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Involvement of pro- and antinociceptive factors in minocycline analgesia in rat neuropathic pain model

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ABSTRACT

In neuropathic pain the repeated minocycline treatment inhibited the mRNA and protein expression of the microglial markers and metalloproteinase-9 (MMP-9). The minocycline diminished the pronociceptive (IL-6, IL-18), but not antinociceptive (IL-1alpha, IL-4, IL-10) cytokines at the spinal cord level. *In vitro* primary cell culture studies have shown that MMP-9, TIMP-1, IL-1beta, IL-1alpha, IL-6, IL-10, and IL-18 are of microglial origin. Minocycline reduces the production of pronociceptive factors, resulting in a more potent antinociceptive effect. This change in the ratio between pro- and antinociceptive factors, in favour of the latter may be the mechanism of minocycline analgesia in neuropathy.

1. Introduction

Studies confirm the complexity of endogenous factors that initiate and regulate neuropathic pain conditions (Austin and Moalem-Taylor, 2010). Substances with diverse mechanisms of action that modulate the neuroimmune system have been shown to relieve neuropathic pain. One of these substances is minocycline, an antibiotic that belongs to the group of semisynthetic tetracyclines and has been used for 30 years (Yong et al., 2004). Minocycline has antiinflammatory, antiapoptotic, and immunomodulatory properties (Golub et al., 1991, 1992; Greenwald and Golub, 1993; Zemke and Majid, 2004; Sapadin and Fleischmajer, 2006; Kielian et al., 2007). Minocycline also has neuroprotective activity (Kim and Suh, 2009), as demonstrated in numerous neurodegenerative disease models in mice and rats (Du et al., 2001; Brundula et al., 2002; Nessler et al., 2002; Thomas and Le, 2004). Many reports revealed that minocycline administration diminished the development of neuropathic pain (Raghavendra et al., 2003; Ledeboer et al., 2005a,b; Mika et al., 2007, 2009).

In 2006, Piao et al. reported that the molecular mechanism of minocycline action consists of inhibiting p38 mitogen-activated protein kinases (p38MAPKs) in microglial cells, while recent publications suggest that minocycline also inhibits the activity of metalloproteinase (MMP-9) (Niimi et al., 2013). These two molecular minocycline targets could be targeted for the development of new treatments for neuropathic pain. p38MAPK has a significant contribution to neuropathic pain, and high levels of p38MAPK are observed in microglia in neuropathic pain models (Jin et al., 2003; Tsuda et al., 2004; Hains and Waxman, 2006; Clark et al., 2007; Hains and Wen et al., 2007). The activation of p38MAPK occurs as the result of the activity of cytokines (IL-1beta, TNFalpha), triggering the production of pronociceptive agents, such as nitrogen oxide, IL-1beta, IL-6, and IL-18 (DeLeo and Yezierski, 2001; Miyoshi et al., 2008). Increased p38 MAPK activity leads to the activation of a number of transcription factors that regulate the expression of genes involved in nociception (Potucek et al., 2006). During neuropathy, neurons and glia exchange signals through chemokines, cytokines and the complement system (Mika, 2008; Austin and Moalem-Taylor, 2010). Antiinflammatory cytokines such as IL-1alpha, IL-4, or IL-10 play an important role in nociception, thereby inhibiting the development of neuropathic pain. Pain develops due to a disturbance in the equilibrium between algesic and analgesic factors. In 2008, Kawasaki et al. demonstrated the crucial role of MMP-9 in the development of neuropathic pain, and the intrathecal administration of MMP-9 leads to the development of allodynia, activation of microglial cells, and p38MAPK upregulation. Administration of the tissue inhibitor of metalloproteinase 1 (TIMP-1) blocked the development of neuropathy. The authors suggest that MMP-9 enhances nociception by transforming inactive proIL-1beta to the active 1beta form.

The use of substances inhibiting microglial activation and formation of proinflammatory cytokines, namely minocycline, pentoxifylline, or propentofylline, inhibits the development of neuropathic pain (Neuner et al., 1994; Lundblad et al., 1995; Sweitzer et al., 2001;

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Raghavendra et al., 2003; Mika et al., 2007, 2008, 2009). Minocycline appears to be a very interesting substance that, on the one hand, reduces microglial activation by inhibiting the activity of p38MAPK in microglial cells (Piao et al., 2006) and, on the other hand, suppresses MMP-9 activity that was recently shown in experimental autoimmune encephalomyelitis models (Niimi et al., 2013).

The objective of our studies was to assess whether and how minocycline, which inhibits the development of neuropathic pain by inhibiting microglia activation (Mika et al., 2010), affects the activation of CD40-positive cells in the spinal cord and DRG during neuropathic pain. Simultaneously, we studied the effect of minocycline on the biosynthesis of proinflammatory cytokines (IL-1beta, IL-6, IL-18) and antiinflammatory cytokines (IL-1alpha, IL-10, IL-4) as well as MMP-9 and MMP-2 and TIMP-1 and TIMP-2. The objective of the *in vitro* studies was to determine whether the above factors involved in the development of neuropathic pain changed in LPS-treated primary microglia cell cultures. In addition, the efficacy of intrathecal MMP-9 inhibitor (MMP-9 INH.I) and p38MAPK inhibitor (SB203580) was compared.

2. Experimental procedures

2.1. Animals

Male Wistar rats (300–350 g) from Charles River (Hamburg, Germany) were housed in cages lined with sawdust under a standard 12/12 h light/dark cycle (lights on at 08:00 h) with *ad libitum* access to food and water. All experiments were performed according to the recommendations of the International Association for the Study of Pain (IASP) (Zimmermann, 1983) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and these experiments were approved by the local Bioethics Committee (Krakow, Poland).

2.2. Surgical preparations

Intrathecal (*i.t.*) catheters were chronically implanted in rats according to the methods of Yaksh and Rudy (1976) under pentobarbital anaesthesia (60 mg/kg; *i.p.*). The catheter (PE 10, INTRAMEDIC, Clay Adams, Becton Dickinson and Company, Rutherford, NJ, USA) was sterilised by immersion in 70% ethanol and fully flushed with sterile water prior to insertion. The catheter was carefully introduced through the atlanto-occipital membrane to the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4–L6). Water for injection or respective drugs were delivered slowly (1–2 min) in a volume of 5 μl through the *i.t.* catheter, followed by 10 μl of water, which flushed the catheter. The injury to the sciatic nerve was performed five days after catheter implantation.

Chronic constriction injury (CCI) was performed according to Bennett and Xie (1988). The right sciatic nerve was exposed under sodium pentobarbital anaesthesia (60 mg/kg; *i.p.*). Four ligatures (4/0 silk) were made around the nerve, distal to the sciatic notch with 1 mm spacing, until a brief twitch in the respective hind limb was observed. After surgery, all rats developed symptoms of long-lasting neuropathic pain, such as allodynia and hyperalgesia.

2.3. Drug administration and experimental scheme

Minocycline hydrochloride (30 mg/kg; Sigma, Schnelldorf, Germany) was dissolved in water for injections and administered preemptively by intraperitoneal (*i.p.*) injections 16 and 1 h before CCI and then twice daily for seven days, as we previously described (Mika et al., 2007, 2009). This method of minocycline administration was used throughout the study and is referred to in the text as "repeated administration". This administration schedule was used because systemic microglia inhibitors attenuate the activation of microglia more efficiently when the inhibitor is injected before injury (Raghavendra et al., 2003; Ledeboer et al., 2005a, b; Mika et al., 2009) and repeatedly. For pharmacological studies, MMP-9 INH.I (5 μg/5 μl; Merc Darmstadt, Germany) and 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; 5 μg/5 μl; Sigma, Schnelldorf, Germany) were *i.t.* administered preemptively 16 and 1 h before CCI, then once daily for seven days. The inhibitors of MMP-9 and p38MAPK were dissolved in DMSO according to the previously published data (Tsuda et al., 2004). The control groups received vehicle according to the same schedule. Naïve animals were processed (behavioural tests and tissue collection) in parallel to the V-CCI and MC-CCI groups with the exception of CCI surgery and anaesthesia.

2.4. Behavioural tests

2.4.1. Tactile allodynia (von Frey test)

Allodynia was measured 60 min after the last administration of minocycline (30 mg/kg) according to our previous study (Mika et al., 2007). Other drugs were examined in the same time point (Fig. 5A). Allodynia was measured using an automatic von Frey apparatus (Dynamic Plantar Aesthesiometer Cat. No. 37400, Ugo Basile, Italy). The animals were placed in plastic cages with wire net floors 5 min before the experiment. The strengths of the von Frey stimuli used in our experiments ranged up to 26 g. The filament was applied to the midplantar surface of the ipsilateral hind paw, and measurements were taken automatically as described by us previously (Mika et al., 2007; Makuch et al., 2013). There was almost no response to the highest strength (26 g) in the naïve animals. No significantly different paw reactions were observed between the contralateral hind paw of CCI-exposed and naïve rats. Therefore, a line was drawn at this value to represent the naïve animals in the figures.

2.4.2. Hyperalgesia (cold plate test)

The cold plate test was conducted 65 min after the last injection of MMP-9 INH.I and SB203580 (Fig. 5A). Hyperalgesia was assessed using the cold plate test (Cold/Hot Plate Analgesia Meter No. 05044, Columbus Instruments, USA) as previously described (Mika et al., 2007; Makuch et al., 2013). The temperature of the cold plate was kept at 5 °C, and the cut-off latency was 30 s. The animals were placed on the cold plate, and the time until the hind paw was lifted was recorded. In the naïve rat group, the reaction of the first hind paw lift was measured. In the rats subjected to nerve injury, the ipsilateral paw reacted first.

2.5. Biochemical tests

2.5.1. Microglial cell cultures and treatments

Primary cultures of microglial cells were prepared from 1-day-old Wistar rat pups as previously described (Zawadzka and Kaminska, 2005). Briefly, cells were isolated from the rats' cerebral cortices and were plated at a density of 3×10^5 cells/cm^2 in a culture medium that consisted of DMEM/Glutamax/high glucose (Gibco, USA) supplemented with heat-inactivated 10% foetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, USA) on poly-L-lysine coated 75 cm^2 culture flasks and were maintained at 37 °C and 5% CO_2. The culture medium was changed after 4 days. The loosely adherent microglial cells were recovered after 9 days by mild shaking and centrifugation. The microglial cells were suspended in a culture medium and plated at a final density of 2×10^5 cells onto 24-well plates for mRNA analysis. Adherent cells were incubated for 48 h in a culture medium before being analysed. Primary microglial cell cultures were treated with LPS [100 ng/ml] (Sigma-Aldrich) for 6 h. Cell specificity was determined in cultures of primary microglia by Western blot assay using an antibody to OX-42 (microglial marker) and by qRT-PCR using primers for *C1q* (a microglial marker) and *GFAP* (an astroglial marker). The homogeneity of the microglial population was high (more than 95% positive for OX-42 and C1q), and our homogeneity was similar to that obtained by Zawadzka and Kaminska (2005).