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European Journal of Pharmacology 484 (2004) 175–181
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Lactoferrin enhances peripheral opioid-mediated antinociception via nitric oxide in rats

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Received 15 September 2003; received in revised form 29 October 2003; accepted 4 November 2003

Abstract

Lactoferrin (LF) is a multifunctional protein found in various biological fluids. However, the peripheral action of lactoferrin remains unknown. In this study, peripherally applied bovine lactoferrin showed antinociceptive effect that was reversed by a μ-opioid receptor antagonist, D-Phe–Cys–Tyr–D-Trp–Orn–Thr–NH_2 (CTOP), or by a nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), but not by an inactive enantiomer of L-NAME, N^G-nitro-D-arginine methyl ester (D-NAME), during phase 1 and phase 2 in the rat formalin test. Peripheral coadministration of a μ-opioid receptor agonist, morphine, with subeffective dose of bovine lactoferrin produced a potentiated antinociceptive effect compared to that of morphine alone during both phases in the formalin test. This potentiated antinociception by morphine with bovine lactoferrin was reversed by CTOP or by L-NAME. These results suggest that bovine lactoferrin exerts an antinociceptive activity via potentiation of the peripheral μ-opioidergic system, and that nitric oxide (NO) is involved in this potentiation.

Keywords: Formalin test; N^G-nitro-L-arginine methyl ester; D-Phe–Cys–Tyr–D-Trp–Orn–Thr–NH_2; Lactoferrin

1. Introduction

Lactoferrin (LF) is a single-chain glycoprotein with a molecular weight (MW) of about 80 kDa that belongs to the family of transferrins (Brock, 2002). Human colostral milk contains 5–7 mg/ml lactoferrin, and mature breast milk contains 1–3 mg/ml lactoferrin (Levay and Viljoen, 1995). Bovids and other species also have lactoferrin in their milk (Masson and Heremans, 1971). That is, the infants of many mammalian species constantly take in exogenous lactoferrin from maternal milk. Lactoferrin can also be detected in blood, saliva, nasal secretions, tears, bronchial mucus, hepatic bile, pancreatic juice, seminal fluid, female cervical mucus, urine, and cerebrospinal fluid (Levay and Viljoen, 1995; Maffei et al., 1999; Masson et al., 1966). Thus, endogenous lactoferrin is also accessible in adult animals.

Lactoferrin has many physiological functions including induction of primary defense against bacterial and viral infection, antitumor activity, immunomodulation, and cell growth regulation (Brock, 2002). Recently, we reported that oral, intraperitoneal, or intrathecal administration of bovine lactoferrin produces a μ-opioid receptor-mediated antinociceptive activity in the rat central nervous system and especially in the spinal cord (Hayashida et al., 2003a). We also reported that intraperitoneal administration of bovine lactoferrin demonstrates an opioid-mediated suppressive effect on anxiety-related behavior induced by maternal separation in rat pups (Takeuchi et al., 2003). These evidences suggest that lactoferrin activates the opioidergic system in the central nervous system. Although various types of opioid ligands have been found in milk or milk digests (Teschemacher and Koch, 1991), bovine lactoferrin did not bind to the μ-opioid receptor or change its binding affinity to the opioid ligands (Takeuchi et al., 2003). However, our previous results show that bovine lactoferrin acts as an enhancer of endogenous opioid signaling via nitric oxide (NO) production in the central nervous system (Hayashida et al., 2003b; Takeuchi et al., 2003), suggesting that lactoferrin is an intriguing natural antinociceptive substance and possesses physiological importance.

In addition to the supraspinal and spinal action of opiates, it is well known that endogenous and exogenous opioid

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doi:10.1016/j.ejphar.2003.11.007

agonists also cause peripheral antinociception (Ferreira and Nakamura, 1979; Levine and Taiwo, 1989; Smith and Buchan, 1984). Several studies reported that peripheral morphine (μ-opioid receptor agonist) antinociception is due to activation of the L-arginine-NO-cGMP pathway (Ferreira et al., 1991; Granados-Soto et al., 1997). On the other hand, it has been reported that a NO releaser enhances the antinociceptive effects of peripherally administered morphine during formalin test in rats (Nozaki-Taguchi and Yamamoto, 1998). Thus, NO exerts a pivotal role in the peripheral opioidergic system that modulates antinociception. Under inflammatory conditions that induce nociception, lactoferrin production is increased in the periphery by the neutrophils (Brock, 2002; Levay and Viljoen, 1995). It is also reported that the level of lactoferrin is increased in the synovial fluid from rheumatoid arthritis patients (Caccavo et al., 1999). Although lactoferrin receptor has been found in the brain (Faucheux et al., 1995; Leveugle et al., 1996; Qian and Wang, 1998), the presence of lactoferrin receptor and its function in the peripheral nerve have not yet been reported. Given that opioids have been known to produce both central and peripheral antinociception, bovine lactoferrin may cause antinociception not only in the central nervous system (Hayashida et al., 2003a,b) but also in the peripheral nervous system.

The aim of the present study was to investigate two aspects of bovine lactoferrin. Firstly, would locally administered bovine lactoferrin produce peripheral antinociception, acting on the peripheral opioidergic system via NO production? Secondly, could bovine lactoferrin potentiate a peripheral morphine-induced antinociception? If so, would NO also be involved in this potentiation by bovine lactoferrin? In this study, we used the formalin test in rats. The formalin test is widely used as a peripheral inflammatory nociceptive test (Malmberg and Yaksh, 1992; Wheeler-Aceto et al., 1990).

2. Materials and methods

2.1. Animals

Male Wistar-Imamichi rats (7 weeks old; body weight: 200–230 g) were used in all the experiments. All the animals were maintained at a controlled temperature (22 ± 2 °C) under a regular light/dark cycle (light period: 7:00 to 19:00 h) 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.

2.2. Drugs and administrations

Bovine lactoferrin (MW = approximately 78,000; Tatua, Morrinsville, New Zealand), recombinant human lactoferrin (MW = approximately 80,000; Tatua), naloxone hydrochloride (naloxone; Sigma, Tokyo, Japan), D-Phe–Cys–Tyr–D-Trp–Orn–Thr–NH_2 (CTOP; Sigma), morphine hydrochloride (morphine; MW = 375.8; Sankyo, Tokyo, Japan), bovine serum albumin (MW = approximately 66,000; Sigma), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma), and N^G-nitro-D-arginine methyl ester hydrochloride (D-NAME; Sigma) were dissolved in saline solution for administration. All control groups were treated with saline. For local peripheral administration, 20 μl of drugs were injected subcutaneously into the right hind paw using a 27 gauge needle 20 min prior to the formalin test. The purity of bovine lactoferrin (95.3%) was certificated by Tatua. We also performed sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on bovine lactoferrin to ensure that no other protein was present in the preparation (data not shown).

2.3. Formalin test

The formalin test was performed according to a method presented in a previous report, with some modifications (Malmberg and Yaksh, 1992). To reduce additional stress to the animal, we chose the minimum concentration (2.0%) and injection volume (50 μl) of formalin that produced a stable flinching behavior in Wistar-Imamichi rats in the preliminary experiment. Rats were given a single subplantar injection of formalin (2.0%, 50 μl) into the right hind paw using a 27 gauge needle before the immediate transfer to a clear perspex observation chamber (base: 20 × 28 cm; height: 15 cm). A mirror placed behind the observation chamber enabled the observer to view the injected hind paw at all times. Nociceptive behavior was quantified by counting the incidents of spontaneous flinching/shaking of the injected paw. Animals were observed individually, and the number of flinches were counted for 1-min periods at 0- to 1-, 5- to 6-, and 5-min intervals during the interval from 10 to 60 min. Immediately after the test, rats were euthanised with an excess dose of pentobarbital (150 mg/kg i.p.).

The data for the phase 1 (0–6 min) and phase 2 (10–60 min) observations were considered separately. In each case, the mean value of saline-treated rats was considered as the control value. We calculated the percentage of analgesia in the respective rats using the following equation:

$$\text{percent analgesia} = (\text{control value} - \text{test value}) / (\text{control value}) \times 100.$$

2.4. Statistics

Data were expressed as the mean ± S.E. Differences between treatment groups were assessed by Student's *t*-test or, when appropriate, one-way analysis of variance followed by Dunnett's posthoc test for multiple comparisons. In all cases, a probability (P) value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Peripheral antinociceptive effects of bovine lactoferrin and morphine in the formalin test

During the formalin test, two phases of spontaneous flinching behavior were observed: Phase 1 started immediately after formalin injection and was decreased at the second observation interval (5–6 min). Phase 2 began at 10 min postinjection, with a maximum response typically observed at approximately 20–30 min (data not shown).

As shown in Fig. 1, peripherally administered bovine lactoferrin (0.128–3.85 nmol/paw) produced a dose-dependent antinociception during both phase 1 and 2. In response to the dose of 0.385 nmol, bovine lactoferrin produced significant antinociceptive effect during both phases ($P<0.05$ vs. control). The peak effects of bovine lactoferrin during phase 1 and 2 were $49.6 \pm 6.3\%$ (3.85 nmol/paw) and $50.0 \pm 2.2\%$ (1.28 nmol/paw), respectively. Thus, we could not calculate the proper ED_{50} values for bovine lactoferrin during both phases. On the other hand, even the highest dose of bovine lactoferrin (3.85 nmol/paw) did not suppress formalin-induced paw oedema (data not shown). No significant antinociception was observed when the highest dose of bovine lactoferrin (3.85 nmol/paw) was injected in the contralateral paw (left paw, data not shown). Thus, in this dose range (0.128–3.85 nmol/paw), bovine lactoferrin produced a peripheral but not a systemic antinociceptive effect. Based on this result, we chose a bovine lactoferrin dose of 1.28 nmol/paw for the subsequent experiments. We also confirmed that recombinant human lactoferrin (1.25 nmol/paw) produced a significant antinociception during both phases ($P<0.005$ vs. control). However, bovine serum albumin (4.5 nmol/paw), another bovine protein, showed no antinociceptive effect.

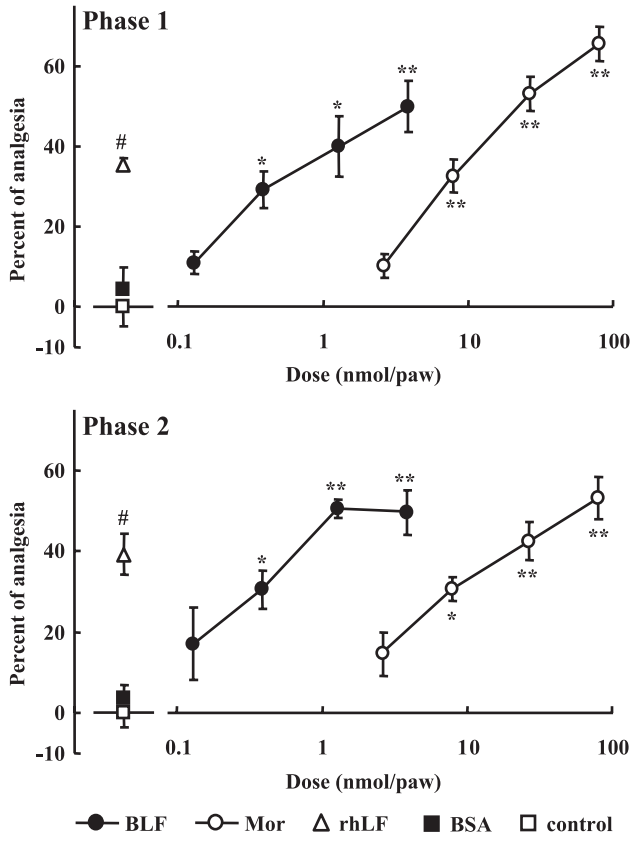

Fig. 1. Peripheral antinociceptive effects of bovine lactoferrin and morphine in the formalin test. All data are presented as the percentage of analgesia during phase 1 and phase 2 of the formalin test. Rats received peripheral administration of saline (control), bovine lactoferrin (BLF, 0.128–3.85 nmol/paw), morphine (Mor, 2.66–79.8 nmol/paw), recombinant human LF (rhLF, 1.25 nmol/paw), or bovine serum albumin (BSA, 4.5 nmol/paw) into the right hind paw ($n=8$ in each group). *$P<0.05$; **$P<0.01$ vs. control (Dunnett's test); #$P<0.005$ vs. control (Student's t-test).

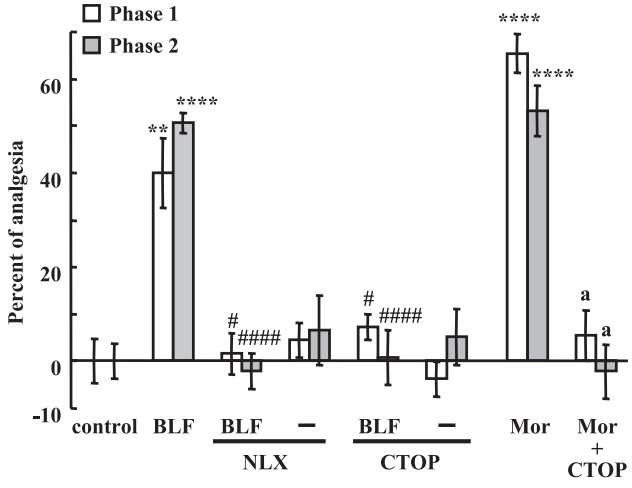

Fig. 2. Effects of opioid receptor antagonists on bovine lactoferrin-induced peripheral antinociception. All data are presented as the percentage of analgesia during phase 1 and phase 2 of the formalin test. Rats received peripheral coadministration of naloxone (NLX, 20 μg/paw) or CTOP (2 μg/paw) with saline (control), bovine lactoferrin (BLF, 1.28 nmol/rat), or morphine (Mor, 79.8 nmol/paw) ($n=8$ in each group). **$P<0.01$; ****$P<0.001$ vs. control (Student's t-test); #$P<0.05$; ####$P<0.001$ vs. BLF (Student's t-test); [a]$P<0.001$ vs. Mor (Student's t-test).

Fig. 1 also shows a dose-dependent antinociceptive effect of peripherally administered morphine (2.66–79.8 nmol/paw). In response to the dose of 7.98 nmol, morphine produced significant antinociception during both phases ($P<0.05$ vs. control). It was also observed that the highest dose of morphine (79.8 nmol/paw) did not produce significant antinociception when injected in the contralateral paw (data not shown). Based on this result, we chose a morphine dose of 79.8 nmol/paw for the subsequent experiments as a positive control.

3.2. Effects of opioid receptor antagonists or a NOS inhibitor on the bovine lactoferrin-induced antinociceptive effect

Peripherally applied bovine lactoferrin (1.28 nmol/paw) produced a significant degree of antinociception during both phase 1 and phase 2 (Figs. 2, 3) ($P<0.01$ vs. controls).

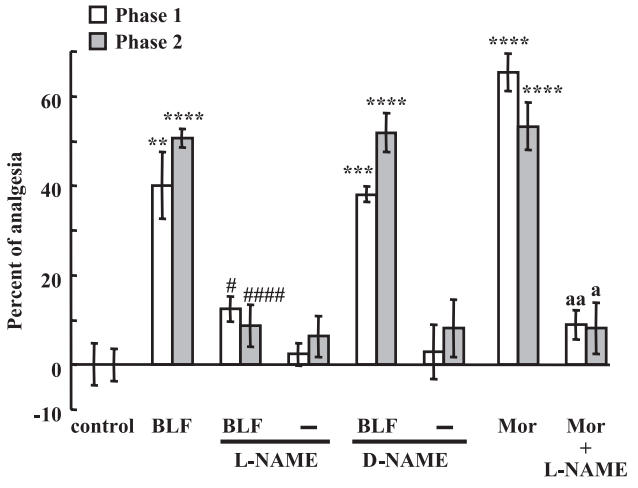

Fig. 3. Effect of a NOS inhibitor on bovine lactoferrin-induced peripheral antinociception. All data are presented as the percentage of analgesia during phase 1 and phase 2 of the formalin test. Rats received peripheral coadministration of L-NAME (100 μg/paw) or D-NAME (100 μg/paw) with saline (control), bovine lactoferrin (BLF, 1.28 nmol/rat), or morphine (Mor, 79.8 nmol/paw) ($n=8$ in each group). $^{**}P<0.01$; $^{***}P<0.005$; $^{****}P<0.001$ vs. controls (Student's t-test); $^{\#}P<0.05$; $^{\#\#\#\#}P<0.001$ vs. BLF (Student's t-test); $^{a}P<0.005$; $^{aa}P<0.001$ vs. Mor (Student's t-test).

Single administration of naloxone (nonselective opioid receptor antagonist; 20 μg/paw) or D-Phe–Cys–Tyr–D-Trp–Orn–Thr–NH_2 (CTOP, selective μ-opioid receptor antagonist; 2 μg/paw) produced no antinociception during either phase (Fig. 2). These doses of opioid antagonists were determined from the preliminary experiment. Peripheral coadministration of naloxone (20 μg/paw) with bovine lactoferrin (1.28 nmol/paw) completely reversed bovine lactoferrin-induced antinociception ($P<0.05$ vs. bovine lactoferrin in both phases). CTOP (2 μg/paw) also significantly reversed bovine lactoferrin-induced antinociception ($P<0.05$ vs. bovine lactoferrin in both phases).

Peripheral injection of a NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 100 μg/paw) and an inactive enantiomer of L-NAME, N^G-nitro-D-arginine methyl ester (D-NAME; 100 μg/paw) produced no antinociception during either phase (Fig. 3). Coadministration of L-NAME (100 μg/paw) with bovine lactoferrin (1.28 nmol/paw) significantly reversed bovine lactoferrin-induced antinociception in both phases on the formalin test ($P<0.05$ vs. bovine lactoferrin). However, coadministration of D-NAME (100 μg/paw) with bovine lactoferrin (1.28 nmol/paw) did not affect bovine lactoferrin-induced antinociception. We also confirmed that morphine-induced antinociception was completely reversed by CTOP (2 μg/paw; $P<0.001$ vs. morphine in both phases) and L-NAME (100 μg/paw; $P<0.005$ vs. morphine in both phases; Figs. 2, 3).

3.3. Potentiation of peripheral μ-opioid receptor-mediated antinociception by bovine lactoferrin

As shown in Fig. 4, doses of 0.128 nmol/paw bovine lactoferrin and 2.66 nmol/paw morphine did not produce significant antinociception per se. However, coadministration of morphine (2.66 nmol/paw) with bovine lactoferrin (0.128 nmol/paw) produced a synergetic antinociceptive effect ($P<0.005$ vs. control, $P<0.05$ vs. bovine lactoferrin, and $P<0.05$ vs. morphine in both phases). This potentiated the antinociception induced by morphine, with the bovine lactoferrin significantly reversed by CTOP (2

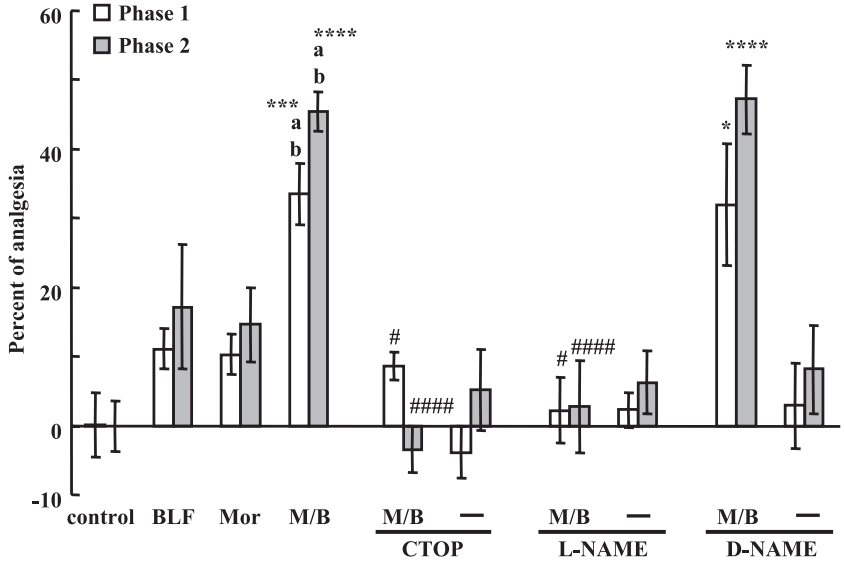

Fig. 4. Potentiation of peripheral μ-opioid receptor-mediated antinociception by bovine lactoferrin. All data are presented as the percentage of analgesia during phase 1 and phase 2 of the formalin test. Rats received peripheral administration of saline (control), bovine lactoferrin (BLF, 0.128 nmol/paw), morphine (Mor, 2.66 nmol/paw), or combination of Mor (2.66 nmol/paw) and bovine lactoferrin (0.128 nmol/paw; M/B) in the presence or absence of CTOP (2 μg/paw), L-NAME (100 μg/paw), or D-NAME (100 μg/paw; $n=8$ in each group). $^{*}P<0.05$; $^{***}P<0.005$; $^{****}P<0.001$ vs. controls (Student's t-test); $^{\#}P<0.05$; $^{\#\#\#\#}P<0.001$ vs. M/B (Student's t-test); $^{a}P<0.05$ vs. BLF (Student's t-test); $^{b}P<0.05$ vs. Mor (Student's t-test).

μg/paw) or L-NAME (100 μg/paw) but not by D-NAME (100 μg/paw).

4. Discussion

In the present study, we demonstrated that bovine lactoferrin exerts an antinociceptive activity via potentiation of the peripheral μ-opioidergic system, and that NO is involved in this potentiation. Recently, we reported that bovine lactoferrin acts as an enhancer of endogenous opioid signaling via NO production in the rat spinal cord (Hayashida et al., 2003b). We also reported that bovine lactoferrin suppressed anxiety-related behavior (body movement and ultrasonic vocalization) induced by maternal separation in rat pups, and that this suppressive function of bovine lactoferrin in the brain was also mediated by NO production (Takeuchi et al., 2003).

In the present study, ispilateral but not contrateral locally administered bovine lactoferrin caused a dose-dependent antinociception (Fig. 1). On the other hand, another bovine protein, bovine serum albumin, did not show an antinociceptive effect. These results suggest that bovine lactoferrin possesses a peripheral antinociceptive effect. It is reported that the presence of lactoferrin receptors have been observed in various peripheral tissues, e.g., immunocytes, liver, intestine, heart, salivary gland, and pancreas (Suzuki and Lonnerdal, 2002). Although lactoferrin receptor has been found in the brain (Faucheux et al., 1995; Leveugle et al., 1996; Qian and Wang, 1998), the presence of LF receptor in the peripheral nerve has not been reported yet. However, present data suggests that lactoferrin possesses a marked inhibitory function in the peripheral nervous system. Thus, it is likely that lactoferrin receptor may exist in the peripheral nerve. Further investigation is required to clarify this point.

The antinociceptive effect of bovine lactoferrin was abolished by naloxone and CTOP, suggesting that the peripheral μ-opioidergic system is involved in bovine lactoferrin-induced antinociception (Fig. 2). We also observed that antinociception induced by peripherally administered morphine was also reversed by CTOP. This result suggests that the activation of the peripheral μ-opioidergic system produced a significant degree of antinociception.

It is reported that peripheral morphine-induced antinociception is mediated by the NO-cGMP pathway (Ferreira et al., 1991; Granados-Soto et al., 1997). It was also found in the present study that coadministration of morphine with a NOS inhibitor significantly suppressed the antinociceptive effect of morphine (Fig. 3). It is reported that a NO precursor, L-arginine, has been shown to produce peripheral antinociception during the formalin test in mice (Kawabata et al., 1994). It is also reported that a NO releaser, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (FK409), dose-dependently enhances the antinociceptive effect of peripherally administered morphine during the formalin test in rats (Nozaki-Taguchi and Yamamoto, 1998). Moreover, other nonenzymatic NO donors, such as sodium nitroprusside and 3-morpholino-sydnonimine, also produce antinociception in a methylene blue (a cGMP phosphodiesterase inhibitor)-reversible manner in hyperalgesic rats (Durate et al., 1990; Ferreira et al., 1991). Thus, NO exerts a pivotal role in the peripheral μ-opioidergic system that modulates antinociception. It is reported that bovine lactoferrin induces NO secretion from macrophages in rats (Sorimachi et al., 1997). Although we did not measure the NO level in the peripheral nerve in this study, we demonstrated the involvement of NO in bovine lactoferrin-induced antinociception in the peripheral opioidergic system. Bovine lactoferrin-induced peripheral antinociception was reversed by a NOS inhibitor (Fig. 3), and a subeffective dose of bovine lactoferrin showed the potentiation of morphine-induced antinociception which was also reversed by a NOS inhibitor (Fig. 4). Based on the present data and the reports described above, we considered that the local administration of bovine lactoferrin enhances the peripheral μ-opioidergic system via NO production.

It is now well known that NOS occurs in three forms: endothelial (e), neuronal (n), and inducible (i). The first two forms are constitutively expressed and Ca^{2+}/calmodulin-dependent, whereas inducible NOS (iNOS) is Ca^{2+}/calmodulin-independent (Anderton et al., 2001). Moreover, It is also well known that neuronal NOS (nNOS) has different splice variants (nNOS-1 and nNOS-2), which mediate different actions. Kolesnikov et al. (1997) reported that nNOS-1 diminishes the antinociceptive actions of the opioidergic system and causes opioid tolerance, whereas nNOS-2 enhances the opioidergic system and causes antinociception without tolerance. On the other hand, spinally applied bovine lactoferrin enhances the μ-opioidergic system via NO production and causes antinociception without tolerance (Hayashida et al., 2003b), suggesting that bovine lactoferrin may stimulate an nNOS-2 system but not an nNOS-1 system in the spinal cord (Teschemacher, 2003). In the present study, peripherally administered bovine lactoferrin also produced antinociception via NO production. Although the mechanism of nNOS activation by bovine lactoferrin has not yet been determined, peripherally administered bovine lactoferrin may stimulate the nNOS system in a manner similar to that of spinally administered bovine lactoferrin.

In the present study, we used lactoferrin that was purified from bovine milk. The lactoferrin 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 C terminus (Teng, 2002). Previously, we reported that intrathecal administration of recombinant human lactoferrin demonstrates a level of antinociceptive activity similar to that of bovine lactoferrin in the rat formalin test (Hayashida et al., 2003a). In this study, we also confirmed that peripherally administered recombinant human lactoferrin produced antinociception during the formalin test (Fig. 1).

Thus, not only bovine lactoferrin but also lactoferrin from different species will produce antinociception.

In the present study, we demonstrated the inhibitory effect of bovine lactoferrin on the nociception induced by formalin-evoked acute inflammation in rats. Under inflammatory conditions that induce nociception, lactoferrin production is increased in the periphery by the neutrophils (Brock, 2002; Levay and Viljoen, 1995). In a related vein, it has been reported that the level of lactoferrin is increased in synovial fluid from rheumatoid arthritis patients (Caccavo et al., 1999). Under some inflammatory conditions, immune-derived opioids cause peripheral antinociception (Cabot, 2001; Stefano et al., 2000; Stein et al., 2001). Thus, under an inflammatory condition, endogenous lactoferrin may reduce peripheral nociception acting in synergy with immune-derived opioids.

In conclusion, we demonstrated the involvement of NO in the potentiation of the rat peripheral μ-opioidergic system by lactoferrin. This peripheral antinociceptive function of lactoferrin involving the NO pathway underscores the physiological importance of the bioactivities of this natural protein. In addition to the antinociceptive effects presented here, lactoferrin has many peripheral functions, including induction of primary defense against bacterial and viral infection, antitumor activity, immunomodulation, and cell growth regulation, that are already known (Brock, 2002). This wide range of lactoferrin activities will potentially be of great benefit to humans and animals with peripheral pain.

Acknowledgements

This investigation was supported in part by Grants-in-Aid for Scientific Research (No. 14360179) from the Ministry of Education, Science, Sports, and Culture of Japan and the Food Science Institute Foundation in Japan to E. Harada.

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