- CHAPTER IV -

CHARACTERISATION OF THE CYTOKINE-MODULATING ACTIVITY OF KETAMINE IN AN EQUINE MACROPHAGE CELL LINE FOLLOWING ENDOTOXIN EXPOSURE

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SUMMARY

Ketamine is widely used in equine anaesthesia. Beside its anaesthetic and analgesic properties, ketamine has been demonstrated to possess cytokine-modulating activity in rodents and humans. Additionally, in an equine macrophage cell line (e-CAS cells) ketamine inhibited the lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production in a concentration-dependent manner. However, the molecular mechanism underlying the cytokine-modulating activity of ketamine in e-CAS cells remains to be elucidated.

Following LPS exposure, synthesis of pro-inflammatory cytokines is regulated downstream of the pathogen-sensing Toll-like receptor 4 (TLR4) through activation of key mediators like mitogen-activated protein kinases (MAPKs: c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase p38 (p38) and extracellular signal-regulated kinase (ERK)), inhibitor of kappa B-alpha (IκB-α) and the transcription factor nuclear factor-kappa B (NF-κB). In the present study, the effect of ketamine (0-36 μM) on LPS-induced (1 μg/mL) TLR4, JNK1/2, ERK1/2, p38, IκB-α and NF-κB expression was determined in e-CAS cells, to elucidate the mechanism via which ketamine inhibits LPS-induced cytokine production. Expression of TLR4 was determined by flow cytometric analyses. Phosphorylated JNK1/2, ERK1/2, p38 and total IκB-α levels were analysed by Western Blotting, and NF-κB expression was investigated by electrophoretic mobility shift assays (EMSAs).

Ketamine inhibited the LPS-induced NF-κB expression in a concentration-dependent manner in e-CAS cells, even when added directly to nuclear extracts of LPS-treated e-CAS cells. No influence of ketamine on TLR4, JNK1/2, ERK1/2, p38 and IκB-α expression could be demonstrated.

The results of the present study demonstrate that the LPS-induced signal transduction pathway in e-CAS cells is only affected by ketamine at the level of NF-κB, whereas TLR4, JNK1/2, ERK1/2, p38 and IκB-α expression remained unaffected in ketamine-treated e-CAS cells. Moreover, the observation that ketamine was able to directly inhibit NF-κB expression when added to nuclear extracts suggests that the cytokine-modulating effects of ketamine can be attributed to a direct interaction between ketamine and the nuclear factor.

1. INTRODUCTION

Ketamine is a dissociative anaesthetic drug whose effect is characterised by superficial sleep of short duration. Next to hypnosis, it produces analgesia, which can be partially ascribed to its antagonistic effect towards N-methyl-D-aspartate (NMDA) receptors.[1]

For decades, ketamine has been in use in equine practice for both the induction and maintenance of general anaesthesia in horses.[2,3] More recently, studies focus on the antinociceptive effects of a subanaesthetic continuous rate infusion of ketamine in conscious horses.[4]

Beside these anaesthetic and analgesic effects, ketamine has been demonstrated to possess cytokine-modulating activity in different species. In mice and rats, ketamine suppressed the lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production both in vitro and in vivo.[5-7] In a human whole blood assay, the LPS-induced production of TNF-α, IL-6 and IL-8 was suppressed in the presence of ketamine.[8,9] In agreement with these studies, we recently demonstrated that ketamine also reduces the LPS-induced TNF-α and IL-6 production in an equine macrophage cell line (e-CAS cells).[10]

Multiple signalling pathways are known to play a pivotal role in upregulating gene expression and synthesis of pro-inflammatory cytokines following LPS exposure. [11-14] Lipopolysaccharide initiates a signalling cascade after binding to and activation of the cell surface receptor, Toll-like receptor-4 (TLR4). Upon stimulation, a downstream phosphorylation cascade activates several intracellular pathways such as the mitogen-activated protein kinase (MAPK) pathways, which include c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase p38 (p38) and the nuclear factor-kappa B (NF-κB) pathway.[15,16]

Nuclear factor-kappa B is a key regulatory transcription factor, which is mainly sequestered in the cytoplasm in an inactive form by association with an inhibitory protein, termed inhibitor of kappa B (IκB). Exposure to LPS results in phosphorylation and degradation of IκB, allowing released NF-κB to translocate to the nucleus, to bind to its cognate DNA element and to activate transcription of target genes.[16]

Ketamine was found to reduce LPS-induced cytokine production by inhibiting NF-κB activity. In vitro, ketamine reduced LPS-induced NF-κB activation in human glioma cells,[17] in rat mononuclear cells[18] and in rat monocytes.[19] In vivo, ketamine suppressed NF-κB activity in the intestines, liver and lungs of endotoxin-

challenged rats,[20] in mouse brain cells[17] and in the hippocampus of the rat following global ischaemia.[21] Yu et al.[18] and Shen et al.[21] could also demonstrate a blocking effect of ketamine on IκB-α degradation. Regarding the influence of ketamine on the MAPK pathways only limited data are available. In the hippocampus of the rat, ketamine pretreatment had no significant effect on JNK1/2 and JNK3 activation following global ischaemia,[22,23] while ketamine pretreatment increased JNK1/2 activation in rat hippocampus following a second insult of global ischaemia.[22]

We have previously reported that ketamine could inhibit LPS-induced TNF-α and IL-6 production in an equine macrophage cell line.[10] However, the molecular mechanisms underlying the cytokine-modulating activity of ketamine in this cell line are still unidentified. Based on results of other studies, a major role for NF-κB is expected, although it can not be entirely excluded that upstream key proteins of the signal transduction pathways might also affect cytokine production independently of NF-κB or impair the activation of NF-κB through interaction with TLR4. Hence, in the current study we investigated systematically the influence of ketamine on TLR4, MAPKs, IκB and NF-κB to identify the molecular target via which ketamine attenuates the LPS-stimulated production of pro-inflammatory cytokines in e-CAS cells.

2. MATERIALS AND METHODS

2.1. CHEMICALS

Ketamine, lipopolysaccharide (LPS; Escherichia coli, O111:B4), nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), dimethylthiazol diphenyl tetrazolium bromide (MTT) and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS), RPMI 1640 medium, penicillin, streptomycin and glutamine were obtained from Biocambrex (Verviers, Belgium). Horse serum (HS), non-essential amino acids and sodium pyruvate were from Gibco Invitrogen (Breda, The Netherlands). Helenalin, SP600125, SB203580 and U0126 were from Biomol (Plymouth, USA). The TNF-α ELISA kit and recombinant equine IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit polyclonal anti-IκB-α was from Biolegend (San Diego, CA, USA). Alamar blue, rabbit anti-phosphorylated JNK1&2 (anti-pJNK1/2) and rabbit anti-phosphorylated p38 were from BioSource (Camarillo, CA, USA). Mouse anti-phosphorylated ERK1&2 (anti-pERK1/2) was purchased

from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Alkaline phosphatase conjugated with polyclonal goat anti-rabbit and conjugated with rabbit anti-mouse immunoglobulins were from Dako Cytomation (Glostrup, Denmark). Bradford protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The NE-PER® Nuclear and Cytoplasmic Extraction Reagents, the LightShift® Chemiluminescent EMSA Kit and the Chemiluminescent Nucleic Acid Detection Module were purchased from Pierce Biotechnology (Rockford, IL, USA).

2.2. CELL CULTURE

The equine bone-marrow-derived macrophage cell line (e-CAS), described by Werners et al.,[24] was grown in RPMI 1640 medium supplemented with 10% HS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 1% non-essential amino acids. Cells were maintained at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. INHIBITION EXPERIMENTS

To investigate whether MAPKs and NF-κB are involved in LPS-stimulated TNF-α and IL-6 production in e-CAS cells, specific signal transduction inhibitors of JNK (SP600125), p38 (SB203580), ERK (U0126) and NFκB (helenalin) were added to LPS-treated e-CAS cells, and subsequently, TNF-α and IL-6 concentrations were measured. For testing, cells were seeded in 24-well plates at a density of 0.2 x 10^6 cells per well (1 mL). Following 24 h of culturing, cells were serum-deprived for 2 h before exposure to LPS (1 µg/mL) and different concentrations of either SP600125 (0-10 µM), SB203580 (10 µM), U0126 (10 µM) or helenalin (0-1 µM). Equal amounts of solvent instead of the inhibitors (0.1% DMSO) or LPS (PBS diluted 1:1000) were added for control. After 24 h of incubation, supernatants were collected and stored at –70 °C until analysis.

2.4. TNF-α ELISA

Concentrations of TNF-α were measured by enzyme-linked immuno-absorbent assay (ELISA) using an equine TNF-α ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). In brief, 96-well plates coated with goat anti-equine TNF-α antibody were incubated with samples or TNF-α standards for 2h at room temperature (RT). A standard curve was established using recombinant equine TNF-α from 0-2000 pg/mL. Plates were washed 3 times followed by incubation with biotinylated goat anti-equine TNF-α antibody for 2 h

at RT. Following 3 washes and 20 min incubation with streptavidin-HRP at RT, plates were incubated with substrate solution for 20 min at RT. The reaction was stopped by adding 2 N H_2SO_4 and absorbance was read at 540 nm using a plate reader. TNF-α concentrations were expressed as pg/mL.

2.5. BIOASSAYS FOR IL-6

The relative IL-6 concentrations were measured by using a bioassay utilising the murine hybridoma cell line B13.29 clone B.9 according to the method of Helle et al.[25] The assay is based on the rate of proliferation of IL-6-dependent B.9 cells, which is quantified by means of the MTT assay. By comparison of the mean inhibitory concentration of serially diluted IL-6 and serial dilutions of the sample, relative IL-6 levels of the samples were determined.

2.6. FLOW CYTOMETRIC ANALYSES OF TLR4 CELL SURFACE EXPRESSION

Cells were grown in 75 cm^2 culture flaks at a density of 0.7 x 10^6 cells per well (20 mL). Following 24 h of incubation, cells were detached by scraping, suspended in medium and pre-incubated with medium containing LPS (1 μg/mL) and ketamine (36 μM) for 24 h. Cells were washed once with PBS and resuspended in medium. Then, TLR4 cell surface expression was measured by staining cells for 20 min with rabbit polyclonal anti-TLR4 (4 μg/mL) at RT and by incubating cells for 20 min with secondary FITC-labeled polyclonal swine anti-rabbit IgG (1.6 μg/mL). Fluorescence was measured using a FACScan® (Becton and Dickinson and Co., Oxnard, CA).

2.7. WESTERN BLOT ANALYSIS OF PHOSPHORYLATED JNK1/2, ERK1/2, P38 AND TOTAL IκB-α LEVELS

Cells were cultured in 6-well plates at a density of 1.0 x 10^6 cells per well (2 mL). After 24 h of culturing, cells were serum-deprived for 2 h prior to testing. For the time-response curves of MAPK and IκB-α, cells were stimulated with blank medium or LPS (1 μg/mL) for 0-24 h or 0-1 h, respectively. Based on the results of the time-response curves, cells were exposed to LPS (1 μg/mL) and ketamine at different concentrations (0-36 μM) for 1 h in case of MAPK analysis. Cells exposed to blank medium, LPS or ketamine (36 μM) alone served as control. To investigate changes in IκB-α levels, cells were incubated with LPS (1 μg/mL) and ketamine (18 μM) for 0, 15, 30 and 60 min.

After exposure, cellular extracts were prepared in lysis buffer [50 mM DTT, 2 mM sodium pyrophosphate, 5 mM EDTA, 10% glycerol, 2% SDS and 63 mM Tris-HCl (pH 6.8)] and protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots containing 10 µg of protein were separated by SDS-PAGE (12.5%) and transferred to polyvinylidene fluoride membranes. Membranes were blocked in blocking solution [150 mM NaCl, 0.1 M Tris-HCl (pH 8.0), 0.03% Tween-20 and 1% BSA] overnight at 4 °C and then incubated with primary antibodies (rabbit polyclonal anti-p38, mouse monoclonal anti-pERK1/2, rabbit polyclonal anti-JNK1/2 and rabbit polyclonal anti-IκB-α; diluted 1:1000) for 1 h at RT. After washing, blots were incubated with secondary antibodies (alkaline phosphatase conjugated polyclonal goat anti-rabbit IgG or rabbit anti-mouse IgG; diluted 1:1000) for 1 h at RT. Immunoreactive protein bands were developed by using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates and quantified through densitometric analysis.

2.8. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) FOR NF-κB

Cells were cultured in 10-cm dishes at a density of 2.0 x 10^6 cells per dish (10 mL) for 24 h. For the time-response curve, cells were exposed to medium or LPS (1 µg/mL) for 0-4 h. Based on these pilot experiments, cells were exposed to LPS (1 µg/mL) and ketamine at different concentrations (0-36 µM) for 1 h. For control, cells were exposed to LPS- or ketamine (36 µM)-containing medium.

To investigate whether ketamine is able to directly interact with NF-κB, ketamine (18 µM) was added to a nuclear extract prepared from LPS-treated e-CAS cells. In control experiments, nuclear extract of LPS-stimulated and non-stimulated e-CAS cells were exposed to blank medium and ketamine or blank medium, respectively.

Nuclear extracts were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA), with minor modifications. In brief, cells were suspended in Cytoplasmic Extraction Reagent I to which protease inhibitors were added (Complete Mini, Roche, Germany) and incubated on ice for 10 min. Cytoplasmic Extractions Reagent II was added and after 1 min of incubation, nuclei were pelleted by centrifugation at 16,000 x g for 5 min at 4 °C. The pellet was resuspended in Nuclear Extraction Reagent to which protease inhibitors were added and incubated on ice for 40 min. The supernatants, containing nuclear extract, were collected by centrifugation at 16,000 x g for 10 min at 4 °C. The

electrophoretic mobility shift assay (EMSA) was performed using the LightShift® Chemiluminescent EMSA Kit according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Briefly, nuclear extract (4 µg) was pre-incubated with binding buffer [0.09% Nonidet P-40, 50 ng/µL poly dI-dC, 170 mM KCl and 2.6 mM DTT] for 10 min on ice. Biotin end-labeled NF-κB oligonucleotides (sense: 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and antisense: 5'-GCC TGG GAA AGT CCC CTC AAC T-3') were added and incubation was continued for 20 min at RT. Mixtures were subjected to 6% polyacrylamide gel electrophoresis in TBE buffer, transferred on nylon membranes (Hybond-N^+) and cross-linked by shortwave (254 nm) ultraviolet radiation. Probe-NF-κB complexes were visualised by Chemiluminescent Nucleic Acid Detection Module. Membranes were exposed to X-ray films and bands were scanned for densitometric analysis. To confirm specificity, competition assays were performed with a 4 and 40-fold excess of non-biotin-labeled and mutated probes (sense: 5'-AGT TGA GGA GAC TTT CCC AGG C-3' and antisense: 5'-GCC TGG GAA AGT CTC CTC AAC T-3').

2.9. CELL VIABILITY

The Alamar Blue ™ (AB) reduction cytotoxicity assay was used as an indicator of cell viability.[26] The assay is based on the reduction of resazurin to fluorescent resorufin. In brief, Alamar Blue solution was added to each well (1:10 dilution) and the plate was incubated at 37 ^oC for 4 h. Thereafter, resorufin formation was measured fluorometrically at 530-560 nm excitation wavelength and 590 nm emission wavelength.

2.10. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS© 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). Data were analysed for statistical significance using one-way analysis of variance (ANOVA), followed by a post-hoc Bonferroni's multiple comparison test. Values were considered to be significant when $p<0.05$. Data are presented as mean ± standard deviation (S.D.).

3. RESULTS

3.1. KETAMINE DOES NOT AFFECT LPS-STIMULATED TLR4 EXPRESSION LEVELS AT THE CELL MEMBRANE

To study whether a reduced LPS-stimulated cytokine production of ketamine-treated e-CAS cells is associated with reduced signalling of TLR4, expression of

TLR4 at the cell surface was studied by FACS analyses. Treatment of e-CAS cells with LPS for 1 h significantly increased TLR4 expression at the cell surface (Fig. 1B), when compared to non-stimulated cells (Fig. 1A). Ketamine did not affect TLR4 expression, neither in non-stimulated (Fig. 1C) nor in LPS-stimulated cells (Fig. 1D).

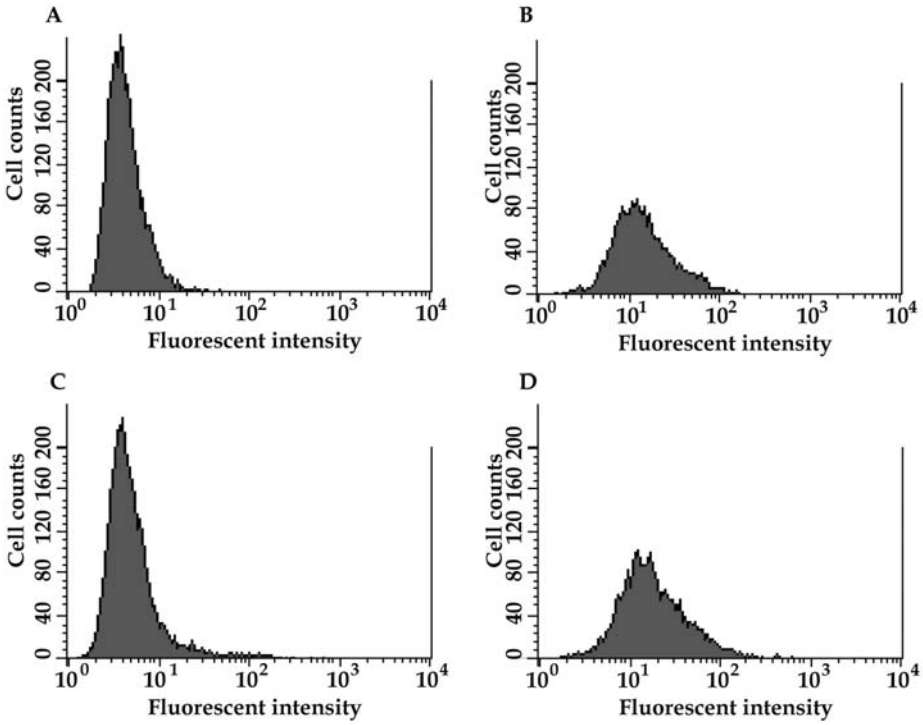


Figure 1. Effect of ketamine on TLR4 expression on the cell surface of non-stimulated (A and C) and LPS-stimulated (B and D) e-CAS cells. Cells were incubated with 36 μM ketamine (C and D) for 24 h. Following incubation, cells were double stained with rabbit polyclonal anti-TLR4 and FITC-labeled polyclonal swine anti-rabbit IgG for 40 min and assayed for TLR4 expression by FACS analysis. Representative FACS of three independent experiments are shown.

3.2. INHIBITORS OF NF-κB AND MAPK PROTEINS AFFECT LPS-STIMULATED CYTOKINE PRODUCTION

To investigate whether NF-κB and MAPKs are involved in LPS-stimulated TNF-α and IL-6 production in e-CAS cells, NF-κB, JNK, ERK and p38 were selectively inhibited in LPS-treated e-CAS cells, and subsequently, TNF-α and IL-6

concentrations were measured. Inhibition of both NF-κB as well as the three MAPK isoforms significantly reduced the LPS-induced TNF-α production in a concentration-dependent manner, whereas LPS-induced IL-6 production was only significantly suppressed following inhibition of NF-κB, p38 and JNK (Figs 2A-D).

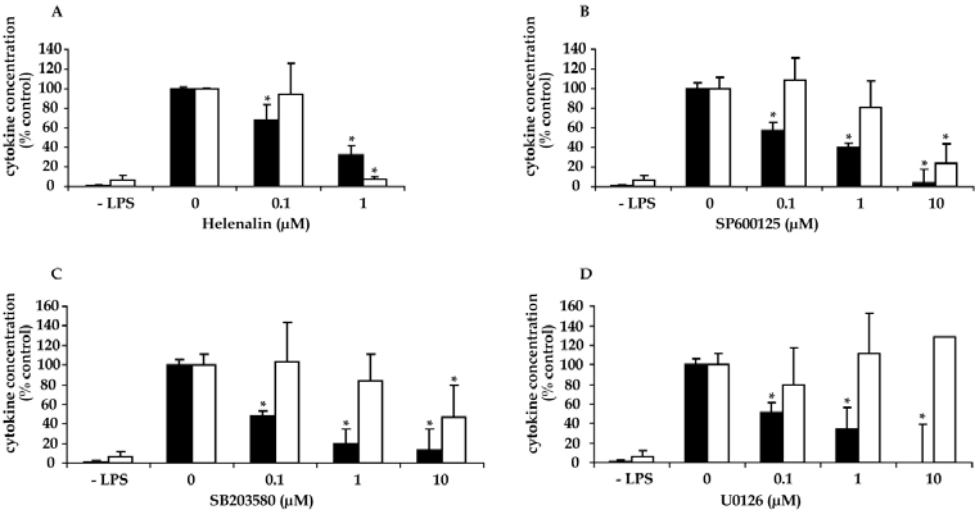


Figure 2. Effect of (A) helenalin, (B) SP600125, (C) SB203580 and (D) U0126 on the LPS-induced TNF-α (■) and IL-6 (□) response in e-CAS cells. Cells were incubated with the specific inhibitors of NF-κB (helenalin; 0-1 μM), JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM), ERK (U0126; 0-10 μM) and LPS (1 μg/mL) for 24 h. Following incubation, TNF-α and IL-6 concentrations were measured in the supernatants by TNF-α ELISA or 7-TD1 bioassay, respectively. Data are expressed as mean ± S.D. of three independent experiments. LPS alone was regarded as a positive control and set at 100%. *statistically different to LPS alone ($p<0.05$).

3.3. KETAMINE DOES NOT AFFECT LPS-INDUCED MAPK PHOSPHORYLATION

To study whether the reduced LPS-stimulated cytokine production in ketamine-treated e-CAS cells is attributable to reduced MAPK activation, Western blot analyses were performed to investigate the phosphorylation status of JNK1/2, ERK1/2 and p38. Cells were treated with LPS for different time-periods and a detectable amount of phosphorylated ERK1/2 was observed in non-stimulated cells. When cells were exposed to LPS (1 μg/mL), phosphorylation of JNK1/2, ERK1/2 and p38 showed a significant level of expression at 60 min following LPS exposure (Fig. 3A). In consideration with these findings, all experiments were

conducted at 60 min following LPS stimulation. When cells were incubated for 1 h with LPS and increasing concentrations of ketamine, ketamine did not reduce the extent of LPS-mediated phosphorylation of JNK1/2, ERK1/2 and p38 (Fig. 3B).

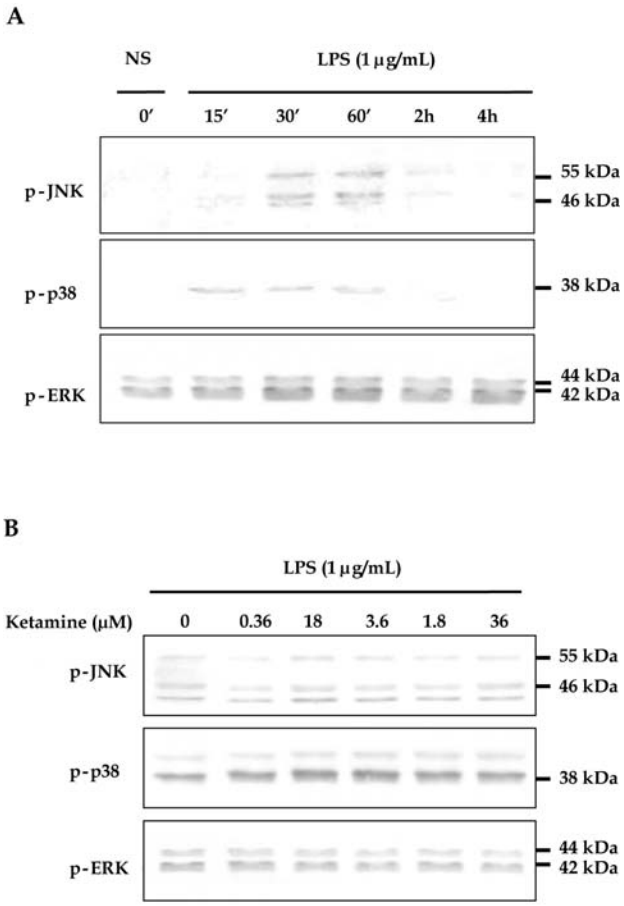


Figure 3. Failure of ketamine to affect LPS-induced MAPK expression in e-CAS cells. (A) Cells were incubated with blank medium or LPS (1 μg/mL) for 0-4 h. Following incubation, cells were lysed and lysates were assayed for p-JNK, p-p38 and p-ERK expression by Western blot analysis. Cells exposed to blank medium served as control (NS = not stimulated). (B) Cells were incubated with LPS (1 μg/mL) and ketamine (0-36 μM) for 1 h. Following incubation, cells were lysed and lysates were analysed for p-JNK, p-p38 and p-ERK expression by Western blot analysis. All Western blots are representatives of three independent experiments.

3.4. KETAMINE DOES NOT AFFECT LPS-INDUCED DOWNREGULATION OF IκB-α EXPRESSION

To study whether the reduced cytokine production of ketamine-treated e-CAS cells is associated with a reduced dissociation of NF-κB from IκB-α, the effect of ketamine on the time-dependent down-regulation kinetics of IκB-α in LPS-treated e-CAS cells was studied by Western blot analysis. In e-CAS cells, IκB-α was virtually absent at 15 min of LPS treatment and significantly increased following 60 min of LPS treatment (Fig. 4). The time-dependent kinetic of disappearance and re-appearance of IκB-α was similar for non-treated and ketamine-treated e-CAS cells.

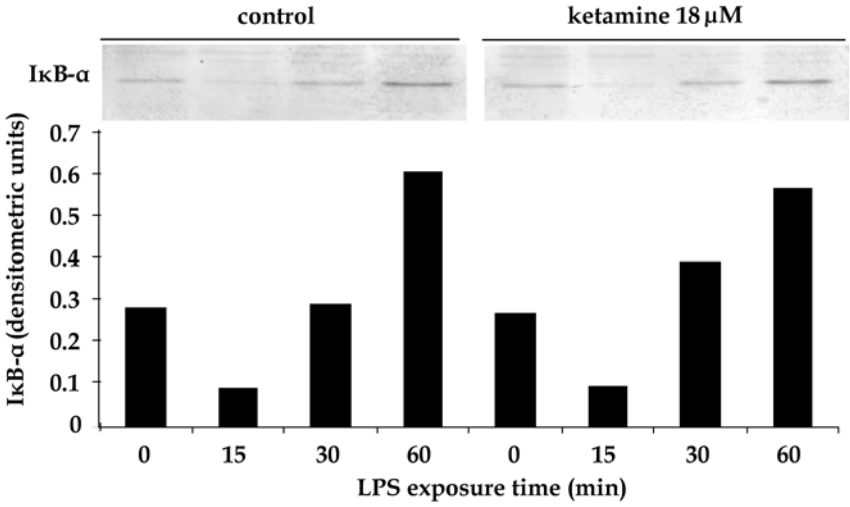


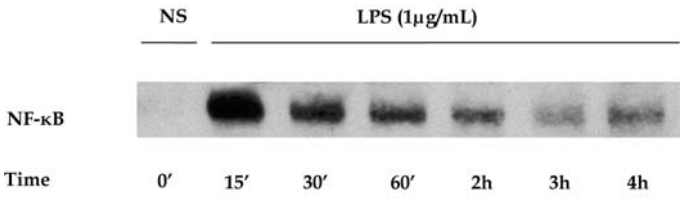
Figure 4. Failure of ketamine to affect LPS-induced IκB-α expression in e-CAS cells. Cells were stimulated with LPS and co-incubated without or with ketamine (18 μM) for indicated times. Following incubation, cells were lysed and lysates were assayed for IκB-α expression by Western blot analysis. Representative Western blots of three independent experiments with densitometric quantification of the IκB-α bands are shown.

3.5. KETAMINE INHIBITS LPS-STIMULATED NF-κB EXPRESSION

To investigate the effect of ketamine on LPS-stimulated NF-κB expression, nuclear extracts were isolated from e-CAS cells and subjected to EMSA. Figure 5A demonstrates that non-treated e-CAS cells showed no NF-κB expression, while addition of 1 μg/mL LPS markedly enhanced NF-κB expression. This increase in NF-κB expression was most significant following 15 min of LPS treatment, whereas longer incubation times with LPS for up to 4 h resulted in a less profound

expression of NF-κB. Appearance of NF-κB expression at 15 minutes following LPS exposure correlates with the disappearance of IκB-α expression (Fig. 4). Moreover, re-appearance of IκB-α is associated with a decrease in NF-κB expression. Figure 5B shows that treatment with ketamine for 60 min attenuated the LPS-stimulated NF-κB expression in a concentration-dependent manner. Direct administration of 1.8 μM ketamine to the nuclear extract obtained from LPS-stimulated e-CAS cells also inhibited NF-κB expression (Fig. 5C). Treatment of non-stimulated cells with ketamine alone did not affect NF-κB expression (data not shown). To demonstrate the specificity of the EMSA, an excess of unlabeled and mutated NF-κB probe was added to the nuclear extract of LPS-treated cells. Figure 5D shows the disappearance of the biotin-labeled probe-NF-κB protein complex following addition of the unlabeled probe. This disappearance is not observed following addition of an excess of unlabeled-probe containing a single mutation.

A


NS
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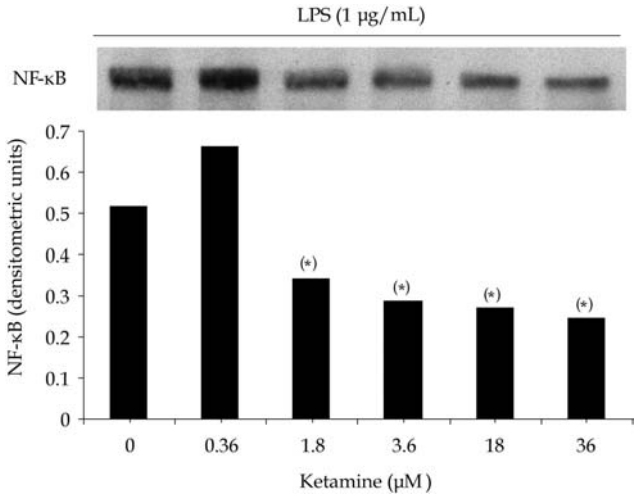
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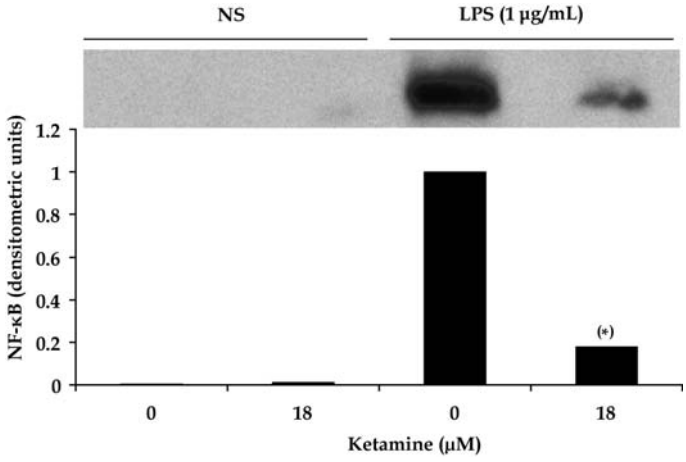
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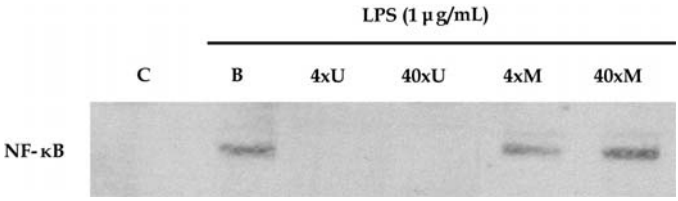

Figure 5. Effect of ketamine on NF-κB expression in LPS-stimulated e-CAS cells. (A) Cells were incubated with blank medium or LPS (1 µg/mL) for 0-4 h. Following incubation, nuclear extracts were prepared and assayed for NF-κB expression by EMSA. Cells exposed to blank medium served as control (NS = not stimulated). (B) Cells were incubated with LPS (1 µg/mL) and ketamine (0-36 µM) for 1 h. Following incubation, nuclear extracts were prepared and assayed for NF-κB expression by EMSA. Cells exposed to LPS alone served as control. (C) Cells were incubated with or without LPS (1 µg/mL) for 1 h. Then, nuclear extracts were prepared and incubation with or without ketamine (18 µM) for 15 min. Following incubation, nuclear extracts were assayed for NF-κB expression by EMSA. Non-stimulated cells served as control (NS = non-stimulated). (D) Cells were incubated with or without LPS (1 µg/mL) for 1 h. Following incubation, nuclear extracts were prepared and incubated with a 4 and 40-fold excess of non-biotin-labeled and mutated probes. Subsequently, nuclear extracts were assayed for NF-κB expression by EMSA. C = control; B = biotin-end-labeled probe; U = unlabeled probe; M = mutated probe. For all experiments, a representative EMSA of three independent experiments is shown. (*) statistically different to LPS alone in the corresponding experiments ($p<0.05$).

4. DISCUSSION

Previously, we could show that in an equine macrophage cell line (e-CAS cells), ketamine inhibits LPS-induced TNF-α and IL-6 levels in a concentration-dependent manner.[10] The aim of the present study was to elucidate the molecular mechanisms underlying the cytokine-modulating activity of ketamine in e-CAS cells. To this end the effect of ketamine on individual components of the LPS signalling cascade were investigated.

Toll-like receptor-4 is a cell surface receptor predominantly expressed in immune cells such as macrophages and neutrophils.[27] TLR4 is activated upon stimulation with LPS and initiates an intracellular signalling cascade.[28] Flow cytometric analysis of e-CAS cells showed a basal expression level of TLR4 on non-stimulated cells, which markedly increased following LPS exposure. Ketamine did not affect the TLR4 expression on LPS-treated e-CAS cells in the tested concentration range, suggesting that the reduced LPS-stimulated cytokine production in e-CAS cells following ketamine exposure is not mediated through an interaction between ketamine and TLR4.

Mitogen-activated protein kinases are a family of serine/threonine kinases which comprise three major subfamilies, JNK, p38 and ERK.[29] In response to LPS, all three MAPKs are involved in regulating transcriptional activation of pro-inflammatory cytokine genes.[13,14] ERK has been demonstrated to be involved in nuclear export of TNF-α mRNA, while p38 and JNK seem to affect the translation of TNF-α mRNA and the TNF-α gene transcription, respectively.[15] Moreover, specific MAPK inhibitors significantly reduced TNF-α production in various experimental models.[15,30] The presented results show that ketamine did not affect the extent of LPS-mediated MAPK phosphorylation in e-CAS cells, and thus the significantly reduced cytokine response in LPS-treated e-CAS cells following ketamine exposure can not be attributed to a reduced MAPK activation. The influence of ketamine on MAPK phosphorylations in LPS-treated macrophages has not been studied before. Regarding ketamine and MAPK interactions, only limited data are described in relation to cerebral ischaemia.[22,23] In these experiments, ketamine pretreatment had no influence on JNK1/2 and JNK3 activation following global ischaemia in the hippocampus of the rat.[22,23] However, ketamine pretreatment reversed the inhibition of JNK1/2 activation following a second insult of global ischaemia.[22] Furthermore, ketamine per se induced MAPK activation and expression of ERK2 in human aortic smooth muscle cells.[31] However, these data are not comparable with the results presented here as entirely different experimental designs (cell type, animal, ketamine pre-treatment, inducers) were used.

Nuclear factor-kappa B is a dimer composed of various combinations of members of the NF-κB/Relish (Rel) family, which include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and C-Rel. In non-stimulated cells, NF-κB is retained in the cytoplasm in an inactive form through association with IκB. Upon stimulation, the activation process of NF-κB can be divided into two phases.[16,32,33] The first phase consists of cytoplasmic signalling pathways leading to degradation of IκB and thus facilitating translocation of liberated NF-κB to the nucleus. The second phase occurs primarily within the nuclear compartment and involves additional regulatory mechanisms that control NF-κB activity, such as regulation of nuclear import of NF-κB dimers, regulation of recruitment of NF-κB dimers to target genes and regulation of NF-κB-mediated transcriptional activity by post-translational modifications including phosphorylation and acetylation of NF-κB proteins, particularly p65.[16,32,33] Up to this date, the reduction of NF-κB expression following ketamine exposure has been ascribed solely to reduced degradation of IκB-α. In LPS-treated rat mononuclear cells, Yu et al.[18] showed a reduced

expression of IκB-α, which markedly increased following exposure to LPS and ketamine at different concentrations (10-5000 μM). In addition, Shen et al.[21] demonstrated that pretreatment with ketamine (50 mg/kg) significantly blocked ischaemia-induced decrement of IκB-α in the hippocampus of the rat. In the present study, however, degradation of IκB-α was unaffected in ketamine-treated e-CAS cells, while ketamine reduced NF-κB expression in a concentration-dependent manner. This observation suggests that the molecular mechanism by which ketamine reduces NF-κB expression in e-CAS cells is related to regulatory events occurring in the second phase. This suggestion is supported by the finding that ketamine was able to inhibit NF-κB when added directly to nuclear extracts. However, the diversity and complexity of these additional regulatory mechanisms make it difficult to exactly indicate in what way ketamine interferes with these mechanisms to suppress NF-κB expression. It has been reported that increase in intracellular calcium concentrations accelerates nuclear import of activated NF-κB dimer and promotes the transcription of NF-κB-driven genes.[34] Lipopolysaccharide has been shown to induce a transient elevation of intracellular calcium. However, as a non-competitive NMDA receptor antagonist, ketamine has been shown to inhibit LPS-induced calcium elevation in rat monocytes.[19] Therefore, the reduced NF-κB expression in e-CAS cells following LPS exposure might be due to reduced nuclear import and reduced transcriptional activity of activated NF-κB dimers.

For these experiments, concentrations of ketamine were selected in consideration of the plasma level of 5 μg/mL (18 μM) following the IV anaesthesia induction dose of 2.2 mg/kg body weight commonly used in horses.[35] The concentration range and concentration-dependent manner by which ketamine inhibited the LPS-induced TNF-α and IL-6 production in e-CAS cells correlate with those at which ketamine suppresses NF-κB expression in these cells. The finding that 18 μM ketamine markedly reduced TNF-α production and NF-κB expression without changing IκB-α expression strengthen the conclusion that IκB-α is not included in the cytokine-modulating cascade of ketamine in e-CAS cells.

In conclusion, the LPS-induced signal transduction pathway in e-CAS cells is only affected by ketamine at the level of NF-κB. Hence, the reduced LPS-stimulated cytokine production in ketamine-treated e-CAS cells is most likely attributable to reduced NF-κB expression. Moreover, the observation that ketamine was able to directly inhibit NF-κB expression when added to nuclear extracts, suggests that the cytokine-modulating activity of ketamine can be attributed to a direct interaction between ketamine and NF-κB. The inhibition of NF-κB and the subsequent pro-

inflammatory mediators[10] is of clinical relevance for equine patients suffering from inflammatory disorders.

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