (KSFM) for 48 hours. Cultures were harvested, lysed in 2 % trichloroacetic acid and assayed for activation of luciferase against an ATP standard curve in a luminescence plate reader (BMG PolarStar Optima).

Western Blotting

Whole cell lysates were obtained from cultures by in situ lysis in sodium dodecyl sulphate (SDS) electrophoresis sample buffer, containing 13 mM dithiothreitol (DTT) and 0.2 % (v/v) protease inhibitors (Protease Inhibitor Cocktail set III, Calbiochem) following two PBS washes. Cells from at least 3 donors were used in all experiments. Mitochondrial and cytosolic fractions were prepared from all (adherent and detached) cells using the MIT1000 kit according to manufacturer's instructions (Merck Millipore). Lysates were sonicated, microcentrifuged and the protein concentration determined by Bradford colorimetric assay. Protein samples (20-40 μg) were resolved by electrophoresis of NuPAGE gels using the Novex system (Invitrogen) and electro-transferred to PVDF-FL membranes (Merck Millipore). Membranes were blocked for 1 hour in Odyssey blocking buffer (Li-Cor), incubated with titrated primary antibody overnight at 4 °C. Primary antibodies raised against Serine473 phosphorylated-Akt (Cell Signalling, 9271, Rabbit, 1:1,000 dilution), Threonine202/Tyrosine204 phosphorylated-

ERK1/2 (Cell Signalling, 9101, Rabbit, 1:1,000 dilution), Serine 9 phosphorylated-GSK3β (Abcam, ab30619, Rabbit, 1:500 dilution), Bcl-2 (Merck Millipore, Clone 100, Mouse, 1:1,000 dilution), Cytochrome c (Santa Cruz, H-104, Rabbit, 0.4 μg/mL), caspase 9 (Cell Signalling, 9502, Rabbit, 1:1,000 dilution), caspase 3 (Cell Signalling, 9662, Rabbit, 1:1,000 dilution), cleaved-poly(ADP ribose) polymerase (cleaved-PARP, Cell Signalling, 5625, Rabbit, 1:1,000 dilution). Densitometry was normalised using the intensity housekeeping proteins β-actin (Sigma, Clone AC15, Mouse, 1:10,000 dilution) or GAPDH (Merck Millipore, Clone 6C5, Mouse, 1:2,000 dilution). Membranes were labelled with the appropriate IRDye conjugated secondary antibody and visualised using an Odyssey Infrared imager (Li-Cor).

Caspase 3/7 assay

NHU cell cultures were seeded at 1.5×10^5 cells/cm^2 in 96-well plates (Primaria^{TM}) and 24 hours after seeding were treated with ketamine in replicates of six. After 72 hours, apoptosis was assessed with the Sensolyte active caspase 3/7 kit used according to the manufacturer's instructions (Anaspec). Briefly, cells were lysed and any active capase-3/7 cleaved a quencher from the AFC fluorophore to generate bright blue fluorescence. Caspase-activity associated fluorescence was measured on a PolarStar Optima plate reader (BMG LabTech) at excitation and emission wavelengths of 355 and 492 nm.

Statistical Analysis

Comparison of means was carried out using the appropriate statistical tests in Graphpad

InStat statistical software (Version 3.05) and repeated measures ANOVA was performed

in SPSS (IBM, v20). For graphical purposes * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Direct exposure of urothelium to ketamine

Organ cultures of human ureters from six donors were used to examine the direct effect of ketamine exposure to the urothelium (Figure 1). Haematoxylin and eosin staining of ureters following 72 hours exposure to 3mM ketamine showed thinning of the epithelium throughout the organ culture in 5/6 donors (eg Donor x, Figure 1), with pyknotic nuclei and karyorrhexis evident as cells were lost from the urothelium (eg Donor y, Figure 1). Immunolabelling of organ cultures with a cleaved-cytokeratin 18 antibody showed immunoreactivity in cells being lost from the epithelium indicating the importance of apoptosis (Figure 1).

To examine the mechanisms of ketamine action further, non-immortalised NHU cell cultures were exposed to ketamine over a 6 day time-course. Ketamine induced a reproducible concentration-dependent effect, being growth inhibitory at ≥ 0.3 mM, cytostatic at 1 mM and toxic at >1 mM (Figure 2A). Counts of proliferating NHU cell cultures after 4 days of exposure found the IC_{50} for ketamine to be 0.93 mM (Figure 2B). Based on this data, the cytostatic/IC_{50} concentration of 1 mM ketamine was used in comparison with a cytotoxic dose of 3 mM for all further studies.

Adherent and detached NHU cells exposed to 3 mM ketamine were collectively lysed and cytoplasmic- and mitochondrial-enriched fractions were prepared to study the localisation of the apoptotic mediator cytochrome c. The cytoplasmic fractions of

ketamine-treated cells showed a significant mean 2.4-fold increase in cytochrome c (Figure 3, A and B) when compared with time-matched controls after 24 hours of exposure (repeated measures ANOVA p<0.05, n=3, Figure 3C). Exposure to 3 mM ketamine caused a rapid reduction in phosphorylation of Akt (serine 473) and ERK1/2 (threonine 202/tyrosine 204), with a consequential reduction of inhibition of GSK3β activity (Figure 3C). The serine 9 phosphorylated form of GSK3β is inactive and 3mM ketamine reduced phosphorylation (Figure 3C) indicating raised GSK3β activity that could contribute to mitochondrial permeability transition pore (MPTP) activation.[12] Inhibition of GSK3β with SB415286 showed a small but significant alleviation of the >1 mM ketamine-induced toxicity at 48 h (ANOVA with Tukey-Kramer Multiple Comparisons post Test p<0.01, n=6, Figure 3D). Furthermore, inhibition of the MPTP with cyclosporin A (CsA) showed a significant concentration-dependent inhibition of toxicity at 48h (ANOVA with Tukey-Kramer Multiple Comparisons post Test p<0.01, n=6, Figure 3E). Co-treatment of NHU cells with 3 mM ketamine and 3 µM CsA led to a 1.4-fold increase in cell viability at 48 hours, compared to cells treated with ketamine alone (n=12 measurements of cells from 2 independent donors).

Exposure to 3 mM ketamine for 72 hours induced significant increases in the abundance of caspase 9 (mean 2.6-fold), caspase 3 (mean 2.1-fold) and cleaved-PARP (mean 48.5-fold) as assessed by densitometry of western blots (Figure 3, F and G). A caspase 3/7 activity assay recorded significant increases in caspase activity detected after 72 hours with ketamine at concentrations ≥3 mM (Figure 3H).

NMDAR expression by human urothelium

Ketamine is an NMDAR antagonist; however, pre-treatment of NHU cells with a combination of D-serine (1 mM) and NMDA (100 µM) was unable to inhibit calcium transients or block ketamine-induced toxicity (data not shown). The potent, specific NMDAR antagonist MK-801 (also known as dizocilpine, which has an NMDAR dissociation constant 100-fold greater than ketamine[13]) did not trigger any toxicity or calcium transients at concentrations ≤100 µm (data not shown). The lack of any effects triggered by specific agonists/antagonists of the NMDAR led to evaluation of the expression of NMDAR (GRIN) isoform transcripts by proliferating and differentiated NHU cell cultures; no expression of GRIN1, 2A, 2B, 2C or 2D was detected (Figure 4). To ensure that lack of GRIN transcripts was not an artefact of culturing urothelial cells, transcript expression was analysed in freshly-isolated urothelium, which was removed from the underlying stroma to prevent contamination with other cell types. No expression of GRIN isoform transcripts was detected in freshly-isolated *in situ* human urothelium (Figure 4).

The role of calcium signalling in the NHU cell toxicity of ketamine

The cytostatic concentration of 1 mM Ketamine was seen to elicit an increase in intracellular $[Ca^{2+}]$ in 95% of NHU cells in culture; this transient was of comparable intensity to that stimulated by 50 µM ATP and all cells that responded to ATP were responsive to ketamine (Figure 5A). The amplitude of the calcium transient in response to ketamine was concentration-dependent and the duration was associated with the

observed toxicity. In NHU cells exposed to ≤1 mM ketamine for 1 min, the calcium signal returned to baseline after an average of 90 seconds, whilst 3 mM ketamine for 1 min invoked a sustained, elevation in intracellular $[Ca^{2+}]$ that remained unresolved after 10 min (Figure 5A).

The role of ATP in driving the observed elevations of cytosolic $[Ca^{2+}]$ was elucidated by co-treatment of 1 mM ketamine with an ectoATPase inhibitor 6-*N*,*N*-diethyl-D-beta-gamma-dibromomethylene-ATP (ARL-67156), which significantly enhanced the amplitude and duration of the calcium transient (Figure 5B). By contrast, pre-treatment and co-administration of the purinergic receptor inhibitor pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) with 1 mM ketamine almost completely abrogated the calcium transient (Figure 5B). Whilst these results demonstrated ATP was critical to the calcium transients stimulated by 1 mM ketamine, it was not possible with PPADS to inhibit the non-resolving elevation of intracellular $[Ca^{2+}]$ triggered by 3 mM ketamine (Figure 5C).

To understand the source and mechanism of ketamine-induced calcium release, cultures were challenged with 1 mM ketamine which, in the presence of 2mM (near-physiological) extracellular $[Ca^{2+}]$, produced repeated calcium transients of similar intensity (Figure 6A). Whilst an initial calcium transient stimulated by 1 mM ketamine was also observed in the absence of extracellular calcium, a second challenge produced a smaller calcium response than the first due to incomplete re-filling of calcium stores in the endoplasmic reticulum (ER, Figure 6B). This was similar to the effect observed upon repeated ATP stimulation in the absence of exogenous calcium and demonstrated that

the cytosolic calcium transients were derived from intracellular stores (Figure 6B). When cultures were pre-treated with the inositol trisphosphate receptor (IP_3R) inhibitor 2-aminoethoxydiphenyl borate (2-APB), in the absence of extracellular calcium, the 1 mM ketamine-stimulated calcium transient was lost, revealing the source of calcium to be the ER (Figure 6C). It was not possible to inhibit the non-resolving elevation of intracellular $[Ca^{2+}]$ triggered by 3 mM ketamine using 2-APB (data not shown).

The prolonged elevation in cytosolic $[Ca^{2+}]$ induced by 3 mM ketamine in 2 mM extracellular $[Ca^{2+}]$ (*cf.* Figure 5A) was reproduced when cultures were stimulated by 3 mM ketamine in the absence of extracellular calcium showing this process was also driven entirely by the release of intracellular calcium stores (Figure 6D). Using the sarco/ER calcium ATPase (SERCA) inhibitor, thapsigargin, to prevent refilling of the ER (and therefore its emptying through calcium leakage), it was observed that the 3 mM ketamine-induced calcium elevation persisted, suggesting this calcium was not derived from a thapsigargin-sensitive region of the ER (Figure 6E).

Neither PPADS, 2-APB nor BAPTA-AM could be titrated to inhibit ketamine toxicity as assessed by Alamar Blue reduction (data not shown).

Mitochondrial Physiology

NHU cells exposed to 3 mM ketamine for 48 hours had a significantly lower resting mitochondrial oxygen consumption rate (OCR) than controls (mean 54.4%, Mann Whitney Test $p<0.001$, Figure 7A). The drop in mitochondrial OCR in response to inhibition of ATP synthase (1 µM oligomycin) indicated the proportion of OCR that

related to the generation of ATP. Subtracting the mitochondrial OCR post-ATP synthase inhibition, from the resting value suggested control cells used more oxygen to generate ATP (mean 2.1-fold, Mann Whitney Test $p < 0.003$, Figure 7A). Addition of the ionophore and uncoupling agent FCCP (1 μM) equalised the proton gradient across the inner mitochondrial membrane and defined the maximum OCR for NHU cell mitochondria (Figure 7A). The maximum mitochondrial OCR in control cells was significantly greater than for ketamine-exposed cells (mean 4.6-fold, Mann Whitney U Test $p < 0.001$).

TMRM labelling of NHU cells showed greater intensity in cultures exposed to 3 mM ketamine for 48 h indicating that mitochondria became hyperpolarised (Figure 7, B and C). A combination of low mitochondrial OCR with high mitochondrial membrane potential suggested ketamine-exposed cells consumed ATP and used ATP synthase to maintain the proton gradient. Cellular ATP assays showed that ATP was significantly depleted following 48 hours of exposure to 3 mM ketamine (76.8% of control cells; t test with Welch correction $p < 0.05$, Figure 7D).

Discussion

The data reported here suggest that ketamine-induced toxicity to urothelial cells is associated with prolonged elevated intracellular $[Ca^{2+}]$ triggered by high concentrations of ketamine (>1 mM). High and sustained intracellular $[Ca^{2+}]$ can lead to mitochondrial stress and ultimately cytochrome c release, which causes caspase activation and apoptosis (reviewed[14]). The role of apoptosis in ketamine-induced cystitis was supported using human urothelial organ cultures which showed pyknotic nuclei, karyorrhexis and immunoreactivity with a cleaved-cytokeratin 18 antibody.

Our study demonstrates for the first time that ketamine induces an increase in intracellular $[Ca^{2+}]$ in urothelial cells via release of calcium from internal stores rather than influx from the extracellular milieu, suggesting disruption of organelle function. The observation that ketamine-induced calcium transients were blocked with 2-APB confirmed that the ketamine-induced calcium response was due to an IP_3-mediated calcium release from the ER. Exacerbation of calcium transients by inhibiting ecto-ATPases (with ARL-67156) and abrogation following P2Y inhibition (by PPADS) suggests that ketamine stimulates ATP release from urothelial cells, which then binds purinergic receptors on the cell surface, allowing IP_3 to trigger calcium release from the ER. The purinergic receptors involved in this process are almost certain to be of the P2Y-family (known to be functional in human urothelium[15]) since P2X receptors act as channels allowing extracellular calcium into the cytoplasm and the ketamine-induced calcium release observed persisted in the absence of extracellular $[Ca^{2+}]$. This proposed P2Y-mediated action of ketamine (illustrated in Figure 8) is supported by similar findings in a

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rat microglial cell line,[16] which is the only other report of intracellular calcium transients triggered by ketamine.

The source of calcium that delivers the sustained elevation observed during 3 mM ketamine exposure remains elusive; however, others have reported thapsigargin-insensitive stores of calcium in the Golgi and high $[Ca^{2+}]$ in some regions of the ER.[17] In addition, it has been reported that thapsigargin-insensitive calcium stores cannot be mobilised by IP_3-release (as triggered by 1 mM ketamine) which might suggest the extra $[Ca^{2+}]$ released by 3 mM ketamine is accessed via a different mechanism.[18] Intense TMRM labelling of ketamine-exposed cells suggests the mitochondria in the majority of adherent cells retain their membrane potential and therefore are unlikely to be the source of the global increase in cytosolic $[Ca^{2+}]$.

The mitochondria of ketamine-exposed urothelial cells displayed extremely low OCR, with only slight inhibition by oligomycin (suggesting little ATP generation) and no detectable increase following FCCP addition (demonstrating a lack of spare capacity for ATP generation). This data supports the concept that the electron transport chain is largely inactive in the mitochondria of ketamine-exposed cells and combined with intense TMRM labelling (showing the inner mitochondrial membranes were hyperpolarized) suggests that the mitochondria had become ATP consumers. By running ATP synthase in reverse, mitochondria can maintain membrane potential and survive stressors that inhibit functioning of the electron transport chain (reviewed[19]). The depletion of cellular ATP in ketamine-exposed cells provides additional support for their inhibited bioenergetics. At a certain threshold, individual mitochondria will run

out of ATP, membrane potential will no longer be maintained by ATP synthase and the MPTP will form, releasing cytochrome c and activating caspases (including 9 and 3) to initiate apoptosis. The involvement of mitochondria in caspase activation in response to ketamine by human lymphocytes and neuronal cells was previously reported,[20] and is an established mechanism of toxicity for other xenobiotics in the urothelium.[21]

Studies of cultured rat[6] and monkey[7] cortical neurons identified the NMDAR as the mediator of ketamine-induced apoptosis-induction in these cells. By contrast, our study found no expression of GRIN transcripts and no response to NMDAR agonists or more specific antagonists by human urothelial cells *in vitro* or *in situ*. Notably, previous reports of NMDAR expression in human and rat urinary tract tested homogenised urothelial/stromal tissue together.[8] In a study of human lymphocytes and neuronal cells, the low stereospecificity of ketamine-induced apoptosis was taken as evidence against the involvement of NMDAR, which has a higher affinity for S-ketamine,[20] suggesting that the significance of non-NMDAR targets of ketamine may not be confined to the urothelium. The initial receptor responsible for stimulating the ATP release remains elusive and is particularly difficult to identify due to the recognised receptor-binding promiscuity of ketamine.

We previously reported nerve hyperplasia present in ketamine cystitis patients which might play a role in pain generation.[5] Here we report urothelial ATP release in response to ketamine exposure which may be another factor in the extreme pain experienced, since ATP acting on afferent nerves in the bladder wall is a widely accepted cause of pelvic pain.[22]

Recreational ketamine consumption tends to be orders of magnitude higher (54% of users have taken >1 g in a session[23]) than in the low (50 mg) oral dose clinical studies on which predicted urine concentrations are based.[24] A young adult male taking 1 g of ketamine, could expect 85% of the drug to be excreted in the urine within 24 h[24] and taking into account the average voiding rate of 6x300 mL/day[25] a urine concentration in excess of 1mM is theoretically possible, suggesting the scale of *in vitro* toxicity reported here (and by others in cancer cell lines[26]) is relevant clinically. Formal support would require accurate quantification of ketamine/metabolite concentrations in the urine of symptomatic recreational users.   Nevertheless, our biological observation of a sustained, calcium-induced, intrinsic apoptotic pathway-driven response that may drive extensive urothelial tissue damage resulting in chronic inflammation, provides valuable clues to explain the reported pathology associated with ketamine cystitis. Moreover, in light of the recent drive by the pharmaceutical sector to produce new generation ketamine derivatives for clinical use as novel antidepressants,[27] this model of bladder cells will be valuable in ensuring that any urological effects are identified and closely monitored.

Acknowledgments

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Figure Legends

Figure 1 Histological assessment of human ureteric organ cultures following 72 hours exposure to 3 mM ketamine showed thinning of the epithelium (shown in the images of donor x) and clear signs of apoptosis including pyknotic nuclei and karyorrhexis (shown in the images of donor y). Immuno-reactivity with a cleaved-cytokeratin 18 antibody (M30 Cytodeath) was observed in cells being lost from the epithelium (arrowed for donor x). n = 6 donors with two representative examples shown. Scale bars = 100 μm.

Figure 2 Effects of ketamine on population growth in cultures of NHU cells. **A:** Concentrations of ketamine between 0.3 and 6 mM were assessed over a 6 day time-course by Alamar blue reduction assay (n=6; error bars represent standard deviation). This graph is representative of repeats in three independent donor cell lines. **B:** Counts from two donor cell lines following 96 hours of exposure to ketamine indicated an IC_{50} for NHU cells of 0.93 mM. The IC_{50} was calculated using a sigmoidal fit and is illustrated as a black line; the 95% confidence interval is shown as dashed lines; n=4 replicates per donor.

Figure 3 Induction of apoptosis in NHU cell cultures by ketamine. **A:** Western blotting and densitometric analysis of NHU cells separated into cytoplasmic and mitochondrial fractions showed elevated cytochrome c in the cytosolic fractions of cultures treated with 3 mM ketamine for 24 hours. Loading controls were Bcl2 for the mitochondrial fraction and GAPDH for the cytoplasmic fraction. **B:** Densitometry analysis showed a

significant mean 2.4-fold change in cytoplasmic cytochrome c content in 3 independent NHU cell lines following 24 hour exposure to 3mM ketamine. **C:** Western blotting of phospho-Akt (active-form), phospho-ERK1/2 (active-form) and S9 phospho-GSK3β (inactive-form) showing early depletion of these forms of the kinases in response to 3 mM ketamine. Abundance quantified by densitometry is shown as a percentage of control cells for each timepoint, normalised to β actin (combined data from n=4 donors). **D:** Inhibition of GSK3β by SB415286 in ketamine-exposed NHU cells was capable of a slight but significant inhibition of toxicity as assessed by Alamar Blue reduction (n=6; error bars represent standard deviation). **E:** Inhibition of the MPTP with cyclosporin A (CsA) in ketamine-exposed NHU cells was capable of a small but significant inhibition of toxicicty as assessed by Alamar Blue reduction (n=6; error bars represent standard deviation). **F:** Western blotting of caspase 9, caspase 3 and cleaved PARP in NHU cells following 72 hours exposure to 3 mM ketamine. **G:** The three markers of apoptosis were all significantly increased by >2-fold in densitometry which was normalised to β actin (n=3 donors, error bars = standard deviation). **H:** Caspase 3/7 activity was assessed in lysates from NHU cell cultures exposed to 0.1 – 6 mM ketamine and normalised to baseline caspase activity in untreated cells. Significant increases in caspase activity were observed following exposure to 3 and 6 mM ketamine. (n=6; error bars represent standard deviation).

Levels of significance in all panels are denoted by asterisks (* = $p<0.05$, ** = $p<0.01$ & *** = $p<0.001$).

Figure 4 RT-PCR of NMDA receptor transcripts (GRIN isoforms). No expression of GRIN isoforms was detected in NHU cells under either proliferating or differentiated cell culture conditions. Furthermore, no GRIN expression was detected in freshly-isolated urothelium following its separation from the underlying stroma. Representative images of one donor (proliferating and differentiated NHU) and one donor P0 urothelium are shown. Uroplakin 2 (UPK2) transcript expression was used to confirm urothelial differentiation and GAPDH was included as an internal housekeeping control to confirm RNA integrity. In addition, no products were detected in reverse transcriptase negative cDNA controls that were generated using each RNA preparation.

Figure 5 Treatment of NHU cells with ketamine resulted in elevation of cytosolic $[Ca^{2+}]$. **A:** The addition of 1 mM ketamine (non-cytotoxic) to a culture of NHU cells produced a transient increase in cytosolic $[Ca^{2+}]$, whereas exposure to 3 mM ketamine (cytotoxic) induced a greater increase of $[Ca^{2+}]$ in the cytoplasm, which did not return to a normal baseline. **B:** The role of purinergic receptors in mediating the urothelial response to 1 mM ketamine was investigated by pre-incubating cultures for 10 min in 100 µM PPADS (purinergic receptor inhibitor) or ARL-67156 (ectoATPase inhibitor). PPADS (dotted line) almost completely blocked the Ca^{2+} transient observed following addition of 1 mM ketamine to NHU cells, whereas ARL-67156 (dashed line) increased both the amplitude and duration of the transient. **C:** Pre-incubating cultures in PPADs was not sufficient to inhibit the $[Ca^{2+}]$ increase triggered by 3mM ketamine ("Ket" = Ketamine). All Figures are traces representative of triplicate experiments.

Figure 6 NHU cell cytosol [Ca^{2+}] was elevated by the release of stores from the endoplasmic reticulum in response to ketamine exposure. **A:** The repeated administration of 1 mM ketamine to NHU cells in the presence of 2 mM extracellular [Ca^{2+}] resulted in repeated transient increases in cytosolic [Ca^{2+}], with no sign of reduced intensity in subsequent transients. **B:** The source of the Ca^{2+} transient was elucidated by performing repeated challenges of urothelial cells with 1 mM ketamine (solid line) and 20 μM ATP (dashed line) in the absence of extracellular [Ca^{2+}]. An initial treatment with either ketamine or ATP created a [Ca^{2+}] transient showing that in both cases Ca^{2+} was released from internal stores. A second stimulation triggered a significantly attenuated transient in response to either ketamine or ATP, indicating incomplete refilling of the internal stores. **C:** To identify the internal store involved in cytosolic Ca^{2+} release by 1 mM ketamine stimulation, the effect of blocking IP_3 receptors on the endoplasmic reticulum (ER) was evaluated. Pre-treatment of urothelial cell cultures with 100 μM 2-APB produced complete inhibition (dashed line) of the 1 mM ketamine-induced Ca^{2+} release (solid line). **D:** The source of the sustained [Ca^{2+}] elevation was confirmed as internal by stimulating the cells with 3 mM ketamine in the absence of exogenous Ca^{2+}. **E:** Thapsigargin was used to inhibit SERCA pumps and effectively empty the ER by allowing Ca^{2+} to leak out. It was observed that the 3mM ketamine-induced calcium elevation persisted suggesting this calcium was not derived from thapsigargin-sensitive regions of the ER. Figures are traces representative of triplicate experiments.

Figure 7 Ketamine exposure caused mitochondrial stress in NHU cells. **A:** NHU cells exposed to ketamine for 48 hours had a significantly lower resting mitochondrial OCR than controls (54.4%, Mann Whitney Test $p<0.001$). The proportion of OCR devoted to physiology other than ATP generation remained the same, whereas the spare mitochondrial capacity in control cells was far greater (4.6-fold, Mann Whitney Test, *** = $p<0.001$).   Data are the means of experiments performed in duplicate on 3 independent cell lines; error bars represent standard deviation. **B:** NHU cells labelled with TMRM show that 3 mM ketamine exposure led to elevated mitochondrial membrane potential (images are representative of experiments performed in cells from three donors, scale bar = 50 μm), which **C:** was confirmed quantitatively by flow cytometry (n= mean of 3 independent donor cell lines; error bars represent standard deviation). **D:** Cellular ATP was significantly (* = $p<0.05$) reduced to 76.8% of control following 3 mM ketamine exposure for 48 hours. Data are the means of duplicate measurements in 3 independent donor cell lines; error bars represent standard deviation.

Figure 8 Schematic diagram summarising the proposed mechanism of ketamine-induced cytotoxicity downstream of initial receptor activation/inhibition and illustrating how the experiments performed in this study support the proposed mode-of-action. Ketamine exposure leads to ATP release from the cells (the ATP signal could be enhanced by preventing its breakdown with ARL-67156) which binds to P_2Y receptors (that were effectively inhibited by PPADS). Activation of P_2Y receptors releases IP_3 which binds IP_3R

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on the endoplasmic reticulum (inhibited using 2-APB) and causes release of stored Ca^{2+} into the cytoplasm. Prolonged elevation of cytoplasmic $[Ca^{2+}]$ triggers further Ca^{2+} release from mitochondria, probably via the activated mitochondrial permeability transition pore (MPTP). Ketamine exposure triggers a reduction in the phosphorylated/active forms of Akt and ERK, promoting an increase in GSK3β-activity (which could be inhibited by SB415286). Acting in concert, GSK3β-activity and sustained elevation of cytosolic Ca^{2+} activate the MPTP (which could be inhibited by cyclosporin A) leading to mitochondrial depolarisation, release of cytochrome c and caspase-mediated apoptosis.