Lactoferrin enhances opioid-mediated analgesia via nitric oxide in the rat spinal cord

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Lactoferrin enhances opioid-mediated analgesia via nitric oxide in the rat spinal cord. Am J Physiol Regul Integr Comp Physiol 285: R306–R312, 2003. First published April 17, 2003; 10.1152/ajpregu.00760.2002.—Lactoferrin (LF) is a multifunctional protein that is found in milk, neutrophils, and other biological fluids, and its receptors have also been identified in the central nervous system. Recently, we found that bovine milk-derived LF (BLF) produced analgesia via a μ-opioid receptor-mediated response in the spinal cord. However, the precise mechanism of this analgesic effect remains unclear. In this study, spinally applied BLF produced analgesia that was reversed by coadministration with a nitric oxide (NO) synthase inhibitor, Nω-nitro-l-arginine methyl ester, during phases 1 and 2 in the formalin test. Spinal coadministration of a μ-opioid receptor agonist, morphine, with a subeffective dose of BLF produced a much more highly potentiated analgesia than that produced by morphine alone during phases 1 and 2 in the formalin test. This potentiated analgesia by morphine with BLF was reversed by a μ-opioid receptor antagonist, d-Phe-Cys-Tyr-d-Trp-Orn-Thr-NH2, or by Nω-nitro-l-arginine methyl ester. In the tail-flick test, continuous spinal infusion of morphine via an osmotic minipump over 6 days resulted in development of tolerance by day 4, but no tolerance of BLF was observed throughout the experiment. These results suggest that BLF acts as an enhancer of the spinal opioidergic system via an NO-mediated mechanism.

Formalin test; tail-flick test; Nω-nitro-l-arginine methyl ester; d-Phe-Cys-Tyr-d-Trp-Orn-Thr-NH2; tolerance

LACTOFERRIN (LF) is a single-chain glycoprotein with a molecular weight of ~80,000 that belongs to the family of transferrins (1). Human collostral milk contains 5–7 mg LF/ml, and mature breast milk contains 1–3 mg LF/ml (19). LF is also present in the milk of bovids and other species (24). That is, the infants of many mammalian species constantly receive exogenous LF from their mother's milk. LF can also be detected in the milk of bovids (35). We also confirmed the presence of BLF in the cerebrospinal fluid after oral or intraperitoneal administration in a preliminary study. This macromolecular transcytosis may be enhanced in inflammatory conditions inducing pain, as suggested by a report that the proinflammatory cytokine tumor necrosis factor-α increased the rate of transendothelial transport of BLF in a blood-brain barrier model (7).

Recently, we found that oral, intraperitoneal, or intrathecal administration of BLF produces a μ-opioid receptor-mediated antinociceptive effect in the rat CNS, especially in the spinal cord (11). It is now clear that many classes of dorsal horn neurons, fibers descending from the brain, and primary afferent fibers exert a powerful modulatory influence on the onward transfer of nociceptive information from the spinal cord to the brain (27). In these modulatory processes, activation of spinal opioid receptors exerts a pivotal role that causes analgesia in animals (12, 21, 28, 29, 38, 43) and humans (2). Thus the pain-modulatory function of LF via the opioid receptor-mediated response would be of substantial physiological importance. Although various types of opioid ligands have been found in milk or milk digests (37), BLF neither bound to the μ-opioid receptor nor changed its binding affinity to the opioid ligands in our preliminary study. Thus we hypothesize that BLF does not act as an opioid agonist but, rather, as an enhancer of endogenous opioid signaling in the spinal cord.

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It is reported that BLF induces nitric oxide (NO) secretion from macrophages in rats (33). Several reports suggest that NO is involved in the central opioidergic system (4, 13, 14, 15, 41). NO is a free radical that is synthesized from l-arginine by NO synthase (NOS). It produces an increase in intracellular cGMP through activation of soluble guanylate cyclase. In the CNS, NO is produced by a constitutive (neuronal) form of NO synthase (nNOS), an enzyme localized to the NO synthase, or by an inducible form of NO synthase, which is localized to glia (5, 26, 31). It has been reported that intrathecal administration of an NOS inhibitor, Nω-nitro-l-arginine methyl ester (l-NNAME), enhances opioid-induced analgesia in the rat tail-flick test and formalin tests (21, 43). Another NOS inhibitor, 7-nitroindazole, also enhances opioid-induced analgesia in the rat tail-flick test after intrathecal administration (21). However, an NO precursor, l-arginine, has been shown to produce analgesia via a central opioidergic mechanism in rats and mice with carrageenin-induced hyperalgesia (13, 14) or in the mouse tail-flick test (15). It has also been reported that an NO donor, nitroglycerin, acts as an adjuvant to morphine (μ-opioid agonist) in cancer patients (17). Together, the available data on the involvement of NO in the central opioidergic system are not consistent and are still a matter of debate. The aim of the present study was to assess three points: 1) Would NO be involved in BLF-induced analgesia? 2) Could spinally applied BLF potentiate a spinal μ-opioid receptor-mediated antinociceptive response? If so, would NO also be involved in this potentiation by BLF? 3) Would BLF cause tolerance? In this study, we used the formalin test and the tail-flick test in rats. The formalin test is widely used as a peripheral inflammatory nociceptive test (23, 40) involving peripheral and central sensitization (18, 23). The tail-flick test is the most used thermal-evoked nociceptive test to assess the development of opioid tolerance in the rat spinal cord (28, 29).

**MATERIALS AND METHODS**

**Animals.** Male Wistar-Imamichi rats (8–9 wk old, 250–330 g body wt) were maintained at a controlled temperature (22 ± 2°C) under a regular light-dark cycle (lights on from 0700 to 1900) with free access to food and water. Behavior tests were conducted during the light period. All experiments were conducted in accordance with the guidelines of the Physiological Society of Japan regarding the care of experimental animals.

**Intrathecal catheterization.** Chronic lumbar intrathecal catheters were implanted in rats under pentobarbital sodium anesthesia (50 mg/kg ip) according to a previous report with modifications (42). The rat was mounted in a conventional stereotaxic instrument. After the overlying skin was shaved and sterilized, the posterior superficial neck muscles were separated to give a clear visual field of the atlantooccipital membrane. A catheter (model SP-10, Natsume, Tokyo, Japan) was inserted through a small incision at the center of the membrane and gently advanced caudally 8.5 cm to the lumbosubarachnoid space. The external end of the intrathecal catheter was capped with a closed tube (model JB-30, Eicom, Kyoto, Japan) and covered by the head skin until the day of the experiment. Only animals showing normal motor function 5–10 days after the surgery were used.

**Drugs and administrations.** BLF (78,000 mol wt; Tatua, Morrinsville, New Zealand), naloxone hydrochloride (naloxone; Sigma, Tokyo, Japan), D-Phe-Cys-Tyr-d-Trp-Orn-Thr-H2 (CTOP; Sigma), morphine hydrochloride (morphine; 60.8 mol wt; Sankyo, Tokyo, Japan), L-NNAME (Sigma), and Nω-nitro-l-arginine methyl ester (l-NNAME; Sigma) were dissolved in saline solution for administration. All control groups were treated with saline. For the spinal application, under light ether anesthesia, a small incision was made for injection of drugs into the catheter covered by the head skin. Drugs were injected at a volume of 5 μl (single injections) or 10 μl (coadministration) and flushed with 10 μl of saline 15 min before the test. For the continuous spinal infusion, we used osmotic minipumps (1 μl/h; model 2001, Alzet, Cuperino, CA) filled with BLF or morphine dissolved in sterilized saline. The osmotic minipumps were implanted subcutaneously on the back of the rat under pentobarbital anesthesia (50 mg/kg ip) and connected to the intrathecal catheters.

**Formalin test.** The formalin test is the most used thermal-evoked nociceptive test after intrathecal administration (21). How- ever, it has been shown to reduce variability in response latencies compared with commercial restraints (30). The end of the tail (5 cm) was placed in a 50°C water bath (49.5–50.5°C). This water-bath temperature was shown to produce a stable noxious stimulus-evoked response in this rat strain in a preliminary experiment. The tail-flick latency was defined as the time required to elicit a flick of the tail. The cutoff time was 30 s. Immediately after control testing, the osmotic minipumps were implanted as described above. Because BLF cannot be dissolved in saline >1.25 μmol/ml, we used this concentration of BLF (1.25 μmol/ml) in this test.

**Tolerance (tail-flick) test.** The tail-immersion version of the tail-flick test was performed according to a previous report with modifications (32). The rat was held in a cloth restrainer during testing. This method of restraint is a less stressful means of containing rats during tail-flick testing and has been shown to reduce variability in response latencies compared with commercial restrainers (30). The rate of the tail (5 cm) was placed in a 50°C water bath (49.5–50.5°C). This water-bath temperature was shown to produce a stable noxious stimulus-evoked response in this rat strain in a preliminary experiment. The tail-flick latency was defined as the time required to elicit a flick of the tail. The cutoff time was 30 s. Immediately after control testing, the osmotic minipumps were implanted as described above. Because BLF cannot be dissolved in saline >1.25 μmol/ml, we used this concentration of BLF (1.25 μmol/ml) in this test.

**Statistics.** For the dose-response analysis, the dose-response lines for phases 1 and 2 of the formalin test were fitted...
using least-squares linear regression analysis, and ED\(_{50}\) values (the dose that produced 50% analgesia) and their 95% confidence intervals (CI) were calculated.

Values are means ± SE. Differences between treatment groups were assessed by Student’s t-test or, when appropriate, ANOVA followed by Dunnett’s post hoc test for multiple comparisons. In all cases, \(P < 0.05\) was considered to indicate statistical significance.

RESULTS

Dose response of BLF on the formalin-evoked nociception. After formalin (2.0%, 50 \(\mu\)l) was injected into the right hindpaw, the rats showed spontaneous flinching/shaking of the injected paw (Fig. 1A). This pain-related behavior can be divided into two phases: an intense initial response beginning immediately after formalin injection and decreasing over the subsequent 5–10 min (phase 1) and a phase beginning at 10 min after injection, with a maximum response typically occurring at 20–30 min after the injection (phase 2). Spinally applied BLF (1.25–1,250 pmol/rat) produced a dose-dependent inhibition of formalin-evoked nociceptive behavior during phases 1 and 2. Spinal administration of a \(\mu\)-opioid receptor agonist, morphine (27 nmol/rat), also produced a marked analgesia in this test. These data on BLF are summarized in Fig. 1B as a percent-age of analgesia. Spinally applied BLF induced significant analgesia at 12.5–1,250 pmol during both phases (\(P < 0.01\) vs. control). ED\(_{50}\) values for BLF were 318.0 (95% CI = 19.4–5,224) pmol/rat in phase 1 and 43.3 (95% CI = 3.1–596) pmol/rat in phase 2. On the basis of this result, we chose a BLF dose of 125 pmol/rat for the subsequent experiments.

Effects of opioid antagonist or an NOS inhibitor on the BLF-induced analgesia. Spinally applied BLF (125 pmol/rat) produced a significant degree of analgesia during phases 1 and 2 (Fig. 2A and C; \(P < 0.001\) vs. controls). Administration of naltrexone, a nonselective opioid antagonist (10 \(\mu\)g/rat), or CTOP, a \(\mu\)-opioid antagonist (1 \(\mu\)g/rat), did not affect formalin-evoked nociception during either phase (Fig. 2A). In accordance with our previous observation (11), coadministration of naltrexone (10 \(\mu\)g/rat) with BLF (125 pmol/rat) completely reversed BLF-induced analgesia (\(P < 0.001\) vs. BLF in both phases). CTOP also completely reversed BLF-induced analgesia (\(P < 0.001\) vs. BLF in both phases). Thus BLF-induced analgesia on the formalin-evoked nociception is mediated by the spinal \(\mu\)-opioid receptor.

Spinal application of an NOS inhibitor, L-NAME (30–300 \(\mu\)g/rat), did not affect phase 1 but produced a dose-dependent analgesia during phase 2 (30 \(\mu\)g/rat, \(P < 0.05\); 300 \(\mu\)g/rat, \(P < 0.01\) vs. controls; Fig. 2B). On the other hand, spinally applied d-NAME (300 \(\mu\)g/rat), the inactive enantiomer of L-NAME, did not affect the formalin-evoked nociception (data not shown). L-NAME at 30 \(\mu\)g/rat induced significant but weak antinociceptive activity only during phase 2; this dose was therefore chosen for the following coadministration experiments. Coadministration of L-NAME (30 \(\mu\)g/rat) with BLF (125 pmol/rat) completely reversed BLF-induced analgesia in phase 1 of the formalin test (\(P < 0.005\) vs. BLF; Fig. 2C). BLF-induced analgesia in phase 2 was also significantly reversed (from 69% to 18% analgesia) by L-NAME at 30 \(\mu\)g/rat, which was antinociceptive by itself (\(P < 0.001\) vs. BLF). However, coadministration of d-NAME (30 \(\mu\)g/rat) with BLF (125 pmol/rat) did not affect BLF-induced analgesia. These results suggest that NO participates in a large part of the BLF-induced analgesia.

Potentiation of spinal \(\mu\)-opioid receptor-mediated analgesia by BLF: involvement of NO. Spinal administration of a \(\mu\)-opioid agonist, morphine (0.27–27 nmol/rat), produced a dose-dependent analgesia during both phases in the formalin test (Fig. 3). The ED\(_{50}\) values were 5.1 (95% CI = 0.7–37.1) nmol/rat in phase 1 and 2.5 (95% CI = 0.3–20.8) nmol/rat in phase 2. Analgesia induced by spinal administration of morphine was greatly potentiated by coadministration of BLF (1.25 pmol/rat) during both phases, although this dose of BLF did not affect formalin-evoked nociception per se (Fig. 1). In the presence of BLF (1.25 pmol/rat), ED\(_{50}\) values for morphine decreased markedly, from 5.1 (95% CI = 0.7–37.1) nmol/rat to 0.10 (95% CI = 0.01–1.0) nmol/rat in phase 1 and from 2.5 (95% CI = 0.3–20.8) nmol/rat to 0.02 (95% CI = 0.002–0.2) nmol/
These results suggest synergy between BLF and morphine.

In the presence of spinally applied naloxone (10 μg/rat) or CTOP (1 μg/rat), the potentiated morphine (0.27 nmol/rat)-induced analgesia by BLF (1.25 pmol/rat) was completely abolished during both phases ($P < 0.001$ in both phases vs. BLF + morphine; Fig. 4). Furthermore, in the presence of spinally applied L-NAME (30 μg/rat), this potentiated analgesia (56% analgesia in phase 1, 87% analgesia in phase 2) was also significantly reversed (7.4% analgesia in phase 1, $P < 0.005$; 20.1% analgesia in phase 2, $P < 0.001$ vs. BLF + morphine). These results suggest that BLF potentiates the spinal μ-opioidergic system and that NO is involved in this potentiation.

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**Fig. 2.** Effects of spinal administration of μ-opioid antagonists or a nitric oxide synthase inhibitor on BLF-induced analgesia in formalin test. Data are presented as percentage of analgesia during phase 1 (open bars) and phase 2 (solid bars) of formalin test (see MATERIALS AND METHODS). A: effects of spinal coadministration of naloxone (NLX, 10 μg/rat) or d-Phe-Cys-Tyr-d-Trp-Orn-Thr-NH₂ (CTOP, 10 μg/rat) with BLF (125 pmol/rat) or saline (control) on BLF-induced analgesia. Six rats were used in each group, except control group ($n = 8$). $*P < 0.001$ vs. control (Student’s t-test). $**P < 0.005$ vs. BLF (Student’s t-test). B: analgesia produced by spinally applied L-N^2-nitro-L-arginine methyl ester (L-NAME, 30–300 μg/rat). Seven rats were used in each group, except saline-treated (control) group ($n = 8$). $*P < 0.05$, $**P < 0.01$ vs. control (Dunnett’s test). C: effects of spinal coadministration of L-NAME (30 μg/rat) or N^2-N^2-nitro-d-arginine methyl ester (d-NAME, 30 μg/rat) with BLF (125 pmol/rat) or saline (control) on BLF-induced analgesia. Six rats were used in each group, except control group ($n = 8$). $*P < 0.05$, $**P < 0.001$ vs. control (Student’s t-test).

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**Fig. 3.** Potentiation of spinal μ-opioid receptor-mediated antinociceptive response by BLF on formalin-evoked nociception. Dose-response curve for spinally applied morphine (2.7–27,000 pmol/rat) or saline (control) in the presence or absence of BLF (1.25 pmol/rat), expressed as percentage of analgesia (see MATERIALS AND METHODS), is shown for phase 1 (A) and phase 2 (B) in formalin test. Seven rats were used in each group. $*P < 0.05$, $**P < 0.01$ vs. control (Student’s t-test).

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**Fig. 4.** Effects of spinal administration of μ-opioid antagonists or an NOS inhibitor on potentiated morphine-induced analgesia by BLF in formalin test. Data are presented as percentage of analgesia during phase 1 (open bars) and phase 2 (solid bars) of formalin test (see MATERIALS AND METHODS). Morphine (270 pmol/rat) with BLF (1.25 pmol/rat; M/B) was spinally administered in the presence or absence of naloxone (NLX, 10 μg/rat), CTOP (10 μg/rat), or L-NAME (30 μg/rat). Six rats were used in each group, except saline-treated (control) group ($n = 8$). $*P < 0.05$, $**P < 0.001$ vs. control (Student’s t-test).
that BLF acts as an enhancer of the spinal opioidergic system.

LF and its receptor have been identified in the CNS, especially in the brain (6, 20, 22, 35). However, whether the LF receptor localizes in the dorsal horn of spinal cord and whether the LF receptor colocalizes with opioid receptors have not been reported. Although present results show that BLF acts in the spinal cord and enhances the spinal opioidergic system, the possibility that BLF may also release opioid peptides, which mediate antinociception, cannot be excluded from the present data. Further investigation is required to clarify these points.

It has been reported that BLF induces NO secretion from macrophages in rats (33). Although we did not measure NO level in the spinal cord in this study, we demonstrated the involvement of NO in BLF-induced potentiation of the spinal opioidergic system (Fig. 4). It is well known that nNOS has different splice variants (nNOS-1 and nNOS-2) that mediate different action. Kolesnikov et al. (16) reported that nNOS-1 diminishes the analgesic actions of the opioidergic system and causes opioid tolerance, whereas nNOS-2 enhances the opioidergic system and causes analgesia without tolerance. BLF enhanced the spinal opioidergic system (Fig. 3) but did not develop tolerance (Fig. 5). Thus BLF may stimulate an nNOS-2 system but not an nNOS-1 system. The limited selectivity of traditional NOS inhibitors cannot explore these opposing actions of nNOS-1 and nNOS-2. In the present study, spinally applied l-NAME significantly reversed the BLF-induced analgesia (Fig. 2C). However, l-NAME itself also produced analgesia during phase 2 in the formalin test (Fig. 2B).

BLF may stimulate an nNOS-2 system, which stimulates an opioidergic system. This mechanism leading to antinociception may be blocked at the level of nNOS-2 by l-NAME. On the other hand, on spinal administration of l-NAME, the activities of the nNOS-1 and nNOS-2 system were blocked, and the antinociceptive potential previously repressed by nNOS-1 appeared and may be the only one that remained as an l-NAME-induced antinociception. Although involvement of NO in the nociception is still a matter of debate, it has been reported that NO exerts an antinociceptive activity via the central opioidergic system in mice and rats (13–15) and also that NO potentiates opioid-induced analgesia in the mouse CNS (41). In addition, it has been reported that a transdermal NO donor, nitroglycerin, potentiates the opioid-induced analgesia in cancer patients (17). On the other hand, NO inhibits the N-type voltage-sensitive Ca channel in human neuroblastoma cells (3). It is well known that the N-type Ca channel is involved in control of neurotransmitters in the peripheral nervous system and the CNS, and this channel is inhibited by the opioid agonists via a G protein-coupling mechanism. In rats, intrathecal administration of an N-type Ca channel blocker potentiates morphine-induced analgesia but does not lead to development of tolerance or cross tolerance to morphine (38). In mice,
intrathecal administration of a Ca channel blocker produces an analgesia that is mediated by a \( \mu \)-opioid receptor (39). In this study, BLF also produced an analgesia that was mediated by a \( \mu \)-opioid receptor (Fig. 24) and potentiated morphine-induced analgesia (Fig. 3) but did not lead to development of tolerance (Fig. 5). On the basis of the present data and the reports described above, it seems likely that the NO produced by BLF may induce analgesia via inhibition of the N-type Ca channel in the spinal cord. Nevertheless, the precise mechanism of BLF-induced antinociception seems to be more complicated and needs further investigation (e.g., experiments using nNOS knockout mice or nNOS splicing variants antisense).

In the present study, we demonstrated that NO is involved in BLF-induced analgesia only at the spinal level. However, because LF is a ubiquitous protein in the periphery (1, 19, 24, 25) and the CNS (9, 20, 22, 35), it is possible to imagine that the NO produced by LF may act in the periphery and the CNS. In some pathological conditions, immune-derived opioids cause peripheral antinociception (34). Endogenous LF, which is mainly produced by neutrophils, and exogenous LF may reduce peripheral pain, acting in synergy with immune-derived opioids. Endogenous and exogenous LF that enter the cerebrospinal fluid via receptor-mediated transcytosis through the blood-brain barrier would be enhanced in inflammatory conditions inducing pain, as suggested by a report that the proinflammatory cytokine tumor necrosis factor-\( \alpha \) increased the rate of transendothelial transport in a blood-brain barrier model (7). Previously, we reported that orally administered BLF entered the cerebrospinal fluid in piglets (10) and calves (35). We also confirmed the presence of BLF in the rat cerebrospinal fluid after oral or intraperitoneal administration in a preliminary study. The endogenous and exogenous LF that are transported into the cerebrospinal fluid from the periphery may also act in the CNS to reduce pain under pathological conditions.

The infants of many mammalian species constantly receive exogenous LF from their mother’s milk (24). Inasmuch as LF is a ubiquitous protein in the periphery (1, 19, 24, 25) and the CNS (9, 20, 22, 35), endogenous LF is also available for the infants and the adult animals. We used LF purified from bovine milk in the present study. The LF of humans, bovids, mice, and pigs share 70% overall amino acid sequence and 100% identity in several stretches of 10–15 amino acids at the COOH terminus (36). We also confirmed that recombinant human LF possesses a level of antinociceptive activity similar to that of BLF in the rat formalin test (11). Thus not only BLF but also LF from different species will produce analgesia.

In summary, this is the first report to provide evidence of the involvement of NO in potentiation of the rat spinal \( \mu \)-opioidergic system by milk-derived BLF. This analgesic function of LF involving the NO pathway underscores the importance of the bioactivities of this ubiquitous protein.

**Perspectives**

In addition to the antinociceptive effects presented here, it is known that LF has many peripheral functions, including induction of primary defense against bacterial and viral infection, antitumor activity, immunomodulation, and cell growth regulation (1). This wide range of LF activity will potentially be of great benefit to patients. The most common source of exogenous LF is milk, which is optimized for the infants of each mammalian species. The multifunctional milk-derived peptide LF per se possesses remarkable antinociceptive activity without tolerance and also dramatically reduces the required dose of morphine, suggesting that LF can be a safely used natural drug for patients with severe pain that requires opioid treatment.

**DISCLOSURES**

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