EFFECTS OF KETAMINE ON PRO-INFLAMMATORY MEDIATORS IN EQUINE MODELS

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EFFECTS OF KETAMINE ON PRO-INFLAMMATORY MEDIATORS IN EQUINE MODELS

HET EFFECT VAN KETAMINE OP ONTSTEKINGSMEDIATOREN BIJ HET PAARD (IN VITRO EN IN VIVO ONDERZOEKEN)

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- CHAPTER I -

SCOPE AND AIM OF THE THESIS

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Ketamine has been in use as an anaesthetic agent in equine medicine since the mid 70s.[1] Initially, ketamine was applied just as an induction agent, producing amnesia, loss of consciousness, analgesia and immobility. In later years, based on these properties, the application of ketamine in equine anaesthesia was extended by using it as an adjunct to inhalation anaesthesia,[2-4] in different total intravenous anaesthesia protocols[5-7] and for local analgesia.[8,9] Currently, studies focus on the antinociceptive effects of subanaesthetic ketamine continuous rate infusions (CRIs) in conscious horses[10] since in human medicine these low dose rate infusions have been proven to be sufficient for post-operative pain management.[11-13] Beside its anaesthetic and analgesic effects, ketamine has been reported to possess anti-inflammatory effects in rodents and humans. In various in vitro and in vivo studies, ketamine reduced the production of distinct inflammatory mediators.[14-17] These results suggest a beneficial role for ketamine in patients suffering from distinct inflammatory diseases. However, the species-specific response in production of inflammatory mediators and the corresponding underlying mechanisms impede the extrapolation of these previous results from either rodents or humans to horses. As yet, no data are available regarding the anti-inflammatory properties of ketamine in horses. Hence, this thesis presents the first experimental work regarding the anti-inflammatory effects of ketamine in both in vitro and in vivo equine models.

In **Chapter II**, a general overview is given regarding ketamine, the studied inflammatory mediators and the key mediators involved in the signal transduction cascade ultimately leading to their production. At the end of each subchapter, the influence of ketamine on the respective mediator in either rodent or human studies is described. Moreover, the presence or otherwise of this respective mediator in horses is depicted.

Chapter III presents the results of the primary in vitro study evaluating the anti-inflammatory effects of ketamine in equines. In this study, an equine macrophage cell line (also referred to as e-CAS cells), developed by Werners et al.,[18] was used, since macrophages play a pivotal role in the pathophysiology of many equine inflammatory disorders. Upon stimulation with lipopolysaccharides (LPS, outer-wall constituents of Gram-negative bacteria), the influence of ketamine on the pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) was investigated.

In rodents and humans, ketamine has been demonstrated to reduce the LPS-induced cytokine response by inhibiting expression of the nuclear transcription factor nuclear factor-kappa B (NF-κB).[19-21] Next to NF-κB, other upstream key mediators like the intracellular mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase p38 (p38) and extracellular signal-related kinase (ERK), and the extracellular Toll-like receptor 4 (TLR4) can be involved in the LPS-induced cytokine response.[22] In **Chapter IV**, the influence of ketamine on TLR4, MAPK and NF-κB expression is investigated to identify the underlying molecular mechanism of the ketamine-induced reduction of pro-inflammatory cytokine production in LPS-treated e-CAS cells.

Upon stimulation by LPS, cells express inducible enzymes like inducible nitric oxide synthase (iNOS)[23] and cyclooxygenase-2 (COX-2)[24] which subsequently induce the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2). Although generally involved in regulating physical homeostasis, overproduction of NO and PGE_2 is correlated with the pathogenesis of inflammatory diseases like arthritis, colitis, neurodegenerative diseases and septic shock.[25,26] In **Chapter V** and **Chapter VI**, the influence of ketamine on respectively iNOS expression and NO production and COX-2 expression and PGE_2 production is studied. Moreover, by inhibiting signalling key mediators like MAPK and NF-κB, the molecular mechanisms involved with iNOS and COX-2 expression in LPS-treated e-CAS cells are investigated.

Like other inflammatory mediators, reactive oxygen species (ROS) play a critical role in regulating homeostasis and represent a major mechanism of host defence against infection. However, under pathophysiologic conditions, oxidative stress may lead to an exaggerated production of ROS, which subsequently leads to cellular destruction and tissue injury.[27] In **Chapter VII**, the influence of ketamine on ROS production in e-CAS cells following exposure to LPS and phorbol myristate acetate (PMA) is described. Generally, cells possess several enzymatic anti-oxidants to protect them from 'home-made' ROS.[28] Glutathione peroxidase is such anti-oxidant which reduces ROS to water by using glutathione (GSH) as substrate. In this study, also the influence of ketamine on GSH-depleted ROS production in LPS/PMA-treated e-CAS cells was studied, to partly identify the mechanism by which ketamine reduces ROS production in stimulated e-CAS cells.

The anti-inflammatory effects of ketamine observed in previous experimental studies performed in rodents and humans, implies that ketamine might also have anti-inflammatory properties in vivo. To be clinically effective in conscious equine

patients in the postoperative period, long-term administration of subanaesthetic dose rates of ketamine seems to be required. Long-term infusions appear to be necessary, since ketamine displays a short elimination half-life,[29] while inflammatory mediators have been detected for hours after onset of inflammation. Moreover, subanaesthetic dose rates are preferred to avoid recumbency and side effects like excitation and catalepsia, regularly associated with ketamine administration. However, before studying the anti-inflammatory effectiveness of this infusion regimen in vivo, its influence on clinical and behavioural parameters in conscious horses was investigated. Hence, in **Chapter VIII**, the pharmacodynamic effects of a long-term subanaesthetic CRI of ketamine administered to healthy conscious horses are evaluated. Moreover, a pharmacokinetic profile of ketamine and its metabolites is described.

To study the influence of ketamine on LPS-induced inflammatory responses in vivo, tissue chamber modeling systems can be used.[30] Upon injection of LPS into the tissue chambers, a marked acute inflammatory response is produced which has been demonstrated to be valid for septic processes at soft tissue level.[31] Moreover, by applying the tissue chamber model, LPS-induced inflammatory responses will be confined largely to the tissue chamber, thereby minimising discomfort to experimental animals.[30] In **Chapter IX**, the anti-inflammatory effects of parenterally administered ketamine on a LPS-induced inflammatory response in tissue chambers of Shetland ponies is investigated. Both a bolus injection of ketamine and a subanaesthetic long-term continuous rate infusion were tested.

Finally, the main results of the aforementioned chapters are summarised and discussed in the concluding chapter of this thesis (**Chapter X**), in which also an outline of the clinical relevance of these results is given.

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- CHAPTER II -

GENERAL INTRODUCTION

CELLULAR TARGETS INVOLVED IN THE ANTI-INFLAMMATORY EFFECTS OF KETAMINE – A LITERATURE REVIEW -

1. KETAMINE

1.1. HISTORY

Ketamine is a dissociative anaesthetic drug which was synthesised in 1962 by Stevens and first used in humans in 1965 by Corssen and Domino.[1] In 1970 ketamine was introduced in veterinary medicine for anaesthesia in the cat[2] and many species followed soon after (for review see 3 and 4). In 1977 the combination xylazine and ketamine was evaluated for anaesthesia in horses.[5] Currently, ketamine is used in a variety of clinical settings regarding equine anaesthesia and analgesia. Besides the application for induction of anaesthesia, ketamine has also been used as an adjunct to inhalant anaesthesia,[6-8] in different total intravenous anaesthesia (TIVA) protocols[9-11] and for epidural analgesia[12] and peripheral nerve blocks.[13] More recently, studies focus on the antinociceptive effects of a subanaesthetic continuous rate infusion (CRI) of ketamine in conscious horses.[14,15]

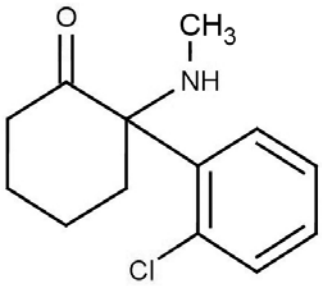


Figure 1. Structural formula of ketamine

1.2. PHYSICOCHEMICAL PROPERTIES

Ketamine is a congener of phencyclidine and is chemically designated as (±) 2-(ortho-chlorophenyl)-2-(methylamino)-cyclohexanone (Fig. 1). Ketamine has a molecular weight of 238 Da, forms a white crystalline salt with a pKa of 7.5 and has relatively high lipid solubility.[16] For veterinary use, commercially available ketamine solution is a racemic mixture consisting of two optical enantiomers, R(-) and S(+)ketamine, in equal amounts and the preservative benzethonium chloride.[17] For human use, also solutions of S(+)-ketamine only, the most active form, are available. Ketamine is prepared in a slightly acidic solution with a pH of 3.5 to 5.5.[16]

1.3. PHARMACODYNAMICS

1.3.1. MECHANISM OF ACTION

Ketamine interacts with multiple binding sites, including N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors, nicotinic and muscarinic cholinergic, adrenergic, γ-aminobutyric acid type A ($GABA_A$), dopamine, serotonin and opioid receptors.[18-20] In addition, interactions with voltage-dependent ion channels such as Na^+, K^+ and L-type Ca^{2+} channels have been described.[19] All of these interactions may play a role in the pharmacological and clinical effects of ketamine. However, non-competitive NMDA receptor antagonism accounts for most of the amnestic, analgesic, psychotomimetic and neuroprotective effects of ketamine.[18,19] By binding to the phencyclidine site of the NMDA receptor, ketamine blocks the open Ca^{2+} channel pore coupled to the NMDA receptor and inhibits glutamate activation of the receptor, thereby inhibiting the excitatory effect of glutamate on neurons in the central nervous system.[18,19]

1.3.2. EFFECTS ON THE CENTRAL NERVOUS SYSTEM

Ketamine produces dose-related unconsciousness and analgesia**.** The primary site of central nervous system action of ketamine appears to be the thalamoneocortical projection system. Electroencephalographic (EEG) studies showed that ketamine selectively depresses neuronal function in especially the association areas of the cortex[21] and the central nucleus of the thalamus.[22] Moreover, depressant effects of ketamine have been noted in nociceptive cells in the medial medullary reticular formation[23] and laminae I and V of the dorsal horn.[24] In conjunction with its depressant effects, ketamine also stimulates parts of the limbic system, including the hippocampus.[21,25] This functional dissociation between the thalamoneocortical and limbic systems produces a characteristic anaesthetic state often referred to as dissociative anaesthesia since ketamine interrupts flow of information from the unconscious to conscious parts of the brain, rather than producing a generalised depression of all brain centres.[3]

Induction of ketamine anaesthesia is often accompanied with increased skeletal muscle tonus generally described as catalepsia. Moreover, limb movements and tonic-clonic spasms of limb muscles occur in absence of external stimuli and are not dose-related. The mechanism of ketamine-induced catalepsia is not clearly understood, but studies indicate that it may be due to a deficiency of dopamine function or an imbalance in cholinergic-dopaminergic function.[26] Patients anaesthetised with ketamine may keep their eyes open, maintain corneal, laryngeal

and pharyngeal reflexes to some extent and show variable degrees of lateral nystagmus.[27,28]

In humans, ketamine produces undesirable psychological reactions like vivid dreaming, extracorporeal experiences and illusions often associated with excitement, confusion, euphoria and fear. Ketamine-induced depression of auditory and visual relay nuclei, leading to misperception of auditory and visual stimuli may be responsible for this reaction.[17] Hallucinatory behaviour, like nystagmus, neighing and slow head and limb movements can also be observed in horses following ketamine anaesthesia. Concomitant administration of benzodiazepines generally attenuates or treats this behaviour.

As a sympathomimetic agent, ketamine significantly increases cerebral blood flow, intracranial pressure and cerebrospinal fluid pressure as a result of cerebral vasodilation and elevated systemic blood pressure.[29,30] Therefore, ketamine should be used with caution in patients with cerebral trauma or intracranial masses.[31] On the other hand, as a non-competitive NMDA receptor antagonist ketamine might have neuroprotective effects, since excessive activation of NMDA receptors has been implicated in the pathogenesis of cerebral ischemia and other neurodegenerative disorders.[19,32] Moreover, during ketamine anaesthesia cerebrovascular autoregulation seems to be preserved.[33]

It has been known since the late 1970s that the ketamine enantiomers exhibit pharmacological and clinical differences.[34] Receptor studies in rodents show that S(+)-ketamine has a 4-fold higher affinity for NMDA receptors than its stereoisomer R(-)-ketamine. In rodents and humans, S(+)-ketamine exhibits 1.5 to 3-fold greater anaesthetic potency than the R(-) compound.[19,34] Moreover, S(+)-ketamine induces less spontaneous movements and psychotomimetic side effects than R(-)-ketamine.[19,33,34]

1.3.3. ANALGESIC EFFECTS

At anaesthetic concentrations ketamine produces profound analgesia. In addition, continuous rate infusions of subanaesthetic ketamine concentrations have proven to be effective as an adjunct to inhalation anaesthesia and in the management of acute postoperative pain. When low dose ketamine infusions are combined with systemic opioid administration, an opioid-sparing effect up to 50% is observed.[20,35] Moreover, by using low dose infusion rates the occurrence of ketamine-related side effects is reduced to a minimum.[20,33,36]

The analgesic potency of ketamine is mainly mediated through binding to NMDA receptors. NMDA receptors are highly involved in the pathogenesis of

hyperalgesia (i.e., a heightened sense of pain), allodynia (i.e., pain caused by a stimulus that does not normally cause pain) and the wind-up phenomenon (i.e., temporal summation of painful stimuli in the spinal cord). As a non-competitive NMDA receptor antagonist ketamine has proven to be useful in reducing these pathological pain states.[36-38] The influence of ketamine on pre-emptive analgesia (blocking pain mediators before pain is initiated) and chronic pain treatment is still controversial.[36,39,40]

It was presumed that ketamine mainly attenuated acute somatic nociception, but recent data, obtained from rodent and human experimental studies, demonstrate that ketamine also significantly attenuates acute visceral nociception.[41-43]

Epidural administration of ketamine produces dose-dependent analgesia in man,[44] dogs[45] and rats.[46,47] In horses, doses of 0.5 - 2.0 mg/kg produced analgesia of the tail, perineum and upper hind limb for 30 to 75 minutes, respectively.[12]

In rodents and humans, S(+)-ketamine exhibits 3-fold greater analgesic potency than the R(-) compound.[19,34] The analgesic potency of (S+)-ketamine is approximately twice that of the racemic mixture.[19,34]

1.3.4. EFFECTS ON THE CARDIOVASCULAR SYSTEM

As a sympathomimetic drug, ketamine stimulates the cardiovascular system, which is characterised by increases in heart rate, mean aortic pressure, pulmonary arterial pressure, central venous pressure and cardiac output.[26] The increase in cardiac output is generally attributed to increases in heart rate and cardiac contractility.[48] In vivo, ketamine-induced increases in blood pressure and cardiac contractility are usually considered to be indirect and mediated through sympathomimetic effects mediated in the central nervous system[49] and inhibition of neuronal reuptake of catecholamines by sympathetic nerve endings.[50] In contrast, in isolated heart muscle preparations of rats and dogs or in sympathoadreno-depleted patients, it is stated that ketamine acts as a direct myocardial depressant.[51-53] The centrally mediated sympathetic responses to ketamine usually override the direct depressant effects of ketamine.[28]

Increase in both blood pressure and heart rate seems paradoxical since increases in blood pressure normally elicit baroreflex-induced decreases in heart rate. Irnaten et al.[54] found that the ketamine-induced increase in heart rate is mediated through decreased activity of parasympathetic cardiac neurons in the brainstem.

The increase in haemodynamic variables is associated with increased cardiac work and myocardial oxygen consumption. The normal heart is able to increase myocardial oxygen supply by increasing cardiac output and decreasing coronary

vascular resistance, so that coronary blood flow is appropriate for the increased oxygen consumption.[55]

Since ketamine preserves cardiovascular function, its use is recommended for anaesthesia of critically ill patients in which there is a risk of cardiac depression and hypotension.[56-58] However, stimulation of the cardiovascular system might be detrimental to patients with little cardiac functional reserve.[3] Some investigators have reported that in patients with shock, the induction of anaesthesia with ketamine can cause marked cardiovascular depression.[59] This likely results from the prior depletion of catecholamine stores in combination with a direct myocardial depressant effect of ketamine now coming to the foreground.[28]

Horses are known to maintain relatively normal haemodynamics when receiving ketamine in combination with xylazine[5] or guaiphenesin for short-term anaesthesia.[60] In halothane anaesthetised horses, incremental ketamine infusions have been demonstrated to improve haemodynamics, especially cardiac output.[6] Cardiac arrhythmias are uncommon in patients under ketamine anaesthesia.[61]

Following epidural administration, ketamine produced a slight but insignificant increase in heart rate, whereas other cardiovascular parameters remained within physiological limits.[12]

1.3.5. EFFECTS ON THE RESPIRATORY SYSTEM

Ketamine has minimal effects on the respiratory system. Following administration, respiration rate is usually maintained or mildly increased, while alveolar ventilation can be transiently decreased, depending on the dose administered.[26] Arterial blood gases are generally preserved when ketamine is used alone. However, by using adjuvant sedatives or anaesthetic drugs, respiratory depression and a subsequent slight increase in arterial carbon dioxide tension ($PaCO_2$) can occur. Ketamine does not affect the central respiratory drive as reflected by an unaltered response to carbon dioxide.[62] At higher dosages, ketamine may produce an apneustic and irregular pattern of breathing.[28]

Ketamine decreases airway resistance, which is probably a result of the sympathomimetic response to ketamine as well as a direct relaxant effect on bronchial smooth muscles.[63] In contrast, upper airway obstruction may occur following ketamine administration, since ketamine can increase salivation and secretion of respiratory tract mucus. Moreover, although pharyngeal and laryngeal reflexes remain relatively intact after ketamine administration, silent aspiration can occur during ketamine anaesthesia thus consequently increasing the risk of aspiration pneumonia.

In horses, the combination of xylazine and ketamine did not affect respiratory rate.[5,11] Only mild respiratory acidosis was observed which can probably be more attributed to ventilation-perfusion mismatch caused by lateral recumbency rather than by the depressant effect of ketamine on the respiratory system per se.[5] Similar results were observed in a study done by Taylor and Luna,[10] where continuous infusion of a detomidine-ketamine-guaiphenesin combination only slightly increased $PaCO_2$ without significantly changing respiratory rate and arterial oxygen tension (PaO_2).
Epidural administration of ketamine at 0.5 – 2.0 mg/kg did not affect respiratory rate and arterial blood gases in horses.[12]
The observed decrease in airway resistance in humans following ketamine administration is believed to occur in horses as well.[64]

1.4. PHARMACOKINETICS

Ketamine can be administered intravenously, intramuscularly, subcutaneously, orally or as in form of an epidural or spinal injection. In horses, the vast majority of clinical use involves intravenous and intramuscular routes, although intramuscular injection of ketamine can induce some tissue irritation since the pH of an aqueous solution of ketamine is 3.5 to 5.5.[16]
Following intravenous bolus injection, ketamine is rapidly distributed. Because of its small molecular weight, a pK_a near the physiological pH, rather low protein binding and high lipid solubility, ketamine rapidly crosses the blood-brain barrier and has an onset of action within 30 seconds following intravenous injection, with a maximal effect occurring in approximately one minute.[28] Termination of effect after a single bolus injection of ketamine is caused by rapid redistribution of the drug from highly vascularised organs (brain and blood) to less perfused tissues in the body.[65]
Ketamine is metabolised extensively by the hepatic cytochrome P_{450} system in all species including the horse. The major pathway involves N-demethylation to form norketamine, which is then hydroxylated and oxidated to form hydroxynorketamine and 5,6-dehydronorketamine, respectively. Subsequently, these products are conjugated to more water-soluble glucuronide derivates and excreted by the kidneys.[17]
The activity of the metabolites of ketamine has not been studied well, but norketamine has been shown to have analgesic properties, although significantly less than the parent compound.[66] Metabolites may also have some slight additive effect to the action of the parent drug.[19]

Ketamine has a wide therapeutic index, although repetitive administration of ketamine has been demonstrated to result in ketamine tolerance in several laboratory animals by inducing multiple forms of hepatic P_{450} enzymes.[67]
Hijazi et al.[68] found that pharmacokinetic variables of ketamine were greater in intensive care patients than in healthy volunteers and in surgical patients. The increase in the volume of distribution was greater than the increase in clearance, resulting in a longer estimated half-life of ketamine in the intensive care group.
The enantiomers of ketamine also differ in their pharmacokinetic profile. In animal laboratory studies, the therapeutic index (i.e. ratio of LD_{50} to ED_{50}) was found to be greater for S(+)-ketamine than for either R(-)-ketamine or the racemic mixture.[66] In humans, following ketamine infusion both higher plasma concentrations of R(-)-ketamine and R(-)-norketamine were found when compared to S(+)-ketamine and S(+)-norketamine, respectively.[66] Overall, plasma clearance of S(+)-ketamine was 16-35% greater than that of R(-)-ketamine.[66,69] This difference is thought to be due to the fact that R(-)-ketamine inhibits the elimination of S(+)-ketamine.[70] S(+)-ketamine is not inverted into R(-)-ketamine.[71] Edwards et al.[66] also found that uptake into most tissues and metabolism in some tissues is enantioselective, whereas Henthorn et al.[69] found no stereoselective influence on peripheral tissue distribution. Moreover, work with human liver microsomes indicated that the rate of N-demethylation was greater with S(+)-ketamine than with R(-)-ketamine or the racemic mixture.[66]
In equines, the pharmacokinetics of ketamine have been investigated after single bolus injection of anaesthetic dosages and following continuous rate infusions of subanaesthetic dose rates in conscious and anaesthetised horses. As in all other species studied, plasma ketamine concentrations in the horse follow a biexponential decline with a rapid initial distribution phase being followed by a slower elimination phase.[72,73] In xylazine premedicated horses, distribution and elimination half-lives measured 2.9 and 42 minutes, respectively, following a single bolus injection of 2.2 mg/kg ketamine.[72] In halothane anaesthetised horses, a similar distribution half-life was found following 2.2 mg/kg ketamine IV, whereas elimination half-life was considerably slower (65.8 ± 3.5 min).[73] Extending ketamine induction by simultaneous infusions of propofol and ketamine increased elimination half-life to 81.1 min.[74] In contrast, subanaesthetic continuous rate infusions in conscious horses revealed a longer distribution half-life and a shorter elimination half-life when compared to the previous described studies.[15] In horses, no differences in plasma concentrations of S(+)- and R(-)-ketamine have been found, whereas plasma concentrations of S(+)-norketamine were continuously

higher than R(-)-norketamine during target-controlled ketamine infusions.[8] Similar to the results found by Henthorn et al.,[69] no differences in tissue distribution of the individual enantiomers could be demonstrated in that study. Also, no stereoselective biotransformation of ketamine to norketamine could be demonstrated in horses.[75]

1.5. CLINICAL USE OF KETAMINE IN HORSES

Ketamine should not be used as a monoanaesthetic in horses, since its anaesthetic effect is often associated with poor muscle relaxation and hallucinatory behaviour.[4] Hence, preanaesthetic sedation is mandatory before ketamine is administered. Acepromazine premedication is inadequate prior to ketamine induction alone since it is not able to attenuate the ketamine-induced muscle rigidity.[4] (Fielding 2006). Most commonly, α_2-agonists, such as xylazine (1.0-1.1 mg/kg IV), detomidine (20 µg/kg IV) and romifidine (80-100 µg/kg IV) are used for this purpose. Administration of an α_2-agonist, followed in 5 to 10 minutes by a bolus injection of ketamine (2.2 - 3.0 mg/kg IV), induces a short period of safe and effective anaesthesia. Lateral recumbency is assumed in 1 to 3 minutes after ketamine injection and when no other anaesthetic is given, duration is about 10-15 minutes and recovery to standing requires 25-30 minutes.[5,76] Anaesthesia can be prolonged by redosing with one third to one half of the original dose of each drug.[4] Alternatively, following premedication, guaiphenesin (55 mg/kg IV) or benzodiazepines (diazepam 0.22 mg/kg IV; midazolam 0.1 mg/kg IV) can be given prior to the bolus injection of ketamine (2.2 mg/kg IV). This regimen improves muscle relaxation and shows less cardiovascular depression when compared to the combination of an α_2-agonist and ketamine only.[4,17,77] The use of benzodiazepines might be preferred to guaiphenesin, since benzodiazepines appear to be more effective in reducing hallucinatory behaviour.[17,77]

For total intravenous anaesthesia following ketamine induction, continuous infusion of the anaesthetic combination detomidine (0.02 mg/mL)-ketamine (2 mg/mL)-guaiphenesin (100 mg/mL) (Triple-drip infusion) at 1 mL/kg/h has been demonstrated to be safe and effective for use in horses.[9,10] Moreover, Mama et al.[11] showed that various infusions of xylazine and ketamine can be used for total intravenous anaesthesia in healthy adult horses.

Beside its use for induction and total intravenous anaesthesia in horses, ketamine is increasingly used during inhalation anaesthesia to reduce the concentration of inhalation anaesthetic required[6] and/or to provide additional analgesia.[8] Muir and

Sams[6] found that plasma ketamine concentrations over 1.0 μg/mL significantly reduced the minimal alveolar concentration (MAC) of halothane by a maximum of 37% in halothane anaesthetised healthy experimental horses. Knobloch et al.[8] showed a significant inhibition of the nociceptive withdrawal reflex under ketamine infusion during isoflurane anaesthesia.
Recently, Fielding et al.[15] studied the clinical and analgesic effects of subanaesthetic continuous rate infusions of ketamine in healthy conscious horses. Infusion rates up to 0.8 mg/kg/h did not cause any behavioural or clinical changes. However, for the infusion rates tested no analgesic effect could be demonstrated.
Ketamine has also been used for local analgesia in horses. Following epidural administration, ketamine dosages of 0.5 - 2.0 mg/kg were effective in producing analgesia of the tail, perineum and upper hind limb for 30 to 75 minutes, respectively.[12] In another study, an abaxial sesamoid block with ketamine ensured adequate analgesia for 15 minutes.[13]

2. POTENTIAL CELLULAR TARGETS FOR KETAMINE

Beside its anaesthetic and analgesic effects ketamine has demonstrated to possess anti-inflammatory properties. In several studies in rodents, ketamine suppressed the lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β) and interleukin-6 (IL-6) response both in vitro and in vivo.[78-86] In a human whole blood assay, the LPS-stimulated production of TNF-α, IL-6 and IL-8 significantly decreased in the presence of ketamine.[87-91] In clinical studies in humans, ketamine given before surgical procedures, such as hysterectomy and coronary artery bypass grafting, significantly reduced IL-6 serum concentrations.[92-94] In addition, in a model of Gram-negative bacterial sepsis in rats, ketamine significantly increased survival compared to rats treated with saline.[82]
In rats and humans, ketamine also attenuated LPS-stimulated neutrophil adhesion both in vitro and in vivo by reducing expression of adhesion molecules on neutrophils and endothelial cells.[89,95-99]
Ketamine also significantly inhibited hypotension and metabolic acidosis and improved survival in rats and dogs injected with LPS.[80,82,100]
Finally, ketamine may also affect inflammatory mediators like nitric oxide (NO), prostaglandin E_2 (PGE_2) and reactive oxygen species (ROS) (see respective subchapters).

2.1. LPS AND ITS SIGNALLING PATHWAYS

Bacterial LPS is a major component of the outer membrane of Gram-negative bacteria. LPS is comprised of three interconnected structures: an inner hydrophobic lipid A component, a core oligosaccharide structure and an outer O-specific polysaccharide chain.[101-103] The O-polysaccharide portion of the complex is composed of repeating polysaccharide units that extend into the environment of the bacterium and acts as an antigen for the production of antibodies.[103] This 'O-antigen' is highly variable among bacterial species, accounting for the serospecificity of Gram-negative bacterial species.[101,103] The core region is composed of a specific arrangement of monosaccharides and 3-deoxy-D-manno-octulosonic acid (KDO) residues`, which remains constant across groups of bacteria.[101-103] Lipid A consists of β(1-6)-linked disaccharides to which amide or ester-linked medium chain fatty acids are attached.[102,103] The lipid A component is very similar among bacterial species and is responsible for the endotoxic properties of the LPS molecule.[101-103]

2.2. LPS AND ITS RESPONSE IN EQUINES

LPS is released from Gram-negative bacteria during periods of bacterial multiplication or death. In healthy horses, large numbers of Gram-negative bacteria, and consequently large amounts of LPS, reside in the intestinal tract.[104] It has been estimated that at least 2.25 gram of free LPS exists in the caecum and ventral colon of healthy, adult horses.[105] Normally, movement of LPS into the systemic circulation is minimised through a highly efficient intestinal mucosal barrier, which is composed of tight junctions between individual epithelial cells, secretions from epithelial cells and resident bacterial populations. Small amounts of LPS may traverse the mucosal barrier, but most is rapidly cleared by Kupffer cells in the liver or neutralised by circulating LPS antibodies.[106] However, when intestinal blood flow is reduced, as may occur in horses suffering from gastrointestinal disorders, the integrity of the mucosal barrier may become impaired. Under these circumstances, LPS readily crosses the damaged mucosal barrier and enters the mesenteric venous system, lymphatic system or peritoneal cavity. When, in addition, the clearance capacity of hepatic Kupffer cells is exceeded, large amounts of LPS will enter the systemic circulation and trigger various cells to produce a plethora of inflammatory mediators, which subsequently induce a myriad of responses, a complex phenomenon generally known as endotoxaemia. The clinical effects associated with equine endotoxaemia include fever, tachycardia, hypotension, hyperpnoea, leukopenia followed by leukocytosis,

metabolic acidosis, hyperglycaemia followed by hypoglycaemia, abdominal discomfort, haemoconcentration, increased vascular permeability, disseminated intravascular coagulation (DIC) and, ultimately, multiple organ dysfunction and death.[106,107]

Besides gastrointestinal disorders, equine endotoxaemia may develop from other Gram-negative infections, such as pleuritis, pneumonia, peritonitis or endometritis.[108]

2.3. MOLECULAR MECHANISMS OF LPS-INDUCED SIGNAL TRANSDUCTION PATHWAYS

2.3.1. LPS-BINDING PROTEIN AND CD14

Once in circulation, LPS forms a high affinity complex with LPS binding protein (LBP).[109-111] This glycoprotein is normally present in plasma at trace concentrations, but its concentration rapidly increases during the acute phase response.[112] The role of LBP is to transfer LPS to the cell surface of monocytes, macrophages and neutrophils where it binds to cluster of differentiation 14 (CD14).[109-112] This receptor exists as a membrane bound form (mCD14) on myeloid cells and a soluble form (sCD14) in plasma. Soluble CD14 functions to enhance LPS responses in cells that do not ordinarily express CD14 such as endothelial and epithelial cells.[110-111] Although CD14 was initially identified as a LPS receptor,[109] it lacks transmembrane and intracellular domains and thus cannot initiate signal transduction by itself.[110-113] However, CD14 plays a pivotal role in LPS-induced signal transduction since blockade of CD14 with monoclonal antibodies prevented cytokine synthesis in whole blood following LPS exposure. Moreover, formation of the CD14/LPS complex significantly reduces the concentration of LPS required for activation of macrophages when compared to unbound LPS alone.[110,111] So far, the role of CD14 in LPS-induced signal transduction appears to be that of binding LPS and presenting it to the LPS receptor complex.[111-113]

2.3.2. LPS RECEPTOR COMPLEX

The LPS receptor complex is composed of Toll-like receptor 4 (TLR4) and myeloid differentiation protein-2 (MD-2).[111,112,114,115] Toll-like receptors (TLRs) are the mammalian homologues of a plasma membrane receptor in Drosophila named Toll.[116,117] TLRs are predominantly expressed by immune cells including monocytes, macrophages and neutrophils.[116,118] TLRs are categorised as pattern recognition receptors (PRRs) as they recognise pathogen associated molecular patterns (PAMPs).[111,119] In mammalian species, at least 11 members of TLRs (TLR1-

TLR11) have been identified. All TLR subtypes contain an extracellular leucine-rich repeat (LRR) motif, a single transmembrane region and an intracellular Toll/interleukin-1 (TIR) domain.[111,112,117] The LRR motifs form a horseshoe structure believed to be directly involved in the recognition of PAMPs.[117] The cytoplasmatic TIR domain is essential for the assembly of downstream signal transduction pathways.[117,120] Different microbial pathogens activate different TLR subtypes. TLR4 is generally considered to be the predominant signal-transducing receptor for LPS, although TLR4 has been demonstrated to be activated by other mediators such as fibronectin and heat shock proteins.[111,116,117]

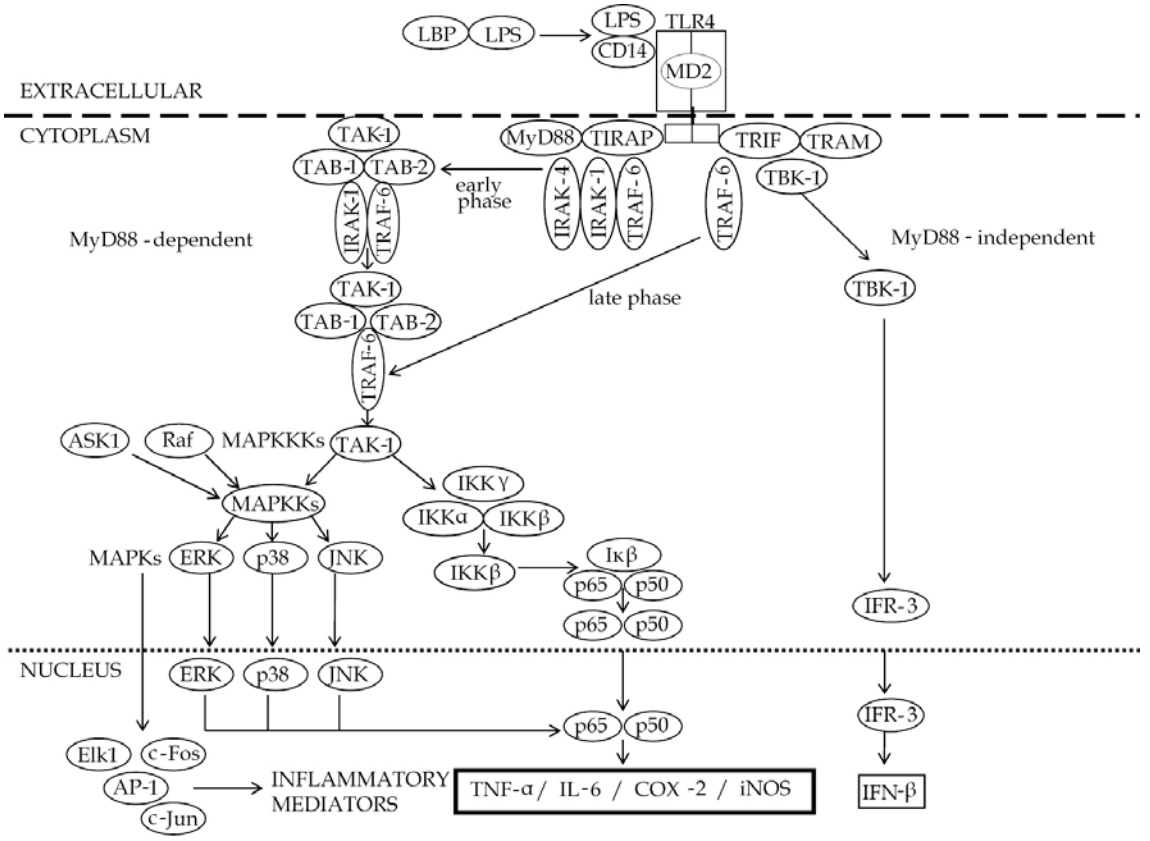
However, TLR4 alone is not able to recognise LPS and induce signalling, but is highly dependent on the presence of the extracellular glycoprotein MD-2.[121,122] MD-2 binds to the lipid A moiety of LPS and the extracellular LRR-domain of TLR4 and thus enables LPS ligand recognition by TLR4.[123] For an efficient LPS response, a physical association between MD-2 and TLR4 is crucial.[124] After ligand binding, TLR4 dimerise and undergo the conformational change required for the recruitment of downstream signalling molecules.[117]

To date, there are no reports on the influence of ketamine on the LPS-activated CD14 protein or LPS receptor complex.

In agreement with previously published reports in rodents, all three components of the LPS receptor complex (i.e., CD14, MD-2 and TLR4) have been identified in horses.[125-127]

2.3.4. DOWNSTREAM SIGNAL TRANSDUCTION PATHWAYS OF TLR4

The TLR4-mediated signal transduction cascade in response to LPS can be divided into two synergistically acting pathways: a myeloid differentiation factor 88 (MyD88)-dependent pathway that leads to an early activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), and a MyD88-independent pathway associated with a delayed activation of NF-κB and MAPK (Fig. 2).[111,112,116,117,128]


LBP
LPS
LPS
CD14
TLR4
MD2
EXTRACELLULAR
CYTOPLASM
TAK-1
TAB-1
TAB-2
IRAK-1
TRAF-6
MyD88 - dependent
MyD88
TIRAP
TRIF
TRAM
TBK-1
early phase
IRAK-4
IRAK-1
TRAF- 6
TRAF-6
MyD88 - independent
late phase
TBK-1
TAK-1
TAB-1
TAB-2
TRAF-6
ASK1
Raf
MAPKKKs
TAK-1
IKKγ
MAPKKs
IKKα
IKKβ
MAPKs
ERK
p38
JNK
IKKβ
Iκβ
p65
p50
p65
p50
IFR-3
NUCLEUS
ERK
p38
JNK
p65
p50
IFR-3
Elk1
c-Fos
AP-1
c-Jun
INFLAMMATORY MEDIATORS
TNF-α / IL-6 / COX -2 / iNOS
IFN-β

In the MyD88-dependent pathway, the adaptor molecules MyD88 and TIR domain-containing adaptor protein (TIRAP) are recruited to the activated receptor complex and associated with interleukin-1 receptor-associated protein kinase 1 (IRAK-1), IRAK-4 and tumour necrosis factor receptor-associated factor 6 (TRAF-6). Following activation, IRAK-1 and TRAF-6 dissociate from this complex and associate with another complex at the plasma membrane composed of transforming growth factor-β-activated kinase 1 (TAK-1) and two TAK-1 binding proteins, TAB-1 and TAB-2. Then IRAK-1 is degraded and the remaining complex, consisting of TRAF-6, TAK-1, TAB-1 and TAB-2, translocates to the cytosol, where ubiquitination of TRAF-6 induces the activation of TAK-1. TAK-1, in turn, phosphorylates mitogen-activated kinase kinases (MAPKKs) and the inhibitors of κB (IκB) kinase (IKK) complex. Stimulated MAPKK activates the transcription factor activating protein 1 (AP-1) through activation of the mitogen activated kinases (MAPKs), c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase p38 (p38). Activation of IKK leads to phosphorylation of inhibitor of kappa B (IκB) and subsequent activation of the transcription factor NF-κB (described below).[111,112,116,117,128]

In the MyD88-independent signal transduction pathway, the adaptor molecules TIR-domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited to the stimulated LPS receptor complex and associated with TRAF-6 and TRAF-family-member-associated NF-κB activator-binding kinase 1 (TBK-1). Activation of TRAF-6 mediates NF-κB activation, whereas stimulation of TBK-1 leads to phosphorylation of the transcription factor interferon-regulatory factor 3 (IRF-3) and the subsequent expression of interferon (IFN)-inducible genes through the Janus activated kinase (JAK) – signal transducer and activator of transcription (STAT) signalling pathway.[111,112,116,117,128]

2.4. MAPKs

MAPKs are a highly conserved family of serine/threonine/tyrosine kinases which comprise three major subfamilies: extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase p38 (p38) and c-Jun N-terminal kinase (JNK).[129] The basic MAPK signalling cascade consists of three sequentially acting protein kinases, i.e. MAPK kinase kinase (MAPKKK, also called MKKK or MAP3K), MAPK kinase (MAPKK, also called MKK or MAP2K) and the specific MAPK. Activation of MAPK pathways is spatially regulated, i.e. activation of one MAPK

does not normally lead to the activation of other MAPKs.[129] However, since each individual MAPK cascade shares a number of upstream and downstream kinases, complex cross-talk and signal convergence occur among MAPK family members.[130] The vast majority of defined substrates for MAPK are transcription factors, among which are AP-1, Elk1, c-Fos, c-Jun and NF-κB.[129,131] However, MAPKs have the ability to activate many other substrates including other protein kinases, phospholipases and cytoskeletal-associated proteins.[129] The fact that MAPKs are able to phosphorylate many different substrates indicates that MAPKs affect many different biological functions.

2.4.1. ERK

The ERK subfamily is made up of 5 isoforms (ERK1-5), of which ERK1 (44 kDa) and ERK2 (42 kDa), commonly referred to as ERK1/2, are most extensively studied. ERK 1/2 are widely expressed in mammalian cells and primarily involved in proliferation, transformation and differentiation.[130,132] Hence, ERK activity is predominantly induced by growth-promoting mitogenic stimuli. However, there is increasing evidence that ERK activation plays a crucial role in LPS- and cytokine-induced inflammatory responses, since ERK has been reported to act as an important regulator of NF-κB activation and NF-κB-dependent gene expression in response to LPS and IL-1.[131,133] Moreover, ERK has demonstrated to be involved in nuclear export of LPS-induced TNF-α mRNA.[134]

In general, ERK activation is initiated by phosphorylation and activation of Raf (MAPKKK) and MEK1/2 (MAPKK),[129,130,135] even though involvement of other members of the MAPKKK family, such as ASK1, Tpl2 and MEKK3 has been described (Fig. 2).[129,134,136]

PD098059 and U0126 have demonstrated to be effective inhibitors of ERK activation. Both compounds do not directly inhibit ERK activation, but interfere with upstream protein kinases.[134]

2.4.2. MAPK P38

The mammalian MAPK p38 subfamily consists of at least four different isoforms, p38α, p38β, p38γ and p38δ.[129] Two isoforms, p38α and p38β, are ubiquitously expressed. In most inflammatory cells, p38α (38 kDa) is the major isoform of p38 MAPK. Expression of p38γ is largely restricted to skeletal muscle, whereas p38δ gene expression is found in the lung, kidney, testes, pancreas and small intestine.[137,138]

MAPK p38 is activated by many stimuli including LPS, pro-inflammatory cytokines (e.g. TNF-α, IL-1), hormones and stresses such as osmotic shock and heat shock.[130,139] Upon stimulation, TAK-1, MAPKKK4 and apoptosis signal-regulating kinase 1 (ASK1) are described as the proximal upstream MAPKKKs that initiate the p38 pathway, whereas MAPKK3, MAPKK4 and MAPKK6 are the immediate upstream MAPKKs to activate p38 (Fig. 2).[136-138]
The main biological response of p38 activation involves the production and activation of inflammatory mediators, such as TNF-α, IL-1, IL-6, IL-8 and COX-2, to initiate leukocyte recruitment and activation. MAPK p38 can regulate expression of pro-inflammatory cytokines at both transcriptional and post-transcriptional level.[134,137,138]
SB203580 is widely used as the specific signal transduction inhibitor of both p38α and p38β. SB203580 potently inhibits the production of pro-inflammatory cytokines such as TNF-α and IL-1 in vitro and in vivo.[137-139] Moreover, administration of SB203580 has beneficial effects in animal disease models such as collagen-induced arthritis and endotoxin-induced septic shock.[138]

2.4.3. JNK

JNK consists of three isoforms (JNK1-3), of which JNK1 (55 kDa) and JNK2 (46 kDa) are widely distributed, whereas JNK3 is mainly located in neuronal tissue. JNK is activated in response to an array of stimuli including LPS, pro-inflammatory cytokines (e.g., TNF-α, IL-1), osmotic stress and ultraviolet irradiation.[130] Upon exposure, JNKs are phosphorylated and activated by the MAPKKs MAPKK4 and MAPKK7, which are activated by 13 distinct upstream MAPKKKs including TAK1, Tpl2, and ASK1 (Fig. 2).[129,136,139] This diversity of MAPKKKs allows a wide range of stimuli to activate the JNK pathway.[132]
Gene disruption experiments in mice have demonstrated that JNK is essential for c-Jun phosphorylation and the subsequent activation of the transcription factor AP-1. JNKs may also enhance the activity of other transcription factors including Elk-1 and NF-κB.[131,139] Moreover, JNKs are required for some forms of stress-induced apoptosis and the regulation of inflammatory genes including cytokines, growth factors, cell surface receptors and adhesion molecules.[139]
The selective inhibitor, SP600125, has been a useful tool in assessing the role of JNK in various disease models in rodents and humans.[139]

2.4.4. MAPKs: THE ROLE OF KETAMINE

Only few reports describe the influence of ketamine on MAPK phosphorylations. Tian et al.[140] and Miao et al.[141] found that pretreatment with ketamine did not significantly influence JNK1/2 and JNK3 activation following global ischemia in the hippocampus of the rat. Boulom et al.[142] demonstrated that ketamine per se induced MAPK activation and expression of ERK2 in human aortic smooth muscle cells.

2.4.5. MAPKs IN EQUINES

Both ERK1 and ERK2 have been identified in different maturation states of equine oocytes,[143,144] while existence of JNK and p38 isoforms is only described in an equine macrophage cell line.[145]

2.5. NF-κB

NF-κB was first described in 1986 as a nuclear factor necessary for immunoglobulin κ light chain transcription in B cells,[146,147] but is now known to exist in virtually all cell types. NF-κB is the key regulatory transcription factor which mediates the transcription of an exceptionally large number of genes including those involved in immune and inflammatory responses.[131,148,149]

Five distinct NF-κB subunits have been identified and cloned in mammalian cells. These include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB and c-Rel.[131] NF-κB1 and NF-κB2 undergo proteolytic processing to liberate p50 and p52, respectively. NF-κB/Rel proteins can exist as distinct homo- or heterodimers, although the most predominant form of NF-κB is the p50/p65 heterodimer. NF-κB/Rel proteins share a highly conserved 300 amino acid long Rel homology domain (RHD), responsible for dimerisation, DNA binding and association with their inhibitory cytoplasmic proteins, termed inhibitor of κB (IκB). Moreover, RHD contains a nuclear localisation sequence (NLS), which promotes NF-κB translocation to the nucleus.[119,131,150,151]

LPS-induced NF-κB activity is biphasic: an early phase occurring at 0.5-2 hours, and a late phase occurring at 8-12 hours post stimulation. LPS and other early released inflammatory mediators like platelet-activating factor (PAF) cause the early phase NF-κB activation, whereas TNF-α and IL-1β mediate the late phase activation.[152,153]

2.5.1. THE INHIBITORY PROTEIN, IκB

In resting cells, NF-κB/Rel dimers are mainly sequestered in the cytoplasm in an inactive form by association with IκB.[119,131,151] The IκB family contains nine known mammalian members, IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3, p105, p105 and molecule possessing ankyrin-repeats induced by lipopolysaccharide (MAIL), among which IκBα is the most abundant.[119,131,154] A common structure for all IκB proteins is the ankyrin repeat domain (ARD), which mediates IκB binding to the NF-κB dimers, masking the NLS on NF-κB proteins.[119,131,151] IκB proteins are different in their structure, preference for binding of NF-κB dimers, biological functions and modes of activation. There are also differences in the mechanism of regulating IκB gene expression. IκBα, IκBβ and IκBε are constitutively expressed, but IκBζ and MAIL are induced by LPS and pro-inflammatory cytokines.[131] Contrasting sharply with the function of other IκBs, MAIL serves as a transcriptional enhancer, enhancing LPS-induced IL-6 expression by over 20-fold. IκBα, IκBζ and MAIL are NF-κB regulated genes. NF-κB activation increases these IκBs, which in turn inhibits NF-κB activation. IκBs inhibit NF-κB activation through three mechanisms: by sequestrating NF-κB dimers in the cytoplasm, facilitating dissociation of DNA-bound NF-κB dimers from their DNA binding sites and exporting NF-κB dimers from the nucleus.[131]

2.5.2. CYTOPLASMIC NF-κB ACTIVATION: CANONICAL AND NON-CANONICAL PATHWAYS

NF-κB is known to be activated by a plethora of activators, including bacteria and their products, pro-inflammatory cytokines and different forms of stress.[131,149] The biological events underlying NF-κB activation can be divided into two phases: a cytoplasmic and a nuclear phase.[131,151,154,155]

The signal transduction pathways leading to cytoplasmic activation of NF-κB are multiple and complex, but have in common that they all lead to the generation of DNA-binding dimers. To date, three major pathways mediating NF-κB activation have been identified: canonical (classical), non-canonical (alternative) and atypical pathways.[119,131,151,154-156] Canonical pathways are stimulated by inflammatory mediators (e.g. LPS, TNF-α, IL-1), which rapidly induce NF-κB activation. In contrast, non-canonical pathways are preferentially induced by B cell activation signals (e.g., lymphokine-β, B-cell activating factor (BAFF)) and mediate a delayed but sustained activation of primarily RelB-containing NF-κB dimers (Fig. 2). The atypical pathway is triggered by DNA damage such as UV irradiation.[119,131,151,154-156]

However, both the non-canonical and atypical pathways will not be further described as they are, based on their inducers, beyond the scope of this thesis.
Activation of NF-κB via the canonical pathway is largely dependent on the β subunit of IKK.[119,131,155,156] IKK is a large protein complex composed of three subunits: IKKα, IKKβ and IKKγ (also called NF-κB essential modulator (NEMO) or IKK associated protein (IKKAP)). IKKα and IKKβ are catalytic subunits, with IKKβ being far more potent in mediating NF-κB than IKKα. Non-catalytic IKKγ functions as a regulatory subunit by associating IKKα/IKKβ dimers to upstream signalling molecules. Upon complex assembly, activated IKKβ rapidly mediates phosphorylation, ubiquitination and degradation of IκB proteins, especially IκBα. Degraded IκBα releases NF-κB and exposes NLS on NF-κB dimers, consequently allowing translocation of NF-κB to the nucleus.[119,131,155,156]

2.5.3. NUCLEAR NF-κB ACTIVATION: ADDITIONAL REGULATORY MECHANISMS

In addition to nuclear translocation, NF-κB activity is controlled by additional regulatory mechanisms.[151,154,155,157,158] These include regulation of nuclear import and export of NF-κB dimers, regulation of the recruitment of NF-κB dimers to the promotor or enhancer sites of NF-κB target genes, regulation of NF-κB transcriptional activity after recruitment and positive or negative feedback mechanisms. Recruitment and transcriptional activity are mainly regulated by posttranslational modifications of NF-κB proteins. Particularly p65 is subjected to a variety of these modifications, including phosphorylation, acetylation, S-nitrosylation and S-glutathionylation.[151,154,155,157,158] It appears that these nuclear events are mainly of relevance to determine strength and duration of the NF-κB transcriptional response.[151]
Upon nuclear activation, NF-κB can mediate transcription of numerous inflammatory genes, which play important roles in the development of endotoxaemia. These genes include cytokines (e.g. TNF-α, IL-1β, IL-6), chemokines, immunoreceptors, inducible enzymes (e.g., inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)), adhesion molecules and acute-phase proteins.[131,149]

2.5.4. NF-κB: THE ROLE OF KETAMINE

There are a few reports on the influence of ketamine on IκB and NF-κB activity. In LPS-treated rat mononuclear cells, Yu et al.[159] showed a reduced expression of IκBα, which markedly increased following exposure to LPS and ketamine at

different concentrations (10-5000 μM). In addition, Shen et al.[160] demonstrated that pretreatment with ketamine (50 mg/kg) significantly blocked ischemia-induced decrement of IκB-α in the hippocampus of the rat. Regarding NF-κB, ketamine reduced LPS-induced NF-κB activation in human glioma cells,[161] in rat mononuclear cells[159] and in rat monocytes[162] in vitro. In vivo, ketamine suppressed NF-κB activity in the intestine, liver and lung of endotoxin-challenged rats,[163] in mouse brain cells[161] and in the hippocampus of the rat following global ischemia.[160]

2.5.5. NF-κB IN EQUINES

In only one study in horses, NF-κB activity has been described. Janicke et al.[164] identified low levels of constitutive NF-κB activity in the nucleus of equine vascular smooth muscle cells and reported on translocation of an additional NF-κB protein 2 hours after stimulation with LPS and interferon-gamma (IFN-γ). Simultaneously, increased expression of COX-2 and limited expression of iNOS was found.

2.6. CYTOKINES

Cytokines are largely inducible proteins with a molecular mass > 5 kDa, which can be secreted by any cell in the body, with the possible exception of erythrocytes, and which can bind to and activate a range of cells. Cytokines highly participate in both homeostatic regulation and pathogenesis of numerous diseases. LPS is able to induce most cells to synthesise a range of cytokines.[102]

Cytokines generally have no enzymatic activity and act at local level. They induce their biological effects only when they bind to their specific receptors on the surface of target cells, inducing specific intracellular signal transduction pathways, and eventually resulting in the switching on of particular sets of genes, including adhesion receptors, acute-phase proteins, lipid-metabolising enzymes, nitric oxide synthase (NOS) and cytokines.[102] The affinity of cytokine receptors for their ligands is extremely high and most cells have only very small numbers of receptors. Furthermore, only a few receptors need to be occupied to produce a maximal response.[102] Thus, very low levels of cytokines can induce biological effects in vivo. However, exaggerated production of cytokines in vivo may cause profound systemic alterations with potentially life-threatening consequences.[102,165]

Generally, cytokines are divided into pro-inflammatory (e.g., TNF-α, IL-1, IL-2, IL-3, IL-5, IL-6, IL-9, IL-11, chemokines, IFNs, platelet-derived growth factor (PDGF)) and anti-inflammatory cytokines (e.g., IL-1 receptor antagonist (IL-1ra), IL-4, IL-10,

IL-13, transforming growth factor β (TGF-β)).[102,166] In the scope of this thesis only TNF-α and IL-6 will be described.

2.6.1. TNF-α

TNF-α, also known as cachexin, consists of two forms: a soluble form (17 kDa) and its membrane bound precursor (26 kDa). The trimeric form of soluble TNF-α is mainly responsible for bioactivity.[165,167]

TNF-α is predominantly produced in mononuclear phagocytes, although it is also produced in numerous other cell types.[167] Upon stimulation by a variety of stimuli (e.g., LPS, synthetic lipid A, IL-1, TNF, leukotrienes, nitric oxide (NO), reactive oxygen species (ROS), parasites, viruses), transcription and translation of the TNF-α gene is rapidly upregulated, enabling the release of large quantities of soluble TNF-α.[167] Expression of TNF-α is mainly regulated by MAPKs and the transcription factors NF-κB and AP-1,[131,134,137] although many other factors at distinct levels have also been demonstrated to be involved in TNF-α expression.[167]

The activities of TNF-α are mediated through at least two types of membrane-associated receptors called type I (TNFR1) and type II (TNFR2) receptors. Each of the TNF receptors is present on virtually all cells, except erythrocytes.[167] TNFR1 is responsible for the major TNF-α bioactivity, including cytotoxicity and activation of neutrophils and endothelial cells, whereas TNFR2 alone is not sufficient to stimulate these functions. However, in combination with TNFR1, TNR2 has been demonstrated to potentiate the effects of TNFR1.[167] Both types of receptors can be released from the membrane and appear in the circulation. These soluble receptors (sTNFRs) bind to TNF-α and function as TNF-binding proteins that neutralise TNF-α activity. However, low amounts of sTNFRs may augment the long-term effects of TNF-α by providing a reservoir of bioactive TNF-α that is slowly released.[167]

Three lines of evidence indicate that TNF-α is the primary mediator of endotoxaemia. First, increased concentrations of TNF-α have been detected in the circulation of endotoxic animals and humans. The kinetic profile of TNF-α is extremely rapid. Following induction of endotoxaemia, plasma concentrations of TNF-α rapidly increase, peak between 90 to 120 minutes and become undetectable within 4 to 6 hours.[165,167,168] Clearance from the circulation is mainly caused by binding to TNF-α receptors and soluble TNF-α binding proteins, following which the whole complex is excreted by the kidneys.[167] Second, systemic administration of purified recombinant TNF-α fully mimics the pathophysiological changes of

LPS-induced endotoxaemia like neutrophil activation, adherence of activated neutrophils to vascular endothelium, upregulation of pro-coagulant factors, downregulation of anti-coagulant factors and phospholipase A_2 activation.[168] Apart from direct effects, the biological effects of TNF-α are largely mediated indirectly by initiating the release of other pro-inflammatory mediators, such as IL-1, IL-6, IFN-γ, prostanoids, leukotrienes, PAF, NO and ROS. Ultimately, this plethora of mediator-induced effects will lead to endotoxaemia which is clinically characterized by fever, hypotension, haemoconcentration, metabolic acidosis, hyperglycaemia followed by hypoglycaemia, diffuse capillary leakage, DIC, hemorrhagic necrosis of tissues, multiple organ failure and death.[167,168] The third and most convincing evidence for the role of TNF-α in endotoxaemia is derived from studies in which monoclonal antibodies directed against TNF-α prevented most of the injurious effects of endotoxaemia.[165,167]

2.6.1.1. TNF-α: ROLE OF KETAMINE

In many experiments with LPS-challenged rodents[78,80-83,86] and humans,[87-91] ketamine significantly reduced TNF-α production in a dose-dependent manner both in vitro and in vivo. No conflicting reports have been found.

2.6.1.2. TNF-α IN EQUINES

In vitro, TNF-α concentration significantly increased in LPS-treated equine peritoneal macrophages when compared to non-stimulated cells. Among the LPS doses tested (0.5 ng/mL, 5 ng/mL and 5 μg/mL), no differences in TNF-α concentrations were found.[169] In another in vitro study, Barton et al.[170] found that LPS-treated peritoneal macrophages obtained from healthy horses and horses suffering from acute gastrointestinal disease both showed a significant increase in TNF-α activity compared to non-treated macrophages, although LPS-induced TNF-α activity was significantly less for macrophages from horses with acute gastrointestinal disease. These results suggest that these macrophages may exhibit early endotoxin tolerance.[170]

In experiments with LPS-challenged horses, plasma TNF-α concentrations significantly increased following bolus injection of 5 μg/kg LPS and low dose infusions of 0.03 μg/kg LPS for 1-4 hours.[171,172] The kinetic profile of equine TNF-α was extremely consistent with those found in other species and independent of the route of LPS administration (i.e., intraperitoneally or intravenously) and the way of LPS administration (i.e., bolus injection or slow infusion).[165,171,172] In response to

LPS, horses seemed depressed, had signs of mild to moderate abdominal pain, developed tachycardia and fever and had leukopenia followed by leukocytosis. Moreover, these symptoms were positively correlated with TNF-α activity.[171,172]
In horses suffering from gastrointestinal disorders, both serum and peritoneal fluid TNF-α activity were significantly higher compared to healthy horses.[173-175] In horses with gastrointestinal disorders, peritoneal fluid TNF-α activity was significantly greater than serum TNF-α activity.[175] Mortality and marked increase in serum TNF-α activity were greater in horses with intestinal inflammatory disorders or strangulating intestinal obstruction than in horses with non-strangulating intestinal obstruction. Serum TNF-α activity and mortality were positively correlated.[173]
Similar results were found in foals, in which serum TNF-α activity was highly correlated with clinical criteria of sepsis. Moreover, an association was apparent between disease severity and serum TNF-α activity.[176]
MacKay[177] found that TNF-α associated cytotoxicity was significantly higher in sera of equine patients suffering from any naturally acquired disease than in sera of healthy horses. Sera from horses with either peritoneal leakage of gastrointestinal tract contents or any bacterial infection were significantly more cytotoxic than sera from horses without these clinical factors. Horses that showed the highest serum TNF-α cytotoxicity were all suffering from gastrointestinal disorders and had a clinical profile suggestive of endotoxaemia.
In vitro, TNF-α antibodies significantly inhibited TNF-α activity in equine peritoneal macrophages.[178] In experiments with LPS-challenged horses, administration of TNF-α antibodies significantly reduced serum TNF-α activity,[179] TNF-α-induced cytotoxicity[172] and the haematological and clinical responses associated with LPS.[179]

2.6.2. IL-6

Interleukin-6 (IL-6), formerly known as interferon-β2, B-cell stimulatory factor-2, hybridoma plasmacytoma growth factor, 26 kDa protein or hepatocyte-stimulatory factor, is a multifunctional cytokine which consists of two N-glycosylation sites that are variably glycosylated. As a result, IL-6 is secreted as a heterogeneous set of proteins with molecular masses ranging from 19-30 kDa.[167,180] Only unglycosylated IL-6 is biologically active.[180]
Under physiological conditions, IL-6 mRNA is constitutively expressed at low levels in several tissues and cell types, including leukocytes, spleen, liver, kidney

and intestines.[167] However, during inflammation, trauma or immunologic challenge, IL-6 is synthesised in nearly all tissues and cell types, with endothelial cells, fibroblasts and mononuclear phagocytes being its major producers.[167,180] Upon stimulation, expression of IL-6 mRNA is mainly regulated by the JAK-STAT pathway and the nuclear factor for IL-6 expression (NF-IL-6).[167,181,182]

The biological effects of IL-6 are mediated through interaction with the IL-6 receptor complex on target cells. IL-6 first binds to a low-affinity ligand-binding subunit which is stabilised and activated when bound by an intracellular signal transducing subunit.[181,183] The kinetic clearance of synthesised IL-6 is biphasic, consisting of a rapid initial elimination ($t_{½}$ ~3 min) and a slower second elimination ($t_{½}$ ~55 min).[180]

IL-6 exerts numerous biological effects. As the major stimulator and regulator of the acute phase response and following tissue trauma and infection, IL-6 induces the biosynthesis of the full spectrum of acute phase proteins in hepatocytes. In contrast, TNF-α and IL-1 stimulate the generation of only a limited subset of acute phase proteins.[167,180] IL-6 also plays an essential role in the functioning of the immune system by stimulating humoral as well as cellular defence mechanisms. Moreover, IL-6 regulates growth and differentiation of various cell types, stimulates haematopoiesis and induces fever through a PGE_2-dependent mechanism.[167,180] Ultimately, the biologic role of IL-6 depends on the level in serum and tissue compartments. Relatively low levels may be beneficial as they stimulate repair mechanisms, including the production of anti-inflammatory cytokines, whereas very high levels accelerate inflammatory disorders and organ dysfunction.[167,180]

Evidence indicates that IL-6 is highly involved in the pathogenesis of endotoxaemia and other inflammatory diseases.[183,184] In experimental studies in humans and rodents, intravenous injection of LPS or TNF-α resulted in a sharp increase in serum IL-6 concentrations,[167] whereas the administration of anti-TNF-α antibodies considerably reduced LPS-induced IL-6 production.[185]

Although generally known as a pro-inflammatory cytokine, IL-6 also exerts anti-inflammatory activities by suppressing LPS-induced synthesis of TNF-α and counteracting the effects of TNF-α and IL-1.[167,186] According to Tilg et al.,[187] these anti-inflammatory properties might be due to the induction of IL-1ra and the release of sTNFR. In contrast to these anti-inflammatory properties, high concentrations of IL-6 have been associated with fatal outcome in humans, making it a useful marker of the pro-inflammatory response.[188,189]

2.6.2.1. IL-6: ROLE OF KETAMINE

In LPS-treated rodents, ketamine significantly inhibited LPS-induced IL-6 production in a dose-dependent manner.[80-83,86] Similar results were found in human whole blood assays, in which LPS-induced IL-6 production decreased in the presence of ketamine.[88,91] Moreover, ketamine attenuated the IL-6 response after various surgical interventions in humans.[92,93]

2.6.2.2. IL-6 IN EQUINES

In vitro, IL-6 activity significantly increased in LPS-treated equine peritoneal macrophages when compared to non-stimulated cells. IL-6 activity peaked at 6 or 12 hours after exposure to 5 or 500 ng/mL and 0.5 ng/mL endotoxin, respectively, and remained high through 24 hours of incubation.[190]

In experiments with LPS-challenged horses, plasma IL-6 concentrations significantly increased following low dose infusions of 0.03-1 μg/kg LPS over 1 hour.[191,192] Depending on the endotoxin concentration infused, serum IL-6 activity was significantly increased above baseline from 2 to 12 hours following the start of infusion. Peak plasma IL-6 activity was observed between 3 and 4 hours after LPS stimulation. The IL-6 response of endotoxin-treated horses started 1 hour after TNF-α appeared in the circulation. In response to LPS, horses became lethargic, had signs of mild to moderate abdominal pain, developed tachycardia and fever and had leukopenia followed by leukocytosis. Significant positive association and linear correlation was only apparent between serum IL-6 activity and rectal temperature.[191]

In horses suffering from gastrointestinal disorders, both serum and peritoneal fluid IL-6 activity were significantly higher when compared to healthy horses.[174,175] IL-6 concentrations were significantly positively correlated with LPS concentrations.[174] In horses with gastrointestinal disorders, peritoneal fluid IL-6 activity was significantly greater than serum IL-6 activity.[175] Blood and peritoneal fluid IL-6 activity was significantly higher in horses with inflammatory or strangulating lesions than in horses with non-inflammatory or non-strangulating lesions. Diagnostic accuracy for non-survival was greatest (80%) when blood IL-6 activity exceeded 60 U/mL.[175]

In experiments with LPS-challenged horses, administration of TNF-α antibody significantly reduced serum IL-6 activity as well as the haematological and clinical responses associated with circulating LPS.[179]

2.7. iNOS

In mammals, three distinct isoforms of nitric oxide synthase (NOS) exist: endothelial NOS (eNOS, NOS I; 135 kDa), neuronal NOS (nNOS, NOS III; 160 kDa) and inducible NOS (iNOS, NOS II; 130 kDa).[193] In general, nNOS and iNOS are soluble, whereas eNOS is membrane bound,[193] although all three can have either cytosolic or membrane bound locations.[194] Recently, the existence of a fourth NOS enzyme, mitochondrial NOS (mtNOS) was reported in rat and mouse.[195] However, it has yet to be determined that this NOS protein is specifically associated with mitochondria, since mtNOS seems more likely to be a membrane-associated iNOS.[194]

The three main isoforms share structural similarities and have nearly identical catalytic mechanisms.[193,194,196,197] Beside the cosubstrates nicotinamide adenine dinucleotide phosphate (NADPH) and O_2, they all require a number of cofactors for activity including haem, tetrahydrobiopterin (H_4B), calmodulin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).

NOS catalyse the biosynthesis of nitric oxide (NO) by a two step oxidative conversion of L-arginine to NO and L-citrulline via the more labile intermediate N-hydroxy-L-arginine, utilising NADPH as an electron donor.[194,197-199] The functional NOS protein is a homodimer, with each subunit comprising of a reductase domain at the C-terminus, an oxygenase domain at the N-terminus and a calmodulin-binding domain in their midregion.[194] The reductase domain contains the FAD and FMN moieties and share extensive homology with cytochrome P_{450} reductase. This domain transfers electrons from NADPH to the oxygenase domain. The oxygenase domain actually catalyses the conversion of arginine into citrulline and NO and contains the binding site for haem, H_4B and arginine. The calmodulin binding domain plays a pivotal role in activation of the different isoforms.[194,197]

Commonly, two isoforms, nNOS and eNOS are constitutively expressed, whereas iNOS is expressed upon cell activation.[193,200,201] However, recent experimental studies demonstrated that all three can be constitutively expressed in some cells or tissues, and all three can be induced, albeit by different stimuli.[194,202] Activation of nNOS and eNOS is calcium-dependent. In response to increases in intracellular calcium concentrations, calcium binds to calmodulin after which the calcium-calmodulin complex binds to the calmodulin binding domain and activates NOS. In contrast, activation of iNOS is independent of intracellular calcium concentrations, as calmodulin is prebound to the molecule even at very low calcium concentrations.[193,194,201,203] As nNOS and eNOS activity is triggered by calcium influx, the activity is transient and results in a low production of NO (low-

output), while iNOS can be active for hours to days and generate 1000-fold higher amounts of NO (high-output).[200,203-205]

nNOS is expressed in the central and peripheral nervous system and in skeletal muscles.[193,203] So far, four splice variants (nNOSβ, nNOSγ, nNOSμ and nNOS-2) have been detected.[194] Cells that express eNOS include vascular endothelial cells and cardiomyocytes. No splice variants of eNOS have been reported.[194] iNOS is inducible in a wide range of cells and tissues[193,194,201] by a variety of stimuli, including bacterial products (e.g., LPS), cytokines (e.g., TNF-α, IL-1β, IFN-γ), viral products, cyclic AMP (cAMP)-elevating agents, protein kinase stimulating agents and growth factors.[193,200,206] Stimuli that induce iNOS expression differ between species and between cell types within the same species.[193] For example, rodent and bovine cells can be more easily triggered than human and pig cells.[201] The majority of human cells require a complex cytokine combination (e.g. TNF-α, IL-1β and IFN-γ) for iNOS induction.[207] In addition, LPS and INF-γ are strong inducers of iNOS in monocytes, whereas TNF-α and IL-1β have only marginal effects in monocytes.[200] In contrast, most tissue-derived cells, such as hepatocytes, mesangial cells and vascular smooth muscle cells are more sensitive to iNOS expression when treated with TNF-α or IL-1β.[193,200,201] iNOS requires a delay of 6-8 hours before the onset of NO production, reflecting the time taken for mRNA and protein synthesis.[200]

Expression of iNOS is regulated by a large array of mechanisms at both transcriptional, posttranscriptional, translational and posttranslational level.[206-208] Once iNOS is expressed, its enzymatic activity can no longer be regulated.[193,207] Overall, the transcription factor NF-κB seems to play a central role in regulating LPS-induced iNOS expression.[209] Besides NF-κB, involvement of transcription factors like interferon regulatory factor 1 (IRF-1), signal transducer and activator of transcription 1 (STAT-1), AP-1 and cAMP-induced transcription factors (e.g., cAMP-responsive element binding protein (CREB), CCAAT-enhancer box binding protein (C/EBP)) have been demonstrated.[201,206,207] Moreover, several studies have now identified the involvement of all three MAPK (i.e., JNK, p38 and ERK) pathways in regulating iNOS expression.[206,207,210,211] iNOS is even able to trigger transcription of his own biosynthetic machinery since iNOS produced NO can activate all three MAPK cascades and the transcription factors NF-κB and AP-1.[200] Yet, a negative feedback on its own expression has also been demonstrated.[204]

2.7.1. NO

Nitric oxide (NO) is a simple diatomic molecule whose physico-chemical and biological properties are determined by its small size (30 kDa), the absence of charge and its single unpaired electron. As an uncharged molecule, it freely crosses cell membranes and it is readily diffusible in body fluids and tissues. Its lone outer electron renders it a radical and therefore chemically reactive. However, for a radical it is relatively stable as it does not react with itself and has a physiological half-life of seconds to minutes. In aqueous solutions NO undergoes oxidation into the relatively unreactive ions, nitrate (NO_3^-) and nitrite (NO_2^-).[203,212-214]

NO acts as a molecular messenger in various physiological and pathological processes in many organ systems.[203,206,215] It has been considered that 'low-output' NO, derived from constitutively active nNOS and eNOS, is an important regulator of physiological homeostasis, whereas 'high-output' NO, produced by iNOS, is correlated with the pathogenesis of inflammatory diseases such as asthma, arthritis, colitis, tumour development, neurodegenerative diseases and septic shock.[205-207]

Under physiological conditions, NO has been reported to be an important free radical scavenger and thus limiting the toxic effects of free radials such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). NO may also have the ability to prevent superoxide production by directly inhibiting NADPH oxidase.[216] In the brain, NO acts a neuromodulator that influences functions like memory, behaviour, nociception, vision and olfaction.[203,204] In the peripheral nervous system, NO acts as a neurotransmitter participating in functions such as smooth muscle control, gastrointestinal motility, and neuroendocriene function.[203,204,214] In skeletal muscles NO functions as a signal transducer regulating both metabolism and muscle contractility. In blood vessels, NO functions as a vasodilator thereby regulating blood flow and pressure.[204,214] Within cardiomyocytes, NO affects calcium currents and, consequently, myocardial contractility.[203,214] Furthermore, it inhibits platelet aggregation, leukocyte adhesion to endothelial cells and stabilises cell membranes resulting in reduced ischaemia-reperfusion injury.[203,204,214,216] In activated macrophages, NO synthesis leads to non-specific cytotoxicity against bacteria, protozoa and tumour cells.[204,205,207]

In addition, large amounts of NO have been demonstrated to inhibit cytokine production in stimulated macrophages by directly inhibiting NF-κB expression,[216] while smaller amounts induced this production.[205]

Under pathophysiological conditions, exaggerated NO production may lead to an array of detrimental effects, among which are excessive vasodilation and loss of systemic resistance, increased vascular permeability and airway and tissue oedema.[217-219] Moreover, increased NO production may induce hepatocyte dysfunction,[220] reduced gastrointestinal motility,[203] neurodegenerative disorders[219] and myocardial depression resulting in negative inotropy.[218]
When NO reacts with free radicals, more potent and highly reactive oxidants like peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) will be formed.[221] Subsequently, these free radicals induce platelet aggregation, disseminated intravascular coagulation, stimulation of lipid peroxidation and inhibition of mitochondrial respiration, finally leading to cellular damage.[203,217] Ultimately, lysis of cells and its organelles might lead to multiple organ dysfunction and death.[221]

2.7.2. iNOS EXPRESSION AND NO PRODUCTION: ROLE OF KETAMINE

In rodents, controversial effects regarding the influence of ketamine on LPS-induced iNOS expression and NO production have been found. In vitro, ketamine inhibited LPS-induced iNOS expression and NO production in a murine macrophage-like cell line[222] and in rat alveolar macrophages.[223] Ex vivo, ketamine blunted iNOS expression in several abdominal organs of endotoxin-challenged rats.[224,225] In contrast, Shibakawa et al.[226] found no significant inhibition of nitrite release in LPS-treated primary glial cell cultures following ketamine exposure.

2.7.3. iNOS EXPRESSION AND NO PRODUCTION IN EQUINES

In vitro, iNOS expression significantly increased in LPS-treated equine alveolar macrophages,[227] articular chondrocytes[228] and e-CAS cells[145] when compared to non-treated cells. In vascular smooth muscle cells only very limited expression of iNOS was found following simultaneous stimulation with LPS and IFN-γ. Basal NO synthesis was demonstrated in non-stimulated equine chondrocytes,[228] synoviocytes,[228] alveolar macrophages[227] and e-CAS cells.[145] Upon stimulation with LPS, NO synthesis significantly increased in equine chondrocytes in a dose-dependent manner.[228] In equine alveolar macrophages, significant increase in NO production was only detected in cells stimulated with LPS concentrations below 1 μg/mL,[227] whereas NO production in equine synoviocytes was neither significantly increased nor decreased by exposure to LPS.[228] In addition, NO was not detected in any supernate from peritoneal macrophage monolayers.[229]
Ex vivo, no substantial difference was detected in iNOS activity between synovial membranes obtained from healthy horses or from horses whose metacarpo-

phalangeal joints were exposed to IL-1β.[230] In vivo, Mirza et al.[231] showed a significant increase in iNOS staining in mucosal leukocytes and vasculature in horses suffering from a strangulating large colon volvulus versus clinically healthy horses. Moreover, higher nitrite concentrations were measured in articular cartilage, subchondral bone and the underlying trabecular bone of the proximal articular surface of the first phalanx of horses suffering from osteoarthritis than in healthy horses.[232] In addition, mares suffering from breeding-induced endometritis showed greater iNOS expression in their uterine biopsies and higher NO concentrations were measured in their uterine secretions compared with resistant mares.[233]

2.8. COX-2

Cyclooxygenase (COX) enzymes, also known as prostaglandin endoperoxide synthases, are fatty-acid oxygenases of the myeloperoxidase superfamily that are responsible for producing prostaglandins.[234] COX enzymes catalyse the sequential oxygenation and reduction of membrane-derived arachidonic acid to form the endoperoxides prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2), respectively.

COX enzymes contain 3 major domains: an N-terminal epidermal growth factor (EGF) domain, a helical membrane binding domain and a large catalytic domain at the C-terminus.[235] The EGF domain functions as a dimerisation domain since it contains conserved disulfide bonds.[234] Via the membrane binding domain, COX enzymes are largely anchored to the luminal side of the endoplasmic reticulum membrane and the nuclear envelope, although they have also been detected in lipid bodies, mitochondria, vesicles and nucleus.[234,236] The membrane binding domain contains 4 helices, which surround the opening where fatty acids enter the enzyme.[235] The catalytic domain has two distinct cyclooxygenase and peroxidase active sites which sequentially catalyse the oxidation and reduction of arachidonic acid, respectively.[234,235] COX enzymes have short catalytic life times, since the enzymes are autoinactivated.[234]

COX enzymes (72 kDa) consist of two isoforms: COX-1 and COX-2, sharing a 61% homology at the amino acid sequence.[234,235] In 2002, a splice variant of COX-1 has been identified in canine and human cerebral cortex and the human heart. This enzyme, designated as COX-3, shows a similar structure but slower enzymatic activity when compared to the parental isoform.[235,237] Splice variants of COX-2 have also been reported, but have failed to show enzymatic activity.[235]

COX-1 is ubiquitously and constitutively expressed in mammalian tissues and cells and is mainly involved in 'housekeeping' functions such as gastric mucosal integrity, crypt cell regeneration after injury, platelet aggregation, renal blood flow and glomerular filtration.[234,237] It is mainly utilised in the immediate prostaglandin biosynthesis.[236]

COX-2 is expressed by cells that mediate inflammation (e.g., macrophages, monocytes, synoviocytes) and has been recognised as the isoform that is primarily responsible for the synthesis of prostaglandins involved in pathological processes, particularly those related to acute and chronic inflammatory disorders.[235,238] In contrast with COX-1, COX-2 is generally present in tissues at very low levels, but highly inducible by stimuli such as LPS, cytokines, mitogens and other growth and differentiation factors.[234,237] Upon stimulation, expression of COX-2 is modulated by an array of transcription factors, among which are NF-κB, AP-1, CREB and C/EBP.[237,238] In addition, all three MAPKs (i.e., JNK, p38 and ERK) have been demonstrated to be involved with COX-2 expression.[210,238,239] The inducible COX-2 is an absolute requirement for delayed prostaglandin synthesis, which lasts for several hours following stimulation.[236] Effective catalysis by COX-2 can proceed at low levels of arachidonic acid (≤ 2.5 μM) derived from endogenous sources, whereas COX-1 requires high levels (> 10 μM) derived from exogenous sources to sustain catalysis.[235,236]

Nowadays, classification of COX into constitutive and inducible isoforms is somewhat oversimplified, since COX-1 can be induced during early stages of acute inflammation and COX-2 is constitutively present in brain and kidney.[237,240] Moreover, data increasingly indicate that COX-2 also has an important role in normal physiological functions, such as regulation of the glomerular filtration rate and gastric mucosal healing.[237]

2.8.1. PGE_2

Prostaglandin H_2 (PGH_2), the end product of COX, does not play a significant role as an inflammatory mediator. Rather, it is isomerised to various bioactive prostanoids (e.g., thromboxane A_2 ($TX A_2$), PGD_2, PGE_2, PGF_2 and PGI_2) by the respective terminal prostanoid synthases.[236]

Prostaglandins act in numerous physiological and pathophysiological processes, such as maintaining gastric mucosal integrity, renal function, vascular homeostasis, thrombosis, mitogenesis, ovulation, parturition, pyresis, algesia and inflammation.[234] Upon liberation, prostaglandins exert their effects by binding to

G-protein-coupled receptors.[240] In vivo, prostaglandins are short-lived (with half-lives of seconds to minutes) and act in an autocrine or a paracrine, rather than an endocrine fashion.[234]

PGE_2 is the most prominent prostaglandin in the onset of inflammation.[235,237] It has been detected in almost all experimental models of inflammation and in many inflammatory disorders in humans.[240] The biologic effects are extremely wide-ranging, but four areas are particularly pertinent to the regulation of the inflammatory response: fever, pain, oedema and regulation of leukocyte function.[240-242]

PGE_2 is a key mediator of the febrile response.[242] Injection of PGE_2 causes an increase in body temperature in many species and levels of PGE_2 are increased in cerebrospinal fluid from febrile animals.[240] PGE_2 mediates fever by acting on the thermoregulatory centre in the anterior hypothalamus.[242] However, the exact moment at which PGE_2 gets involved in mediating the febrile response following LPS stimulation is still unclear.[242] Fever promptly develops following LPS exposure, but synthesis of PGE_2 by COX-2 is significantly delayed. Since a local production of PGE_2 in the hypothalamus could not be demonstrated immediately after LPS injection, Blatteis et al.[242] postulated that PGE_2 is mainly responsible for the late and sustained phase of fever and that other components (e.g., norepinephrine) initiate the early phase.

PGE_2 plays an important role in mediating the pain response. Monoclonal antibodies to PGE_2 inhibit the pain response by 80%.[240] PGE_2 lowers the pain threshold and thus induces hyperalgesia during tissue injury and inflammation. Nishihara et al.[241] demonstrated that PGE_2-induced hyperalgesia is mediated by the N-methyl-D-aspartate (NMDA) receptor, since PGE_2-induced hyperalgesia was inhibited by both competitive and non-competitive blockers of these receptors.

PGE_2 induces both vasoconstriction and vasodilation, depending on the specific vascular bed involved.[168] As part of these vascular effects, PGE_2 has profound influence on the occurrence of oedema associated with inflammation, since PGE_2 increases blood flow to the inflamed site by arteriolar dilation. Oedema is not induced by injection of PGE_2 alone, but in the presence of an agent that increases vascular permeability to plasma proteins (e.g., LPS), a synergistic increase in the extent of plasma leakage, and thus oedema formation, can be observed. In addition, treatment with anti-PGE_2 antibody significantly reduced carrageenan-induced oedema.[240]

PGE_2 also shows immunosuppressive effects by modulating leukocyte function. In vitro, PGE_2 inhibits neutrophil activation, as has been measured by reduced

chemotaxis and superoxide production.[243] Moreover, PGE_2 decreases both TNF-α and IL-1 production in LPS treated macrophages.[244,245] This effect occurs also in vivo, since administration of COX inhibitors to mice[246] and humans[247] enhances the release of TNF-α in response to LPS exposure.

2.8.2. COX-2 AND PGE_2: THE ROLE OF KETAMINE

In vitro, ketamine inhibited the LPS-induced PGE_2 production in primary cell cultures of rat glial cells.[266] In vivo, pretreatment with ketamine attenuated hepatic COX-2 expression in LPS-treated rats,[248] whereas no influence on gastric mucosal COX-2 expression was found in rats anaesthetised with the combination ketamine/xylazine.[249] Nishihara et al.[241] found that PGE_2-induced hyperalgesia is mediated by NMDA receptors, since the non-competitive NDMA receptor antagonist ketamine inhibited PGE_2-induced hyperalgesia, although only following higher doses of PGE_2.

2.8.3. COX-2 AND PGE_2 IN EQUINES

In vitro, COX-2 expression significantly increased in LPS-treated equine alveolar macrophages[227] and vascular smooth muscle cells[164,250] when compared to non-treated cells. Upon stimulation with LPS or IL-1β, equine chondrocytes,[251,252] peritoneal macrophages[229] and vascular smooth muscle cells[164] have been shown a significant increase in PGE_2 synthesis, compared to non-stimulated cells.

In experimental in vivo studies, increased plasma PGE_2 concentrations have been associated with colitis[253] and ischemia-reperfusion injury in the large colon of horses.[254] Increased levels of PGE_2 have also been detected in the synovial fluid of LPS-induced synovitis of the carpal joint.[255]

In vivo, significantly higher PGE_2 concentrations have been measured in synovial fluid collected from osteoarthritic joints[256] and joints with osteochondritis dissecans[257] than in synovial fluid obtained from non-affected joints.

2.9. ROS

Phagocytic cells like neutrophils, monocytes and macrophages, produce and release reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), in response to phagocytosis.[221,258,259] Under pathophysiological circumstances, cells other than phagocytes may produce ROS.[221] Upon stimulation by either endogenous factors (e.g., cytokines, platelet-activating factor (PAF), complement fragments, eicosanoids) or exogenous influences (LPS, bacterial peptides, UV light irradiation, toxic chemicals and

drugs), the multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) reduced oxidase system is assembled and activated, consequently catalysing the reduction of oxygen to superoxide anion.[221,258,260-262] Next to the NADPH oxidase pathway, superoxide anion can be enzymatically generated by cytochrome P_{450s}, hypoxanthine/xanthine oxidase, lipoxygenase, cyclooxygenase and through the oxidation of catecholamines. Moreover, superoxide anion can be non-enzymatically generated as a side product of mitochondrial respiration where oxygen is reduced to water by the acceptance of four electrons.[221,254,258,261]

Normally, 1-2% of total oxygen consumption may be converted to superoxide anion.[261] Formation of superoxide anion leads to a cascade of other ROS. First, superoxide anion is rapidly converted by superoxide dismutase (SOD) to hydrogen peroxide, which is then, in a spontaneous reaction catalysed by Fe^{2+} (Fenton reaction), converted to the highly reactive hydroxyl radical.[221,254,258,259]

Superoxide is only moderately reactive with other biological molecules. However, when produced in combination with NO, superoxide anion interacts with NO to form peroxynitrite ($ONOO^-$). The peroxynitrite molecule itself is not very reactive, but its acid form, peroxynitrous acid (ONOOH) is a strongly oxidizing and cytotoxic product.[221,263] Thus, rapid conversion of superoxide anion to hydrogen peroxide is important.

Hydrogen peroxide is relative unreactive, although it can be converted by myeloperoxidase (MPO) (i.e., an oxidative enzyme in neutrophil azurophilic granules) to hypochlorous acid (HOCl), which is a strong oxidant that can chlorinate amino acids, peptides and proteins, and oxidise thiol functions of many proteins.[258,264] Reaction of HOCl with hydrogen peroxide yields another radical, singlet oxygen ($^1O_2$).[258]

The hydroxyl radical is so reactive that it will instantaneously extract a hydrogen atom from the closest molecule, producing water and another radical. This resulting radical reacts with a second molecule to continue the chain reaction. Polyunsaturated fatty acids are susceptible targets. So, extraction of a hydrogen atom from a polysaturated fatty acid may initiate the process of lipid peroxidation.[221,258]

Mammalian cells possess several enzymatic anti-oxidants to protect them from ROS formed during normal cellular metabolism. Superoxide dismutase catalyses the conversion of superoxide anion to hydrogen peroxide. Catalase catalyses the reduction of hydrogen peroxide to water and oxygen to prevent secondary generation of hydroxyl radicals. Glutathione peroxidase also reduces hydrogen peroxide to water, using glutathione as the substrate. In addition, since redox-

active metals, such as Fe^{2+}, catalyse formation of hydroxyl radicals, concentrations of these metal ions are kept low by binding to storage and transport proteins (e.g., ferritin, transferrin, lactoferrin).[221,254,258,261] Moreover, non-enzymatic antioxidants, such as α-tocopherol, ascorbate and beta carotene function to protect against oxidant injury.[221,254,261]

Under physiological conditions, the respiratory burst (i.e., ROS production due to transient consumption of oxygen) is kept in control and represents a major mechanism of host defence against infection. However, under pathophysiological conditions, oxidative stress, defined as an imbalance between radical-generating and radical-scavenging systems, may generate exaggerated amounts of ROS, which subsequently lead to cellular destruction and tissue injury.[221,258]

ROS-induced tissue injury can be mediated by both direct and indirect mechanisms.[221,254] An important direct mechanism of ROS involves the peroxidation of membrane phospholipids, which cause cell damage by way of disruption and lysis of cells and their organelles. Other direct mechanisms are hydroxyl-induced oxidation of nucleic acids and proteins which subsequently lead to DNA strand breaks and inactivation of enzymes.[221,254] Indirect tissue injury initiated by ROS involves disruption of intracellular calcium homeostasis. Calcium accumulates because of release from the endoplasmic reticulum and mitochondriae and from influx of extracellular calcium into the cytosol secondary to inactivation of cell membrane-associated calcium-ATP-ase. Increased cytosolic calcium concentrations activate phospholipase A_2, an enzyme which releases arachidonic acid from cell membrane phospholipids, thereby leading to further structural cell and tissue damage.[221,254] Ultimately, ROS-induced tissue injury may lead to multiple organ dysfunction and death.[254]

In humans, ROS have been implicated in various disorders such as arthritis,[265] asthma,[266] carcinogenesis, drug toxicity and inflammation.[267]

Next to their bactericidal function in host defence, ROS play an essential role in intracellular signal transduction pathways.[221,261] Exposure to ROS induces activation of MAPK cascades (i.e., JNK, p38 and ERK) and transcription factors like NF-κB and AP-1, albeit largely cell-specific and stimulus-dependent.[140,221,261,268] For example, NF-κB is not activated by hydrogen peroxide in monocytic cell lines and peripheral T cells. Moreover, hydrogen peroxide enhances NF-κB-mediated COX-2 expression and PGE_2 production in human primary monocytes only following priming or activation by LPS.[268] Chandel et al.[269] even found that LPS activates NF-

κB and TNF-α gene expression through a ROS-independent mechanism in murine macrophage J774.1 cells.
The mechanism by which ROS potentially modulates MAPKs and several transcription factors is still under extensive debate.

2.9.1. ROS: ROLE OF KETAMINE

Regarding the influence of ketamine on ROS production, conflicting results have been found. In human polymorphonuclear leukocytes (PMNs), ketamine significantly reduced N-formyl-methionyl-leucyl-phenylalanine (FMLP)/ opsonised zymosan[270] or FMLP/phorbol 12-myristate 13-acetate (PMA)-induced[89] ROS production in a dose-dependent manner. In contrast, no influence of ketamine on ROS synthesis in PMA-treated,[271] FMLP-treated[99] and opsonised zymosan-treated[272,273] human PMNs could be demonstrated.

2.9.2. ROS IN EQUINES

In vitro, both ROS produced by PMA-stimulated equine neutrophils, as well as ROS present in the supernatant of PMA-stimulated equine neutrophils, were highly cytotoxic to equine endothelial cells in culture.[274] This indicates that under pathological conditions with an important activation of neutrophils, damage induced by ROS can spread to cells and tissues away from the primary inflammatory focus.[274]
In vivo, ROS have been demonstrated to play a pivotal role in the pathogenesis of gastrointestinal ischemia-reperfusion injury in horses in both experimental and clinical studies.[254,275] Following one hour of intestinal ischemia, an increased xanthine oxidase (XO) concentration was detected in the equine small intestine, whether inconsistent results were found with respect to XO concentrations in the equine large colon.[254] In another study, Grülke et al.[275] found a significantly higher plasma level of the oxidative enzyme MPO in horses operated for strangulating obstruction of the large intestine compared with horses suffering from a non-strangulating displacement of the large intestine. These results indicate that PMN activation might play a major role in the pathogenesis of acute abdominal disease and endotoxaemic shock.

3. CONCLUSIONS

The presented literature review suggests that ketamine may exert an anti-inflammatory effect also in horses. As possible targets of its action, interference with pathogen-associated signalling pathways (represented by LPS), leading to the

release of pro-inflammatory mediators, could be identified. Further studies should address the effect of ketamine on the individual components of the inflammatory cascade.

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- CHAPTER III -

KETAMINE INHIBITS LPS-INDUCED TUMOUR NECROSIS FACTOR-ALPHA AND INTERLEUKIN-6 IN AN EQUINE MACROPHAGE CELL LINE

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SUMMARY

Ketamine is widely used in equine anaesthesia. Beside its anaesthetic and analgesic properties, ketamine possesses cytokine-modulating activity. However, to date no data are available regarding the inhibitory effect of ketamine on the cytokine response in horses. In horses, cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) play a pivotal role in the pathogenesis of equine endotoxaemia following gastrointestinal disorders. Hence, the objective of this study was to assess the influence of ketamine on LPS-induced TNF-α and IL-6 formation in an equine macrophage cell line (e-CAS cells). Results demonstrate a cytokine-modulating activity of ketamine in an equine cell line, suggesting a beneficial role for ketamine in the treatment of equine endotoxaemia.

1. INTRODUCTION

Ketamine is a dissociative anaesthetic drug whose effect is characterised by superficial sleep combined with marked analgesia.[1] For decades, ketamine has been used for the induction and maintenance of general anaesthesia in horses.[2,3] More recently, it has also been used to provide postoperative analgesic support. Moreover, ketamine preserves cardiovascular function,[4] from which it is advocated for anaesthesia in cardiovascular depressed patients, including horses suffering from endotoxaemia.

Endotoxaemia is a serious complication in horses suffering from gastrointestinal disorders. Circulating endotoxins may stimulate mononuclear phagocytes to produce and release inflammatory mediators such as eicosanoids, platelet activating factor and cytokines.[5,6] In horses, the pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), play a pivotal role in the pathogenesis of endotoxaemia following gastrointestinal disorders.[5,7]

It has been previously shown that ketamine modulates cytokine levels in rodents[8,9] and humans.[10,11] However, to date, there are no reports on ketamine modulating the cytokine activity in equine patients. Hence, the aim of the present study was to determine the influence of ketamine on LPS-induced TNF-α and IL-6 concentration in an equine macrophage cell line. For comparison, a human monoblastoid cell line was used.

2. MATERIALS AND METHOD

2.1. CHEMICALS

Ketamine, lipopolysaccharide (LPS; Escherichia coli, O111:B4), phorbol myristate acetate (PMA) and methyl-thiazolyl tertrazolium (MTT) were purchased from Sigma chemicals (St. Louis, MO, USA). Phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin, streptomycin and glutamine were obtained from Biocambrex (Verviers, Belgium). Fetal calf serum (FCS) and non-essential amino acids were obtained from Gibco Invitrogen (Breda, The Netherlands). Alamar Blue (AB) was purchased from BioSource International (Etten-Leur, The Netherlands) and horse serum (HS), was prepared in our own laboratory, according to standard procedures.

2.2. CELL LINES AND CULTURE CONDITIONS

Two cell lines were used: the equine bone-marrow-derived macrophage cell line (e-CAS), developed by Werners et al.,[12] and the human monoblastoid tumour cell line (U937 cells; ECACC No. 85011440, UK).

The e-CAS cells were seeded in 24-well plates (0.2 x 10^6 cells/mL) and incubated in RPMI 1640 medium supplemented with 20% HS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 1% non-essential amino acids for 24 h at 37°C, 5% CO_2 prior to testing.

The U937 cells were cultured in RPMI 1640 medium, supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin, according to the protocol described by Wang et al.,[13] with minor modifications. The cells were plated in 24-well plates (0.2 x 10^6 cells/ mL) and incubated at 37°C, 5% CO_2 for 24 h. Subsequently, the cells were stimulated to differentiate along the monocyte/macrophage lineage by the addition of PMA (50 ng/well) for 24 h at 37°C prior to use.

2.3. EXPOSURE TO LPS AND KETAMINE

Following the initial incubation period, the medium was removed and replaced with fresh RPMI 1640 medium containing LPS (1 µg/mL) and ketamine at different concentrations (0, 0.36, 1.8, 3.6, 18 and 36 µM), or ketamine (36 µM) alone. After 4 and 24 h of incubation, the supernatants were collected for TNF-α analysis. Samples for IL-6 analysis were taken after 24 h of incubation only. All samples were stored at -70°C prior to analysis.

2.4. CELL VIABILITY

Cell viability following LPS and ketamine exposure was measured using the Alamar Blue (AB) reduction assay as described by Bull et al.[14]

2.5. TNF-α AND IL-6 BIOASSAY

Concentrations of TNF-α in cell supernatants were measured by using a porcine kidney cell line (PK-15) according to the method of Bertoni et al.[15] Concentrations of IL-6 were measured using a murine B cell hybridoma cell line (7-TD1), described by Okada et al.[16] The TNF-α-induced cytotoxicity in PK-15 cells and the IL-6 induced proliferation of 7-TD1 cells, were determined using the MTT assay.[17] Cytokine concentrations in supernatants were quantified by comparison of

calculated EC_{50} values from samples with EC_{50} values from the TNF-α and IL-6 standard curve, respectively.

2.6. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS® 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). All data are expressed as mean ± standard deviation (S.D.) of four independent experiments carried out in duplicate. Data were analysed for statistical significance using one-way analysis of variance (ANOVA), followed by a post hoc Bonferroni's multiple comparison test. Lipopolysaccharide alone was regarded as a positive control and set at 100%. Values were considered significantly different to control values when $p<0.05$.

3. RESULTS

3.1. EFFECT OF KETAMINE ON LPS-INDUCED CYTOKINE RESPONSE IN E-CAS CELLS

In e-CAS cells, TNF-α concentrations were significantly decreased by ketamine concentrations of 18 and 36 μM, compared to LPS alone following 4 h of incubation (Fig. 1A). After 24 h, a significant decrease in TNF-α concentration was also observed with 10-fold lower ketamine concentrations (Fig. 1B). Data indicate that ketamine significantly suppresses the IL-6 concentrations in a dose-dependent manner (0.36-36 μM), compared to the control (Fig. 1C). Neither TNF-α nor IL-6 data exhibited a true dose-response relationship. In the absence of LPS, ketamine had no effect on TNF-α and IL-6 formation. Moreover, data obtained with the AB assay demonstrated that cell viability was not significantly influenced under the given experimental conditions (Fig. 1D).

3.2. EFFECT OF KETAMINE ON LPS-INDUCED CYTOKINE RESPONSE IN U937 CELLS

In U937 cells, after both 4 and 24 h of incubation, TNF-α concentrations were significantly decreased by ketamine treatment (Figs 2A and 2B), although data did not exhibit a true dose-response relationship. At 24 h after incubation, ketamine significantly decreased IL-6 concentration only at 36 μM (Fig. 2C). Ketamine alone had no effect on TNF-α and IL-6 formation. Again, no loss of cell viability was observed following 24 h exposure to ketamine (Fig. 2D).

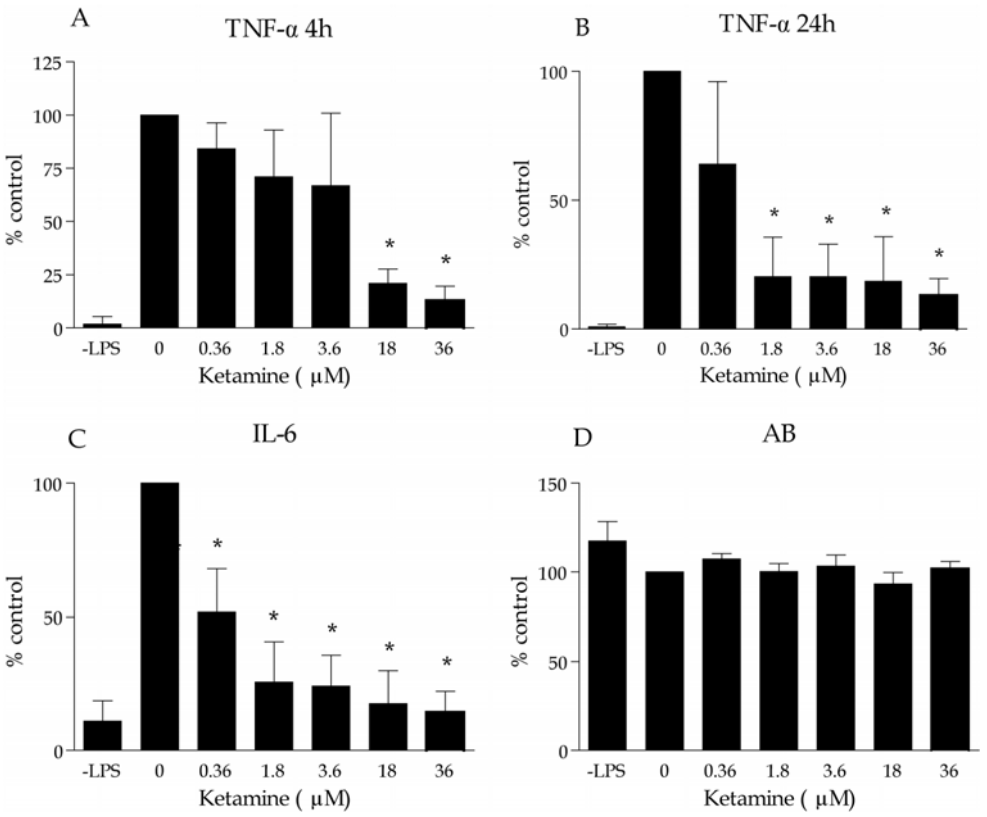

Figure 1. Effect of ketamine on LPS-induced (A) TNF-α (4 h), (B) TNF-α (24 h), (C) IL-6 (24 h) and (D) viability (24 h) in e-CAS cells after exposure to LPS and ketamine, or ketamine alone (-LPS). Data expressed as mean ± S.D. of four independent experiments. * statistically different to LPS alone ($p<0.05$).

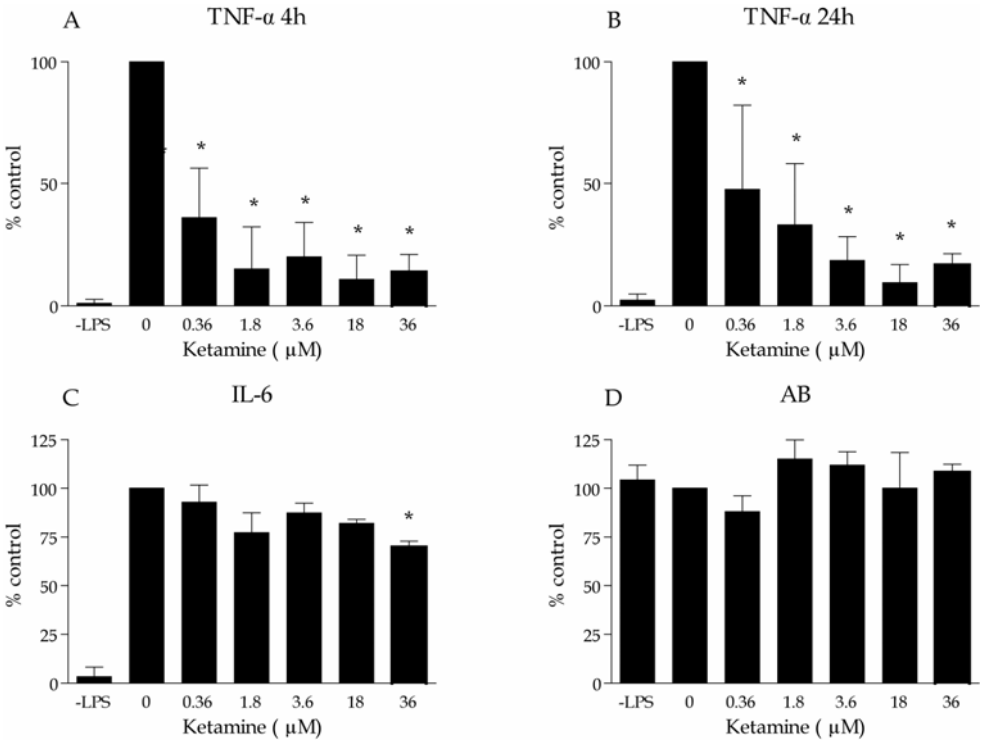

Figure 2. The influence of ketamine on LPS-induced (A) TNF-α (4 h), (B) TNF-α (24 h), (C) IL-6 (24 h) and (D) viability (24 h) in U937 cells after exposure to LPS and ketamine, or ketamine alone (-LPS). Data expressed as mean ± S.D. of four independent experiments. * statistically different to LPS alone ($p<0.05$).

4. DISCUSSION

In the present study, the effect of ketamine on the cytokines TNF-α and IL-6 was investigated since these cytokines have been shown to play a pivotal role in the pathogenesis, progress and outcome of equine endotoxaemia.[5,7]

Ketamine was selected as a drug to be investigated for a variety of reasons. Ketamine is frequently used in equine anaesthetic procedures, both per- and postoperatively.[2] As a N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine provides somatic analgesia even at subanaesthetic doses.[4] Its observed visceral analgesic properties make it specifically indicated for horses suffering from gastrointestinal disorders. Moreover, ketamine preserves cardiovascular function.[4] Hence, its use is indicated in horses suffering from endotoxaemia. Although other anaesthetic agents used in equine anaesthesia, such as barbiturates and volatile anaesthetics, have been shown to suppress the LPS-induced cytokine response in other species,[11,18] ketamine has been most widely studied in different species.

The ketamine concentrations used in this study were selected in consideration of the plasma level of 5 μg/mL (18 μM) following the IV induction dose of 2.2 mg/kg body weight commonly used in horses.[19] The ketamine dose range tested aimed at determining the minimal effective concentration that suppresses the TNF-α response. This is of clinical relevance since lower plasma concentrations are desirable in the postoperative period to circumvent the anaesthetic and excitatory effects of ketamine.

To avoid the use of in vivo challenge experiments and due to known species difference in the LPS response, an equine macrophage cell line was used. A cell line was chosen rather than primary cells, since it has the advantage of a more uniform response pattern.[12] The well-established human macrophage derived cell line (U937 cells) was used as a reference.

This study demonstrates that ketamine suppresses LPS-induced TNF-α concentration in both e-CAS and U937 cells in a dose-dependent manner, although a true dose-response relationship could not be established. These results correlate with ex vivo studies carried out in human blood cells[10] and murine peritoneal macrophages.[8] However, in the present investigation, inhibition of cytokine concentration was achieved at lower ketamine concentrations, which could be due to a higher sensitivity of the equine cells to ketamine.

The effect of ketamine on IL-6 concentrations was only evident in e-CAS cells, since no consistent significant decrease in the U937 cells could be demonstrated. This

latter finding correlates with data from a human whole blood assay described by Larsen et al.,[11] where ketamine failed to significantly inhibit IL-6 production following LPS exposure, although TNF-α production was decreased. In contrast, Weigand and colleagues[20] showed a significant decrease in IL-6 concentration in human whole blood following ketamine treatment, albeit at 30-fold higher concentrations (100-1000 μM). The discrepancies observed between equine and human macrophage cell lines in IL-6 suppression strengthen the necessity to conduct experiments with cells derived from the animal species under consideration.

The mechanism by which ketamine suppresses cytokine concentration has not been defined in this study. However, recent research has shown that ketamine decreases cytokine formation by reducing nuclear factor kappa B (NF-κB) activation in rats.[21] Since NF-κB is the major transcription factor triggering cytokine production, its inhibition leads to a decrease in cytokine formation.

In conclusion, this study demonstrates that ketamine inhibits the LPS-induced TNF-α and IL-6 response in a dose-dependent manner in an equine macrophage cell line even at subanaesthetic concentrations. These results suggest a potential role for ketamine in the treatment of equine patients suffering from gastrointestinal disorders and subsequent endotoxaemia.

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- 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).

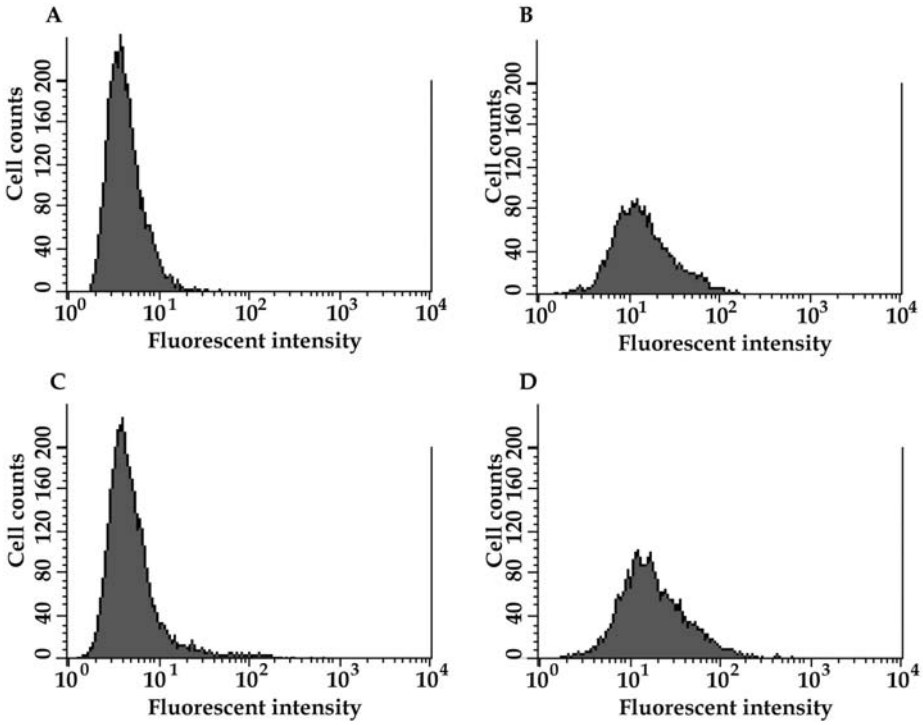


Figure 1. Effect of ketamine on TLR4 expression on the cell surface of non-stimulated (A and C) and LPS-stimulated (B and D) e-CAS cells. Cells were incubated with 36 μM ketamine (C and D) for 24 h. Following incubation, cells were double stained with rabbit polyclonal anti-TLR4 and FITC-labeled polyclonal 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).

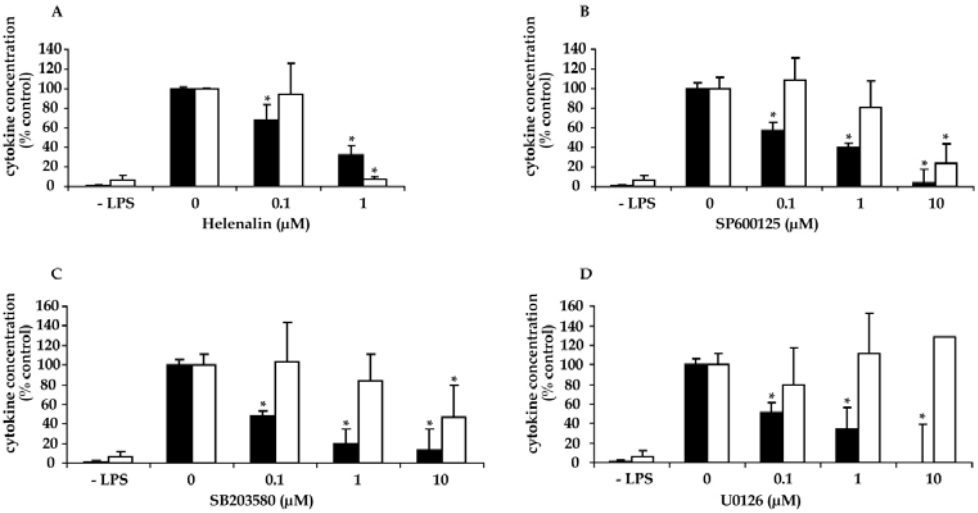


Figure 2. Effect of (A) helenalin, (B) SP600125, (C) SB203580 and (D) U0126 on the LPS-induced TNF-α (■) and IL-6 (□) response in e-CAS cells. Cells were incubated with the specific inhibitors of NF-κB (helenalin; 0-1 μM), JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM), ERK (U0126; 0-10 μM) and LPS (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).

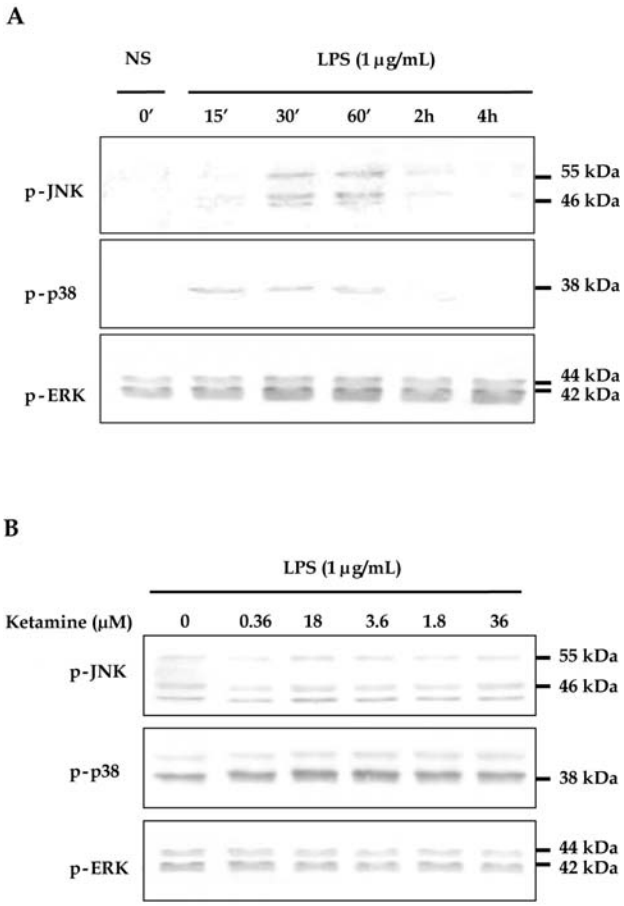


Figure 3. Failure of ketamine to affect LPS-induced MAPK expression in e-CAS cells. (A) Cells were incubated with blank medium or LPS (1 μg/mL) for 0-4 h. Following incubation, cells were lysed and lysates were assayed for p-JNK, p-p38 and p-ERK expression by Western blot analysis. Cells exposed to blank 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.

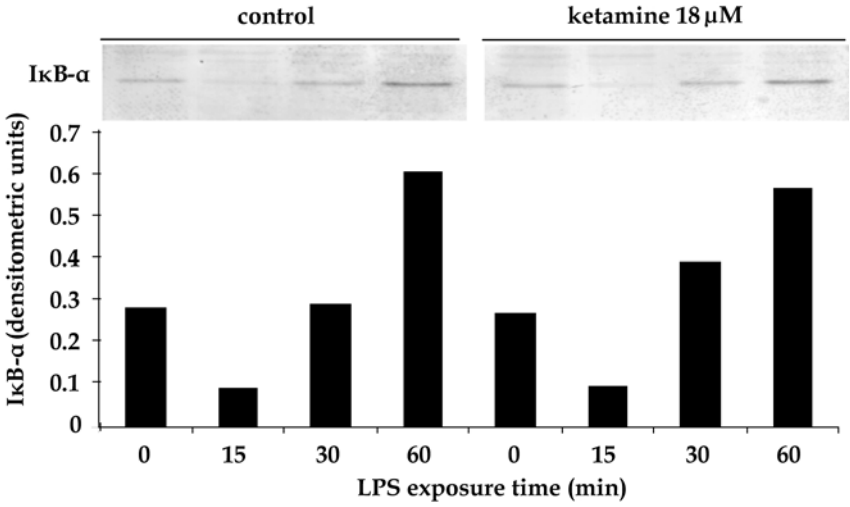


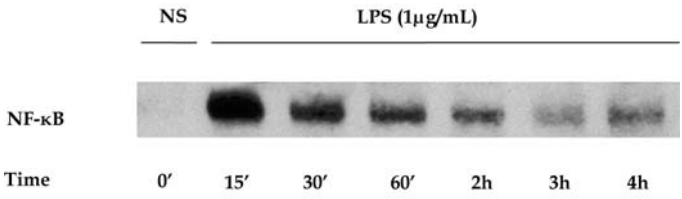
Figure 4. Failure of ketamine to affect LPS-induced IκB-α expression in e-CAS cells. Cells were stimulated with LPS and co-incubated without or with ketamine (18 μM) for indicated times. Following incubation, cells were lysed and lysates were assayed for IκB-α expression by Western blot analysis. Representative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

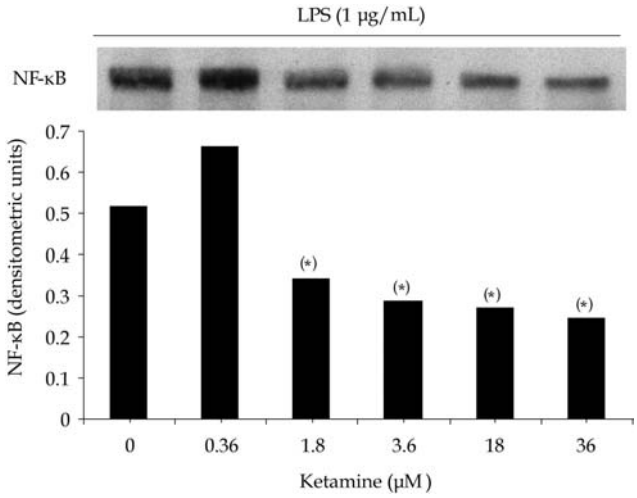
To investigate the effect of ketamine on LPS-stimulated NF-κB expression, nuclear extracts were isolated from e-CAS cells and subjected to EMSA. Figure 5A demonstrates that non-treated e-CAS cells showed no NF-κB expression, while addition of 1 μg/mL LPS markedly enhanced NF-κB expression. This increase 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.

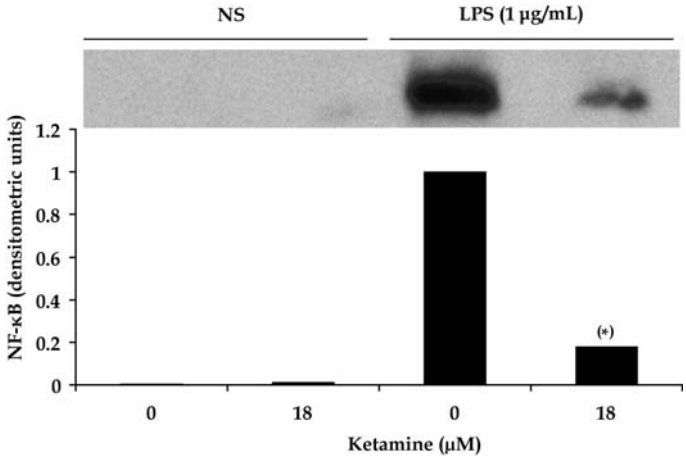
A


NS
LPS (1µg/mL)
NF-κB
Time
0′
15′
30′
60′
2h
3h
4h

B


LPS (1 µg/mL)
NF-κB
NF-κB (densitometric units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(*)
(*)
(*)
(*)
0
0.36
1.8
3.6
18
36
Ketamine (µM)

C


NS
LPS (1 µg/mL)
NF-κB (densitometric units)
1.2
1
0.8
0.6
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18
0
18
Ketamine (µM)

D

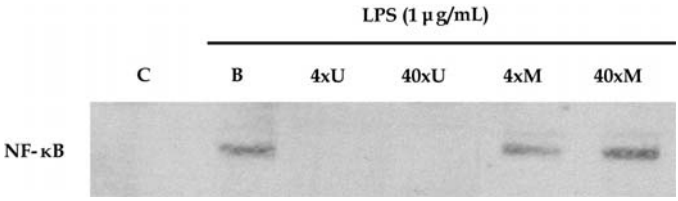

Figure 5. Effect of ketamine on NF-κB expression in LPS-stimulated e-CAS cells. (A) Cells were incubated with blank medium or LPS (1 µg/mL) for 0-4 h. Following incubation, nuclear extracts were prepared and assayed for NF-κB expression by EMSA. Cells exposed to blank medium served as control (NS = not 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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- CHAPTER V -

EFFECTS OF KETAMINE ON INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION AND NITRIC OXIDE SYNTHESIS IN AN EQUINE MACROPHAGE CELL LINE FOLLOWING LPS EXPOSURE

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SUMMARY

Ketamine has been found to reduce lipopolysaccharide (LPS)-induced tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) concentrations in an equine macrophage cell line (e-CAS cells). This effect could be attributed to a direct inhibition of nuclear factor-kappa B (NF-κB) expression. NF-κB is one of the regulatory transcription factors associated with the expression of pro-inflammatory mediators, including cytokines, and inducible enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Inducible NOS and its product nitric oxide (NO) are involved in various inflammatory disorders. Inhibiting NO production by blocking iNOS expression reduces the clinical signs of inflammation.

In the present study, the effect of ketamine (0-1000 µM) on LPS-induced iNOS expression and NO production was investigated in e-CAS cells. Furthermore, the intracellular signalling pathways, c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase p38 (p38), extracellular signal-regulated kinase (ERK) and NF-κB were selectively inhibited, with the aim to elucidate the mechanisms underlying LPS-induced iNOS expression and NO production. For comparison, RAW 274.6 cells were used.

The results show that ketamine does not affect the LPS-induced iNOS expression and NO production in e-CAS cells at any of the concentrations tested. Inhibition experiments revealed that following LPS activation, different mitogen-activated protein kinases (MAPKs), including JNK, p38, ERK, as well as NF-κB, contribute to the increased iNOS expression and NO production in e-CAS cells. In previous experiments with e-CAS cells, ketamine has been found to inhibit the LPS-induced signalling pathways only at the level of NF-κB,[1] which alone seems not to be sufficient to suppress iNOS induction. Hence, despite its potency to reduce LPS-induced TNF-α and IL-6 production, the role of ketamine in iNOS and NO associated equine inflammatory disorders might be limited.

1. INTRODUCTION

Ketamine is a dissociative anaesthetic agent which is frequently used in equine practice for both the induction and maintenance of general anaesthesia in horses.[2,3] As a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist it produces superficial sleep combined with profound analgesia.[2-4] Ketamine preserves respiratory and cardiovascular functions and is therefore advocated for use in cardiovascular depressed patients.[5] Furthermore, ketamine has been found to possess cytokine-modulating effects in rodents[6-8] and humans.[9,10] Previously, we demonstrated that in an equine macrophage cell line (e-CAS cells), ketamine inhibits the LPS-induced release of both tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by directly inhibiting nuclear factor-kappa B (NF-κB) expression in a concentration-dependent manner, even at subanaesthetic concentrations[1,11] NF-κB is a transcription factor, which is normally sequestered in the cytoplasm as a dimer, coupled to its inhibitory protein, inhibitor of kappa B (IκB). Exposure of responsive cells 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 initiate the transcription of multiple pro-inflammatory mediators such as cytokines, adhesion molecules, and enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).[12-15] Alternatively, the transcription of many of these pro-inflammatory mediators can be induced by mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase p38 (p38) and c-Jun N-terminal kinase (JNK).[16,17]

Inducible NOS (iNOS) catalyses the production of nitric oxide (NO) by conversion of L-arginine to L-citrulline.[12,18] It is absent in resting cells, but in response to LPS, expression of iNOS increases, thereby generating large amounts of NO within several hours and for as long as 5 days.[12,19-21] Excessive NO production by LPS-induced iNOS may lead to an array of detrimental effects such as vasodilation, increased vascular permeability and oedema.[22-24] In addition, excessive NO production might result in the formation of peroxynitrite ($ONOO^-$), that acts as a radical and thus contributes to tissue damage.[25,26] Therefore, inhibiting NO production by blocking iNOS expression reduces the pathophysiological consequences of (local) inflammatory processes.

Considering the effect of ketamine on LPS-induced TNF-α and IL-6 production by directly inhibiting NF-κB, it was hypothesised that ketamine might also reduce LPS-induced iNOS expression and NO production. In previous experimental

studies, ketamine has been found to exert conflicting effects on LPS-induced iNOS expression and NO production. For example, in vitro, ketamine inhibited LPS-induced iNOS expression and NO production in a murine macrophage-like cell line[27] and in rat alveolar macrophages.[28] Moreover, ketamine blunted iNOS expression in several abdominal organs of endotoxin-challenged rats.[29,30] In contrast, in an ex vivo study performed by Shibakawa et al.,[31] no significant inhibition of nitrite release in LPS-treated primary glial cell cultures following ketamine exposure was found.

In the present study, the influence of ketamine on LPS-induced iNOS expression and NO production in an equine macrophage cell line was investigated. Furthermore, the intracellular signalling pathways, JNK, p38, ERK and NF-κB were selectively inhibited, with the aim to elucidate the mechanisms underlying LPS-induced iNOS expression and NO production. To support the validity of the e-CAS model, a murine macrophage-like cell line (RAW 264.7) was used for comparison.

2. MATERIALS AND METHOD

2.1. CHEMICALS

Ketamine, lipopolysaccharide (LPS; Escherichia coli, O111:B4), sodium pyrophosphate, ethylenediaminotetraacetic acid (EDTA), Igepal (CA-630), sodium dodecyl sulphate (SDS), Tris, glycerol, bromphenolblue, bovine serum albumin (BSA), nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), dithiothreitol (DTT), sulphanilamide, N-(1-Naphtyl)ethylenediamine.2HCL (NED) were purchased from Sigma chemicals (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, glutamine and phosphate buffered saline (PBS) were obtained from Biocambrex (Verviers, Belgium). Horse serum (HS), fetal bovine serum (FBS), non-essential amino acids and sodium pyruvate were from Gibco Invitrogen (Breda, The Netherlands). Phosphoric acid was obtained from Boom B.V. (Meppel, The Netherlands). Alamar Blue (AB) was purchased from BioSource International (Etten-Leur, The Netherlands). Helenalin, SP600125, SB203580 and U0126 were from Biomol (Plymouth, USA). Polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, Massachusetts, USA). Dimethyl Sulfoxide (DMSO) was from Fisher Emergo B.V. (Landsmeer, The Netherlands). Tween-20 and sodium nitrite were from Merck KGaA (Darmstadt, Germany). Bradford Protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). MgCl2.6H20 was from VWR International (Amsterdam, The Netherlands). Rabbit

polyclonal anti-iNOS was from Cayman Chemical Company (Tallinn, Estonia). Alkaline phosphatase conjugated with polyclonal goat anti-rabbit immunoglobulin was purchased from Dako Cytomation (Glostrup, Denmark).

2.2. CELL LINES AND CULTURE CONDITIONS

Two cell lines were used: the equine bone-marrow-derived macrophage cell line (e-CAS cells) described by Werners et al.,[32] and the murine macrophage-like cell line (RAW 264.7; ATCC No. TIB-71).

The e-CAS cells were grown in RPMI 1640 medium supplemented with 10% HS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 1% non-essential amino acids. Cells were maintained at 37 ^{o}C, in a humidified atmosphere of 5% CO_2/ 95 % air.

The RAW 264.7 cells were cultured in DMEM, supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine and 1 mM sodium pyruvate. Cells were maintained at 37 ^{o}C, in a humidified atmosphere of 5% CO_2/ 95 % air as well.

To determine NO production and relative expression of iNOS protein, cells were seeded in 24-well plates at a density of 0.2 x 10^6 cells per well (1 mL) and in 6-wells plates at a density of 1.0 x 10^6 cells per well (2 mL), respectively. Following 24 h of culturing, different experiments were performed as described below.

2.3. TIME RESPONSE CURVE

To determine the time-dependent NO production in e-CAS cells, cells were incubated with serum-free RPMI 1640 medium containing 1 µg/mL LPS. Cells exposed to medium only served as control. Nitric oxide production and cell viability were determined every 4 h following 4 - 48 h of incubation.

2.4. EXPOSURE TO LPS AND KETAMINE

To study the influence of ketamine on LPS-induced iNOS expression and NO production, e-CAS cells were exposed to serum-free medium containing LPS (1 µg/mL) and ketamine concentrations at a range of 0-36 µM or 0-1000 µM, comprising clinically relevant as well as high experimental concentrations, respectively. Based on the results of the time-response curves, iNOS expression, NO production and cell viability were determined following 24 h of exposure.

For control experiments, RAW cells were exposed to 1 µg/mL LPS and the experimental concentration range of ketamine (0-1000 µM).

2.5. INHIBITION EXPERIMENTS

To investigate the potential involvement of MAP kinases and NF-κB in LPS-induced iNOS expression and NO production, cells were exposed to serum-free medium containing LPS (1 μg/mL) and specific inhibitors of JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM), ERK (U0126; 0-10 μM) or NF-κB (helenalin; 0-1 μM). To the control samples, equal amounts of inhibitor solvent (0.1% DMSO) and/or the LPS solvent (PBS) were added. iNOS expression, NO production and cell viability were determined following 24 h of incubation.

In RAW 264.7 cells, parallel inhibition experiments were only performed for LPS-induced iNOS expression in the presence and absence of the above mentioned inhibitors

2.6. DETERMINATION OF NITRIC OXIDE

Nitric oxide production was determined by measuring nitrite (NO^{2-}) accumulation in the culture medium using the spectrophotometric Griess assay.[33] Briefly, 200 μl of supernatant was transferred to a 96-well plate and incubated with 20 μl sulphanilamide (1 % w/v) in phosphoric acid (5%) for 10 minutes at room temperature (RT). Subsequently, 20 μl N-(naphtyl)-ethylenediamine dihydrochloride (1.4 % w/v) was added and 2 minutes later the optical density of the reaction mixture was read at 540 nm using a microplate reader (Bio Rad Model 3550 Microplate Reader). Nitrite concentrations were calculated by using a standard curve of sodium nitrite ($NaNO_2$) dissolved in culture medium.

2.7. WESTERN BLOT ANALYSIS

Expression of iNOS protein was determined by Western blot analysis. In brief, after exposure to the indicated test compounds (LPS and inhibitors), cellular extracts were prepared in RIPA buffer [150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl (pH 8)]. Protein concentrations were measured using the Bradford protein assay according to the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA, USA). Cell pellets were resuspended in sample buffer [12.5% glycerol, 1.25 % SDS, 0.004% bromophenol blue, 95 mM DTT and 62.5 mM Tris-HCl (pH 8.8)]. Aliquots containing 40 μg of protein were separated by SDS-PAGE (8%) 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-iNOS diluted

1:1000) for 1 h at RT. After washing, blots were incubated with secondary antibodies (alkaline phosphatase conjugated polyclonal goat anti-rabbit IgG diluted 1:1000) for 1 h at RT. Immunoreactive protein bands were developed by using nitroblue tetrazolium and bromochloroindolyl phosphate as substrates and quantified through densitometric analysis.

2.8. CELL VIABILITY

The Alamar Blue ™ (AB) reduction cytotoxicity assay was used as an indicator of cell viability.[34] 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°C for 4 h. Thereafter, resorufin formation was measured fluorometrically at 530-560 nm excitation wavelength and 590 nm emission wavelength.

2.9. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS© 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). Data of NO production are presented as mean ± standard deviation (S.D.) of three independent experiments carried out in triplicate. One-way analysis of variance (ANOVA) was used to test for significant differences between group means. When appropriate, post hoc multiple comparisons were performed to test for significant differences between experimental groups (Dunnett's). Data were considered to be statistically significant when $p<0.05$.

3. RESULTS

3.1. TIME-DEPENDENT NO PRODUCTION IN LPS-STIMULATED E-CAS CELLS

To determine the time response for NO production in LPS-treated e-CAS cells, NO production and cell viability were determined every 4 h following 4 - 48 h of LPS (1 μg/mL) exposure. Over time, a significant increase in LPS-induced NO production was demonstrated (Fig. 1). An apparent steady state level was observed between 16 and 24 h of exposure, after which a second increase in NO production was documented. Cell viability remained above 90% until 28 h of LPS exposure. Thereafter, viability significantly decreased towards the end of the experiment ($p<0.05$). Based on these results, iNOS expression, NO production and cell viability were determined at 24 h of LPS exposure in the further experiments with e-CAS cells.

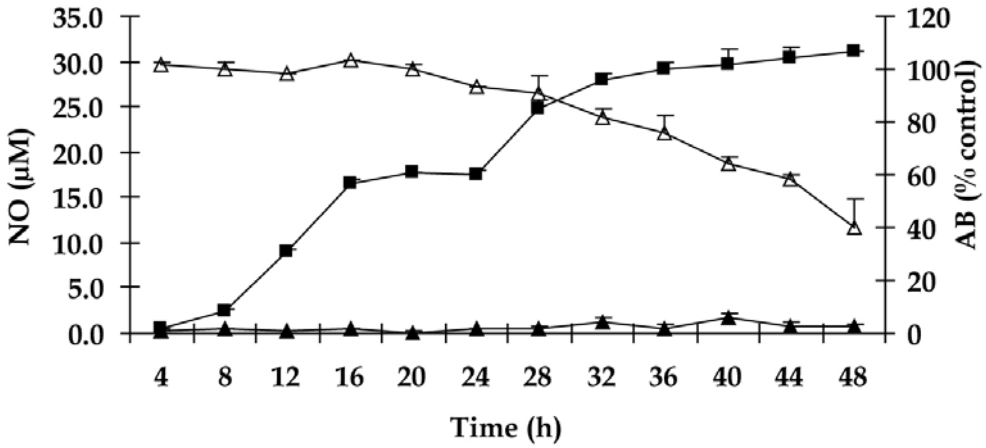

Figure 1. Time-response curve of NO production in e-CAS cells. Cells were incubated with serum-free RPMI containing no (▲) or 1 μg/mL (■) LPS. NO production and cell viability (△) were determined every 4 hrs following 4 - 48 h of incubation. Data are expressed as mean ± S.D. of two independent experiments carried out in duplicate.

3.2. CELL VIABILITY

In all forthcoming experiments, cell viability was also tested by using the Alamar Blue cytotoxicity assay, but no significant differences were found (data not shown).

3.3. INFLUENCE OF KETAMINE ON LPS-INDUCED iNOS EXPRESSION AND NO PRODUCTION IN E-CAS CELLS

To study the influence of ketamine on LPS-induced iNOS expression and NO production in e-CAS cells, cells were initially exposed to LPS (1 μg/mL) in the presence of a clinically relevant ketamine concentration range (0-36 μM). Exposure to LPS significantly increased iNOS expression and NO production (from 2.2 ± 0.5 to 25.2 ± 0.7 μM) in e-CAS cells. At the concentrations tested, ketamine did not affect the LPS-induced iNOS expression and NO production.

Subsequently, cells were exposed to high experimental ketamine concentrations (0-1000 μM), but again no effect on LPS-induced iNOS expression and NO production could be observed (Fig. 2). Control experiments revealed that ketamine alone, in the absence of LPS, did not alter iNOS expression or NO production (data not shown).

3.4. EFFECT OF KETAMINE ON LPS-TREATED RAW 264.7 CELLS

Western blot analysis showed that the iNOS protein is undetectable in resting RAW 264.7 cells, but significantly increased in the presence of LPS. Like in e-CAS cells, none of the ketamine concentrations tested significantly inhibited LPS-induced iNOS expression in RAW 264.7 cells (Fig. 3A). In non-stimulated RAW 264.7 cells, NO production was low, varying between 0.1 and 0.3 µM. After stimulation with LPS (1 µg/mL), NO production significantly increased to 10.0 ± 0.6 µM. When RAW 264.7 cells were incubated with LPS and high experimental concentrations of ketamine, significant inhibition of NO production was only observed at 1000 µM (Fig. 3B).

3.5. EFFECT OF INHIBITORS OF NF-κB AND MAPK PROTEINS ON LPS-INDUCED iNOS EXPRESSION AND NO PRODUCTION IN E-CAS AND RAW 264.7 CELLS

In e-CAS cells, a concentration-dependent inhibition of LPS-induced iNOS expression by helenalin (NF-κB inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and U0126 (ERK inhibitor) could be determined (Fig. 4). Co-incubation of inhibitors with LPS reduced the LPS-stimulated NO production in e-CAS cells in a similar concentration-dependent manner as observed for the iNOS expression (Fig. 5). Helenalin significantly inhibited NO production in LPS-treated e-CAS cells at 0.1 and 1 µM by 22% and 81%, respectively. The MAPK inhibitors, SP600125, SB203580 and U0126, significantly blocked LPS-induced NO production at only 10 µM by 50%, 30% and 36%, respectively. Control experiments confirmed that in absence of LPS, none of the inhibitors induced iNOS expression or NO production (data not shown).

Similarly, in RAW 264.7 cells, inhibition of NF-κB and the three MAPK isoforms significantly suppressed the stimulatory effect of LPS on iNOS expression (Fig. 6).

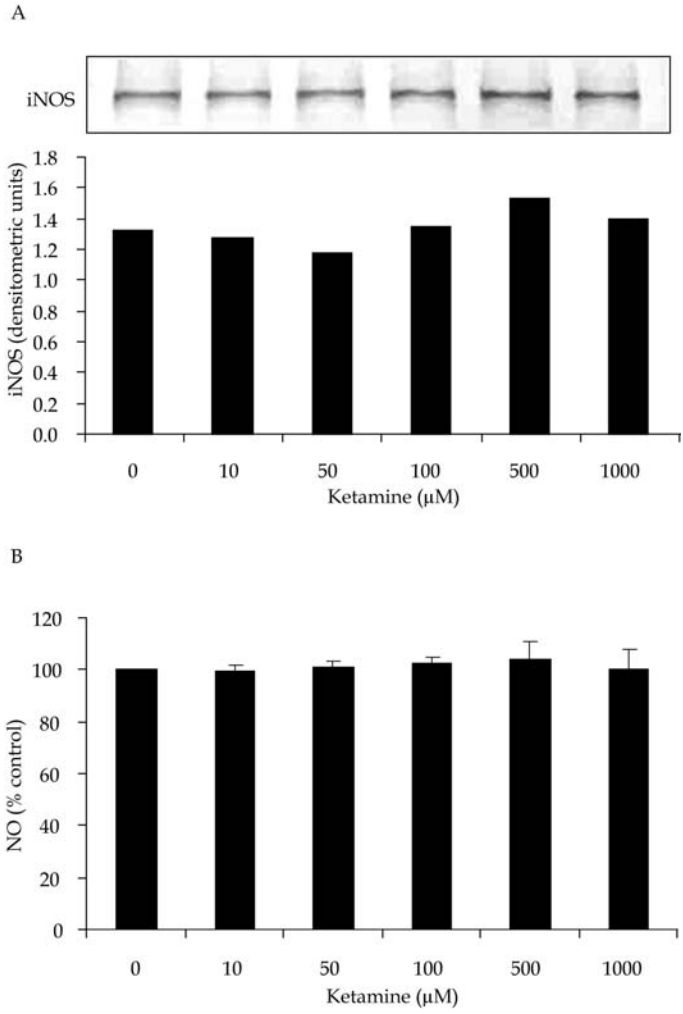

Figure 2. Ketamine at high experimental concentrations does not affect LPS-induced (A) iNOS expression and (B) NO production in e-CAS cells. (A) Cells were incubated with LPS (1 μg/mL) and ketamine (0-1000 μM) for 24 h. Following exposure, cells were lysed and lysates were analysed for iNOS expression by Western blotting. A representative Western blot of three independent experiments with densitometric quantification of the iNOS bands is shown. (B) Cells were incubated with LPS (1 μg/mL) and ketamine (0-1000 μM) for 24 h. Following exposure, supernatants were analysed for NO production by Griess reaction. LPS alone was regarded as a positive control and set at 100%. Data are expressed as mean ± S.D. of three independent experiments carried out in triplicate.

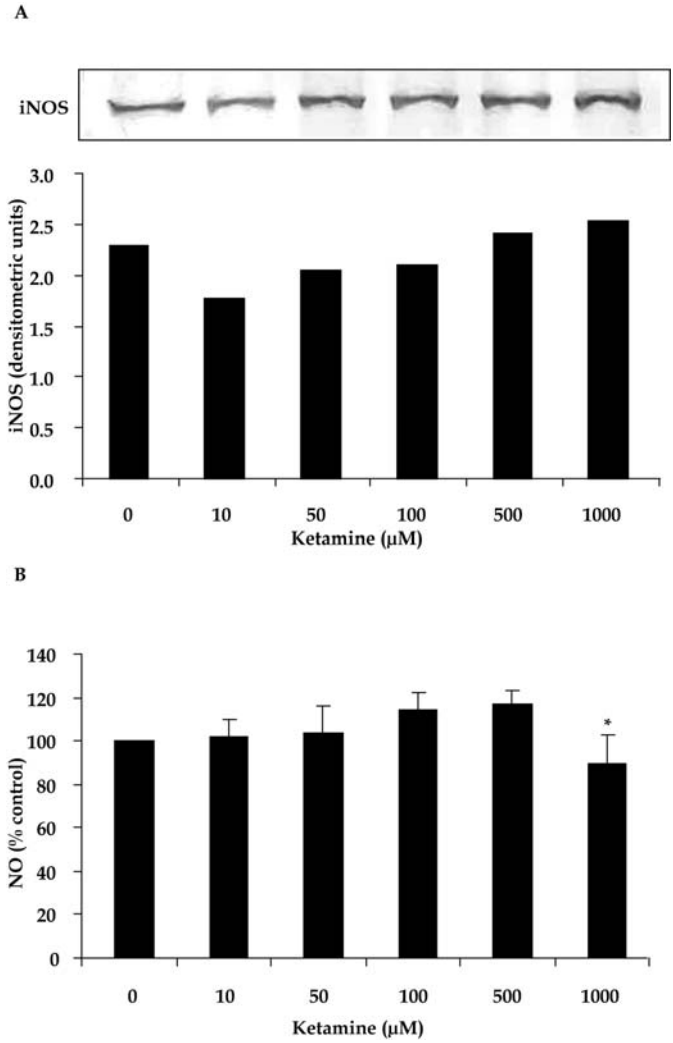

Figure 3. Ketamine at high experimental concentrations does not affect LPS-induced (A) iNOS expression and (B) NO production in RAW 264.7 cells. (A) Cells were incubated with LPS (1 μg/mL) and ketamine (0-1000 μM) for 24 h. Following exposure, cells were lysed and lysates were analysed for iNOS expression by Western blotting. A representative Western blot of three independent experiments with densitometric quantification of the iNOS bands is shown. (B) Cells were incubated with LPS (1 μg/mL) and ketamine (0-1000 μM) for 24 h. Following exposure, supernatants were analysed for NO production by Griess reaction. LPS alone was regarded as a positive control and set at 100%. Data are expressed as mean ± S.D. of three independent experiments carried out in triplicate. * statistically different to LPS alone ($p<0.05$).

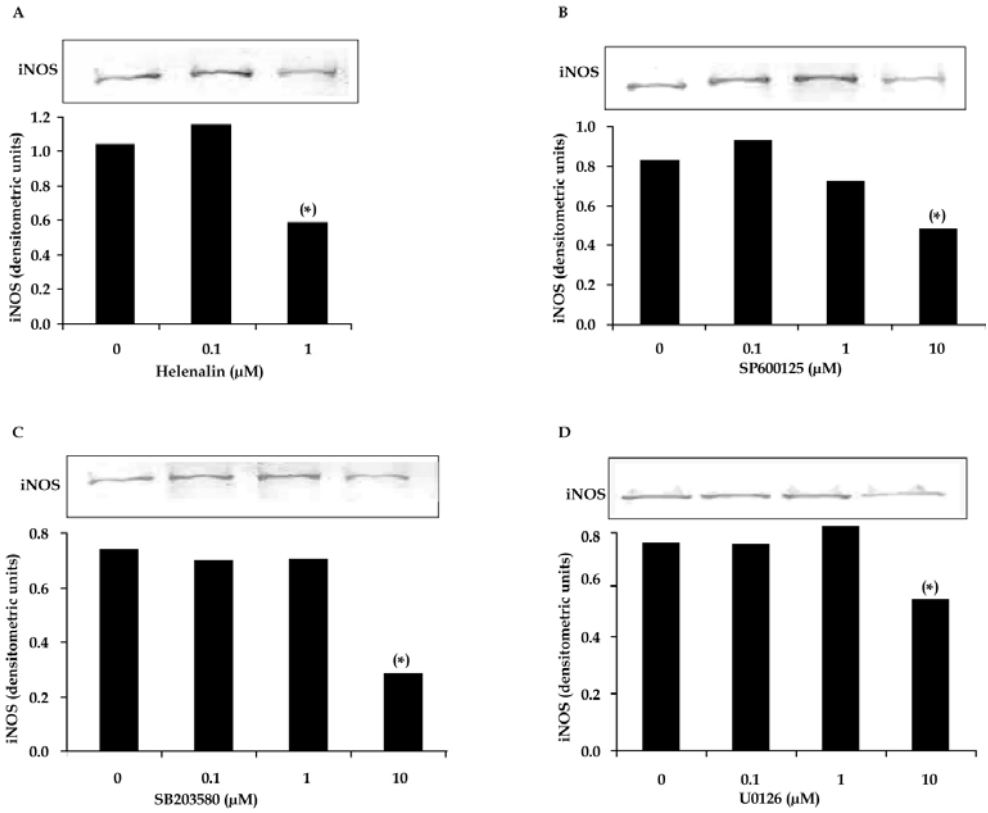

Figure 4. Inhibition of LPS-induced iNOS expression in e-CAS cells by NF-κB and MAPK inhibitors. Cells were incubated with LPS (1 μg/mL) and signal transduction inhibitors against (A) NF-κB (helenalin; 0-1 μM), (B) JNK (SP600125; 0-10 μM), (C) p38 (SB203580; 0-10 μM) or (D) ERK (U0126; 0-10 μM). Following 24 h of exposure, cells were lysed and lysates were analysed for iNOS expression by Western blotting. Representative Western blots of three independent experiments with densitometric quantification of the iNOS bands are shown. (*) statistically different to LPS alone in the corresponding experiments ($p<0.05$).

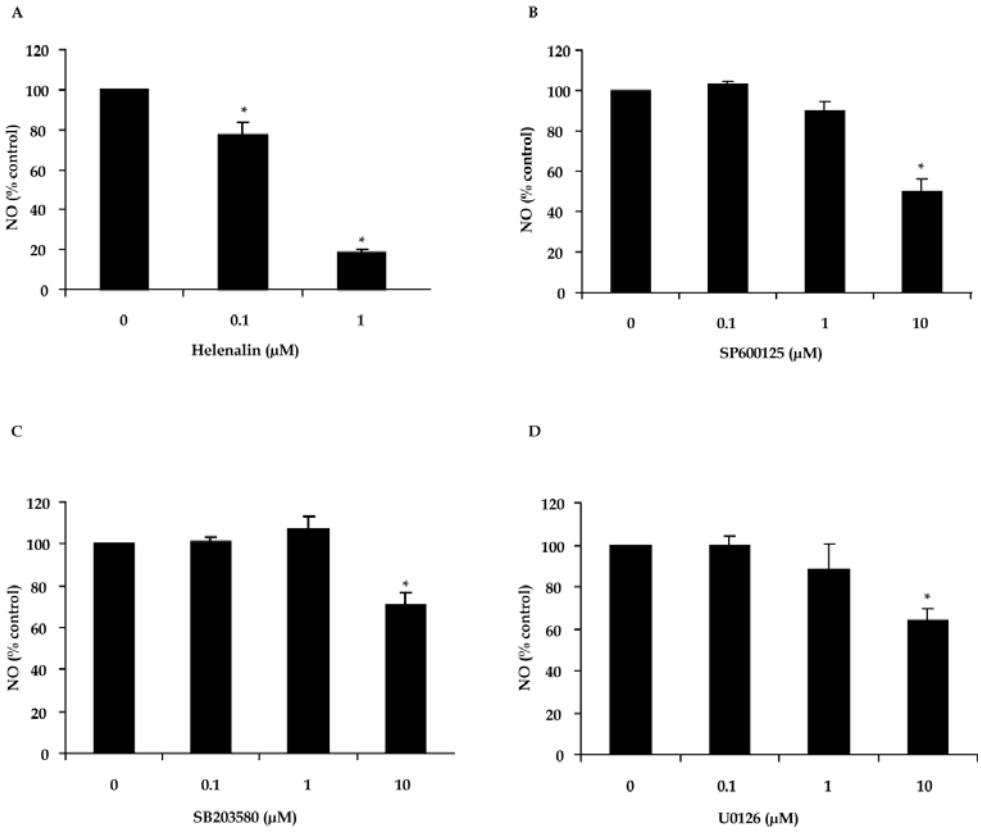

Figure 5. Inhibition of LPS-induced NO production in e-CAS cells by NF-κB and MAPK inhibitors. Cells were incubated with LPS (1 μg/mL) and signal transduction inhibitors against (A) NF-κB (helenalin; 0-1 μM), (B) JNK (SP600125; 0-10 μM), (C) p38 (SB203580; 0-10 μM) or (D) ERK (U0126; 0-10 μM). Following 24 h of exposure, supernatants were analysed for NO production by Griess reaction. Data are expressed as mean ± S.D. of three independent experiments carried out in triplicate. LPS alone was regarded as a positive control and set at 100%. * statistically different to LPS alone ($p<0.05$).

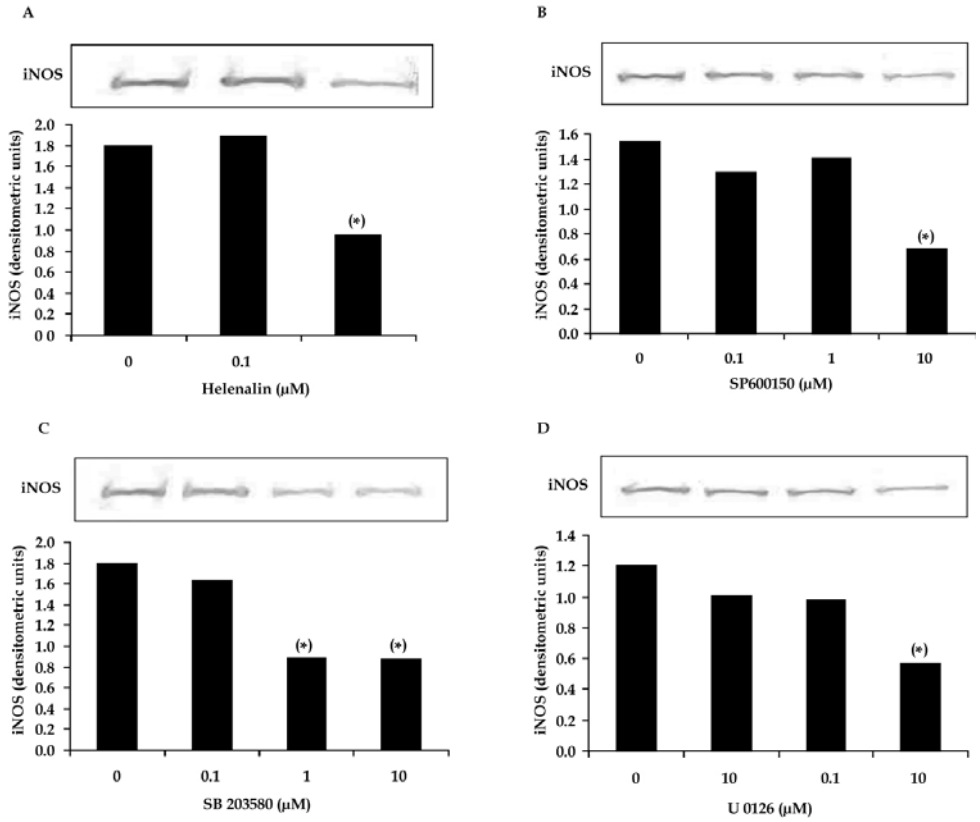

Figure 6. Inhibition of LPS-induced iNOS expression in RAW 264.7 cells by NF-κB and MAPK inhibitors. Cells were incubated with LPS (1 μg/mL) and signal transduction inhibitors against (A) NF-κB (helenalin; 0-1 μM), (B) JNK (SP600125; 0-10 μM), (C) p38 (SB203580; 0-10 μM) or (D) ERK (U0126; 0-10 μM). Following 24 h of exposure, cells were lysed and lysates were analysed for iNOS expression by Western blotting. Representative Western blots of three independent experiments with densitometric quantification of the iNOS bands are shown. (*) statistically different to LPS alone in the corresponding experiments ($p<0.05$).

4. DISCUSSION

In rodents and humans, iNOS and its product NO have been shown to be involved in many inflammatory disorders, such as arthritis, colitis, neurodegenerative diseases and septic shock.[16,19] In analogy to rodents and humans, it has been suggested that both iNOS and NO play a significant role in equine inflammatory diseases as well. Indeed, in vitro experiments showed that iNOS expression and NO production are significantly increased in LPS-treated equine alveolar macrophages,[35] and in articular chondrocytes.[36] In vivo, Mirza et al.[37] showed a significant increase in iNOS staining in mucosal leukocytes and mucosal vasculature in horses suffering from a strangulating large colon volvulus. Moreover, NO concentrations determined in articular cartilage, subchondral bone and the underlying trabecular bone were shown to be higher in horses suffering from osteoarthritis than in healthy horses.[38] In addition, both iNOS expression and NO production were shown to be higher in uterine biopsies and secretions of mares suffering from breeding-induced endometritis than in mares resistant to it.[39] The findings of these experiments suggest that inhibiting NO production by blocking iNOS expression might be beneficial in limiting the inflammatory response in horses.

Previous characterisation of e-CAS cells had already indicated that these cells are capable of expressing iNOS and producing NO in response to LPS.[32] In the present study, a time-dependent increase in NO production could be demonstrated. The delayed increase in NO production at 8 h following LPS treatment is consistent with the preceding onset of LPS-induced iNOS expression observed in the experimental studies carried out in murine macrophages.[40] The second increase following an apparent steady-state level between 16 and 24 h after LPS stimulation is thought to be related to an intracellular stress-response resulting from serum deprivation over a longer period. The latter is also reflected by simultaneous reduction in cell viability. Based on these data, the 24 h time point was selected to measure NO production in e-CAS cells in the further experiments.

The clinically relevant ketamine concentrations used in this study were selected in consideration of the plasma level of 5 μg/ml (18 μM) following the IV induction dose of 2.2 mg/kg body weight commonly used in horses.[41] Moreover, at these concentrations ketamine has been shown to reduce NF-κB expression in e-CAS cells.[1] Since NF-κB regulates iNOS expression, initially the clinically relevant ketamine concentrations were tested. As these ketamine concentrations did not affect LPS-induced iNOS expression and NO production in e-CAS cells, high

experimental ketamine concentrations up to 1000 μM were tested to study if ketamine could affect LPS-induced iNOS expression and NO production at all.

The fact that ketamine did not inhibit LPS-induced iNOS expression and NO production at any of the concentrations tested in e-CAS cells is in contrast with results obtained from other experimental studies. In vitro, ketamine (10-100 μM) reduced iNOS expression and NO production in a concentration-dependent manner in LPS-treated rat alveolar macrophages.[28] Moreover, in J774 macrophages, ketamine concentrations of 30-600 significantly decreased NO production in response to LPS and interferon-γ (IFN-γ).[27] In vivo, ketamine blunted iNOS expression in the stomach, ileum and liver of LPS-treated rats.[29,30] The disparities between the literature reports and our findings might be due to interspecies differences and specific sensitivity of individual cell types and organs, but are more likely reflecting differences in the experimental protocols used. The prototypical inducer of iNOS expression is LPS, but IFN-γ is frequently added to increase iNOS expression and NO production in LPS-primed cells.[40,42] However, the molecular pathway by which LPS induces iNOS expression and NO production differs from that of IFN-γ. Lipopolysaccharide binds to CD14 and activates the transcription factor NF-κB via the IKK-IκB pathway, whereas IFN-γ binds to IFN-γ receptors at the cell membrane and stimulates the transcription factor IFN regulatory factor 1 via the JAK-STAT signalling pathway.[15,16,42] Hence, differences in outcome might be due to the fact that ketamine differently affects the distinct pathways involved in regulating iNOS expression.

The inhibition experiments presented in this paper indicate that both NF-κB and the MAPKs, JNK, p38, and ERK, are involved in the LPS-induced regulation of iNOS expression and NO production in e-CAS cells. Similar results were found in RAW 264.7 cells used in this study. These data correlate with previous inhibition experiments performed in other cell culture models.[43-47] However, in previous experiments with e-CAS cells, ketamine was found to significantly inhibit the LPS-induced signalling pathways only at the level of NF-κB.[1] This finding and the fact that NF-κB alone is not sufficient to suppress iNOS expression, explains the failure of ketamine to significantly reduce iNOS expression and the subsequent NO production.

In conclusion, ketamine does not affect the MAPK and NF-κB-mediated iNOS expression and subsequent NO production in LPS-treated e-CAS cells, which is in line with previous data showing that ketamine affects the LPS-induced signal transduction pathway only at the level of NF-κB.[1]

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- CHAPTER VI -

EFFECTS OF KETAMINE ON LPS-INDUCED COX-2 EXPRESSION AND PGE_2 PRODUCTION IN AN EQUINE MACROPHAGE CELL LINE

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SUMMARY

Ketamine has been reported to reduce lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production in an equine macrophage cell line (e-CAS cells) by directly inhibiting nuclear factor-kappa B (NF-κB) expression.[1] NF-κB is a key regulatory transcription factor associated with the expression of various pro-inflammatory mediators, including cytokines and inducible enzymes like cyclooxygenase-2 (COX-2). Prostaglandin E_2 (PGE_2), the main product of COX-2, significantly contributes to the clinical symptoms of inflammation. Hence, COX-2 inhibitors such as the non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to reduce PGE_2 production. Considering the effect of ketamine on LPS-induced NF-κB expression, it was hypothesised that ketamine might have also an effect on LPS-induced COX-2 expression.

To this end, we studied the effect of ketamine (0-1000 μM) on LPS-induced (1 μg/ml) COX-2 expression and PGE_2 production in e-CAS cells. Furthermore, the intracellular signalling molecules, c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase p38 (p38), extracellular signal-regulated kinase (ERK), and NF-κB were selectively inhibited, to elucidate the molecular mechanism underlying LPS-induced COX-2 expression and subsequent PGE_2 production in e-CAS cells. For comparison RAW 264.7 cells were used.

In LPS-treated e-CAS cells, ketamine did not affect COX-2 expression at any of the concentrations tested. Ketamine significantly reduced LPS-induced PGE_2 production only at the highest ketamine concentration (1000 μM) investigated. The inhibition experiments suggest that COX-2 expression and the subsequent PGE_2 production are mainly regulated at the level of JNK, p38 and ERK in the e-CAS cell model and that activated NF-κB does not significantly contribute to COX-2 induction in e-CAS cells. In previous experiments with e-CAS cells, ketamine was found to inhibit the LPS-induced signalling pathways only at the level of NF-κB,[1] which is in line with the finding that ketamine does not inhibit COX-2 expression in LPS-treated e-CAS cells. Hence, despite its potency to reduce LPS-induced TNF-α and IL-6 production, the role of ketamine in COX-2 and PGE_2 associated equine inflammatory disorders might be limited.

1. INTRODUCTION

Ketamine is commonly used in a variety of equine anaesthesia procedures. Besides the induction of anaesthesia,[2] ketamine can be used as an adjunct to inhalant anaesthesia,[3-5] in total intravenous anaesthesia (TIVA) protocols,[6-8] for epidural analgesia[9] and peripheral nerve blocks.[10] More recently, studies focus on the antinociceptive effects of a subanaesthetic continuous rate infusion (CRI) of ketamine in conscious horses.[11] Besides its anaesthetic and analgesic effects, ketamine has been found to possess cytokine-modulating effects in rodents[12-14] and humans.[15,16] Previously, we demonstrated that in an equine macrophage cell line (e-CAS cells), ketamine inhibits the lipopolysaccharide (LPS)-induced release of both tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) production by directly inhibiting nuclear factor kappa B (NF-κB) expression in a concentration-dependent manner, even at subanaesthetic concentrations.[1,17] NF-κB is a key regulatory transcription factor, which is primarily sequestered in the cytoplasm in an inactive form by association with its inhibitory protein, 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 initiate the transcription of multiple pro-inflammatory mediators such as cytokines, adhesion molecules and enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).[18-20] Alternatively, the transcription of many these pro-inflammatory mediators can be regulated by mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase p38 (p38) and c-Jun N-terminal kinase (JNK).[21]

COX enzymes catalyse the biosynthesis of prostanoids such as prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 (TXA_2), by sequential oxygenation and reduction of membrane-derived arachidonic acids. COX enzymes (72 kDa) consist of two isoforms: COX-1 and COX-2, sharing a 61% homology at the amino acid sequence.[22,23] COX-1 is ubiquitously expressed in mammalian cells and mainly involved in 'housekeeping' functions like maintaining gastric mucosal integrity, initiating platelet aggregation, regulating renal blood flow and glomerular filtration.[22] COX-2 is an inducible enzyme primarily expressed by cells that mediate inflammation such as macrophages, monocytes and neutrophils.[24] In response to inflammation, COX-2 expression results in an increased synthesis of prostaglandin E_2 (PGE_2), which contributes to the clinical syndrome of inflammation including vasodilation, oedema, fever and hyperalgesia.[25-27] Hence, to reduce PGE_2 production in clinical human and animal

patients, COX-2 inhibitors as non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment of inflammatory disorders.[28]
Considering the effect of ketamine on TNF-α and IL-6 release by directly inhibiting NF-κB, it was hypothesised that ketamine might also reduce LPS-induced COX-2 expression and the subsequent PGE_2 production. Hence, these effects as well as the involvement of JNK, p38, ERK, and NF-κB on LPS-induced COX-2 expression and PGE_2 production were investigated in an equine macrophage cell line (e-CAS cells). To support the validity of the e-CAS model, RAW 264.7 cells were used for comparison.

2. MATERIALS AND METHODS

2.1. CHEMICALS

Ketamine, lipopolysaccharide (LPS; Escherichia coli, O111:B4), bovine serum albumin (BSA), Tris, ethylenediaminotetraacetic acid (EDTA), Igepal (CA-630), sodium deoxycholate, sodium dodecyl sulphate (SDS), glycerol, bromphenolblue, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), dithiothreitol (DTT) and anti-PGE_2 antiserum were purchased from Sigma chemicals (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, glutamine and phosphate buffered saline (PBS) were obtained from Biocambrex (Verviers, Belgium). Horse serum (HS), fetal bovine serum (FBS), non-essential amino acids and sodium pyruvate were from Gibco Invitrogen (Breda, The Netherlands). Alamar blue was purchased from BioSource International (Etten-Leur, The Netherlands). Helenalin, SP600125, SB203580 and U0126 were from Biomol (Plymouth, USA). Polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, Massachusetts, USA). Dimethyl Sulfoxide (DMSO) was from Fisher Emergo B.V. (Landsmeer, The Netherlands). Tween-20 was from Merck KGaA (Darmstadt, Germany). Bradford Protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). MgCl2.6H20 was from VWR International (Amsterdam, The Netherlands). Rabbit polyclonal anti-COX-2 was from Abcam (Cambridge, UK). Alkaline phosphatase conjugated with polyclonal goat anti-rabbit immunoglobulin was purchased from Dako Cytomation (Glostrup, Denmark). [^{3}H]PGE_2 was obtained from Amersham-Pharmacia Biotech (Veenendaal, The Netherlands). Ultima Gold was from Perkin Elmer (Wellesley, MA, USA).

2.2. CELL LINES AND CULTURE CONDITIONS

Two cell lines were used: the equine bone-marrow-derived macrophage cell line (e-CAS cells) described by Werners et al.,[29] and the murine macrophage-like cell line (RAW 264.7; ATCC No TIB-71).

The e-CAS cells were 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 ^{o}C, in a humidified atmosphere of 5% CO_2/ 95 % air.

The RAW 264.7 cells were cultured in DMEM, supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine and 1 mM sodium pyruvate. Cells were maintained at 37 ^{o}C, in a humidified atmosphere of 5% CO_2/ 95 % air as well.

To determine the relative expression of COX-2 protein, cells were cultured in 6-wells plates at a density of $1.0x10^6$/well (2 mL). For measuring PGE_2 production, cells were seeded in 24-well plates at a density of $0.2x10^6$/well (1 mL). Following 24 h of culturing, different experiments were performed as described below.

2.3. TIME RESPONSE CURVE

To determine the time-dependent PGE_2 production in e-CAS cells, cells were incubated with serum-free RPMI 1640 medium containing 1 µg/mL LPS. Cells exposed to medium only served as control. Production of PGE_2 and cell viability was determined every 4 hrs following 4 - 48 h of incubation.

2.4. EXPOSURE TO LPS AND KETAMINE

To study the influence of ketamine on LPS-induced COX-2 expression and PGE_2 production, e-CAS cells were exposed to serum-free medium containing LPS (1 µg/mL) and ketamine concentrations at a range of 0-36 µM or 0-1000 µM, comprising clinically relevant as well as high experimental concentrations, respectively. Based on the results of the time-response curves, COX-2 expression, PGE_2 production and cell viability were determined following 24 h of exposure.

For control experiments, RAW cells were exposed to 1 µg/mL LPS and the high concentration range of ketamine (0-1000 µM).

2.5. INHIBITION EXPERIMENTS

To investigate the potential involvement of MAP kinases and NF-κB on LPS-induced COX-2 expression and PGE_2 production, cells were exposed to serum-free medium containing LPS (1 µg/mL) and specific inhibitors of JNK (SP600125; 0-10

μM), p38 (SB203580; 0-10 μM), pERK (U0126; 0-10 μM) or NF-κB (helenalin; 0-1 μM). To control samples, equal amounts of inhibitor solvent (0.1% DMSO) and/or LPS solvent (PBS) were added. COX-2 expression, PGE_2 production and cell viability were determined following 24 h of incubation.

2.6. WESTERN BLOT ANALYSIS

Expression of COX-2 protein was determined by Western blot analysis. In brief, after exposure, cellular extracts were prepared in RIPA buffer [150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl (pH 8)]. Protein concentrations were measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Cell pellets were resuspended in sample buffer [12.5% glycerol, 1.25 % SDS, 0.004% bromophenol blue, 95 mM DTT and 62.5 mM Tris-HCl (pH 8.8)]. Aliquots containing 40 μg of protein were separated by SDS-PAGE (10%) 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-COX-2 diluted 1:500) for 1 h at room temperature (RT). After washing, blots were incubated with secondary antibodies (horse radish peroxidase conjugated polyclonal goat anti-rabbit 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.

2.7. PGE_2 RADIO-IMMUNOASSAY

Production of PGE_2 was measured by radio-immunoassay (RIA). In brief, anti-PGE_2 antiserum and [^{3}H]PGE_2 were added to each standard or sample. The tubes were incubated for 18-24 h at 4^{o}C. After incubation, dextran-coated charcoal suspension (20 mg/ml charcoal, 4 mg/ml dextran) was added and tubes were centrifuged (3000 rpm, 4^{o}C, 15 min) to separate bound and unbound fractions. Supernatants, containing the bound fraction, were transferred into scintillation-vials and scintillation fluid (Ultima Gold) was added. After mixing, samples were analysed using a liquid scintillation counter (Tri-carb 2900TR, Packard). Concentrations of PGE_2 were determined by comparing the values of the samples with a standard curve.

2.8. CELL VIABILITY

The Alamar Blue ™ (AB) reduction cytotoxicity assay was used as an indicator of cell viability.[30] 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 ^{o}C for 4 h. Thereafter, resorufin formation was measured fluorometrically at 530-560 nm excitation wavelength and 590 nm emission wavelength.

2.9. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS® 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (S.D.) obtained from three independent experiments. One-way analysis of variance (ANOVA) was used to test for significant differences between group means. When appropriate, post hoc multiple comparisons were performed to test for differences between experimental groups (Dunnett's). Data were considered to be statistically significant when $p<0.05$.

3. RESULTS

3.1. TIME-DEPENDENT PGE_2 PRODUCTION IN LPS-STIMULATED E-CAS CELLS

To determine the time response for PGE_2 production in LPS-treated e-CAS cells, PGE_2 production and cell viability were determined every 4 hrs following 4 - 48 h of LPS (1 μg/mL) exposure. Over time, a significant increase in LPS-induced PGE_2 production was demonstrated (Fig. 1). Cell viability remained above 90% until 28 h of LPS exposure. Thereafter, viability significantly decreased towards the end of the experiment. Based on these results, COX-2 expression, PGE_2 production and cell viability were determined following 24 h of LPS exposure in the further experiments with e-CAS cells.

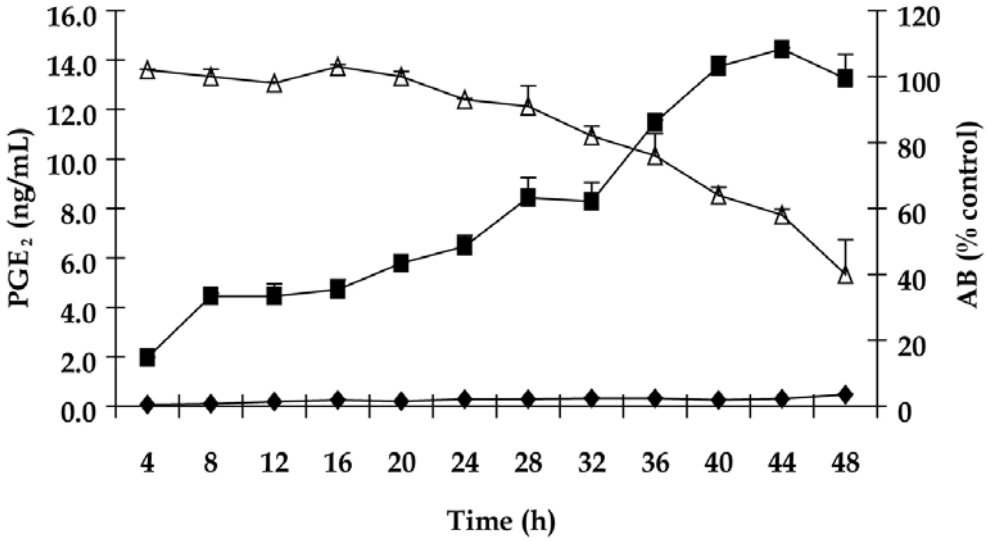

Figure 1. Time response curve of PGE_2 production in e-CAS cells. Cells were incubated with serum-free RPMI 1640 medium containing no (♦) or 1 μg/mL (■) LPS. PGE_2 production and cell viability (△) were determined every 4 h following 4 - 48 h of incubation. Data are expressed as mean ± S.D. of two independent experiments carried out in duplicate.

3.2. CELL VIABILITY

In all forthcoming experiments, cell viability was also tested by using the Alamar Blue cytotoxicity assay, but no significant differences were found (data not shown).

3.3. INFLUENCE OF KETAMINE ON LPS-INDUCED COX-2 EXPRESSION IN E-CAS AND RAW 264.7 CELLS

To study the influence of ketamine on LPS-induced COX-2 expression in e-CAS cells, cells were initially exposed to LPS (1 μg/mL) in the presence of a clinically relevant ketamine concentration range (0-36 μM). Western blot analysis showed that COX-2 expression was undetectable in non-stimulated e-CAS cells, but significantly increased in the presence of LPS (1 μg/mL). At clinical concentrations, ketamine did not significantly affect LPS-induced COX-2 expression in e-CAS cells. Subsequently, LPS-treated e-CAS and RAW 264.7 cells were exposed to high experimental ketamine concentrations (0-1000 μM). Again, no significant influence on COX-2 expression was found (Figs 2A and 3A).

Control experiments revealed that in absence of LPS, ketamine did not induce COX-2 expression in the tested concentration range (data not shown).

3.4. EFFECT OF NF-κB AND MAPK INHIBITORS ON LPS-INDUCED COX-2 EXPRESSION IN E-CAS AND RAW 264.7 CELLS

In both e-CAS and RAW 264.7 cells, helenalin (NF-κB inhibitor) did not significantly influence LPS-induced COX-2 expression (Figs 4A and 6A). In contrast, SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and U0126 (ERK inhibitor) significantly reduced COX-2 expression in LPS-treated e-CAS cells and RAW 264.7 cells (Figs 4 and 6, BCD, respectively). In LPS-treated e-CAS cells, SP600125 significantly inhibited COX-2 expression at 10 μM, while SB203580 and U0126 significantly blocked LPS-induced COX-2 expression at 1 and 10 μM (Fig. 4 BCD, respectively). Similar results were found in LPS-treated RAW 264.7 cells, although U0126 significantly blocked COX-2 expression only at 10 μM (Fig. 6 BCD).

Control experiments confirmed that in absence of LPS, none of the inhibitors induced COX-2 expression in e-CAS and RAW 264.7 cells (data not shown).

3.5. INFLUENCE OF KETAMINE ON LPS-INDUCED PGE_2 PRODUCTION

Exposure to LPS significantly increased PGE_2 production in e-CAS cells (from 0.17 ± 0.05 to 3.21 ± 0.24 ng/mL) and RAW 264.7 cells (from 0.04 ± 0.04 to 2.9 ± 0.7 ng/mL) when compared to non-treated cells.

Similarly to the effect of ketamine on LPS-induced COX-2 expression, ketamine at clinically relevant concentrations did not significantly inhibit LPS-induced PGE_2 production.

Following exposure to high experimental concentrations, PGE_2 production was only significantly reduced in e-CAS cells at a concentration of 1000 μM (Fig. 2B). In RAW 264.7 cells, no significant influence of experimental ketamine concentrations on LPS-induced PGE_2 production was found (Fig. 3B).

3.6. INFLUENCE OF MAPK AND NF-κB INHIBITORS ON LPS-INDUCED PGE_2 PRODUCTION

Corresponding to the influence of the selective inhibitors on LPS-induced COX-2 expression, PGE_2 production was only significantly reduced following inhibition of JNK, p38 and ERK in LPS-treated e-CAS and RAW 264.7 cells (Figs 5 and 7, BCD, respectively). Again, inhibition of NF-κB did not significantly reduce LPS-induced PGE_2 production in e-CAS and RAW 264.7 cells (Figs 5A and 7A).

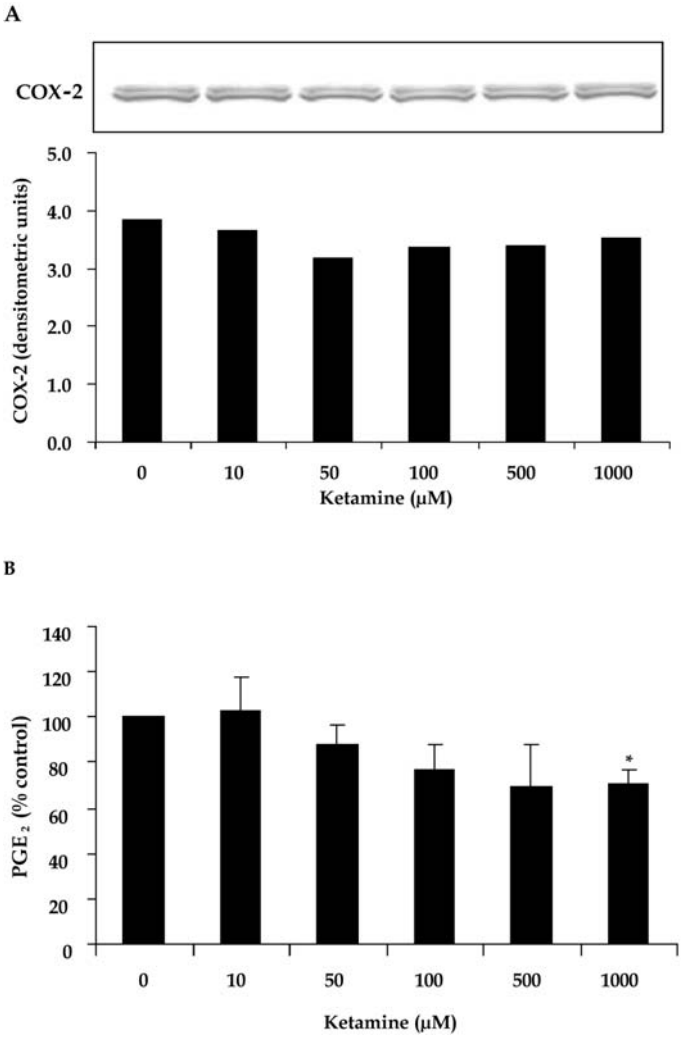

Figure 2. Effect of high experimental ketamine concentrations on LPS-induced (A) COX-2 expression and (B) PGE_2 production in e-CAS cells. (A) Cells were stimulated with LPS (1 μg/mL) and ketamine (0-1000 μM) for 24 h. Following incubation, cells were lysed and lysates were assayed for COX-2 expression by Western blot analysis. A representative Western blot of three independent experiments with densitometric quantification of the COX-2 bands is shown. (B) PGE_2 production in e-CAS cells was measured by radio-immunoassay (RIA) after 24 h of exposure to LPS (1 μg/mL) and ketamine (0-1000 μM). LPS alone was regarded as a positive control and set at 100%. Data are expressed as mean ± S.D. of three independent experiments carried out in triplicate. * statistically different from LPS alone ($p<0.05$).

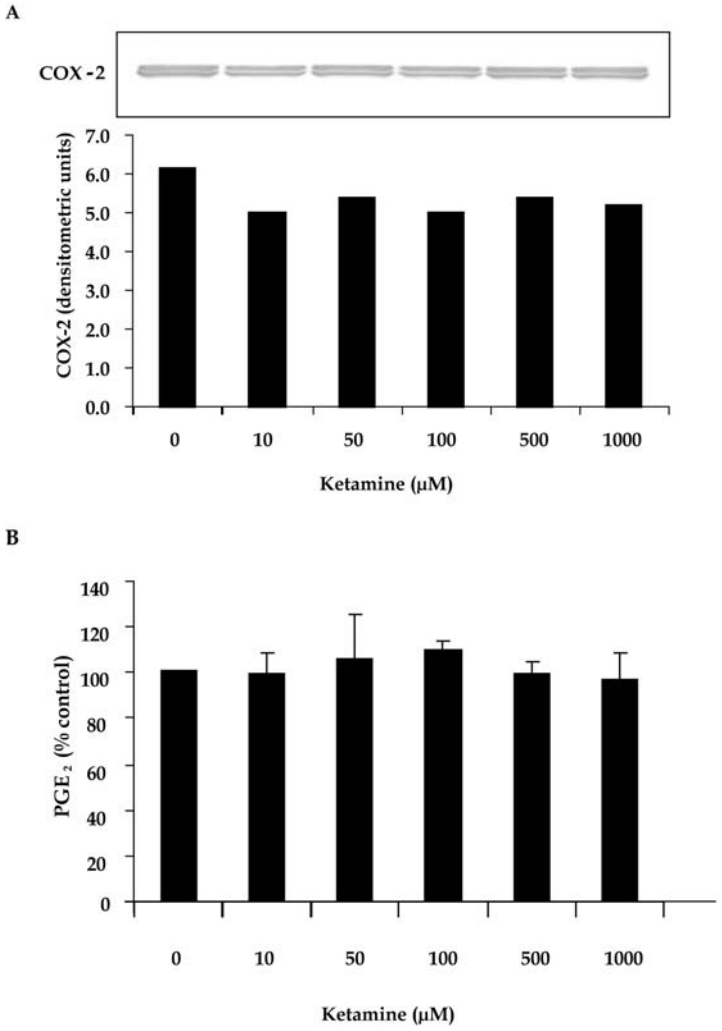

Figure 3. Effect of high experimental ketamine concentrations on LPS-induced (A) COX-2 expression and (B) PGE_2 production in RAW 264.7 cells. (A) Cells were stimulated with LPS (1 μg/mL) and ketamine (0-1000 μM) for 24 h. Following incubation, cells were lysed and lysates were assayed for COX-2 expression by Western blot analysis. A representative Western blot of three independent experiments with densitometric quantification of the COX-2 bands is shown. (B) PGE_2 production in RAW 264.7 cells was measured by radio-immunoassay (RIA) after 24 h of exposure to LPS (1 μg/mL) and ketamine (0-1000 μM). LPS alone was regarded as a positive control and set at 100%. Data are expressed as mean ± S.D. of three independent experiments in triplicate.

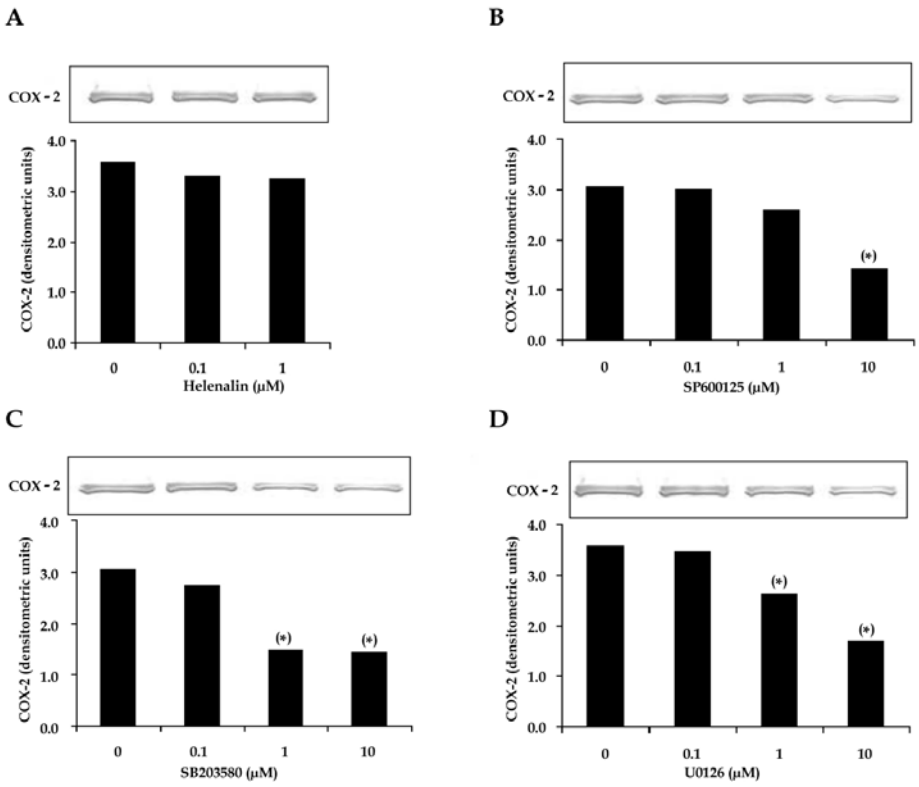

Figure 4. Effect of (A) helenalin, (B) SP600125, (C) SB203580 and (D) U0126 on LPS-induced COX-2 expression in e-CAS cells. Cells were incubated with the specific inhibitors of NF-κB (helenalin; 0-1 μM), JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM) and ERK (U0126; 0-10 μM) and LPS (1 μg/mL) for 24 h. Following incubation, cells were lysed and lysates were assayed for COX-2 expression by Western blot analysis. Representative Western blots of three independent experiments with densitometric quantification of the COX-2 bands are shown. (*) statistically different to LPS alone in the corresponding experiments ($p<0.05$).

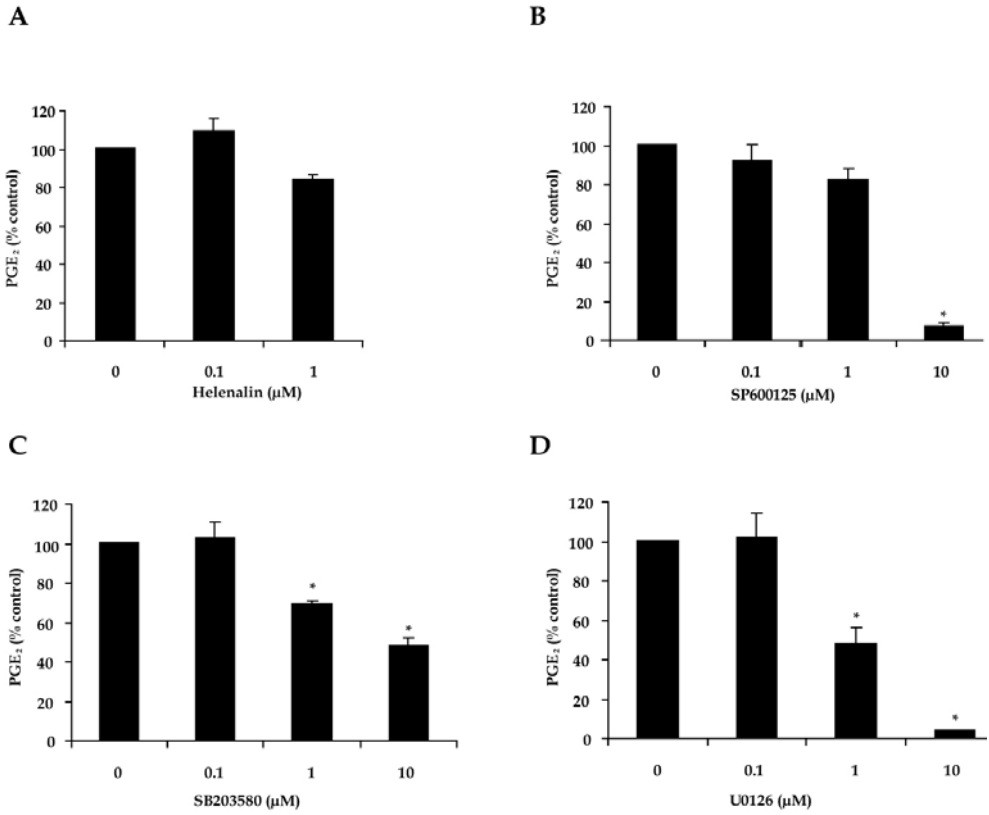

Figure 5. Effect of (A) helenalin, (B) SP600125, (C) SB203580 and (D) U0126 on LPS-induced PGE_2 production in e-CAS cells. Cells were incubated with the specific inhibitors of NF-κB (helenalin; 0-1 μM), JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM) and ERK (U0126; 0-10 μM) and LPS (1 μg/mL) for 24 h. Following incubation, PGE_2 production in RAW 264.7 cells was measured by radio-immunoassay (RIA). LPS alone was regarded as a positive control and set at 100%. Data are expressed as mean ± S.D. of three independent experiments in triplicate. *statistically different from LPS alone ($p<0.05$).

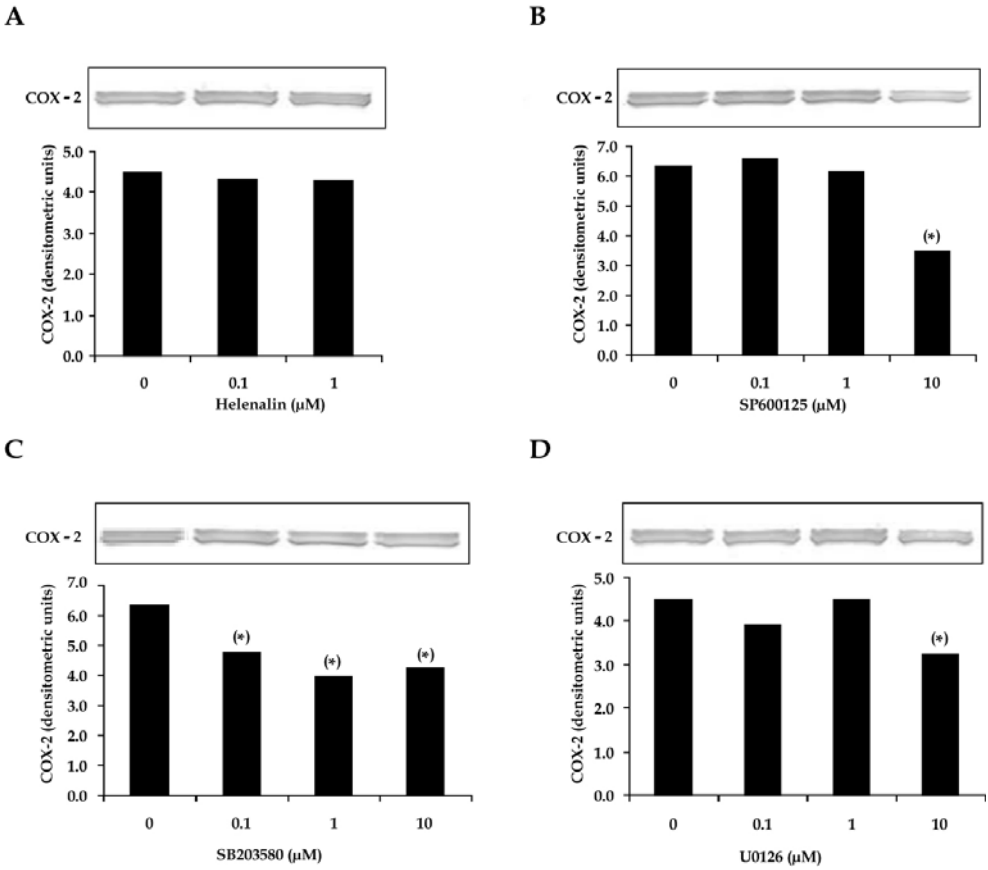

Figure 6. Effect of (A) helenalin, (B) SP600125, (C) SB203580 and (D) U0126 on LPS-induced COX-2 epxression in RAW 264.7 cells. Cells were incubated with the specific inhibitors of NF-κB (helenalin; 0-1 μM), JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM) and ERK (U0126; 0-10 μM) and LPS (1 μg/mL) for 24 h. Following incubation, cells were lysed and lysates were assayed for COX-2 expression by Western blot analysis. Representative Western blots of three independent experiments with densitometric quantification of the COX-2 bands are shown. (*) statistically different to LPS alone in the corresponding experiments ($p<0.05$).

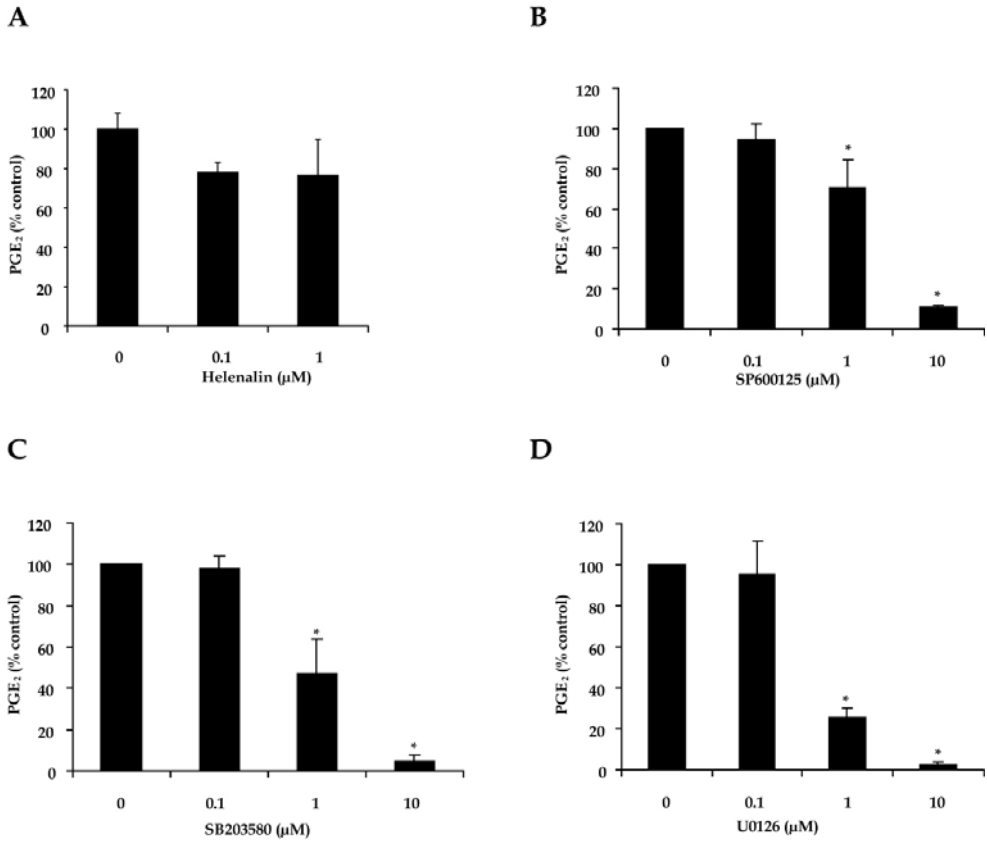

Figure 7. Effect of (A) helenalin, (B) SP600125, (C) SB203580 and (D) U0126 on LPS-induced PGE_2 production in RAW 264.7 cells. Cells were incubated with the specific inhibitors of NF-κB (helenalin; 0-1 μM), JNK (SP600125; 0-10 μM), p38 (SB203580; 0-10 μM) and ERK (U0126; 0-10 μM) and LPS (1 μg/mL) for 24 h. Following incubation, PGE_2 production in RAW 264.7 cells was measured by radio-immunoassay (RIA). LPS alone was regarded as a positive control and set at 100%. Data are expressed as mean ± S.D. of three independent experiments in triplicate. *statistically different from LPS alone ($p<0.05$).

4. DISCUSSION

Expression of COX-2 and subsequent PGE_2 synthesis have been associated with different inflammatory conditions such as colitis,[31] ischemia-reperfusion injury,[32] synovitis,[33] osteoarthritis,[34] osteochondrosis dissecans[35] and equine endotoxaemia.[36]

Inhibitors of COX-2, like the non-steroidal anti-inflammatory drugs (NSAIDs), are commonly used to reduce COX-2 expression and subsequent PGE_2 production, resulting in reduction of the clinical signs of inflammation.[37] In the present study, it was hypothesised that ketamine might reduce COX-2 expression as well, since COX-expression has been described to be regulated by NF-κB and ketamine has been found to possess cytokine-modulating effects by directly inhibiting NF-κB expression.[1,17,38,39] However, in both e-CAS and RAW 264.7 cells, ketamine did not reduce COX-2 expression at any of the tested concentrations.

It was previously assumed that LPS-induced COX-2 expression is controlled by an array of signalling molecules, including NF-κB and the three MAPKs (i.e., JNK, p38 and ERK).[21,24,40] However, the results of the present study demonstrate that LPS-induced COX-2 expression is not regulated via the NF-κB pathway, which is in line with the results found by Wadleigh et al.[41] The inhibition experiments in e-CAS and in RAW 264.7 cells revealed that LPS-induced COX-2 expression and PGE_2 production are predominantly regulated by the MAPKs ERK, p38 and JNK. In a previous experimental study regarding LPS-treated e-CAS cells, ketamine was found to significantly affect the LPS-induced signal transduction pathway only at the level of NF-κB.[1] This finding and the fact that COX-2 expression is not regulated by NF-κB, explains the failure of ketamine to significantly reduce LPS-induced COX-2 expression in the present study. However, since the highest concentration of ketamine (1000 μM) did significantly reduce PGE_2 production in LPS-treated e-CAS cells, an effect of this ketamine concentration on LPS-induced MAPK expression can not be fully excluded.

The clinically relevant ketamine concentrations used in this study were selected to mimic the plasma level of 5 μg/ml (18 μM) following the IV induction dose of 2.2 mg/kg body weight commonly used in horses.[42] Moreover, at these concentrations ketamine has been shown to reduce NF-κB expression in e-CAS cells.[1] Since NF-κB regulates COX-2 expression, initially clinically relevant ketamine concentrations were tested. As these ketamine concentrations did not affect LPS-induced COX-2 expression and PGE_2 production in e-CAS cells, high experimental ketamine

concentrations up to 1000 μM were tested to study if ketamine could affect LPS-induced COX-2 expression and PGE_2 production at all.

Ketamine is primarily used for its anaesthetic and analgesic properties. The analgesic potency of ketamine is achieved by its binding to N-methyl-D-aspartate (NMDA) receptors at which ketamine acts as an antagonist, resulting in the reduction of pathological pain states such as hyperalgesia, allodynia and the 'wind-up' phenomenon.[43-45] Increased PGE_2 concentrations are known to mediate hyperalgesia. Nishihara et al.[25] found that ketamine inhibits PGE_2-induced hyperalgesia via a NMDA-dependent mechanism. In addition, the results of the present study strongly suggest that the reduced PGE_2-induced hyperalgesia following ketamine exposure is not mediated by inhibiting COX-2 expression.

In conclusion, in LPS-treated e-CAS cells COX-2 expression is not regulated via the transcription factor NF-κB, but via the three classical MAPK pathways. Moreover, ketamine does not affect LPS-induced COX-2 expression in e-CAS cells. These findings are in line with previous data showing that ketamine affects the LPS-induced signal transduction pathway only at the level of NF-κB.

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- CHAPTER VII -

KETAMINE REDUCES PRODUCTION OF REACTIVE OXYGEN SPECIES IN AN EQUINE MACROPHAGE CELL LINE FOLLOWING STIMULATION WITH LPS AND PMA

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SUMMARY

Ketamine is commonly used in equine anaesthesia. Next to its anaesthetic and analgesic effects, ketamine has also been found to exhibit potent cytokine-modulating and antioxidative properties in rodents and humans. In addition, in an equine macrophage cell line (e-CAS cells), ketamine reduced lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production in a concentration-dependent manner.[1] In the present study, the antioxidative effects of ketamine in e-CAS cells were studied. Initially, two different ROS-inducers, LPS (1 μg/mL), phorbol myristate acetate (PMA; 1 μM) and the combination LPS (1 μg/mL)/PMA (1 μM) were tested. The capacity of ketamine (0-36 μM) to suppress reactive oxygen species (ROS) production in e-CAS cells was investigated by using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as a molecular probe. Furthermore, to determine the mechanism underlying the effect of ketamine on stimulated ROS production, the influence of ketamine on intracellular glutathione (GSH) concentrations was measured.

In e-CAS cells, a significant increase in ROS formation was documented only following exposure to the combination LPS/PMA. Ketamine significantly and dose-dependently reduced ROS formation in LPS/PMA-stimulated e-CAS cells. In addition, ketamine significantly increased total intracellular GSH concentrations in LPS/PMA-treated e-CAS cells in a dose-dependent manner. These results suggest that the antioxidative properties of ketamine in e-CAS cells are most likely related to an indirect, GSH-sparing effect of ketamine.

1. INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), are produced during normal cellular metabolism. Phagocytic cells like neutrophils, monocytes and macrophages respond to infectious agents with an increased ROS production, often referred to as respiratory burst, which plays a pivotal role in the innate host defence.[2,3] Under physiological conditions, ROS production is counterbalanced by various enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, that equilibrate the intracellular redox potential.[2-5] However, following infection or toxic cell injury, an excessive production of ROS occur, which interacts with cellular macromolecules resulting in cell function impairments, DNA damage and finally apoptosis and necrosis.[2,3]

In rodents and humans, ROS have been associated with inflammatory diseases like arthritis,[6] asthma,[7] cardiac injury[8] and sepsis.[9] Moreover, numerous toxins have been found to induce ROS production. In horses, ROS production has been primarily implicated in the pathogenesis of gastrointestinal ischaemia-reperfusion injury.[4,10] The application of ROS scavengers is considered to be of therapeutic advantage in many diseases, particularly as part of a multimodal therapy concept.

Ketamine, a dissociative anaesthetic agent, is commonly used in equine anaesthesia. Besides the induction of anaesthesia,[11] ketamine is used as an adjunct to inhalant anaesthesia,[12-14] in total intravenous anaesthesia (TIVA) protocols[15-17] and for local analgesia.[18,19] More recently, studies focussed on the antinociceptive effects of low dose ketamine infusions administered to conscious horses[20] and its anti-inflammatory properties.[21] In the evaluation of these anti-inflammatory effects, ketamine was found to reduce the production of inflammatory mediators like cytokines (i.e., tumour necrosis factor-alpha and interleukin-6 (IL-6), nitric oxide (NO) and ROS in rodents[22-24] and humans.[25-28]

In the present study, the effect of ketamine on stimulated ROS production in an equine macrophage cell line and the mechanisms underlying the effect of ketamine on stimulated ROS production were investigated.

2. MATERIALS AND METHOD

2.1. CHEMICALS

Ketamine, lipopolysaccharide (LPS; Escherichia coli, O111:B4), phorbol 12-myristate 13-acetate (PMA), L-buthionine-(S,R)-sulfoximine (BSO), glutathione (GSH) standard, glutathione reductase, dimethylthiazol diphenyl tetrazolium

bromide (MTT), triethanolamine (T-1377), Triton-X, sulfosalicylic acid, nicotinamide adenine dinucleotide phosphate (NADPH) and 5-5'dithio-bis (2-2)-nitrobenzoic acid (DTNB) were purchased from Sigma chemicals (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin, streptomycin, glutamine and phosphate buffered saline (PBS) were obtained from Biocambrex (Verviers, Belgium). Horse serum (HS), non-essential amino acids and sodium pyruvate were from Gibco Invitrogen (Breda, The Netherlands). The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Invitrogen Paisley, UK). Isopropanol was from Boom B.V. (Meppel, The Netherlands).

2.2. CELL CULTURE

The equine bone-marrow-derived macrophage cell line (e-CAS cells), described by Werners et al.,[29] 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 ^{o}C, in a humidified atmosphere of 5% CO_2/ 95 % air.

2.3. ROS INDUCERS AND DETERMINATION OF ROS PRODUCTION

Since ROS production in equine mononuclear cells is highly dependent on the inducer used, initially two inducers, i.e., LPS, PMA and the combination LPS/PMA were tested, before the effect of ketamine on stimulated ROS production was investigated. The intracellular production of ROS was measured by using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as a molecular probe. After diffusion into cells, intracellular esterases hydrolyse H_2DCF-DA molecules into H_2DCF, which can react with a broad range of oxidants. Upon oxidation, the probe becomes fluorescent and this effect can be quantified by using a fluorescence reader. The determination of ROS was based on the method described by Trayner et al.[30] with minor modifications. In brief, cells were seeded in 96-well plates at a density of 2.5x10^4 cells per well (100 μL). Following 48 h of culturing, cells were incubated in serum- and phenol red-free RPMI medium containing H_2DCF-DA (10 μM) and LPS (1 μg/mL), PMA (1 μM) or the combination LPS (1 μg/mL)/PMA (1 μM). Following 1 h of incubation, H_2DCF-DA and inducers were removed by washing the cells once with PBS (200 μL). Subsequently, cells were stimulated with one of the inducers tested for an additional 4 h. Fluorescence was then measured with a Cytofluor model 2300 microplate fluorimeter (Millopore, Bedford, MA,

USA) using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Production of reactive oxygen species (ROS) was expressed as the ratio of fluorescence of the treated samples over the response in the appropriate controls [(fluorescence$_{treatment}$/fluorescence$_{control}$) x 100%, after correction for background absorbance].

2.4. EXPOSURE TO LPS/PMA AND KETAMINE

Based on the results of the experiments described above, the influence of ketamine on ROS production was tested in LPS/PMA-treated e-CAS cells. Briefly, cells were incubated in serum- and phenol red-free RPMI medium containing H_2DCF-DA (10 μM), LPS (1 μg/mL), PMA (1 μM) and ketamine (0-36 μM) for 1 h. Subsequently, cells were washed once with PBS (200 μL) to remove the probe and again exposed to LPS (1 μg/mL), PMA (1 μM) and ketamine (0-36 μM) in fresh serum- and phenol red-free RPMI medium. After 4 h of incubation, fluorescence was measured as described above.

2.5. DETERMINATION OF INTRACELLULAR GLUTATHIONE LEVELS

To determine whether ketamine reduces ROS production in LPS/PMA-treated e-CAS cells by increasing intracellular glutathione (GSH) concentrations, total intracellular GSH was measured based on the method described by Griffith.[31] In brief, cells were seeded in 6 well-plates at a density of 1.0 x 10^6 cells per well (2 mL). Following 24 h of culturing, cells were incubated in serum- and phenol red-free RPMI medium containing LPS (1 μg/mL)/PMA (1 μM) and ketamine (0-36 μM) for 4 h. Subsequently, cell lysates were prepared in lysis buffer [Triton-X 0.05% (w/v), EDTA 0.05 mM in PBS] and total cellular protein amount was determined using the technique described by Lowry et al.[32] Remaining samples were mixed with sulfosalicylic acid (2.5%), incubated on ice for 5 min and centrifuged at 12,000 x g for 5 min at 4 °C. Next, supernatants were neutralised by adding triethanolamine (8 %).

GSH levels were determined in a mixture containing 0.24 mM NADPH and 6 mM 5-5'dithio-bis (2-2)-nitrobenzoic acid (DTNB). After incubation for 5 min at 30 °C, the cell lysates or GSH standards (0, 0.5, 1, 2 and 3 μM) were added and the reaction was initiated by addition of glutathione reductase (0.5 U/sample). Increase in absorption was measured at 412 nm and 30 °C. Specific activity was calculated by comparison to the standard curve and expressed as μmol GSH/mg total cellular protein.

To investigate whether ketamine induces GSH synthesis, the influence of ketamine on intracellular GSH content was measured in GSH-depleted cells following pre-incubation with buthionine sulfoximine (BSO). In brief, following 24 h of culturing in 6-well plates at a density of 1.0 x 10^6 cells per well, cells were pre-incubated with serum- and phenol red-free RPMI medium containing BSO (25 μM) for 4 h. Thereafter, cells were washed and exposed to fresh medium containing ketamine (0-36 μM) only for an additional period of 4 h. Subsequently, cell lysates were prepared and the intracellular GSH production was measured as described above.

2.6. CELL VIABILITY

The mitochondrial-dependent reduction dimethylthiazol diphenyl tetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability.[33] After measuring ROS production, 3 mg/mL MTT solution was added and plates incubated for 30 min at 37 ºC in a humidified atmosphere. The reaction was terminated by discarding the incubation solutions, followed by dissolving the formed formazan product in 100 μL acidic isopropanol (containing 0.5% SDS and 0.04 M HCl). Absorbance was measured with a Biorad 3550 microplate reader (Biorad, Veenendaal, The Netherlands). Cell viability was expressed as the relative formazan formation in treated samples as percentage of the formazan formation in solvent-treated controls [(A595treated cells/A595 of appropriate control) x 100%, after correction for background absorbance].

2.7. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS® 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (S.D.) obtained from at least two independent experiments carried out in quadruplicate. One-way analysis of variance (ANOVA) was used to test for significant differences between group means. When appropriate, post hoc multiple comparisons were performed to test for significant differences between experimental groups (Dunnett's). Data were considered to be statistically significant when $p<0.05$.

3. RESULTS

3.1. ROS INDUCERS

A significant increase in ROS production was only determined following co-stimulation with LPS (1 μg/mL) and PMA (1 μM), compared to cells exposed to medium only (Fig. 1A). Data obtained with the MTT assay demonstrated that cell

viability was not significantly influenced following exposure to any of the inducers tested (Fig. 1B). Based on these results, the effect of ketamine on ROS production was studied in LPS/PMA-treated e-CAS cells.

3.2. KETAMINE INHIBITS ROS PRODUCTION IN LPS/PMA-TREATED E-CAS CELLS

Data indicate that ketamine significantly reduces ROS production in LPS/PMA-stimulated e-CAS cells in a concentration-dependent manner (0-36 μM), compared to LPS/PMA alone (Fig. 2A). Again, no loss of cell viability was observed (Fig. 2B). Control experiments revealed that ketamine alone, in absence of LPS, did not affect ROS production (data not shown).

3.3. EFFECT OF KETAMINE ON INTRACELLULAR GSH CONCENTRATION

To investigate whether ketamine reduces ROS production in LPS/PMA-treated e-CAS cells by increasing intracellular GSH concentrations, total intracellular GSH content was measured following exposure to LPS/PMA and ketamine. In LPS/PMA-treated e-CAS cells, ketamine significantly increased intracellular GSH content in a concentration-dependent manner (Fig. 3A).

To investigate whether ketamine increases the intracellular GSH content by inducing GSH synthesis, the influence of ketamine on total intracellular GSH concentrations was measured in GSH-depleted cells following pre-incubation with BSO. As shown in Fig. 3B, BSO significantly reduced the intracellular GSH production with 54% in the concentration tested. However, ketamine did not affect GSH synthesis at any of the tested concentrations. Ketamine alone, in absence or presence of BSO did not affect the intracellular GSH concentration (data not shown).

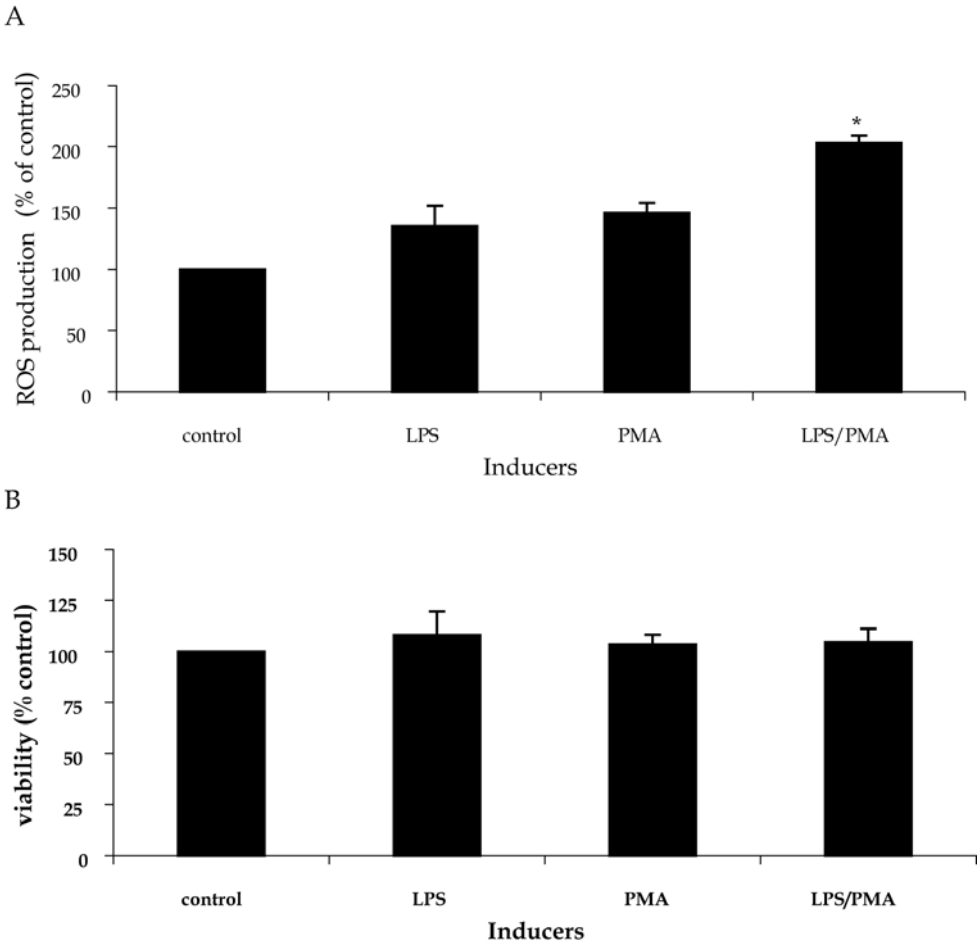

Figure 1. The effect of LPS, PMA and the combination LPS/PMA on (A) ROS production and (B) viability of e-CAS cells following 4 h of exposure. Data are expressed as mean ± S.D. of three independent experiments carried out in quadruplicate. * statistically different from non-stimulated cells (control; $p<0.05$).

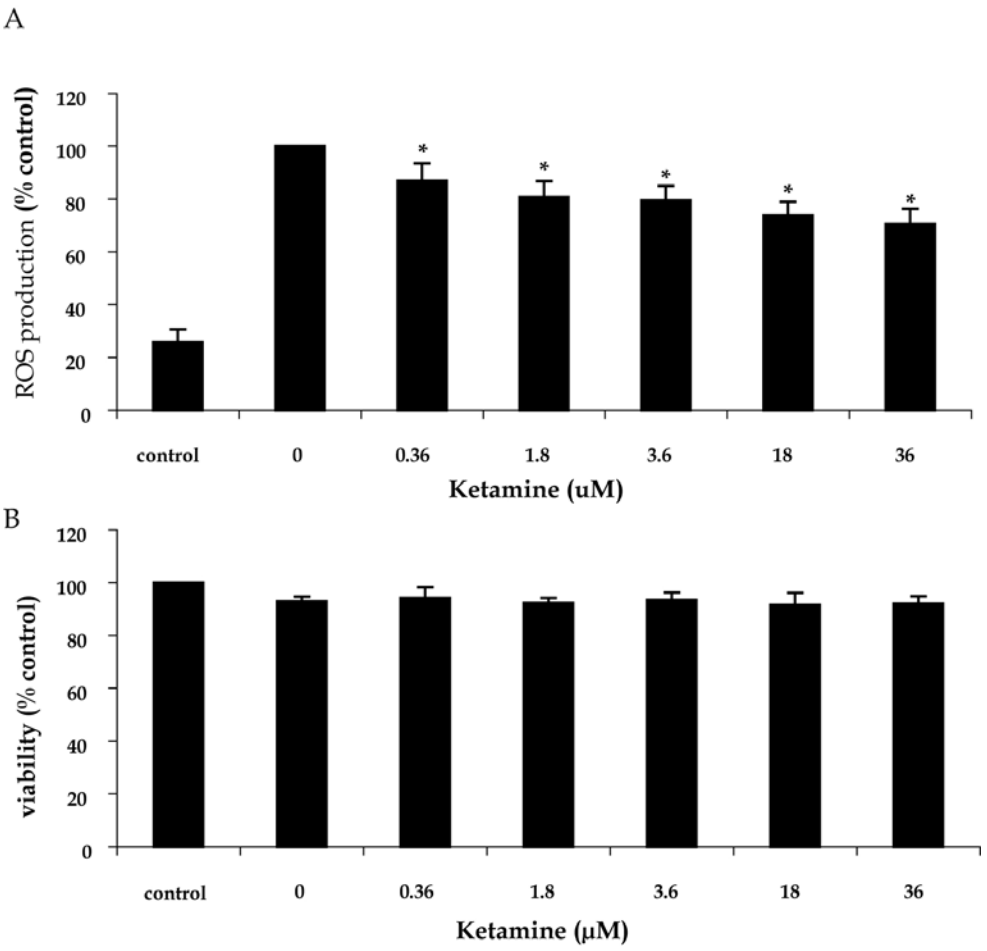

Figure 2. The effect of ketamine on LPS/PMA-induced (A) ROS production and (B) viability of e-CAS cells following 4 h of exposure. Data are expressed as mean ± S.D. of four independent experiments carried out in quadruplicate. * statistically significant differences ($p<0.05$) between ketamine-treated cells and cells treated only with LPS/PMA (controls).

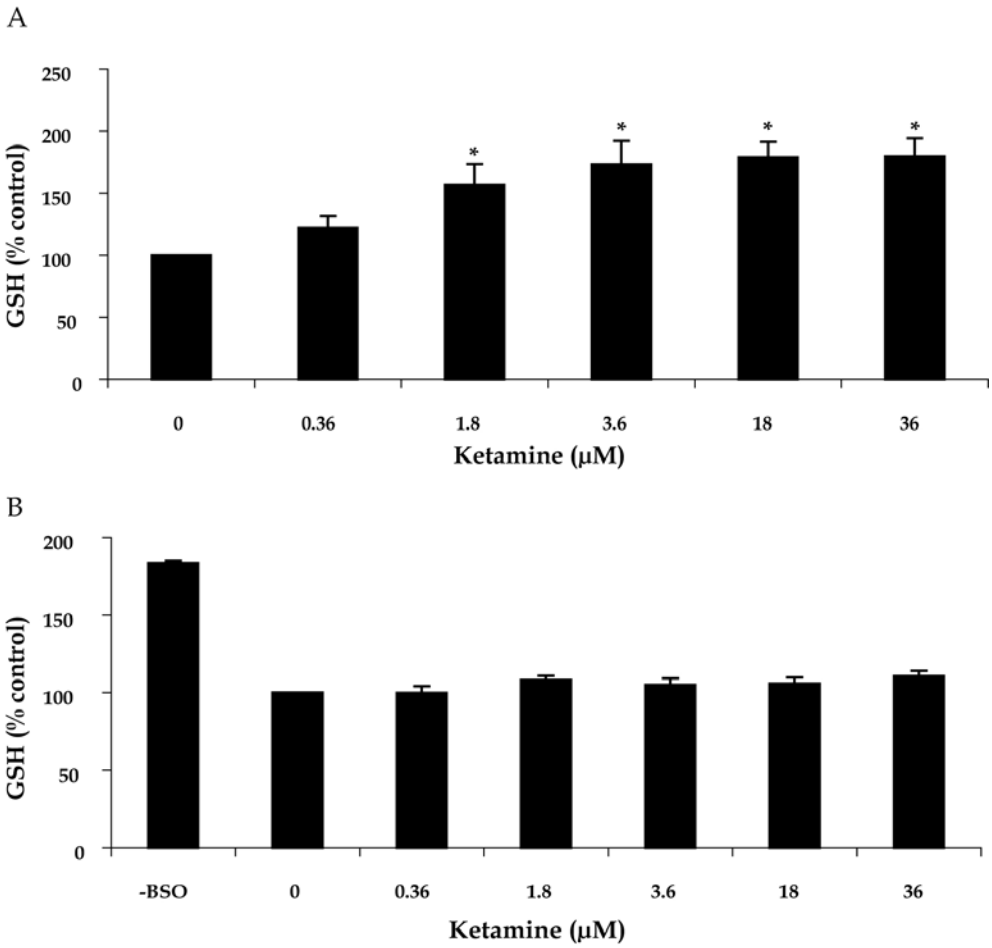

Figure 3. The effect of ketamine on (A) total intracellular GSH in LPS/PMA-treated e-CAS cells and on (B) GSH synthesis in GSH-depleted e-CAS cells following pre-incubation with BSO. Data are expressed as mean ± S.D. of two independent experiments carried out in duplicate. * statistically significant differences ($p<0.05$) between ketamine-treated and non-stimulated cell (controls).

4. DISCUSSION

Phagocytic cells like neutrophils, monocytes and macrophages, produce and release ROS upon stimulation by either endogenous factors (e.g., cytokines, lipid mediators and various breakdown products) or exogenous influences (e.g., LPS, UV light irradiation, toxic chemical and drugs).[2,3,8] This 'respiratory burst' is catalysed by the multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) reduced oxidase system.[2,3,5,34,35] Alternatively, ROS can be generated by the mitochondrial electron transport chain, by xanthine oxidase, lipoxygenase, cyclooxygenase and cytochrome P_{450}s, as well as by auto-oxidation of various substances, particularly catecholamines.[2-5,8]

Mammalian cells possess several endogenous mechanisms, including superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione, ubiquinone and vitamins C and E, to protect themselves against ROS formed during the normal cellular metabolism.[2-5,8] When ROS formation exceeds the antioxidant capacity of a cell, excessive ROS formation will lead to cellular destruction and tissue injury due to lipid peroxidation, inactivation of enzymes and breaking of DNA strands.[2,3] Ultimately, the ROS-induced tissue injury may lead to multiple organ dysfunction and death. Hence, inhibition of exaggerated ROS formation will be beneficial in limiting ROS-induced cellular injury and mutational events.

In contrast to the numerous studies on ROS production in human or rodent cell lines (or isolated primary cell cultures), data on ROS production in equine cells are scarce. One of the few examples are the investigations of Benbarek et al.,[36] who describe LPS-induced ROS production in a whole blood assay, but failed to observe ROS production in isolated equine neutrophils stimulated with LPS. In line with these results, we also found that LPS alone did not stimulate ROS formation in e-CAS cells.

Macrophages produce ROS at a lower rate than neutrophils. Hence, in vitro stimulation of ROS production generally requires application of additional stimulators like N-formyl-methionyl-leucyl-phenylalanine (FMLP), opsonised zymosan or phorbol myristate acetate (PMA) next to LPS.[37-40] In the present study, PMA was chosen as an additive to LPS, since it has been reported to be the most effective activator.[37] Similar to LPS, PMA alone did not induce ROS formation, but a significant increase in ROS formation was observed only when cells were co-stimulated with LPS and PMA. Similar results have been found by Bochsler et al.,[38] as pre-incubation of equine polymorphonuclear leukocytes with LPS followed by PMA significantly increased the secretion of superoxide anions. In consideration of

these findings, the effect of ketamine on ROS production was studied in e-CAS cells co-stimulated with LPS and PMA.

The ketamine concentrations used in this study were selected on the basis of the peak plasma level of 5 μg/ml (18 μM) following the IV induction dose of ketamine of 2.2 mg/kg body weight commonly used in horses.[41] Ketamine significantly reduced LPS/PMA-induced ROS formation in a concentration-dependent manner in e-CAS cells. Similarly, ketamine was found to significantly reduce FMLP/opsonised zymosan[39] or FMLP/PMA-induced[26] ROS production in human polymorphonuclear leukocytes (PMNs) in a dose-dependent manner, whereas no influence of ketamine on ROS synthesis was found in other studies including PMA-treated,[42] FMLP-treated[43] and opsonised zymosan-treated[44,45] human PMNs. The contrasting results from these latter studies may be due to the fact that only one single stimulator was used.

Reduction of ROS concentration in stimulated cells can be achieved either by application of direct radical scavengers or by stimulation of intracellular defence mechanisms. According to the results of the present study, ketamine reduces ROS formation in e-CAS cells by modulation of the intracellular GSH concentration. Cellular GSH content can increase via either a direct or indirect mechanism. The direct mechanism includes the induction of GSH synthesis, whereas the indirect mechanism is based on a rapid GSSG reduction (redox cycling). In the presence of ROS, GSH is converted into its reduced form GSSG by glutathione peroxidase to neutralise ROS.[46,47] Since ketamine did not induce GSH synthesis in GSH-depleted e-CAS cells, it can be assumed that ketamine indirectly increases the total intracellular GSH content by scavenging ROS, thus stabilising the intracellular redox potential. Indeed, ketamine has been described to be a weak radical scavenger, due to its phenol-moiety in early studies.[48] The effect of ketamine on other cellular enzymes involved in ROS production remains to be elucidated.

In conclusion, we demonstrate here that ketamine reduces ROS formation in stimulated e-CAS cells by indirectly increasing the intracellular GSH content. This effect is clinically desirable as it prevents tissue injury following infections or during tissue reperfusion after hypoxia.

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- CHAPTER VIII -

PHARMACODYNAMIC EFFECTS AND PHARMACOKINETIC PROFILE OF A LONG-TERM CONTINUOUS RATE INFUSION OF RACEMIC KETAMINE IN HEALTHY CONSCIOUS HORSES

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SUMMARY

Ketamine possesses analgesic and anti-inflammatory activity at sub-anaesthetic doses, suggesting a benefit of long-term ketamine treatment in horses suffering from pain, inflammatory tissue injury and/or endotoxaemia. However, data describing the pharmacodynamic effects and safety of continuous rate infusion (CRI) of ketamine and its pharmacokinetic profile in non-premedicated horses are missing. Therefore, we administered to six healthy horses a CRI of 1.5 mg/kg/h ketamine over 320 min following initial drug loading. Cardiopulmonary parameters, arterial blood gases, glucose, lactate, cortisol, insulin, non-esterified fatty acids, and muscle enzyme levels were measured, as were plasma concentrations of ketamine and its metabolites using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Levels of sedation and muscle tension were scored. Respiration and heart rate significantly increased during the early infusion phase. Glucose and cortisol significantly varied both during and after infusion. During CRI, all horses scored 0 on sedation. All but one horse scored 0 on muscle tension, with one mare scoring 1. All other parameters remained within or close to physiological limits without significant changes from pre-CRI values. Mean plasma concentration of ketamine during the 1.5 mg/kg/h ketamine CRI was 235 ng/mL. Decline of its plasma concentration-time curve of both ketamine and norketamine following the CRI was described by a two-compartmental model. The metabolic cascade of ketamine was norketamine, hydroxynorketamine and 5,6-dehydronorketamine. The ketamine median elimination half-lives ($t_{1/2\alpha}$ and $t_{1/2\beta}$) were 2.3 and 67.4 min, respectively. The area under the ketamine plasma concentration-time curve (AUC) was 76.0 μg•min/mL. Volumes of C_1 and C_2 were 0.24 and 0.79 L/kg, respectively. It was concluded that a ketamine CRI of 1.5 mg/kg/h can safely be administered to healthy conscious horses for at least 6 h, although a slight modification of the initial infusion rate regimen may be indicated. Furthermore, in the horse, ketamine undergoes very rapid biotransformation to norketamine and hydroxynorketamine and 5,6-dehydronorketamine were the major terminal metabolites.

1. INTRODUCTION

Ketamine [(±) 2-(ortho-chlorophenyl)-2-(methylamino)-cyclohexanone], a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, has been in use in equine practice for both the induction and maintenance of general anaesthesia in horses for many years.[1,2] It causes a characteristic anaesthetic state often referred to as dissociative anaesthesia. Based on the observation that NMDA receptors are involved in the modulation of intractable visceral and somatic pain,[3-5] ketamine as one of the few clinically available NMDA receptor antagonists drugs has been tested and found to be effective as an analgesic in both laboratory and human clinical studies.[6-16]

Experimental evidence is surfacing that indicates that ketamine also exhibits potent anti-inflammatory actions including reduced chemotactic activation of neutrophils and suppression of leukocyte-dependent production of cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-8 through either direct or indirect mechanisms.[17-22] In an equine macrophage cell line, ketamine inhibited LPS-induced TNF-α and IL-6 responses in a concentration-dependent manner,[23] demonstrating that this drug could have cytokine-modulating effects in horses suffering from systemic inflammation and endotoxaemia. Considering the concentration-dependent influence and the fact that elevated plasma cytokine levels have been detected in horses for several hours after onset of endotoxaemia,[24,25] one must assume that ketamine has to be administered over extended periods of time and at relatively high doses to be clinically effective.

The analgesic and anti-inflammatory effects of ketamine, shown at sub-anaesthetic doses in other species, may prove useful in pain treatment and/or intra- and postoperative intensive care of the horse. Despite those attractive properties, the risk of side effects, mainly affecting the central nervous system (excitement/sedation) and increased skeletal muscle tone (catatonia), may preclude a wider use of ketamine for long-term treatment in the conscious horse.

We undertook the present study in preparation for future investigations analysing the immunomodulatory role of ketamine and its metabolites in vivo and focused on three main goals. First, we aimed to determine the safety of a continuous rate infusion (CRI) of racemic ketamine in conscious horses by evaluating its effects on central and peripheral nervous activity, cardiopulmonary function, metabolic and endocrinological homeostasis, choosing an infusion dose likely to achieve plasma concentrations that are known to exhibit analgesic and cytokine-modulating effects. Secondly, we aimed to expand the current knowledge regarding the metabolism of ketamine by using a modified version of a recently developed liquid

chromatography-mass spectrometry (LC-MS/MS) technique[26] to identify all metabolites that are formed in the horse. Thirdly, we aimed to determine the pharmacokinetic parameters for ketamine following steady-state infusion as those data are not yet reported for the conscious horse and are important for future dose tailoring.

2. MATERIALS AND METHODS

2.1. ANIMALS

Six healthy, adult Dutch Warmblood horses (four mares, two geldings, age 13 ± 6 years, body weight 600 ± 35 kg) in good body condition were studied after approval by the Utrecht University Ethics and Animal Research Committee. No food or water was withheld before or during the experiment.

2.2. ANIMAL PREPARATION AND INSTRUMENTATION

Two hours prior to experimentation, horses were restrained in stocks and a 12-G, 8 cm polytetrafluoroethylene catheter (Intraflon 2®, Vygon Nederland BV, Veenendaal, The Netherlands) was placed aseptically into the left and right jugular veins. Using lidocaine-prilocaine cream (EMLA®, Astra Pharmaceutica BV, Zoetermeer, The Netherlands) and an aseptic technique, a 14-G, 4.5-cm over the needle catheter (Arterial Cannula with FloSwitch®, Becton & Dickenson, Swindon, UK) was inserted percutaneously into the left facial artery for arterial blood sampling and arterial blood pressure (ABP) measurements. Respiration rate (RR) was determined on visual observation. The heart rate (HR) was recorded using a digital heart rate monitor (Polar HorseTrainer S610™, Polar Electro Europe BV, Fleurier, Switzerland). Systolic, diastolic and mean ABPs (SAP, DAP, MAP) were monitored using a disposable blood pressure transducer (DTXTM Plus DT-XX®, Becton & Dickinson Critical Care Systems Pte Ltd., Singapore, China) connected to a multichannel recorder (Hewlett-Packard®, Utrecht, The Netherlands). After restricting the horse's head height to its normal, relaxed standing position, the zero level of the transducer was set at the estimated level of the heart base. Transducers were calibrated before each experimental period for each horse.

2.3. EXPERIMENTAL PROTOCOL AND MEASURED PARAMETERS

All studies were started at 10:00 a.m. to avoid the possible variation imposed by circadian rhythm changes which have been identified in horses placed in a stable environment and accustomed to routine patterns of management.[27] Following

complete instrumentation, horses were allowed to adapt to the new environment for 60 min. A jugular venous infusion of racemic ketamine (Narketan® 100 mg/mL, Vétoquinol BV, 's-Hertogenbosch, The Netherlands), dissolved in 3 L of 0.9 % NaCl solution, was started in all animals at 10:00 a.m. (t = 0 min) at a rate of 4.8 mg/kg/h using a computer-controlled infusion device (ARGUS414®, Adquipment Medical B.V., Spijkenisse, The Netherlands). The infusion rate was then reduced in a step-wise fashion every 10 min to 3.6, 3.0, 2.4, and finally 1.5 mg/kg/h was maintained for 320 min (Fig. 1).

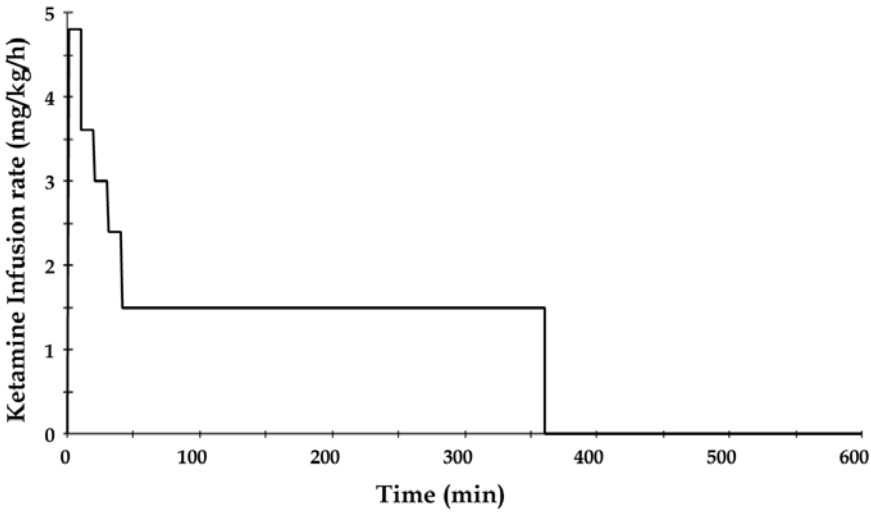

Figure 1. An intravenous infusion (CRI) of ketamine was started in each horse at 10:00 a.m. (time 0) at a loading rate of 4.8 mg/kg/h and then reduced in a step-wise fashion every 10 min till finally a rate of 1.5 mg/kg/h was achieved, which then was kept constant for 320 min.

All parameters including RR, HR, SAP, DAP, MAP, with the exception of muscle enzymes, were determined 15 min before starting ketamine infusion to collect baseline values. Baseline plasma concentrations of muscle enzymes were determined 24 h before start of infusion. Subsequent measurements of physiological and blood parameters were conducted at variable intervals. The level of sedation and muscle tension were subjectively scored each time RR and HR were recorded. A five-point scale (0-4) was used to score the level of sedation: 0 = no sedative effect, 1 = reduced alertness with no other signs, 2 = drowsiness and slight drop of the head, 3 = marked drowsiness and drop of the head, 4 = recumbency.[28] A four-point scale (0-3) was used

to score muscle tension: 0 = muscle relaxation present in trunk and limbs, 1 = muscle twitching present in some regions of trunk and limbs, 2 = muscle twitching present over the majority of trunk and limbs, 3 = muscle rigidity present over the majority of trunk and limbs.[29]

Blood samples for arterial pH (pH), partial pressures of O_2 (P_aO_2) and CO_2 (P_aCO_2) and venous glucose and lactate levels were collected in heparinised syringes (2 mL) and immediately analysed using a blood gas analyzer (ABL System 605®, Radiometer, Copenhagen, Denmark). Heparinised venous blood samples were collected for measurement of plasma lactate dehydrogenase (LDH), creatine phosphokinase (CK) and aspartate aminotransferase (AST) levels. Blood samples were centrifuged immediately following collection and then, plasma was harvested and stored at -20 °C until analysis. Plasma concentrations of LDH, CK, and AST were determined later by a colorimetric technique. Venous heparinised blood samples (8 mL) for cortisol, insulin and non-esterified fatty acids (NEFA) determinations were centrifuged immediately following collection, plasma was harvested and then stored at -20 °C until analysis. Plasma insulin and cortisol concentrations were measured by use of commercial radioimmunoassay kits (Coat-A-Count® Insulin and Coat-A-Count® Cortisol; Diagnostic Products Corporation, Los Angeles, USA), and NEFA concentrations were measured by colorimetry using a Randox kit (Randox Laboratories Ltd, Ardmore, UK). All three kits were validated for samples obtained from horses.[30]

Two blood samples for measurement of ketamine and its metabolites in plasma were collected prior to drug administration and at 9, 19, 29, 39, 45, 50, 90, 150, 210, 270, 330, 359, 361, 363, 365, 368, 372, 380, 395, 410, 430, 450, 480, 510, 540, 570, and 600 min after start of ketamine infusion. Samples were centrifuged, serum harvested and then stored at -70 °C prior to LC-MS/MS analysis.

2.4. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS/MS) ANALYSIS

Dichloromethane (Cert ACS/HPLC grade), isopropanol (ACS/HPLC grade), ammonium hydroxide (ACS grade), 16 x 125 screw cap culture tubes, and 16 x 100 screw cap tubes were obtained from Scientific Equipment Company (SECO, Aston, PA, USA). Formic acid (Supra-Pure, 99%) was obtained from VWR Scientific (Bridgeport, NJ, USA). Water and acetonitrile (Optima grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Calibration standards for ketamine HCl, norketamine HCl, and d_4-ketamine (internal standard) were obtained from Cerriliant Corp. (Austin, TX, USA). All reagents, solutions, and standards were prepared from these materials.

Unknowns, calibrators, and controls were prepared by adjusting 0.1 mL samples to pH of 9.0 with 2 mL of pH 9.0 ammonium formate placed in screw cap tubes. Internal standard d4-ketamine (100 µL) was added to all tubes (unknowns, calibrators and controls) except negative control. Calibrators and controls were prepared by using 0.1 mL aliquots of previously prepared spiked plasma (1-1000 ng/mL). All tubes were then extracted with 5 mL of dichloromethane:isopropanol (10:1) on gentle mixing (rotorack) for 10 min. The sample tubes were centrifuged for 5 min at 2500 x g, after which the organic solvent was transferred to clean, dry, labeled culture tubes and evaporated at 50 ^{o}C under a gentle stream of nitrogen. The dried samples were reconstituted with 0.1 mL 0.1 % formic acid, and then transferred to clean, labeled auto sampler vials, fitted with 0.2 mL limited volume inserts and capped.

Previously screened negative equine plasma sample was used for the creation of calibrators and positive controls. Ten concentrations, ranging from 1 to 1000 ng/mL, were prepared. Aliquots of 0.1 mL of each sample were used to maintain the analysis within the linear range of the instrumentation with sensitivity sufficient for the current studies.

Analysis was conducted on an integrated liquid chromatograph-mass spectrometer. The liquid chromatograph with auto sampler was a Surveyor® (Thermo Electron Corp., San Jose, CA, USA) and the mass spectrometer was a Deca XP plus® (Thermo Electron Corp., San Jose, CA, USA) operated in positive ion electrospray mode. The chromatographic column was an ACE 5 C-18 analytical column (3 x 50 mm, 5 micron) and the integrated guard column an ACE 3 C-18 (2.1 x 12.5 mm). The LC mobile phase was 2.33 mM formic acid (pH 6.0) and acetonitrile, run over a 10-min convex gradient from 100 % aqueous to 100 % organic at 0.2 mL/min. The mass spectrometer utilised atmospheric pressure ionisation (API) in positive ion electrospray mode, and data were acquired by reconstructed ion chromatograms of targeted precursor ions in MS mode. Data reconstruction and quantification was performed using Xcalibur® software, version 1.3 (Thermo Electron Corp., San Jose, CA, USA).

Chromatographic conditions were determined in MS mode by extraction of the relevant precursor ion species [M + H^+] for the respective analytes. Peak purity was determined by examination of the relative isotopic abundance of the precursor ion and the chlorine isotope [M + H^+ + 2] ion. This was necessary to determine full chromatographic separation of isobaric analytes with either [M + H^+] or [M + H^+ + 2] masses at the masses of m/z 224, 226, 240, and 242.

The MS mode allowed the determination of analytes for which no reference compound exists for the construction of calibration curves. For calibration of hydroxynorketamine and 5,6-dehydronorketamine the norketamine calibration curve was utilised. The ionisation efficiencies of MS mode precursor ion production are more parallel than the tandem MS/MS mode, which would be dependent upon both the efficiencies of product ion generation, as well as the product ions chosen for quantitative determination. Standard operating procedures for the quantification of analytes met requirements for accreditation by the American Association for Laboratory Accreditation and International Guidelines (ISO/IEC 17025, Geneva, Switzerland, 1999).

2.5. PHARMACOKINETIC ANALYSIS

Plasma concentration-time curve of ketamine and its metabolites, norketamine, hydroxynorketamine, and 5,6-dehydronorketamine following IV infusion of ketamine were analysed using standard linear compartmental analysis (WinSAAM).[31,32] Pharmacokinetics of ketamine and its metabolites were described by a six-compartment model (Fig. 2). The infusion of ketamine was into compartment 1 (C_1) and the distribution of ketamine and norketamine were described by two-compartment inter-compartmental transfer rate constants $k_{1,2}$, $k_{2,1}$, $k_{3,4}$ and $k_{4,3}$.

The rate of metabolism and appearance of norketamine from ketamine was estimated by $k_{1,3}$ and the rate of direct elimination and metabolism of norketamine to hydroxynorketamine and 5,6-dehydronorketamine were described by $k_{3,0}$, $k_{3,5}$, and $k_{3,6}$, the sum of which in effect describes the total elimination of norketamine. The elimination of hydroxynorketamine and 5,6-dehydronorketamine from C_5 and C_6 was described by fractional rate constants $k_{5,0}$ and $k_{6,0}$.

The inter-compartmental fractional transfer rates were directly estimated by using the following equations:

$$\dot{Y} = -LY + U \qquad (1)$$

where Y is a matrix describing the quantity of drug present in each of the compartments considered, $\dot{Y}$ is the derivative of Y, L is a matrix of the fractional rate constants describing the inter-compartmental flow of drug, and U is a matrix of the instantaneous input rates into each compartment. L is related to the eigenvalues and eigenvectors by:

$$L = A \alpha A^{-1} \qquad (2)$$

where A is the matrix of eigenvectors (boundary conditions), α is a matrix of the eigenvalues (exponential slope constants) and A^{-1} is the inverse of A.[33] The WinSAAM software is uniquely refined to permit direct translation between the exponential and equivalent compartmental model forms. The eigenvalues were estimated using WinSAAM utilising equations 1 and 2. Half-lives ($t_{1/2}$) were calculated as the natural $\log_2$ divided by the fractional rate constants.

The weights W_k, applied in the fitting process utilised the fractional standard deviation (FSD) of the data, and were in the form of $W_k = 1/[C \times Q_{O(k)} \times 2]$, in which $Q_{O(k)}$ is the kth observed datum and C is its FSD. The fitting process (iterations) ceased when the improvement in the sums of squares of the last iteration is < 1%.[33]

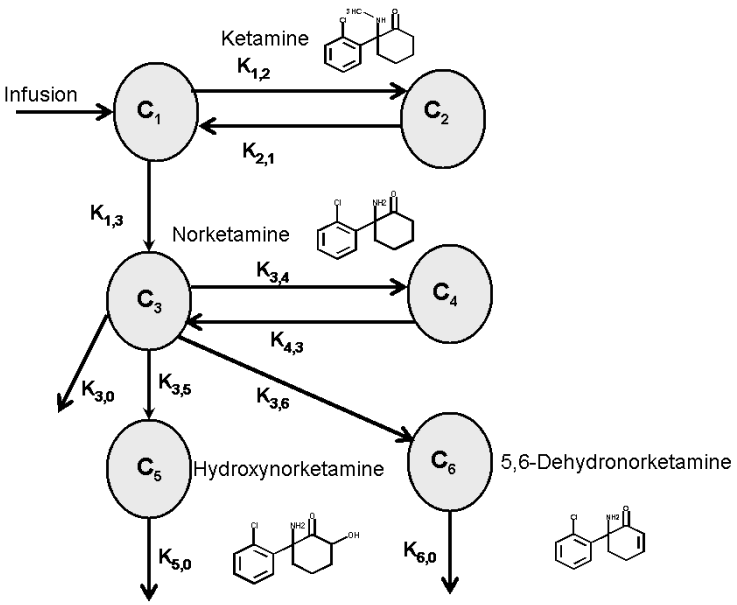

Figure 2. The six-compartment model describing the distribution of ketamine and its metabolites following the intravenous infusion of ketamine. The infusion of ketamine was into compartment 1 (C_1) and the distribution and elimination of ketamine and norketamine were described by two-compartment inter-compartmental transfer rate constants $k_{1,2}$, $k_{2,1}$, $k_{3,4}$ and $k_{4,3}$. The rate of metabolism and appearance of norketamine from ketamine was estimated by $k_{1,3}$. The rate of direct renal elimination of norketamine and metabolism of norketamine to hydroxynorketamine and 5,6-dehydronorketamine were described by $k_{3,0}$, $k_{3,5}$, and $k_{3,6}$, the sum of which in effect describes the total elimination of norketamine. The elimination of hydroxynorketamine and 5,6-dehydronorketamine from C_5 and C_6 were described by fractional rate constants $k_{5,0}$ and $k_{6,0}$. Volumes (V) of C_3, C_5, and C_6 were set equal to C_1.

The total area under the plasma ketamine and metabolites concentration curves (AUC_0^{610}) from 0 to 610 min was calculated using the trapezoid rule. Time to maximum plasma concentration (T_{max}) and maximum plasma concentration (C_{max}) were obtained directly from the experimental data. Volumes (V) of C_3, C_5, and C_6 were set equal to C_1. The volumes of C_2 and C_3 were calculated as:

$$V_2 = V_1 \times k_{1,2}/k_{2,1} \text{ and } V_3 = V_1 \times k_{3,4}/k_{4,3}$$

Clearance (Cl) from each compartment was estimated as:

$$Cl = V \times k$$

2.6. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS© 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). Pharmacodynamic data and the plasma concentrations of ketamine and its metabolites, norketamine, hydroxynorketamine, and 5,6-dehydronorketamine, were analysed using a repeated measures analysis of variance and the Huynh-Feldt test statistic to adjust for sphericity and were expressed as mean ± standard deviation (S.D.). When significant a paired t-test was used to compare differences with baseline values. Pharmacokinetic parameter estimates of ketamine and metabolites were expressed as median and range. Differences were considered to be significant when $p<0.05$.

3. RESULTS

3.1. CARDIOVASCULAR, METABOLIC AND ENDOCRINE EFFECTS

In all horses RR and HR significantly increased during the early infusion phase but after 20 min, when the ketamine infusion rate was decreased to 3.6 mg/kg/h, values returned to baseline and remained stable for the remainder of the experiment, reaching a significant minimum for RR at 30 h (Table 1). SAP, DAP, MAP and arterial blood gas tensions were collected in five horses only as in one horse arterial catheterisation was not successful. Despite initial mild blood pressure increases and subsequent increases towards the end of ketamine infusion period, none of the time points were statistically significant from control (Table 1). The pH, P_aO_2 and P_aCO_2 were within physiological ranges throughout most of the infusion period, only P_aCO_2 showed significant difference from baseline at 360 min of ketamine infusion.

Plasma lactate, insulin and NEFA concentrations remained within physiological limits, with the only exception of a significant decrease of plasma NEFA concentrations at 30 h post-infusion (Table 2). Throughout the entire experiment, plasma glucose concentrations remained slightly above the physiological range (3.9-5.6 mmol/L). Plasma cortisol concentrations varied throughout the experimental period.

Table 1. Cardiopulmonary and blood-gas parameters prior to, during, and following ketamine continuous rate infusion in conscious horses

Time	RR† (min^{-1})	HR† (min^{-1})	SAP† (mmHg)	DAP (mmHg)	MAP† (mmHg)	pH	P_aO_2 (kPa)	P_aCO_2† (kPa)
Baseline	16 ± 5	37 ± 3	144 ± 21	89 ± 9	112 ± 16	7.42 ± 0.01	13.6 ± 1.0	5.3 ± 0.5
10 min	21 ± 5*	51 ± 9*	165 ± 14	97 ± 18	119 ± 11			
20 min	22 ± 4	53 ± 13*	165 ± 20	95 ± 6	126 ± 13			
30 min	22 ± 3	51 ± 18	165 ± 26	89 ± 5	126 ± 16			
60 min	20 ± 5	44 ± 9	151 ± 16	87 ± 12	115 ± 11	7.43 ± 0.04	13.7 ± 1.9	5.2 ± 0.4
90 min	17 ± 6	41 ± 8	148 ± 24	83 ± 10	109 ± 10			
120 min	17 ± 5	40 ± 4	144 ± 14	87 ± 14	108 ± 10	7.43 ± 0.01	13.1 ± 1.0	5.2 ± 0.3
150 min	17 ± 4	37 ± 4	135 ± 7	86 ± 9	108 ± 11			
180 min	16 ± 4	38 ± 4	143 ± 10	87 ± 12	108 ± 10	7.43 ± 0.02	13.9 ± 2.3	5.2 ± 0.6
210 min	17 ± 4	39 ± 7	132 ± 10	82 ± 9	105 ± 6			
240 min	15 ± 5	37 ± 3	139 ± 7	85 ± 16	106 ± 9	7.44 ± 0.02	13.2 ± 0.7	5.2 ± 0.4
270 min	16 ± 4	39 ± 4	143 ± 10	89 ± 6	110 ± 6			
300 min	19 ± 6	40 ± 5	149 ± 16	90 ± 15	112 ± 9	7.42 ± 0.01	12.8 ± 0.8	5.5 ± 0.4
330 min	17 ± 4	41 ± 5	150 ± 17	94 ± 12	117 ± 10			
360 min	17 ± 2	41 ± 5	157 ± 15	94 ± 12	120 ± 12	7.44 ± 0.04	14.9 ± 1.7	4.8 ± 0.3*
370 min	17 ± 5	43 ± 4	148 ± 16	89 ± 9	113 ± 11			
380 min	17 ± 2	40 ± 4			113 ± 22			
390 min	17 ± 4	40 ± 6			115 ± 10			
14 h	14 ± 3	35 ± 3						
30 h	14 ± 5*	34 ± 2						

Values are expressed as mean ± S.D..
RR = respiration rate; HR = heart rate; SAP = systolic arterial pressure; DAP = diastolic arterial pressure; MAP = mean arterial pressure; PaO_2 = arterial partial pressure of oxygen; P_aCO_2 = arterial partial pressure of carbon dioxide.
† Parameters changing significantly from baseline over time based on repeated measures analysis of variance and Huynh-Feldt tests.
* Values significantly different (based on paired t-test) from baseline recorded 15 min prior to start of ketamine infusion: $p<0.05$.

Table 2. Lactate, glucose, insulin, cortisol, non-esterified fatty acid (NEFA), and muscle enzyme levels prior to, during, and following ketamine continuous rate infusion in conscious horses

Time (h)	Lactate (mmol/L)	Glucose† (mmol/L)	Insulin (pmol/L)	Cortisol† (nmol/L)	NEFA† (mmol/L)	LDH (IU/L)	AST (IU/L)	CK (IU/L)
Baseline	0.7 ± 0.3	7.2 ± 0.8	393 ± 339	287 ± 91	0.23 ± 0.16	538 ± 151	165 ± 50	152 ± 63
1	0.7 ± 0.3	5.9 ± 1.1*	218 ± 172	201 ± 56*	0.38 ± 0.09			
2	0.8 ± 0.3	6.0 ± 0.5*	250 ± 169	178 ± 46*	0.28 ± 0.16			
3	0.7 ± 0.3	5.9 ± 0.3*	235 ± 160	144 ± 34*	0.20 ± 0.07	683 ± 465	179 ± 50	175 ± 94
4	0.6 ± 0.2	5.8 ± 0.2*	187 ± 151	152 ± 60*	0.20 ± 0.08			
5	0.5 ± 0.1	5.7 ± 0.5*	155 ± 110	168 ± 76*	0.22 ± 0.07			
6	0.6 ± 0.2	5.8 ± 0.4*	127 ± 88	214 ± 40	0.27 ± 0.11	438 ± 130	163 ± 63	144 ± 62
7	0.6 ± 0.2	5.8 ± 0.6*	104 ± 85	271 ± 49	0.27 ± 0.12			
8	0.7 ± 0.1	6.0 ± 0.9*	148 ± 125	201 ± 55*	0.25 ± 0.17			
10	0.8 ± 0.1	6.0 ± 0.3*	151 ± 75	133 ± 48*	0.11 ± 0.05	567 ± 137	165 ± 33	158 ± 43
14	0.7 ± 0.1	5.6 ± 0.4*	134 ± 56	121 ± 32*	0.08 ± 0.01			
22	0.7 ± 0.1	5.8 ± 0.7*	192 ± 100	204 ± 33	0.10 ± 0.02			
30	0.8 ± 0.2	5.8 ± 0.3*	183 ± 74	135 ± 50*	0.07 ± 0.03*	440 ± 185	189 ± 41	130 ± 40

Values are expressed as mean ± S.D..
NEFA = non-esterified fatty acids; LDH = lactate dehydrogenase; CK = creatine kinase; AST = aspartate aminotransferase.
† Parameters changing significantly from baseline over time based on repeated measures analysis of variance and Huynh-Feldt tests.
* Values significantly different (based on paired t-test) from baseline recorded 15 min prior to start of ketamine infusion: $p<0.05$.

3.2. BEHAVIORAL EFFECTS

All horses started shifting weight from one front leg to the other within 5-10 min after start of infusion and continued to show this behavior for 20-30 min. During ketamine CRI, none of the horses displayed any sedative effects. On the contrary, upon subjective evaluation, three of six horses showed an increase in alertness during the 6-h infusion period.

3.3. SKELETAL MUSCLE

All but one horse scored 0 on muscle tension. One mare scored 1 at 20 and 30 min following start of ketamine infusion. There were no significant changes in plasma LDH, CK and AST concentrations 3 h after the start of infusion (Table 2).

3.4. KETAMINE PHARMACOKINETICS AND METABOLISM

The median and range of the inter-compartmental fractional rate constants and pharmacokinetic parameter estimates for the IV infusion of ketamine and its metabolites are shown in Tables 3 and 4. The plasma concentration of ketamine progressively decreased as the rate of infusion was decreased and this change was significant ($p<0.02$) at the end of the 2.4 mg/kg/h infusion period (Table 5; Fig. 3). Conversion of ketamine to norketamine was quantified at the first sampling time and there was a significant increase in the norketamine plasma concentrations ($p<0.02$) at the end of first infusion period of ketamine (4.8 mg/kg/h). The norketamine concentrations gradually declined and paralleled the concentration changes of ketamine (Fig. 4). Conversion of norketamine to hydroxynorketamine and 5,6-dehydronorketamine was also rapid and continuous over the CRI (Table 5; Figs 3 and 4). There were significant increases in the plasma concentration of hydroxynorketamine ($p<0.04$) and 5,6-dehydronorketamine ($p<0.01$) at the end of the second infusion period of ketamine (3.6 mg/kg/h) (Table 5). There were no significant differences in the plasma concentrations of hydroxynorketamine and 5,6-dehydronorketamine at the end of the CRI.

Once the CRI was discontinued, ketamine concentrations decreased rapidly, reaching plasma levels approximately one-tenth of those measured during CRI within 90 min. A six-compartment model best described the decline of the plasma concentration-time curve of ketamine and its metabolites (Fig. 2). A biexponential equation best described the data for ketamine and norketamine. The initial distributive (α) phase of ketamine was characterised by an estimated half-life of 2.3 min ($t_{1/2\alpha}$) and the slower elimination (β) phase by a half-life of 67.4 min ($t_{1/2\beta}$) (Table 3). Thus, ketamine distributed very rapidly from the central (C_1; plasma and blood-rich tissues) to the peripheral tissue compartments (C_2) with an inter-compartmental transfer rate constant $k_{1,2}$ of 0.046 min^{-1}. The apparent volumes of C_1, C_2 and C_4 were estimated as 242.2, 786.2, and 755.1 mL/kg, respectively, and the estimated clearances of C_1, C_3, C_5, and C_6 are also shown (Table 3). Like the parent drug, norketamine rapidly interchanged between the central and peripheral compartments (C_3 and C_4). The elimination of the secondary metabolites hydroxynorketamine and 5,6-dehydronorketamine were described by a mono-exponential equation (Fig. 3; Table 4).

Table 3. Pharmacokinetic parameter estimates of ketamine and metabolites following an IV infusion of ketamine in n = 6 horses

Parameter	Median	Range
Ketamine		
$t_{1/2\alpha}$ (min)	2.34	1.67-2.86
$t_{1/2\beta}$ (min)	67.45	59.2-85.6
$t_{1/2kt\,1}$ (min)	2.96	2.29-3.31
V_1 (mL/kg)	242.2	163.7-335.8
V_2 (mL/kg)	786.2	329.4-1437.7
Cl_1 (mL/min/kg)	53.0	36.6-88.7
AUC_0^{610} (μg•min/mL)	76.0	48.0-105.3
Norketamine		
$t_{1/2\alpha}$ (min)	10.46	5.0-15.3
$t_{1/2\beta}$ (min)	160.69	80.2-307.6
$t_{1/2kt\,3}$ (min)	7.53	5.4-9.0
V_4 (mL/kg)	755.1	547.3-1558.2
Cl_3 (mL/min/kg)	23.3	15.9-42.9
AUC_0^{610} (μg•min/mL)	160.8	94.1-232.9
Hydroxynorketamine		
$t_{1/2ke5}$ (min)	56.95	33.6-64.4
Cl_5 (mL/min/kg)	3.1	2.0-6.8
AUC_0^{610} (μg•min/mL)	372.9	304.5–449.3
5,6-Dehydronorketamine		
$t_{1/2ke6}$ (min)	43.26	27.6-46.5
Cl_6 (mL/min/kg)	4.4	2.6-8.1
AUC_0^{610} (μg•min/mL)	396.6	251.1-568.2

$t_{1/2\alpha}$ and $t_{1/2\beta}$ = compartmental half-lives; $t_{1/2kt}$ = transfer half-lives from C_1 and C_3; $t_{1/2ke}$ = elimination half-lives from C_5 and C_6; V_1 and V_2= apparent volumes of C_1 and C_2; AUC = area under the curve; Cl = clearance from V_1, V_3, V_5, and V_6.

Table 4. Median and range of calculated intercompartmental transfer and elimination rate constants (k) and fractional standard deviation of the estimates (FSD) of the six-compartment model describing the pharmacokinetics of ketamine and metabolites following the IV infusion of ketamine in n = 6 horses

Parameter	Median	Range	FSD
Ketamine			
$k_{1,2}$ (min^{-1})	0.046	0.015-0.060	0.034
$k_{2,1}$ (min^{-1})	0.013	0.009-0.015	0.015
$k_{1,3}$ (min^{-1})	0.235	0.209-0.303	0.017
Norketamine			
$k_{3,4}$ (min^{-1})	0.033	0.015-0.140	0.057
$k_{4,3}$ (min^{-1})	0.010	0.006-0.019	0.058
$k_{3,5}$ (min^{-1})	0.032	0.021-0.062	0.019
$k_{3,6}$ (min^{-1})	0.044	0.036-0.066	0.018
$k_{3,0}$ (min^{-1})*	0.013	0.000-0.038	0.068
Hydroxynorketamine			
$k_{5,0}$ (min^{-1})	0.012	0.011-0.021	0.016
5,6-Dehydronorketamine			
$k_{6,0}$ (min^{-1})	0.016	0.015-0.025	0.016

Table 5. Plasma concentrations (mean ± S.D.) of ketamine (KET) and its metabolites norketamine (NKET), hydroxynorketamine (HNK), and 5,6-dehydronorketamine (DHNK) at the end of each infusion period (n = 6 horses)

Infusion Rate (mg/kg/h)	Time (min)	KET (ng/mL)	NKET (ng/mL)	HNK (ng/mL)	DHNK (ng/mL)
4.8	9	407 ± 183	351±154*	184 ± 57	159 ± 62
3.6	19	368 ± 87	471 ± 137	315 ± 63*	347 ± 74*
3.0	29	352 ± 88	538 ± 115	460 ± 91	535 ± 99
2.4	39	288 ± 80*	510 ± 120	506 ± 83	649 ± 144
1.5	372	230 ± 120	489 ± 137	920 ± 169	1001 ± 238

*Values significantly different from measurements at previous infusion rate and sampling time, respectively: $P < 0.05$.

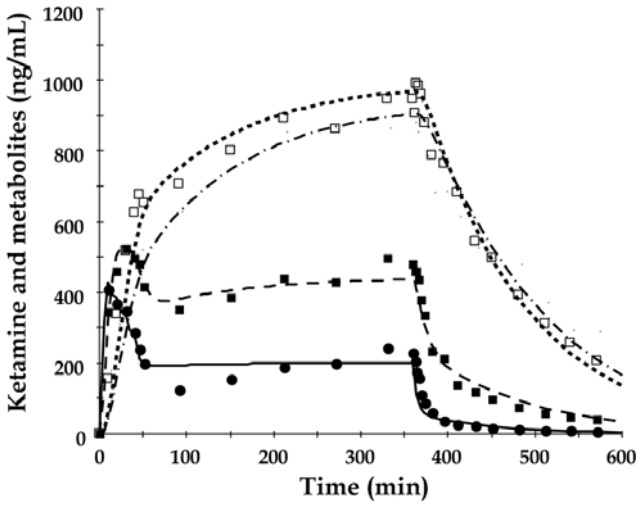

Figure 3. Plasma concentrations and the corresponding curves of best fit of ketamine (●—), norketamine (■---), hydroxynorketamine (○— ▪ — ▪ —), and 5,6-dehydronorketamine (□▪▪▪▪▪) during and following IV ketamine infusion. Symbols represent mean values from n = 6 horses.

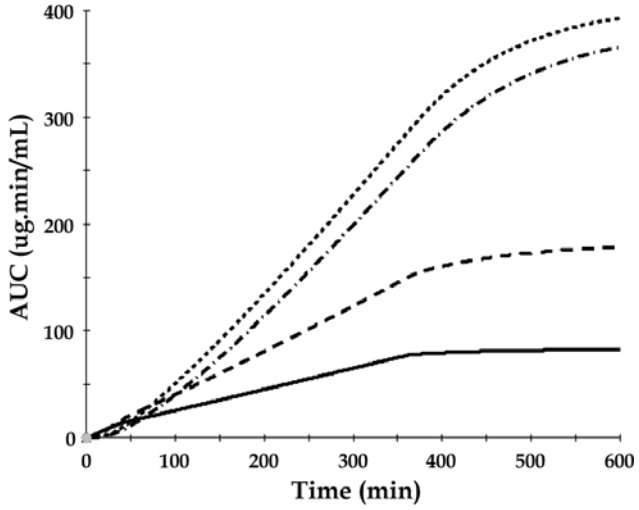

Figure 4. Area under the curve (AUC) values of ketamine and its metabolites during and following IV ketamine infusion. The AUC reflects the relative amount of metabolites to the parent compound (bottom to top: ketamine, norketamine, hydroxynorketamine, and 5,6-dehydronorketamine). Data from n = 6 horses.

4. DISCUSSION

4.1. PHARMACODYNAMIC EFFECTS OF RACEMIC KETAMINE DURING LONG-TERM INFUSION IN CONSCIOUS HORSES

The present data indicate that in healthy conscious horses ketamine at a rate of 1.5 mg/kg/h can be safely administered over prolonged periods of time. The CRI rate of 1.5 mg/kg/h chosen in this study was based on previously published data, in which horses were receiving an IV induction dose of 2.2 mg/kg and returned to consciousness when plasma ketamine concentrations fell to or below 1 μg/mL.[34] Thus, to be prepared for future investigations analysing dose-dependent immunomodulatory effects of ketamine in the horse, we aimed at achieving relatively high plasma concentrations, yet without the risk of causing loss of consciousness. A 0.8 μg/mL plasma concentration appeared to be the upper limit, as it was close to but still safely below those associated with anaesthetic effects. Applying the previously reported pharmacokinetic variables in the horse[34] (Table 6), a computer program for therapeutic drug monitoring (Beal SL, Sheiner LB: NONMEM Users Guides, NONMEM Project Group, University of California, San Francisco, CA, USA) predicted a ketamine CRI dose of 1.5 mg/kg/h as appropriate for achieving this goal. To reach steady-state concentrations, a CRI can either be preceded by a bolus injection or by an initially higher infusion rate, followed by a stepwise decrease of the dosage. We opted for the latter, as it carried the least risk of resulting in peak plasma concentrations in excess of 1 μg/mL.

During the 1.5 mg/kg/h ketamine CRI plasma ketamine concentrations averaged 235 ng/mL (range: 118-277 ng/mL; Figs 3 and 4), which were far below the plasma concentrations predicted by the computer program. This significant discrepancy might have been caused by a variety of reasons. Foremost, pharmacokinetic properties may change when ketamine is administered in combination with other drugs, leading to altered metabolism, distribution and excretion of the compound.

Table 6. Pharmacokinetic data for racemic ketamine from comparative references

	Kaka et al., 1979[35] (n = 4)	Waterman et al., 1987[34] (n = 10)	Schwieger et al., 1991[41] (n = 4)		Domino et al., 1982[†,46] (n = 7)
Species	Horse	Horse	Dog		Human
Ketamine dose	2.2 mg/kg IV	2.2 mg/kg IV	10 mg/kg IV	0.3 mg/kg/min CRI[*]	2.0-2.2 mg/kg IV
V_1 (mL/kg)	212 ± 24	492 ± 74	810 ± 135	n.d.	50 ± 17
V_2 (mL/kg)	611 ± 131	n.d.	n.d.	2888 ± 460	157 ± 47
V_{SS} (mL/kg)	1625 ± 103	611 ± 131	1370 ± 205	n.d.	2114
Cl (mL/min/kg)	26.6 ± 3.5	31.1 ± 2.3	18.1 ± 2.9	13.9 ± 2.5	18.6
$t_{1/2\alpha}$ (min)	2.9	2.89 ± 0.25	7.7 ± 0.9	n.d.	0.49 ± 0.08
$t_{1/2\beta}$ (min)	42	65.84 ± 3.46	121.9 ± 4.6	140.6 ± 19.8	158 ± 36
$k_{1,2}$ (min^{-1})	0.093 ± 0.020	0.164 ± 0.024	n.d.	n.d.	n.d.
$k_{2,1}$ (min^{-1})	0.032 ± 0.006	0.042 ± 0.005	n.d.	n.d.	n.d.

Data are presented as mean ± S.D..
V_1 and V_2 = the estimated volumes of distribution for the central and peripheral compartments, respectively; V_{SS} = the volume of distribution at steady-state; $t_{1/2\alpha}$ and $t_{1/2\beta}$ = the compartmental half-lives; $k_{1,2}$ and $k_{2,1}$ = the corresponding inter-compartmental transfer rate constants; Cl = an estimate of the total body clearance rate; n.d. = not determined.
[*] Initial ketamine loading dose of 26 mg/kg over 20 min, followed by a continuous rate infusion (CRI) for 5 h.
[†] Data were analysed using a three-compartment model.

Although plasma concentrations were far below the estimated concentration of 800 ng/mL, evaluation of the recorded physiological and behavioral parameters suggests that the CRI rate chosen approaches the upper limit of what may be tolerated under clinical circumstances. Especially the infusion rates of 4.8 mg/kg/h and 3.6 mg/kg/h, administered early during the infusion period, were associated with significant increases in RR, HR and ABP (Table 1). In addition, at these infusion rates, horses showed mild behavioral changes in the form of shifting weight from one front leg to the other, and some muscle twitching was recorded in both front legs of one horse. In addition, increased alertness observed in three of six horses during the 6-h ketamine infusion, points towards increased central nervous activity caused by ketamine and warrants caution when infusing the drug at higher rates.

High baseline plasma glucose and cortisol concentrations might indicate a stress response induced by experimental environment and manipulations performed prior to ketamine infusion. One may also speculate that the increase in plasma cortisol concentration, rather than the expected diurnal decrease, and the remaining elevated plasma glucose concentrations during the experiment were the result of the ketamine CRI, even though other factors not controlled for in these experiments might have been responsible as well. As an indirect sympathomimetic drug, ketamine may cause elevated endogenous catecholamine levels and hence stimulate the release of plasma cortisol, subsequently leading to hyperglycemia caused by increased gluconeogenesis and/or insulin resistance. In order to evaluate any acute impact of ketamine on skeletal muscle tissue integrity, plasma concentrations of LDH, AST and CK were measured. Creatine phosphokinase has a high specificity for muscle damage. Therefore, the facts that no enzyme showed a significant change of the plasma concentration, and that both AST and CK remained within physiological limits, suggests that ketamine, when administered at the infusion rates used in this study, had no significant impact on skeletal muscle activity and tissue integrity.

4.2. PHARMACOKINETICS

Clinically, ketamine is used primarily as a short-acting anaesthetic, most commonly administered in conjunction with α_2-adrenoceptor agonists. Thus, in previous equine studies, the drug was administered at anaesthetic doses (≥ 2.2 mg/kg) following an IV injection of either xylazine (1.1 mg/kg) or detomidine (20 μg/kg) and its pharmacokinetic profile was determined under conditions of inhalant or total intravenous anaesthesia.[34-36] Inhalant anaesthetics (e.g., halothane) and anaesthetic adjuvants (e.g., diazepam, secobarbital, alfentanil, xylazine) are

known to delay redistribution and hepatic metabolism of ketamine in various animal species. Chronic ketamine administration is associated with induction of hepatic drug-metabolising cytochrome P_{450} (CYP_{450}) enzymes in laboratory animals.[34,37,38] Moreover, protein binding of ketamine,[39] stability of ketamine in blood samples,[40] and accuracy of the analytical technique used to measure ketamine concentrations in plasma can be of influence. Thus, extrapolating previously obtained pharmacokinetic data for the use of long-term infusion of ketamine in otherwise non-premedicated horses may not be appropriate. The present study is therefore the first to investigate in awake horses the distribution and metabolism of ketamine when administered in the form of a long-term infusion (1.5 mg/kg/h).

Pharmacokinetic variables ($t_{1/2\alpha}$, $t_{1/2\beta}$, V_1, V_2, Cl) determined for infusion of ketamine were not very different from those previously reported in horses after IV bolus administration[34-36] (compare Tables 3 and 6). This observation coincides with findings in dogs,[41] in which clearance (Cl) and elimination half-lives ($t_{1/2\beta}$) were similar between animals receiving a single IV dose or a CRI of ketamine (Tables 3 and 6). Plasma concentrations only reached a stable plateau after 300 min of ketamine CRI (Fig. 3), confirming that for a true steady-state to occur continuous drug infusion must be maintained for a period of at least five elimination half-lives.[42-43] The average plasma ketamine concentration during 1.5 mg/kg/h ketamine infusion was 235 ng/mL and thus was far below levels needed to produce anaesthesia (i.e., 1.2 ± 0.2 μg/mL),[35] but was within the range of concentrations that exhibit analgesic effects in humans[15,16] and anti-inflammatory actions in vitro.[23] Similar to the results obtained in previous pharmacokinetic studies in horses[34-36] and other species,[37] following termination of the CRI plasma ketamine concentrations declined biexponentially, and the disposition of ketamine and norketamine in all six horses was best described using a two-compartment model, which had previously been described for ketamine in the horse,[34,35] dog,[44] cat,[45] and other species including man as well.[37] As with an IV bolus during CRI of ketamine the initial distribution phase from the central (plasma and vessel-rich tissues; C_1) to the peripheral tissue compartments (C_2) occurs with a very short half-life ($t_{1/2\alpha}$) of approximately 2-3 min, followed by a much slower elimination phase, representing both metabolism and renal elimination and occurring with a half-life ($t_{1/2\beta}$) of about 1 h. The apparent volume of distribution of the peripheral compartments (C_2 and C_4) was approximately 3.2 times greater than that of C_1 (Table 3), which has been also observed in other species such as the dog,[44] cat,[45]

and human.[46,47] The large volume of the second compartment is characteristic for the strong lipid solubility of weak bases such as ketamine[37] and hence confirms the high affinity of the drug for many tissues other than plasma.

Ketamine is metabolised extensively by the hepatic CYP_{450} enzyme system in all species including the horse.[37,48,49] However, total body clearance (Table 3) appears to exceed hepatic blood flow in the horse[35] and renal ketamine excretion occurs only in the immediate period post-IV administration,[50] indicating that also extrahepatic pathways in organs such as kidney, lung, and gut may participate in the elimination of ketamine from plasma, as has been demonstrated in the rat.[51] In many species including the horse,[37,49] initial hepatic biotransformation via N-demethylation produces norketamine (metabolite I) that then can be hydroxylated at one or more positions in the cyclohexanone ring to form hydroxynorketamine, which in turn can be conjugated to more water-soluble glucuronide derivatives and be excreted by the kidneys (Fig. 2). Since the hyroxylated metabolites of norketamine are unstable ex vivo at higher temperatures, they were thought to rapidly undergo further oxidation (thermal dehydration) to 5,6-dehydronorketamine, which had been identified as the predominant final metabolite also found in equine serum and urine following IV ketamine administration.[50,52]

Although called metabolite II, [34,37] the in vivo existence of 5,6-dehydronorketamine as a true metabolite has long been questioned by investigators suggesting that formation of 5,6-dehydronorketamine could be the result of an analytical artifact associated with the gas-liquid chromatography-mass spectrometry (GC-MS) that was commonly used at the time for detection and quantification of ketamine and metabolites in plasma and urine.[26,37] It was thought that under the harsh chemical conditions of the GC method, involving benzene extraction of a strongly alkalinised sample followed by heptafluorobutyric anhydride derivatisation in the presence of pyridine, hydroxynorketamine and other α- and β-hydroxy ketones would be easily converted to the respective dehydro compounds.[26] The use of the more benign LC-MS in this study has been shown to eliminate much of the thermal component of 5,6-dehydronorketamine generation. The difference between the two available methodologies can be quantified by comparison of 5,6-dehydronorketamine levels obtained by GC-MS and LC-MS/MS. Additionally, the lack of any GC-MS reports of hydroxynorketamine speaks directly to the facility with which this conversion can take place. The elevated temperatures of GC-MS instrumentation and the harsh conditions of chemical derivatisation both seem more than sufficient to produce 5,6-dehydronorketamine from hydroxy-

norketamine. The present study shows high levels of both hydroxynorketamine and 5,6-dehydronorketamine, which have parallel slopes of appearance and disappearance (Fig. 3). However, even using our LC-MS/MS method to minimise the thermal conversion, ion source conditions and electrospray ion formation and ejection processes may still be sufficient to facilitate the enol-mediated dehydration process, probably leading to some degree of artificial formation of 5,6-dehydronorketamine in addition to in vivo-generated 5,6-dehydronorketamine metabolite. The detection by our methodology of both enolic positional hydroxylations (which are chromatographically resolved – but are reported as a single species) and measurement of consistently higher 5,6-dehydronorketamine than hydroxynorketamine levels (Figs 3 and 4) lend further support to this idea and underscore some limitations of the LC-MS/MS methodology that could result in an overestimation of the quantity of 5,6-dehydronorketamine formed during biotransformation.

Independent of the possible analytical bias toward the production of 5,6-dehydronorketamine, the LC-MS/MS technique allowed us for the first time to describe the complete metabolic cascade of ketamine in the horse (Figs 2 and 4; Tables 3 and 4). Already in the first blood sample drawn after starting the ketamine infusion (i.e., at 9 min) all three metabolites (norketamine, hydroxynorketamine, and 5,6-dehydronorketamine) were detected. During the initial loading dose infusion of ketamine, norketamine was the predominant metabolite detected, however, by the time the infusion was changed to a continuous infusion at 1.5 mg/kg/h, hydroxynorketamine and 5,6-dehydronorketamine had become the predominant metabolites detected in plasma, with concentrations rapidly surmounting plasma concentrations of the parent compound ketamine and metabolite norketamine. These findings coincide with previous data in the horse, demonstrating the detection of norketamine in plasma as early as 2-5 min and peaking at 10-20 min following IV ketamine bolus administration.[34,49] Rapid metabolism of ketamine to norketamine and 5,6-dehydronorketamine has also been described in other species including sheep,[53] dog,[44] and cat.[45]

In order to better determine the rate at which norketamine and other metabolites are formed, we administered in one additional adult horse an IV bolus of 1 mg/kg ketamine over a period of 10 min and collected blood samples at 1, 2, 4 and 8 min and extended the collection period to 12 and 24 h post-ketamine administration. Plasma concentrations of norketamine were quantifiable at the 1-min sampling period and of hydroxynorketamine and 5,6-dehydronorketamine at the 2-min sampling period (Fig. 5). Given these observations and the predicted distribution

half-life ($t_{1/2\alpha}$) of ketamine of 2.34 min (Table 3), we can conclude that the conversion of ketamine to norketamine must start within compartment C_1, the plasma/extracellular fluid space (Fig. 2). As for ketamine, the plasma concentration-time curve for norketamine also declined in a biexponential fashion, suggesting the use of a similar two-compartment model for norketamine, with C_3 representing the central and C_4 the peripheral compartment of distribution. Individual distribution half-lives ($t_{1/2\alpha}$; $t_{1/2\beta}$) and AUC_0^{610} values, however, were quite different between ketamine and its first metabolite (Table 2). The model assumes that norketamine rapidly distributes to compartment C_3, which is thought to be of equal volume size as C_1, and then interchanges with compartment C_4. The median inter-compartmental transfer constant $k_{1,3}$ was 0.235 min, indicating how rapidly ketamine is metabolized to norketamine. Our model suggests that norketamine is eliminated both by conversion to hydroxynorketamine and 5,6-dehydronorketamine and by renal excretion of the intact metabolite.[50]

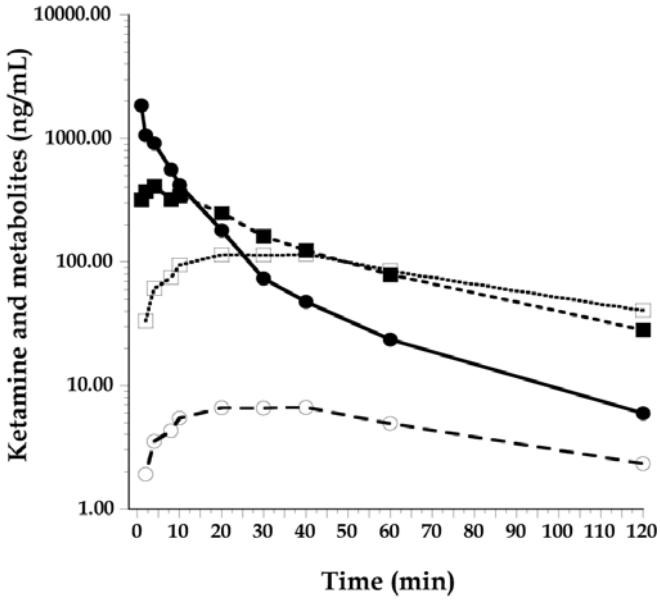

Figure 5. Plasma concentrations (0 to 120 min) of ketamine (●—), norketamine (■---), hydroxynorketamine (○— — —), and 5,6-dehydronorketamine (□▪▪▪▪▪) following a single IV administration of ketamine (1 mg/kg) in one horse.

In developing our six-compartment model to describe the distribution and metabolism of ketamine and its metabolites, we invoked the principle of parsimony. By this, we mean that we employed the minimum number of compartments, rate constants and assumptions that were necessary to describe all of the main features of the available experimental data and be consistent with the known pharmacokinetics ketamine. Our model does describe all the main features evident in the disposition curves of ketamine, norketamine, hydroxynorketamine and 5,6-dehydronorketamine during and following ketamine infusion, and R^2 values for ketamine and all metabolites in all individual horses were in excess of 0.90. The robustness of our model is further evidenced by the fact that it could well describe the pattern of the disposition curves of ketamine, norketamine, hydroxynorketamine and 5,6-dehydronorketamine following a short-term (10 min) infusion of ketamine (Fig. 5).

Despite the general success of our model, it must be acknowledged that during the period between 1 and 6 h into the continuous rate infusion our model tends to under-predict the concentrations of ketamine and norketamine. There may be several reasons for this under-prediction. First, in compartmental modeling of drug kinetics, it is generally assumed that when a drug is injected or infused into a compartment (generally the blood), it instantaneously disperses completely throughout that compartment. In reality, thorough mixing of drug throughout blood and extracellular fluid may take more than 1 min. Thus, immediately after a bolus injection or during continuous infusion of drugs, plasma concentrations of the drug will necessarily be higher than predicted by models. Secondly, in our model we assumed that the initial volumes of distribution for ketamine (C_1), norketamine (C_3), hydroxynorketamine (C_5) and 5,6-dehydronorketamine (C_7) are all equivalent. The general success of our modeling would suggest that this assumption is a good approximation. However, if this assumption is not true, then this could account for some of the discrepancies that we allude to. In support of this possibility, it is known that the metabolites of ketamine, especially hydroxynorketamine and 5,6-dehydronorketamine are more hydrophilic than ketamine and consequently their initial volumes of distribution may be somewhat larger than that of ketamine.[37,51] The third possibility that we must consider is that there could exist pathways of distribution and metabolism that we have not modeled. For example, one may contemplate the possibility that some norketamine, hydroxynorketamine or 5,6-dehydronorketamine may be reconverted to ketamine. Another possibility is that the metabolism of norketamine might also occur from C_4.[37,51]

The compartmental model presented here provides a complete conceptualisation of the distribution and metabolism of ketamine and its metabolites. Furthermore, the model provides a means of making predictions of various regimens of ketamine administration and serves as a basis on which to make additional hypotheses regarding the metabolism of ketamine. We suggest that the model could be tested by further experimentation. For example, our assumption that there is no catabolism of norketamine to ketamine could be tested by an experiment in which norketamine is injected into the blood. The disposition of both ketamine and norketamine can be described by two-compartment models, but the disposition of the more watersoluble metabolites hydroxynorketamine and 5,6-dehydronorketamine can be described by just single exponential curves. Thus, we can speculate that compartments C_2 and C_4 may represent the sequestration of ketamine and norketamine, respectively within fatty tissue.

The contribution of ketamine's metabolites to the pharmacological effects of ketamine in the horse is unknown. Studies in sheep and humans suggest that norketamine still exhibits up to 10% of the anaesthetic activity of the parent compound, while 5,6-dehydronorketamine has only about 1% of that activity.[37,53] Whether these metabolites have also analgesic or anti-inflammatory effects cannot be answered at this time.

In summary, a CRI of 1.5 mg/kg/h ketamine can safely be administered to healthy conscious horses for at least 6 h, although a slight modification of the initial infusion rate regimen may be indicated. Future experiments have to reveal the safety and efficacy of long-term ketamine administration in equine patients suffering from severe pain and/or systemic inflammatory disease and the importance of ketamine's very rapid biotransformation to norketamine, hydroxynorketamine and 5,6-dehydronorketamine in those patients.

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- CHAPTER IX -

INVESTIGATION OF THE ANTI-INFLAMMATORY EFFECTS OF KETAMINE IN A TISSUE CHAMBER MODEL IN SHETLAND PONIES

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SUMMARY

Ketamine is frequently used in equine anaesthesia. Besides its anaesthetic and analgesic effects in vivo, ketamine has previously been reported to possess cytokine-modulating properties in an equine macrophage cell line.[1] These results suggest that ketamine might also have appreciable anti-inflammatory effects in vivo.

In the present study, the influence of parenterally administered ketamine on a lipopolysaccharide (LPS)-induced, local inflammatory response in tissue chambers implanted in Shetland ponies was investigated. Two dose regimens were tested: a single bolus injection of ketamine (SI group) and a ketamine CRI protocol, comprising a loading dose followed by a continuous rate infusion of ketamine for 6 h (CRI group). For comparison, a saline infusion protocol was used (C group). White blood cell count, tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) concentrations in tissue chamber fluid were measured to assess the influence of ketamine on the local inflammatory response. Simultaneously, plasma concentrations of ketamine and its metabolites were determined.

Administration of ketamine, either as bolus or as CRI, did not significantly affect any of the investigated parameters. This lack of effect is thought to be attributable to the fact that only a small fraction (0.5 - 0.6 μM) of the circulating ketamine entered the tissue chambers. These fractions might be too low to generate anti-inflammatory effects since the minimal effective dose in in vitro experiments was 1.8 μM.[1]

However, in equine patients, suffering from systemic infections or reperfusion injury, ketamine could still be effective as the measured serum concentrations exceed the minimal effective concentration determined in the e-CAS model.

1. INTRODUCTION

Ketamine, a dissociative anaesthetic agent, is commonly used in a variety of clinical settings in equine anaesthesia. Besides the induction of anaesthesia,[2] ketamine can be used as an adjunct to inhalant anaesthesia,[3-5] in total intravenous anaesthesia (TIVA) protocols[6-8] and for systemic and local analgesia.[9,10] Recent studies focus on the antinociceptive effects of subanaesthetic ketamine infusions administered to conscious horses.[11] As ketamine preserves respiratory and cardiovascular functions, its use is recommended for anaesthesia of cardiovascular depressed patients.[12]

More recently, accumulated evidence suggests that beside its anaesthetic and analgesic effects, ketamine suppresses the production of inflammatory mediators like tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2), as demonstrated in in vitro and in vivo studies in rodents[13-16] and humans.[17-19] Previously we demonstrated that in an equine macrophage cell line (e-CAS cells) ketamine also inhibited the lipopolysaccharide (LPS)-induced TNF-α and IL-6 production in a dose-dependent manner, even at subanaesthetic concentrations.[1] These results suggest that ketamine has also appreciable anti-inflammatory effects in equine patients.

The purpose of the present study was to investigate the influence of parenterally administered ketamine on an LPS-induced local inflammatory response in tissue chambers implanted in Shetland ponies. This model is commonly used to assess the effect of anti-inflammatory agents since the localised inflammatory response does not impair the animal's health and well-being.[20]

Both the effects of a single intravenous ketamine injection and a long-term ketamine infusion protocol, comprising a loading dose followed by a ketamine continuous rate infusion (CRI) for 6 h, were investigated. Besides studying the influence of ketamine per se, the results of both dose regimens were compared to differentiate between both short-term as well as long-term ketamine treatments. White blood cell count, TNF-α, IL-6 and PGE_2 concentrations were determined in tissue chamber fluid to assess the influence of ketamine on the local inflammatory response. Concentrations of ketamine and its metabolites in both tissue chamber fluid and plasma samples were determined to study the kinetics and rate of drug penetration into tissue chambers.

The ponies were assigned to three treatment groups in a Latin square cross-over design. This implies that all animals were treated three times with LPS. Since

repeated LPS administration might evoke endotoxin tolerance, individual LPS responses were subjected to statistical analysis.

2. MATERIALS AND METHODS

The experiments described in this study were approved by the Ethical Committee on Animal Experimentation of the Utrecht University and conducted in compliance with the Dutch Act on Animal Experiments.

2.1. PONIES

Eight Shetland ponies (all geldings, age 7 ± 2.4 years, bodyweight 151 ± 25.5 kg) were used. All ponies were considered to be clinically healthy on the basis of routine physical examination and hematologic screening. Ponies were vaccinated and treated with anthelmintics prior to the experiments. During each experiment, ponies were stabled in individual boxes and fed a diet of haylage or fresh grass.

2.2. TISSUE CHAMBER IMPLANTATION

The implantation and use of tissue chambers in Shetland ponies has been previously described by Ensink et al.[21] Briefly, under general anesthesia, custom made round tissue chambers (inner diameter 4.4 cm, depth 1.8 cm, total volume 27 mL) were aseptically implanted in the subcutaneous tissue of the mid-neck region on both sides of each pony. Peri-operative antimicrobial treatment included 6.6 mg/kg gentamycin (Gentamycin®, Eurovet Animal Health BV, Bladel, The Netherlands) and 20.000 IU/kg sodium penicillin (Benzylpenicilline Natrium®, Eurovet Animal Health BV, Bladel, The Netherlands) intravenously prior to the start of surgery, and 20.000 IU/kg procain penicillin (Depocilline®, Intervet, Boxmeer, The Netherlands) intramuscularly at the end of the surgical procedure. Peri-operative analgesia consisted of 0.1 mg/kg methadone IV (Methadon HCl® 10 mg/mL, Eurovet Animal Health BV, Bladel, The Netherlands) and 1.1 mg/kg flunixine meglumine IV (Bedozane®, Eurovet Animal Health BV, Bladel, The Netherlands) at the time of pre-anaesthesic medication. To ensure the proper embedding of the tissue chambers, ponies were allowed to recover from implantation for at least 6 weeks prior to the start of the experiments.

2.3. EXPERIMENTAL DESIGN

To test all dose regimens of ketamine in each pony, the ponies were assigned to three treatment groups in a Latin square cross-over design with an 8-week recovery period between each individual experiment.

2.4. LPS STIMULATION AND TREATMENT PROTOCOLS

Two hours before experimentation, an 8 cm 14-gauge polytetrafluoroethylene catheter (Intraflon 2®, Vygon Nederland BV, Veenendaal, The Netherlands) was inserted under aseptic conditions into the right and left jugular veins to facilitate simultaneous treatment and blood sampling, respectively. Thereafter, ponies were allowed to adapt to the experimental environment for at least 1 hour.

At t = 0 h, an inflammatory response was provoked by injecting 3 mL LPS (20 μg/mL; Escherichia coli, O111:B4) in both tissue chambers. Immediately after inoculation, intravenous treatment with ketamine was started. The treatment consisted of a bolus injection of 2.2 mg/kg ketamine (Narketan® 100 mg/mL, Vétoquinol BV, 's-Hertogenbosch, The Netherlands) IV, directly followed by a ketamine continuous rate infusion (CRI) of 1.5 mg/kg/h for 6 h (CRI-group). Alternatively, only a single bolus injection of 2.2 mg/kg ketamine IV directly followed by a 0.9% NaCl CRI for 6 h (SI-group) was given. In control experiments, a bolus injection of 0.9% NaCl IV was directly followed by a 0.9% NaCl CRI for 6 h (C-group). When using 0.9% NaCl, equal volumes compared to ketamine volumes used, were administered.

Between t=-10 min and t=20 min, a sling attached to an overhead hoist was used to support ponies during the induction phase. To standardise experimental conditions, the sling was used for the control experiments as well. After removing the sling, ponies were restrained in stocks for the entire experimental period and allowed to consume haylage and water.

2.5. SAMPLING

Before each experiment, the area around all tissue chambers was examined for the presence of abnormalities, such as wounds and signs of inflammation.

At specific time points prior to and after LPS stimulation, 1 mL tissue chamber fluid was aspirated aseptically from both tissue chambers. Samples of the left tissue chamber were collected for measurement of ketamine concentrations and its metabolites. Tissue chamber fluid from the right tissue chamber was collected for white blood cell (WBC) and differential cell counts, and for TNF-α, IL-6 and PGE_2 analyses. Samples were aliquoted in either micro tubes containing 16 IU heparin (ketamine, TNF-α, IL-6 and total leukocyte and differential count) or tubes containing 50 IU heparin and 10.73 μg indomethacin (PGE_2 analysis). Immediately after sampling the tissue chambers, venous blood samples were collected to determine plasma WBC and its differentiation and plasma concentrations of

ketamine and its metabolites. Total WBC and differential cell count were determined within 30 min after sampling. Venous blood samples for determining the ketamine concentrations were centrifuged at 400 rpm for 10 min at 0°C and supernatants as well as tissue chamber fluids were stored at -70°C until analysis.

2.6. CLINICAL PARAMETERS

Heart rate and rectal temperature were recorded one hour before and at preselected intervals following LPS inoculation. Simultaneously, the area around the tissue chambers was checked for swelling. Behaviour and appetite were noted during the entire experiment.

2.7. TOTAL WHITE BLOOD CELL AND DIFFERENTIAL COUNTS

The total number of WBC per mL tissue chamber fluid was counted by using the Coulter Counter® (Beckman-Coulter, Mijdrecht, The Netherlands). For counting WBCs in venous blood samples the Medonic CA 530® (A. Menarini Diagnostics, Valkenswaard, The Netherlands) was used. The relative number of PMNs and macrophages were determined microscopically after May-Grünwald Giemsa staining.

2.8. 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, 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.

2.9. IL-6 BIOASSAY

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.[22] 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.

2.10. PGE_2 RADIO-IMMUNOASSAY

Concentrations of PGE_2 were measured by radio-immunoassay (RIA). In brief, anti-PGE_2 antiserum (Sigma Chemical Co., St Louis, MO, USA) and [^{3}H]PGE_2 (Amersham-Pharmacia Biotech, Veenendaal, The Netherlands) were added to each standard or sample. The tubes were incubated for 18-24 h at 4^{o}C. After incubation, dextran-coated charcoal suspension (20 mg/mL charcoal and 4 mg/mL dextran) was added and tubes were centrifuged (3000 rpm, 4^{o}C, 15 min) to separate bound and unbound fractions. Supernatants, containing the bound fraction, were transferred into scintillation-vials and scintillation fluid (Ultima Gold, Perkin Elmer, Wellesley, MA, USA) was added. After mixing, samples were analysed using a liquid scintillation counter (Tri-carb 2900TR®, Packard). Concentrations of PGE_2 were determined by comparing the values of the samples to the standards.

2.11. KETAMINE AND METABOLITES

Concentrations of ketamine, norketamine, hydroxynorketamine and 5,6-dehydronorketamine in both tissue chamber fluid and plasma samples were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in detail elsewhere.[23]

2.12. STATISTICAL ANALYSES

Statistical analysis was performed using the SPSS© 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). To study the influence of ketamine on LPS-induced inflammatory responses, data of different ketamine groups (i.e., CRI, SI and C) were analysed using a repeated measures analysis of variance and the Huynh-Feld test statistics to adjust for sphericity, and were expressed as mean ± standard deviation (S.D.). When significant, a paired samples t-test was used to compare the differences with baseline values. Differences were considered to be significant when $p<0.05$.

3. RESULTS

3.1. INFLUENCE OF KETAMINE ON TOTAL WHITE BLOOD CELL COUNT AND DIFFERENTIAL COUNTS

Compared to baseline values, the influx of WBC into the tissue chambers was significantly increased in all treatment groups from 8 h following LPS exposure until the end of the experiment. Administration of ketamine, either as bolus or as CRI, did not significantly influence the LPS-induced WBC influx into the tissue chambers when compared to the saline-treated control group (Fig. 1A). Differential counts of tissue chamber WBC revealed a significant increase in polymorphonuclear neutrophils towards the end of each experiment.

In plasma, the number of WBC in the SI and C-group remained within physiological limits throughout the entire experiment. In the CRI-group, WBC slightly increased to 11.2 ± 1.6 G/L at 8 h following LPS exposure with a subsequent decrease to baseline values after 24 h of exposure (Fig. 1B). No differences in the number of WBC were observed between control and ketamine-treated animals. Differential counts of WBC were not significantly affected in any of the experiments.

3.2. INFLUENCE OF KETAMINE ON LPS-INDUCED TNF-α, IL-6 AND PGE_2 PRODUCTION

In all groups, TNF-α, IL-6 and PGE_2 significantly increased upon inoculation of LPS into the tissue chambers (Fig. 2). Peak concentrations of TNF-α, IL-6 and PGE_2 were reached at 6, 12 and 8 h, respectively. Again, no significant influence of ketamine administration on LPS-induced TNF-α, IL-6 and PGE_2 production could be demonstrated (Fig. 2).

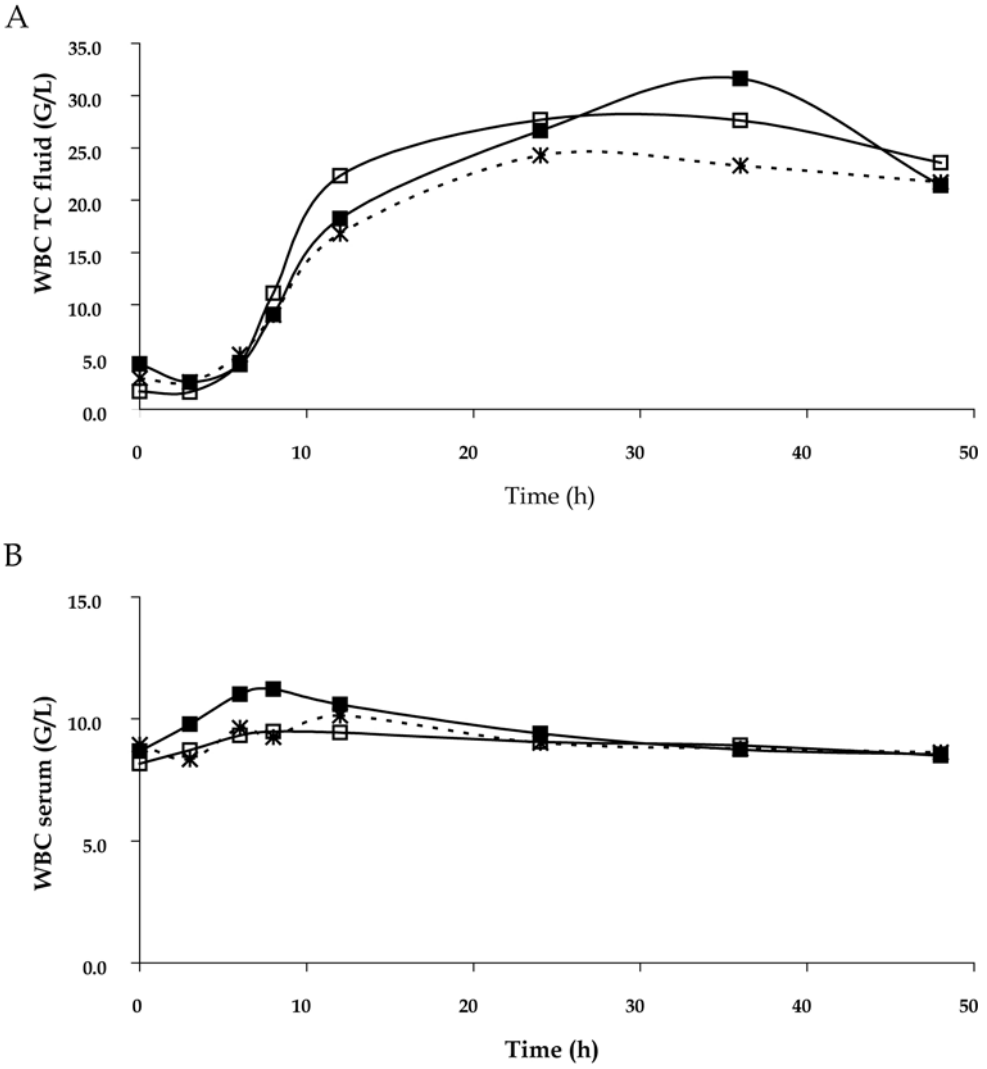

Figure 1. The effect of ketamine CRI (■), ketamine SI (□) and saline (∗) on total white blood cell count in (A) tissue chamber fluid and (B) serum.

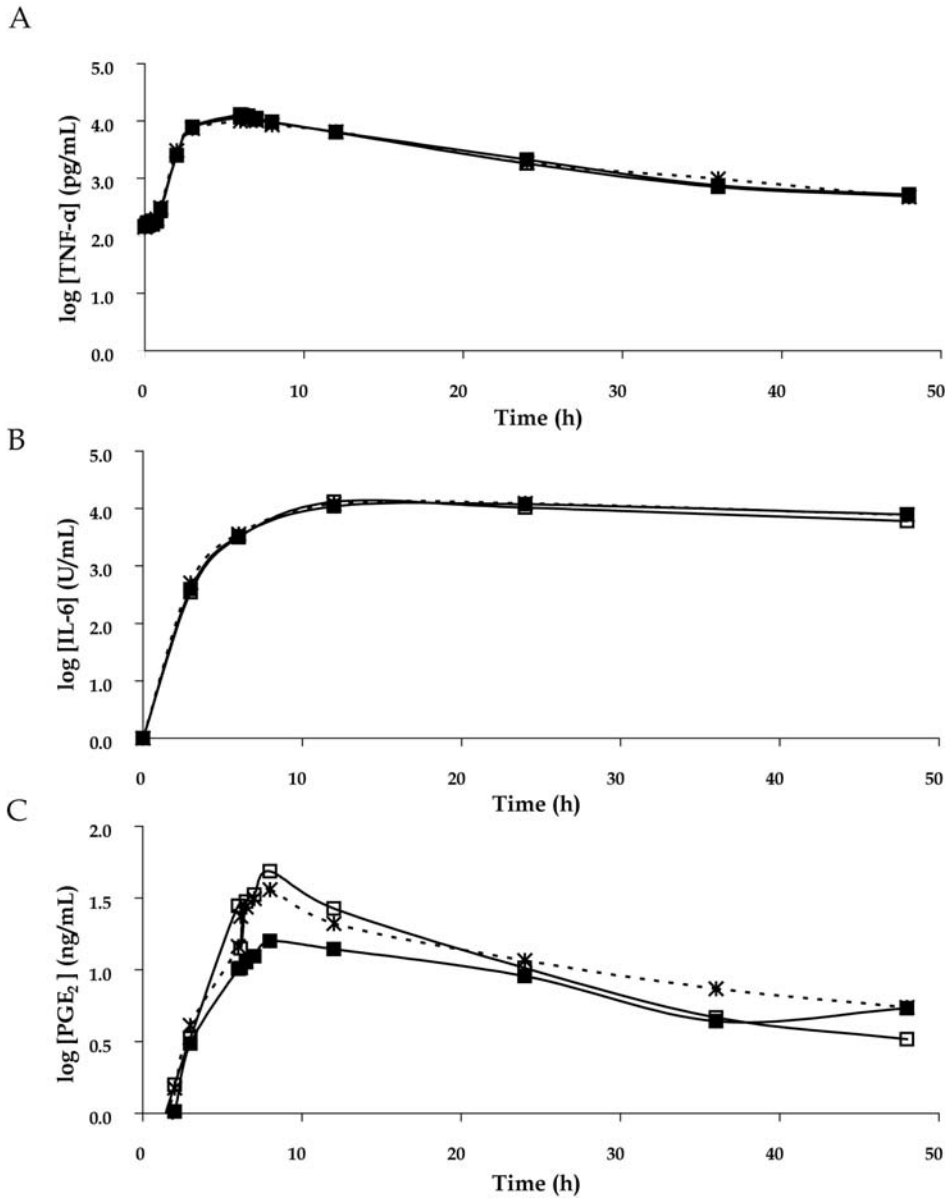

Figure 2. The effect of ketamine CRI (■), ketamine SI (□) and saline (∗) on (A) TNF-α, (B) IL-6 and (C) PGE_2 concentrations in the tissue chamber fluid.

3.3. INFLUENCE OF KETAMINE ON CLINICAL PARAMETERS

Following the bolus injection of ketamine, induction was achieved within 30 s and lasted for 3 to 5 min. Overall, in three individual cases catatonia was observed, one in the SI and two in the CRI group.

In the control group, heart rate remained stable and within the physiological range (28-40 beats per min) throughout the entire experiment. In contrast, in both the CRI and SI-group, heart rate significantly increased to a peak value of 51 ± 6 bpm at 15 min following ketamine injection. Thereafter, rates returned to physiological values in the SI group and remained stable for the remainder of the experiment. In the CRI group, heart rate also decreased following 15 min of the bolus injection, but remained slightly above the physiological range until the end of the ketamine CRI. Then, baseline values were reached within 15 min post-infusion.

Rectal temperature did not significantly differ between the studied groups.

Before each experiment, the area around the tissue chambers did not show any abnormalities. Swelling was first seen approximately 3 h after inoculation and remained visible till the end of the experiment. However, none of the ponies was reluctant to move the neck and abscess formation of the tissue chambers did not occur.

3.4. KETAMINE CONCENTRATIONS IN PLASMA AND TISSUE CHAMBER FLUID

In the CRI group, the peak plasma concentration of 576 ± 305 ng/mL at 5 min following ketamine bolus injection decreased to a minimum of 101 ± 64 ng/mL at 2 h during ketamine CRI (Fig. 3A). Thereafter, the plasma ketamine concentration slightly increased again towards the end of infusion. During ketamine CRI, no obvious steady state level was reached. The average plasma ketamine concentration was 155 ± 54 ng/mL. Once the ketamine CRI was discontinued, the plasma ketamine concentration decreased rapidly to approximately one-tenth of the initial value within 2 h. In tissue chamber fluid, a peak concentration (139 ± 67 ng/mL) was reached at 30 min following ketamine injection. Subsequently, an apparent steady state level at 126 ± 9 ng/mL was observed till the end of the ketamine CRI. After discontinuing the ketamine CRI, the ketamine concentration in the tissue chamber fluid gradually decreased to baseline values until the end of the experiment.

In the SI group, the peak plasma concentration of 685 ± 147 ng/mL progressively decreased to 14 ± 5 ng/mL within 2 h (Fig. 3B). In tissue chamber fluid, a maximum concentration of 177 ± 41 ng/mL was reached between 45 min and 2 h

following ketamine injection, which gradually decreased until the end of the experiment.

3.5. KETAMINE METABOLITES IN PLASMA AND TISSUE CHAMBER FLUID

In the CRI group, the concentration of norketamine remained high above the concentration of the parent compound in both plasma and tissue chamber fluid (Figs 4A and 4B). Plasma concentrations of hydroxynorketamine and 5,6-dehydronorketamine nearly equalled the plasma concentrations of ketamine. Although plasma concentrations of all metabolites reached an apparent steady state during ketamine CRI, concentrations in tissue chamber fluid still increased till the end of ketamine CRI (Figs 4A and 4B).

In the SI group, plasma concentrations of the metabolites remained below or slightly above the plasma concentration of ketamine (Fig. 4C). In the tissue chamber fluid, the concentration of all metabolites remained below the concentration of ketamine (Fig. 4D).

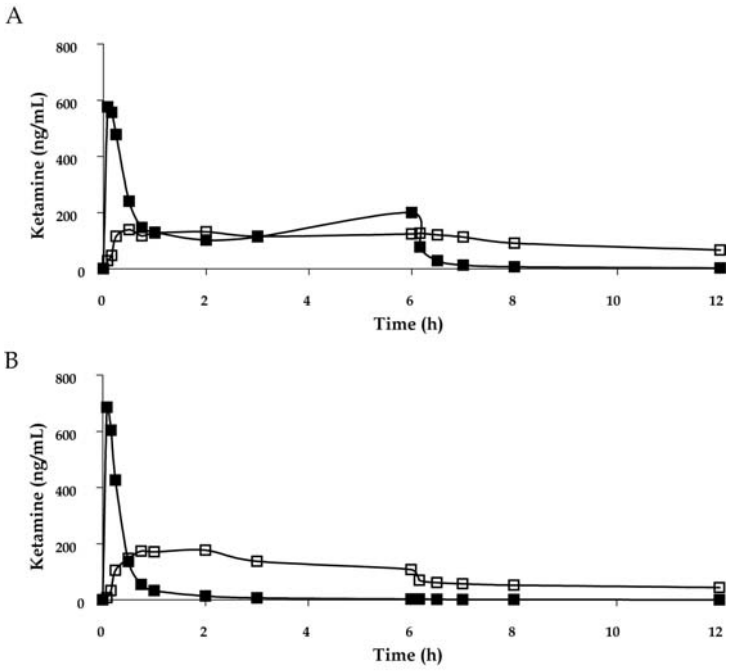

Figure 3. The mean ketamine concentration in plasma (■) and tissue chamber fluid (□) following (A) ketamine CRI and (B) a single injection of ketamine.

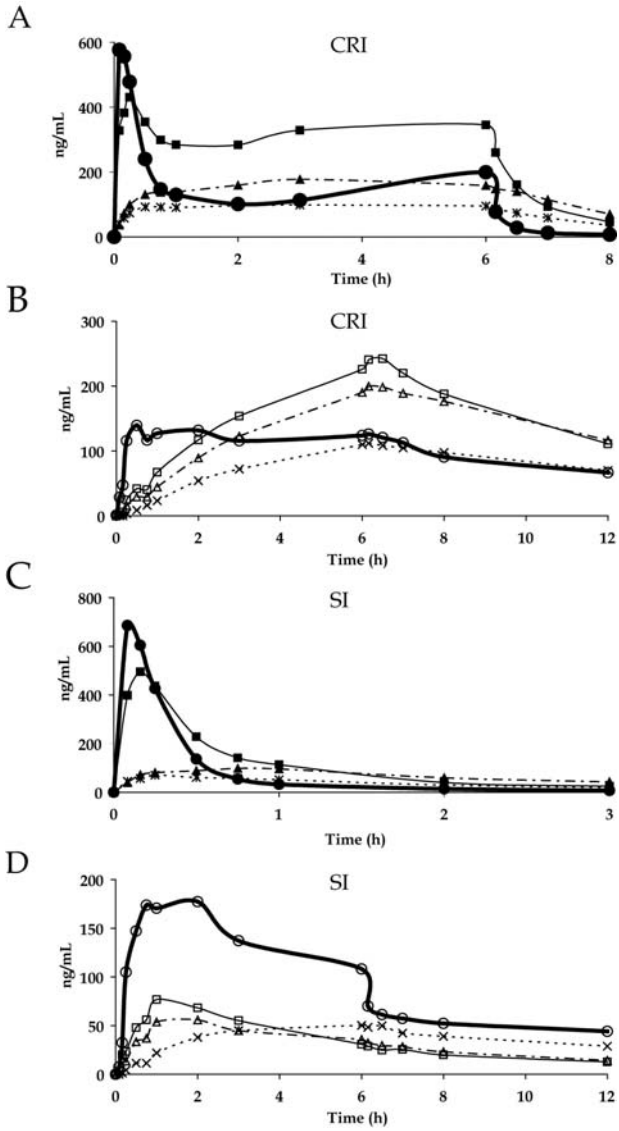

Figure 4. The mean concentrations of ketamine (●/○), norketamine (■/□), hydroxynorketamine (▲/△) and 5,6-dehydronorketamine (∗/×) in plasma (closed symbols in A and C) and tissue chamber fluid (open symbols in B and D) following ketamine CRI (A and B) and a single ketamine injection (C and D).

4. DISCUSSION

Previously, we demonstrated that ketamine exerts anti-inflammatory effects in an equine macrophage cell line (e-CAS cells) by reducing LPS-induced TNF-α and IL-6 production in a concentration-dependent manner.[1] The aim of the present study was to evaluate these anti-inflammatory effects of ketamine in vivo by using an LPS-inoculated equine tissue chamber model.

Considering the concentration-dependent influence and the fact that elevated plasma cytokine levels have been detected in horses for several hours after onset of inflammation, it was hypothesised that ketamine needed to be administered over extended periods of time and at relatively high doses to be effective. To verify the hypothesis regarding long-term ketamine treatment, a bolus injection followed by a ketamine CRI was compared to a bolus injection of ketamine only. For the bolus injection the commonly used induction dose of 2.2 mg/kg body weight was selected. The dose rate of the ketamine CRI tested is based on the premise that it should allow its administration to conscious horses in the post-operative intensive care. The risk of hypnotic and excitatory side effects limits the maximal dose rate of the ketamine CRI in conscious horses. Recently, it has been demonstrated that a ketamine CRI of 1.5 mg/kg/h could be administered safely to healthy conscious horses for at least 6 h.[23]

In contrast to the results obtained in vitro, ketamine did not influence LPS-induced TNF-α, IL-6 and PGE_2 production in the equine tissue chamber model. Moreover, no effect of ketamine on WBC count in tissue chamber fluid could be detected. This discrepancy between the in vitro findings and the present results might be due to the fact that only a limited fraction of ketamine entered the tissue chamber. Following intravenous injection, ketamine rapidly entered the tissue chamber, corresponding well with the rapid distribution ($t_{1/2\alpha}$ = 2-3 min)[23-25] and the low protein binding of ketamine (50%).[24] However, the peak concentrations in tissue chamber fluid remained below 25% of the peak concentrations measured in plasma, despite the generally large volume of distribution.[23-25] This low tissue chamber concentration is associated with a delayed diffusion of ketamine into the tissue chambers and the extremely rapid elimination ($t_{1/2\beta}$ = 65-67 min).[23,25] Moreover, due to its low protein binding and as a weak base, ketamine is presumably not being trapped in the tissue chambers but rapidly redistributed. Hence, the final ketamine concentration in the tissue chamber fluid (range 126-177 ng/mL; 0.5-0.6 μM) was considered to be too low to generate a measurable anti-inflammatory activity. In e-CAS cells, TNF-α concentrations were only

significantly suppressed by ketamine concentrations exceeding 1.8 μM. However, higher dose rates of ketamine could not be tested since a previous pharmacokinetic study on a long-term ketamine CRI in conscious healthy horses showed that at concentrations exceeding 1.5 mg/kg/h considerable undesirable side effects, mainly related to the central nervous system (excitement / cardiovascular stimulation) and increasing skeletal muscle tone (catatonia), will occur.[23]

Ketamine is metabolised extensively by the hepatic cytochrome P_{450} system. The major pathway involves N-demethylation to form norketamine, which is then hydroxylated and oxidated to form hydroxynorketamine and 5,6-dehydronorketamine, respectively.[26] In the present study, all three metabolites were measured in both plasma samples and tissue chamber fluid. However, the pharmacodynamical effects of these metabolites have not been fully elucidated yet.

The tissue chamber model has been proven to be a valid model for simulating inflammatory reactions at soft tissue level following LPS stimulation.[27] Applying the tissue chamber model, LPS-induced inflammatory responses will be restricted mainly to the tissue chamber, thereby minimising discomfort to experimental animals.[28] Similarly, in the present study, the LPS-induced acute inflammatory responses remained largely confined to the tissue chambers. Upon stimulation by LPS, a significant increase in WBC count and TNF-α, IL-6 and PGE_2 concentrations in the tissue chamber fluid was measured, whereas WBC in plasma and rectal temperature remained within or slightly above the physiological range. Furthermore, none of the ponies gave any impression of malaise.

In the present study, substantial inter- and intra-individual differences in the response to LPS were observed. In humans and rodents, this variability in LPS response is ascribed to changes in the coding sequence of the LPS receptor complex.[29-31] In addition, differences in plasma concentrations of LPS binding protein, which presents LPS to the LPS receptor complex, might explain the variability in LPS response.[32] In horses, inter-individual differences in the LPS response have previously been described.[33] However, the altered response to LPS could not be related to mutations observed in parts of the LPS receptor complex.[34] Finally, development of endotoxin tolerance might also be responsible for differences in the LPS response. However, the statistical analysis of the three independent experiments performed in this study showed no significant difference between the three experiments for any of the studied parameters.

In conclusion, following the administration of clinically tolerable dosages, ketamine does not reach tissue chamber concentrations which are effective in

reducing LPS-induced TNF-α, IL-6 and PGE_2 concentrations in tissue chamber fluid. Further experiments should be conducted to measure the systemic effect of ketamine, preferentially in equine patients suffering from inflammatory diseases that receive ketamine as part of the general anaesthesia protocol or in the intensive care.

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- CHAPTER X -

SUMMARISING DISCUSSION AND CONCLUSIONS

KETAMINE: AN OLD DRUG WITH NEW TRICKS?

Ketamine, first described in 1966 by Corssen and Domino,[1] is used in human and veterinary anaesthesia for inducing amnesia, loss of consciousness, analgesia and immobility.[2-5] In contrast to other anaesthetics, such as hypnotics and volatile anaesthetics, ketamine produces somatic and visceral analgesia even at subanaesthetic concentrations.[6-12] Since ketamine preserves cardiovascular function, its use is recommended for anaesthesia of critically ill patients in which there is a risk of cardiac depression and hypotension.[13-15]

In equine anaesthesia and analgesia, ketamine is commonly used in a variety of clinical procedures. Besides its application for induction of anaesthesia,[16] ketamine has also been used as an adjunct to inhalant anaesthesia,[17-19] in different total intravenous anaesthesia (TIVA) protocols,[20-22] and for epidural analgesia[23] and peripheral nerve blocks.[24] More recently, studies focus on the antinociceptive effects of a subanaesthetic ketamine continuous rate infusion (CRI) administered to conscious horses.[25]

Since the mid 90s, potential anti-inflammatory effects of ketamine, such as cytokine-modulating effects and anti-oxidative properties, have attracted attention and have been studied intensively in rodents[26-31] and humans.[32-37] However, no data are available on the anti-inflammatory effects of ketamine in horses suffering from inflammatory diseases. This thesis summarises experimental work conducted into the anti-inflammatory effects of ketamine in different in vitro and in vivo equine models.

CYTOKINE-MODULATING EFFECTS OF KETAMINE IN AN EQUINE MACROPHAGE CELL LINE

For the in vitro experiments, an equine bone-marrow-derived macrophage cell line, referred to as e-CAS cells, was used to investigate the potential anti-inflammatory effects of ketamine in horses in vitro (**Chapter III-VII**). Although primary equine cells like PBMCs,[38] peritoneal macrophages,[39-41] alveolar macrophages,[42,43] vascular smooth muscle cells[44] and chondrocytes[45] can easily be isolated, and have frequently been used to study several anti-inflammatory therapeutic approaches, a cell line over primary cells was chosen, since the former has the advantage of a more uniform response pattern.[46]

Previously, e-CAS cells have intensively been studied by Werners et al.[47] In particular, the equine-specific signalling pathways have been characterised in detail in these cells. However, since the e-CAS model is a rather new model, both a human monoblastoid tumour cell line (U937 cells) (**Chapter III**) and a murine

macrophage cell line (RAW 264.7 cells) (**Chapter V-VI**) were used to confirm the outcome observed with the e-CAS model.

In all experiments, lipopolysaccharide (LPS) was applied as the primary stimulator of the inflammatory response, since LPS is a homogeneous reagent with uniform properties[48] and has been implicated in the pathogenesis of various inflammatory diseases in several species, including horses.[49-54] Upon stimulation with LPS, cells of the innate immune system produce and release inflammatory mediators, such as cytokines, acute phase proteins and prostanoids, which can potentially be used as markers for the early detection of inflammation. Evidence suggests that tumour necrosis factor-alpha (TNF-α) is the primary mediator of the LPS-induced effects,[55] whereas the interleukin-6 (IL-6) concentration predominantly has a prognostic value in horses suffering from inflammatory disorders.[56,57] Hence, both cytokines were used as markers when studying the anti-inflammatory effects of ketamine in equine models.

In all in vitro studies in this thesis, ketamine concentrations ranging between 0-36 μM were tested. This range was selected in consideration of the peak plasma level of 5 μg/mL (18 μM) resulting from the standard IV induction dose in horses of 2.2 mg/kg body weight.[58] Lower concentrations were included to determine the minimal effective concentration that exerts anti-inflammatory effects in vitro. This is of possible clinical relevance since lower plasma concentrations are desirable in the post-operative period to circumvent the hypnotic and excitatory side effects of ketamine in conscious horses. In some cases, high experimental concentrations up to 1000 μM were tested as well to investigate whether ketamine would be able to affect the inflammatory parameters at all.

In the LPS-stimulated e-CAS cells, ketamine significantly suppressed the TNF-α and IL-6 concentrations in a concentration-dependent manner (**Chapter III**). This initial finding prompted us to start with a series of experiments aiming at the understanding of the molecular mechanisms involved in the cytokine-modulating effects of ketamine.

MOLECULAR MECHANISM UNDERLYING THE CYTOKINE-MODULATING EFFECTS OF KETAMINE

Following LPS exposure, multiple signalling pathways are known to be involved in upregulating gene expression and synthesis of pro-inflammatory cytokines.[59-62] Following binding to and activation of the cell surface receptor Toll-like receptor 4 (TLR4), LPS initiates a complex intracellular signalling cascade.[63-65] The most

prominent signalling pathways encompass the phosphorylation of mitogen-activated protein kinases (MAPKs) and the activation of nuclear factor-kappa B (NF-κB).[66,67] MAPKs represent a highly conserved family of serine/threonine/tyrosine kinases comprising three major subfamilies: c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase p38 (p38) and extracellular signal-regulated kinase (ERK). Upon phosphorylation, MAPKs translocate to the nucleus and activate transcription factors like activating protein-1 (AP-1), Elk1, c-Fos, c-Jun and NF-κB.[67,68]

NF-κB, representing a group of structurally related transcriptional proteins that form dimers of distinct composition, is considered to be a key regulatory transcription factor, which is involved in numerous cell functions, including the cell differentiation and maturation, and control of cell proliferation and apoptosis (for review see 67). The most prominent NF-κB protein is the p50/p65 heterodimer, which has been found to bind to a distinct DNA sequence, regulating the transcription of more than 200 genes, including those related to the expression of inflammatory mediators. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form by association with the inhibitory proteins of the inhibitor of kappa B (IκB) family, such as IκBα, IκBβ, IκBγ, IκBζ and molecule possessing ankyrin-repeats induced by LPS (MAIL). Of these isoforms, IκBα is most abundant and constitutively expressed, whereas IκBζ and MAIL are induced by LPS and pro-inflammatory cytokines. Exposure to a variety of stimuli, including LPS, results in activation of the IκB kinase complex (IKK), which in turn causes rapid phosphorylation and degradation of IκBs. Subsequently, released NF-κB translocates into the nucleus, where it binds to its cognate DNA sequence, resulting in the transcription of multiple inflammatory genes.[69]

The finding that ketamine suppresses the LPS-induced cytokine production in e-CAS cells could, in principle, be attributed to the impairment of either extracellular, intracellular or intranuclear signalling molecules or any combination thereof. Hence, we studied in a serial approach the different phases of LPS signalling, including the effect of ketamine on LPS-binding to TLR4 (extracellular), on the MAPK pathway (intracellular) and on NF-κB (intranuclear) activation (**Chapter IV**). Results show that ketamine significantly inhibited the LPS-induced signal transduction cascade only at the level of NF-κB. Activation of NF-κB can be divided grossly into two phases: a first phase comprising the dissociation of NF-κB from IκB, and a second phase that involves various additional regulatory mechanisms, such as nuclear uptake and export, and affinity to the DNA

responsive element that determines the final strength and duration of the NF-κB transcriptional response.[69-72] Western blot analysis indicated that, in contrast to previous in vitro studies in rodents,[73,74] from our e-CAS cells experiments no influence of ketamine on IκB could be detected. Hence, the results described in Chapter IV suggest that ketamine inhibits NF-κB expression by affecting the regulatory events occurring in the second phase. This is supported by the finding that ketamine also inhibited NF-κB expression when added directly to the nuclear extract. At present, this is the first report in which reduced NF-κB expression following ketamine exposure could be ascribed to a direct interaction between NF-κB and ketamine.

EFFECT OF KETAMINE ON INDUCIBLE ENZYMES

NF-κB is known to be involved in the transcription of enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS),[67,75] that account for many of the clinical symptoms associated with the inflammatory response. Since ketamine directly inhibits NF-κB expression, it was hypothesised that ketamine might also reduce COX-2 and iNOS expression and subsequently inhibit the production of prostaglandin E_2 (PGE_2) and nitric oxide (NO).

However, in e-CAS cells ketamine did not reduce COX-2 expression, not even following exposure to concentrations up to 1000 μM (**Chapter VI**). Since these results were unexpected to some extent, we additionally investigated the molecular mechanism underlying LPS-induced COX-2 expression by selectively inhibiting the JNK, p38 and ERK, as well as the NF-κB pathway. We found that COX-2 expression was only regulated by the three MAPKs, as their inhibition resulted also in the inhibition of COX-2 expression. Inhibition of NF-κB did not reduce COX-2 expression. This latter finding correlates with findings by Wadleigh et al.,[76] who described that the transcriptional activation of COX-2 in LPS-stimulated RAW 264.7 cells is independent of NF-κB. Hence, the fact that ketamine inhibits the LPS-induced signalling pathway only at the level of NF-κB explains why ketamine did not reduce COX-2 expression and the subsequent PGE_2 production in LPS-treated e-CAS cells.

Similarly, ketamine did not reduce iNOS expression, neither at clinically relevant nor at high experimental concentrations (**Chapter V**). Corresponding to these results, no effect on LPS-induced NO production was found. However, the inhibition experiments in this study revealed that iNOS expression is regulated by the MAPKs as well as the NF-κB pathway. These results and the fact that ketamine

inhibits the LPS-induced signal transduction pathway only at the level of NF-κB suggests that MAPKs are more important in regulating iNOS expression than NF-κB. Moreover, these results show that MAPKs can regulate iNOS expression independently of NF-κB.

ANTI-OXIDATIVE EFFECTS OF KETAMINE

Next to LPS, NF-κB is known to be activated by various other stimuli, including reactive oxygen species (ROS).[77] This implies that blocking ROS formation should, theoretically, result in reduced activation of NF-κB and, consequently, reduced cytokine production. In stimulated e-CAS cells, ketamine significantly reduced ROS formation (**Chapter VII**), NF-κB expression (**Chapter IV**) and cytokine production (**Chapter III**) in a concentration-dependent manner. Although no data are available regarding the direct interaction between ROS formation and NF-κB expression in e-CAS cells, it is considered unlikely that the reduced NF-κB expression in e-CAS cells is caused by reduced ROS formation. LPS alone significantly induced NF-κB expression, but resulted only in a marginal increase in ROS formation. Only when e-CAS cells were co-stimulated with LPS and phorbol myristate acetate (PMA), a significant increased ROS formation was observed. However, the significant increase in NF-κB expression was not preceded by nor accompanied with an increased ROS formation. Expression of NF-κB activity was most significantly at 15 min of LPS exposure, whereas ROS formation only significantly increased following 2 h of stimulation. Hence, in e-CAS cells, ketamine reduced the LPS-induced NF-κB expression through a ROS-independent pathway.

LONG-TERM KETAMINE CRI AND ITS NON-ANAESTHETIC EFFECTS IN VIVO

The finding that ketamine reduced TNF-α and IL-6 production in LPS-treated e-CAS cells indicates that ketamine has cytokine-modulating effects in vivo. Considering the concentration-dependency and the fact that elevated plasma cytokine levels have been detected in horses for several hours after onset of inflammation, it was hypothesised that ketamine may need to be administered for a prolonged period of time and at relatively high dose rates to be clinically effective in modulating inflammatory mediators. During general anaesthesia relatively high dose rates can be used. However in the intensive care, when ketamine is administered to conscious horses, the risk of hypnotic and excitatory side effects limits the dose rate of the ketamine CRI. The results of **Chapter VIII**

show that a ketamine CRI of 1.5 mg/kg/h can safely be administered to conscious healthy horses for at least 6 h. The pharmacokinetic profile of this CRI was not very different from those previously reported in horses after single IV bolus administration.[58,78,79] In equine patients, in which pathophysiological conditions may lead to a different pharmacokinetic profile, delayed total body clearance of ketamine may result in increasing plasma concentrations and, subsequently, in ketamine-associated undesirable side effects. Administration of the ketamine CRI studied over more than six hours could potentially be associated with side effects, since in neither of the studies described in Chapter VIII and IX steady-state plasma levels were reached at the time the infusions were stopped.
Ketamine is metabolised extensively by the hepatic cytochrome P_{450} enzyme system.[80,81] The major pathways involve N-demethylation to produce norketamine, which is then hydroxylated and oxidated to hydroxynorketamine and 5,6-dehydronorketamine, respectively. In both in vivo studies, all three metabolites were identified by using liquid chromatography-tandem mass spectrometry. This newly developed technique has been shown to be more accurate in detecting ketamine metabolites, in particular for 5,6-dehydronorketamine, when compared to the former technique of gas-liquid chromatography-mass-spectrometry.[82]

To evaluate the cytokine-modulating effects of ketamine in vivo, a tissue chamber model was used (**Chapter IX**). In this model the LPS-induced inflammatory response is confined to the tissue chamber and does not affect the overall clinical health status of the animal, thus meeting the modern ethical standards of animal experiments. As anticipated, LPS induced an inflammatory response characterised by an invasion of white blood cells and an increased concentration of pro-inflammatory mediators. However, when the tissue chamber fluid was analysed, ketamine has been shown not to reduce the LPS-induced production of TNF-α, IL-6 and PGE_2. This most likely is due to the low ketamine concentrations in the tissue chamber fluid. Following bolus injection and CRI of ketamine, only a limited fraction of ketamine entered the tissue chamber. This was considered to be associated with a very rapid elimination of ketamine ($t_{1/2\beta}$ = 65-67 min) in combination with a limited diffusion of ketamine into the tissue chambers. In addition, due to its low protein binding and as a weak base, ketamine has presumably not been trapped in the tissue chambers but rapidly redistributed. The low tissue fluid concentrations (0.5 μM) might explain the discrepancy found between the cytokine-modulating effects found in vitro (**Chapter III**) and in vivo

(**Chapter IX**), since in vitro LPS-induced TNF-α concentrations were only significantly suppressed by ketamine concentrations exceeding 1.8 μM.
Horses (Chapter VIII) and ponies (Chapter IX) did not significantly differ in the level of plasma concentrations of ketamine or norketamine measured during CRI. However, with respect to both hydroxynorketamine and dehydronorketamine plasma concentrations, significantly higher levels were measured in ponies than in horses during ketamine CRI. These findings most likely reflect the known species- and breed-differences in the activity of biotransformation enzymes found in other animal species.[83,84]

CLINICAL RELEVANCE OF KETAMINE ADMINISTRATION TO HORSES SUFFERING FROM INFLAMMATORY DISEASES

The finding that ketamine significantly reduces TNF-α, IL-6 and ROS formation in stimulated e-CAS cells suggests that ketamine might be beneficial in horses suffering from inflammatory disorders. The rapid biotransformation and elimination of single ketamine injections as well as the limited affinity to proteins, preventing accumulation of the active compound in the inflamed tissue, indicate that ketamine does not have the favourable pharmacokinetic profile to suppress a systemic inflammatory response, as seen in patients with gastrointestinal disorders and endotoxaemia. However, in these patients, the observed anti-inflammatory properties of ketamine can be regarded as a favourable side effect following its application as anaesthetic and analgesic agent during surgery and in the post-operative intensive care.

MAIN CONCLUSIONS

The major conclusions of this thesis can be summarised as follows:

- Ketamine significantly inhibits the LPS-induced TNF-α and IL-6 production in an equine macrophage cell line in a dose-dependent manner (Chapter III).
- Ketamine inhibits the LPS-induced intracellular signal transduction pathways only at the level of NF-κB (Chapter IV).
- Ketamine inhibits NF-κB expression through a direct interaction between ketamine and NF-κB (Chapter IV).
- Ketamine does not reduce the MAPK and NF-κB-mediated iNOS expression and the subsequent NO production in an equine macrophage cell line following LPS exposure (Chapter V).

- Expression of COX-2 in an equine macrophage cell line is predominantly regulated via the three classical MAPK pathways without a major contribution of NF-κB (Chapter VI).
- Ketamine does not reduce the MAPK-mediated COX-2 expression and the subsequent PGE_2 production in an equine macrophage cell line following LPS exposure, which implies also that the analgesic effects of ketamine are not mediated by COX-2 expression (Chapter VI).
- Ketamine reduces ROS formation in an equine macrophage cell line (Chapter VII).
- Ketamine increases the intracellular GSH concentrations, but does not induce GSH synthesis (Chapter VII).
- A ketamine CRI of 1.5 mg/kg/h can safely be administered to healthy conscious horses for at least 6 hours (Chapter VIII).
- Ketamine is rapidly metabolised into norketamine, hydroxynorketamine and 5,6-dehydronorketamine (Chapter VIII).
- Ketamine does not exhibit cytokine-modulating or antioxidative properties in LPS-inoculated tissue chambers in Shetland ponies (Chapter IX).

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- NEDERLANDSE SAMENVATTING -

KETAMINE: EEN NIEUWE DIMENSIE VAN EEN BEKENDE STOF?

Ketamine is een anestheticum dat in de humane en veterinaire anesthesie voornamelijk wordt gebruikt voor het induceren van hypnose, pijnstilling en immobilisatie. In tegenstelling tot andere anesthetica, zoals hypnotica en inhalatie-anesthetica, geeft ketamine somatische en viscerale pijnstilling zelfs na toediening van heel lage doseringen. Daar ketamine als sympaticomimeticum het cardiovasculaire systeem stabiliseert, wordt het gebruik van ketamine wel aanbevolen voor patiënten met een verhoogd risico op cardiovasculair falen.
In het kader van de algehele anesthesie van paarden wordt ketamine veelvuldig toegepast in diverse anesthesie protocollen. Behalve voor de inductie van een algehele anesthesie, wordt ketamine ook gebruikt in combinatie met inhalatie-anesthesie, bij intraveneuze anesthesie en voor epidurale pijnstilling. De laatste jaren wordt m.n. veel onderzoek verricht naar de pijnstillende effecten van laag gedoseerde ketamine infusen toegediend aan paarden, die bij bewustzijn zijn.
Sinds halverwege de jaren '90, hebben onderzoeken bij muizen, ratten en mensen aangetoond dat ketamine niet alleen anesthetische en pijnstillende effecten heeft, maar ook ontstekingsremmende eigenschappen bevat, zoals het remmen van de productie van ontstekingmediatoren, bijvoorbeeld cytokinen en zuurstofradicalen. Het feit dat bij het paard deze ontstekingsremmende effecten van ketamine nog nooit zijn onderzocht, was voor ons aanleiding om hier onderzoek naar te verrichten. Dit proefschrift geeft een overzicht van de verschillende in vitro en in vivo experimenten die zijn uitgevoerd om de ontstekingsremmende effecten van ketamine bij het paard te onderzoeken.

HET EFFECT VAN KETAMINE OP DE PRODUCTIE VAN CYTOKINEN IN EEN PAARDEN-MACROFAGEN-CELLIJN

Om de ontstekingsremmende eigenschappen van ketamine bij het paard in vitro te kunnen bestuderen is gebruikt gemaakt van een paarden-macrofagen-cellijn, ook wel e-CAS cellijn genoemd. Hoewel primaire cellen, zoals monocyten, macrofagen, vaatwand- en kraakbeencellen eenvoudig uit een paard kunnen worden geïsoleerd en veelvuldig zijn gebruikt voor het bestuderen van de ontstekingsremmende effecten van diverse farmaca is, voor de in vitro onderzoeken beschreven in dit proefschrift, toch gekozen voor het gebruik van een cellijn, omdat het reactiepatroon in een cellijn over het algemeen veel uniformer verloopt.
Experimenten voorafgaand aan dit ketamine-onderzoek hebben de karakteristieken van de e-CAS cellijn reeds duidelijk in kaart gebracht. Vooral de intracellulaire signaal transductie paden zijn in deze cellijn al intensief bestudeerd.

Daar de e-CAS cellijn echter een tamelijk nieuwe cellijn is, is besloten om de meeste in vitro experimenten niet alleen in de e-CAS cellijn uit te voeren, maar ook in bekende macrofagen-cellijnen afkomstig van mensen (U937) en muizen (RAW 264.7). De resultaten van deze cellijnen versterken op deze manier de resultaten verkregen met de e-CAS cellijn.
Om een ontstekingsreactie op te wekken is in alle experimenten gebruik gemaakt van LPS (lipopolysacchariden). LPS is een gifstof afkomstig van de buitenste celwand van Gram-negatieve bacteriën en speelt een belangrijke rol in het ontstaan van ontstekingsreacties bij diverse diersoorten waaronder het paard. Stimulatie van macrofagen met LPS leidt tot de productie van ontstekingsmediatoren, zoals cytokinen, acute fase eiwitten en prostanoïden, die alle gebruikt kunnen worden voor de vroege detectie van een ontsteking. Van de cytokinen, die worden gevormd, zijn TNF-α (tumor necrosis factor alpha) en IL-6 (interleukine 6) het meest bestudeerd. TNF-α is de eerste cytokine, die wordt gevormd in met LPS gestimuleerde cellen, terwijl IL-6 voornamelijk een prognostische waarde lijkt te hebben. Op basis van deze feiten zijn voor het bestuderen van de invloed van ketamine op de cytokinenproductie bij het paard zowel TNF-α als IL-6 gekozen.
De ketamine concentraties in de in vitro experimenten variëren tussen de 0-36 μM. Deze concentraties zijn gebaseerd op de plasma ketamine spiegel van 5 μg/ml (18 μM), die wordt bereikt na de intraveneuze toediening van de voor het paard gebruikelijke inductie dosis van 2,2 mg/kg lichaamsgewicht. Om de minimaal effectieve ketamine concentratie te achterhalen, waarbij nog steeds een ontstekingsremmend effect kan worden waargenomen, zijn ook concentraties lager dan 18 μM getest. Deze lagere ketamine concentraties kunnen in klinisch opzicht vooral bij bewustzijn zijnde paarden van grote waarde zijn, omdat met behulp van deze lagere concentraties de met ketamine gepaard gaande ongewenste neveneffecten, zoals hypnose en excitatie, vrijwel niet voorkomen. Wanneer na toediening van deze relatief lage ketamineconcentraties geen enkel effect werd waargenomen, werden ketamineconcentraties tot 1000 μM getest om te kijken of ketamine überhaupt enig effect zou hebben op de betreffende onstekingsmediator.
In hoofdstuk 3 is het eerste in vitro experiment aangaande de ontstekingsremmende effecten van ketamine bij het paard beschreven. De resultaten van dit onderzoek tonen aan, dat ketamine op een concentratie-afhankelijke manier de productie van TNF-α en IL-6 in LPS gestimuleerde e-CAS cellen remt. Deze resultaten waren voor ons aanleiding een onderzoek op te zetten naar de moleculaire mechanismen, die hieraan ten grondslag liggen.

MOLECULAIRE MECHANISMEN ACHTER DE CYTOKINEN-MODULERENDE EFFECTEN VAN KETAMINE

Na stimulatie met LPS wordt de productie van cytokinen in een cel gereguleerd door vele intracellulaire signaal transductie paden. Binding van LPS aan de, op de buitenkant van een cel gelegen, TLR4 (Toll-like receptor 4) receptor initieert de activatie van deze complexe intracellulaire cascade. In deze cascade spelen twee paden een belangrijke rol, nl. het MAPK (mitogen-activated protein kinase) pad en het NF-κB (nuclear factor kappa B) pad. Het MAPK pad bestaat uit drie afzonderlijk paden, het JNK (c-Jun N-terminal kinase), p38 (MAPK p38) en het ERK (extracellulair signal-regulated kinase) pad. Deze MAPKs bevinden zich in het cytoplasma van een cel en verplaatsen zich na activatie naar de kern waar ze op hun beurt weer een groot aantal transcriptie factoren zoals AP-1 (activating protein 1), Elk-1, c-Fos, c-Jun en NF-κB activeren.

NF-κB wordt gezien als een van de hoofdtranscriptie factoren in een cel. NF-κB bestaat uit een groep van eiwitten, die paren van diverse samenstelling vormen. Het meest bekende eiwitpaar binnen de NBF-κB groep is het p50/p65 paar. NF-κB speelt een prominente rol in de regulatie van diverse celfuncties. Na binding aan DNA reguleert NF-κB de transcriptie van meer dan 200 genen, waaronder die van ontstekingsmediatoren. Onder normale omstandigheden bevindt NF-κB zich in een inactieve vorm in het cytoplasma van de cel. NF-κB is inactief, doordat het in het cytoplasma gebonden is aan IκB (inhibitor of kappa B). Door stimulatie met LPS wordt in het cytoplasma het IKK (IκB kinase) complex geactiveerd. Geactiveerd IKK zorgt voor de phosphorylatie en degradatie van IκB waardoor NF-κB vrij komt. De vrije vorm van NF-κB verplaatst zich vervolgens naar de kern waar de eerder beschreven transcriptie van genen plaatsvindt.

Ketamine remt de productie van de cytokinen TNF-α en IL-6 in e-CAS cellen. De vraag is nu: via welke mechanismen remt ketamine de cytokinen productie in e-CAS cellen? Remt ketamine de productie van cytokinen door beïnvloeding van factoren buiten de cel, in de cel of in de kern? Of zelfs een combinatie daarvan? Om deze vragen te beantwoorden hebben we het effect van ketamine op de LPS gestimuleerde expressie van TLR4 (buiten de cel), MAPK, IκB (in de cel) en NF-κB (in de kern) in e-CAS cellen onderzocht (hoofdstuk 4). De resultaten tonen aan, dat ketamine de LPS gestimuleerde intracellulaire signaal paden alleen remt op het niveau van NF-κB. Activatie van NF-κB kan grofweg in 2 fasen worden verdeeld: de eerste fase bestaat uit het loskomen van IκB, de tweede fase uit het transport

van NF-κB naar de kern, een verdere activatie van NF-κB in de kern en de binding aan DNA. Met behulp van Western blots hebben we aangetoond dat ketamine geen invloed heeft op de expressie van IκB in e-CAS cellen. Dit in tegenstelling tot resultaten gevonden bij muizen en ratten waar ketamine wel de expressie van IκB leek te beïnvloeden. Het feit dat ketamine in e-CAS cellen geen invloed heeft op de expressie van IκB, suggereert dat ketamine de expressie van NF-κB remt door de tweede fase van de NF-κB activatie te beïnvloeden. Deze bevinding wordt versterkt door het feit dat ketamine ook in staat was de expressie van NF-κB te remmen wanneer het rechtstreeks aan het nucleair extract werd toegevoegd. Tot op heden is dit het enige artikel dat de door ketamine verminderde expressie van NF-κB wijdt aan een directe interactie tussen ketamine en NF-κB.

HET EFFECT VAN KETAMINE OP LPS GESTIMULEERDE ENZYM EXPRESSIE

NF-κB is betrokken bij de transcriptie en expressie van legio mediatoren, waaronder cytokinen. Naast cytokinen reguleert NF-κB ook de expressie van enzymen, zoals COX-2 (cyclooxygenase 2) en iNOS (inducible nitric oxide synthase). Onder normale omstandigheden zijn COX-2 en iNOS niet of nauwelijks in een cel aanwezig, maar na stimulatie door LPS neemt in een cel de productie van deze enzymen toe. COX-2 en iNOS produceren op hun beurt, respectievelijk PGE_2 (prostagladine E_2) en NO (nitric oxide), ontstekingsmediatoren, die een belangrijke rol spelen bij de klinische symptomen van een ontsteking. Het feit dat ketamine de expressie van NF-κB kan remmen, suggereert dat ketamine ook de expressie van deze enzymen en daarmee de productie van hun bijbehorende ontstekingsmediatoren kan remmen.

De resultaten van hoofdstuk 6 geven echter aan dat ketamine niet in staat is de expressie van COX-2 in LPS gestimuleerde e-CAS cellen te remmen, zelfs niet na blootstelling aan hoge ketamine concentraties tot 1000 μM. Daar NF-κB door vele onderzoekers wordt gezien als een van de belangrijkste transcriptie factoren van een cel en ketamine heeft aangetoond de expressie van NF-κB te verminderen, was het resultaat van dit onderzoek toch enigszins onverwacht. Om dit onverwachtte resultaat te kunnen verklaren, hebben we de aanname dat de expressie van COX-2 in e-CAS cellen door NF-κB wordt gereguleerd verlaten en de moleculaire mechanismen die ten grondslag liggen aan de expressie van COX-2 in e-CAS cellen verder onderzocht. Remming van zowel MAPK als NF-κB gaf aan, dat de expressie van COX-2 en de productie van PGE_2 in LPS gestimuleerde e-CAS cellen alleen via de drie MAPK paden wordt gereguleerd en niet via het NF-κB pad. Gezien het feit

dat ketamine niet de expressie van de MAPKs kan verminderen, maar alleen die van NF-κB verklaart nu waarom ketamine niet in staat bleek de expressie van COX-2 en de daarbij behorende productie van PGE_2 in e-CAS cellen te remmen.
Net als bij COX-2 remt ketamine ook niet de expressie van iNOS en daarbij behorende productie van NO in LPS gestimuleerde e-CAS cellen (hoofdstuk 5). Remming van zowel de MAPK paden als het NF-κB pad gaf echter aan, dat de expressie van iNOS en de productie van NO via beide paden verloopt. Dit suggereert, dat de MAPKs een grotere rol spelen dan NF-κB bij de regulatie van de iNOS expressie dan tot nu toe werd aangenomen. Sterker nog de remmings-experimenten geven zelfs aan, dat de MAPKs de expressie van NF-κB onafhankelijk van NF-κB kunnen reguleren.

ANTI-OXIDATIEVE EIGENSCHAPPEN VAN KETAMINE

Behalve door LPS kan NF-κB ook door zuurstofradicalen worden geactiveerd. Dit suggereert, dat het remmen van de productie van zuurstofradicalen automatisch resulteert in een verminderde expressie en activatie van NF-κB en daarmee in een verminderde productie van cytokinen. Ketamine kan zowel de productie van zuurstofradicalen (hoofdstuk 7), als de expressie van NF-κB (hoofdstuk 4) en de productie van cytokinen (hoofdstuk 3) in LPS gestimuleerde e-CAS cellen remmen. Ondanks het feit, dat er niets bekend is over de link tussen de productie van zuurstofradicalen in e-CAS cellen enerzijds en de expressie van NF-κB in e-CAS cellen anderzijds, lijkt het heel onwaarschijnlijk, dat de verminderde productie van zuurstofradicalen in deze cellen wordt veroorzaakt door een verminderde expressie van NF-κB. Ten eerste hebben de resultaten uitgewezen, dat LPS alleen wel in staat is de expressie van NF-κB te activeren, maar niet om de productie van zuurstofradicalen te stimuleren. Dit laatste was alleen mogelijk wanneer LPS in combinatie met een tweede activator, te weten PMA (phorbol myristate acetate), werd gebruikt. Ten tweede werd de expressie van NF-κB nooit voorafgegaan door een verhoogde productie van zuurstofradicalen. Sterker nog, de expressie van NF-κB in LPS gestimuleerde e-CAS cellen was al significant op 15 minuten na de stimulatie met LPS, terwijl de productie van zuurstofradicalen pas 2 uur na de stimulatie met LPS en PMA significant werd. Hieruit kan worden geconcludeerd dat de door ketamine verminderde expressie van NF-κB in LPS gestimuleerde e-CAS cellen onafhankelijk is van de productie van zuurstofradicalen.

LANGDURIGE KETAMINE TOEDIENING EN DE NIET-ANESTHETISCHE EFFECTEN VAN KETAMINE IN VIVO

Het gegeven dat ketamine de productie van TNF-α en IL-6 in LPS gestimuleerde e-CAS cellen remt, suggereert, dat ketamine ook de cytokinenproductie in vivo zou kunnen remmen. De concentratie-afhankelijke remming in vitro en het feit dat verhoogde plasmaspiegels van cytokinen zijn gemeten gedurende een aantal uren na het ontstaan van een ontsteking, doet vermoeden, dat ketamine gedurende lange tijd en in relatief hoge doseringen moet worden toegediend om klinisch een ontstekingsremmend effect te kunnen bereiken. Tijdens een algehele anesthesie kunnen relatief hoge doseringen worden toegediend. In de intensive care, wanneer ketamine aan bij bewustzijn zijnde paarden moet worden toegediend, is de ketamine dosering beperkt door de mogelijk optredende ongewenste effecten, zoals liggen en excitatie. De resultaten van hoofdstuk 8 geven aan dat een ketamine infuus van 1,5 mg/kg/uur gedurende tenminste 6 uur veilig aan gezonde en bij bewustzijn zijnde paarden kan worden toegediend. Het pharmacokinetische profiel van dit infuus verschilde weinig van de eerder gevonden pharmacokinetische waarden behorende bij een eenmalige intraveneuze injectie van ketamine. De bij patiënten aanwezige pathologische veranderingen kunnen echter leiden tot een verandering van het pharmacokinetisch profiel. Dit kan betekenen, dat bij deze patiënten ketamine vertraagd wordt afgevoerd met als gevolg verhoogde plasmaspiegels en de daarbij optredende ongewenste effecten. Daarnaast zou toediening van een ketamine infuus gedurende meer dan 6 uur eveneens kunnen leiden tot het optreden van deze ongewenste effecten, omdat bij de infuus-experimenten beschreven in dit proefschrift (hoofdstuk 7 en 8) op 6 uur na infusie van ketamine nog steeds geen steady-state in plasmaspiegels was bereikt.

Ketamine wordt in de lever gemetaboliseerd door het cytochrome P_{450} enzymsysteem. Demethylatie van ketamine produceert de eerste metaboliet, norketamine, dat op zijn beurt wordt gehydroxyleerd en geoxideerd tot de metabolieten hydroxynorketamine en 5,6-dehydronorketamine. In de twee in vivo experimenten van dit proefschrift zijn deze metabolieten bepaald met behulp van de combinatie vloeistofchromatografie en tandem massa spectrometrie, een relatief nieuwe techniek die veel nauwkeuriger lijkt te zijn dan de oude techniek bestaande uit de combinatie gas-vloeistof chromatografie en massa spectrometrie.

Om de ontstekingsremmende effecten van ketamine in vivo te kunnen bestuderen is gebruik gemaakt van het weefselkamermodel. Een voordeel van dit model is, dat

de door LPS geïnduceerde ontstekingsresponse beperkt blijft tot de weefselkamer en dat op deze manier niet het algeheel welbevinden van het te onderzoeken dier wordt beïnvloed. Zoals verwacht werd de door LPS geïnduceerde ontstekingsreactie in de weefselkamer gekenmerkt door een infiltratie van witte bloedcellen en een verhoogde concentratie van onstekingsmediatoren. Onderzoek van weefselkamervloeistof wees echter uit, dat ketamine niet in staat bleek de door LPS gestimuleerde productie van TNF-α, IL-6 en PGE_2 te remmen. Dit resultaat is zeer waarschijnlijk het gevolg van het feit, dat te lage ketamine concentraties in de weefselkamervloeistof werden bereikt. Zowel na een enkelvoudige bolus injectie als na een continu infuus gedurende 6 uur kwam slechts een fractie van de in het lichaam aanwezige ketamine in de weefselkamer. Dit wordt toegeschreven aan de snelle eliminatie halfwaardetijd van ketamine uit het lichaam ($t_{1/2\beta}$ = 65-67 min) in combinatie met de beperkte diffusie van ketamine vanuit het lichaam naar de weefselkamer. Bovendien lijkt ketamine, wanneer het zich eenmaal in de weefselkamer bevindt, zeer makkelijk daaruit te diffunderen, omdat het een zwakke base met een lage eiwitbinding is. In vitro werd een significant verminderde cytokineconcentratie gemeten vanaf een ketamineconcentratie van 1,8 μM. In de weefselkamervloeistof bedroeg de hoogste ketamine concentratie slechts 0,5 μM. Deze beduidend lagere ketamine concentratie in vivo is daarmee mogelijk een verklaring voor het achterwege blijven van een onstekingsremmend effect in vivo.

Tijdens de ketamine infusen is geen verschil in ketamine en/of norketamine plasmaspiegels gemeten tussen paarden (hoofdstuk 8) en ponies (hoofdstuk 9), terwijl de hydroxynorketamine en 5,6-dehydroketamine spiegels bij ponies veel hoger was dan bij paarden. Dit verschil is mogelijk toe te schrijven aan een verschil in biotransformatie tussen rassen.

KLINISCHE RELEVANTIE VAN EEN KETAMINE INFUUS AAN PAARDEN MET EEN INFECTIEUZE AANDOENING

De bevinding dat ketamine de productie van TNF-α, IL-6 en zuurstofradicalen in e-CAS cellen significant remt, suggereert dat het gebruik van ketamine van waarde zou kunnen zijn voor paarden met een infectieuze aandoening. Echter, de snelle afbraak en eliminatie van ketamine, de lage eiwitbinding en derhalve de matige ophoping van ketamine in ontstoken weefsel, geven aan dat ketamine mogelijk niet het juiste pharmacokinetisch profiel heeft om een ontstekingsreactie bij dergelijk paarden te onderdrukken. Toch zijn de resultaten van de in vitro studies

zodanig, dat verder onderzoek naar de ontstekingsremmende effecten van ketamine tijdens een anesthesie procedure of in de intensive care gerechtvaardigd is.

CONCLUSIES

Op grond van de resultaten verkregen met de experimenten beschreven in dit proefschrift kan het volgende worden geconcludeerd:

- ketamine remt de door LPS geïnduceerde TNF-α en IL-6 productie in e-CAS cellen op een concentratie-afhankelijke wijze
- ketamine remt de door LPS geïnduceerde intracellulaire signaal transductie paden enkel en alleen op het niveau van NF-κB
- ketamine remt de expressie van NF-κB middels een directe interactie met NF-κB
- ketamine remt niet de door MAPK en NF-κB gemedieerde iNOS expressie en NO productie in LPS gestimuleerde e-CAS cellen
- expressie van COX-2 wordt voornamelijk gereguleerd via de drie MAPK paden.
- ketamine remt niet de door MAPK gemedieerde COX-2 expressie en PGE_2 productie, wat suggereert, dat de analgetische effecten van ketamine worden gemedieerd op een COX-2 onafhankelijk manier
- ketamine remt de productie van zuurstofradicalen in e-CAS cellen
- ketamine verhoogt de intracellulaire glutathion concentratie, maar induceert op zichzelf geen glutathion synthese
- een ketamine infuus van 1,5 mg/kg/uur kan gedurende tenminste 6 uur veilig worden toegediend aan gezonde, wakkere paarden
- ketamine wordt snel gemetaboliseerd tot zijn metabolieten norketamine, hydroxynorketamine en 5,6-dehydronorketamine
- ketamine heeft geen ontstekingsremmende eigenschappen in met LPS geïnfecteerde weefselkamers in Shetland ponies

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N

O

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U

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- CURRICULUM VITAE -

Daniëlle Lankveld was born on August 4th 1969 in Wisch, The Netherlands. In 1986, she passed her final exams at the 'Isala College' grammar school in Silvolde, after which she started her veterinary medicine study at Utrecht University. She graduated 'with honours' on August 31th 1995. The next day, she started as a resident in Equine Surgery at the former Department of General and Large Animal Surgery. After completing this residency in September 2000, she started work in the Division of Anaesthesiology and Intensive Care of the Department of Equine Sciences. In 2004, she started this PhD study under supervision of prof. dr. J. Fink-Gremmels of the Division of Veterinary Pharmacology, Pharmacy and Toxicology and prof. dr. L.J. Hellebrekers and dr. P. van Dijk of the Division of Anaesthesiology and Intensive Care of the Department of Equine Sciences. The results of this PhD study are presented in this thesis.

Daniëlle Lankveld werd geboren op 4 augustus 1969 te Wisch. In 1986 werd, na het behalen van het V.W.O. diploma aan het Isala College te Silvolde, begonnen met de studie diergeneeskunde aan de Universiteit Utrecht. Op 31 augustus 1995 behaalde zij het dierenartsdiploma 'met genoegen' en op 1 september 1995 trad zij als specialist in opleiding chirurgie paard in dienst van de toenmalige vakgroep Algemene Heelkunde en Heelkunde der Grote Huisdieren. Na afronding van deze specialisatie stapte zij in september 2000 over naar de discipline anesthesiologie en intensive care van de Hoofdafdeling Gezondheidszorg Paard. In 2004 is zij begonnen met een promotieonderzoek bij deze Hoofdafdeling. Dit onderzoek werd verricht onder begeleiding van prof. dr. J. Fink-Gremmels van de discipline veterinaire farmacologie, farmacie en toxicologie en prof. dr. L.J. Hellebrekers en dr. P. van Dijk van de discipline anesthesiologie en intensive care. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

- LIST OF ABBREVIATIONS -

AB	Alamar Blue
ABP	arterial blood pressure
ANOVA	analysis of variance
AP	activating protein
API	atmospheric pressure ionisation
ARD	ankyrin repeat domain
ASK	apoptosis signal-regulating kinase
AST	aspartate aminotransferase
BAFF	B-cell activating factor
BSO	buthionine sulfoximine
CD	cluster of differentiation
C/EBP	CCAAT-enhancer box binding protein
CK	creatine phosphokinase
COX	cyclooxygenase
CREB	cyclic AMP-responsive element binding protein
CRI	constant / continuous rate infusion
CYP_{450}	cytochrome P450
Da	dalton
DAP	diastolic arterial pressure
DIC	disseminated intravascular coagulation
EEG	electroencephalographic
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
eNOS	endothelial NOS
ERK	extracellular signal-regulated kinase
FACS	flow cytometric analysis
FAD	flavin adenine dinucleotide
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FMN	flavin mononucleotide
GABA	gamma-aminobutyric acid
GC-MS	gas-liquid chromatography-mass spectrometry
GSH	glutathione
H_4B	tetrahydrobiopterin
HNK	hydroxynorketamine
HPLC	high-performance liquid chromatography
HR	heart rate
IFN	interferon
IκB	inhibitor of kappa B
IKK	IκB kinase
IKKAP	IKK associated protein

IL	interleukin
IL-1ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated protein kinase
IRF	interferon-regulatory factor
JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
kDa	kilo dalton
KDO	3-deoxy-D-manno-oct-2-ulosonic acid
LBP	lipopolysaccharide binding protein
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAC	minimal alveolar concentration
MAIL	molecule possessing ankyrin-repeats induced by LPS
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MD	myeloid differentiation protein
MEK	MAPK-ERK kinase
MEKK	MAPK-ERK kinase kinase
MPO	myeloperoxidase
mtNOS	mitochondrial NOS
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphatase
NEFA	non-esterified fatty acid
NEMO	NF-κB essential modulator
NF-IL-6	nuclear factor for IL-6 expression
NF-κB	nuclear factor-kappa B
NLS	nuclear localisation sequence
NMDA	N-methyl-D-aspartate
nNOS	neuronal NOS
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
p38	mitogen-activated protein kinase p38
$PaCO_2$	arterial carbon dioxide tension
PAF	platelet-activating factor
PAMP	pathogen associated molecular pattern

PaO_2	arterial oxygen tension
PDGF	platelet-derived growth factor
pERK	phosphorylated ERK
PG	prostaglandin
pJNK	phosphorylated JNK
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
p-p38	phosphorylated p38
PRR	pattern recognition receptor
RHD	Rel homology domain
ROS	reactive oxygen species
RR	respiration rate
SAP	systolic arterial pressure
sCD	soluble CD
SD	standard deviation
SI	single injection
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
sTNFR	soluble TNF receptor
TAB	TAK binding protein
TAK	transforming growth factor-β-activated kinase
TANK	TRAF-family-member-associated NF-κB activator
TBK	TANK-binding kinase
TIR	Toll / interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TIVA	total intravenous anaesthesia
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	TNF receptor
TRAF	tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
TX	thromboxane
XO	xanthine oxidase