Pathophysiology and modulation of inflammation during mechanical ventilation and surgery

Evolvement of the concept of balanced anesthesia

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Unus pro omnibus, omnes pro uno

Één voor allen, allen voor één.

(Alexandre Dumas)

Voor de musketiers:

Agnes Kalma

Yolanda van der Wal

Contents

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1

General introduction and outline of the thesis

New developments of anesthesiology

Historical overview of artificial lung ventilation

Anesthesia, literally meaning 'without sensations' allowed surgeons to perform their work without a patient experiencing pain.[1] The ancient Greeks already experimented with poppy extracts. However, the first attempt of intravenous anesthesia is probably in 1656 in Oxford, where a dog was injected with a mixture of alcohol and opium and woke up successfully.[2, 3] A major milestone of modern anesthesia is the first demonstration of ether gas anesthesia in 1846 in Boston by William Thomas Green Morton where a surgical procedure could take place without a pain sensation.[1, 2] After this event endotracheal anesthesia was performed for the first time in a human by Friedrich Trendelenberg in 1869 with the use of an inflatable cuff, his work later extended by Eisenmenger.[4] Alfred Kristein was the first to use direct laryngoscopy in Berlin in 1895.[4] The first attempts at mechanical ventilation began in 1893 by George E. Fell, with the Fell's bellow, later modified by O'dwyer in 1896.[4] The use of mechanical ventilation started with the introduction of the whole body negative pressure device, extensively used during the poliomyelitis epidemic in the 1950s.[4] Rudolph Matas was the first to introduce positive pressure breathing during thoracic surgery at the beginning of the twentieth century, although this principle was not very popular at the time and was only further explored in the late 1930s.[4] By the 1950s a lot of positive pressure ventilators had been developed and in the 1960s and 1970s anesthesiologists began to recognize the pathophysiological mechanisms of respiratory failure and the concept of blood gas analysis also due to the development of long term mechanical ventilation in intensive care units.[4] Lung injury after mechanical ventilation was divided into three main types of trauma. Firstly 'barotrauma', initially demonstrated in 1974 and consisting of the development of a pressure gradient between the alveolus and the bronchovascular sheet due to mechanical ventilation with high peak airway pressure. This could result in lung edema, rupture of the alveolus and consequently air leakage.[5] After this discovery multiple studies showed that not only peak pressure but overdistention of the lung was deemed the main determinant in lung injury, hence the second form of trauma, and identified as a 'volutrauma'.[6, 7] Additionally, mechanical ventilation results in mild morphological changes and a pulmonary and systemic inflammatory response, the so called 'biotrauma', which is the third form of positive ventilation lung trauma.[8-10]

Historical overview of acute and chronic pain

As early as in the mid 1800s the knowledge about pain was already divided into an acute, chronic and palliative component.[11] Opiates were standard treatment in the early 1900s for acute and chronic pain.[11] Widespread use of short acting opioids, during anesthesia and surgery occurred from the

1960s.[2] The local anesthetic cocaine was isolated in 1856 and in 1884 Kollar showed that it provided reliable corneal anesthesia. The first neuraxial block, which means that a local anesthetic was placed around the nerves of the central nervous system, was performed in 1884. Procaine was introduced in 1905 and the first percuteanous brachial plexus blocks were described in 1911.[2] Neuromuscular blocking drugs have interested physicians during the 19th century, however it was only in the 1930s and 1940s that the use of skeletal muscle paralyses was integrated in anesthesia.[2] The use of intravenous anesthetics with barbiturates began in 1932 but propofol was clinically introduced in 1977.[1] In the early twentieth century Graham, Gaylord and Simpson were the first to suggest the influence of anesthesia on the immune system.[12]

Development of the concept of balanced anesthesia

All the vigorous work in the development of anesthesia led to the concept of balanced anesthesia, initially posed by John Lundy in 1926 from the Mayo clinic. It encompasses the use of multiple drugs to produces anesthesia, analgesia, skeletal muscle relaxation and attenuation of autonomic reflexes. In the twentieth and twenty-first century we learned a lot more about immune responses during anesthesia subsequently mechanical ventilation and surgery. The key topic is that an inflammatory response is a homeostatic response to injury or infection and homeostasis or balance is a fundamental requirement for life.[13] The definition of homeostasis is '**The tendency of a system, especially the physiological system of higher animals, to maintain internal stability, owing to the coordinated response of its parts to any situation or stimulus that would tend to disturb its normal condition or function'**. Stimuli in the context of anesthesia are for example mechanical ventilation and surgery in which an acute phase response and especially *dysregulation* of this acute phase response can lead to a wide variety of chronic diseases but also can play a role in pain after surgery and the development of chronic pain.[14-18]

Hereby we thus will extend the topic of balanced anesthesia to the modulation and maintenance of homeostasis of the inflammatory response in anesthesia and surgery.

The acute phase response

The human immune system consists of the innate and the adaptive immune system. The innate immune systems acts as a non specific first responder to attack foreign material, pathogens and reacts to tissue damage; the so called 'acute phase response'. The adaptive immune system is responsible for pathogen specific attacks and the development of immunity.[15, 19]

In mammals the symptoms of an acute phase response include fever, lassitude, inhibiton of gastric function, tachycardia and activation of immune cells including lymphocytes and neutrophils.[20]

The acute phase response consists of complex interactions between immune cells and programming of gene expression in response to cytokines.[15] Generally, pattern recognition receptors (PRR's) play a critical role in sensing the pathogen-associated molecular patterns (PAMP's) and danger-associated molecular patterns (DAMP's). Well studied PRR's are, for example Toll-like receptors and Nod-like receptors (NLRs).[21] Via several downstream signaling pathways TLR's promote function by transcription of inflammatory molecules via Nuclear Factor (NF)-κB dependent inflammatory gene expression. PRR's are expressed on a variety of cells including neutrophils, which play a pivotal role in the acute phase response.[22] Neutrophils adhere to the vascular wall after sensing danger and transmigrate to the site of damage, where they release inflammatory molecules.[19, 23, 24] Cytokines regulate local inflammatory responses and create cell to cell communication.[25] There are several pro- and anti-inflammatory cytokines. Important pro-inflammatory cytokines during acute phase response are interleukin (IL)-1β, Il-6, IL-8, tumor necrosis factor (TNF)-α.[20, 26] IL-10 is a well known anti-inflammatory cytokine which limits the immune response during infections.[27, 28] IL-10 is also known to decrease the synthesis of pro-inflammatory cytokines in acute phase response as IL-1α, IL-1β, IL-6 and TNF-α by neutrophils.[29] The main goal however remains to restore homeostasis, where anti-inflammatory and pro-inflammatory cytokines are probably of equal importance.[26, 30] Figure 1. provides a simplified overview of an acute phase response that can occur on a systemic or local level. The next paragraphs will describe the acute phase response after mechanical ventilation and surgery.

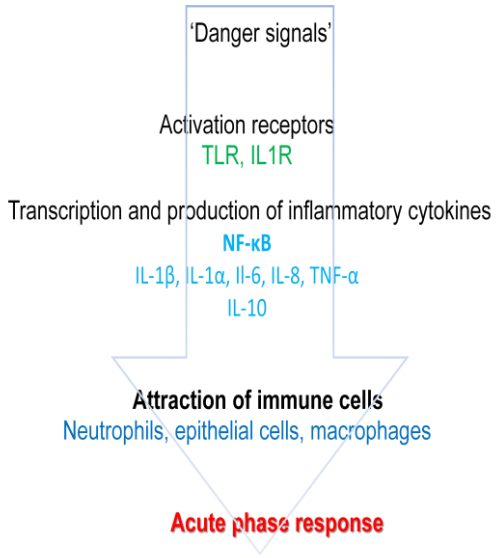

Figure 1. This figure provides a simplified overview of an innate acute phase response. TLR=toll like receptor, IL1R = Interleukin 1 receptor.

Inflammatory responses after mechanical ventilation

After mechanical ventilation an acute phase response occurs that can lead to ventilator-induced lung injury.[31] Ventilator induced lung injury is characterized by a sterile inflammatory response including release of pro-inflammatory cytokines and recruitment of inflammatory cells possibly resulting in progressive lung injury, hence part of the so called 'biotrauma'.[31, 32] In the lung, cytokines are generated by bronchiolar and alveolar epithelial cells, alveolar macrophages and neutrophils.[33-35] Experimental studies demonstrate that even lung protective mechanical ventilation with low tidal volume and application of positive end expiratory pressure induces an inflammatory response.[10, 36, 37] Precise mechanisms behind ventilator induced cytokine responses are not yet completely understood, however several mechanisms have been proposed, for example mechanotransduction which means cytoskeletal alteration without ultrastructional damage.[38] Furthermore stress failure of the alveolar barrier and of plasma membrane and effects on pulmonary vasculature independent of stress can initiate the enhanced generation of cytokines.[7, 8, 39] Several studies have shown that alveolar cells produce cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IL10.[10, 34] Low tidal volume ventilation attenuates, but does not abrogate the inflammatory responses.[40, 41] Several studies have also shown an association between mechanical ventilation and a systemic inflammatory response, influenced by ventilator settings.[10, 42] Leukocytes play an important role in the ventilator induced inflammatory response.[43, 44] Leucocytes are primarily attracted by ketatinocyte-derived chemokine (KC; IL-8 homologue) but for activation cytokines are probably needed.[45, 46] Previous investigations suggest that the pattern recognition receptor, Toll-like receptor 4 (TLR4), plays an important role in the ventilator induced inflammatory response by activation of NF-κB, a transcription factor, and consequently transcription of pro-inflammatory cytokines.[47, 48] Figure 2. Illustrates the TLR4 receptor pathway which leads to transcription of inflammatory cytokines.

Interleukin (IL)-1β also plays a pivotal role in the pathogenesis of the ventilator induced lung injury by promoting recruitment and transmigration of neutrophils.[49] Bound to its receptor IL-1R, which is present on nearly all cells, IL-1β binding leads to an inflammatory cascade.[50] IL-1β however is secreted as an inactive molecule, pro-IL-1β, that has to be cleaved at the amino-terminal by caspase-1 or extracellular serine protease before it can activate the IL-1 receptor.[50, 51]

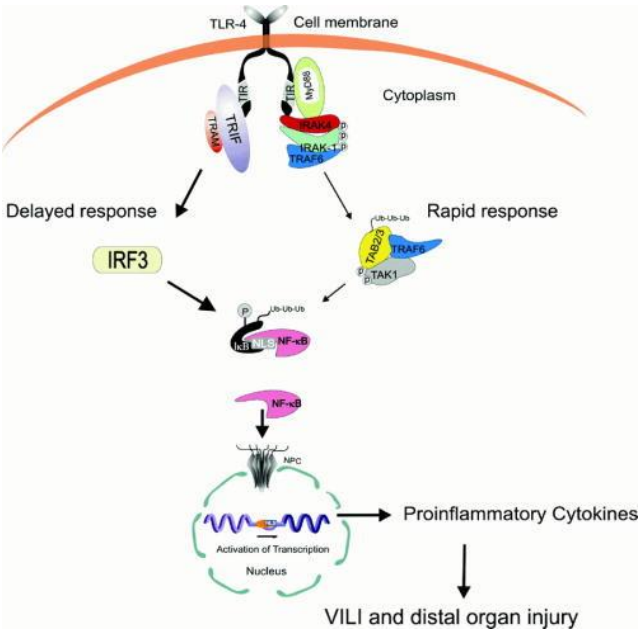

Figure 2. This figure illustrates the TLR4 receptor pathway which leads to transcription of inflammatory cytokines. Reprinted with permission of M. Vaneker.

The inflammatory response during surgery and the influence of anesthetic drugs

An acute phase response is also mediated after surgery and encompasses a complex set of physiological changes with a very important role of the immune system responding on a local and systemic level.[52] Important cytokines involved seem to be IL-6, TNFα, IL-1, interfon (IFN)- γ, IL-8 and IL-10.[12, 26, 30] The severity of the injury seems to correlate with the inflammatory response.[12, 52, 53] The main goal remains to restore homeostasis, where anti-inflammatory and pro-inflammatory cytokines are probably equally important.[26, 30] The immune response during surgery does not differ with the use of anesthetic technique, thus regional or general anesthesia.[54, 55] Certain anesthetic drugs can determine the extent of the inflammatory response. The anesthetic drugs discussed below are used in the different experiments compromising this thesis.

The first volatile anesthetic ether was used as early as in 1846 in William Thomas Green.[1, 2] From the 1950's there was progress in research of organic fluoride compound leading to the development of the modern fluorinated hydrocarbon class volatile anesthetics, including isoflurane.[56] Isoflurane is widely used in humans and animals as an agent to provide sleep during general anesthesia.[57, 58] Several studies have shown that isoflurane can attenuate pulmonary and systemic inflammatory acute phase responses. Isoflurane decreases neutrophil influx, IL-1β after mechanical ventilation.[59]

Part of the anti-inflammatory effects of isoflurane may be due to inhibition of the NF-κB pathway.[60]

Ketamine was first synthesized in the early 1960s and in 1965 its anesthetic properties were identified as a profound drug providing analgesia and amnesia.[61] Ketamine is a N-Methyl-D-aspartate (NMDA) receptor antagonist, this receptor presents at spinal and supraspinal locations and is involved in transmission of nociceptive signals.[61] It is widely used in humans and animals as an anesthetic and analgesic.[62, 63] Ketamine can inhibit production of pro-inflammatory cytokines by reduction of NF-κB or suppression of natural killer (NK) cells, neutrophils and macrophages.[12, 63] In humans the anti-inflammatory effects of ketamine remain controversial.[62]

Lidocaine is a local amide anesthetic first synthesized in 1943 and was used for many years as a local anesthetic agent before it was used as an intravenously administered drug for treatment of arrhythmias.[64] Since the early 1980's there has been increasing interest in the systemic administration of lidocaine in treatment of acute and chronic pain.[65] It is widely used during anesthesia in humans and animals.[63, 66]
Lidocaine acts as an anti-hyperalgesic and anti-inflammatory agent.[17, 67] *In vitro* research showed that lidocaine attenuates priming of human neutrophils by inhibition of G-protein coupled receptors[68, 69] Furthermore lidocaine attenuated activated endothelial interleukin (IL)-1, 6 and 8 concentrations and intracellular adhesion molecule-1 (ICAM-1), important for transport of immune cells to site of inflammation.[70, 71] In different *in vivo* models intravenous lidocaine reduced levels of tumor necrosis factor (TNF)-α, IL-1β IL-6 and Il-8,[72-74] and systemic lidocaine attenuates acute lung injury in rabbits.[75, 76] The requirements for additional anesthetics are diminished.[77, 78] Human research reveals an attenuation of Il-6, IL-8 and IL-1 receptor antagonist in plasma at the end of abdominal surgery showing it has anti-inflammatory effects.[79-81]

Medetomidine is an α_2-adrenergic agonist first used as anesthetic and analgetic drugs in veterinary medicine in the 1980's, the *S*-enantiomer of medetomidine used in veterinary and human medicine is dexemdetomidine.[82] Mainly macrophages seem to be affected in the acute phase response by stimulation of their sympathetic adrenergic receptors and medetomide can modulates the TLR4-NF-κB pathway and attenuates pro-inflammatory cytokines.[63] Anti-inflammatory effects seem to be dose-dependent.[83] The exact mechanisms of anti-inflammatory effects are not yet completely elucidated. In human research on α_2-adrenergic agonists it has been proposed that the drugs indeed affect macrophages and clinically patients had a decrease of number of days of mechanical ventilation and improved survival.[84]

Pain after surgery

Nociceptive pain

Pain after tissue damage results in an inflammatory response and neuroplasticity usually returns to its normal state after the inflammation is resolved.[85] Interestingly the inflammatory acute phase response and especially *dysregulation* of this acute phase response can lead to a wide variety of chronic diseases but it can also play a role in pain after surgery and the development of chronic pain.[14-18] The definition of nociception is: 'the neutral process of encoding and processing noxious stimuli'.[86] Nociception can induce a transformation of function and structure in the nervous system, the so called neuroplasticity.[85] Surgery induces restriction of normal functioning and nociceptive pain, generated by powerful stimuli that activate Aδ and C fibers.[87, 88] Nociceptive and inflammatory processes are involved in the development of persistent or chronic pain after surgery.[89-91] Peripheral and central neuroplastic changes may explain the chronicity after wound healing has already occurred.[90] Several mechanisms are known to play a role in neurplasticity including NMDA receptor and kinases and inflammatory mediators.[89, 90] Moderate to severe pain after surgery induces a reduction of the quality of life of patients in the immediate postoperative period [92], which can be an important reason for delayed discharge [16] and a higher risk of developing chronic pain.[91] Subsequently, persistent postsurgical pain reduces the quality of life, leads to unanticipated hospital admissions and has a huge socio-economic impact.[47]

Neuropathic pain

Nerve damage during surgery can lead to neuropathic pain which is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system. Neuropathic pain presents as a constant, burning pain with spontaneous sharp exacerbations and allodynia, a worsening of pain upon normal sensory triggers and there is an increased sensitivity to pain, hyperalgesia. [93] Sensory testing is an important diagnostic tool in determination of neuropathic pain and allodynia and hyperalgesia can be determined. [94] Multiple tests have been described to test Aβ, C and Aδ fibers including mechanical or pressure and thermal tests.[88, 95]

The pathophysiology of nerve damage describes increased sodium channel expression, ectopic electrical activity and altered neuroplasticity.[90] The variety of changes in the peripheral and central nervous system can be described firstly by spontaneous firing of peripheral sensory fibers.[85] Secondly, in the spinal cord the ongoing nociceptive signals from the injured nerves can trigger central sensitization. Thirdly, supraspinally changes occur that shift inhibitory pain to facilitation which means that pain can be accentuated.[96] Finally, there appears to be a direct connection between inflammatory responses and neuropathic pain in animal models, proinflammatory cytokines

such as tumor necrosis factor-α (TNF-α), interleukin 1 β (IL-1β), and interleukin 6 (IL-6) can induce acute or short-term hyperalgesia and chronic hyperalgesia and allodynia.[97] After peripheral nerve injury the immune response includes macrophages localizing to damaged nerve fibers but also localization upstream in the dorsal root ganglion and subsequently activation of microglia and astrocytes in the central nervous system.[98, 99] Animal models of pain are contributing to the identification of the aetiology of persisting pain states and potential targets for treatment.[100] There is a continuing need for animal models that represent the clinical situation.

Objectives of thesis

Since the pathophysiology of neuropathic pain after surgery is incompletely understood and there are multiple hits to the patients before this pain state occurs there is a need for stable translational models to investigate these processes, their acute phase responses but also to investigate possible therapies or modulators of these processes.

Modulation and investigation of the pathophysiology of the inflammatory processes involved in surgery, anesthesia and perioperative nerve damage could provide valuable insights in the treatment and perhaps prevention of postoperative neuropathic pain. Figure 3. demonstrates a hypothesis on the balance that exists between the input and outcome in relation to mechanisms and modulatory therapy.

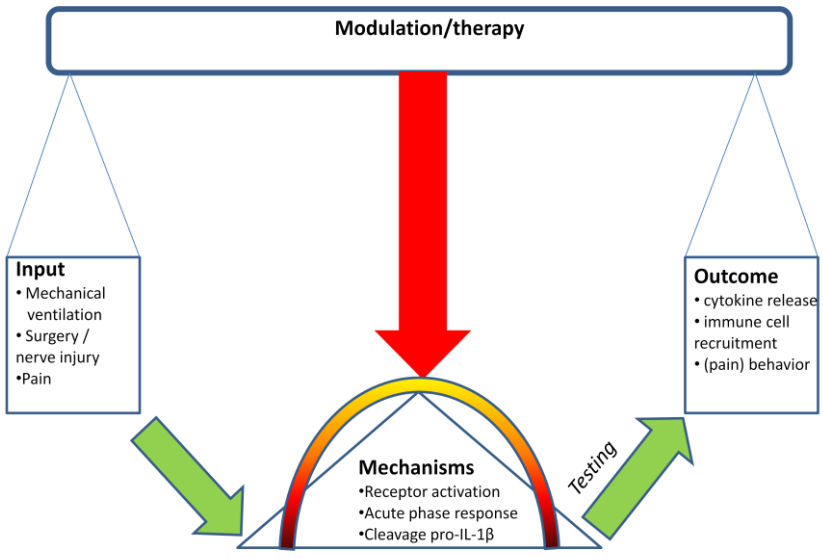

Figure 3. Pathophysiology and modulation of inflammation during mechanical ventilation and surgery affecting outcome

This thesis thereby aims to identify some of the mechanisms responsible for the responses after mechanical ventilation and surgery measured by inflammatory and behavioral outcome parameters

and explores possibilities of modulation of inflammatory responses after mechanical ventilation and surgical induced nerve injury.

Consequently, with respect to our hypothesis on anesthesiological homeostasis, the following research questions are identified (figure 3).

- What is the role of IL-1β, caspase-1 and neutrophil factors in the mechanical ventilation induced inflammatory response in mice?
- Do resveratrol and intravenous lidocaine attenuate the mechanical ventilation induced inflammatory response in mice?
- What is the current state of knowledge on the *in vitro* mechanisms and *in vivo* efficacy of intravenous lidocaine in acute and chronic pain?
- Can we develop a murine model of neuropathic pain behavior?
- Can we develop a murine 'two hit' model of neuropathic pain?
- Does lidocaine attenuate the postoperative inflammatory response and development of neuropathic pain in mice?

Outline of this thesis

In chapter 2 we investigate the role of IL-1β, caspase-1 and neutrophil factors in the mechanical ventilation induced inflammatory response in mice.

In chapter 3 we investigate the mechanical ventilation induced pulmonary and systemic inflammatory response and the modulatory effects of resveratrol in healthy mice.

In chapter 4 we investigate the mechanical ventilation induced pulmonary and systemic inflammatory response and the modulatory effects of intravenously administered lidocaine in healthy mice.

Chapter 5 provides a review on the literature of the modulatory mechanism of lidocaine in vitro and in vivo on pain mechanisms.

Chapter 6 describes the development of a murine model of neuropathic pain.

Chapter 7 describes the development of a ‘two hit’ murine model of nerve injury and investigates the effect of intraperitoneally administered lidocaine in a two hit model of surgery and nerve injury.

In chapter 8 we discuss the results of this thesis research in an integrated scientific context and several recommendations for future research will be provided.

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2

IL-1beta processing in mechanical ventilation-induced inflammation is dependent on neutrophil factors rather than caspase-1

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Abstract

Introduction

Mechanical ventilation can cause ventilator-induced lung injury, characterized by a sterile inflammatory response in the lungs resulting in tissue damage and respiratory failure. The cytokine interleukin-1β (IL-1β) is thought to play an important role in the pathogenesis of ventilator-induced lung injury. Cleavage of the inactive precursor pro-IL-1β to form bioactive IL-1β is mediated by several types of proteases, of which caspase-1, activated within the inflammasome, is the most important. Herein, we studied the roles of IL-1β, caspase-1 and neutrophil factors in the mechanical ventilation-induced inflammatory response in mice.

Methods

Untreated wild-type mice, IL-1αβ knockout and caspase-1 knockout mice, pralnacasan (a selective caspase-1 inhibitor)-treated mice, anti-keratinocyte-derived chemokine (KC)-treated mice and cyclophosphamide-treated neutrophil-depleted wildtype mice were ventilated using clinically relevant ventilator settings (tidal volume 8 ml/kg). The lungs and plasma were collected to determine blood gas values, cytokine profiles and neutrophil influx.

Results

Mechanical ventilation resulted in increased pulmonary concentrations of IL-1β and KC and increased pulmonary neutrophil influx compared with non-ventilated mice. Ventilated IL-1αβ knockout mice did not demonstrate this increase in cytokines. No significant differences were observed between wild-type and caspase-1-deficient or pralnacasan-treated mice. In contrast, in anti-KC antibody-treated mice and neutropenic mice, inflammatory parameters decreased in comparison with ventilated non-treated mice.

Conclusions

Our results illustrate that IL-1 is indeed an important cytokine in the inflammatory cascade induced by mechanical ventilation. However, the inflammasome/caspase-1 appears not to be involved in IL-1β processing in this type of inflammatory response. The attenuated inflammatory response observed in ventilated anti-KC-treated and neutropenic mice suggests that IL-1β processing in mechanical ventilation-induced inflammation is mainly mediated by neutrophil factors.

Introduction

Mechanical ventilation is a life-saving therapy, although it can also cause ventilator-induced lung injury (VILI).[1] VILI is characterized by a sterile inflammatory response in the lungs resulting in tissue damage that may sustain respiratory failure. The mechanical ventilation-induced inflammatory response can also spread systemically, which in severe cases can result in multi-organ dysfunction syndrome (MODS).[2] Even protective ventilation strategies that do not cause direct mechano-induced tissue damage (baro- or volutrauma) have been shown to elicit the release of pro-inflammatory cytokines, recruitment of leukocytes and subsequent lung injury.[3, 4] The mechanisms behind this so-called 'biotrauma' have not yet been completely elucidated.

Previous studies have demonstrated that the TLR4/TRIF pathway is important in the mechanical ventilation-induced inflammatory response.[4, 5] Furthermore, it is becoming increasingly clear that the pro-inflammatory cytokine interleukin-1β (IL-1β) plays a key role in the pathogenesis of the inflammatory response and VILI by promoting neutrophil recruitment and by increasing epithelial injury and permeability.[6-8] Through recognition by the IL-1 receptor (IL-1R), not only the secreted IL-1β but also the cell-associated family member IL-1α may stimulate production of other inflammatory cytokines via IL-1R-associated kinases (IRAKs) and thereby positively amplify the inflammatory response.[9] However, up till now, this has not been studied in the context of mechanical ventilation-induced inflammation.

Upon activation of the innate immune system, e.g. via TLRs, IL-1β is synthesized as an inactive precursor molecule, pro-IL-1β, that cannot bind and activate the IL-1R.[10] In order to process pro-IL-1β and form bioactive IL-1β, proteolytic cleavage of the N-terminal 116 amino acids from the precursor is required. Caspase-1 is the major protein implicated in cleavage of pro-IL-1β.[10, 11]

Caspase-1 exists as an inactive zymogen in cells of myeloid origin (e.g. tissue macrophages, dendritic cells) which needs to be activated to perform its proteolytic tasks.[9] Caspase-1 is also known to be expressed in a wide range of other cell types including lung fibroblasts and epithelial cells.[12, 13] The inflammasome is a protein platform that is responsible for the activation of caspase-1.[10, 14] A broad range of infectious and autoimmune diseases that involve IL-1β have been associated with inappropriate activation of the inflammasome [12, 14, 15], while in several other disease models in which IL-1β plays a crucial role, the inflammasome appears not to be involved.[16, 17] IL-1β processing in these models might rely on neutrophil serine proteases, like elastase, granzyme A, cathepsin G or proteinase-3.[10, 18-20] Hitherto, the role of caspase-1 in processing of IL-1β in the mechanical ventilation-induced inflammatory response is unknown.

We studied the roles of IL-1β, caspase-1 and neutrophil factors in the mechanical ventilation-induced inflammatory response in mice ventilated with clinically relevant ventilator settings.

Materials and methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health. They have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Animals

Age-matched wild-type C57Bl/6 mice and extensively backcrossed caspase-1 knockout mice or IL-1αβ knockout mice (aged 8 to 14 weeks, weight 25 ± 4 g) with C57Bl/6 background were used in this study. The mice were housed in a light- and temperature-controlled room under specific pathogen-free (SPF) conditions. Standard pelleted chow (1.00% Ca, 0.22% Mg, 0.24% Na, 0.70% P, 1.02% K, SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*. These conditions are similar to previous studies in which this mouse model was used.[4, 5, 21, 22]

Experimental design

IL-1αβ knockout experiments

IL-1 can induce inflammation via activation of the IL-1 receptor. To study whether IL-1 is indeed involved in initiation and/or propagation of the inflammatory cascade induced by mechanical ventilation, mechanically ventilated IL-1αβ$^{-/-}$ mice (*n* = 8) were compared with ventilated wild-type mice (*n* = 8). As controls, non-ventilated IL-1αβ$^{-/-}$ (*n* = 8) and wild-type mice (*n* = 8) were used.

Caspase-1 experiments

Caspase-1 is able to cleave the inactive precursor pro-IL-1β to form the active cytokine IL-1β. To study the role of caspase-1 in the mechanical ventilation-induced inflammatory response, mechanically ventilated caspase-1 knockout mice (*n* = 8) and ventilated wild-type mice treated with the selective caspase-1 inhibitor pralnacasan (100 mg/kg) (*n* = 8) were compared with ventilated untreated wild-type mice (*n* = 8).[23, 24] As controls, non-ventilated caspase-1$^{-/-}$, pralnacasan-treated wild-type and untreated wild-type mice (*n* = 8 per group) were used.

Anti-KC antibody experiments

Apart from caspase-1, neutrophil serine proteases are also able to process IL-1β.[8] In order to investigate whether the attraction of neutrophils by the chemo-attractant keratinocyte-derived chemokine (KC) is involved in the inflammatory response elicited by mechanical ventilation, mechanically ventilated wild-type mice treated with an intraperitoneal dose of 100 μg of a neutralizing monoclonal anti-KC antibody (R&D Systems, Minneapolis, MN, USA) 1 h before induction of anaesthesia (*n* = 8) were compared with ventilated untreated wild-type mice (*n* = 8). As controls, non-ventilated untreated wild-type mice (*n* = 8) were used.

Neutrophil depletion experiments

Neutrophil serine proteases are able to process IL-1β.[8] In order to study the possible role of neutrophil factors in IL-1β processing in the mechanical ventilation-induced inflammatory response, mechanically ventilated neutrophil-depleted wild-type mice (*n* = 8) were compared with ventilated untreated wild-type mice (*n* = 8). As controls, non-ventilated wild-type mice (*n* = 8) were used. The neutrophil-depleted group was neutrophil-depleted with cyclophosphamide as described previously.[25, 26]

Experimental procedures

The mice were anaesthetized using an intraperitoneal injection of 7.5 μl per gram body weight of KMA mix (25.5 mg/ml ketamine, 40 μg/ml medetomidine, 0.1 mg/ml atropine in saline). Subsequently, the animals were orally intubated, an arterial line was placed in the arteria carotis, and the mice were mechanically ventilated (*MiniVent®*, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). The ventilation settings used were based on measured tidal volume and respiratory rate during spontaneous ventilation in C57Bl/6 mice [27]: a tidal volume of 8 ml/kg body weight and a frequency of 150/min. All animals received 4 cm H_2O positive end-expiratory pressure (PEEP), and fraction of inspired oxygen was set to 0.4. In order to maintain anaesthesia, boluses of 5.0 μl per gram body weight maintenance KMA mix (3.6 mg/ml ketamine, 4 μg/ml medetomidine, 7.5 μg/ml atropine in saline) were given every 30 min via an intraperitoneally placed catheter. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C using a heating pad. After the 4-h ventilation period, the mice were sacrificed by exsanguination under anaesthesia. The control mice were anaesthetized, but not ventilated, and sacrificed shortly after induction of anaesthesia. Tissue and blood were sampled in order to determine blood gas values (only in ventilated mice), cytokine production and neutrophil influx.

Lipopolysaccharide (LPS) was measured in the mechanical ventilation circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD, USA; detection limit: 0.06 IU/ml) to rule out contamination and LPS-induced pulmonary inflammation.

Tissue harvesting

Plasma was isolated by centrifugation at 13,000*g* for 5 min and stored at –80°C. Immediately after exsanguination, the heart and lungs were carefully removed *en block* via midline sternotomy. The right middle lung lobe was fixed in 4% buffered formalin solution overnight at room temperature. The right lung was snap-frozen in liquid nitrogen and stored at –80°C. The left lung was snap-frozen and placed in 500 µl lysis buffer containing PBS, 0.5% Triton X-100 and protease inhibitor (complete EDTA-free tablets, Roche, Woerden, The Netherlands). Subsequently, the lungs were homogenized using a polytron and subjected to two rapid freeze-thaw cycles using liquid nitrogen. Finally, homogenates were centrifuged (10 min, 16,000*g*, 4°C), and the supernatant was stored at −80°C until further analysis.

Pulmonary neutrophil influx

After overnight incubation in 4% buffered formalin solution, the right middle lung lobe was dehydrated and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4-µm thickness were used. Enzyme histochemistry using chloracetatesterase (LEDER staining) was used to visualize the enzyme activity in the neutrophils. Neutrophils were counted manually (ten fields per mouse), and after automated correction for air/tissue ratio, the average number of neutrophils per square centimetre per mouse was calculated.

Biochemical analysis

KC (murine equivalent of human IL-8) in the lung homogenate was determined by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). The lower detection limit is 160 pg/ml. IL-1β in the lung homogenate was determined using a radioimmunoassay (RIA) as described previously.[28] In the samples of the IL-1αβ (Figure 1) and caspase (Figure 2) experiments, total protein concentrations in the lung homogenates were determined using a BCA protein assay (Thermo Fisher Scientific, Etten-Leur, The Netherlands), and cytokine concentrations in the homogenates were normalized for protein concentration and therefore expressed as nanogram cytokine per microgram protein. In the anti-KC (Figure 3) and neutrophil depletion (Figure 4)

experiments, cytokine concentrations in the lung homogenate were not normalized for total protein content due to insufficient sample volume and therefore expressed as picogram per millilitre.

Statistical analysis

Data were not normally distributed (determined using the Kolmogorov-Smirnov and Shapiro-Wilk tests) and therefore expressed as median and range or median and interquartile range (IQR). Differences between groups were analyzed using the Kruskal-Wallis and Dunn's *post hoc* tests. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). *P* values <0.05 were considered significant.

Results

Mean arterial pressure remained stable and above 65 mmHg in all animals throughout the mechanical ventilation period. Blood gas values that were obtained at the end of the ventilation period did not indicate substantial lung injury (Table 1).

Table 1

	Median	**IQR**
pH	7.36	7.25 to 7.38
pCO_2	4.73	4.17 to 5.18
PO_2	15.3	14.6 to 17.5
BE	−5.5	−7.3 to –4.0
HCO_3	20.2	18.3 to 20.7
TCO_2	21.0	19.8 to 21.5
sO_2%	99%	98 to 99
Lac	0.98	0.90 to 1.16

Table 1. Blood gas values after 4 hours of ventilation. Values (median and IQR) from a representative ventilated group (wild-type ventilated mice used as the control group for caspase-$1^{-}/_{-}$ and pralnacasan-treated mice).

Involvement of IL-1 in the mechanical ventilation-induced inflammatory response

After 4 h of mechanical ventilation, pulmonary levels of pro-inflammatory cytokine KC significantly increased in wild-type mice compared with non-ventilated wild-type mice. In contrast, ventilated IL-1αβ knockout mice did not show an increase in pulmonary cytokines compared with non-ventilated IL-1αβ knockout mice (Figure 1).

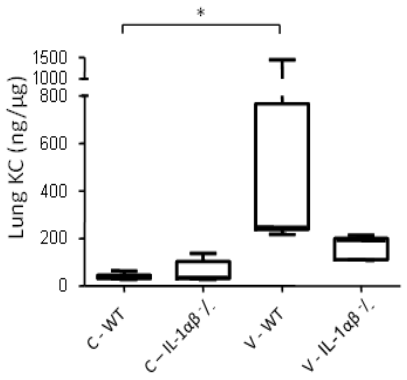

Figure 1. Involvement of IL-1 in the mechanical ventilation-induced inflammatory response. KC **levels in lung homogenates expressed as nanogram cytokine per microgram total protein. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. Results of analysis in the non-ventilated (C) and ventilated (V) wild-type (WT) mice and IL-1αβ knockout (¯/_) mice are shown. * Indicates p<0.05.**

Involvement of caspase-1 in the mechanical ventilation-induced inflammatory response

Pulmonary neutrophil influx significantly increased in mechanically ventilated mice compared with non-ventilated wild-type and caspase-1¯/_ mice, but no differences were observed between wild-type mice, caspase-1¯/_ mice or pralnacasan-treated mice. Similar to the results described above, 4 h of mechanical ventilation resulted in increased IL-1β and KC concentrations in lung homogenates in all groups. However, no significant differences in lung cytokine levels were observed between wild-type mice, caspase-1¯/_ mice or pralnacasan-treated mice. (Figure 2)

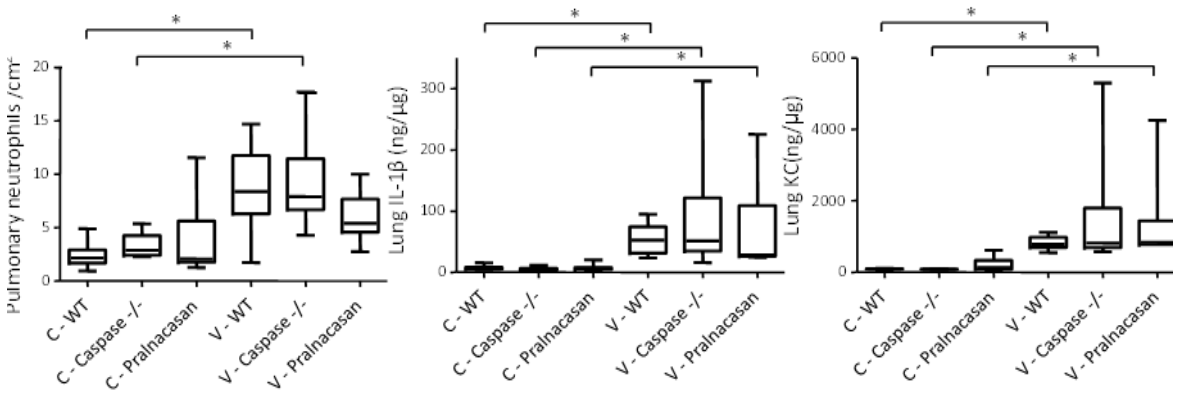

Figure 2. Involvement of caspase-1 in the mechanical ventilation-induced inflammatory response. Pulmonary neutrophil counts expressed as the number of neutrophils per square centimetre tissue and IL-1β and KC levels in lung homogenates expressed as nanogram cytokine per microgram total protein. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. Results of analysis in the non-ventilated (C) and ventilated (V) wild-type (WT) mice, caspase-1 knockout (¯/_) mice and pralnacasan-treated mice are shown. * Indicates p<0.05.

Involvement of neutrophil factors in the mechanical ventilation-induced inflammatory response

To determine whether neutrophil factors are involved in the mechanical ventilation-induced inflammatory response and IL-1β processing, we investigated the effects of treatment with an antibody against KC. KC is one of the major factors involved in neutrophil attraction to the site of inflammation (chemo-attractants). Mechanical ventilation resulted in increased levels of pulmonary neutrophils (Figure 3). This increase was abrogated by pre-treatment with an anti-KC antibody. Furthermore, the mechanical ventilation-induced increase in pulmonary IL-1β levels was less pronounced in anti-KC-treated mice compared with untreated mice, although this did not reach statistical significance (Figure 3).

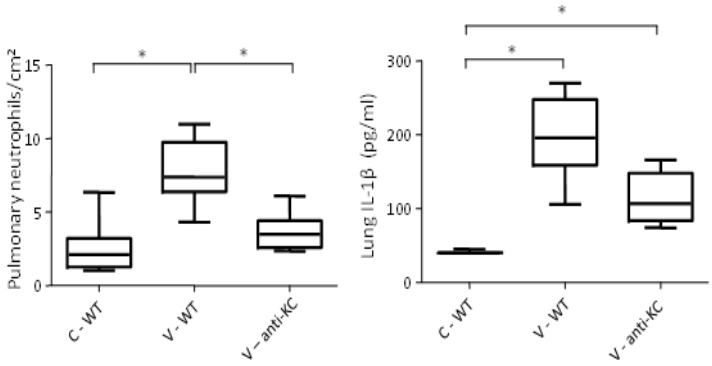

Figure 3. Involvement of KC in the mechanical ventilation-induced inflammatory response. Pulmonary neutrophil counts expressed as the number of neutrophils per square centimetre tissue and IL-1β concentration expressed as picogram cytokine per millilitre lung homogenate. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. Pulmonary neutrophils and IL-1β concentration in the non-ventilated (C) and ventilated (V) untreated wild-type mice (WT) and anti-KC antibody-treated wild-type (anti-KC) mice are shown. * Indicates p<0.05.

To further confirm the role of neutrophil factors, we investigated the effects of mechanical ventilation following neutrophil depletion using cyclophosphamide. The effect of cyclophosphamide was visually inspected, and no pulmonary neutrophils were present (data not shown). As depicted in Figure 4, the mechanical ventilation-induced increase in pulmonary IL-1β and KC concentrations was diminished in neutrophil-depleted mice.

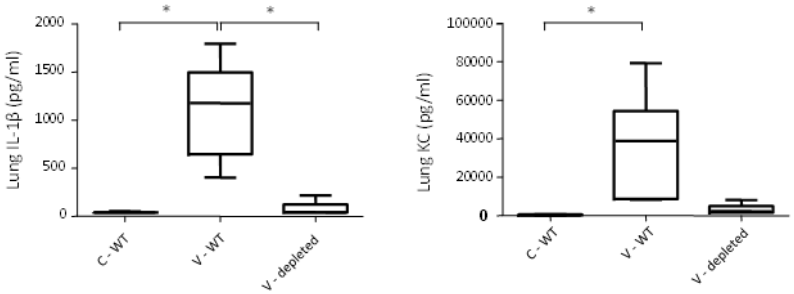

Figure 4. Effects of neutrophil depletion on the mechanical ventilation-induced inflammatory response. IL-1β and KC concentrations expressed as picogram cytokine per millilitre lung homogenate, measured in the non-ventilated (C) and ventilated (V) untreated wild-type (WT) and cyclophosphamide-treated neutrophil-depleted mice. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. * Indicates p<0.05.

Our hypothesis regarding the role of IL-1β processing in the inflammatory response following mechanical ventilation is illustrated in Figure 5. We present the following hypothesis based on our results and previous findings. Mechanical ventilation causes mechanotransduction and cell and/or tissue damage. This causes the release of danger-associated molecular patterns (DAMPs) that activate TLR4 and possibly other pattern recognition receptors. Ligation of these receptors induces production of cytokines, most importantly IL-1β. Subsequently, KC is produced, leading to neutrophil recruitment to the lungs. Pro-IL-1β processing to bioactive IL-1β could occur intracellularly by caspase-1, although in our model, it only plays a minor role in IL-1β bioactivation, not excluding that it may be involved at the onset of the inflammatory process, when very few neutrophils are present. The majority of pro-IL-1β is excreted in the inactive form and then cleaved by factors released by neutrophils, such as neutrophil serine proteases. Finally, active IL-1β present extracellularly binds to the IL-1R, which in turn leads to the production of more cytokines and hence positive amplification of the inflammatory response. As such, a positive feedback loop is activated which could be an explanation for the extensive inflammatory response observed following mechanical ventilation. Numbers 1 to 4 represents the experiments performed in this study and corresponds to the figure numbers in this paper. References [4] and [22] refer to previous studies performed by our grou

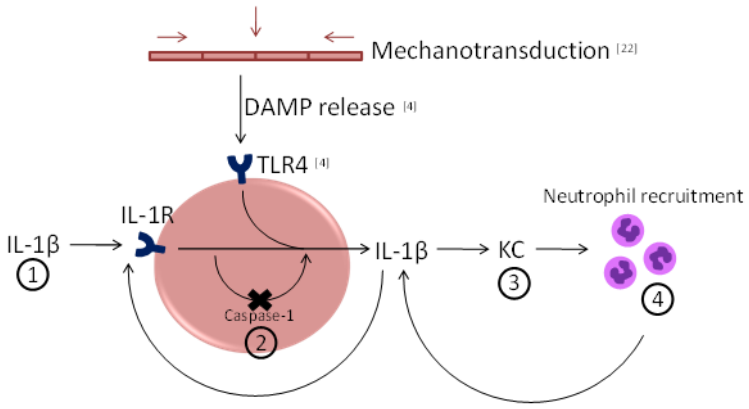

Figure 5. Hypothesis regarding the role of IL-1β processing in the inflammatory response following mechanical ventilation.

Discussion

Consistent with previous results published by our group [4, 5, 22] and others [29, 30], the present study shows that mechanical ventilation using clinically relevant settings induces a pulmonary inflammatory response in mice. In addition, our data is in support of previous findings that IL-1 plays

an important role in initiation and/or propagation of the mechanical ventilation-induced inflammatory response and suggests that processing of IL-1β in mechanical ventilation-induced inflammation occurs via the release of neutrophil factors and not through caspase-1-dependent mechanisms.

Our finding that caspase-1 does not play a significant role in mechanical ventilation-induced inflammation is in contrast to a recent study where the NLRP3 inflammasome was found to play an important role in the mechanical ventilation-induced inflammatory response and VILI.[31] Several differences between their study and ours might explain the different results. First, in the previous study, ASC and NLRP3 (components of the inflammasome upstream of caspase-1) knockout mice were used, and it was shown that mechanical ventilation activated caspase-1 in a NLRP3-dependent fashion. Nevertheless, it is very well possible that ASC and NLRP3 play other roles in the mechanical ventilation-induced inflammatory cascade than merely activating caspase-1. As abrogation and inhibition of caspase-1 by either a knockout approach or pralnacasan treatment did not have any effect in our model, the role of caspase-1/the inflammasome appears not to be as crucial as suggested. Second, differences between wild-type and ASC or NLRP3 knockout were only found at a high tidal volume of 15 ml/kg, known to cause extensive lung damage [22], while no effects were found at a low tidal volume of 7.5 ml/kg, which is more representative of the current clinical practice and similar to that used in the present study. This suggests that the inflammasome might play a more important role at higher tidal volumes which lead to apparent lung injury but not in mechanical ventilation-induced inflammation at clinically relevant ventilator settings. Interestingly, a more recent study from the same group showed that pre-treatment with allopurinol or uricase (both degraders of known inflammasome-activating factors [32]) did not decrease mechanical ventilation-induced inflammation, which is in support of a caspase/inflammasome-independent mechanism.[33] As beneficial effects of uricase and allopurinol were observed in terms of alveolar barrier dysfunction, it appears plausible that ASC and NLRP3 are involved in VILI via inflammation-independent mechanisms.

The pronounced influx of neutrophils in the lung observed in our experiments suggests a major role for these inflammatory cells in the inflammatory cascade following mechanical ventilation. Our findings that treatment with an antibody against KC or depletion of neutrophils reduced the mechanical ventilation-induced production of IL-1β and KC indicate an important role for neutrophils in initiation and/or propagation of the inflammatory response. In this respect, pro-IL-1β cleavage in our model is probably achieved through neutrophil factors, such as the serine proteases proteinase-3 (PR-3), elastase or cathepsin G, leading to bioactive IL-1β and propagation of the inflammatory response through binding of the IL-1-receptor, which in turn leads to production of other

inflammatory cytokines such as KC.[10, 34, 35] Several other IL-1β-mediated inflammatory responses are described to be partly or completely independent of the inflammasome and caspase-1 and possibly dependent on neutrophil factors, including proteinase-3 and cathepsin G .[35] Future studies should focus on the confirmation of our hypothesis and the identification of these neutrophil factors.

Our study has several limitations. First, we used cyclophosphamide to deplete neutrophils. While this is a widely used method [25, 26, 36, 37], cyclophosphamide treatment may also result in depletion of other cell types that play a role in mechanical ventilation-induced inflammation.[38, 39] Nevertheless, our data of mice treated with an anti-KC antibody underline the importance of neutrophils in this process. Second, no histological slides to perform neutrophil counts were collected in the IL-1αβ^{-}/$_{-}$ experiments to investigate whether these knockout mice were still able to recruit neutrophils. Finally, we cannot exclude the possibility that next to mechanical ventilation, the procedures related to the instrumentation/ventilation (e.g. intubation, arterial cannulation) also induce inflammation to a certain extent. However, we have previously shown that the inflammatory response is aggravated when mice are ventilated with these parameters for a longer period of time or when higher tidal volumes are used, suggesting that the inflammatory response is mainly ventilation-induced.

In conclusion, our results indicate that IL-1 signalling is important in mechanical ventilation-induced inflammation. We show that following mechanical ventilation, IL-1β bioactivation is not caspase-1 dependent but appears to be mediated by neutrophil factors, leading to a positive amplification loop and further propagation of the inflammatory response. Further elucidation of the precise mechanism of IL-1β processing in mechanical ventilation-induced inflammation could provide novel targets for the future treatment of VILI.[40]

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3

Resveratrol attenuates NF-κB following mechanical ventilation in healthy mice.

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Abstract

Introduction

Mechanical ventilation (MV) can result in inflammation and subsequent lung injury. Toll Like Receptor (TLR) 4 and NF-κB are proposed to play a crucial role in the MV-induced inflammatory response. Resveratrol exhibits anti-inflammatory effects *in vitro* and *in vivo*, supposedly by interfering with TLR4 signaling and NF-κB. In the present study we investigated the role of resveratrol in MV-induced inflammation in mice.

Methods

Resveratrol (RVT) (10 mg/kg, 20 mg/kg, and 40 mg/kg) or vehicle was intraperitoneally administered one hour before start of MV (four hours, tidal volume 8ml/kg, positive end expiratory pressure 1,5 cmH_2O, and FiO_2 0.4). Blood and lungs were harvested for cytokine analysis. DNA binding activity of transcription factor NF-κB was measured in lung homogenates.

Results

MV resulted in elevated pulmonary concentrations of IL-1β, IL-6, KC (keratinocyte-derived chemokine) and NF-κB DNA binding activity. Resveratrol at 10, 20 and 40 mg/kg reduced NF-κB's DNA-binding activity following MV compared with ventilated controls. However, no differences in cytokine release were found between resveratrol treated and control ventilated mice. Similarly, in plasma, MV resulted in elevated concentrations of TNF-α, KC and IL-6 but resveratrol did not affect cytokine levels.

Conclusions

Resveratrol abrogates the MV-induced increase in pulmonary NF-κB activity, but does not attenuate cytokine levels. This implies a less prominent role for NF-κB in MV-induced inflammation than previously assumed.

Introduction

Mechanical ventilation (MV) could be life saving in patients with acute respiratory failure. However, a large body of evidence suggests that MV can result in an inflammatory response resulting in lung injury or so-called ventilator-induced lung injury (VILI).[1, 2] Studies have shown that besides aggravating existing lung injury, MV can also induce injury in healthy lungs.[1, 3, 4] An inflammatory response, characterized by release of inflammatory cytokines and influx of immune cells such as neutrophils, contributes to the development of lung injury.[2, 5-7] To date, no effective therapy exists to attenuate the MV-induced inflammatory response.

Toll-like receptors (TLR) are pattern recognition receptors that play a pivotal role in innate immunity. [8-11] Recently, major advances have been made concerning the elucidation of the mechanisms behind MV-induced inflammation in which a crucial role for TLR4 has been identified.[12] TLR4 signaling leads to activation of NF-κB,[12-14] and subsequent production of inflammatory cytokines.[15, 16] TLR4 can activate NF-κB by different downstream signaling pathways: (TRIF) Toll/interleukin-1 receptor domain-containing adapter-inducing interferon-beta and (MyD88) myeloid differentiation factor 88.[14, 17] As increased activation of NF-κB and pro-inflammatory cytokines play a major role in MV- induced lung injury, [7, 18] inhibition of NF-κB activation and cytokine production could be an effective strategy to prevent or attenuate MV induced inflammation.

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a polyphenol found in plants and grapes and exhibits multifaceted physiological effects including anti-inflammatory and protective effects on different organ systems, including the lungs.[14, 19-21] These anti-inflammatory effects of resveratrol are mediated by inhibition of TLR4-signaling, supposedly by inhibition of TRIF or MyD88 [14, 19], and decreased NF-κB activation. [22-26] Therefore, resveratrol could represent a novel therapeutic option to reduce MV-induced inflammation. In the present study, we investigated the effects of resveratrol on NF-κB activation and cytokine production induced by MV in healthy mice to test the hypothesis that resveratrol attenuates MV-induced NF-κB activation and cytokine production.

Materials and methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and The National Institutes of Health.

Animals

All studies were performed in C57BL6 male mice in our established MV mice model.[4, 12, 17] Mice were housed in a light and temperature controlled room under specific pathogen free (SPF) conditions. Standard pelleted chow (1.00 % Ca; 0.22 % Mg; 0.24 % Na; 0.70 % P; 1.02 % K; SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*.

Experimental design

Five groups of mice, unventilated (CON, n = 16), ventilated (V-CON, n = 16) and mice treated with different doses of resveratrol (RVT) were studied. Resveratrol (Ergomax, Nijmegen the Netherlands), 10 mg/kg (V-RVT 10, n = 8), 20 mg/kg (V-RVT 20, n = 8) and 40 mg/kg (V-RVT 40, n = 8) was administered intraperitoneal one hour prior to the start of MV were compared with asses NF-κB binding activity and cytokine response prior to and after MV. The ventilated control group (V-CON) received equal volume of NaCl 0.9% intraperitoneal. In MV mice, intra-arterial carotid blood pressure was measured throughout the experiment. Arterial blood gas analysis (iSTAT, Abbott, Birmingham, United Kingdom) was performed after 4 hours of MV (data not shown).

Lipopolysacharide was measured in the ventilation circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit: 0.06 IU/ml) to rule out contamination with lipopolysacharide in our experimental setting. No lipopolysacharide could be detected in air, tubing or ventilator (data not shown).

Mechanical ventilation

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine and atropine (KMA): 7.5 µl per gram of body weight of induction KMA mix (consisting of 1.26 ml ketamine, 100 mg/ml; 0.2 ml medetomidine, 1 mg/ml; 1 ml atropine, 0.5 mg/ml; and 5 ml NaCl, 0.9%). Animals were orally intubated and mechanically ventilated (*MiniVent®*, Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany) for 4 hours. The following MV settings

were used: tidal volume 8 ml/kg and frequency 170 / min, 1,5cm H_2O positive end-expiratory pressure and fraction of inspired oxygen was set to 0.4. These setting are within the normal range of tidal volume and respiratory rate measured during spontaneous ventilation in C57BL6 mice.[27]

To maintain anesthesia, 5.0 µl per gram of body weight boluses of maintenance KMA mix (consisting of 0.72 ml ketamine, 100 mg/ml; 0.08 ml medetomidine, 1 mg/ml; 0.3 ml atropine, 0.5 mg/ml; and 18.9 ml NaCl, 0.9%) were administered every 30 minutes *via* an intraperitoneally placed catheter. Rectal temperature was monitored continuously and maintained between 36.0 ºC and 37.5ºC using a heating pad.

Tissue harvesting

Blood was collected by exsanguination, centrifuged (5 minutes, 14000 rpm), and plasma was stored at -80°C for cytokine analysis. Immediately after exsanguination, heart and lungs were carefully removed *en block* via midline sternotomy. The right upper and lower lobes were snap frozen in liquid nitrogen and stored at -80 ºC. The left lobes were snap-frozen and placed in 500 µL lysisbuffer containing PBS, 0.5% triton X-100 and protease inhibitor (complete EDTA-free tablets, Roche, Woerden, The Netherlands). Subsequently, the left lobes were homogenized using a polytron and subjected to two rapid freeze-thaw cycles using liquid nitrogen. Finally, homogenates were centrifuged (10 minutes, 14000 rpm, 4 °C) and the supernatant was stored at -80° C until cytokine analysis.

Cytokine analysis

Tumor necrosis factor (TNF)-α, interleukin (IL)-6 and keratinocyte-derived chemokine (KC) (murine equivalent of human IL-8) in lung homogenate were determined by enzyme-linked-immunosorbent assay (ELISA) (for IL-6; CytoSet, BioSource, CA; for TNF-α and KC; ELISA-Kit, R&D Systems, Minneapolis, MN). Lower detection limits: TNF-α: 32 pg/ml; IL-6: 160 pg/ml and KC: 160 pg/ml.

A simultaneous Luminex® assay was used to determine plasma cytokine levels of TNF-α, IL-6, KC and IL-1β (Milliplex, Millipore, Billerica, MA).

IL-1β in lung homogenate was determined using a radioimmunoassay (RIA) as described previously.[28]

Total protein concentrations in the lung homogenates were determined using a BCA protein assay (Thermo Fisher Scientific, Etten-Leur, The Netherlands). Cytokine concentrations in the homogenates were normalized for protein concentration.

NF-κB's DNA-binding activity

NF-κB's DNA-binding activity in the right upper lobes was determined by electrophoretic mobility shift assay. Lung tissue (20 mg) was homogenized in 5ml ice-cold buffer (HEPES) 10mM, 1.5mM $MgCl_2$, 10mM KCl and 0.6% Nonidet-P40, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulphonylfluoride (Sigma-Aldrich, Zwijndrecht, The Netherlands) and centrifuged for 30s at 350 g (4°C). The supernatant was then incubated on ice for 5 min and centrifuged for 5 min at 6000g (4°C). The pellet was resuspended in 200µl buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCL and 1.2 M sucrose, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulphonylfluoride (Sigma-Aldrich)) and centrifuged for 30 min at 13000g (4°C). Then the pellet was resuspended in 66 µl buffer (HEPES 20mM, 1.5mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 25% glycerol, 0.5mM dithiothreitol, 0.2mM phenylmethylsulphonylfluoride, 2.0 mM benzamidine and 5.0 µg/ml leupeptine (Sigma-Aldrich), incubated on ice for 20 min and centrifuged for 2 min at 6000g (4°C). The supernatants were used as nuclear extracts. Protein concentrations in these extracts were determined by using the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands).

Double stranded oligonucleotides containing an NF-κB consensus binding site (5'-AGTTGAGGGGACTTTCCCAGGC-3') were radiolabeled with 32[P]-adenosine triphosphate using T4 polynucleotide kinase (Promega, Madison, WI). Labeled NF-κB oligonucleotides were mixed with nuclear extracts (10 µg) and incubated at room temperature for 20 min. Then, these samples were loaded on a 4% polyacrylamide gel. After electrophoresis for 45 min, the gel was dried and exposed for 24 hours to an X-ray film. The bands on the film were quantified using optical densitometry software (GeneTools, Syngene, Cambridge, United Kingdom).

Statistical Analysis

We performed a sample size calculation based on previous investigations considering a difference of 40% in cytokine levels of cytokines between ventilated and control mice with a type 1 error of 5% (α=0.05) and a power of 80% (β=0.2).[4, 12, 17] This resulted in a group size of 8 animals per group.

NF-κB's binding activity before and after MV was expressed as mean (%) of the unventilated control group and analyzed using an unpaired t-test with Welch's correction.

Shapiro-Wilk test showed that cytokine data were not normally distributed, data were also not log normally distributed. Differences between control *versus* resveratrol were therefore studied using Mann Whitney-U tests and expressed as median with interquartile range depicted as column bar

graphs. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). P-values < 0.05 were considered significant.

Results

Cardiopulmonary physiology

The mice exhibited stable hemodynamic variables during MV. Mean arterial pressure was within normal limits and remained above 65 mmHg, which was in line with our previous data in this model. [4, 12, 17] Blood gas values remained within normal range after 4 hours of MV (data not shown). Three mice died during instrumentation (one in the ventilated control (V-CON) group, and two in the ventilated resveratrol 40 mg/kg (V-RVT 40) group.

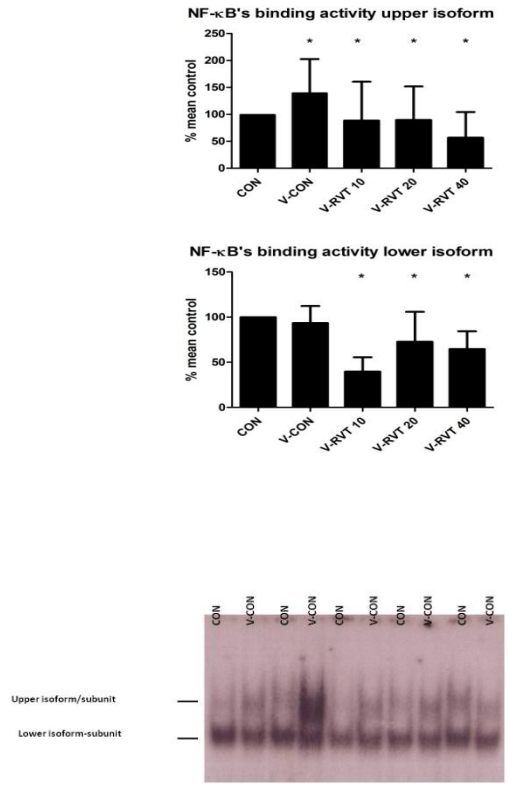

Figure 1. Upper panel: NF-κB activity in lung tissue divided in upper band, lower band an total activity and expressed as percentage of the mean of the unventilated mice (CON). The figure shows NF-κB activity in lung tissue of unventilated mice (CON) compared with control ventilated mice (V-CON) and ventilated mice receiving different dosages of resveratrol (RVT) 10 mg/kg (10), 20 mg/kg (20) and 40 mg/kg (40). A difference is found in the upper isoform/subunit measured by NF-κB's binding activity between CON compared with V-CON. No significance is obtained in the lower isoform/subunit or total NF-κB activity between CON compared with V-CON. NF-κB activity was lower (P < 0.05) between V-CON compared with V- RVT 10, V- RVT 20 and V- RVT 40 in both isoform/subunits. Data are expressed as optical densities obtained using an electrophoretic mobility shift assay analysis and expressed as mean (SD). (* = P<0.05) Lower panel: A representative example of the x-ray obtained from the electrophoretic mobility shift assay used to determine NF-κB's DNA binding activity in the CON and V-CON groups.

MV induced NF-κB's DNA-binding activity

Mechanical ventilated mice (V-CON) showed increased DNA- binding activity of the upper NF-κB isoform/subunit compared with unventilated mice (CON). The activity of the lower subunit was not different between ventilated and unventilated mice (Figure 1). Resveratrol prevented up regulation of DNA-binding activity of the 'upper and lower' isoform of NF-κB following MV in all dosage groups of resveratrol (RVT) compared with ventilated control mice (V-CON). (Figure 1).

Cytokine release induced by MV

MV induced a pulmonary pro-inflammatory response as indicated by elevated concentrations of IL-1β, KC and IL-6 in ventilated mice (V-CON) compared with unventilated control mice (CON). No differences were found in pulmonary levels of TNF-α after MV. Resveratrol (any dose tested) did not affect the attenuation of cytokine in the lung by MV (Figure 2).

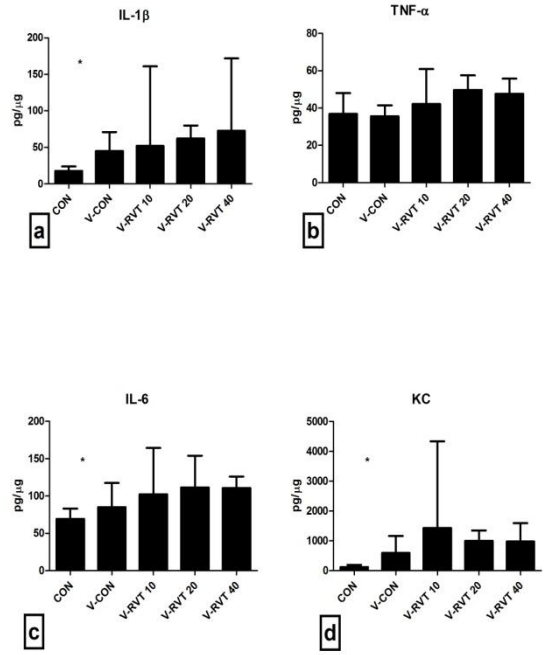

Figure 2. Cytokine levels in lung homogenates. Levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, keratinocyte derived chemokine (KC), in unventilated (CON) and ventilated control (V-CON) mice compared with ventilated mice receiving resveratrol (V-RVT) in different dosages. RVT was given in dosages of 10 mg/kg (10), 20 mg/kg (20) and 40 mg/kg (40).
(*panels A–D*). V-CON mice showed increased IL-1β (*P* < 0.05) and KC (*P* < 0.05) compared with CON mice. No differences were observed within the different ventilated groups. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

In plasma, MV resulted in elevated concentrations of KC, IL-6 and TNF-α in ventilated control mice (V-CON) compared with unventilated control mice (CON). Resveratrol (any dose tested) did not affect cytokine response in the plasma elicited by MV(Figure 3).

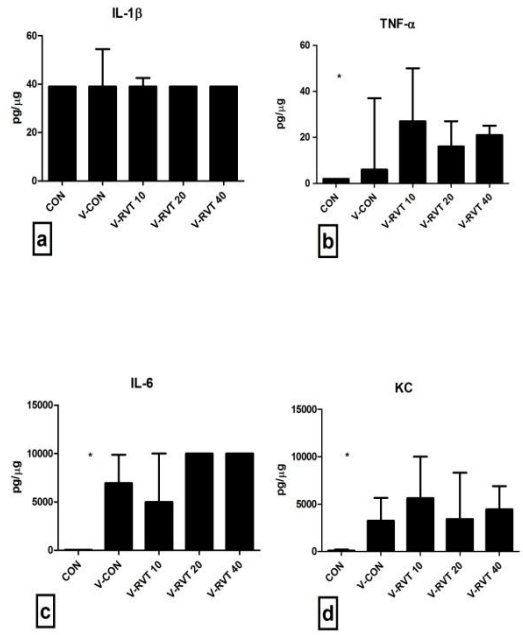

Figure 3. Cytokine levels in plasma. Levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, keratinocyte derived chemokine (KC), in unventilated (CON) and ventilated control (V-CON)

mice compared with ventilated mice receiving resveratrol (V-RVT) in different dosages. RVT was given in dosages of 10 mg/kg (10), 20 mg/kg (20) and 40 mg/kg (40).

(*panels A–D*). V-CON showed increased TNF-α, IL-6 and KC (*P* < 0.05) compared with CON mice. No differences were observed within the different ventilated groups. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

Discussion

The present study is to our knowledge the first to show that resveratrol inhibits pulmonary DNA-binding activity of NF-κB in healthy mice following MV. However, pretreatment of resveratrol did not attenuate the induction of pulmonary of systemic cytokines elicited by MV.

Resveratrol was investigated in several lung injury mouse models. Li et al. investigated the effect of resveratrol in mice with respiratory syncytial virus and found inhibition of pulmonary expression of TNF-α, IL-1β and IL-6.[29] In an LPS induced acute lung injury model, Cao et al. found resveratrol to

attenuate the production of IL-1β and suppress the nuclear translocation of NF-κB in lung tissue.[21] Therefore we hypothesized that resveratrol could inhibit the inflammatory response following MV. In the present study we found resveratrol to inhibit production of NF-κB, but no reduction of inflammatory cytokines was found. Not only did we prove our hypothesis wrong, our data also indicate that NF-κB is not the only transcription factor in the acute phase inflammatory response upon MV in our model.
It is however possible that the MV induced inflammatory response in our model has a different mechanism than the lung injury mouse models.

Previously we found an attenuation of NF-κB activity and cytokine levels after MV in TLR4-TRIF knock-out mice and showed the importance of the TLR4-TRIF pathway.[17] Resveratrol has been shown to inhibit TLR4 signaling and attenuate NF-κB.[14, 19] In the present study we found no decrease in cytokine levels after pre-treatment with resveratrol. These findings could suggest that resveratrol reduces NF-κB activity via a different pathway than TLR4.
We also found a more pronounced inflammatory response after MV in plasma than in pulmonary cytokines measured by picogram per microgram. A possible explanation for this would be that the systemic acute phase response is more pronounced after 4 hours of MV than the inflammatory pulmonary response measured in picogram per microgram with lung protective tidal volumes.

According to the literature different isoforms of NF-κB exist. Kirchner et al. described a role for both the p50-p50 homodimer and the p50-p65 heterodimer in rabbit lungs, where p50-p50 homodimers were shown to inhibit NF-κB-driven transcription.[30, 31] We did observe an increase of one particular NF-κB isoform/subunit, however we were unable to identify which isoform was increased, since there was no material left to perform such analysis. More research is needed to identify the involvement of certain isoforms of NF-κB in the inflammatory response after MV, however our results indicate that only one isoform seemed to participate in ventilation induced pro-inflammation.

A limitation of this study concerns time of administration of resveratrol. We administered resveratrol in equivalent dosages described in literature but just prior to MV. [32, 33] *In vivo* effects of resveratrol have been observed with longer (pre)treatment of resveratrol, varying from 72 hours to several weeks.[21, 29, 34, 35] There is not much data on resveratrol in human clinical trials, although a growing number of trials have started recently (clinicaltrials.gov).[36]

Conclusion

Our data show that resveratrol pretreatment attenuates MV-induced NF-κB's DNA-binding activity, but not cytokine production. This suggests that NF-κB does not play a pivotal role in the MV-induced acute phase inflammatory response.

The absence of an effect of resveratrol on MV-induced inflammation makes it an unlikely therapeutic option to limit MV induced inflammation.

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4

Lidocaine increases the anti-inflammatory cytokine IL-10 following mechanical ventilation in healthy mice.

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Abstract

Introduction

Mechanical ventilation (MV) induces an inflammatory response that may result in (acute) lung injury. Lidocaine, an amide local anesthetic, has anti-inflammatory properties *in vitro* and *in vivo*, possibly due to an attenuation of pro-inflammatory cytokines, ICAM-1 and reduction of neutrophils influx. We hypothesized an attenuation of MV-induced inflammatory response with intravenously administered lidocaine.

Methods

Lidocaine (Lido) (2, 4 and 8 mg/kg/h) was intravenously administered during four hours of mechanical ventilation (MV) with a tidal volume of 8ml/kg, positive end expiratory pressure 1,5 cmH_2O and FiO_2 0.4. We used one ventilated control (CON) group receiving vehicle. After MV, mice were euthanized and lungs and blood were immediately harvested and cytokine levels and ICAM-1 levels were measured in plasma and lung homogenates. Pulmonary neutrophils influx was determined in LEDER stained slices of lungs. Anesthetic need was determined by painful hind paw stimulation.

Results

Lidocaine treated animals (Lido 2, 4 and 8 mg/kg/h) showed higher IL-10 plasma levels compared with control animals (CON). Lidocaine treatment with 8 mg/kg/h (Lido 8) resulted in higher IL-10 in lung homogenates. No differences were observed in pro-inflammatory cytokines, ICAM-1 and pulmonary influx between the different ventilated groups.

Conclusions

Intravenously administered lidocaine increases levels of plasma IL-10 with infusion from 2, 4 and 8 mg/kg/h and pulmonary levels of IL-10 with 8 mg/kg/h, in a murine mechanical ventilation model. Intravenously administered lidocaine appears to reduce anesthetic need in mice.

Introduction

For patients with acute respiratory failure, mechanical ventilation (MV) can be life saving. However, a large body of evidence suggests that MV can result in lung injury, so-called ventilator-induced lung injury.[1, 2] It is widely assumed that an inflammatory response, characterized by release of inflammatory cytokines and influx of immune cells such as neutrophils contributes to the development of lung injury.[2-4] To date, no effective therapy exists to attenuate the MV-induced inflammatory response.

Lidocaine is an amide local anesthetic and a non-specific sodium channel blocker that is mostly used for the treatment of acute and chronic pain. It was demonstrated that low dose intravenous lidocaine acts as an anti-hyperalgesic and anti-inflammatory agent.[5, 6] Extensive *in vitro* research showed that lidocaine attenuates priming of human neutrophils by inhibition of G-protein coupled receptors[7, 8] Furthermore lidocaine attenuated activated endothelial interleukin (IL)-1, 6 and 8 concentrations and intracellular adhesion molecule-1 (ICAM-1), important for transport of immune cells to site of inflammation.[9, 10] In different *in vivo* models intravenous lidocaine reduced levels of tumor necrosis factor (TNF)-α, IL-1β IL-6 and Il-8.[11-13] Also systemic lidocaine was found to attenuate acute lung injury in rabbits.[14, 15] An additional effect of lidocaine infusion is that the requirements for anesthetics are diminished.[16, 17] In human research an attenuation in inflammatory response (measured by IL-6, IL-8 and an IL-1 receptor antagonist) in plasma has been found at the end of abdominal surgery in response to lidocaine.[18-20]

Since lidocaine seems to have prominent anti-inflammatory effects we aim to investigate the role of intravenously administered lidocaine at different dosages of 2,4 and 8 mg/kg/h during 4 hours of mechanical ventilation in healthy mice in an established acute phase model.[21-23]

We hypothesize that intravenously administered lidocaine attenuates the inflammatory response following MV.

Methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and The National Institutes of Health.

Animals

All studies were performed in C57BL6 male mice in our established MV mice model.[21-23] Mice were housed in a light and temperature controlled room under specific pathogen free (SPF) conditions. Standard pelleted chow (1.00 % Ca; 0.22 % Mg; 0.24 % Na; 0.70 % P; 1.02 % K; SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*.

Experimental design

Four groups of mice (N= 8 / group, randomly allocated) were studied after MV: control mice with vehicle (CON) and three groups of mice treated with different doses of lidocaine 2% (Lido)(Fresenius Kabi, Zeist, the Netherlands), 2 mg/kg/hour (Lido 2), 4 mg/kg/hour (Lido 4) and 8 mg/kg/hour (Lido 8). Lidocaine was administered intravenously *via* an indwelling intravenous tail-catheter just before MV and continued during 4 hours. The control group (CON) received an equal volume of NaCl 0.9% intravenously. Intra-arterial carotid blood pressure and heart rhythm was measured throughout the experiment. Arterial blood gas analysis (iSTAT, Abbott, Birmingham, United Kingdom) was performed after 4 hours of MV (data not shown).

Lipopolysaccharide was measured in the ventilation circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit: 0.06 IU/ml) to rule out contamination with lipopolysaccharide in our experimental setting. No lipopolysaccharide could be detected in air, tubing or ventilator.

Mechanical ventilation and anesthetic need

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine and atropine (KMA): 7.5 µl per gram of body weight of induction KMA mix (consisting of 1.26 ml ketamine, 100 mg/ml; 0.2 ml medetomidine, 1 mg/ml; 1 ml atropine, 0.5 mg/ml; and 5 ml NaCl, 0.9%). Animals were orally intubated and mechanically ventilated (*MiniVent®*, Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany) for 4 hours. The following MV settings were used: tidal volume 8 ml/kg and frequency 170 / min, 1,5cm H_2O positive end-expiratory pressure and fraction of inspired oxygen was set to 0.4. These setting are within the normal range of tidal volume and respiratory rate measured during spontaneous ventilation in C57BL6 mice.[24]

To maintain anesthesia, 5.0 µl per gram of body weight boluses of maintenance KMA mix (consisting of 0.72 ml ketamine, 100 mg/ml; 0.08 ml medetomidine, 1 mg/ml; 0.3 ml atropine, 0.5 mg/ml; and

18.9 ml NaCl, 0.9%) was administered when showed a positive reaction after manually administered painful hind paw stimulation, via an intraperitoneally placed catheter. Painful hind paw stimulation was performed every 30 minutes and represented anesthetic need. Rectal temperature was monitored continuously and maintained between 36.0 ºC and 37.5ºC using a heating pad.

Tissue harvesting

Blood was collected by exsanguination, centrifuged (5 minutes, 14000 rpm), and plasma was stored at -80°C for cytokine analysis. Immediately after exsanguination, heart and lungs were carefully removed *en block* via midline sternotomy. The right upper and lower lobes were snap frozen in liquid nitrogen and stored at -80 ºC. The left lobes were snap-frozen and placed in 500 µL lysisbuffer containing PBS, 0.5% triton X-100 and protease inhibitor (complete EDTA-free tablets, Roche, Woerden, The Netherlands). Subsequently, the left lobes were homogenized using a polytron and subjected to two rapid freeze-thaw cycles using liquid nitrogen. Finally, homogenates were centrifuged (10 minutes, 14000 rpm, 4 °C) and the supernatant was stored at -80° C until cytokine analysis.

Cytokine analysis

A simultaneous Luminex® assay was used to determine plasma cytokine levels of TNF-α, IL-6, IL-10, KC and IL-1β (Milliplex, Millipore, Billerica, MA).

Tumor necrosis factor (TNF)-α, interleukin (IL)-6 and keratinocyte-derived chemokine (KC) (murine equivalent of human IL-8) in lung homogenate were determined by enzyme-linked-immunosorbent assay (ELISA) (for IL-6 and IL10; CytoSet, BioSource, CA; for TNF-α and KC; ELISA-Kit, R&D Systems, Minneapolis, MN). Lower detection limits: IL-1α and IL-1β 40 pg/ml; TNF-α: 32 pg/ml; IL-6: 160 pg/ml; IL-10: 16 pg/ml and KC: 160 pg/ml.

IL-1β and IL-1α in lung homogenate were determined using a radioimmunoassay (RIA) as described previously.[23] Total protein concentrations in the lung homogenates were determined using a BCA protein assay (Thermo Fisher Scientific, Etten-Leur, The Netherlands). Cytokine concentrations in the homogenates were normalized for protein concentration.

ICAM-1 analysis

Concentration of mouse sICAM-1 was determined in plasma and lung tissue using the quantikine mouse sICAM (CD54) ELISA (MIC100) kit. (R & D systems, Abingdon, United Kingdom). Lower detection limits: 24,8 ng/ml.

Pulmonary neutrophil influx

After overnight incubation in 4% buffered formalin solution, the right middle lung lobe was dehydrated, and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4µm-thickness were used. Enzyme histochemistry using chloracetatesterase (LEDER staining) was used to visualize the enzyme activity in the neutrophils. Neutrophils were counted manually (10 fields per mouse, blinded), and after automated correction for air/tissue ratio, the average number of neutrophils/μm^2 per mouse was calculated.[23]

Statistical analysis

We performed a sample size calculation based on previous investigations considering a difference of 40% in cytokine levels between ventilated and control mice with a type 1 error of 5% (α=0.05) and a power of 80% (β=0.2).[21-23] This resulted in a group size of 8 animals per group.
Shapiro-Wilk tests showed that data were not normally or log normally distributed.

Data are therefore expressed as median with interquartile range (IQR) and depicted as column bar graphs. Differences between control *versus* lidocaine groups were studied using Mann Whitney tests. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). P-values < 0.05 were considered significant.

Results

Cardiopulmonary physiology

All mice in this experiment exhibited stable hemodynamic variables during MV (P>0.05).

Mean arterial pressure was within normal limits and remained above 65 mmHg (except in one mouse measured in the Lido 8 group), which was in line with previous data from our lab in this model. [21-23] We did not observe arrhythmic changes in the different MV groups (except one mouse measured

in the Lido 8 group). Blood gas values remained within normal range after 4 hours of MV and no differences were observed within the ventilated groups (data not shown). Four mice, one in each group, died during the experiment (N=7 in each group remaining). Two mice died during instrumentation (CON, Lido 2, no measurement on hemodynamics obtained yet), one mouse died in its cage before the experiment started, without apparent reason (Lido 4). One mouse died before the end of the experiment (Lido 8) from severe hypotension and bradycardia resulting in death.

Cytokine analysis in plasma

Cytokine analysis in plasma revealed no significant differences between the ventilated groups in IL-1β, IL-6, TNF-α and KC. However, IL-10 analysis showed a significant increase in all the lidocaine groups, 2, 4 and 8 mg/kg/h (Lido 2, Lido 4 and Lido 8) in comparison with the control group (CON) (figure 1).

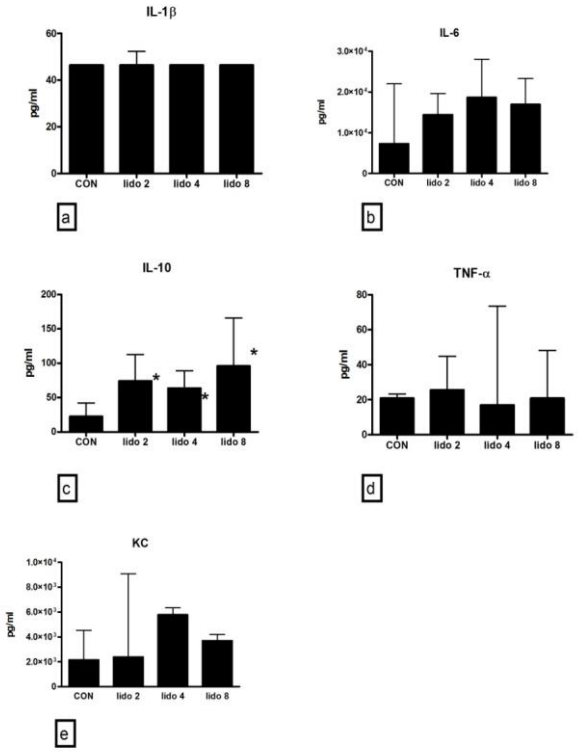

Figure 1. Cytokine levels in plasma. Levels of interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α and keratinocyte derived chemokine (KC) in ventilated control (CON) compared with ventilated lidocaine mice (Lido) receiving lidocaine in different dosages. Lidocaine was given in dosages of 2, 4 and 8 mg/kg/h. (*panels a-e*). Lido 2,4 and 8 showed increased IL-10 compared with CON. No differences were observed within the different ventilated groups of IL-1β, IL-6, IL-10, TNF-α and KC. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

Cytokine analysis in lungs

IL-10 analysis showed a significant increase between the control group (CON) in comparison with the lidocaine 8 mg/kg/h group (Lido 8) but not in comparison with the lidocaine 2 mg/kg/h and 4 mg/kg/h group (figure 2).

Cytokine analysis in lung homogenates revealed no significant differences between the different ventilated groups in IL-1β, IL-6, TNF-α, KC and IL-1α.

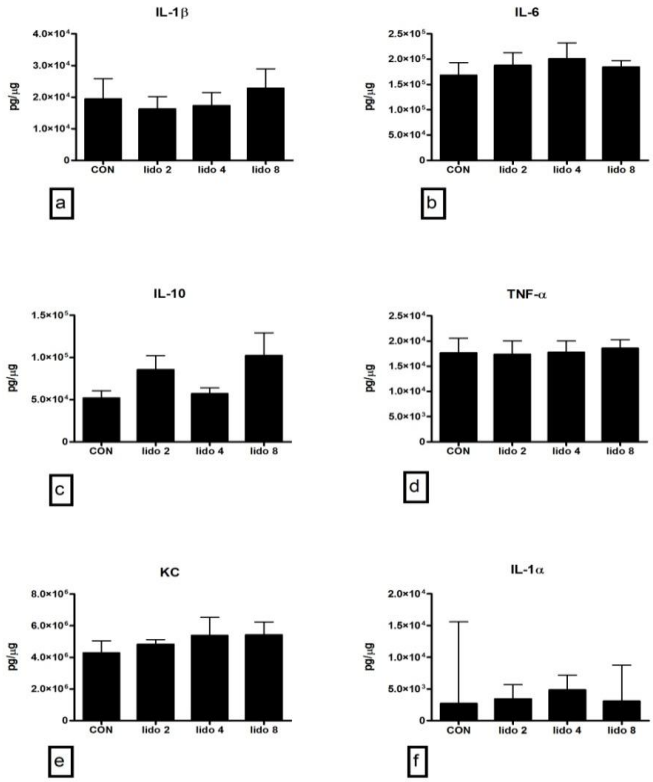

Figure 2. Cytokine levels in lung homogenates. Levels of interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, keratinocyte derived chemokine (KC) and IL-1α, in ventilated control (CON) compared with ventilated lidocaine mice (Lido) receiving lidocaine in different dosages. Lidocaine was given in dosages of 2, 4 and 8 mg/kg/h. (*panels a-f*). Lido 8 showed increased IL-10 compared with CON. No differences were observed within the different ventilated groups of IL-1β, IL-6, IL-10, TNF-α, KC and IL-1α. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

ICAM-1 analysis

ICAM-1 analysis in plasma and lung homogenates showed no significant differences between the different ventilated groups (figure 3).

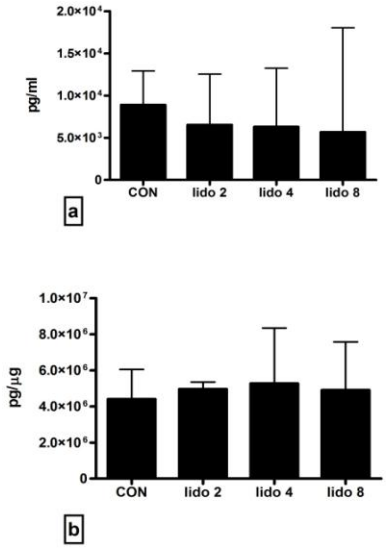

Figure 3. Intracellular adhesion molecule (ICAM)-1 levels in plasma (*panel a*) and lung homogenates (*panel b)*. Levels of in ventilated control (CON) compared with ventilated lidocaine mice (Lido) receiving lidocaine in different dosages. Lidocaine was given in dosages of 2, 4 and 8 mg/kg/h. No differences of ICAM-1 were observed between the different ventilated groups. Data are expressed as median with interquartile range (IQR).

Pulmonary neutrophil influx

No significant differences between pulmonary neutrophil influx measured per μm^2 were observed between the different ventilated groups (figure 4).

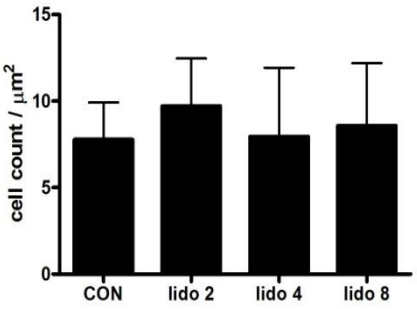

Figure 4. Pulmonary neutrophils influx in lung. Neutrophil influx was measured by cell count in ventilated control (CON) compared with ventilated lidocaine mice (Lido) receiving lidocaine in different dosages. Lidocaine was given in dosages of 2, 4 and 8 mg/kg/h. No differences were observed between the different ventilated groups. Data are expressed as median with interquartile range (IQR).

Anesthetic need

Mice showed a decrease in anesthetic need in lidocaine 2 and 8 mg/kg/h (lido 2 and 8) group, compared with control mice (CON) (figure 5).

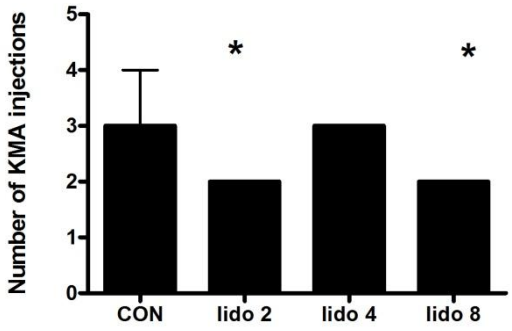

Figure 5. Anesthetic need in mice. Anesthetic need was determined by painful hind paw stimulaton every 30 minutes. If the mouse retracted his hindpaw, 5.0 µl per gram of body weight boluses of maintenance KMA mix (consisting of 0.72 ml ketamine, 100 mg/ml; 0.08 ml medetomidine, 1 mg/ml; 0.3 ml atropine, 0.5 mg/ml; and 18.9 ml NaCl, 0.9%) was administered. The number of KMA injections was measured in the different ventilated groups receiving lidocaine in dosages of 2, 4 and 8 mg/kg/h. (Lido 2, 4 and 8) compared with ventilated control (CON). Differences were observed between CON compared with lido 2 and lido 8. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

Discussion

This study is the first to show that intravenously administered lidocaine caused an increase in pulmonary and systemic IL-10 levels following MV in healthy mice compared with control animals.

IL-10 is a well known anti-inflammatory cytokine which limits the immune response during infections and is produced by nearly every type of cell in the immune system.[25, 26] IL-10 is known to decrease the synthesis of pro-inflammatory cytokines in acute phase response as IL-1α, IL-1β, IL-6 and TNF-α by neutrophils.[27] In mouse lung fibroblast exposed to mechanical stretch, IL-10 inhibited inflammatory cytokines.[25] A low lung concentration of IL-10 in patients with acute lung injury is an indication for development of adult respiratory distress syndrome (ARDS).[28] Administration of IL-10 has shown protective effects in LPS induced lung injury.[29] Interestingly, inhaled IL-10 attenuates biotrauma and mortality in a ventilator-induced lung injury model in rats.[30]

We did not observe an attenuation of pro-inflammatory cytokine levels, pulmonary ICAM-1 levels or pulmonary neutrophil influx. A possible explanation for this could be that although IL-10 is known to

attenuate inflammation, the acute phase response in our MV model is only a mild inflammatory response.

We did not include an unventilated group whereas the placement of an indwelling tail catheter in an awake mouse provides extreme stress which could lead to false high cytokine levels. Furthermore previous investigations have shown that cytokine levels of unventilated mice are below or extremely close to detection limits.[21-23] Dosage of lidocaine, especially 8 mg/kg/h, is relatively high. In comparison, dosages of 2 mg/kg/h can be considered safe in humans, 4 mg/kg/h is relatively high and 8 mg/kg/h is considered too high in humans.[31] Previous research has shown an ED50 for central nervous system and cardiac toxicity in mice of approximately 19,5 and 21,2 mg/kg.[32] . The cardiac side effects of lidocaine, contributed by the blockage of voltage-gated sodium channels, appear at plasma levels higher than 10 µg/ml in humans.[33] Considering the high ED50 for lidocaine in mice and extensive animal research in lidocaine toxicity with similar dosage we did not measure plasma levels of lidocaine and we have strong indications we stayed under critical plasma levels of lidocaine. One mouse however died in the 8 mg/kg/h group, because of uncontrollable hypotension, which could possibly indicate an overdose of lidocaine. In lung homogenates a significant increase of IL-10 was observed only at 8 mg/kg/h lidocaine (Lido 8), suggesting a possible dose related effect. Mice in our experiment showed a decrease in anesthetic need with lidocaine administration which is consistent with previous experiments.[16, 17] Although a decreased anesthetic need did not lead to an attenuation of other cytokine levels in our experiment an influence on the level of IL-10 cannot completely be ruled out.

In conclusion, low dose intravenously administered lidocaine in MV increases levels of plasma IL-10 with infusion from 2, 4 and 8 mg/kg/h and pulmonary levels of IL-10 with 8 mg/kg/h, in a murine mechanical ventilation model indicating a modulatory role of lidocaine in inflammatory response. After 4 hours of MV no effects were found on pro-inflammatory cytokines, neutrophil influx or ICAM-1 levels. More research has to be performed to elucidate the exact role of lidocaine in ventilator induced pulmonary inflammation and cytokine levels during time, and since we only ventilated mice for 4 hours, the full impact of lidocaine on the MV induced inflammatory response, cannot be fully described.

Lidocaine could prove to be an interesting therapeutic in multiple hit models. Furthermore intravenously administered lidocaine decreases anesthetic need.

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5

The *in vitro* mechanisms and *in vivo* efficacy of intravenous lidocaine on the neuroinflammatory response in acute and chronic pain: a review of current knowledge

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Abstract

Introduction

The neuroinflammatory response plays a key role in several pain syndromes. Intravenous lidocaine has beneficial effects in acute and chronic pain. This review delineates the current literature concerning *in vitro* mechanisms and *in vivo* efficacy of intravenous lidocaine on the neuroinflammatory response in acute and chronic pain.

Methods

We searched PUBMED and the Cochrane Library for *in vitro* and *in vivo* studies from July 1975 to August 2014. *In vitro* articles providing an explanation for the mechanisms of action of lidocaine on the neuroinflammatory response in pain were included. Animal or clinical studies were included concerning intravenous lidocaine for acute or chronic pain or during inflammation.

Results

Eighty eight articles concerning systemic administration of lidocaine were included: 36 *in vitro* studies evaluating the effect on ion channels, receptors and inflammation; 31 animal studies concerning acute and chronic pain and inflammatory models; 21 clinical studies concerning acute and chronic pain.

Low dose lidocaine inhibits *in vitro* voltage gated sodium channels, the glycinergic system, some potassium channels and Gαq- coupled protein receptors. Higher dosages lidocaine block voltage gated calcium channels, potassium channels and NMDA receptors. Animal studies demonstrate lidocaine to have analgesic effects in acute and neuropathic pain syndromes and anti-inflammatory effects early in the inflammatory response. Clinical studies demonstrate lidocaine to have a clear advantage in abdominal surgery and in some neuropathic pain syndromes.

Conclusions

Intravenous lidocaine has analgesic, anti-inflammatory and antihyperalgesic properties. It attenuates the neuroinflammatory response in perioperative pain and chronic neuropathic pain.

Background and objective

The neuroinflammatory response plays a key role in several acute and chronic pain syndromes. Even though pain is typically categorized as either inflammatory or neuropathic many similarities exists between these two conditions [1-5].
After tissue injury, pro-inflammatory cytokines and chemokines are synthesized and immune cells migrate to the injury site. This "inflammatory soup" reduces firing tresholds of A-δ and C-fiber nociceptors and causes acute pain [6]. Chronic pain can exist despite tissue healing and results from persistently generated impulses to the central nervous system due to ongoing inflammation or nerve injury [7, 8]. These induce ligand- and voltage gated ion channels in the peripheral and central nervous system. Prolonged peripheral input causes glutamate release and subsequent N-methyl-D-aspartic acid (NMDA) receptor activation, resulting in hyperexcitability and modification of the central nervous system [7, 9]. Concurrently, microglia and astrocytes in the dorsal horn and spinal cord are activated resulting in an enhanced release of proinflammatory cytokines and algesic mediators contributing to development and persistence of chronic pain [3, 10].

Lidocaine is the only local anesthetic considered safe for intravenous use, as a result of the extensive experience for anti-arrhythmic therapy [11]. Lidocaine is well known for its ability to block sodium channels. However, blockade of potassium- and calcium channels, G protein-coupled receptors, NMDA-receptors and the glycinergic system may contribute to its efficacy in the neuroinflammatory response in pain conditions [12]. Over the last years, an increasing number of *in vitro* publications have emerged concerning the different target mechanism contributing to the analgesic efficacy of systemic lidocaine. Additionally, an increasing number of *in vivo* publications have emerged concerning the administration of lidocaine as a perioperative analgesic and for relieving chronic pain conditions.

The first aim of this review is to present an overview of the preclinical studies concerning the various target mechanisms of lidocaine, which elucidate its effect on the neuroinflammatory response in acute and chronic pain. The second aim is to present an overview of the clinical studies concerning the efficacy of systemic lidocaine on the neuroinflammatory response in acute and chronic pain.

Databases and data treatment

Search

A systematic literature search was performed. We used the electronic database PUMBED from July 1975 till November 2014 for *in vitro* studies explaining mechanisms of action of lidocaine. We searched the databases PUBMED and the Cochrane Library from July 1975 till November 2014 for studies regarding the effects of intravenous lidocaine on nerve injury and inflammation in acute and chronic pain. Furthermore, reference lists were searched for relevant articles. The search was restricted to articles written in English or Dutch and published as 'full paper'.

We used the following terms for our search: 'lidocaine', 'lignocaine' 'intravenous lidocaine OR systemic lidocaine', 'intravenous lidocaine OR systemic lignocaine'. Additional search terms included: 'chronic and acute pain', 'hyperalgesia', 'inflammation', 'cytokines', 'perioperatieve', 'peroperative'.

In vitro articles were included providing a specific explanation of the mechanisms of action of lidocaine on the neuroinflammatory response in acute and chronic pain. In vitro articles evaluating the effect of lidocaine on ionchannels (sodium-, potassium-, and calcium channels), receptors (NMDA-, G-protein coupled- and glycine receptors) and on the inflammatory response were included.

Animal or clinical studies were included concerning systemic lidocaine administration for acute or chronic pain or during inflammation. Abstracts of possible relevant studies were independently assessed. A data collection was assembled consisting of reviews, randomized controlled clinical trials, intervention and some retrospective studies in humans and animals. Studies were excluded for the following reasons: evaluation of locoregional, epidural or local applied lidocaine, pain or inflammatory response was not used as an outcome measure, the effectiveness of lidocaine treatment on pain or the inflammatory response was not the aim of the study. Other reasons for exclusion were as follows: studies were already described in included systematic reviews, the manuscripts concerned case studies or opinion articles, or articles were of poor methodological quality. Studies were considered to be of poor methodological quality when the aim was not clearly described; the research design, selection of participants or data collection were inadequate; and analysis of data, the description of results and conclusions were not accurate or clearly described.

Analysis

In vitro literature concerning lidocaine was categorized in three groups evaluating the effect on: 1) ion channels (sodium-, calcium- and potassium channels); 2) receptors (G-protein coupled, NMDA and glycine); 3) inflammatory response. We compared these studies regarding their general characteristics: cell type, technique, lidocaine concentration and results. Clinical studies concerning systemic lidocaine administration were subdivided in animal and clinical studies. Animal studies were categorized in three groups: 1) acute pain; 2) chronic pain; 3) anti-inflammatory effects. We compared these studies regarding the following characteristics: type and number of animals, pain syndrome, lidocaine dose and results. Clinical studies were categorized in two groups: 1) acute pain; 2) chronic pain. We compared these studies regarding the following characteristics: study design, number of trials or patients included, pain syndrome, lidocaine dose and results.

Results

A total of 88 articles were included. 36 articles were *in vitro* studies, of which 18 studies concerning ion channels, 13 studies concerning receptors and 5 additional studies concerning the inflammatory response. 31 were animal studies, of which 12 studies concerning acute pain, 7 studies concerning chronic pain and 12 studies evaluated the effect of lidocaine in inflammatory models.
Reviews, RCT published after these reviews and interesting retrospective studies, evaluated the clinical efficacy of lidocaine in acute (10 articles) and chronic (11 articles) pain.
The results are presented in table 1 to 7.

Mechanism of action of lidocaine: *In vitro* research

Ion channels (Table 1)

Sodium channels

Voltage-gated sodium channels (VGSC) are compromised of a pore-forming α subunit, $Na_v1.1$ - $Na_v1.9$, with one or more smaller β subunits, β1-β4. The α subunits regulates Na+ currents [13] and the β subunits are multifunctional channel modulators, members of the immunoglobulin superfamily and cell adhesion molecules [14]. Each isoform has its own electrophysiological characteristics and are expressed in specific neuronal tissue. Changes in expression of VGSC isoforms occur in chronic neuropathic and inflammatory pain conditions [9, 13, 15-17].
After depolarization, VGSC eventually assume a nonconducting state through a distinct process

known as inactivation and cannot reopen during a certain period. After a train of depolarizations, the availability of channels to reopen declines, which is called use-dependence. Lidocaine preferentially binds to the inactivated state, thereby enhancing use-dependence and suppressing cellular excitability, particularly at high rates of stimulation [18]. Lidocaine produces a tonic block of sodium currents after depolarization [19].

Lidocaine decreases conduction in Nav 1.4, by enhancing the transition to slow inactivation [20-22]. This implies that lidocaine induces a conformational gating change of Nav 1.4 linked to a stable inactivated state. Lowering extracellular sodium concentration enhances use-dependent blockade [21]. Chevrier et al. [23] studied Nav 1.7 and Nav 1.8 expressed in *Xenopus* oocytes. Lidocaine enhances tonic and use-dependent block and the transition to slow inactivation in both channels, although Nav 1.8 is 4.4-fold more sensitive to lidocaine than Nav 1.7. Sheets et al. [24] showed a decreased transition to slow inactivation in Nav 1.7, which opposes Chevrier's results. An explanation for this can be that Sheets et al. studied Nav 1.7 in Human Embryonic Kidney cells (HEK cells) and Chevrier et al. in *Xenopus* oocytes; additionally, Chevrier et al. applied other lidocaine dosages and stimulation pulses. However, it can be concluded that lidocaine seems to have differing potencies on subtypes of VGSC. This is confirmed for tetrodotoxin sensitive (TTXs) and tetrodotoxin resistant (TTXr) VGSC. TTXs VGSC are found to be 5-fold more sensitive for lidocaine. Nevertheless, stimulating TTXr at higher frequencies reduces the inhibitory concentration to block 50% (IC50) of sodium channels, implicating a slower activation and higher stimulation threshold of TTXr neurons [25]. Aδ- and C-fibers can be divided according to their response to sustained depolarization into: tonic, adapting and single spike neurons. Lidocaine suppresses tonic firing and adapting firing neurons by interacting with VGSC [26].

Lidocaine influences inflammation, mediated by VGSC. Huang et al. [27] revealed that lidocaine dose dependently inhibits the expression of inducible nitric oxide (iNOS) and cationic amino acid transporter (CAT-2) in lipopolysaccharide (LPS) stimulated murine macrophages, presumably by blocking VGSC. Upregulation of iNOS and subsequent nitric oxide overproduction is a critical factor in the sequence of sepsis. This research group further explored the role of lidocaine on toll-like receptor 4 (TLR4) and nuclear factor (NF)-κB and mitogen-acitvated protein kinases (MAPK's) since these mediate iNOS mediated inflammation, and demonstrated lidocaine to attenuate activation of TLR4, NF-κB and MAPK's in activated macrophages, with involvement of VGSC [28].

Calcium channels

Voltage gated calcium channels (VGCC) are involved in neuronal excitement and diverse physiological functions and can be subdivided into low voltage-activated T-type and high voltage-activated L-, N- and R-type channels. Changes in the biophysical properties and expression levels of VGCC are observed in neuropathic pain [29].

Lidocaine inhibits calcium currents in amphibian neurons in a dose- and voltage dependent manner [30, 31]. At an IC 50 for blocking sodium currents (100µM), 35% of the calcium currents were blocked in frog neurons [31]. However, the concentration needed for blocking calcium currents in snail neurons was about ten times higher (1mM) [30]. These opposing results may be attributed to the different applied external Ca^{2+}concentration (2mM in frog, and 10mM in snail one), since the efficacy of lidocaine is reduced for increased external Ca^{2+}concentrations [30].

Studies in mammalian neuronal preparations revealed a dose-dependent inhibition of high voltage activated VGCC and more specifically L-type VGCC. Lidocaine dosages needed for VGCC blockade were relatively high (1-10mM) compared with VGSC blockade (60-200µM) [32, 33].

Potassium channels

Potassium channels are important regulators of membrane potentials, action potential shape, and firing adaptation in excitable tissues including sensory neurons [34]. Various potassium channels are involved in pain modulation and inflammation: voltage-gated potassium channels (VGPC), voltage independent potassium channels, tandem pore domain potassium channels (2P K+ channels) and ATP-sensitive potassium channels. Lidocaine inhibits K^+ currents in various neuronal preparations, including transient K^+ currents in rat dorsal horn neurons [19] and sustained K^+ currents in rat dorsal root ganglion neurons [35] and amphibian sciatic nerves [36]. Although the affinity of lidocaine for VGPCs is 6-fold lower compared with VGSCs, blockade of VGPCs seems to contribute to the broadening of the action potential in the presence of lidocaine. Inhibition of the outward potassium currents causes partial depolarization and leads to an increased amount of inactivated sodium channels. Inactivated sodium channels are more sensitive to lidocaine. Thus, inhibition of outward potassium currents promotes sodium channel inactivation [36]. Kindler et al. [37] investigated members of 2P K+ ion channel family, which are widely expressed in the central nervous system and the molecular entities of background or leak potassium conductances involved in the control of resting membrane potential and firing pattern of excitable cells. Lidocaine inhibited tandem pore weak inward rectifying K channel (TWIK)-related acid-sensitive K+ channel 2 (TASK-2) in a dose-dependent manner (IC 50 = 1mM).

Lidocaine suppresses tonic firing Aδ- and C-fibers by interacting with VGPC [26].
The flicker potassium channel is a voltage independent potassium channel found in most of the thin nerve fibers and generates the resting membrane potential of these fibers. In a frog sciatic nerve, lidocaine blocks the flicker potassium channel intracellularly (IC 50 = 219μM) and binding is pH-dependent, *id est* improved binding at higher pH-values and reduced binding at low pH-values [38]. Lidocaine modulates mitochondrial adenosine triphosphate (ATP)-sensitive potassium channels resulting in a reduction of cytokine-induced cell injury in vascular smooth muscle and endothelial cells. Cell survival improved as the cells were incubated with increasing dosages of lidocaine [39].

Receptors (Table 2)

G- coupled protein receptors

The G-protein-coupled receptors (GPCRs) consist of a large family (nearly 2000 GCPRs), which are of fundamental importance for intra- and intercellular communication pathways [40]. Following injury, a variety of inflammatory mediators are released activating GCPRs expressed on sensory neurons. Downstream GPCR signaling, diverse intracellular enzymes are activated, which converge upon ion channels that transduce noxious input or modulate basal excitability of nociceptors [41]. Specifically the α-subunit of the Gq family plays an important role in pain modulation and inflammation [41, 42] and seems to be a target of lidocaine.
Lidocaine inhibits m1 and m3 muscarinic receptors in clinically relevant dosages. Dosages needed for inhibition of m1 (IC 50 = 18nM) is about a 21-fold less for m3 muscarinic (IC 50 = 370nM) signaling, but still both dosage are significantly less compared with sodium channel blockade (60-200μM) [43, 44]. Intracellular binding to the α-subunit of the Gq-protein subunit is similar for m1 and m3 receptor. The m1 receptor has an additional major extracellular binding site for lidocaine, which clarifies its sensitivity for lidocaine [43, 44]. Inhibition of m3 muscarinic receptors is not restricted to *Xenopus* GCPR [40]. Binding of lidocaine to m1 and m3 receptor has a time dependent biphasic response. Lidocaine initially inhibits m1 and m3 receptors, but after 8 hours it enhances m1 and m3 signaling. This enhancement may be attributed to an interaction with an extracellular receptor domain and subsequent modulation of PKC activity and receptor phosphorylation [45].
Lidocaine inhibits immune modulatory Gαq-coupled receptors, such as lysophosphatidic acid (LPA), platelet activating factor (PAF) or tromboxane A2 (TXA2) receptors. Interestingly, after prolonged lidocaine administration an increased inhibitory potency is exhibited [46-48]. LPA is an intracellular phospholipid mediator which is released at injury sites and has chemoattractive and priming effects towards human polymorphonuclear neutrophil (hPMN). PAF plays a pivotal role in inflammatory disorders and also can effectively prime hPMNs. hPMN are of great importance in host defense, as

they move actively to the site of inflammation, where a multicomponent enzyme complex generates oxygen metabolites. Priming of hPMN potentiates the response to a subsequent activating stimulus and is a critical component of hPMN-mediated tissue injury. Lidocaine inhibits LPA en PAF mediated priming of hPMN in clinically relevant concentrations [48, 49]. TXA2 is a potent platelet aggregator and vasoconstrictor, however dosages needed for inhibition are relatively high (IC 50 = 1,1mM) [47].

N-methyl-D-aspartate receptor

The N-methyl-D-aspartate (NMDA) receptors are involved in rapid excitatory neurotransmission and modulation of nociceptive information, contributing to the development of hyperalgesia and chronic pain [9, 50]. NMDA receptors are protein complexes composed of two classes of co-assembling subunits: the essential subunit GluN1 and the modulating subunit GluN2 (A-D).
Hahnenkamp et al. [51] and Gronwald et al. [52] expressed human GluN1/GluN2A NMDA receptors in *Xenopus* oocytes and stimulated these with glutamate/glycine. Lidocaine inhibits the NMDA receptor in a dose dependent manner via an intracellular binding site. Addition of structural derivates of lidocaine, revealed NMDA receptor binding is stereoselective. Various mechanism of NMDA receptor inhibition have been proposed; altering the receptor phosphorylation state as a result of inhibition of the PKC pathway[52], binding to various sites on the NMDA receptor [53], or modulation of the glycinergic system [54].
Lidocaine dosages needed for NMDA receptor blockade are relatively high (NMDA rec IC 50 = 0,8 – 1,2 mM) compared with sodium channel blockade (60-200µM).

G*lycinergic system*

Glycine serves a dual role in central neurotransmission. It is not only an obligatory inhibitory neurotransmitter, but also a coagonist at the excitatory NMDA receptor. These actions depend on extracellular glycine levels, which are regulated by glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2). GlyT1 is responsible for removal of glycine from the synaptic cleft, whereas GlyT2 is required for re-uptake of glycine into nerve terminals, allowing neurotransmitter reloading of synaptic vesicles [55]. During high neuronal activity, glycine released from inhibitory interneurons escapes from the synaptic cleft, reaches nearby NMDA receptors by so-called spillover and facilitates NMDA receptor currents [56, 57]. Lidocaine exerts a biphasic response on the glycine receptor. Low dose lidocaine (10µM) enhances and high dose (1mM) inhibits glycinergic signaling [58].
Werdehausen et al. [54] revealed that not lidocaine, but its metabolites mediate the actions on glycinergic transmission by inhibiting GlyT1 in clinically relevant concentrations (55µM). Inhibition of

glycine re-uptake reduces glycine binding to the NMDA receptor and subsequent activation.

Anti-inflammatory effects

The inflammatory response leads to an increase in acute phase proteins and the release of vasoactive mediators from mast cells and platelets followed by activation of the kinin-, complement- and cytokine- systems. These inflammatory substances sensitize the central nervous system and induce pain [12, 59].

The inflammatory effects of lidocaine are mediated by inhibition of VGSC, Gq-receptors and ATP-sensitive potassium channels. Lidocaine attenuates the expression of iNOS en CAT-2 in LPS stimulated murine macrophages and the activation of TLR4, NF-κB and MAPK's by inhibiting VGSC [27, 28, 60, 61]. Additionally, inhibition of Gq-protein coupled receptors reduces PAF and LPA induced priming of hPMN [48, 49] and modulation of cytokine induced cell injury is mediated by ATP-sensitive potassium channels [39]. Moreover, other studies demonstrate lidocaine to decrease cytokine release in epithelial cells and neutrophils [62] and attenuate the expression of activated endothelial interleukin-1 (IL-1), IL-6 and IL-8 concentrations and intracellular adhesion molecule-1 (ICAM-1) during reperfusion injury [63, 64].

Mechanism of action of lidocaine: *In vivo* research

Research in animals

Acute pain (Table 3)

Woolf et al. [65] and Sotgiu et al. [66] investigated the effects of lidocaine on neuronal activity and the site of action of systemic lidocaine in rats. Intravenous lidocaine (1-5mg/kg) suppresses polysynaptic C-fiber evoked flexor response to noxious heat and chemicals, without blocking the conduction block at the peripheral nerve [65]. Intravenous lidocaine reduces responsiveness of wide dynamic range neurons to noxious evoked activity, but not to spontaneous activity or non-noxious stimuli [66]. In an acute pain rat model using a formalin test, pretreatment with lidocaine significantly reduces thermal hyperalgesia measured by paw withdrawal [67]. Lidocaine or its metabolites have a modulating effect on glycinergic signaling, since agonists of the glycine binding site at the NMDA-receptors inhibit the nociceptive effects [68]. These studies implicate a selective effect of lidocaine on nociceptive transmission at the level of the spinal cord. It should be noted that an effect on supraspinal pathways further has to be elucidated.

Small studies evaluating the effect of lidocaine on acute pain in dogs, demonstrate lidocaine to

provide intermediate analgesic effects. Lidocaine has comparable results to meloxicam [69] or has no extra analgesic effect in addition to opioids during ovariectomy [70], although it reduced preoperative inhalation anesthetic need [70]. During ocular surgery, lidocaine provides similar post-operative analgesia as morphine [71]. Furthermore, no anti-nociceptive effects were observed in conscious dogs, although they did show signs of mild to moderate sedation [72]. During orthopedic or soft tissue surgery, lidocaine significantly reduced supplemental intra-operative analgesic usage [73]. Dogs received a bolus of 1-2mg/kg followed by an infusion of 1,5-3mg/kg/h.
Two studies showed a reduction in length of stay (LOS) and incidence of postoperative ileus in horses, which is often a fatal complication [74, 75], although no difference in pain assessment was obtained [75]. However, a recently performed study found no beneficial effects of lidocaine treatment in 36 horses scheduled for a laparotomy for colic [76].

Chronic (neuropathic) pain (table 4)

Various dosages of lidocaine provide analgesia in rat models based on nerve constriction injury. Lidocaine silences ectopic neuroma and dorsal root ganglion discharge without affecting nerve conduction. The median effective dose of lidocaine for blocking dorsal root ganglion cells was significantly lower than for neurons at the injury site [77]. Lidocaine attenuates allodynia after nerve ligation and reduces ectopic discharges in injured afferent fibers [78]. Sotgiu et al. [79] found lidocaine (4mg/kg) to reduce receptive field and hyperesthetic sensation for 35 min. Dorsal horn neurons were more sensitive compared with ganglionic neurons [79]. Agonists of the glycine binding site at the NMDA-receptors inhibit nociceptive effects, indicating a general glycine-like action of lidocaine or some of its metabolites [68]. Thus, reduction of peripheral and central excitability is most likely a component of the analgesic properties of lidocaine.
Constant rate infusion of lidocaine results in a prolonged alleviation of allodynia in nerve-ligated rats without affecting motor function [67, 80] The long lasting analgesic effects were not achieved after intrathecal or perineural administration [80]. Lidocaine has a threshold value (2,1 µg/ml) and a ceiling effect for relief of allodynia [81].

Anti-inflammatory effects (Table 5)

Lidocaine exerts anti-inflammatory effects during the acute inflammatory response. Timing of lidocaine administration seems to be a critical factor in its efficacy. Pretreatment with lidocaine in a rabbit endotoxemia model induced by *Escheria coli* reduces the release of complement, cytokine release and activation of PMN and improves lung mechanics [82]. Early posttreatment slightly attenuates endotoxin-induced lung edema, without affecting chemical mediators in brochoalveolar

lavage fluid [83]. In another endotoxin rabbit model, systemic lidocaine administered immediately after endotoxin injection reduces release of Il-6 and Il-8 and improves hemodynamics [84]. Acute lung injury induced by phospholipase A2 and trypsin is attenuated when phospholipase A2 is co-administered with lidocaine [85] Similar dosages were used (bolus 2-3mg/kg + continuous rate infusion (CRI) 2mg/kg/h) in these rabbit studies.

CRI infusion of lidocaine (2,5-5mg/kg/hr for 7days) during septic peritonitis in mice reduces tumor necrosis factor α (TNF-α), ICAM-1 and chemokines, improves organ dysfunction and reduces mortality [86].

In an endotoxemia model in horses induced by lipopolysaccharide (LPS), systemic lidocaine infusion reduced discomfort and levels of TNF-α in plasma and peritoneal fluid [87]. Rats receiving an intravenous bolus or aerosolized lidocaine before treatment with LPS had lower levels of cytokines in bronchoalveolar fluid of IL-1β and TNF-α, without changes in plasma cytokine level [88].

Studies evaluating lidocaine administration during reperfusion injury show moderately positive effects depending on the species and reperfusion injury model used. Lidocaine attenuates ischemic injury, improves mucosal barrier [89], and reduces mucosal cyclooxygenase 2 and plasma levels of prostaglandin E2 in the equine jejenum after surgery [90]. Lidocaine (3-4 µg/ml) reduces reperfusion injury and improves gas exchange in lung allografts in dogs by inhibiting PMN adhesion and subsequent neutrophil migration. Neutrophils mediate postischemic tissue injury by oxygen radical and proteolytic enzyme release [91]. Pretreatment with systemic lidocaine reduces infarct size after reperfusion in a murine myocardial ischemia model most likely due to a reduction of hypoxia induced apoptosis. Lidocaine had no effects on leucocyte rolling or adhesion [92]. In a porcine model of myocardial ischemia, systemic lidocaine had no effect on myocardial damage. However, retrograde infusion before coronary reperfusion reduces myocardial infarct size [93].

Clinical studies

Acute pain (Table 6)

Sun et al. [94] performed a meta-analysis of 21 trials concerning lidocaine administration during abdominal surgery. Lidocaine reduces pain scores, opioid consumption, postoperative nausea and vomiting (PONV), LOS and duration of ileus after open and laparoscopic surgery. Some studies show an attenuation of postoperative rise of proinflammatory cytokines. Vigneault et al. [95] and Mc Carthy et al. [96] confirmed the beneficial effects in gastrointestinal surgery, although lidocaine has no or minimal impact on postoperative analgesia or outcome in cardiac surgery, gynecologic procedures, tonsillectomy and total hip arthroplasty. Mc Carthy et al performed a systematic review

of 16 trials, which described specifically the effects of lidocaine on different subtypes of surgery. Vigneaults meta-analysis included 29 trials, was more extensive and provided a comprehensive understanding of intravenous lidocaine on perioperative outcomes.
Randomized control trials published after this meta-analysis confirms the beneficial effects of perioperative lidocaine administration in gastrointestinal surgery [97-99]. However, Yon et al. [99] found no difference in PONV and LOS in patients undergoing gastrectomy.
Lidocaine has differing efficacy in other types of surgery. Lidocaine has no influence on LOS, postoperative pain, return of bowel function or inflammatory and stress response measured by plasma cortisol levels and CRP in major laparoscopic renal surgery [100]. Lidocaine improves pain scores from 5.5 to 4.4 on a 11-point Likert scale, reduces 48h opioid requirements by approximately 25% and patients exhibit greater physical scores at 1 and 3 months after major spine surgery [101]. Grigoras et al. [102] tested the efficacy of a lidocaine infusion in 36 patients undergoing breast surgery. No difference in intraoperative or postoperative opioid consumption was found. Nevertheless, postoperative pain scores were reduced at 4 hours and a significant reduction of chronic pain and hyperalgesia was observed at 3 months follow up (11,8 % vs. 47,4 %). However, Terkawi et al. [103] found no difference in pain scores, opioid consumption, PONV and LOS in 71 patients undergoing breast surgery. Long term effects were not evaluated in this study.

Chronic (neuropathic) pain (Table 7)

Challapalli et al.[104] performed a Cochrane review of 30 RCT concerning the efficacy of intravenous lidocaine and its oral analogues on relieving neuropathic pain in 2005. The treatment intervention was lidocaine in16 trials, mexilitine in 12 trials, sequential mexilitine and lidocaine in one study and tocainide in one study. Lidocaine and mexilitine were more effective compared with placebo in decreasing neuropathic pain. In a subgroup analysis, lidocaine tended to be more effective for relieving neuropathic pain caused by diabetes, trauma or cerebrovascular diseases. No serious adverse effects were observed during these studies. The number of studies was relatively small and their methodology not always consequent. Minor side effects occurred in about 35% of the patients compared with 12% of the patients allocated to placebo. The most common adverse effects were sleepiness, fatigue, nausea, dizziness, perioral numbness and metallic taste. Since 2005, many other trials have been published that explored the use of systemic lidocaine for chronic (neuropathic) pain. Three randomized crossover trials, examining the effect on neuropathic symptoms and pain, found differing results in neuropathic pain syndromes. Lidocaine (5mg/kg in 30minutes) relieves spontaneous pain and brush evoked dysesthesia in 24 patients with spinal cord injury, without effect

on cold allodynia, pinprick hyperalgesia and pain evoked by repetitive pinprick [105]. In a small group of 13 patients with nerve injury, lidocaine (5mg/kg) reduces brush evoked pain and cold allodynia and has a tendency to reduce spontaneous pain, although not statistically significant due to the small study population. The presence of mechanical allodynia did not predict response to lidocaine [106]. Conversely, lidocaine (5mg/kg in 30min) had minimal to no effect on spontaneous pain, brush evoked pain or cold allodynia in 20 patients with nerve injury pain. Lidocaine reduced pain evoked by repetitive pinprick [107].

Tremont-Lukats et al. [108] investigated lidocaine (5 mg/kg in 6h) versus placebo in 31 patients with variable causes of peripheral neuropathic pain in a randomized cross-over trial. Lidocaine was more effective than placebo in relieving neuropathic pain, and pain reduction persisted for 4h after discontinuation of infusion. Lidocaine (5 or 7,5mg/kg in 4h) reduces significantly severity and quality of pain for 14 days and persisted for up to 28 days in 15 patients with intractable diabetic neuropathy [109]. No difference in analgesic effect was found between saline, lidocaine 1mg/kg and lidocaine 5mg/kg in patients with neuropathic pain as a result of failed back surgery syndrome [110].

Some retrospective studies imply a beneficial effect of lidocaine in chronic pain syndromes. Thomas et al.[111] did a retrospective chart review in 768 patients acutely admitted to a hospice. Patients receiving intravenous lidocaine for pain relief were evaluated. A great deal of them had neuropathic pain symptoms (78%) and 52% of the 61 patients had opioid refractory pain. Significant pain reduction was achieved in 50 patients (82%), partial response in 5 patients (8%) and no response in 6 patients (10%). Lidocaine infusion during 5 days reduces pain, thermal and mechanical allodynia and inflammatory symptoms for 3 months in 76% of patients presenting with CRPS. In general, by 6 months, CRPS factors had returned to baseline [112]. Lidocaine in unknown dosages reduced pain in 68 cases of refractory chronic daily headache for averagely 8,5 days [113]. Adolescents receiving lidocaine (2,4 -3,6 mg/kg/h) for refractory headache or neuropathic pain had reduced pain scores during 80% of the infusions. Only minor side effects were reported, which resolved quickly after discontinuation of infusion [114].

Conclusion

In vitro studies

In this review, we found that following *in vitro* research, lidocaine exerts its different effects on the neuroinflammatory response by inhibiting ion channels and receptors. Although, comparison of lidocaine dosages between *in vitro* studies is not always feasible due to the different cell types and methodology used, lidocaine seems to inhibit ion channels and receptors in various potencies. *In*

vitro, higher dosages are required for inhibition of VGCC, NMDA receptor and some of the potassium channels, as for VGSC, GCPR and glycinergic transmission. Nevertheless, partial inhibition of potassium channels, VGCCs and NMDA-receptor can contribute to analgesic and anti-inflammatory effects or side-effects [115].
Additionally, extrapolation of dosages from *in vitro* to *in vivo* situation encounters some difficulties. Whereas intravenous lidocaine *in vivo* induces favorable effects at clinically relevant concentration (1-15µM or 0,3 - 4,5µg/ml), *in vitro* concentrations to block ion channels or receptors are frequently higher to achieve the desired effect. One difference between *in vivo* and *in vitro* studies is the exposure time to lidocaine: exposure in most *in vitro* settings is 10-30 minutes, compared with hours or days *in vivo*. Hollman et al. [46] and Picardi et al. [45] found an enhanced effect of lidocaine on Gαq coupled protein receptors in *in vitro* setting during a prolonged exposure time. Another difference is that during *in vitro* experiments frequently just a single cell is observed which complicates translation to the *in vivo* situations.

Interestingly, lidocaine has slight differing effects on isoforms of specific ion channels. For example, Nav 1.8 is more sensitive to inhibition of lidocaine compared with Nav 1.7. Both Nav 1.7 and Nav 1.8 play specific roles in the neurobiology of neuropathic pain and are upregulated in inflammatory and neuropathic pain states [17]. An increased expression of Nav 1.8 is observed in neuromas [116, 117]. An increased expression of Nav 1.3, 1.7, 1.8 and 1.9 isoforms are observed in neuronal cell bodies following inflammatory lesions [116, 118]. Although lidocaine blocks all sodium channel isoforms, the little differences in sensitivity could be an additional explanation for its efficacy on ectopic discharges in neuropathic pain. Tanelian et al. [119] found lidocaine to inhibit tonic discharges in acutely injured Aδ and C-fibers in clinically relevant concentrations (5,7µg/ml), suppression of nerve conduction was achieved at 50-fold higher concentrations (250µg/ml). An increased expression of sodium channels in dorsal root ganglia and around the injury site of injured axons contributes to spontaneous firing of nerve fibers after injury [77].

In vivo studies

In the selected *in vivo* studies, the effects of lidocaine on the neuroinflammatory response in acute and chronic pain are presented. Animal studies demonstrate lidocaine to affect hyperexcitable neuroma, dorsal root ganglion neurons and dorsal horn neurons without affecting normal nerve conduction [77-79]. Dorsal horn neurons are more sensitive to lidocaine compared with peripheral neurons [79]. It should be noted that an effect on supraspinal pathways further has to be elucidated. The high susceptibility of hyperexcitable neurons to lidocaine may be attributed to their frequency dependency and to the changed expression of sodium channels during nerve injury [17], which may

render them subject to exaggerated blockade by lidocaine.
Lidocaine has anti-inflammatory effects during the acute phase of the inflammatory response in endotoxemia and reperfusion injury animal models. Pro-inflammatory cytokines and neutrophil migration are reduced, when lidocaine is administered early in the inflammatory cascade. Inflammatory effects are mediated by blockade of GCPR, VGSC and ATP-sensitive K+ channels. Limitation in these animal studies is the diverse endotoxemia models or reperfusion models used, which exert differences in immune response. Secondly, different dosing regimens were used between studies and long-term effects were not recorded. It is not clear whether attenuation of the hyperinflammatory response induces subsequent immune suppression. Thus, future animal studies are needed to further evaluate the underlying mechanisms and register long term-efficacy

Clinical studies reveal systemic lidocaine to reduce pain scores, opioid consumption, PONV, LOS in abdominal surgery in animal and clinical studies. The pronounced analgesic effect in abdominal surgery may be explained by the central antihyperalgesic effect of lidocaine[68]. Hyperalgesia is found in patients undergoing bowel surgery with peritoneal irritation, which induces inhibitory gastrointestinal reflexes. Intravenous lidocaine may shorten the duration of ileus by reducing opioid consumption, by preventing inflammatory processes and by decreasing sympathetic tone [120]. However, for other types of surgery the efficacy of intravenous lidocaine remains ambiguous. Cardiac or major orthopedic surgery involves substantial tissue disruption and a subsequent inflammatory response, but lidocaine exerts no beneficial effect in this type of surgery. Though, recent studies demonstrate lidocaine to have prolonged analgesic effects during complex spine surgery [101] and to reduce chronic pain after breast surgery [102]. Most studies concerning perioperative administration of lidocaine do not report long term results, so no definite conclusion can be made on this topic.

Lidocaine provides analgesia in neuropathic pain and reduces neuropathic symptoms. However relief of clinical symptoms varies between causes of neuropathic pain, presumably due to the distinct underlying pathofysiological mechanisms.
Studies evaluating lidocaine administration for chronic pain syndromes encounter several limitations. A problem in placebo controlled studies is that most people experience some minor side effects, therefore bias in these studies cannot be ruled out. Secondly, long term analgesic effects of intravenous lidocaine for chronic neuropathic pain have not been conclusively documented. Thirdly, some studies are retrospective, which implicate a risk of bias. Finally, the main limitation of most studies is the small study size.

A limitation in all clinical studies is the varying dosing regimens. A bolus of 1,5 - 2 mg/kg is administered followed by a continuous infusion of 1,5 - 2 mg/kg/h is the most common dose in the perioperative situation. However, duration of infusion vary widely, ranging from discontinuation at the end of surgery untill 24 hours postoperative. For neuropathic pain relief mostly a bolus of 5 mg/kg lidocaine is administered over a period ranging from 30 minutes till 6 hour. However, in some studies continuous infusion is administered for a longer period.
Most likely, perioperative administration is sufficient because modulatory action on the initiation of the inflammatory response primarily takes place during surgery and sustained lidocaine concentrations in cerebrospinal fluid extend beyond infusion time [121]. However, in vitro studies show a time dependent enhancement on GCPR [45, 46] .

Although precise therapeutic plasma levels and duration of infusion of lidocaine are still not that well defined, the optimal therapeutic range for pain treatment seems to be between 1-5 µg/ml [12, 119, 122-124]. Bolus administration of 2 mg/kg and a continuous infusion of 2-5 mg/kg/h have shown to reach plasma levels of 1-4 µg/ml [121]. After a bolus injection or continuous administration for up to 12 h, the half-life of lidocaine is about 100 minutes and shows linear pharmacokinetics [125]. Following prolonged infusion, lidocaine exhibits time-dependent, or nonlinear pharmacokinetics [126]. Minor side effects like light-headedness, periorbital numbness, vertigo or sedation can develop in therapeutic concentrations.
Clinical features of lidocaine toxicity include symptoms of central nervous and cardiovascular depression. Plasma levels of 5-10 µg/ml cause prolongation of conduction time and increased diastolic threshold [127], at levels higher than 7.5 µg/ml seizure activity can become noticeable. Plasma levels above 10µg/ml cause marked central nervous system and cardiovascular depression [127]. Lidocaine toxicity primarily arises from blockade of VGSC, though conceivably inhibition of ATP-dependent potassium channels plays a role [128, 129]. Cardiovascular compromised people or patients with a reduced liver or kidney function are more at risk for developing toxicity symptoms [127].

We recommend more trials to be performed, with larger study size and impeccable methodology to determine the effect of intravenous lidocaine on the neuroinflammatory response in acute and chronic pain. Since similar toxic plasma levels in animals and humans have been described in literature, further research in animals concerning the precise mechanism responsible for reduction in inflammation and pain could be extrapolated to the clinical setting. More research has to be done assessing the effect of the metabolites of lidocaine.

Although amide local anesthetics share common properties, the scope of this review was intravenous lidocaine. Lidocaine is the only local anesthetic registered for intravenous use in the Netherlands. Considering the potential of lidocaine infusion, investigation of the efficacy of other amide local anesthetics seems only a matter of time. More after, other routes than intravenous lidocaine administration exert beneficial effects, however this is beyond the scope of this review.

In summary, lidocaine exerts *in vitro* inhibitory effects on VGSC, some potassium channels, the glycinergic system and Gαq-protein pathways and in higher dosages inhibitory effects of potassium channels, VGCC and the NMDA-receptor. Animal studies show pain relieving effects in neuropathic pain syndromes and anti-inflammatory effects during the first phase of hyperinflammatory responses. Clinical studies demonstrate lidocaine to have beneficial effects in abdominal surgery and in some neuropathic pain syndromes.

Tables

Table 1: *In vitro* studies evaluating effects of lidocaine on voltage gated sodium-and calcium channels and subtypes of potassium channels

Sodiumchannels

Reference	Cells	Technique	Effect lidocaine on	Lidocaine Concentration	Results
Olscheweski 1998 **[19]**	Rat dorsal horn neurons	Patch clamp	Na+ currents after depolarization	1µM – 10mM	LDC IC 50 = 112 µM LDC produces a tonic block Na+-currents which is use-dependent
Balser 1996**[20]**	Xenophus oocytes + HEK cells	Voltage clamp	Mutation in III-IV Na_v 1.4: disabling fast inactivation	30µM- 1000µM	Dose dependent current reduction, IC 50 = 74 µM, indicating augmentation slow inactivation LDC modifies coupling between (in-) activation as an effector of the allosteric gating process
Chen 2000**[21]**	HEK cells + Xenophus oocytes	Voltage clamp	Kinetics Na_v 1.4	50µM - 200µM	Lowering extracellular Na-concentration augments use-dependent LDC block Outer pore (P-loop) mutation reduces LDC use-dependent block LDC increases slow inactivation
Fukuda 2005**[22]**	HEK cells	Voltage clamp	Kinetics Na_v 1.4	100µM	LDC stabilize structural rearrangements coupled to slow inactivation
Chevrier 2004**[23]**	Xenophus oocytes	Voltage clamp	Kinetics Na_v 1.7 and Na_v 1.8	10-100-300 µM	Na_v 1.8 is more sensitive to LDC: Na_v 1.8 EC 50 = 104 µM, Na_v1.7 EC 50 = 450 µM; tonic block and use-dependent block of Na_v 1.8 is 4,4-fold more enhanced as for Na_v1.7. LDC increases transition to slow inactivation Na_v 1.8>Na_v1.7. LDC modulates gating properties
Sheets 2011**[24]**	HEK cells	Voltage clamp	Kinetics Na_v 1.7	0,1, 0,3, 1, 3, 10 and 30mM	Voltage and pulse duration determine concentration response curve: IC 50 2-727 µM LDC enhances recovery of Na_v 1.7 from prolonged depolarization LDC decreases transition of Na_v 1.7 to the slow inactivated state. Use-dependent inhibition
Scholz 1998**[25]**	Dorsal horn neurons rats	Patch clamp	Kinetics TTXs and TTXr Na+-channels	50-300 µM	IC50 TTXs = 42 µM, IC50 TTXr = 210 µM TTXr show higher affinity for LDC in the inactivated state. A low concentration of LDC blocks trains of action potentials better at high frequencies in TTXr
Wolf 2014**[26]**	Dorsal horn neurons rats	Patch clamp	Tonically or adapting firing and single spike neurons	100 µM (50% of VGSC are blocked)	LDC blockade of adapting firing neurons is mediated by VGSC
Huang 2006**[27]**	Murine macrophages + LPS	PCR + electro-phoresis	LPS induced upregulation of iNOS and CAT-2	5, 50, 500 µM	Lidocaine attenuates in a dose dependent manner LPS-induced up regulation of iNOS and CAT-2, probably mediated by a VGSC, since veratridine (=VGSC-activator) inhibits this effect
Lee 2008**[28]**	Murine macrophages + LPS	Immuno-blotting assay	LPS induced upregulation (upstream iNOS) of TLR-4, NF-κβ, MAPKs	50 µMol/L	Lidocaine inhibits endotoxin induced activation of TLR-4, NF-κβ and MAPKs, probably mediated by VGSC, addition of veratridine reduces LDC effects

Calcium channels

Akaike 1982**[30]**	Helix neurones	Voltage clamp	Calcium current (I_{ca})	0,1mM – 10mM	Dose-dependent inhibition Ca+ current. Internal and external applied LDC reduces Ca+ current
Oyama 1988 **[31]**	Frog dorsal root ganglia	Voltage clamp	Calcium current	1µM – 10mM	Threshold for depressing I_{Ca} = 10µM (for I_{Na} 1µM) Blockade I_{Ca} is voltage- and dose-dependent. LDC blocks Ca^{2a} – channel in open state. At IC50 for blockade I_{Na} (100µM), I_{Ca} is 35% reduced
Sugiyama 1994**[32]**	Rat dorsal root ganglion	Patch clamp	Calcium current	10µM -100mM	LDC blocks high voltage I_{Ca} at high concentrations +/- 1-10mM

Potassium channels

Brau 1998 **[36]**	Sciatic nerve of Xenopus Laevis	Patch clamp	Voltage dependent K^+ channels	10µM – 1mM	IC 50 1118µM for blocking outward voltage dependent K^+ current. (IC 50 Na^+ = 204µM) Direct interaction of LDC with ion channel
Olscheweski 1998**[19]**	Rat dorsal horn neurons	Patch clamp	Rapidly inactivating K+ currents	1µM – 10mM	LDC IC 50 = 163 µM Delayed rectifier K= currents are almost insensitive for LDC
Komai 2001**[35]**	Isolated rat dorsal root ganglion neurons	Patch clamp	K+ currents	1-10mM	IC 50 = 2,2 mM LDC inhibits sustained and slow-inactivating K+ currents
Wolf 2014**[26]**	Dorsal horn neurons rats	Patch clamp	Tonic or adapting firing and single spike neurons	100 µM (50% of VGSC are blocked)	LDC blockade of tonic firing neurons is mediated by VGPC
Brau 1995**[38]**	Demyelinated tibial and peroneal nerve of Xenopus Laevis	Patch clamp	Basic kinetics of LDC on flicker K+ channel	300µM	LDC IC 50 = 219 µM Blockade flicker K+ channel mainly by an extracellular binding site, is pH dependent LDC blocks the flicker K+ channel by impeding gating, not conduction
Kindler 2003**[37]**	Xenophus Laevis HEK cells	Voltage clamp	TASK-1 TASK-2	50-2000µM	LDC IC 50 = 1000µM LDC has a greater sensitivity for TASK-2 as for TASK-1
Klaver 2003**[39]**	Rat vascular muscle cells+ human vascular endothelial cells + TNFα, INFY, Il-1β	Cellculturing + staining + microscopic and spectrophoto metric analysis	ATP-senitive K+ channel	5-100µM	Lidocaine attenuates cytokine induced cell injury in dose dependent manner (5µM 10% vs 100µM 60% cell survival). Decrease LDH release in both cell types Effects appear to be modulated by ATP-sens K+ channels (5-hydroxydecanoate = inh ATP-sens K+ abolish effects)

LDC lidocaine, IC 50 half maximal inhibitory concentration, EC 50 half maximal effective concentration, HEK human embryonic kidney, TTXs tetrodotoxin-sensitive, TTXr tetrodotoxin-resistant, VGSC voltage gated sodium channel, LPS lipopolysacchariden, iNOS inducible nitric oxide, CAT-2 cationic amino acid transporter-2, TLR-4 toll-like receptor 4, NF-κβ nuclear factor κβ, MAPKs mitogen-activated protein kinases, VGPC voltage gated potassium channel, TASK-1 TASK-2 TWIK (tandem pore weak inward rectifying K channel)-related acid-sensitive K+ channel 1 or 2, TNFα tumor necrosis factor α, INFγ interferon γ, Il-1β interleukin-1 β, LDH lactate dehydrogenase

Table 2: *In vitro* studies evaluating effects of lidocaine on G-protein coupled receptors (GPCR), N-methyl-D-aspartate (NMDA) receptors and glycinergic signaling

Reference	Cells	Technique	Effect lidocaine on	Lidocaine Concentration	Results
-protein coupled receptors					
Hollmann 2000**[43]**	Xenopus oocytes	Voltage clamp	M1 receptor	0,1nM – 10mM	LDC IC 50 = 18nM Non-competitive binding extracellular polar site on muscarinic receptor and intracellular on the coupled G-protein
Hollmann 2001**[44]**	Xenopus oocytes	Voltage clamp	M3 receptor	0,1nM – 10mM	LDC IC 50 = 370nM. Non-competitive binding intracellular Gαq -protein. M3 lacks extracellular binding site for LDC
Hollmann 2002**[40]**	Xenopus oocytes injected with mouse Gq	Voltage clamp	LPA and M3 Gq signaling in mammalian Gq protein	100µM -10mM	LDC reduced response LPA and M3 signaling via intracellular binding site LDC IC 50 LPA inhibition = 148µM
Hollmann 2004**[46]**	Xenopus oocytes + primed and activated hPMN	Voltage clamp + cytochrome c assay	Time-dependent effect Gαq protein		LDC attenuated Gαq rec signaling in reversible and time-dependent manner (= reduction LPA- signaling)
Picardi 2014**[45]**	Xenopus oocytes	Voltage Clamp	M1 and M3 receptor Gαq-signaling and time dependent	M1: 18nM M3: 370nM	Biphasic effect LDC on M1 and M3: t= 30-120min inhibition; t= >8h increased response, (dependent on PKC activity and receptor phosphorylation). Effect is mediated by Gαq
Honemann 2004**[47]**	Xenopus oocytes Human K562 cells	Voltage clamp + fluorometric determ IC Ca	TXA2-signaling	1µM – 100mM	LDC IC 50 = 1,1mM LDC attenuates TXA2 signaling via intracellular binding site Pathway coupled with Gα11 and Gαq
Fischer 2001**[49]**	hPMN	Cytochrome c-assay	Priming and activating of LPA on hPMN	1µM and 0,1mM	LPA act as a priming and chemo-attractant toward hPMN, not as activator. LDC attenuates chemotactic en metabolic response of hPMN probably by reducing LPA via an effect on Gαq Concentration and time dependent effect
Hollmann 2001**[48]**	hPMN	Cytochrome c-assay	PAF priming of hPMN	1-100µM	LDC inhibit PAF mediated priming of hPMN, which is PLC and PKC dependent and mainly Gq-mediated
NMDA receptor					
Hahnenkamp 2006**[51]**	Xenopus oocytes	Voltage clamp	NMDA receptor	1nM – 0,1mM	LDC inhibit NMDA receptor signaling via intracellular binding site and by influencing PKC dependent pathway (PKC activates NMDA-rec) Binding is not charge dependent
Sugimoto 2003**[53]**	Xenopus oocytes	Voltage clamp	NMDA receptor + site of action	1µM -10mM	LDC IC 50 = 1,2mM. NMDA receptor mutagenesis reveals various binding sites are involved
Gronwald 2012**[52]**	Xenopus oocytes	Voltage clamp	Structural features needed for inhibition NMDA receptor	0,1µM – 1mM + several LDC-analogues	LDC IC 50 = 0,8mM Position and length aliphatic side chain in aromatic part strongly influences inhibition and potency
Glycine					
Hara 2007**[58]**	Xenopus oocytes	Voltage clamp	Glycine receptor $GABA_A$ and $GABA_C$ receptor	0,1µM – 1mM	Biphasic response on glycine receptor: LDC low concentrations (10µM) enhances, whereas >1mM inhibits glycine receptor. High dosages LDC inhibits $GABA_A$ (580µM); No effect $GABA_C$
Werde-Hausen 2012**[54]**	Primary rat astrocytes Xenopus oocytes	Tetrazolium hydroxide assay + Voltage clamp	LDC + metabolites: GlyT1 function	1µM – 1mM	LDC reduced glycine uptake only in high dosages (1mM) LDC metabolites reduces uptake in clinically relevant concentrations EC 50 55µM

LDC lidocaine, M1 muscarinic 1, M3 muscarinic 3, IC 50 half maximal inhibitory concentration, Gαq Gq-protein α-subunit. LPA lysophosphatidic acid, t time, TXA2 thromboxane A2, IC Ca intracellular concentration of calcium, hPMN human polymorphonuclear leucocytes, PAF platelet-activating factor, PLC phospholipase C, PKC protein kinase C, $GABA_A$ γ-aminobutyric acid A, $GABA_C$ γ-aminobutyric acid C, GlyT1 glycine transporter 1

Table 3: Animal studies concerning the efficacy of lidocaine in acute pain

Acute pain animal

Reference	Participants	Evaluate effect lidocaine on	Study-medication	Results
Woolf 1985**[65]**	10 Rats	Nociceptive processing primary afferents spinal cord	I: LDC 1, 2, 5, and10mg/kg II: tocainide 50 and 100mg/kg	Polysynaptic C-fiber evoked flexor response to noxious heat and chemicals are suppressed (prolonged action tocainide), without blocking conduction in Aβ, Aδ and C primary afferents.
Sotgiu 1991**[66]**	20 Rats Cross-over	Responsiveness WDR neurons	LDC B 3-4mg/kg, control in same rat	LDC reduces in WDR neurons noxious evoked activity, no reduced response to non-noxious stimuli and spontaneous activity
Abram 1994**[67]**	25 Rats I: n=8, II: n=7, III: n=5, IV: n=5	Formalin sc : dose- and timing effect of LDC	I: saline (II-IV =LDC) II: B 1,5mg + CRI 0,75mg/h, III and IV: B 3mg + CRI 1,5mg/h	Reduction of hyperalgesia if pretreatment with LDC, not with post-treatment LDC (IV)
Muth-Selbach 2009**[68]**	48 Rats 8 groups n=6	Formalin sc in rats receiving intrathecal strychnine, d-serine,, l-serine or saline	I: LDC B10mg/kg + CRI 5,4mg/kg/h, II: saline	Reduction of acute pain Modulating effect on glycine signaling and NMDA-receptor
Tsai 2013**[69]**	27 Dogs 3 groups n=9	Ovariohysterectomy	I: Meloxicam 0,2mg/kg II: LDC B 1mg/kg + CRI 1,5mg/kg/h III: Meloxicam + LDC	Similar analgesic effect till 12h postoperative, no advantage meloxicam + LDC. Little more sedative effect in LDC group
Columbano 2012**[70]**	24 dogs 4 groups n=6	Ovariectomy	I: Buprenorfine 0,02mg/kg, II: Fentanyl 4µg/kg + CRI 8µg/kg/h, III: Bupr + LDC 2mg/kg + CRI 3mg/kg/h, IV: Fent + LDC	Peroperative total anesthetic dose, autonomic responses, postoperative behavioral and pain scores were similar. III: minor reduction in peroperative sevoflurane use
Smith 2004**[71]**	12 Dogs 3 groups n=4	Intra-oculair surgery	I: LDC B 1mg/kg + CRI 1,5mg/kg/h, II: Saline III: Morfine B 0,15mg/kg + CRI 0,1mg/kg/h	LDC has a similar analgesic effect as morphine, no difference in intra-ocular pressure, aqueous flare, cell count (pilot)
MacDougall 2009**[72]**	6 Dogs Cross-over	Vital signs conscious dogs in 5 dosing regimens	LDC B 2mg/kg + CRI I: 0,6mg/kg/h; II: 1,5mg/kg/h III: 3mg/kg/h; IV: 4,5mg/kg/h V: 6mg/kg/h	No difference in nociceptive tresholds in all groups Respiratory rate decreased in II, IV, and V. Blood pressure increased after 4h in V. Sedation increased + occasional nausea with increasing dose
Ortega 2011**[73]**	41 Dogs I: n=20 II: n=21	Orthopedic or soft tissue surgery	I: LDC B 2mg/kg + CRI 3mg/kg/h II: Saline	LDC decreases intraoperative supplemental analgesics and sympathetic response to surgical stimuli
Torfs 2009**[74]**	126 Horses Retrospective	Small intestinal surgery	Prokinetic drugs: LDC, metoclopramide, erytromycin or combinations	Postoperative ileus is highly associated with death in horses. Significantly reduced risk of postoperative ileus in horses receiving prophylactic LDC
Malone 2006**[75]**	32 Horses I: n=17 II: n=15	Ileus	I: LDC B 1,3mg/kg + CRI 3mg/kg/h for 24h II: Saline	LDC reduces reflux and hospital stay,
Nannarone 2014**[76]**	36 Horses	Laparotomy for colic	I: LDC B 1,5mg/kg + CRI 3mg/kg/h II: LDC CRI 3mg/kg/h	No advantage of a loading dose of LDC prior to CRI on peroperative vital signs and quality of recovery

LDC lidocaine, WDR wide dynamic range, B bolus, CRI constant rate infusion, n number, sc subcutaneous, bupr buprenorfine, fent fentanyl

Table 4: Animal studies concerning the efficacy of lidocaine in chronic pain

Chronic pain animal				
Reference	**Particpants**	**Evaluate effect lidocaine on**	**Study-medication**	**Results**
Devor 1992 **[77]**	Rats	Ectopic neuroma and DRG discharg	LDC gradually increasing dosages: 0,125mg – 10mg	LDC suppresses ectopic discharge at nerve injury site and DRG cells. ED 50 for blocking DRG discharge = 0,37mg (1mg/kg); for neuroma = 1,9mg (6mg/kg)
Abdi 1998 **[78]**	36 Rats I-III: n=8 IV-V: n=6	Ligation L5 and L6 spinal nerves; measuring ectopic discharges and mechanical allodynia	I: Saline IP II: Amitriptyline 1,5mg/kg IP III: Gabapentin 50mg/kg IP IV: Saline IV V: LDC 10mg/kg for 10min	AMI, GBP, LDC increase mechanical treshold. LDC: quick onset and long lasting effect LDC reduces ectopic discharges more as AMI; GBP does not
Sotgiu 1994 **[79]**	15 Rats	Activity in ganglionic and dorsal horn neurons after ligation sciatic nerve	LDC B 4mg/kg	LDC reduces hyperactivity in dorsal horn > ganglionic neurons
Muth-Selbach 2009 **[68]**	48 Rats 8 groups n=6	Ligation sciatic nerve in rats receiving ITC strychnine, d-serine, l-serine or saline	I: LDC B10mg/kg + CRI 10,8mg/kg/h, II: saline	LDC reduces response to a thermal stimulus Modulating effect on glycine and NMDA-receptor
Abram 1994 **[67]**	6 Rats Cross-over	Ligation sciatic nerve	Study I: LDC B 0,6mg + CRI 0,3mg/h Study II: saline or LDC B 0,6mg + CRI 0,3mg/h or LDC B 0,06mg + CRI 0,03mg/h	LDC reduces hyperalgesia. Paw withdrawal normalizes 24h postinfusion of LDC Lower dose needed as for acute pain
Chaplan 1995 **[80]**	12 Rats I: n=6 II: n=6	Ligation L5 and L6: different dosing regimens and studies	I: Receiving LDC IV aimed at plasma conc 2-2,5µg/ml, max dose 15-30mg/kg/h; LDC ITC 0,5mg; LDC 1mg perineural II: Saline	LDC: no analgesic effect without constriction injury. ITC/perineural LDC no relief tactile allodynia. LDC IV induces a prolonged relief of tactile allodynia after 30min steady state (EC50 0,75mcg/ml), without affecting motor function.
Sinnot 1999 **[81]**	40 Rats I: n=8 II-V: n=7-10 VI: n=5 VII: n=5	Ligation L5 and L6 spinal nerves I: evolution allodynia II-V: dose-effect VI: no nerve ligation VII: control	II-VI: aimed at steady plasma concentrations varying from 1,1µg/L till 9,7µg/L VII: saline	LDC partially relieves allodynia, Treshold value 2µg/L and ceiling effect with wide variability

LDC lidocaine, B bolus, ITC intrathecal, CRI constant rate infusion, DRG dorsal root ganglion, n number, IV intravenous, IP intraperitoneal, EC 50 half maximal effective concentration, ami amitriptyline, GBP gabapentin

Table 5: Animal studies concerning the anti-inflammatory effects of lidocaine

Antinflammatory effects animal

Reference	Evaluate effect of IV lidocaine on	Participants	Study-medication	Results
Mikawa 1994**[82]**	E. Coli endotoxemia model	N = 27 rabbits	I: Saline II: E. Coli endotoxin III: II LDC 2B mg/kg + CRI 2mg/kg/h	LDC improves lung mechanics, reduces PMN count, albumin, C3a, C5a, TNFα, IL-1β, TXB2 in BALF
Nishina 1995**[83]**	E. Coli endotoxemia model	N = 32 rabbits	I: LDC B 2mg/kg + CRI 2mg/kg/h II: Saline	LDC improves slightly W/D ratio lung; no reduction chemotaxine (C3a, C5a, cytokine, AA metabolites) when administered after endotoxin
Taniguchi 2000**[84]**	E. Coli endotoxemia model	N = 32 rabbits	I: Endotoxin; II: Saline, no endotoxin; III: LDC B 3mg/kg + CRI 2mg/kg/h + no endotoxin IV: Endotoxine + LDC	LDC: more stable hemodynamics. IL-6 + IL-8 does not increase when administered immediately after endotoxin
Kiyonari 2000**[85]**	Acute lung injury model induced by PLA2 and trypsin	N = 21 rabbits	I: LDC B 2mg/kg + CRI 2mg/kg/hr; II: saline III: Non lung injury	LDC attenuates neutrophil and platelet count + improve lung mechanics; no effect complement
Gallos 2004**[86]**	Septic peritonitis: caecal ligation + puncture	N = 138 mice	I: LDC 5mg/kg/h 7days II: LDC 2,5mg/kg/h 7d III: Bupivacaine 0,5mg/kg/h 7d IV: Bupivacaine 1mg/kg/h 7d V: Saline	I and IV: Improved survival + reduced liver and kidney injury + reduction TNF-α, ICAM-1 and chemokine
Peiro et al 2010**[87]**	LPS induced endotoxemia	N = 12 horses	I: saline II: LDC B 1,3mg/kg + CRI 3mg/kg/h	LDC inhibits TNF-α + stable hemodynamics. No effect IL-6 or infiltration inflammatory cells abdominal cavity
Flondor 2010**[88]**	LPS induced endotoxenia model	N = 36 rats	I: Ae LDC 4mg/kg; II: Ae LDC 0,4mg/kg; III: Iv LDC 4mg/kg; IV: LPS or sham	I + III: reduces IL-1β + TNF-α in BAL, reduced nitrite. No influence plasma cytokine levels
Cook et al 2008**[89]**	Ischemic injury in jejenum	N = 24 horses	I: saline; II: flunixin meglumine II LDC B 1,3mg/kg + CRI 3mg/kg/h; IV: LDC+ flunixin	LDC improves mucosal barriere
Cook et al 2009**[90]**	Ischemic injury in jejenum	N = 24 horses	I: saline; II: flunixin meglumine II LDC B 1,3mg/kg + CRI 3mg/kg/h; IV: LDC + flunixin	Reduced plasma PG E2 and mucosal COX-2 expression in jejenum after ischemia. Reduced neutrophil migration caused by flunixin
Schmid et al 1996 **[91]**	Reperfusion injury in lung allografts	N = 13 dogs	I: LDC flush + CRI 4mg.kg.h II: placebo	I: gas exchange improved, PMN count reduced, PMN CD11b expression reduced. No effect wet/dry (W/D) ratio
Kaczmarek 2009**[92]**	Reperfusion after coronary artery ligation	N = 20 mice N = 20 mice N = 18 mice	I: saline II: LDC B1mg/kg + CRI 0,6mg/kg/h	LDC reduces infarct size by reducing apoptosis, size of neutrophil infiltration reduces, neutrophil density not, no effect leucocyt rolling/firm adhesion
Lee et al 1998**[93]**	Reperfusion after coronary artery ligation	N = 34 pigs	I: control, II: saline, III: LDC iv, IV: LDC local iv, V: arginine iv, VI: arginine local iv	Retrograde delivery in great cardiac vein of arginine and LDC reduces infarct size, Iv LDC does not

N number, E. Coli Escheria Coli, LDC lidocaine, B bolus, CRI constant rate infusion, PMN polymorphonuclear leucocytes, C3a complement 3a, C5a complement 5a, TNFα tumor necrosis factor α, IL-1β interleukine-1 β, TXB2 thromboxane B2, BALF brochoalveolar lavage fluid, W/D ratio wet/dry ratio, AA metabolites arachnidonic acid metabolites, IL-6 interleukin-6, IL-8 interleukin-8, PLA2 phospholipase A2, ICAM-1 intercellular adhesion molecule 1, LPS lipopolysaccharide, Ae aerosolized, BAL bronchoalveolar lavage, Iv intravenous, PG E2 prostaglandin E2, COX-2 cyclooxygenase 2

Table 6: Clinical studies concerning the efficacy of lidocaine in acute pain

Acute pain human

Reference	Study design	Patients	Evaluate effect lidocaine on	Study-medication	Results
McCarthy 2010**[96]**	Review of RCT	16 Trials 764 (LDC 395, control 369)	Abdominal surgery, ambulatory surgery, CABG, tonsillectomy, THP	LDC B 100mg or 1,5-2mg/kg + CRI 1-3mg/kg/h	LDC reduces pain scores and opioid consumption, LOS, improves bowel function in open + laparoscopic abdominal surgery. Reduces pain scores + opioid ambulatory surgery. No effect CABG, THP, tonsillectomy.
Vigneault 2011**[95]**	Meta-analysis	29 Trials 1754	Abdominal surgery, ambulatory surgery, cardiothoracic surgery tonsillectomy, THP	LDC B 100mg or 1,5-2mg/kg + CRI 1-3mg/kg/h	LDC reduces pain scores and opioid consumption, LOS, improves bowel function in abdominal surgery
Sun 2012**[94]**	Meta-analysis	21 trials 1108 (LDC 548, control 560)	15 trials: open abdominal surgery 6 trials: laparoscopic abdominal surgery	B LDC 100mg or 1,5-2mg/kg + CRI 1,5-2mg/kg/h or 2-3mg/min up to 1, 4, 24hr postoperative	LDC reduces pain scores, PONV, opioid consumption, duration of ileus. Reduced LOS after open surgery. Reduced IL-8 postop 18/21: no side effect of LDC; 3/21: mild side effects
Yang 2014**[98]**	RCT	N = 72 I: 22 II: 26 III: 24	Laparoscopic cholecystectomy	I: LDC intraperitoneal 3,5mg/kg; II: LDC B 1,5mg/kg + CRI 2mg/kg/h during surgery; III: placebo	I + II: reduced pain scores, opioid consumption. Improved patient satisfaction No side-effects reported
Tikuisis 2014**[97]**	RCT	N = 64 I: 32 II: 32	Laparoscopic colon surgery	I: LDC B 1,5mg/kg + CRI 2mg/kg/h intraop 24h postop II: bolus	I: lower pains scores 24h, reduced LOS, reduction time to first diet, and bowel movement
Yon 2014**[99]**	RCT	N = 36 I: 17 II: 19	Gastrectomy	I: LDC B 1,5mg/kg + CRI 2mg/kg/h during surgery II: Placebo	I: lower pain scores until 24h postop. opioid consumption and CRP on day 3. No difference in PONV, LOS
Wuethrich 2012**[100]**	RCT	N = 64 I: 32 II: 32	Laparoscopic renal surgery	I: LDC B 1,5mg/kg + CRI 2mg/kg/h intraop 1,3mg/kg/h 24 h postop II: Placebo	No difference in: pain scores, opioid consumption, PONV, LOS, return bowel function, CRP levels
Farag 2013**[101]**	RCT	N = 116 I: 57 II: 58	Complex spine surgery	I: LDC CRI 2mg/kg/h peroperative + PACU (max 8h) II: placebo	I: Reduced pain scores + opioid consumption. Lower 30 day complication rate + higher QOL-scores + functioning month 1&3. No difference PONV + LOS
Grigoras 2012**[102]**	RCT	N = 36 I: 17 II: 19	Oncologic breast surgery	I: LDC B 1,5mg/kg + CRI 1,5mg/kg/h till 1h postop II: Placebo	I: Reduced VAS score at 4hour postop. Significant reduction chronic pain and hyperalgesia at 3months follow up. I&II: similar opioid consumption and PONV
Terkawi 2014**[103]**	RCT	N = 71 I: 37 II: 34	Oncologic breast surgery	I: LDC B 1,5mg/kg + CRI 2mg/kg/h till 2h postop II: Placebo	No difference in: pain scores, opioid consumption, PONV, LOS, fatigue

LDC lidocaine, CABG coronary artery bypass grafting, THP total hip replacement, B bolus, CRI constant rate infusion, LOS length of stay, IL-8 interleukin 8, PONV postoperative nausea and vomiting, CRP C-reactive protein, PACU post anesthesia care unit , QOL-scores quality of life scores, intraop intraoperative, postop postoperative

Table 7: Clinical studies concerning the efficacy of lidocaine in chronic pain

Chronic pain human

Reference	Study design	Participants	Evaluate effect lidocaine on	Study-medication	Results
Challapalli 2005**[104]**	Cochrane review	16 trials LDC 12 trials mexelitine 1trial mex + LDC; 1trial tocainide	Chronic neuropathic pain: CRPS, MS, periph. neuropathy, plexopathy, central pain, amputation pain, fibromyalgia	LDC B 1-5mg/kg +/- CRI	LDC and mexilitine were superior to placebo. No difference in efficacy or adverse effects compared with carbamazepine, morfine, amantadine, gabapentin
Finnerup 2005**[105]**	RCCT	N = 24	Spinal cord injury, n=12 allodynia n=12 without allodynia	LDC 5mg/kg in 30min or saline	LDC reduces spontaneous pain in all patients and brush evoked dysesthesia. No reduction cold allodynia, pinprick hyperalgesia, pain evoked evoked by repetitive pinprick
Gormsen 2009**[106]**	RCCT	N = 13	Peripheral nerve injury: mechanical allodynia or pinprick hyperalgesia	I: NS1209 322mg IV; II: LDC 5mg/kg in 4h; III: Saline	LDC and NS1209 reduces brush evoked pain and cold allodynia, not spontaneous pain
Gottrup 2006**[107]**	RCCT	N = 20	Nerve injury with spontaneous pain, allodynia and pinprick hyperalgesia	LDC 5mg/kg in 30min or saline. Ketamine B 0,1mg/kg + 0,4mg/kg/h or saline.	LDC had minimal effect on spontaneous pain, reduced pain evoked by pinprick stimuli. No reduction brush evoked pain, cold allodynia
Tremont-Lukats 2006**[108]**	RCCT	N = 31	Neuropathic pain: CRPS n=23, polyneuropthy n=5, Radicular pain n=3 Plexopathy n=1	LDC 1, 3, 5 mg/kg in 6h or saline	LDC 5mg/kg/h reduced pain significantly and pain reduction persisted for 4h after stop infusion Mild adverse events
Viola 2006**[109]**	RCCT	N = 15	Painful diabetic neuropathy	LDC 5mg/kg in 4h, 7,5mg/kg or saline	LDC reduces pain for 14days up to 28days. LDC 7,5mg/kg gives a slightly better response
Park 2012**[110]**	RCCT	N = 18	Failed back surgery syndrome	LDC 1mg/kg/h 1h, 5mg/kg/h 1h or placebo	Reduction pain in all groups, no significant differences between groups
Thomas 2004**[111]**	Retrospec-tive study	768 evaluated 82 IV LDC 61 of them included	Intractable pain or opioid refractory pain associated with advanced cancer	LDC B 1-2mg/kg + CRI 1mg/kg/h	Pain reduction: 82% major response, 8% partial response, 10% no response. 78% neuropathic characteristics 52% were opioid refractory. No sign adverse effects, 30% experienced somnolence
Schwartzman 2009**[112]**	Retropec-tive study	N = 49	CRPS	LDC CRI 5 day with gradually increasing dose	76% pain reduction of 25% for 3 months. Effective in reducing thermal and mechanical allodynia. Reduced inflammatory components CRPS. No severe complications
Rosen 2009**[113]**	Retrospec-tive study	N = 68	Chronic daily headache	Not mentioned	Average NRS 7,9 pretreatment, after LDC NRS 3,9 lasting 8,5days Low incidence adverse effects
Mooney 2014**[114]**	Retrospec-tive study	N = 15 Adolescents	Refractory headache or neuropathic pain	LDC CRI 2,4-3,6 mg/kg/h	58 infusions: 80% had pain reduction, more pronounced response if NRS>6 and 3 infusions. 80% experienced minimal side effects; 20% moderate

LDC lidocaine, mex mexilitine, CRPS complex regional pain syndrome, MS multiple sclerosis, B bolus, CRI constant rate infusion, RCCT randomized controlled cross-over trial, N number, NS 1209, NRS numeric rating scale

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6

Behavior of neuropathic pain in mice following chronic constriction injury comparing silk and catgut ligatures.

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In revision.

Abstract

Introduction

Neuropathic pain is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system and is common after surgery. Neuropathic can persist without an obvious injury. In this study we aim to validate a murine chronic constriction injury model in mice as a model for neuropathic pain research and determine if silk or catgut ligatures induced most stable neuropathic pain behavior.

Methods

In this study mice underwent chronic constriction or sham surgery. Mice were tested on cutaneous hyperalgesia with the cumulative reaction time in the acetone test, on allodynia with the cumulative reaction time and number of lifts in the cold plate test and the maximal force before withdrawal in von Frey test.

Results

In the acetone test neuropathic pain was seen in CCI mice, but not in sham mice. Hyperalgesia was present postoperatively in CCI mice compared with preoperatively. In the cold plate test cumulative reaction time and number of lifts were higher in the ipsilateral hind paw than in the contralateral hind paw and sham mice. Postoperative measurements were higher than preoperatively. In the von Frey test the postoperative measurements were lower in the ipsilateral hind paw than preoperatively, while the contralateral hind paw showed an increase in maximal force before withdrawal. The contralateral hind paw showed more difference with sham mice than the ipsilateral hind paw. Silk ligatures showed more stable neuropathic pain behavior. In the acetone test, the cold plate test and the von Frey test the mice scored higher on neuropathic pain having silk ligatures, compared with catgut ligatures.

Conclusions

In this study we validated a murine CCI model for neuropathic pain behavior. In the murine CCI model it appears that silk ligatures demonstrate more stable neuropathic pain behaviors than catgut ligatures in de CCI model.

Introduction

Neuropathic pain is defined as pain arising as a consequence of a lesion or disease affecting the somatosensory system and is common after surgery.[1] Neuropathic pain presents as a constant, burning pain with spontaneous sharp exacerbations and worsening upon normal sensory triggers.[2] The grading system of neuropathic pain is based on certain criteria, explained by Treede.[1] The criteria consist of the distribution of pain coupled to the medical history and clinical investigation with supplemental sensory testing. Depending on the number of criteria that match with the patient, neuropathic pain is confirmed or excluded.[1] Sensory testing is an important diagnostic tool in determination of neuropathic pain and allodynia and hyperalgesia should be determined.[3]

Neuropathic pain is associated with poor physical and mental health and adversely affects quality of life.[4, 5] The prevalence of neuropathic pain in the human population ranges from 1% to 17.9%.[6] considering the above neuropathic pain adds to the burden of direct and indirect medical cost for our society [5], as there are direct medical costs, loss of the ability to work, loss of caregivers' ability to work and possibly greater need for institutionalization or other living assistance.[7]
The treatment of neuropathic pain mostly consists of oral analgesics such as tricyclic anti-depressants (TCAs) and anti-epileptic drugs (AEDs) [8], to decrease the symptoms of neuropathic pain. However, the therapeutic response on the pharmacological treatment of neuropathic pain is rather poor, as few patients receive efficacious dosages of medication.[2, 7]

Because the mechanisms of neuropathic pain are insufficiently understood, [9, 10] it seems pivotal to investigate the course and cause of neuropathic pain and development of treatment and perhaps prevention strategies. Therefore ideally we want to study a neuropathic pain animal model extrapolatable to the clinical situation. Often a chronic constrictive injury model is used in rats to study neuropathic pain which include thermal and mechanical allodynia testing.[11, 12] In this study we aim to validate a chronic constriction model in mice. A murine model can lead to a better understanding of the course of neuropathic pain, and will lead to an improvement of accuracy and variability of the chronic constriction model, because of the possibility to use transgenic mice.[12]

The material that is used for ligatures can have an effect on the outcome of the observed sensory abnormalities.[13] In chronic constriction injury, either catgut or silk ligatures are used. In rats, catgut is commonly used as ligature material [14]. Catgut leads to a development of an inflammatory reaction and consequentially a loss of most A-fibres and some C-fibers, but few cell bodies.[15, 16]

In mice, however, the preferable ligature material is not known. In this research both silk and catgut ligatures were compared, to check for efficacy in inducing neuropathic pain behavior.

In this study we aim to validate a model of neuropathic pain in mice and investigate whether silk or catgut ligature material is more effective in inducing neuropathic pain.

Material and methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and The National Institutes of Health.

Study Population

All studies were performed in C57BL/6J male mice (Charles River). Mice were aged 6 weeks upon arrival were first acclimatized. Mice were housed in a light and temperature controlled room under specific pathogen free (SPF) conditions. Standard pelleted chow (1.00 % Ca; 0.22 % Mg; 0.24 % Na; 0.70 % P; 1.02 % K; SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*.

Experimental design

This experiment was used to validate the chronic constriction injury (CCI) model in mice (n=45) to induce neuropathic pain. Thereby it will be checked if the preferable ligature material used in rats, which is catgut [14], differs from the preferable ligature material used in mice. In the experiment postoperative testing was done in both sham-group (n=5) and CCI-group (n=40), with either catgut (n=20) or silk (n=20) ligatures.

Surgical procedure

Both sham and CCI-mice were being operated. Before surgery, the mice got rimadyl subcutaneously according to their weight (0.1 ml rimadyl per 10 gram). The mice were anesthetized using isofluran inhalation (1-4%). Under a dissecting microscope, the left common sciatic nerve was exposed at the level of the mid-thigh by dissecting through the biceps femoris. In contrary to the sham-mice, in which no ligatures were placed, in de CCI-mice, proximal to the nerve trifurcation (while taking care to preserve epineural circulation), three ligatures (either silk 6.0 or catgut 6.0) were loosely tied around the sciatic nerve, at about 1 mm spacing, until they elicited a brief twitch in the related hind paw. The muscle layer was then stitched and the incision in the shaved skin layer was closed using clips. The sham-operated animals were used as controls and had only sciatic exposure without

ligation. Also after surgery the mice got rimadyl subcutaneously according to their weight on day 1 and day 2 once a day. On day 10, the clips were removed. After the experiment, when the mice were euthanized, the nerve histology was studied by removing connective tissue and ligatures.

General well-being

The first week after surgery, animals will be weighted daily. When the animal lost too much weight (>30% directly after surgery or 20% not directly after surgery, starting weight mean of approximately 24 grams) or did not recover within 1 week the humane endpoint had been reached and the animal will be excluded from the experiment and consequently postoperative pain testing. They were also tested on activity, state of the surgical wound and eventual damage on the left feet or toes (by autotomy).

Postoperative testing

Responses to thermal and mechanical stimuli were tested in all mice. Sham mice were tested before surgery (baseline) and 3, 7, 10, 14 and 21 days after surgery. CCI-mice were tested before surgery (baseline) and 3, 7, 10, 14, 21 and also 28 days after surgery

Thermal and chemical hyperalgesia were tested using the acetone spray test. After habituation for at least 15 minutes in plexiglass cubicles with a wire mesh metal floor, the plantar area of the left hind paw was exposed to acetone. For one minute the mouse was scored on lifting up the paw, scratching to the paw and touching the left hip or paw. The duration of the reaction was measured and analysed as cumulative reaction time.[17]

Thermal allodynia was measured using the Cold Plate test. The mice were exposed to a temperature of 2-2.5ºC to regain the best response. Measurements were performed on both the ipsilateral and contralateral hind paws. Mice were scored for 5 minutes on scratching with a paw, lifting up the paw, lifting up the paw shortly in the same place and licking on the toes. The amount of lifting of the hind paw was measured and analysed as number of lifts. Also the duration of reaction was measured, analysed as cumulative reaction time.[11, 18] However, the cold plate test became defective, so in some groups the number of mice with catgut ligatures is lower.

Mechanical allodynia was measured using the Von Frey test, preoperatively and on day 4, 7, 11, 14, 18, 21 and 27. Mechanical allodynia is induced by application of pressure to the skin.[19] The mice were placed in a test cage with a wire mesh metal floor and the rigid tip of a von Frey filament (punctate stimulus) was applied to the skin of the midplantar area of the hind paw until it bends.

Different filaments, ranging from 0.145 to 5.1 gram (table 1), made of nylon, were used that exerted an increasing force, starting below the threshold of detection (hair number 7 or 8; 0.145-0.320 gram) and increasing until the animal removed its paw. Withdrawal threshold of ipsilateral and contralateral paws was measured 3-5 times and the maximal force before withdrawal was the mean of the evaluations.[20]

Table 1.

Number of the hair	Weight of the hair (g)
4	0.03
6	0.09
7	0.15
8	0.32
9	0.39
10	1.1
11	1.7
12	3.3
13	5.1
14	8.3

Table 1. Number of the von Frey hair with the corresponding weight in grams.

Statistical Analysis

Results are presented as mean values ± S.E. All statistical analyses were performed with IBM SPSS Statistics 20 (SPSS, Chicago, IL). Because of some missing data in the CCI group statistical analysis of post- and pre-measurements in the acetone test, cold plate test and the von Frey test were done using linear mixed models. The dynamic mechanical allodynia is shown in percentage of mice that responded to the different 3 hairs. For the analysis of ligature material and the differences between CCI and sham mice an ANOVA-test is performed. A p-value of 0.05 is considered statistically significant. To determine the experimental group size a calculation on data is performed based on previously published information [21], using the following formula: $n = 1 + 2C(s/d)2$ [22] to compute sample size for continuous variables where s is an estimation of the standard deviation of the variable, d is the magnitude of the difference we wish to be detected, and C is a constant dependent on the value of alpha and beta selected. $C = 10.5$ for $\alpha = 0.5$ and $1-\beta = 0.9$, then sample size is $n = 1 + 21 \times (5/10)2 = 6.25$. This analysis showed that to detect differences of 10% with a power of 90% and

statistical significance at the $p < 0.05$ level, 7 mice per group are needed. Since we expect that 10–30% of animals that will undergo chronic construction injury will not have successful neuropathic symptoms 10 animals per group will be needed to produce statistically valid results.

Results

General well-being

There were no complications after surgery and no animals had to be excluded from the study. One mouse got a staphylococcus infection at the end of the experiment, which was treated. The fur of all the mice was clean, shining and well groomed.

Directly after surgery, CCI-mice showed a characteristic posture of the left hind paw, with a curve downward and decreased musculature and thereby made abnormal movements with the left hind paw. After approximately one week, the enlarged movements were still seen and the left hind paw was still curved downwards, but the other abnormal movements mostly disappeared. Some mice showed mild signs of autotomy, as they gnaw or bite their paws or toes, which could indicate that the ligation of the sciatic nerve was too tight. There were occasional signs of stress present, but no abnormal aggression amongst the mice was seen. The activity of the mice was generally normal. Both sham as CCI-mice gained weight during the experiment. After the experiment it was confirmed that constrictions were still present.

Acetone test

To test thermal hyperalgesia the acetone test was performed in sham and CCI-mice (Figure 1). According to the acetone test an increase in cumulative reaction time in sham mice was seen. This increase was significant postoperatively compared with preoperatively on day 18 and 21. In CCI mice a significant increase in cumulative reaction was seen preoperatively compared with day 7, in silk ligatured mice more than in catgut ligatured mice. From day 7 the cumulative reaction time decreased in the silk group, but was still higher than preoperatively, which indicates neuropathic pain following CCI in mice according to the acetone test. In the catgut ligatured mice the cumulative reaction time increased until day 14 but then showed a decline on day 18. All postoperative measurements in catgut ligatured group were significantly higher than preoperatively. However, there was never a significant difference between sham mice and CCI mice.

Cold plate test

With the cold plate test cumulative reaction time as well as number of lifts are measured in left and right hind paw (Figure 2). Sham mice and the contralateral hind paw showed an equal cumulative reaction time, while cumulative reaction time increased in CCI mice, in silk ligatured mice more than catgut ligatured mice. The cumulative reaction time in the silk ligatured mice was still increasing on day 21, while catgut ligatured mice showed a decrease in cumulative reaction time on day 14. A difference in sham mice and the right hind paw is shown on day 14 ($p=0.007$) and day 18 ($p=0.037$). In comparison the ipsilateral hind paw and the sham mice were different on day 7, day 10, day 18 and day 21.

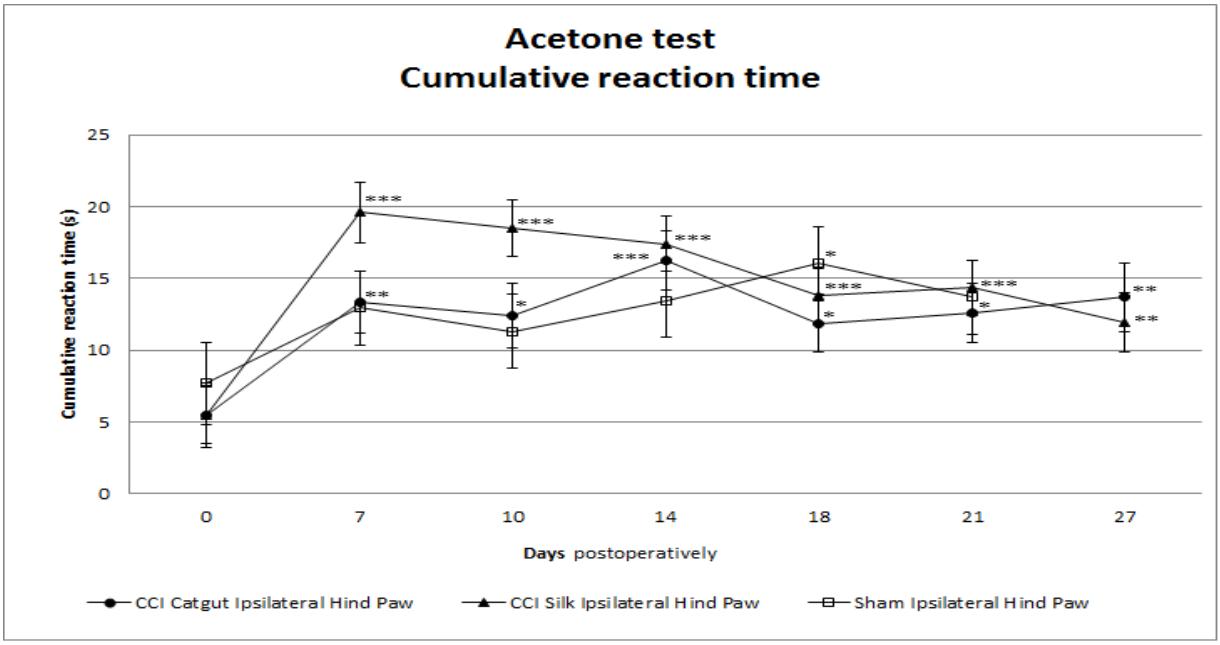

Figure 1. Thermal and chemical hyperalgesia (cumulative reaction time ±SE) measured in CCI mice and sham mice with acetone test preoperatively and postoperatively. Statistics were done with linear mixed models in which: * $p<0.05$ as compared with preoperative measurement, ** $p<0.01$ as compared with preoperative measurement, *** $p<0.001$ as compared with preoperative measurement. Measurements on day 3 and day 33 were left out, because of missing data. A significant increase was seen in both CCI mice, silk and catgut ligatures, from day 0 until day 7. In silk a significant decrease is seen after day 7.

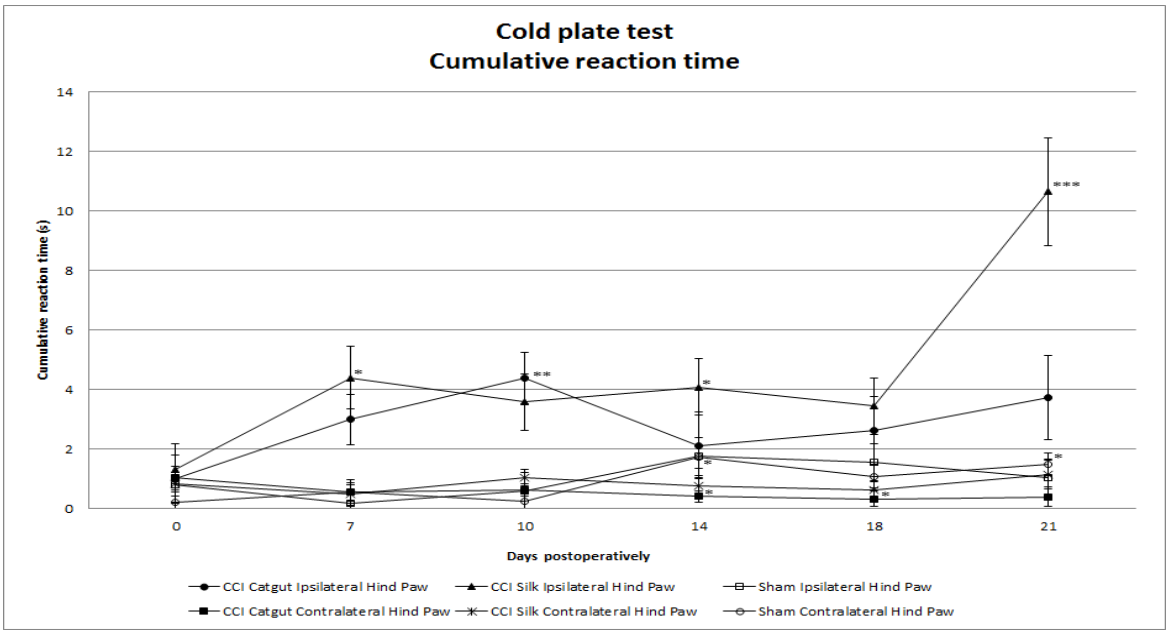

Figure 2. Thermal allodynia measured in CCI mice and sham mice with the cold plate test in cumulative reaction time ±SE preoperatively and postoperatively in both ipsilateral (left) and contralateral (right) hind paw. Statistics were done with linear mixed models in which: * p<0.05 as compared with preoperative measurement, ** p<0.01 as compared with preoperative measurement, *** p<0.001 as compared with preoperative measurement. In CCI-mice with silk ligatures a significant increase is seen from preoperatively to day 10. From day 10 until day 14 a decrease in cumulative reaction time was seen, though this difference was not significant. In CCI-mice with silk ligatures a significant increase of cumulative reaction time was seen on day 7, day 14 and day 21 compared with the preoperative cumulative reaction time.

Thermal allodynia was also tested by the cold plate test considering the number of lifts (Figure 3). Sham mice and right hind paw remained constant over time. Sham mice even showed a significant decrease in number of lifts of day 7 and 10. The number of lifts in CCI mice significantly increased. The catgut ligatured CCI mice showed an increase in number of lifts, until a decrease in number of lifts was seen on day 14, on which the postoperative measurement was not significantly different to the preoperative measurement. In silk ligatured mice a constant increase in number of lifts was seen and the number of lifts was still increasing on day 21. Neuropathy in mice according to cumulative reaction time as well as number of lifts significantly increased from day 7 in the left hind paw of CCI mice and decreased from day 7 in the right hind paw of CCI mice. The number of lifts of sham mice and the contralateral hind paw differed from the ipsilateral hind paw in all postoperative measurements, but not in the preoperative measurement, which indicates neuropathic pain following the cold plate test.

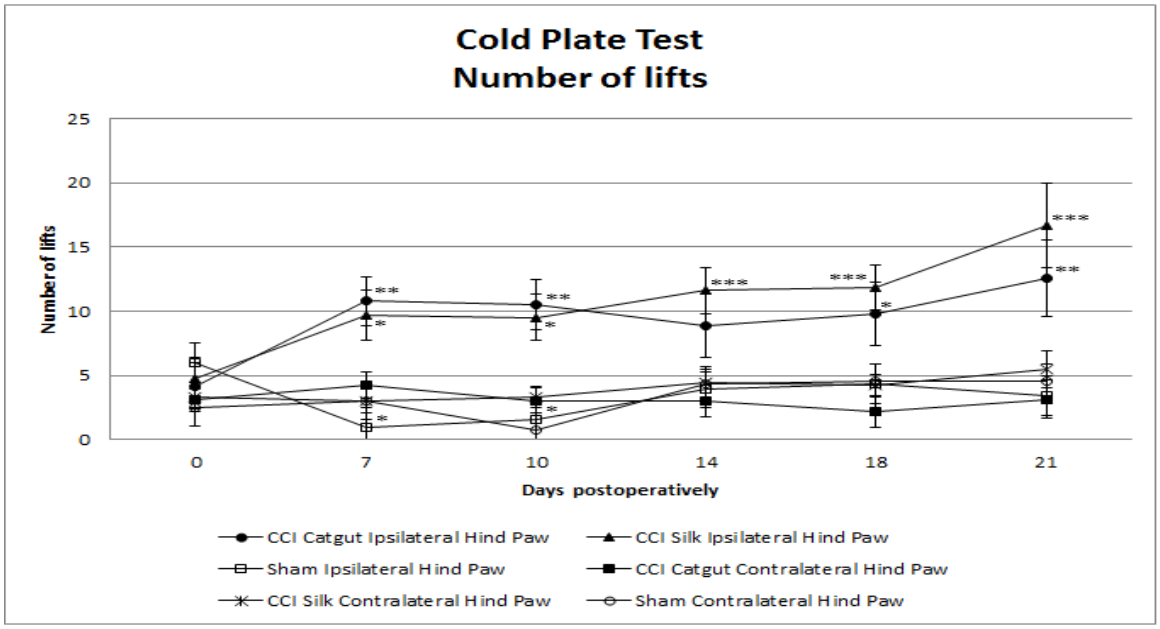

Figure 3. Thermal allodynia measured in CCI mice and sham mice with cold plate test in number of lifts ±SE preoperatively and postoperatively in both ipsilateral (left) and contralateral (right) hind paw. Statistics were done with linear mixed models in which: * p<0.05 as compared with preoperative measurement, ** p<0.01 as compared with preoperative measurement, *** p<0.001 as compared with preoperative measurement. A significant increase was seen from preoperatively to day 7 in both silk and catgut ligatured mice considering the left hind paw. In CCI mice with silk ligatures also a significant increase was seen from day 18 to day 21. In the CCI mice considering the right hind paw and the sham-mice the number of lifts remained constant.

Von Frey test

Mechanical allodynia was tested in the von Frey test (Figure 4). Sham mice showed overall a low force before withdrawal, but on day 11 sham mice showed a peak in maximal force before withdrawal. The right hind paw showed an increase in maximal force before withdrawal. The left hind paw showed a significant decrease in maximal force before withdrawal, in silk ligatured mice more than in catgut ligatured mice. In catgut ligatured mice an increase in maximal force before withdrawal was seen until day 11. On day 14 a drop was seen, and after that a small increase was seen. Postoperatively there were no significant differences compared with preoperatively. In the silk ligatured mice a significant decrease in maximal force before withdrawal was seen on day 7. From day 7 the maximal force before withdrawal remained constant. The maximal force before withdrawal was significantly different in the contralateral hind paw than the sham mice. Between the ipsilateral hind paw and the sham mice only differences were seen preoperatively, on day 7 and day 10.

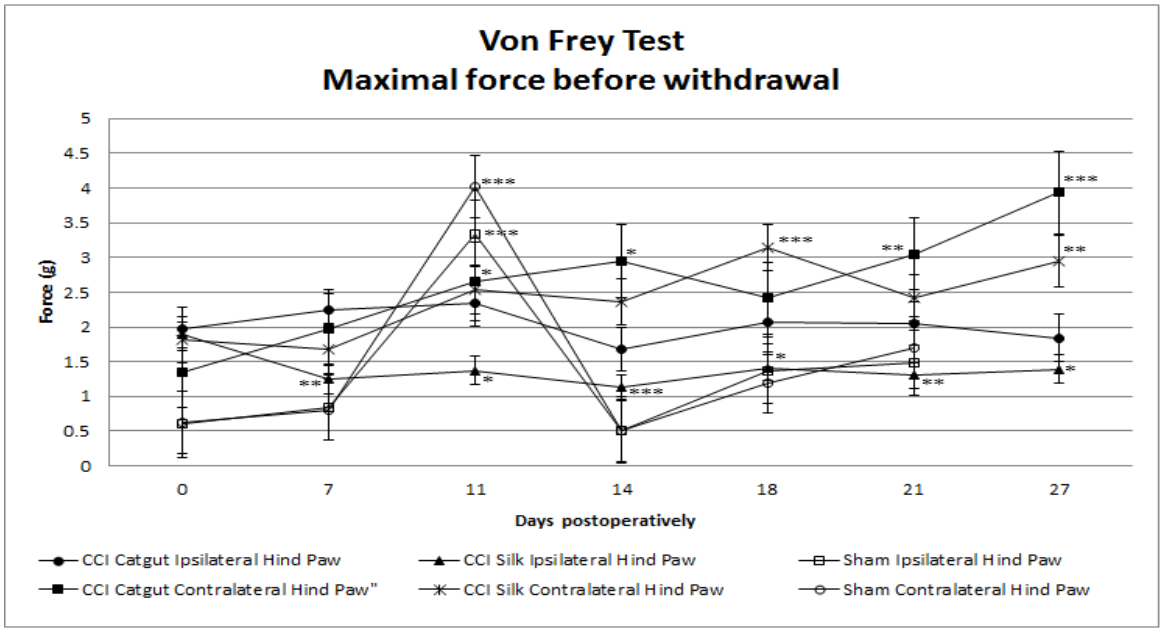

Figure 4. Mechanical allodynia measured in CCI mice and sham mice with the Von Frey test in maximum force before withdrawal ±SE preoperatively and postoperatively in both ipsilateral (left) and contralateral (right) hind paw. Statistics were done with linear mixed models in which: * p<0.05 as compared with preoperative measurement, ** p<0.01 as compared with preoperative measurement, *** p<0.001 as compared with preoperative measurement. Considering the right hind paw the CCI mice, both catgut and silk ligatured, showed a significant increase in the maximal force before withdrawal. Considering the left hind paw no significant difference was found in the catgut ligatured mice. In the silk ligatured mice a significant decrease was found in all postoperative measurements compared with the preoperative measurements.

Discussion

In this study we demonstrated that a CCI model in mice can induce neuropathic pain behaviors comparable to neuropathic pain signs and symptoms in humans. Chronic constriction injury in mice seems to present significant quantitative changes proportional to external stimulation in thermal and chemical hyperalgesia, thermal allodynia and mechanical allodynia. Neuropathy was developed from day 7 postoperatively and in most animals neuropathy was still observed until day 21-27 days postoperatively.

We tested two ligature materials, silk and catgut. Silk seems to be preferable compared with catgut as ligature material in mice.

Neuropathic pain behavior

After surgery abnormal movements were seen in all groups, which disappeared mostly after one week. No mice had to be excluded from the experiment due to extensive weight loss, disease or autotomy. In some mice a slight reddening of the plantar surface of the toes was seen, these mice were not excluded from the experiment. It could however indicate that the ligature of the sciatic

nerve was too tight. According to cumulative reaction time, measured with the acetone test, thermal and chemical hyperalgesia were present from day 7 in CCI mice with both silk and catgut ligatures. Sham mice were barely responsive to acetone application, but CCI mice showed associated aversive behavior as licking of the affected paw, limping with the left paw and enlarged movements. The acetone test was only performed in the ipsilateral hind paw, so no comparison with the contralateral hind paw could be made. Also according to both cumulative reaction time and number of lifts, measured with the cold plate test, cold allodynia was present in CCI mice with silk and catgut ligatures from day 7, compared with the contralateral hind paw and the sham mice. And also the von Frey test showed neuropathy in CCI mice with silk catgut ligatures from day 7.
The CCI-model has its limitations. For example, to obtain validated results, environmental factors should be eliminated. Concerning the acetone test the duration of exposure to cold is dependent on the spread and the evaporation of acetone, because of the ambient temperature and the body temperature of the mouse.[23, 24] Furthermore the landing of the acetone of the plantar surface of the left hind paw is technique-dependant, and causes differences in mice.[17] Also the liquid itself may elicit a chemical, olfactory or mechanical stimulus that may, independent of the temperature, elicit a flexion reflex.[17, 23, 24] Concerning the cold plate testing, not all tests could be performed due to technical difficulties, especially in the catgut-ligatured mice. Concerning the von Frey testing, the bending forces applied by Von Frey filaments are significantly influenced by ambient humidity and slightly by temperature. Also washing and drying can significantly affect the bending forces.[25] It is important the experimenter waits for the animal to hold its paw in the right position as weight bearing of the limb might be a confounding factor in determining von Frey withdrawal thresholds. Therefore also the increased weight of the CCI mice during the experiment could be a determent factor in the von Frey test.[26] In further research it might be useful to use an electronic von Frey meter, because of the difference in increase of the forces.

Silk versus catgut

In the acetone test, the cold plate test and the von Frey test a trend toward more neuropathic behavior was shown in mice with silk ligatures compared with catgut ligatures. We suspect that these results are not significant because fewer mice with catgut ligatures were measured than initially powered due to technical difficulties. Robinson et al. showed catgut ligation caused cold allodynia, chemical hyperalgesia and mechanical hyperalgesia for at least 56 days post-surgery following partial sciatic nerve ligation (PNL) in rat. Silk ligatures caused the same deficits, but several of these deficits diminished over time 21-28 post surgery. In contrary to the rat model, where catgut is mostly used presumably because it induces an inflammatory response, in mice, catgut does not seem to be as effective. Perhaps catgut in mice does not induce an inflammatory response where silk ligature

material does.[13] However this research just measured neuropathic pain for 27 days, sham even 21 days, so the long term effect of ligature material on neuropathy is not studied and inflammatory parameters have not been studied. More research is therefore needed to prove our hypothesis that silk is more effective than catgut in mice in a CCI model.

In conclusion this study demonstrated that a murine chronic constriction injury model to study neuropathic pain behavior can be a valuable model for testing of neuropathic pain and observational studies. Because mice are genetically modifiable, chronic constriction injury research in mice could create important opportunities in for example the role of inflammatory receptors or channel pathology compared with other animal models for the further discovering and testing of the mechanisms of neuropathic pain and possible new treatment targets.

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7

Intraperitoneally administered lidocaine attenuates thermal allodynia in a murine second hit chronic constriction injury (CCI) model.

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Submitted.

Abstract

Introduction

Neuropathic pain is defined as pain arising after nerve injury and is common after surgery.

Methods

Mice underwent mechanical ventilation (MV) and were allocated to receive sham (MV-sham) or chronic constriction injury (MV-CCI) surgery. Postoperative systemic cytokines were determined on day 0 and 16 and sensory testing was performed on day 0, 3, 7 and 16 by cold plate test (number of lifts (NOL) and cumulative reaction time (CRT)) and von Frey test.

Results

MV-Sham showed an increase in interleukin (IL)-1β and tumor necrosis factor (TNF)-α compared with MV, MV-CCI lido in keratinocyte derived chemokine (KC) compared with MV. MV-CCI showed a difference between the left and right paw on day 7, MV-CCI lido on day 7 and 16. The NOL on the left paw was lower in MV-sham compared with MV-CCI and lower in the MV-CCI lido compared with MV-CCI mice on day 16.

The left and right hind paw were different in CCI group on day 3 and 7. In MV-CCI lido the left and hind paw were different on day 7. The CRT was higher in MV-CCI mice than MV-sham mice on day 16 and in MV-CCI mice than in MV-CCI lido mice. The left hind paw scored lower on maximal force before withdrawal on day 16 in the CCI lido group than the right hind paw.

Conclusions

We demonstrated that nerve injury and not systemic inflammatory response seems mandatory for development of neuropathic pain in a 'second hit' model. Lidocaine attenuates cold allodynia in mice.

Introduction

The response to surgical injury is a combination of complex physiological and behavioral changes with an important role for the local and systemic immune system.[1] The severity of the injury seems to correlate with the inflammatory response [1, 2] involving Interleukin- (IL) 6, TNFα, IL-1 and IL-8. [2] After injury the host defense is aimed at restoring homeostasis, and the release of anti-inflammatory and pro-inflammatory cytokines seems equally important.[3] Interestingly, dysregulation of the inflammatory response can play a role in pain after surgery and the development of chronic pain.[4-8] Mechanical ventilation without surgery can result in an inflammatory response too, characterized by release of inflammatory cytokines and influx of immune cells such as neutrophils.[9-12] Moderate to severe pain after surgery can lead to chronic pain and a reduction in the quality of life in the immediate postoperative period [13], which is an important reason for delayed discharge.[14]

Neuropathic pain is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system and is common after surgery. [15] Nerve damage is characterized by an increased sodium channel expression, ectopic electrical activity and altered neuroplasticity.[16] In animal models, the inflammatory response is linked with the development neuropathic pain. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 induce acute or short-term and chronic hyperalgesia and allodynia.[17] Neuropathic pain presents as a constant, burning pain with spontaneous sharp exacerbations and worsening upon normal sensory triggers. [18] Sensory testing is an important diagnostic tool in determining neuropathic pain, which can objectivity allodynia and hyperalgesia.[19]

Lidocaine is an amide local anesthetic and aspecific sodium channel blocker used for treatment of acute and chronic pain. Low dose systemic lidocaine has anti-hyperalgesic and anti-inflammatory properties.[7, 20] Lidocaine attenuates activated endothelial interleukin (IL)-1, 6 and 8 concentrations and ICAM-1 expression *in vitro* [21, 22] and reduces levels of tumor necrosis factor (TNF)-α, IL-1β IL-6 and Il-8 in animal endotoxemia models.[23-25] Lung mechanics are improved in acute lung injury in rabbits by lidocaine.[26, 27] The antihyperalgesic effect of lidocaine in neuropathic pain syndromes results from a decrease in ectopic discharges in injured neurons and its selective inhibition of hyperexcitable peripheral and central neurons to noxious stimuli.[28-30]

Since mechanisms of neuropathic pain after surgery are insufficiently understood, [31, 32] investigating the course and cause of neuropathic pain is pivotal for subsequent development of treatment and prevention strategies. In this study we investigate the postoperative inflammatory

response after chronic constriction injury (CCI) versus sham, and we determine mechanical and thermal allodynia after MV and CCI versus sham in a murine model.
We study the effect of intraperitoneally administered lidocaine on the postoperative inflammatory response and the development of thermal and mechanical allodynia.
We additionally used MV to enhance extrapolation to the clinical 'second hit' situation in which patients are being ventilated and undergo surgery.

Material and methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed according tothe guidelines of the Dutch Council for Animal Care and The National Institutes of Health.

Study Population

Studies were performed in C57BL/6J male mice (n=64) (Charles River). Mice were acclimatized to their environment and were aged between 8-12 weeks for the start of the experiment. Mice were housed in a light and temperature controlled room under specific pathogen free (SPF) conditions. Standard pelleted chow (1.00 % Ca; 0.22 % Mg; 0.24 % Na; 0.70 % P; 1.02 % K; SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*.

Experimental design

This experiment consists of two parts. First we investigated the inflammatory systemic response after surgery, comparing sham and CCI with or without systemic low dose lidocaine (bolus 1,5 mg/kg and 2 mg/kg/hour (figure 1).

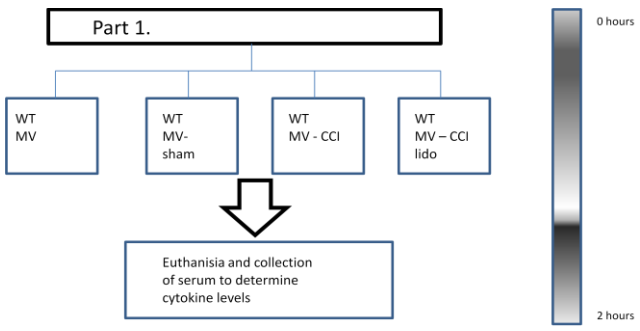

Figure 1. First part of experiment, lasting 2,5 hours. WT=wildtype, MV=Mechanical ventilation, CCI=chronic constriction injury.

Secondly, we investigated the effect of low dose lidocaine on the development of neuropathic pain with postoperative sensory testing (see paragraph 'postoperative testing') done in both sham-group and CCI-group (n=), with silk ligatures, during 16 days (figure 2). All mice were euthanized by exsanguination and cytokine levels were determined.

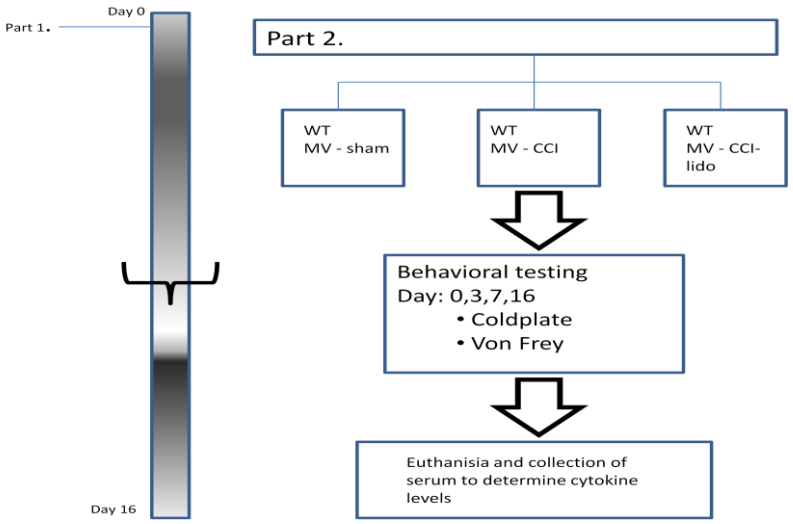

Figure 2. Second part of experiment lasting 16 days. WT=wildtype, MV=Mechanical ventilation, CCI=chronic constriction injury.

Mechanical ventilation and surgical procedure

Both sham and CCI-mice were operated. Before surgery, the mice received carprofen subcutaneously according to their weight (0.1 ml carprofen per 10 gram). The mice were anesthetized using isofluran inhalation (1-4%). After oral intubation with a 20 gauche catheter, the mice were mechanically ventilated (*MiniVent®*, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). The ventilation settings used were based on measured tidal volume and respiratory rate during spontaneous ventilation in C57Bl/6 mice [27]: a tidal volume of 8 ml/kg body weight and a frequency of 150/min. All animals received 4 cm H_2O positive end-expiratory pressure (PEEP), and fraction of inspired oxygen was set to 0.4. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C using a heating pad. Under a dissecting microscope, the left common sciatic nerve was exposed at the level of the mid-thigh by dissecting through the biceps femoris. In the CCI-mice three ligatures (silk 6.0) were loosely tied around the sciatic nerve proximal to the nerve trifurcation (while taking care to preserve epineural circulation), at about 1 mm spacing, until they elicited a brief twitch in the related hind paw. The muscle layer was then stitched and the incision in the shaved skin layer was closed using clips. Sham-mice had only sciatic exposure without ligation

and were used as controls. After surgery the mice received carprofen subcutaneously once a day according to their weight on day 1 and day 2. On day 10, the clips were removed. Lipopolysaccharide (LPS) was measured in the mechanical ventilation circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD, USA; detection limit: 0.06 IU/ml) to rule out contamination and LPS-induced pulmonary inflammation.

General well-being

In the second part of the experiment in the first week after surgery, animals were weighted daily. When the animal lost too much weight (>30% directly after surgery or 20% in the days after surgery, starting weight mean of approximately 24 grams) or did not recover within 1 week the humane endpoint had been reached and the animal was to be excluded from the experiment and consequently postoperative pain testing. They were also tested on activity, state of the surgical wound and eventual damage on the left feet or toes (by autotomy).

Cytokine analysis

A simultaneous Luminex® assay was used to determine plasma cytokine levels of TNF-α, IL-6, IL-10, KC and IL-1β (Milliplex, Millipore, Billerica, MA).

TNF-α, IL-6 and KC (murine equivalent of human IL-8) in lung homogenate were determined by enzyme-linked-immunosorbent assay (ELISA) (for IL-6 and IL10; CytoSet, BioSource, CA; for TNF-α and KC; ELISA-Kit, R&D Systems, Minneapolis, MN). Lower detection limits: IL-1β 40 pg/ml; TNF-α: 32 pg/ml; IL-6: 160 pg/ml; IL-10: 16 pg/ml and KC: 160 pg/ml.

Postoperative testing

Responses to thermal and mechanical stimuli were tested in all mice before (baseline) and 3, 7 and 16 days after surgery.

Thermal allodynia was measured using the Cold Plate test. The mice were exposed to a temperature of 2-2.5ºC to regain the best response. Measurements were performed on both the ipsilateral and contralateral hind paws. Mice were scored for 5 minutes on scratching with a paw, lifting up the paw, jumping, lifting up the paw in the same place and licking on the toes. The amount of lifting of the hind paw was measured and analysed as number of lifts. Also the amount of jumps was counted and the duration of reaction was measured and analysed as cumulative reaction time. [33, 34]

Mechanical allodynia was measured using the Von Frey test, before surgery, on day 3, 7 and 16. Mechanical allodynia was induced by application of pressure of the skin (table 1). [35] Mice were placed in a test cage with a wire mesh metal floor and the rigid tip of a von Frey filament (punctate stimulus) was applied to the skin of the midplantar area of the hind paw until it bended. Different filaments, ranging from 0.145 to 5.1 gram (table 1), made of nylon, were used that exerted an increasing force, starting below the threshold of detection (hair number 7 or 8; 0.145-0.320 gram) and increasing until the animal removed its paw. Withdrawal threshold of ipsilateral and contralateral paws was measured 3-5 times and the maximal force before withdrawal was the mean of the evaluations. [36]

Number of the hair	Weight of the hair (g)
4	0.03
6	0.09
7	0.15
8	0.32
9	0.39
10	1.1
11	1.7
12	3.3
13	5.1
14	8.3

Table 1. The number of the hair used in the von Frey test with corresponding weight of the hair in grams (g).

Statistical Analysis

For both parts of the experiment separate power analyses were performed.

First part: We performed a sample size calculation based on previous investigations considering a difference of 40% in cytokine levels between ventilated and control mice with a type 1 error of 5% (α=0.05) and a power of 80% (β=0.2).[12, 37, 38] This resulted in a group size of 8 animals per group. Shapiro-Wilk tests showed that data were not normally or log normally distributed. Data are therefore expressed as median with interquartile range (IQR) and depicted as column bar graphs. Differences between control *versus* lidocaine and between ventilated groups were studied using Mann Whitney tests. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). P-values < 0.05 were considered significant.

Second part: The experimental group size calculation for the behavioral test is performed based on previously published information [39], using the following formula: n = 1 + 2C(s/d)2 [40] to compute sample size for continuous variables where s is an estimation of the standard deviation of the variable, d is the magnitude of the difference we wish to be detected, and C is a constant dependent on the value of alpha and beta selected. C = 10.5 for α = 0.5 and 1-β = 0.9, then sample size is n = 1 + 21 x (5/10)2 = 6.25. This analysis showed that to detect differences of 10% with a power of 90% and statistical significance at the $p < 0.05$ level, 7 mice per group are needed. Therefore all groups originally started with n = 8. Results of the behavioral tests are presented as mean values ± S.E. All statistical analyses were performed with Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). Pre-operative measurements were compared with post-operative measurements using Mann Whitney tests. Comparisons between left and right hind paw were made by a paired samples T-test. A p-value of < 0.05 is considered statistically significant.

Results

Cytokine analysis

First part experiment:

MV-Sham showed an increase in IL-1β compared with MV. MV-CCI lido showed an increase in KC compared with MV. MV-Sham showed an increase in TNF-α compared with MV. No differences were observed in IL-6 and IL-10 between the different groups after 2 hours of MV (figure3.)

Second part experiment:

No differences in cytokine levels were observed betweenthe different groups after 16 days (figure 4.).

Cold plate test – number of lifts

The number of lifts was determined in the cold plate test. Differences between left (CCI) and right were determined. The differences between the groups were also investigated.

left versus right paw

We tested the number of lifts in the left (CCI) as well as right hind paw.

We observed no differences between the left and right hind paw on day 0, 3, 7 and 16 in the sham group. In the CCI group a difference was found between the left and right paw on day 7. In the CCI lido group a difference was found between the left and right paw on day 7 and 16 (figure 5.).

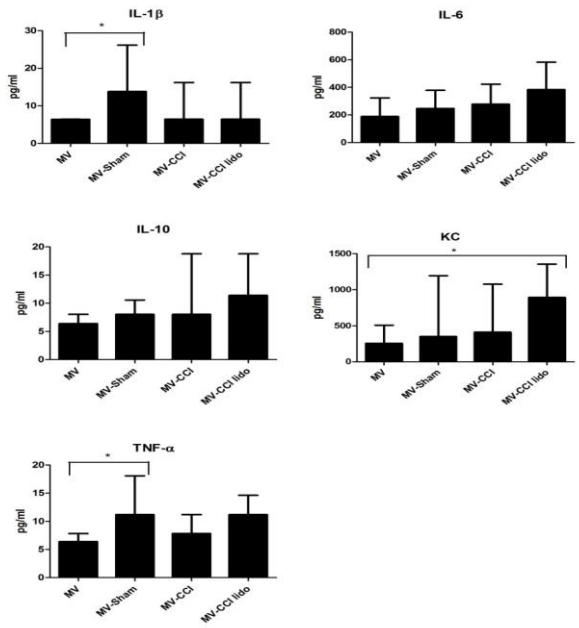

Figure 3. Cytokine levels in plasma after 2 hours. Levels of interleukin (IL)-1β, IL-6, IL-10, keratinocyte derived chemokine (KC) and tumor necrosis factor (TNF)-α keratinocyte derived chemokine (KC), in ventilated mice (MV) compared with MV sham mice (MV-sham), MV chronic constriction injury (CCI) mice (MV-CCI) and MV-CCI mice receiving lidocaine at 1,5 mg per kilogram per hour (MV-CCI lido). MV-Sham showed an increase in IL-1β compared with MV. MV-CCI lido showed an increase in KC compared with MV. MV-Sham showed an increase in TNF-α compared with MV. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

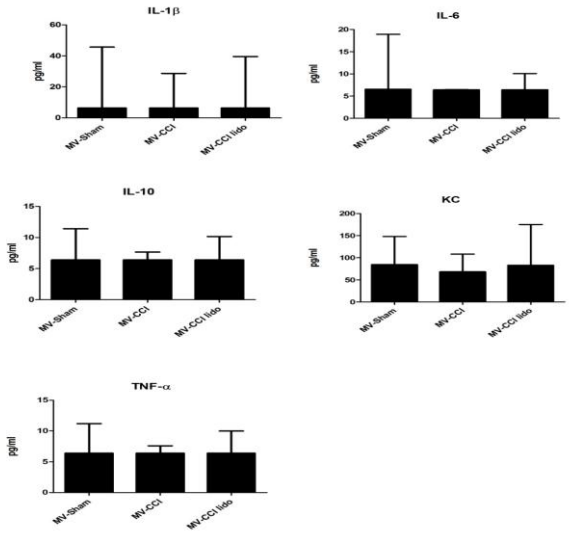

Figure 4.

Cytokine levels in plasma after 16 days. Levels of interleukin (IL)-1β, IL-6, IL-10, keratinocyte derived chemokine (KC) and tumor necrosis factor (TNF)-α keratinocyte derived chemokine (KC), in ventilated (MV) sham mice (MV-sham), MV chronic constriction injury (CCI) mice (MV-CCI) and MV-CCI mice receiving lidocaine at 1,5 mg per kilogram per hour (MV-CCI lido). No differences were observed between the different groups. Data are expressed as median with interquartile range (IQR). (* = P < 0.05)

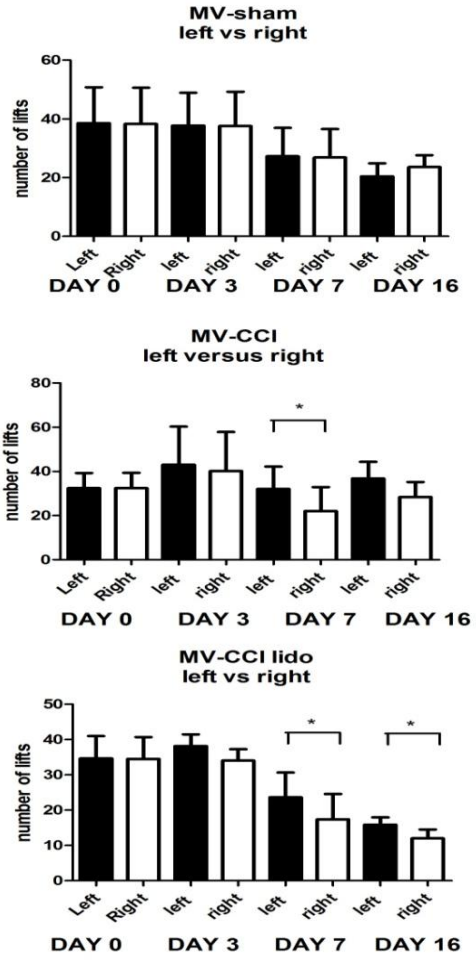

Figure 5 . Number of lifts (NOL) comparing left versus right on day 0, day 3, day 7 and day 16. MV=mechanical ventilation, CCI=chronic constriction injury, lido=lidocaine. Data are expressed as mean with standard error of the mean (SEM). (* = P < 0.05)

Difference between groups

The number of lifts on the left paw was lower in the sham group compared with CCI and lower in the CCI lido group compared with the CCI mice on day 16 (Figure 6.).

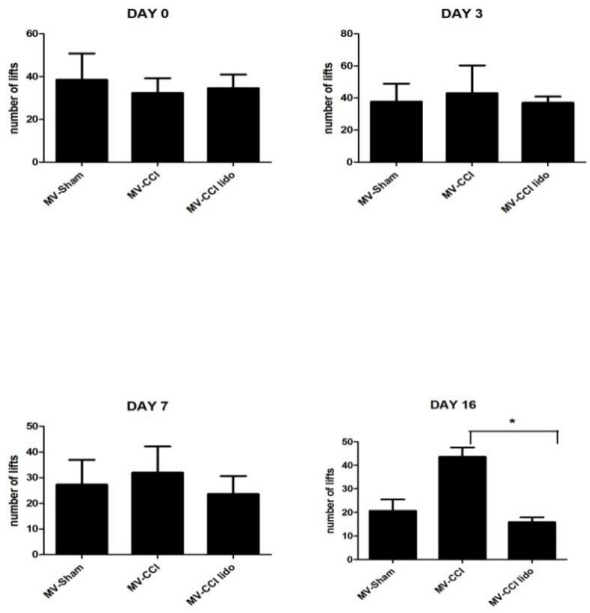

Figure 6. Number of lifts (NOL) measured by cold plate comparing different groups on day 0, day 3, day 7 and day 16. MV=mechanical ventilation, CCI=chronic constriction injury, lido=lidocaine. Data are expressed as mean with standard error of the mean (SEM). (* = P < 0.05)

Cold plate test – cumulative reaction time

The cumulative reaction time was determined in the cold plate test. Differences between left (CCI) and right were determined. The difference between the groups was also investigated.

Left versus right paw

We observed no differences between the left and right hind paw on day 0, 3, 7 and 16 in the sham group. The left and right hind paw were different in CCI group on day 3 and day 7.

In the CCI lido group the left and hind paw were different on day 7 (figure 7.).

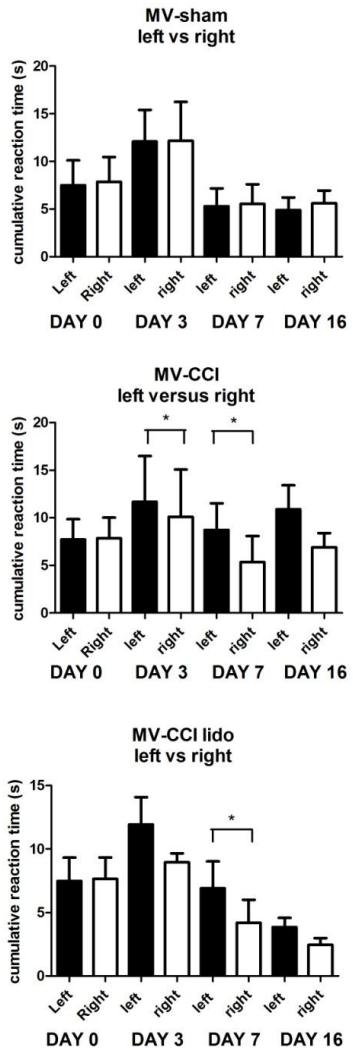

Figure 7. Cumulative reaction time (CRT) measured by cold plate comparing left versus right on day 0, day 3, day 7 and day 16. MV=mechanical ventilation, CCI=chronic constriction injury, lido=lidocaine. Data are expressed as mean with standard error of the mean (SEM). (* = P < 0.05)

Difference between groups

The cumulative reaction time was higher in CCI mice than sham mice on day 16. Cumulative reaction time was also higher in CCI mice than in CCI mice that received lidocaine on day 16 (figure 8.).

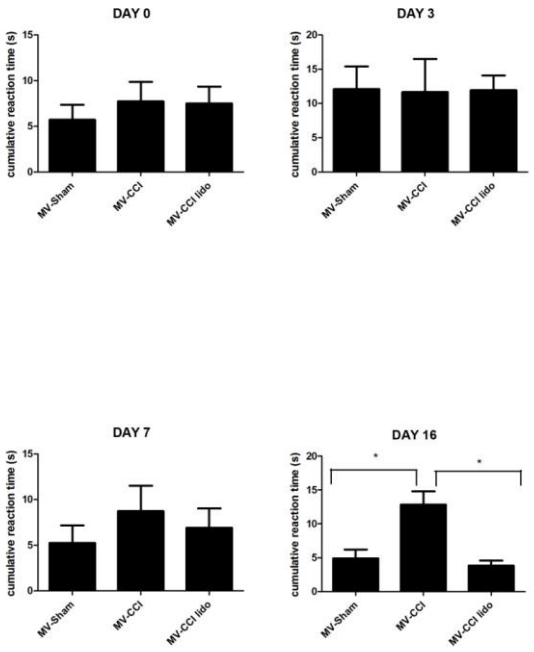

Figure 8. Cumulative reaction time (CRT) measured by cold plate comparing different groups on day 0, day 3, day 7 and day 16. MV=mechanical ventilation, CCI=chronic constriction injury, lido=lidocaine. Data are expressed as mean with standard error of the mean (SEM). (* = P < 0.05)

Von Frey test – maximal force before withdrawal

Left versus right

The maximal force before withdrawal was tested in the von Frey test. The left hind paw scored lower on maximal force before withdrawal on day 16 in the CCI lido group than the right hind paw (figure 9).

Differences between groups

No differences were shown comparing the different groups (figure 10.).

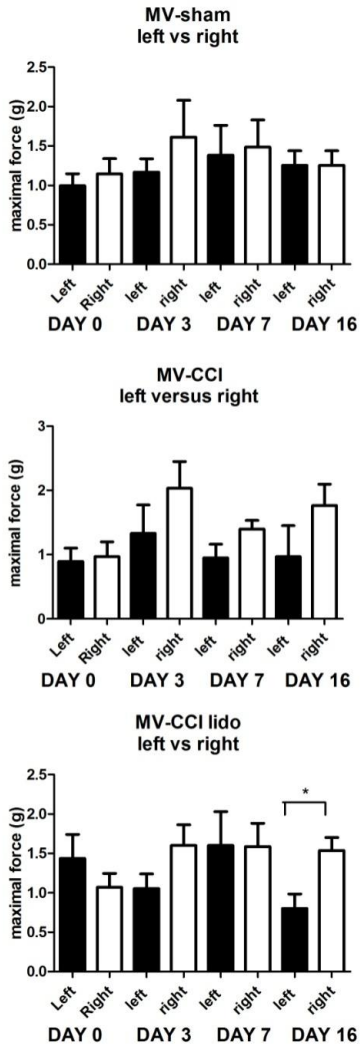

Figure 9. Maximal force of withdrawal by von Frey comparing left versus right on day 0, day 3, day 7 and day 16. MV=mechanical ventilation, CCI=chronic constriction injury, lido=lidocaine, g=gram. Data are expressed as mean with standard error of the mean (SEM). (* = P < 0.05)

General well being

Part 1. In the MV group one mouse died during intubation. In the MV CCI group one mouse died during ventilation. In the MV CCI lido group one mouse died during intubation.

Second part experiment. In the MV sham group 1 mouse was excluded from the experiment with paralysed hind extremities and in the CCI group 2 mice died the day after surgery. No autotomy was observed.

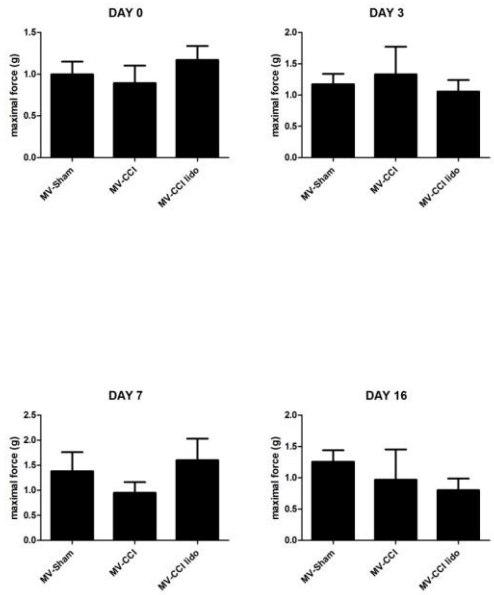

Figure 10. Maximal force of withdrawal measured by von Frey comparing different groups on day 0, day 3, day 7 and day 16. MV=mechanical ventilation, CCI=chronic constriction injury, lido=lidocaine, g=gram. Data are expressed as mean with standard error of the mean (SEM). (* = P < 0.05)

Discussion

To our knowledge this is the first experiment showing a decrease in thermal allodynia after perioperative i.p. administration of lidocaine in an experiment creating a 'second hit' pain model with MV and surgery (sham/CCI).

Anesthetic technique

We used isoflurane as monoanesthetic and carprofen, a non-steroidal anti inflammatory drug administered before surgery and on day 1 and 2. We observed some hiccupping during monoanesthesia with isoflurane and a somewhat longer recovery time compared with previous experiments where we performed CCI in spontaneous breathing mice. Previous literature describes an acute phase response to MV but also to anesthetic drugs.[41] Isoflurane has been shown to attenuate the inflammatory response after MV.[42] No medication was used that could interfere with the neuropathy.

In the CCI lido group we did not investigate anesthetic need although lidocaine administration can decrease anesthetic need.[43-45] Further studies are needed to assess the effect of other anesthetics

in this murine model, for example ketamine, dexmedetomidine and opioids that are known for their anti-inflammatory and pain modulating effects.[2, 46]

Cytokine release

Part 1 of the experiment

We found a systemic increase of cytokines IL-1β and TNF-α after surgery (MV-sham) and KC after surgery (MV-CCI lido) in comparison with MV. These findings support our 'second hit' model. Previous investigations have shown that cytokine levels of unventilated mice are below or extremely close to detection limits, therefore we did not include an unventilated group.[12, 37, 38] The entire procedure lasted only 2 hours and not all measured cytokines can reach peak concentrations in such a short period of time.[47, 48] In a previous investigation we have shown an increase of IL-10 after 4 hours of MV with systemic administration of lidocaine.[45] We did not show an increase in cytokine levels comparing sham operation to CCI. Although severity of injury seems to correlate with systemic inflammatory response and perhaps even with the amount of postoperative pain.[1, 2, 6]

Part 2 of the experiment

No differences in systemic cytokine levels were observed after 16 days. Cytokine levels were almost below detection limits, indicating a return to preoperative status consisted with previous investigations.[12, 37, 38]

Neuropathic pain behavior

We compared sham with CCI and CCI with lidocaine administration creating a model of postsurgical neuropathic pain. In this model CCI but not sham surgery leads to the development of neuropathy in mice. Perioperative administration of lidocaine can lead to a decreased thermal allodynia. Mice receiving lidocaine had significantly lower symptoms of neuropathy during the cold plate test.

Sensory testing

We used cold plate testing to assess cold allodynia and von Frey testing to assess mechanical allodynia.

Cold allodynia is thought to be mediated by C and Aδ fibers, mechanical allodynia by Aβ fibers through peripheral and central sensitisation, although the exact underlying mechanisms are not completely understood.[49] Cold allodynia in mice can mimic cold allodynia observed in patients.[50] Cold plate testing has high behavioral variability and is mainly used for neuropathy models.[51, 52] Von Frey testing to determine mechanical allodynia can reliably be used in mice.[51, 52] During cold plate testing we did found high baseline values in the cold plate tests with high variability (figure 5,7). We believe that habituation is a contributing factor in the gradual decline of our measurements and

perhaps a longer period of acclimatization should be applied to research with cold plate testing in mice. [53] Concerning the von Frey testing, the bending forces applied by Von Frey filaments can be influenced by ambient humidity, temperature, washing and drying.[54] Increased weight of the CCI mice during the experiment could also be a conflicting factor in the von Frey test.[55]

Left versus right hind paw

No differences between left and right hind paw were observed in the sham operated mice, however an increase in thermal and mechanical allodynia was objectivised in the CCI treated mice. A sham operation did not lead to altered sensory testing. Therefore we conclude that although a systemic inflammatory response is caused by sham operation and MV, nerve injury is needed for altered sensory testing in our experiment. Perhaps a more extended surgical procedure or an infectious component without neurological damage, will alter sensory testing after surgery as has been shown in a model of inflammatory pain.[56] Further research is needed to identify the role of inflammatory / infectious disease and its role in the development of neuropathic pain.

Lidocaine

Lidocaine acts on voltage-gated sodium channels in the damaged nerve and inhibits the release of nociceptive mediators by keratinocytes, G-protein coupled receptors and glycinergic system. [57, 58] Some studies indicate perioperative intravenous lidocaine to reduce postoperative complications and neuropathic pain. [58-60] . Low dose systemic lidocaine acts like an anti-hyperalgesic and anti-inflammatory agent.[7, 20] Lidocaine targets neuropathic pain possibly by a decrease in ectopic discharges and prevention of central hyperalgesia.[28-30] Our study shows that cold allodynia is attenuated by lidocaine on day 16 measured by number of lifts (NOL) and in cumulative reaction time (CRT) in the cold plate tests. These findings insinuate lidocaine to have a greater effect on Aδ fibers and C fibers than on Aβ fibers and to prevent central hyperalgesia since it has been posed that mechanical allodynia is caused by central sensitization.[49]

We did not find a significant difference in the von Frey tested groups, however this could be attributed to a power problem since two mice died in the CCI group. Further research is needed to elucidate the mechanism contributing to the attenuation of sensory testing by lidocaine.

Since mice are genetically modifiable, chronic constriction injury research in mice could create opportunities in exploring the role of inflammatory receptors or channel pathology in neuropathic pain, and subsequent development of new treatment targets.

In conclusion this study demonstrated that not a systemic inflammatory response, but nerve injury is mandatory for development of neuropathic pain in a murine ‘two hit’ model. Lidocaine attenuates cold allodynia in healthy mice.

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8

General discussion and recommendations for future research

This thesis aimed to identify some of the mechanisms responsible for the responses after mechanical ventilation and surgery measured by inflammatory and behavioral outcome parameters and explores possibilities of modulation of inflammatory responses after mechanical ventilation and surgical induced nerve injury.

Consequently, with respect to our hypothesis on homeostasis, the following research questions were identified.

- What is the role of IL-1β, caspase-1 and neutrophil factors in the mechanical ventilation induced inflammatory response in mice?
- Do resveratrol and intravenous lidocaine attenuate the mechanical ventilation induced inflammatory response in mice?
- What is the current state of knowledge on the *in vitro* mechanisms and *in vivo* efficacy of intravenous lidocaine in acute and chronic pain?
- Can we develop a murine model of neuropathic pain behavior?
- Can we develop a murine 'two hit' model of neuropathic pain?
- Does lidocaine attenuate the postoperative inflammatory response and development of neuropathic pain in mice?

IL-1beta processing in mechanical ventilation-induced inflammation is dependent on neutrophil factors rather than caspase-1.

In chapter 2 we found that cleavage of pro-1β is dependent rather on neutrophil proteasen than caspase-1. Our findings resulted in the following hypothesis: Mechanical ventilation causes mechanotransduction and cell and/or tissue damage. This causes the release of danger-associated molecular patterns (DAMPs) that activate TLR4 and possibly other pattern recognition receptors. Ligation of these receptors induces production of cytokines, most importantly IL-1β. Subsequently, KC is produced, leading to neutrophil recruitment to the lungs. The majority of pro-IL-1β is excreted in the inactive form and then cleaved by factors released by neutrophils, such as neutrophil serine proteases. Finally, active IL-1β extracellularly binds to the IL-1R, which in turn leads to the production of more cytokines and hence positive amplification of the inflammatory response. As such, a positive feedback loop is activated which could be an explanation for the extensive inflammatory response observed following mechanical ventilation.

Previous investigations suggest that the pro-inflammatory cytokine IL-1β plays a key role in the pathogenesis of the inflammatory response after mechanical ventilation by promoting neutrophil recruitment and by increasing epithelial injury and permeability.[1-3] It has been assumed that upon activation of the innate immune system, e.g. via Toll Like Receptors (TLRs), IL-1β is synthesized as an inactive precursor molecule, pro-IL-1β, that cannot bind and activate the IL-1R.[4] In order to process pro-IL-1β and form bioactive IL-1β, proteolytic cleavage from the precursor is required. Caspase-1 is the major protein implicated in cleavage of pro-IL-1β. [4, 5] Our finding that caspase-1 does not play a significant role in mechanical ventilation-induced inflammation is in contrast to a recent study where the Nod-Like Receptor (NLR)P3 inflammasome was found to play an important role in the mechanical ventilation-induced inflammatory response.[6] Several differences might explain our results. First components of the inflammasome, upstream of caspase-1, in knockout mice were used, and it was shown that mechanical ventilation activated caspase-without involvement of these components. Nevertheless, it is possible that they play other roles in the mechanical ventilation-induced inflammatory cascade than merely activating caspase-1. As abrogation and inhibition of caspase-1 by either a knockout approach or pralnacasan treatment did not have any effect in our model, the role of caspase-1/the inflammasome appears not to be as crucial as suggested. Second, differences between wild-type and knockout mice were only found at a high tidal volume, known to cause extensive lung damage [7], while no effects were found at a low tidal volume. This suggests that the inflammasome might play a more important role at higher tidal volumes but not in mechanical ventilation-induced inflammation at clinically relevant ventilator settings. A recent study from the same group showed that pre-treatment with allopurinol or uricase (both degraders of known inflammasome-activating factors [8]) did not decrease mechanical ventilation-induced inflammation, which is in support of a caspase/inflammasome-independent mechanism.[9] Our findings that treatment with an antibody against KC or depletion of neutrophils reduced the mechanical ventilation-induced production of IL-1β and KC indicate an important role for neutrophils in initiation and/or propagation of the inflammatory response. Pro-IL-1β cleavage in our model is probably achieved through neutrophil factors as the serine proteases proteinase-3 (PR-3), elastase or cathepsin G, leading to bioactive IL-1β and propagation of the inflammatory response.[4, 10, 11] Several other IL-1β-mediated inflammatory responses are described to be partly or completely independent of the inflammasome and caspase-1 and possibly dependent on neutrophil factors as proteinase-3 and cathepsin G .[11] Future studies should focus on the confirmation of our hypothesis and the identification of these neutrophil factors, they could provide a possible therapeutic target to influence homeostasis.

Resveratrol attenuates NF-κB-binding activity but not cytokine production in mechanically ventilated mice

Resveratrol inhibits pulmonary DNA- binding activity of NF-κB in healthy mice following mechanical ventilation. However, pretreatment of resveratrol did not attenuate the induction of pulmonary of systemic cytokines elicited by mechanical ventilation.

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a polyphenol found in plants and grapes and exhibits multifaceted physiological effects including anti-inflammatory and protective effects on different organ systems.[12-14] *In vitro* and *in vivo* studies have implicated that the anti-inflammatory effects of resveratrol are mediated by inhibition of TLR4-signaling, supposedly by inhibition of Toll/interleukin-1 receptor domain-containing adapter-inducing interferon-beta (TRIF) or myeloid differentiation factor 88 (MyD88) [12, 13] and attenuation of NF-κB.[15-17]

A crucial role for TLR4 has been identified in the mechanical ventilation-induced inflammatory response.[18] TLR4 signaling leads to activation of NF-κB,[13, 18, 19] and subsequent production of inflammatory cytokines.[20, 21] TLR4 can activate NF-κB by different downstream signaling pathways: TRIF and MyD88.[13, 22] The anti-inflammatory effects of resveratrol are mediated by inhibition of TLR4-signaling, supposedly by inhibition of TRIF or MyD88 [12, 13], and decreased NF-κB activation. [15-17, 23, 24]

Resveratrol was investigated in several lung injury mouse models. Li et al. investigated the effect of resveratrol in mice with respiratory syncytial virus and found inhibition of pulmonary expression of TNF-α, IL-1β and IL-6.[25] In an LPS induced acute lung injury model, Cao et al. found resveratrol to attenuate the production of IL-1β and suppress the nuclear translocation of NF-κB in lung tissue.[26]

A limitation of this study concerns time of administration of resveratrol. We administered resveratrol in equivalent dosages described in literature but just prior to mechanical ventilation. [27, 28] *In vivo* effects of resveratrol have been observed with longer (pre)treatment of resveratrol, varying from 72 hours to several weeks.[25, 26, 29, 30]

In our model resveratrol inhibits production of NF-κB, but not inflammatory cytokines. Furthermore this indicates that NF-κB is not the only transcription factor in the acute phase inflammatory response upon mechanical ventilation. This study suggest that resveratrol reduces NF-κB activity via a different pathway than TLR4. According to the literature different isoforms of NF-κB exist. Kirchner et al. described a role for both the p50-p50 homodimer and the p50-p65 heterodimer in rabbit lungs, where p50-p50 homodimers were shown to inhibit NF-κB-driven transcription.[31, 32] We did observe an increase of one particular NF-κB isoform/subunit, but were unable to identify the isoform. More research is needed to identify the involvement of certain isoforms of NF-κB in the inflammatory response after MECHANICAL VENTILATION, however our results indicate that only one

isoform participates in ventilation induced inflammatory response. Identification of this isoform could provide valuable insights in the pathofysiological mechanisms responsible for the increase of NF-κB in our model and provide possible targets for influencing homeostasis.

Lidocaine increases the anti-inflammatory cytokine IL-10 following mechanical ventilation in healthy mice.

In chapter 4 we found that different doses of intravenously administered lidocaine in a murine model of mechanical ventilation increases levels of plasma and pulmonary IL-10, indicating a modulatory role of lidocaine in inflammatory response. No effects were found on pro-inflammatory cytokines, neutrophil influx or ICAM-1 levels. We hypothesized that low dose intravenously administered lidocaine acts as an anti-hyperalgesic and anti-inflammatory agent [33, 34] and therefore may proof to be an important therapeutic to modulate the inflammatory response after mechanical ventilation.

Extensive *in vitro* research showed that lidocaine attenuates priming of human neutrophils by inhibition of G-protein coupled receptors.[35, 36] Furthermore lidocaine attenuated activated endothelial interleukin (IL)-1, 6 and 8 concentrations and intracellular adhesion molecule-1 (ICAM-1), important for transport of immune cells to site of inflammation.[37, 38] In different *in vivo* models intravenous lidocaine reduced levels of tumor necrosis factor (TNF)-α, IL-1β IL-6 and Il-8.[39-41] Also systemic lidocaine was found to attenuate acute lung injury in rabbits.[42, 43] An additional effect of lidocaine infusion is that the requirements for anesthetics are diminished.[44, 45] In human research an attenuation in inflammatory response (measured by IL-6, IL-8 and an IL-1 receptor antagonist) in plasma has been found at the end of abdominal surgery in response to lidocaine.[46-48]

IL-10 is a well known anti-inflammatory cytokine which limits the immune response during infections and is produced by nearly every type of cell in the immune system.[49, 50] IL-10 is known to decrease the synthesis of pro-inflammatory cytokines in acute phase response as IL-1α, IL-1β, IL-6 and TNF-α by neutrophils.[51] In mouse lung fibroblast exposed to mechanical stretch, IL-10 inhibited inflammatory cytokines.[49] A low lung concentration of IL-10 in patients with acute lung injury is an indication for development of adult respiratory distress syndrome (ARDS).[52] Administration of IL-10 has shown protective effects in LPS induced lung injury.[53] Interestingly, inhaled IL-10 attenuates biotrauma and mortality in a ventilator-induced lung injury model in rats.[54] We did not observe an attenuation of pro-inflammatory cytokine levels, pulmonary ICAM-1 levels or pulmonary neutrophil influx. Again a possible explanation could be that although IL-10 is known to attenuate inflammation, the acute phase response in our mechanical ventilation model is only a mild inflammatory response. In our opinion we stayed below toxic levels of lidocaine, however extrapolation remains difficult.

Mice in our experiment showed a decrease in anesthetic need with lidocaine administration which is consistent with previous experiments.[44, 45] Although a decreased anesthetic need did not lead to an attenuation of other cytokine levels in our experiment an influence on the level of IL-10 cannot completely be ruled out. We believe lidocaine could be used as a modulatory agent during mechanical ventilation however, more research has to be performed to elucidate the exact role of lidocaine in ventilator induced pulmonary inflammation and cytokine levels during a longer period of mechanical ventilation with lung protective ventilator setting.

Intravenous lidocaine has analgesic, anti-inflammatory and antihyperalgesic properties, it also attenuates the neuroinflammatory response in perioperative pain and chronic neuropathic pain.

In chapter 5 we provide an overview of the evidence on in vitro and in vivo efficacy of intravenous lidocaine on the inflammatory response in acute and chronic pain. Low dose lidocaine inhibits *in vitro* voltage gated sodium channels, the glycinergic system, some potassium channels and G- coupled protein receptors. Higher dosages lidocaine block voltage gated calcium channels, potassium channels and NMDA receptors. Animal studies demonstrate lidocaine to have analgesic effects in acute and neuropathic pain syndromes and anti-inflammatory effects early in the inflammatory response. Clinical studies demonstrate lidocaine to have a clear advantage in abdominal surgery and in some neuropathic pain syndromes. Intravenous lidocaine has analgesic, anti-inflammatory and antihyperalgesic properties. It attenuates the (neuro) inflammatory response in perioperative pain and chronic neuropathic pain. We recommend more clinical trials to be performed, with larger study size, different dosages of administered lidocaine, and impeccable methodology to determine the effect of intravenous lidocaine on the neuroinflammatory response in acute and chronic pain. More research has to be done assessing the effect of the metabolites of lidocaine. Lidocaine does however seems a valuable therapeutic agent to modulate the neuroinflammatory response, perioperative pain and chronic neuropathic pain.

Behavior of neuropathic pain in mice following chronic constriction injury comparing silk and catgut ligatures.

In chapter 6 we developed an improvement program to optimize an animal translational pain behavior model for neuropathic pain after surgery. This model is a murine model of CCI comparing silk and catgut ligatures. We found that silk seemed to be preferable to catgut.

Neuropathic pain is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system and is common after surgery. [55] Because the mechanisms of neuropathic pain are insufficiently understood, [56, 57] it seems pivotal to investigate the course and cause of neuropathic pain and development of treatment and perhaps prevention strategies. Therefore ideally we want to study a neuropathic pain animal model that can be translated to the clinical situation. Often a chronic constrictive injury model is used in rats to study neuropathic pain which include thermal and mechanical allodynia testing [58, 59]. A murine model facilitates a better understanding of the course of neuropathic pain, and improves accuracy and decreases variability of the chronic constriction model, because of the possibility to use transgenic mice. [59] The material that is used for ligatures can have an effect on the outcome of the observed sensory abnormalities. [60] In chronic constriction injury, either catgut or silk ligatures are used. In rats, catgut is commonly used as ligature material [61]. Catgut induces a development of an inflammatory reaction and consequentially a loss of most A fibers and some C fibers, but few cell bodies. [62, 63] In mice, however, the preferable ligature material is not known. In our study silk seemed to be preferable compared with catgut as ligature material in mice, however more research has to be performed to confirm this hypothesis. In further research it might be useful to use an electronic von Frey meter, because of the difference in increase of the forces.

In this study we demonstrated that a chronic constrictive injury (CCI) model in mice can induce neuropathic pain behaviors comparable to neuropathic pain signs and symptoms in humans. We can use this model in future research to test our translational modulatory hypothesis and it could be used with transgenic models to provide more insights in the mechanisms that contribute to the development of neuropathic pain.

Intraperitoneally administered lidocaine attenuates thermal allodynia in a murine second hit chronic constriction injury model.

In chapter 7 we developed an improvement program to optimize an animal translational pain behavior model for neuropathic pain after surgery and mechanical ventilation thereby creating a ‘second hit’ translational model. We found that intraperitoneally administered lidocaine does not attenuate the postoperative inflammatory response but does reduce cold allodynia. We also found a systemic increase of cytokines IL-1β, TNF-α and KC after surgery. These findings support our ‘second hit’ model.

Translational pain model

CCI but not sham surgery led to the development of neuropathy in mice. Mice receiving lidocaine had significantly lower symptoms of neuropathy during the cold plate test.

We used cold plate testing to assess cold allodynia and von Frey testing to assess mechanical allodynia.

Cold allodynia is thought to be mediated by C and Aδ fibers, mechanical allodynia by Aβ fibers through peripheral and central sensitisation, although the exact underlying mechanisms are not completely understood.[64] Cold allodynia in mice can mimic cold allodynia observed in patients.[65] Cold plate testing has high behavioral variability and is mainly used for neuropathy animal models.[66, 67] Von Frey testing to determine mechanical allodynia can reliably be used in mice.[66, 67]

We conclude that nerve injury is needed for altered sensory testing in our experiment. Perhaps a more extended surgical procedure or an infectious component without neurological damage, will alter sensory testing after surgery as has been shown in a model of inflammatory pain.[68] Further research is needed to identify the role of inflammatory / infectious disease and its role in the development of neuropathic pain. Our study shows that cold allodynia is attenuated by lidocaine insinuating that lidocaine has a greater effect on Aδ fibers and C fibers than on Aβ fibers and to prevent central hyperalgesia since it has been posed that mechanical allodynia is caused by central sensitization.[64]

Further research is needed to elucidate the mechanism contributing to the attenuation of sensory testing by lidocaine. Further studies are also needed to assess the effect of other anesthetics in this murine model, for example ketamine, dexmedetomidine and opioids that are known for their anti-inflammatory and pain modulating effects.[69, 70]

Inflammatory response

After injury the host defense is aimed at restoring homeostasis, and the release of anti-inflammatory and pro-inflammatory cytokines seems equally important.[71] Interestingly, dysregulation of the inflammatory response can play a role in pain after surgery and the development of chronic pain.[33, 72-75] Mechanical ventilation without surgery can result in an inflammatory response too, characterized by release of inflammatory cytokines and influx of immune cells such as neutrophils.[7, 76-78] The entire procedure lasted only 2 hours and not all measured cytokines can reach peak concentrations in this time window. Additionally, the effect of lidocaine may not be observed after such a short period of time.[79, 80] We did not show an increase in cytokine levels comparing sham operation to CCI. Although severity of injury seems to correlate with systemic inflammatory response and perhaps even with the amount of postoperative pain.[70, 74, 81] However, further research should focus on the neuroinflammatory responses in the central nervous system.

General conclusion

In the introduction of this thesis we explained the development of the concept of balanced anesthesia, which encompasses the use of multiple drugs to produce anesthesia, analgesia, skeletal muscle relaxation and attenuation of autonomic reflexes. This thesis provides some insights in the responses during anesthesia and surgery. The key topic is that an inflammatory response is a homeostatic response to injury or infection and homeostasis or balance is a fundamental requirement for life.[82] Hereby we have extended the topic of balanced anesthesia to the modulation of the inflammatory response in anesthesia and surgery. We have determined that in mechanical ventilation with lung protective settings serine proteasen play an important role and that not only NF-κB is responsible for transcription of inflammatory cytokines, furthermore certain subtypes of NF-κB may be of greater importance than other. Modulation of the inflammatory response after mechanical ventilation, but also in a translational second hit model, by resveratrol and lidocaine, has revealed altered outcomes, measured by DNA binding activity of NF-κB, increased IL-10, altered sensory testing and attenuation of anesthetic need.

This thesis provided valuable insights in on the balance that exists between the input and outcome in relation to mechanisms and modulatory therapy. We present the figure from the introduction of this thesis again with the findings of this thesis.

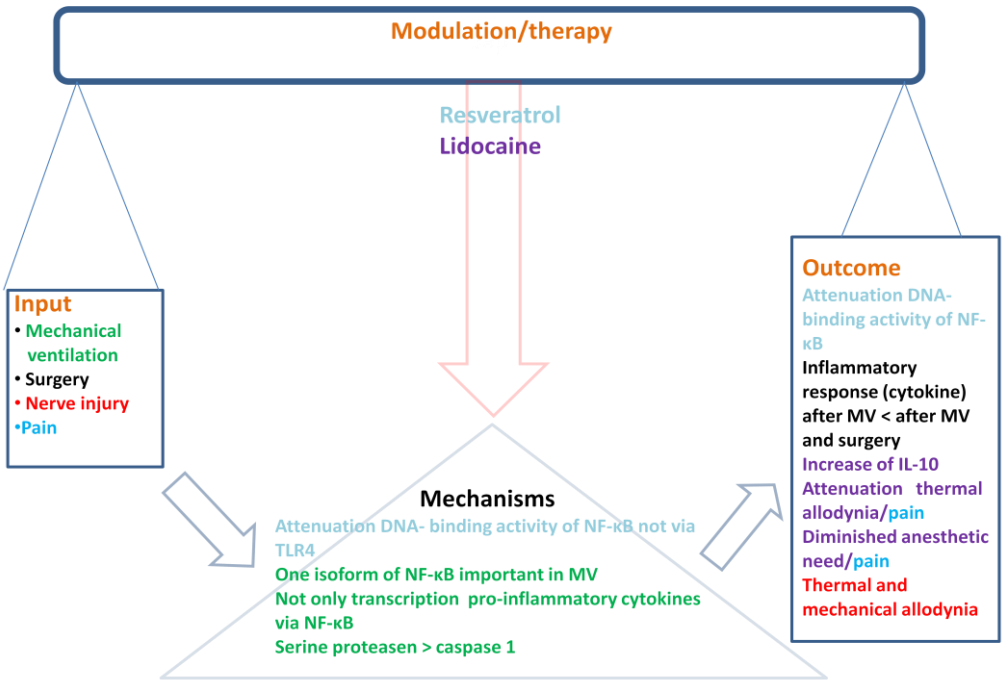

Figure 1. Pathophysiology and modulation of inflammation during mechanical ventilation and surgery affecting outcome

Recommendations for further research

We designed an animal model that reflects the clinical setting during a regular anesthesia with mechanical ventilation and surgery with or without nerve injury. In this model we investigated pathophysiology of inflammatory responses but also provided modulation therapy thereby trying to balance outcome parameters. However this model of modulation should ultimately include more features.

Firstly we strongly recommend extended research that takes species and gender in account. But also transgenic mouse models require further investigation. An interesting question for example is what role of Toll like receptor 4 is in the development of pain in our murine model. Secondly we also recommend to investigate the effects of different types of anesthesia, for example we used isoflurane in our 'second hit' model. Further research should aim to investigate the (modulatory) effect of other anesthetics as for example ketamine or medetomide. Thirdly forthcoming research should include animal research with different etiologies of neuropathic pain and inflammation to elucidate exact mechanisms responsible for chronic pain after surgery.

Finally, future research should also include patient cohorts with different etiologies of neuropathic pain and inflammation. Therapy should include pre-emptive treatment and include patient cohorts where different etiologies of neuropathic pain are equally distributed.

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9

Summary / Samenvatting

Summary

The aim of this thesis was to identify some of the mechanisms responsible for the responses after mechanical ventilation and surgery measured by inflammatory and behavioral outcome parameters and explores possibilities of modulation of inflammatory responses after mechanical ventilation and surgical induced nerve injury.

In chapter 1 we provide a brief overview on the development of anesthesia and mechanical ventilation and describe the evolution of the concept of balanced anesthesia. We also provide a brief introduction on the pathyofysiology of the acute phase response after mechanical ventilation and surgery and describe the mechanisms of pain after surgery and neuropathic pain. The effects of the anesthetic drugs isoflurane, lidocaine, ketamine and metedomidine are briefly highlighted. We pose a hypothesis on the balance that exists between the input and outcome in relation to mechanisms and modulatory therapy.

In chapter 2 we describe the role of IL-1β, caspase-1 and neutrophil factors in the mechanical ventilation induced inflammatory response in mice. The cytokine IL-1β is thought to play an important role in the pathogenesis of ventilator-induced lung injury. Cleavage of the inactive precursor, pro-IL-1β, is needed to form bioactive IL-1β. This can be mediated by several types of proteases. Our results illustrate that IL-1 is indeed an important cytokine in the inflammatory cascade induced by mechanical ventilation, but caspase-1 appears not to be involved in IL-1β processing in this type of inflammatory response. Our results suggest that this is mainly mediated by neutrophil factors.

In chapter 3 we investigated the modulatory effects of resveratrol on the mechanical ventilation induced inflammatory response in healthy mice. Resveratrol, a polyphenol found in plants and grapes exhibits anti-inflammatory effects *in vitro* and *in vivo*, supposedly by interfering with TLR4 signaling and NF-κB. Resveratrol abrogates the mechanical ventilation induced increase in pulmonary NF-κB activity, but does not attenuate cytokine levels. These results imply a less prominent role for NF-κB in mechanical ventilation induced inflammation than previously assumed.

In chapter 4 we investigate the effects of intravenously administered lidocaine on the mechanical ventilation induced inflammatory response. Lidocaine, an amide local anesthetic, has anti-

inflammatory properties *in vitro* and *in vivo*, possibly due to an attenuation of pro-inflammatory cytokines, ICAM-1 and reduction of neutrophils influx. Intravenously administered lidocaine increases levels of plasma and pulmonary IL-10 indicating a modulatory role of lidocaine on inflammation. Intravenously administered lidocaine also appears to reduce anesthetic need in mice.

In Chapter 5 we reviewed the literature concerning the modulatory mechanism and effect of lidocaine on neuroinflammatory responses in acute and chronic pain. Intravenous lidocaine has analgesic, anti-inflammatory and antihyperalgesic properties. It attenuates the neuroinflammatory response in perioperative pain and chronic neuropathic pain.

In chapter 6 we developed a translation murine model of neuropathic pain. We created a chronic constriction injury of the sciatic nerve with silk and catgut ligatures and performed sensory testing of mechanical and thermal allodynia. Silk seems to be the preferred ligature material.

In chapter 7 we described the development a 'two hit' murine model of nerve injury and investigated the effect of intraperitoneally administered lidocaine in a two hit model of surgery and nerve injury. Perioperative administered lidocaine attenuated thermal allodynia in our 'two hit'murine model, indicating a modulatory role for lidocaine in the development of pain.

In chapter 8 we will discuss the results of this thesis research in an integrated scientific context and proposed several recommendations for future research.

Samenvatting

Het doel van dit proefschrift was om een aantal van de mechanismen die verantwoordelijk zijn voor de reacties na mechanische ventilatie en een operatie te identificeren, mede door het meten van inflammatoire parameters en gedragsmatige uitkomstmaten. Tevens wilden we de mogelijkheden verkennen tot modulatie van ontstekingsreacties na mechanische ventilatie en chirurgisch geïnduceerd zenuwletsel.

In hoofdstuk 1 geven we een kort overzicht van de ontwikkeling van anesthesie en mechanische ventilatie en beschrijven we de ontwikkeling van het concept van gebalanceerde anesthesie. We geven een korte inleiding over de pathofysiologie van de acute fase respons na mechanische ventilatie en chirurgie en beschrijven de mechanismen van pijn na een operatie en van neuropathische pijn. De effecten van de anesthetica isofluraan, lidocaïne, ketamine en metedomidine zijn kort belicht. Wij stellen een hypothese op over de balans die bestaat tussen de verschillende mechanismen, modulerende therapie en de uitkomsten.

In hoofdstuk 2 beschrijven we de rol van IL-1β, caspase-1 en neutrofiele factoren in de mechanische ventilatie geïnduceerde ontstekingsreactie bij muizen. Het cytokine IL-1β speelt een belangrijke rol bij de pathogenese van ventilator-geïnduceerde longbeschadiging. Splitsing van de inactieve precursor, pro-IL-1β is nodig om bioactief IL-1β vormen. Dit kan worden gemedieerd door verschillende soorten proteasen. Onze resultaten laten zien dat IL-1β inderdaad een belangrijk cytokine is in de inflammatoire cascade veroorzaakt door mechanische ventilatie, maar caspase-1 lijkt niet betrokken bij IL-1β verwerking in ons model. Onze resultaten suggereren dat dit juist voornamelijk wordt gemedieerd door neutrofiele factoren.

In hoofdstuk 3 onderzochten we de modulerende effecten van resveratrol op de mechanische ventilatie geïnduceerde ontstekingsreactie in gezonde muizen. Resveratrol, een polyfenol gevonden in planten en druiven vertoont anti-inflammatoire effecten *in vitro* en *in vivo*, vermoedelijk door te interfereren met TLR4 signalering en NF-kB. Resveratrol reduceert de mechanische ventilatie geïnduceerde toename van pulmonale NF-κB-activiteit, maar niet het cytokinegehalte. Deze resultaten impliceren een minder prominente rol voor NF-κB in mechanische ventilatie geïnduceerde ontsteking dan eerder werd aangenomen.

In hoofdstuk 4 onderzochten we het effect van intraveneus toegediend lidocaïne op de mechanische ventilatie geïnduceerde ontstekingsreactie. Lidocaïne, een amide lokaal anestheticum, bezit anti-inflammatoire eigenschappen in vitro en in vivo. Intraveneus toegediend lidocaïne verhoogt het

niveau van plasma en pulmonale IL-10 en dit zou kunnen wijzen op een modulerende rol van lidocaïne op de inflammatoire respons in muizen na mechanische ventilatie. Intraveneus toegediend lidocaïne geeft ook een vermindering van anesthesie behoefte bij muizen.

In hoofdstuk 5 beoordeelden we de literatuur over de modulerende mechanismen en de effecten van lidocaïne op neuro-inflammatoire reacties in acute en chronische pijn. Intraveneus toegediende lidocaine heeft analgetische, anti-inflammatoire en antihyperalgetische eigenschappen. Het dempt de neuroinflammatoire reactie in peri-operatieve en chronische neuropathische pijn.

In hoofdstuk 6 hebben we een extrapoleerbaar muizenmodel van neuropathische pijn ontwikkeld. We creëerden een chronisch constrictie model van de nervus ischiadicus met een ligatuur van zijde en kattendarm en verrichtten sensorische testen om mechanische en temperatuur geïnduceerde allodynie op te wekken. Zijde lijkt het ligatuur materiaal van voorkeur.

In hoofdstuk 7 beschreven we de ontwikkeling van een 'second hit' muizenmodel van chirurgisch zenuwletsel en onderzochten het effect van peri-operatief intraperitoneaal toegediend lidocaïne in dit model. Perioperatief toegediend lidocaïne vermindert thermische allodynie in onze 'second hit' muizen model, dit impliceert een modulerende rol van lidocaïne in de ontwikkeling van pijn.

In hoofdstuk 8 hebben we de resultaten van dit proefschrift besproken in een geïntegreerde wetenschappelijke context en gaven we aanbevelingen voor toekomstig onderzoek.

10

Appendices

- Dankwoord
- Bibliografie
- Publicaties

Chapter 10

Dankwoord

Tijdens de totstandkoming van dit proefschrift zijn er veel mensen geweest die daar een aandeel in hebben gehad. Deze mensen hebben veelal ook een belangrijke rol gespeeld in mijn persoonlijke ontwikkeling als wetenschapper, arts maar ook als mens.

Als eerste wil ik mijn promotoren bedanken.

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Bibliografie

Selina Elisabeth Ingrid van der Wal werd geboren op 7 mei 1981 te Arnhem. In 1999 haalde zij haar gymnasium diploma aan het Olympus college te Arnhem om hierna te starten met de studie Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen. In 2003 rondde zij deze studie succesvol af met als hoofdvakken pathobiologie en reproductietoxicologie. Hier is haar liefde voor de wetenschap waarschijnlijk ontwaakt. Zij is echter wel vlot doorgestroomd naar de studie geneeskunde alwaar zij in 2005 haar doctoraal en 2007 haar artsexamen haalde. Tijdens haar opleiding werkte zij als student-assistent op de interne geneekunde en was zij hoofdredacteur bij de studenteneditie van het nederlands tijdschrift voor geneeskunde.

Na haar artsexamen gehaald te hebben is zij begonnen als arts-assistent op de afdeling intensive care in het Universitair medisch centrum te Nijmegen. Na anderhalf jaar mocht zij eind 2008 beginnen als arts-assistent op de anesthesiologie. Vanaf begin 2010 is zij begonnen met haar promotietraject. Tijdens deze periode heeft zij een professionele en persoonlijke ontwikkeling doorgemaakt. In 2012 won zij de best oral presentation tijdens de anesthesiologen dagen van de Nederlandse vereniging voor anesthesiologie met een presentatie van haar onderzoek en in 2014 begeleidde zij de winnaar. In 2014 won zij samen met Sandra Radema de Paul Speth prijs.

In februari 2013 rondde zij haar specialisatie tot anesthesioloog af om direct daaropvolgend een fellowship Pijn en Palliatieve geneeskunde te volgen en af te ronden begin 2014. Vanaf 2013 tot op heden is zij met veel plezier werkplek manager van de neurochirurgie en plastische chirurgie. Vanaf begin 2014 is zij werkzaam als anesthesioloog-pijnarts in het Radboud Universitair medisch centrum.

In de toekomst zal zij het onderzoek naar het moduleren van inflammatoire processen die betrokken zijn bij anesthesie en het ontstaan en persisteren van pijn voortzetten in samenwerkingsverband met onder andere de oncologie. Tevens zal zij zich blijven inzetten als pijnarts en anesthesioloog in het Radboud universitair medisch centrum.

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4. S. van der Wal, S. van den Heuvel, S. Radema, B. van Berkum, M. Vaneker, M. Steegers, G.J. Scheffer, K. Vissers. The in vitro mechanisms and in vivo efficacy of intravenous lidocaine on the neuroinflammatory response in acute and chronic pain: A review of current knowledge. Submitted.
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Awards

2012	Best oral presentation Nederlandse vereniging voor anesthesiologie: The role of IL-1beta in mechanical ventilation
2014	Best oral presentation (begeleider): intravenous lidocaine reduces the anti-inflammatory cytokine IL-10 after mechanical ventilation in healthy mice
2014	Paul Speth prijs

Posters

2013	World institute of pain: a case report of low dose intravenous lidocaine in chronic chemotherapy induced neuropathy