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Research report

Bovine lactoferrin reduces extra-territorial facial allodynia/hyperalgesia following a trigeminal nerve injury in the rat

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ABSTRACT

There is an urgent clinical need for an effective therapeutic agent to treat neuropathic pain. This study explored whether intrathecal administration of bovine lactoferrin (bLF), in combination with signal transduction pathway inhibition or an inflammatory cytokine production, results in reduced allodynia/hyperalgesia in the whisker pad area following mental nerve transection (MNT) in rats. Rats were intrathecally infused with bLF, lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS), an antagonist of Toll-like receptor 4 (TLR4), or interleukin (IL)-18 binding protein (BP). bLF attenuated allodynia/hyperalgesia and blocked upregulation of phosphorylated (p)-p38 mitogen-activated protein kinase (MAPK), p-nuclear factor (NF)-κB p65, p-IκB kinase, and IL-18 in the trigeminal subnucleus caudalis (Vc). Microglia expressed p-p38 and astrocytes expressed p-NF-κB p65 in the Vc following MNT. LPS-RS had the same effects as bLF, except for attenuation of p-NF-κB p65. IL-18 BP attenuated allodynia/hyperalgesia and IL-18 upregulation in the Vc. These results suggest that bLF suppresses IL-18 production, which is involved in allodynia/hyperalgesia following MNT, by inhibiting TLR4-derived p38 MAPK activation in microglia. Additionally, binding of bLF to tumor necrosis factor receptor-associated factor 6 might result in inhibition of p38 MAPK and NF-κB activation. The findings suggest that bLF could serve as a potent therapeutic agent for neuropathic pain.

1. Introduction

Neuropathic pain has been defined as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" by the International Association for the Study of Pain. However, its mechanism is still unknown. Thus, the epidemiology and classification of neuropathic pain remain an open research topic (Smith B.H. and Torrance, 2012). Meanwhile, an estimated 6.9–10% of the population suffers from pain with neuropathic characteristics (van Hecke et al., 2014; Yawn et al., 2009). Traditional pain medications are not very effective for neuropathic pain (Dworkin et al., 2010; Moore et al., 2014). Thus, there is an urgent clinical need for an effective therapeutic agent to treat neuropathic pain.

The whisker pad area (WP) in the rat, which is innervated by the maxillary nerve, exhibits mechanical allodynia/hyperalgesia following mental nerve transection (MNT) (Murasaki et al., 2013; Takahashi et al., 2011). Previous studies from our laboratory have focused on glial cell functions in the trigeminal subnucleus caudalis (Vc) to provide a better understanding of MNT-induced extra-territorial mechanical allodynia/hyperalgesia (Murasaki et al., 2013; Takahashi et al., 2011). Following MNT, inflammatory cytokines, such as interleukin (IL)-1β or tumor necrosis factor (TNF)-α, are released from glial cells at the dorsal portion of the Vc and induce extra-territorial facial pain (Murasaki et al., 2013; Takahashi et al., 2011). Furthermore, peripheral nerve injury results in increased expression of phosphorylated (p)-p38 mitogen-activated protein kinase (MAPK) and p-transcriptional

Abbreviations: bLF, bovine lactoferrin; MNT, mental nerve transection; LPS-RS, lipopolysaccharide from Rhodobacter sphaeroides; TLR4, toll-like receptor 4; IL-18, interleukin-18; BP, binding protein; MAPK, mitogen-activated protein kinase; Vc, trigeminal subnucleus caudalis; WP, whisker pad area; TNF α, tumor necrosis factor α; NF-κB, transcriptional factor nuclear factor-κB.

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factor nuclear factor-κB (NF-κB) p65, which are involved in inflammatory cytokines production in the spinal dorsal horn or Vc (Ito et al., 2013; Lee et al., 2009; Miyoshi et al., 2008). IL-18 upregulation in spinal microglia, which depends on ligand-gated ion channel purinergic receptors and p-p38 MAPK, plays an important role in bone cancer-related pain (Yang et al., 2015). Taken together, these studies suggest that inflammatory cytokines released from glial cells and cell signaling peptides following MNT are likely involved in allodynia/hyperalgesia onset at intact skin territories adjacent to the denervated region.

Lactoferrin (LF) belongs to the transferrin family of iron-binding proteins, and has a molecular mass of approximately 80 kDa. The transferrin family molecules are widely found in various secretory fluids, such as nasal secretions, tears, saliva, and milk (Adlerova et al., 2008). Interestingly, LF exhibits diverse biological actions, such as anti-inflammatory, antibacterial and anticancer effects (Azzam et al., 2007; Cornish et al., 2006; Farnaud and Evans, 2003; Zhang et al., 2014), and establishes an innate immune system that provides host protection with anti-inflammatory activities (Spadaro et al., 2008). Several *in vitro* studies have shown that LF or LFP-20, a porcine lactoferrin peptide, suppress p-p38 MAPK, p-NF-κB p65, and inflammatory cytokines such as IL-1β and TNF-α (Inubushi et al., 2012; Zong et al., 2015). The use of LF is considered safe, because it is a food-derived protein, and its therapeutic effects are currently being analyzed.

This report is the first to examine the therapeutic effects of intrathecal administration of bovine LF (bLF) on MNT-induced extra-territorial facial pain. Analysis of the analgesic mechanisms of bLF at the molecular level suggest that bLF is a candidate therapeutic agent for neuropathic pain following peripheral nerve injury.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (200–250 g; Charles River Laboratories, Yokohama, Japan) were used. Animals were kept in separate cages in a controlled environment (lights on 8:00–20:00, 22 °C) with food and water freely available. The animal model adhered to the International Association for the Study of Pain ethical guidelines (Zimmermann, 1983) and the ARRIVE guidelines for animal research (Kilkenny et al., 2011), and was approved by the Institutional Animal Care and Use Committee of Hiroshima University (Approval number A1411). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were randomly allocated to experimental groups and analyzed in good health. All experiments were conducted under blind conditions as previously described (Guo et al., 2007). The total number of animals used in each experiment, and the number of animals in each experimental group are shown in Table 1.

2.2. Surgery

MNT was performed as previously described (Takahashi et al., 2011). Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The mental nerve was then exposed and ligated with 5–0 silk. A section (1–2 mm) distal to the ligation and distal nerve stump was resected.

2.3. Behavioral tests

Tactile allodynia/hyperalgesia was assessed as previously described (Takahashi et al., 2011). Briefly, a series of calibrated von Frey filaments with bending forces ranging from 0.4 to 60 g were applied to the WP. Active withdrawal of the head from the probing filament was defined as a stimulus response. Response frequencies [(number of responses/number of stimuli) × 100%] to a range of von Frey filament forces were determined to construct a stimulus-response curve. Nonlinear regression analysis of this curve produced an EF_{50} value, which was defined as the von Frey filament force (g) that produces a 50% response frequency. Drug effects on EF_{50} were quantified ipsilaterally, but not contralaterally, because results from our previous study showed no allodynia/hyperalgesia in the contralateral WP following MNT (Takahashi et al., 2011).

Table 1
Number of rats in this experiment.

Experiments	Sample size (n)	Group (n)	Rats (n)
Behavioral test	4–5 rats	9	43 rats
Western blotting	3–5 rats	13	53 rats
Immunofluorescence staining	2 rats	1	2 rats
Immunoprecipitation	3 rats	3	9 rats
Total number of rats			107 rats

2.4. Drug delivery

To avoid systemic drug effects, the drugs were delivered into the cerebrospinal fluid space around the Vc as previously described (Takahashi et al., 2011). Briefly, the rats were injected with a bolus volume of bLF (1 or 10 mg/mL, 20 μL; Sunstar Inc., Osaka, Japan), ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS, a Toll-like receptor 4 (TLR4) antagonist, 1.0 or 10 μg/mL, 20 μL; InvivoGen, San Diego, CA, USA) or recombinant human IL-18 binding protein (BP) (10 or 100 μg/mL, 10 μL; R&D Systems Inc., Minneapolis, MN, USA) via a PE10 catheter (Intramedic, Hellerup, Denmark) implanted into the cerebral spinal fluid space around the Vc. All drugs were dissolved in 0.15 M NaCl. The control group was injected with saline or albumin (200 μg/rat).

2.5. Immunofluorescence staining

Immunofluorescence staining was performed as previously described (Takahashi et al., 2011). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 250 ml of 0.9% saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brain stem was cut into 30-μm thick sections. Free-floating sections were blocked with Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) for 1 h, and then incubated overnight at 4 °C with anti-p-p38 MAPK (1:500; #9211; Cell Signaling Technology, Danvers, MA, USA), anti-p-NF-κB p65 (1:100; #3033; Cell Signaling Technology), anti-Iba1 (1:400; sc-28530; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), or anti-GFAP (1:500; AB5804; Millipore, Bedford, CA, USA). After repeated washes in 0.1 M phosphate-buffered saline, the sections were incubated for 2 h with Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11008; Invitrogen, San Diego, CA, USA) or Alexa Fluor 568-conjugated goat anti-mouse IgG (A-11001; Invitrogen). Digital images were captured using a confocal laser-scanning microscope (Lsm5 Pascal; Carl Zeiss, Oberkochen, Germany).

2.6. Western blotting

Brain sample preparation and western blotting were performed as previously described (Ito et al., 2013). Briefly, the rats were anesthetized with halothane and quickly decapitated 24 h after drug administration. The dorsal part of the Vc was removed and homogenized in solubilization buffer. The homogenates were