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Research report

Opioid mediated suppressive effect of milk-derived lactoferrin on distress induced by maternal separation in rat pups

Takashi Takeuchi[a,*], Ken-ichiro Hayashida[a], Hideaki Inagaki[b], Masayoshi Kuwahara[b], Hirokazu Tsubone[b], Etsumori Harada[a]

[a]*Department of Veterinary Physiology, Faculty of Agriculture, Tottori University, Tottori 680-0945, Japan*
[b]*Department of Comparative Pathophysiology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*

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Abstract

The present study assessed the effects of bovine milk-derived lactoferrin (bLf) on distress activities induced by maternal separation in 5- to 18-day-old rat pups. The rat pups were injected with BSA (100 mg/kg, i.p.; control) or bLf (100 mg/kg, i.p.) 30 min before the behavioral test. Distress activity was estimated by means of recording body movements or ultrasonic vocalizations (USVs). After 5 min of maternal separation, bLf significantly ($P<0.01$) suppressed body movements, particularly in the 10-day-old pups. This suppressive effect of bLf was reversed by pretreatment with naloxone, CTOP, and norBNI at doses of 0.1–1 mg/kg. Additionally, USVs were also suppressed by bLf, which was reversed by pretreatment with naloxone. Inhibition of nitric oxide synthase (NOS) with nitro-L-arginine methyl ester (L-NAME) dose dependently (3–10 mg/kg) suppressed separation-induced USV production in 10-day-old pups. Interestingly, the suppressive effect of bLf was completely reversed by pretreatment with a low dose (1 mg/kg) of L-NAME, which did not affect the USVs with single application. These findings demonstrate that milk-derived bLf suppresses distress induced by maternal separation via an opioid-mediated mechanism. Furthermore, bLf possibly activates NOS, and an elevated nitric oxide may cause some modification of the opioid system.

Keywords: Milk-derived bovine lactoferrin; Distress; Maternal separation; Opioid; Nitric oxide

1. Introduction

Lactoferrin (Lf) was originally discovered as an iron-binding protein present in milk [30]. A difference of Lf content exists among mammals. Human milk has the highest Lf level (1–5 mg/ml), and guinea pig, mouse, and mare milk has 0.1–1 mg/ml, while cow, goat, and sow milk has 0.01–0.1 mg/ml. Rat, rabbit, and dog milk have a very low Lf level (less than 0.05 mg/ml) [36]. In later studies, the protein was shown to have a much wider distribution; e.g. in tears, saliva, etc., as well as in granules of neutrophils [22,23]. Lf is a glycoprotein, with a molecular weight of about 80 kDa (670–690 amino acid residues), and between Lfs from different species, the sequence identities are ~70% [3]. Lf is a multifunctional protein that has antibacterial, antifungal, antiviral, anti-tumor, anti-inflammatory, and immunoregulatory properties. Recently, we found that administered bLf was transported into cerebrospinal fluid (CSF) via plasma in neonatal and weaned pigs [14]. However, the physiological function of Lf is unknown within the brain, especially in neonatal animals.

Maternal separation of the neonatal rat has been found to produce immediate and enduring distress activities, including ultrasonic vocalizations (USVs), rising behavior, locomotion, etc. These reactions to social isolation are shown only during a limited preweaning period [29]. It has been reported that the maternally separated rat pups in the examination of distress conditions showed a marked increase in rising behavior and crossed more squares and had more head raises [17]. This report clearly demonstrated that separated pups are more active than are their mothered littermates. In addition, rate of myoclonic twitching has been reported as a distress activity, while it was not correlated with rate of USVs [24].

It is well known that the isolation-induced USVs may be

*Corresponding author. Tel.: +81-857-31-5645; fax: +81-857-31-5425.
E-mail address: takeuchi@muses.tottori-u.ac.jp (T. Takeuchi).

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used by pups as distress calls, promoting orientation, approach, and retrieval behavior in lactating dams [1]. Milk may potentially influence the spontaneous rate of distress vocalization emitted by infant rats when they are left by the mother [5]. Modulation of distress vocalization may influence maternal return to the nest or may have deleterious consequences to the infants by advertising their location to predators [5]. Pharmacologically, isolation-induced USVs are attenuated by anxiolytic compounds [19] and nitric oxide synthase inhibitor. Inhibition of nitric oxide synthase (NOS) with nitro-L-arginine methyl ester (L-NAME) effectively suppresses isolation-induced ultrasound production in 10- and 11-day-old rat pups [7].

In addition, evidence is accumulating that endogenous brain opioid systems seem to be involved in the modulation of separation-induced distress vocalization in several animal species, such as guinea pigs, mice, and chicks [16,25,28]. In this regard, the powerful effects of opioids on a variety of developmental processes as well the high sensitivity of young animals to drugs influencing the brain opioid system have been demonstrated [38]. It has been shown that β-casomorphins, which are opioid peptides derived from enzymatic digestion of milk protein (β-casein), significantly affect separation-induced distress vocalization in young chicks [26], and the effects of their prolonged administration on ultrasonic calling has also been reported in rat pups [10]. However, the physiological role of Lf in stress responses is still unknown.

The present study assessed the effects of bovine milk-derived lactoferrin (bLf) on the distress induced by maternal separation in 5- to 18-day-old rat pups. In particular, suckling rats were treated with bLf because they are respectively the most potent natural structures present in milk. Furthermore, a possible mechanism for the suppressive effects of bLf including opioid- and NO-mediated regulatory systems is introduced.

2. Materials and methods

2.1. Animal and housing

A total of 202 Wistar-Imamichi strain rat pups aged from 5 to 18 days and 10 adult rats were used in this study. Wistar-Imamichi rats were obtained from the Institute of Animal Reproduction (Ibaragi, Japan) and housed in a temperature-controlled animal facility with a 12:12 h light–dark cycle (light 07:00–19:00 h). Newborn rats were culled to 10 pups per dam within 12 h of birth. The pups were kept with their dams and were fed food and water ad libitum. The average weight on day 10 was 18.64±0.36 g. Both sexes of pups were used randomly, and each pup was studied once only. Each litter contributed only one pup/experimental condition.

The experimental procedure used in the present study was approved by the Animal Welfare Committee in accordance with the guidelines issued by the Tottori University. All animals were killed with an ether anesthesia at the completion of the studies.

2.2. Apparatus

A body movement was recorded by detecting vibrations produced by the pups. Advantages of this method are the higher sensitivity and conventionally monitoring over a long time course. The pups move accompanying with vibrations, and detecting vibrations is more sensitive than recording the exploring locomotion or cyclonic twitching. In brief, the rat pups aged from 5 to 18 days were put on a thin plastic tray, which was set upon a water-filled balloon. The vibratory movements were recorded by monitoring the oscillation of water pressure. The magnitude of changes in the water pressure was calculated by means of a computer connected with an interface (MacLab, Data Science Inc.), thereafter indicated as an integrated value (Fig. 1A).

Ten-day-old pups were used for measuring USV activity. Testing for USV activity took place in a Plexiglas chamber (26×15×12 cm) with a small Plexiglas cylinder (9 cm in diameter) on the floor. Ultrasonic vocalizations were recorded via an ultrasonic microphone (SF-12DC, Aco, Tokyo, Japan) connected to an amplifier (DAF-1010, Dia Medical System, Tokyo, Japan). The broadband output of the amplifier was then fed into a digitizer (DAF-1020, Dia Medical System, Tokyo, Japan). This digitizer contained band pass filters that were set to 25–35 and 35–45 kHz. When input was detected at one of these frequencies, the digitizer produced a pulse for the duration of the signal. The output of the digitizer was connected via a DAT recorder (PC-204, Sony, Tokyo, Japan) for offline analysis. The data were transferred to a Macintosh computer on which a custom program written using software (MacLab, Data Science Inc.) recorded the occurrence of each ultrasonic vocalization during 10-min intervals (Fig. 1B).

2.3. Drugs

Bovine serum albumin (BSA), nitro-L-arginine methyl ester (L-NAME), nitro-D-arginine methyl ester (D-NAME), naloxone, D-Phe–Cys–Tyr–D-Trp–Orn–Thr–Pen–Thr–NH_2 (CTOP), nor-binaltorphimine (norBNI), and naltrindole were purchased from Sigma (St. Louis, MO), bovine lactoferrin was from Wako Pure Chemical Co., Ltd. (Osaka, Japan), and [^{3}H]DAMGO (Try–D-Ala–Gly–*N*-methyl–Phe–Gly-ol) and [^{3}H]U-69,593 were from Amarsham Pharmacia Biotech (UK).

2.4. Experimental protocol

On the day of the experiments, pups were transported together with their mother in their home cages to the experimental room and left undisturbed for at least 30 min.

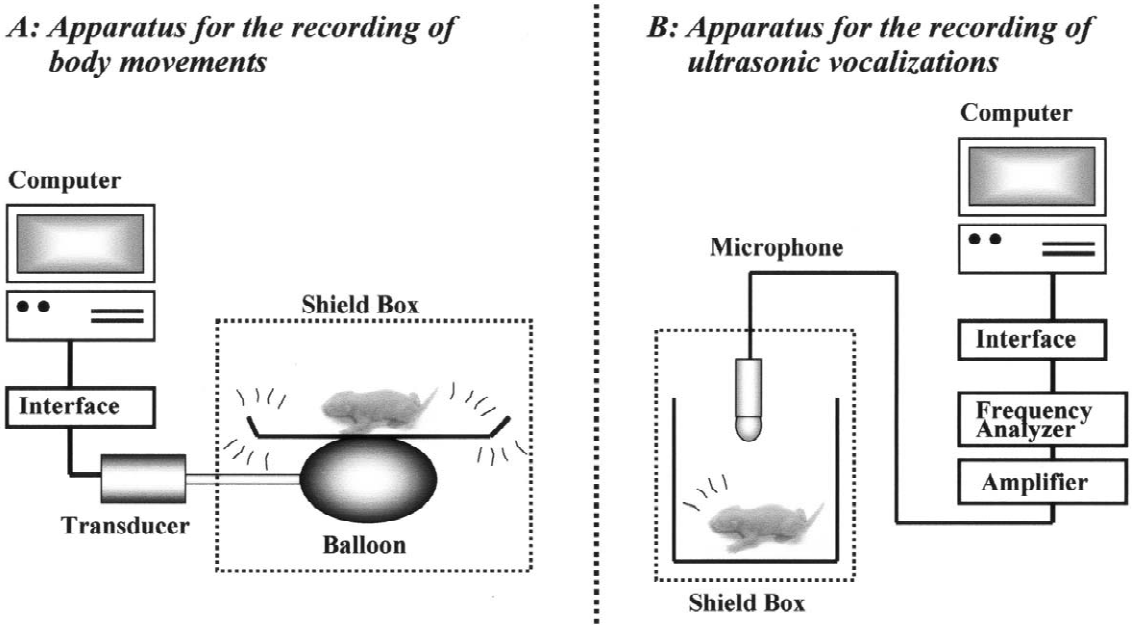

Fig. 1. Apparatus for recording a distress activity induced by maternal separation in rat pups. (A) Body movement was recorded by detecting vibrations produced by the pups. The vibratory movements were recorded by monitoring the oscillation of water pressure. The magnitude of changing in the water pressure was calculated by means of a computer connected with an interface. (B) USVs were recorded via an ultrasonic microphone connected to an amplifier. The broadband output of the amplifier was then fed into a digitizer and transferred to a computer.

The pups were then weighed, marked, and intraperitonealy injected with the drugs (BSA 100 mg/kg, bLf 100 mg/kg, naloxone 0.1–1.0 mg/kg, CTOP 0.1–1.0 mg/kg, norBNI 0.1–1.0 mg/kg, naltrindole 0.1–1.0 mg/kg, L-NAME 1.0–10.0 mg/kg, D-NAME 1.0 mg/kg). The concentration of bLf was determined preliminarily in the range 10–100 mg/kg. After injections, pups were returned to their home cage nests with their mother and littermates for 30 min. Then, each pup was individually placed into the testing chamber for body movement or USV recording. Distress activities were monitored in terms of body movement for 1 h or USVs for 10 min. Each pup then had its body temperature measured using a thermometer (BAT-12, Sensortek, Clifton, NJ, USA).

2.5. Corticosterone assay

Blood samples were collected from 10-day-old pups by heart puncture. These samples were centrifuged at 1000 *g* at 4 °C for 15 min, the plasma separated from the red blood cells and transferred to individual tubes. Plasma corticosterone levels were determined by means of a RIA kit (Amarsham Pharmacia Biotech, UK) in the presence or absence of bLf administration (100 mg/kg, i.p.) following 1 h of maternal separation.

2.6. Radioligand receptor assay

Opioid receptor binding assay was modified from that described by Petrillo et al. [27] with slight modifications. In brief, adult rats that did not experience the maternal separation were killed by cervical dislocation. Brain membrane protein was prepared from whole brains without the cerebellum by homogenization in 0.32 M sucrose solution and repeated centrifugations. The sedimented pellet was resuspended in 0.05 M Tris–HCl buffer (pH 7.4). Incubation mixtures (0.5 ml), which were contained in the final membrane protein 100 μg, were pre-incubated for 10 min at 37 °C to saturate bindings with endogenous opioids. Then the incubation was continued for a further 10 min with different concentrations of ligands, including [^{3}H]DAMGO (spec. act. = 50 Ci/mmol) and [^{3}H]U-69,593 (spec. act. = 41 Ci/mmol) for detection of μ- and κ-subtype opioid receptors, respectively. The specific binding of two radioligands were examined in the range 0.25–8 nM. To assess the effects of bLf on binding affinity with opioid receptors, the mixture was incubated in the presence or absence of bLf (1 nM). The concentration of bLf was determined preliminarily in the range of 10^{-12} to 10^{-5} M. Samples were cooled to 4 °C, filtered through GF-B filters (Whatman, USA), and the filters were then washed under a vacuum with three 5-ml ice cold Tris–HCl buffer solutions. Radioactivity was determined using standard scintillation counting techniques. Dissociation constants (K_d) and the relative number of binding sites per mg protein (B_{max}) were calculated based on Scatchard plot analyses.

2.7. Statistical analysis

Data are expressed as mean ± S.E. Differences between

treatment groups were assessed by Student's *t*-test or, where appropriate, ANOVA followed by Fisher's post-hoc test for multiple comparisons. In all cases a probability (P) value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Developmental changes in distress induced by maternal separation

Fig. 2A shows the value of total activities of body movements during each 20-min period for 1 h in 5- to 18-day-old pups. Body movement in rat pups given BSA (100 mg/kg, i.p.) 30 min before the test showed a higher value with age and reached a maximal level at 10 days old. Although the pups given bLf (100 mg/kg, i.p.) did not show clear effects regarding distress activities until 7 days after birth, a significant suppressive effect of bLf was observed during 0–40 min in the 10-day-old pups ($P<0.05$). In 18-day-old pups, the locomotion activity increased in both groups, and bLf did not suppress this excessive locomotion. bLf showed the strongest effect on 10-day-old pups in regard to the body movement induced by the maternal separation; thus we used the 10-day-old pups for further experiments.

A number of USVs during 10 min of maternal separation in 10-day-old pups is shown in Fig. 2B. The bLf significantly suppressed the USVs of pups at a dose of 100 mg/kg as well as body movement ($P<0.05$).

3.2. Time course of body movement in the 10-day-old pups

Within the first 10 min of recording, BSA-treated pups revealed the highest levels of body movement (Fig. 3). The BSA-treated pups showed a suppression of body movement after these 10 min, and maintained higher levels until 60 min. The bLf treatment had a weak influence on body movement during the first 5 min, and was thereafter strongly suppressed at 10 min, which was maintained up to 60 min. In particular, a significant ($P<0.01$) suppressive effect of bLf was confirmed from 5 to 10-min intervals in the 10-day-old pups. ANOVA confirmed the significant effect of bLf on body movement ($F_{1,118}=23.6$, $P<0.01$). At the end of the test, the body temperature of pups was at 31.5±0.5 °C in the BSA-treated group and 31.8±0.6 °C in the bLf-treated group.

3.3. Effects of opioid receptors antagonists on distress

Naloxone, a non-selective antagonist of opioid receptor, blocked the suppressive effect of bLf on the body movement and USVs in 10-day-old pups in a dose dependent manner during each 20 min for 1 h (Figs. 4A,B). Interestingly, naloxone induced excessive distress activity even in the BSA-injected group, and CTOP, a selective antagonist

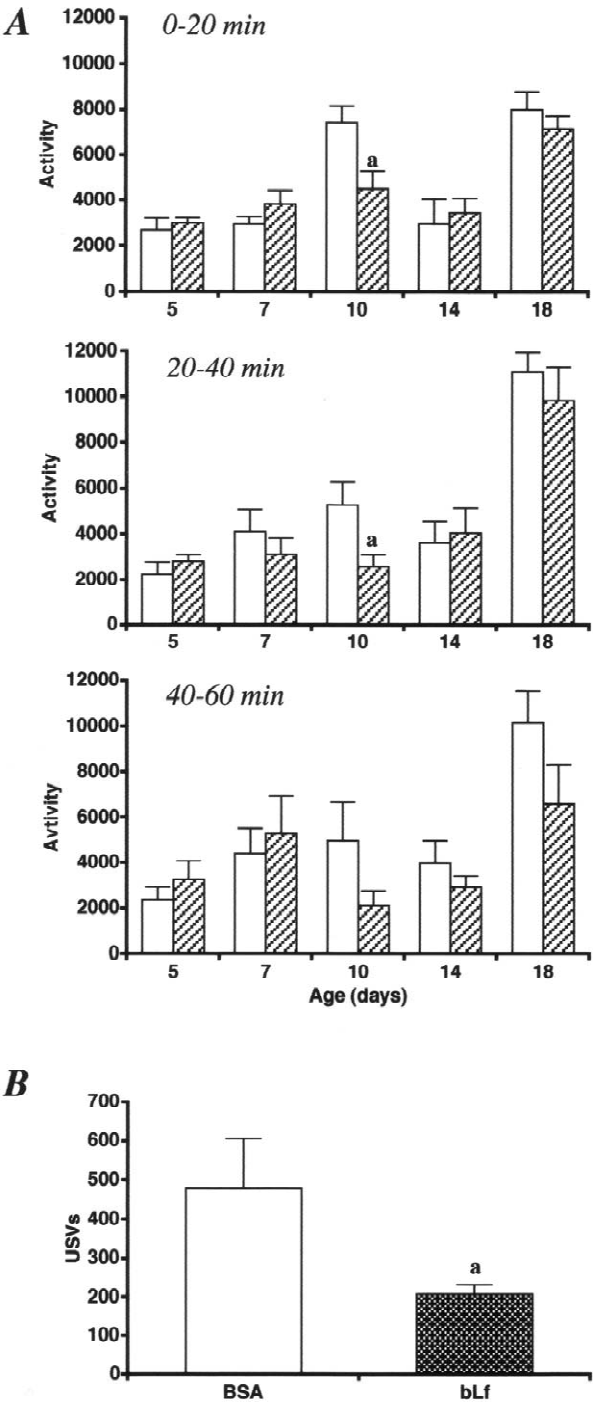

Fig. 2. Developmental changes in the distress activities. The body movement is shown in terms of total activities during 20-min periods in 5- to 18-day-old pups (A). USV activity is shown as the number of occurrences of each USV during 10-min intervals (B). The pups were given BSA (open column, 100 mg/kg, i.p.) or bLf (shaded column, 100 mg/kg, i.p.) 30 min before the test. Each bar shows the mean±S.E. from five pups. [a]Significant difference from the BSA-administered group at $P<0.05$.

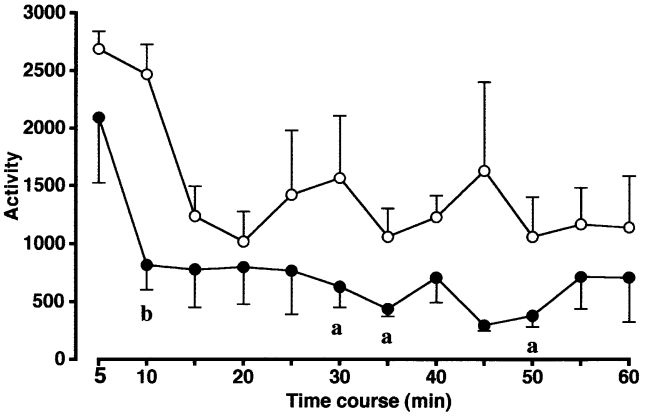

Fig. 3. Time course of body movement during every 5-min interval in the 10-day-old pups. The pups were given BSA (open circle, 100 mg/kg, i.p.) or bLf (closed circle, 100 mg/kg, i.p.) 30 min before the test. Each data point shows the mean±S.E. from five pups. [a,b]Significant difference from the BSA-administered group at $P<0.05$ or $P<0.01$, respectively.

to μ-type receptor, and norBNI, a selective antagonist to κ-type receptor, blocked the bLf effect dose-dependently as well as did naloxone, while norBNI revealed the highest sensitivity in this model. However, naltrindole, a selective antagonist to δ-type receptor, was not clearly influenced by the bLf treatment (Fig. 4A).

3.4. Effects of NOS inhibitors on USVs

We tried to assess the correlation of NO production with the suppressive effect of bLf in the maternally separated pups, because it has been reported that inhibition of NOS with L-NAME effectively suppressed isolation-induced ultrasound production in 10- or 11-day-old rat pups [7]. At first, we checked the influence of L-NAME on the BSA-treated 10-day-old pups (Fig. 5). Pre-treatment with L-NAME suppressed USVs in a dose-dependent manner (3.0–10 mg/kg). This trend was in accordance with the results of previous reports [7]. To examine the involvement of NO in the bLf-induced anti-stress effect, we treated pups with a combination of bLf and L-NAME at a low dose. The L-NAME significantly ($P<0.05$) reversed the bLf-induced suppression at 1.0 mg/kg, which did not show any effect in the BSA-treated pups. However, an inactive isomer of the NOS inhibitor, D-NAME did not affect the bLf-induced suppression in 10-day-old pups at 1.0 mg/kg.

3.5. Plasma corticosterone level

Changes in the plasma corticosterone level induced by maternal separation are shown in Fig. 6. The concentration of corticosterone in non-stressed 10-day-old pups was 31.15±4.29 ng/ml. Following maternal separation for 1 h,

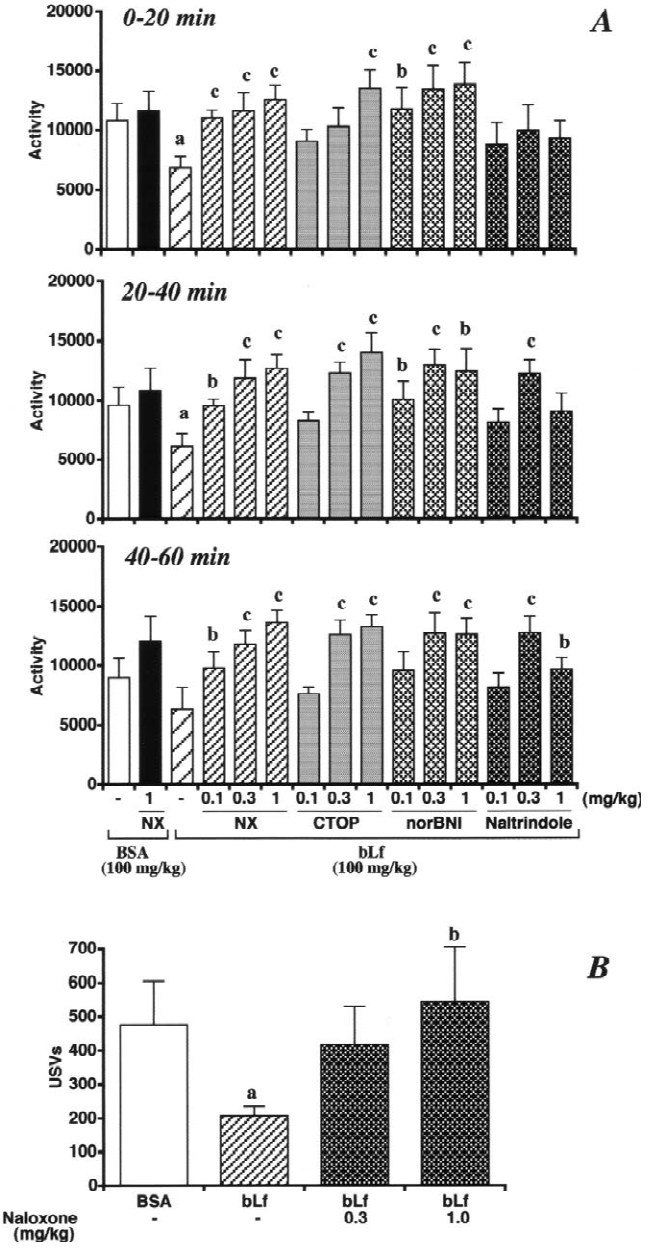

Fig. 4. Effects of opioid receptor antagonists on the distress activities in 10-day-old pups. Total values of the body movements during each 20-min interval are represented (A). Naloxone (NX, 0.1–1.0 mg/kg), non-selective opioid receptor antagonist, or CTOP, norBNI, and naltrindole, the selective antagonists, were intraperitonealy injected 30 min before the test. USV activity is represented as the total number of USVs during 10-min intervals (B). Naloxone (0.3 or 1.0 mg/kg) was intraperitonealy injected 30 min before the test. Each bar represents the mean±S.E. from five pups. [a]Significant difference from BSA alone at $P<0.05$; [b,c]significant difference from bLf alone at $P<0.05$ or $P<0.01$, respectively.

the corticosterone level significantly increased to 60.82±9.88 ng/ml ($P<0.01$), and this increment was significantly decreased to 32.67±6.21 ng/ml by pretreatment with bLf at 30 min before the maternal separation ($P<0.01$).

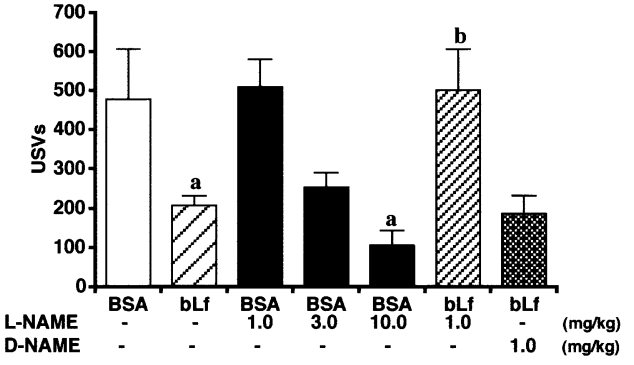

Fig. 5. Effects of nitric oxide synthase inhibitor on USVs in 10-day-old pups. The number of USVs during 10 min of maternal separation is represented. The nitric oxide synthase (NOS) inhibitor, L-NAME (1.0–10 mg/kg, i.p.), and the inactive isomer of NOS inhibitor, D-NAME (1.0 mg/kg, i.p.), were injected with BSA or bLf 30 min before the test. Each bar represents the mean±S.E. from four to six pups. [a]Significant difference from BSA alone at $P<0.05$; [b]significant difference from bLf alone at $P<0.05$.

3.6. Analyses of bLf with specific binding to opioid receptor in the brain

We examined the possibility of bLf modification of the binding reaction of endogenous opioid to μ- and κ-subtypes opioid receptors in the brain. Brain membrane was prepared from whole brain without the cerebellum in adult rats. Specific binding of two radioligands ([^{3}H]DAMGO, and [^{3}H]U-69,593) to μ- (Fig. 7A) and κ-subtypes (Fig. 7B), examined in the range 0.25–8.0 nM, was saturable in the presence or absence of bLf at 1 nM. The bLf did not express clear influences on the specific binding of μ- and κ-subtypes in the brain membrane. The values of K_d and B_{max} of μ- and κ-subtypes in opioid receptors are shown in Table 1. The K_d value in [^{3}H]U-69,593 binding was slightly increased from 13.92±4.35 to 9.75±1.83 nM by the treatment with bLf. B_{max} was slightly reduced from 180.4±41.2 to 131.9±16.6 (fmol/mg protein) by bLf treatment. However, no significant difference was seen between the control and bLf treatment.

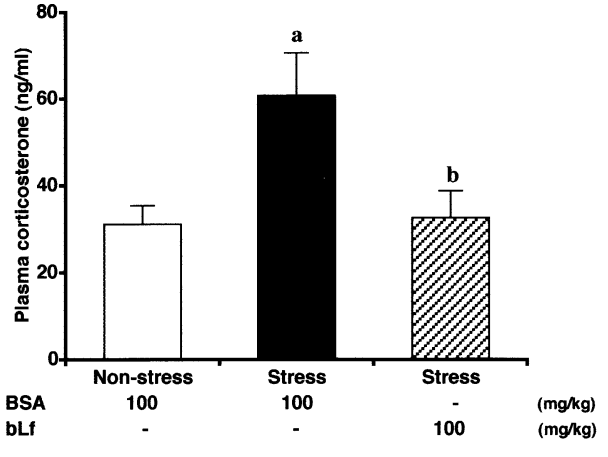

Fig. 6. Effect of pre-treatment with bLf on the plasma corticosterone level in the maternally separated 10-day-old pups. Blood samples were collected from the pups after 1 h of maternal separation. Each bar represents the mean±S.E. from nine pups. [a]Significant difference from non-stress with BSA-administered group at $P<0.01$; [b]significant difference from stress with BSA-administered group at $P<0.05$.

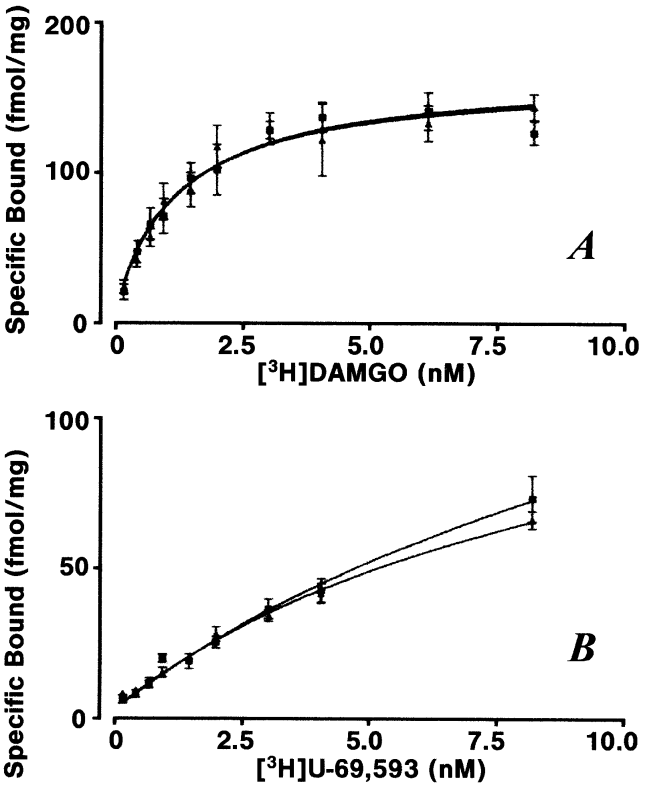

Fig. 7. Effect of treatment with bLf on the specific binding of [^{3}H]-DAMGO or [^{3}H]-U-69,593 with brain membrane of adult rats. Brain membrane protein (100 μg) was incubated with specific radioligands, [^{3}H]DAMGO (A) or [^{3}H]U-69,593 (B) in the range of 0.25–8 nM with the presence (■) or absence (▲) of bLf at 1 nM. Data points represent the mean±S.E. from four experiments.

4. Discussion

In the present study, the data demonstrates a novel suppressive function of bLf for the distress activity induced by maternal separation, particularly in 10-day-old rat pups. Furthermore, our results suggest that the suppressive effect of bLf is mediated by opioid and NO system in the brain. In this regard, bLf is a novel substance activating NOS.

We recorded two different types of behavior, body movement and USVs, in order to estimate distress conditions, and the results regarding the two behaviors showed strong similarities. It has been reported that the exploratory locomotion of maternally separated rat pups in the examination of distress conditions showed a marked increase in rising behavior and crossed more squares and had more

Table 1
Scatchard analysis of [^{3}H]DAMGO and [^{3}H]U-69,593 binding in the rat brain membrane treated with bLf

Treatment	[^{3}H] DAMGO		[^{3}H]U-69,593	
	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)
Control	1.10±0.21	160.2±10.3	13.92±4.35	180.4±41.2
bLf (1 nM)	1.09±0.24	159.0±11.8	9.75±1.83	131.9±16.6

Each value represents mean±S.E. from four experiments.

head raises [17]. This report clearly demonstrated that separated pups are more active than are their mothered littermates. Furthermore, normally mothered pups showed a rapid decline in activity over 6 min, while separated pups more nearly maintained their initial high levels of activity [17]. This suggests that the 2-week-old rat pups are apparently capable of exhibiting increased levels of locomotion and exploratory behavior depending on maternal separation.

A similar suppressive effect on separation-induced distress vocalization in young chicks [26] and rat pups [10] has been found in β-casomorphins, which are opioid peptides derived from the enzymatic digestion of milk protein. β-Casomorphins acts as opioid receptor agonists, and are thus called opioid peptides. However, bLf did not affect the specific binding of ligands ([^{3}H]DAMGO, and [^{3}H]U-69,593) to both μ- and κ-sites at a dose of 1 nM (Fig. 6). Therefore, taken together, the evidence suggests that bLf may enhance an endogenous opioid system under distress conditions. In addition, bLf did not show significant suppression at the first 5-min interval of maternal separation, but it dramatically suppressed distress activity during 5–10-min periods (Fig. 3). These time-dependent effects of bLf may be explained by the time lag in the activation of an endogenous opiate (e.g. β-endorphin, enkephalin, dynorphin) induced by the stress.

In the rodent brain, the concentrations of binding sites for several opioids increase during ontogenesis, whereas binding affinities do not change [8,9,32,35,39]. Petrillo et al. [27] have reported that there occurred no changes in the binding affinities, and the concentration (pmol/g protein) of the κ-site is the first to reach adult levels, namely between 7 and 14 days after birth. Adult levels of μ-sites are attained between 14 and 21 days after birth. Our results regarding the observations of body movements, using selective opioid receptor antagonists, were in accordance with this previous report [27]. In fact, the suppressive effects of bLf were blocked more by norBNI, a selective antagonist to the κ-sites, than by the μ-site as well at the lower dose. Together with these findings, it is suggested that the κ-sites of opioid receptor function as effectively in the 10-day-old pups as do the μ-sites. Furthermore, bLf enhances both κ- and μ-sites related to the opioid system. A similar physiological role of κ opioid receptor during the developmental period is known in analgesia [4]. Thus, the κ opioid receptor may be a principal opioid-mediated regulatory system for anxiety or pain in young pups.

Campbell et al. [7] have reported that the inhibition of NOS decreased USV production dose-dependently in isolated rat pups. Studies on the neuroanatomical development of the NO system have shown that NOS is present from embryonic day 10 [20], and that the distribution of NADPH-diaphorase activity (a neuronal marker for NOS) is adult-like in the first few postnatal weeks in the rat [37]. These reports suggest that the NO system develops early in ontogeny, and is functionally active in 2-week-old pups. Dunn et al. [11] have also reported that a selective nNOS inhibitor, 7-nitroindazole displayed anxiolytic activity, as measured by an elevated plus-maze. Furthermore, they suggested that the anxiolytic activity of 7-nitroindazole was independent of benzodiazepine receptor activation [11].

Homayoun et al. [18] reported the involvement of endogenous opioids and NO in the anticonvulsant effects of stress against pentylenetetrazole (PTZ)- or electroconvulsive shock-induced seizures in mice. They mentioned that pretreatment with non-specific NOS inhibitor, L-NAME (1–30 mg/kg), blocked stress-induced anticonvulsant effects. Lower doses of naloxone (0.3 mg/kg) and L-NAME (2 mg/kg) showed additive effects in blocking the stress-induced anticonvulsant properties. Furthermore, a low dose of morphine (0.5 mg/kg) showed potentiation with stress in increasing the PTZ seizure threshold [18]. This potentiation was reversed by either naloxone or L-NAME at low doses. They concluded that NO synthesis is involved in opioid-dependent stress-induced anticonvulsant effects against electrical and PTZ-induced convulsions [18]. In the present study we confirmed that the suppressive effect of bLf was reversed by either naloxone or L-NAME at low doses. In particular, a low dose (1 mg/kg) of L-NAME, which showed no effect on distress activity alone, blocked the anti-stress effect of bLf. In addition, our data demonstrate that the suppression of USVs caused by BSA, bLf, or L-NAME is not a result of any drug-induced alterations in body temperature. It should be noted, however, that maternal separations are known to affect other physiological systems including heart rate [17].

These finding taken together show that bLf may activate NOS, and in turn increased NO can modulate the opioid-dependent anti-stress effect. Because bLf is more effective under stressful conditions, that might be related to the activated endogenous opioid system. This is in accordance with the results of a previous report in which L-NAME did not alter the susceptibility of non-stressed animals to either

PTZ or electrical seizures [18]. Furthermore, it has been reported that NO and opioids are coupled in a variety of processes including antinociception [12], tolerance and dependence to morphine [21], thermoregulation [6], and gastroprotection [13]. Morphine also stimulates NO production in the vasculature of rat median eminence [33]. Thus, NO production may play an important role in opioid-dependent anti-stress and other effects.

Recently, it has been suggested that following acute stress, NO is produced mainly through constitutive NOS, while iNOS may play an important role during chronic stress [34]. The dual effects of lower and higher doses of NOS substrate or NO donors have been reported [31] and excessive NO is thought to inhibit its own synthesis [2]. These mechanisms may explain the reversed effect of L-NAME dose-dependently in the USVs production in our experiment, although further research is needed in this regard. In addition, it is necessary to investigate the relation of bLf with neuroactive steroids, such as allopregnanolone and pregnenolone sulfate, because pregnenolone sulfate, a precursor of progesterone has behavioral effects that are opposite those of allopregnanolone, a metabolite of progesterone, via negative or positive modulation of $GABA_A$ receptor [40,41].

The passage of macromolecules contained in plasma into the brain is limited by the blood–brain-barrier (BBB) and blood–CSF barrier. These barriers to proteins appear to be well formed in fetal and newborn animals, because mature, tight junctions have been observed between cerebral endothelial cells and the choroids plexus. Recently, Harada et al. [14,15] demonstrated the transfer of bLf or porcine Lf into CSF from systemic circulation after oral administration or natural suckling in neonatal or weaned pigs. Furthermore, we also detected bLf in plasma after intraduodenal infusion of bLf (1 g/kg) in our preliminary rat experiment. This transported bLf from lumen into the circulation was further transported into the CSF even in the adult rats (in preparation). These findings suggest that not only a homologeous but also a heterologous Lf can be absorbed from the intestine and be transported into the brain in adult rats. Thus, Lf plays an important role in physiologically regulated brain function, especially under distress conditions. In particular, the pups under the distress condition may be protected from various damage by Lf treatment. However, it is still unknown how Lf successfully reaches neurons, and up-regulates NOS activity within the brain. Further research is required to clarify these points.

In summary, the present study demonstrates that bLf has a suppressive effect on acute distress induced by maternal separation in rat pups. bLf possibly activates NOS, and an elevated NO can modulate the opioid-dependent anti-stress mechanism. These findings suggest the possible application of the safety substance from milk as a way of controlling distress.

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