Bovine lactoferrin has a nitric oxide-dependent hypotensive effect in rats

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Hayashida, Ken-ichiro, Takashi Takeuchi, Takeshi Ozaki, Hirohiko Shimizu, Kunio Ando, Atsushi Miyamoto, and Etsumori Harada. Bovine lactoferrin has a nitric oxide-dependent hypotensive effect in rats. Am J Physiol Regul Integr Comp Physiol 286: R359–R365, 2004. First published October 16, 2003; 10.1152/ajpregu.00214.2003.—Lactoferrin (LF) is a multifunctional protein that is found in milk, neutrophils, and other biological fluids. Under inflammatory conditions, LF production is increased in the periphery by neutrophils. However, the cardiovascular function of LF is still unknown. In the present study, we investigated the effect of bovine LF (BLF) on the mean blood pressure (MBP) and heart rate (HR) in urethane-anesthetized rats and the vascular function of BLF in the rat thoracic aorta. Intravenous injection of BLF produced dose-dependent decreases in MBP but did not affect HR, while the opioid agonist morphine decreased both MBP and HR. The hypotensive effect of BLF was not altered by naloxone methiodide, which cannot pass through the blood-brain barrier, but was significantly reduced by naloxone hydrochloride, which does pass through the blood-brain barrier. BLF-induced hypotension was completely blocked by the NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) but not by the inactive enantiomer of L-NAME, N\textsuperscript{G}-nitro-D-arginine methyl ester (D-NAME). BLF-induced hypotension was not altered by the muscarinic ACh receptor antagonist atropine or the cyclooxygenase inhibitor diclofenac. BLF produced relaxation in endothelium-intact vessels but not endothelium-denuded aortic rings precontracted with phenylephrine. The relaxation evoked by BLF was completely blocked by L-NAME but not by D-NAME or the ATP-sensitive potassium channel blocker glibenclamide. These results suggest that BLF causes hypotension via an endothelium-dependent vasodilation that is strongly mediated by NO production and that BLF-induced hypotension also may be mediated by the central opioidergic system.

\[ \text{N}^\text{G}-\text{nitro-L-arginine methyl ester; naloxone hydrochloride; naloxone methiodide; endothelium cell} \]

EPIDEMIOLOGICAL EVIDENCE implies that the consumption of milk and dairy products is inversely related to blood pressure (BP) and the risk of hypertension. The National Health and Nutrition Examination Survey I, an epidemiological study conducted in the United States from 1971 to 1975 involving over 20,000 people, found that a diet low in dairy products was predictive of hypertension (28). Other large population studies have also reported an inverse association between the consumption of milk and BP (1, 17, 34). In addition, milk supplementation has been shown to lower BP in some clinical studies (5, 7). There is some evidence of an inverse association between the intake of total protein or animal protein and BP (31, 34).

Based on this evidence, we were interested in lactoferrin (LF), a natural iron-binding protein in milk. LF is a single-chain glycoprotein with a molecular mass of \( \sim 80 \text{kDa} \) that belongs to the family of transferrins (6). LF has many peripheral functions, inducing primary defense against bacterial and viral infection, antitumor activity, immunomodulation, and cell growth regulation (6). However, there are no published studies on the cardiovascular effects of LF so far. Under inflammatory conditions, LF production is increased in the periphery by neutrophils (6, 23) and in the central nervous system (CNS) by the microglia (16). LF is also transported from blood to the cerebrospinal fluid through the blood-brain barrier via receptor-mediated transcytosis (14). It is also reported that orally administered bovine milk-derived LF (BLF) inhibits formalin-evoked nociception (19), carrageenan-induced inflammation with immunomodulation (46), and vascular endothelial growth factor-mediated angiogenesis (30) in adult rats. Thus not only endogenous but also exogenous LF are effective in adult mammals, and the investigation of the cardiovascular action of LF should have physiological importance.

It is reported that LF binds to specific high- and low-affinity sites on the capillary endothelial cells (EC) (14). After binding to EC, LF crosses from the apical to the abluminal surface without being degraded, possibly via caveolae (14). On the other hand, it is well known that EC produces different vasodilating agents, such as nitric oxide (NO), prostacyclin (PGI\textsubscript{2}), and endothelium-derived hyperpolarizing factor, and plays an important role in the control of vascular tone. Although the actions of LF on/in the EC are not clear yet, it is reported that BLF increases NO production in the macrophages (36). It is also reported that EC contains the \( \mu \)-opioid receptor that is coupled to NO release and vasodilation (38). Indeed, there are several reports that intravenously administered opioid agonists cause NO-dependent vasodilation (8, 9, 10). Moreover, opioids also act in the CNS and cause systemic hypotension (11, 39). On the other hand, LF and its receptor have also been identified in the CNS (13, 15, 16, 24, 25, 32, 33, 41). Recently, we reported that BLF produces \( \mu \)-opioid receptor-mediated antinociceptive activity (19), and this BLF-induced analgesia is mediated by NO production (20). Although various types of opioid ligands have been found in milk or milk digests (43), BLF did not bind to the \( \mu \)-opioid receptor nor did it change its binding affinity to the opioid ligands (40). Thus BLF does not act as an opioid agonist but rather as an enhancer of endogenous opioid signaling (20).
In this study, we investigated the effect of the intravenous administration of BLF on BP and heart rate (HR) in urethane-anesthetized rats and the vascular function of BLF in the rat thoracic aorta. The possible mechanism of action was also studied pharmacologically.

MATERIALS AND METHODS

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

Drugs
For in vivo experiments, BLF (mol wt ~78,000; Tatabu, Morrisville, New Zealand), bovine serum albumin (BSA; mol wt ~66,000; Sigma, Tokyo, Japan), naloxone hydrochloride (Sigma), naloxone methiodide (Sigma), morphine hydrochloride (Sankyo, Tokyo, Japan), dihydrogenica (Sigma), acetylcyanone hydrochloride (ACH; Daiichi, Tokyo Japan), atropine sulfate (atropine; Fuso, Osaka, Japan), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma), and Nνω-nitro-D-arginine methyl ester hydrochloride (D-NAME; Sigma) were dissolved in saline solution for administration at a volume of 1 ml/kg. For in vitro experiments, BLF, ACh, L-NAME, D-NAME, L-phenylephrine hydrochloride (Sigma), and sodium nitroprusside dihydrate (SNP, Sigma) were dissolved in saline solution for administration at a volume of 1 ml/kg. 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and the changes in MBP by BSA or BLF are summarized in Fig. 1B. As shown in Fig. 1, A and B, BSA increased MBP slightly but not significantly compared with the baseline value (from 111.0 ± 4.3 to 114.0 ± 4.8 mmHg). In contrast, the intravenous administration of BLF dose dependently lowered the MBP. We also observed that the hypotensive response to BLF is repeatable (data not shown). Based on our results, we chose a BLF dose of 384 nmol/kg for the subsequent experiments.

Time course of the cardiovascular effect of BLF. In the second set of experiments, the time course of the effects on HR and MBP of an μ-opioid agonist, morphine (133 nmol/kg iv), and BLF (384 nmol/kg iv) were measured. Baseline values of MBP were 138.2 ± 8.5 mmHg in the BLF-treated group and 133.1 ± 11.0 mmHg in the morphine-treated group; baseline values of HR were 317.0 ± 16.8 and 306.2 ± 23.0 beats/min, respectively (Fig. 2). Morphine caused a marked decrease of both MBP (−44.3 ± 10.1%) and HR (−67.8 ± 16.8%) at 10 s after administration, after which MBP and HR recovered rapidly. In contrast, the maximum hypotensive effect of BLF was observed at 60 s after the administration (−31.0 ± 4.0%), and the MBP showed a slower recovery. However, BLF did not affect HR.

Role of the opioidergic system. In the third set of experiments, the effects of opioid antagonists on responses to BLF (384 nmol/kg iv) and morphine (133 nmol/kg iv) were investigated. Responses were measured 5 min after intravenous administration of opioid antagonists, namely naloxone hydrochloride, which passes through the blood-brain barrier, and naloxone methiodide, which cannot pass through it. Neither opioid receptor antagonists altered the MBP when injected at a dose of 10 mg/kg iv. However, the lowering of MBP in response to morphine (−39.2 ± 3.6%) was significantly blocked by naloxone hydrochloride (−6.6 ± 1.2%) and naloxone methiodide (−7.6 ± 1.8%) (Fig. 3). The hypotensive effect of BLF was not altered by naloxone methiodide. The low dose (2 mg/kg) of naloxone hydrochloride did not affect the BLF response; however, the higher dose of 10 mg/kg of naloxone hydrochloride significantly reversed the hypotensive response to BLF from −29.3 ± 3.0 to −14.1 ± 4.5%.

Role of NO production. In the fourth set of experiments, the role of NO release in mediating hypotensive effects of BLF (384 nmol/kg iv), ACh (100 ng/kg iv), and morphine (133 nmol/kg iv) was investigated. The actual values of MBP are represented in Fig. 4A, and the changes in MBP are summarized in Fig. 4B. Responses were measured following intravenous administration of the NO synthase inhibitor l-NAME (50 mg/kg), and the NO synthase inhibitor was found to increase MBP (Fig. 4A). In the presence of l-NAME, decreases in MBP in response to BLF, ACh, and morphine were significantly reduced (Fig. 4B). On the other hand, the inactive enantiomer of l-NAME, d-NAME (50 mg/kg iv), did not affect the hypotensive effect of BLF.

Role of muscarinic ACh receptor and PG production. In the fifth set of experiments, the involvement of a muscarinic ACh receptor in mediating the hypotensive effects of BLF (384 nmol/kg iv) and ACh (100 ng/kg iv) was investigated. Responses were measured 5 min after intravenous administration of the muscarinic ACh receptor antagonist atropine (0.1 mg/kg), which by itself did not alter MBP. In the presence of atropine, however, ACh-induced hypotension was completely abolished by atropine. In contrast, the hypotensive effect of BLF was not affected by atropine (Fig. 5).

In the last series of experiments, the role of PG release in mediating the hypotensive effect of BLF (384 nmol/kg iv) was investigated 15 min after the administration of a cyclooxygenase inhibitor, diclofenac (3 mg/kg iv). The administration of diclofenac (3 mg/kg iv) did not alter MBP. This dose of diclofenac (3 mg/kg iv)
Diclofenac was chosen based on the report that diclofenac (3 mg/kg iv) completely inhibits PG production after intravenous injection of arachidonic acid in rats (18). Diclofenac did not affect BLF-induced hypotension (data not shown).

**In Vitro Experiment**

**BLF-induced relaxation in the rat thoracic aorta.** To clarify the vascular function of BLF, we performed isolated vascular ring myography. First, we investigated whether BLF causes relaxation of the rat thoracic aorta. The responses to BLF (1–10 μM) in the endothelium-intact and -denuded aortic rings precontracted with phenylephrine (1 μM) are presented in Fig. 6A and summarized in Fig. 6B. BLF caused concentration-dependent relaxation in the endothelium-intact aorta. Endothelial denudation completely abolished the BLF-induced relaxation.

**Role of NO and ATP-sensitive potassium channel.** We then investigated the role of NO in the BLF-induced relaxation of the aortic rings. In the presence of L-NAME (100 μM), BLF-induced relaxation was completely abolished (Fig. 7A). This concentration of L-NAME also abolished ACh (5 μM)-induced relaxation. On the other hand, D-NAME (100 μM) did not affect BLF-induced relaxation.

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Fig. 4. Influence of nitric oxide (NO) synthase inhibitor on the hypotensive effects of BLF, ACh, and morphine. Actual values in MBP (A) and changes in MBP (B) in response to the intravenous injection of BLF (384 nmol/kg), ACh (100 ng/kg), and morphine (133 nmol/kg) in the presence or absence of Nω-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg iv) or Nω-nitro-D-arginine methyl ester (D-NAME; 50 mg/kg iv) are shown. *P < 0.05, ****P < 0.001 vs. control (unpaired t-test).

Fig. 5. Influence of a muscarinic ACh receptor antagonist on the hypotensive effects of BLF and ACh. Changes in MBP in response to the intravenous injection of BLF (384 nmol/kg) and ACh (100 ng/kg) in the presence or absence of atropine (0.1 mg/kg iv) are shown. ****P < 0.001 vs. control (unpaired t-test).

Fig. 6. BLF-induced relaxation in the rat thoracic aorta. Typical responses to BLF in endothelium-intact (E+) and endothelium-denuded (E−) aortic rings precontracted with phenylephrine (Phe) are represented in A, and data are summarized in B (see MATERIALS AND METHODS). ****P < 0.001 vs. endothelium-denuded group (unpaired t-test). SNP, sodium nitroprusside.
The hypotensive effect of intravenously administered morphine is mainly mediated by the peripheral opioidergic system, while the hypotensive effect of intravenously administered BLF is mediated by the central opioidergic system. The present data also suggest that the hypotensive effect of BLF is not mediated by the activation of peripheral opioid receptors, including the μ₂-opioid receptor in the EC. In addition to the peripheral tissues (e.g., EC), an LF receptor has also been identified in the CNS (13, 33). It is reported that BLF enters into the cerebrospinal fluid via receptor-mediated transcytosis (14). However, the maximum hypotensive effect of BLF was observed at 60 s after the intravenous injection in this study (Fig. 2). It is unlikely that intravenously administered BLF enters the CNS and acts on its receptor within 60 s. BLF may influence the central opioidergic system from outside the blood-brain barrier, although its mechanism is difficult to explain from the present data. The precise mechanism of BLF-induced hypotension seems to be rather complicated and to require further investigation.

We next investigated the role of NO in mediating BLF-induced hypotension. NO is known as an endothelium-derived relaxing factor that causes vasodilation and controls vascular tone. It is reported that the blood pressure-buffering effect of endogenous NO is mediated by endothelial NO synthase (eNOS) (37). In the present study, BLF-induced hypotension was completely abolished by L-NAME (Fig. 4). We also found that BLF induced relaxation in the rat thoracic aorta precontracted with phenylephrine (Fig. 6). Endothelium denudation completely abolished BLF-induced relaxation. L-NAME also completely abolished the BLF-induced relaxation (Fig. 7A). These results suggest that BLF induces vasodilation via activation of eNOS, which produces NO in the CNS (44). The hypotensive effect of BLF is not mediated by the muscarinic ACh receptor (Fig. 5) or the peripheral opioid receptor, which are well known to cause vasodilation via NO production from eNOS (3). BLF may activate eNOS via its receptor-mediated mechanism or via direct interaction with eNOS, as in the case of the heat shock protein 90 (44). In addition to the peripheral hypotensive effect of NO, several studies suggest that NO is involved in the central regulation of the cardiovascular functions (2, 12, 21, 22). However, since the maximum hypotensive effect of BLF was observed at 60 s after the intravenous injection in this study, it is unlikely that intravenously administered BLF produces NO in the CNS within 60 s.

It is reported that NO activates the ATP-sensitive potassium channel in the vascular smooth muscle cell (29). The activation of the ATP-sensitive potassium channel causes the hyperpolarization of the membrane potential and is well known to reduce vascular excitability. Thus we investigated the role of the ATP-sensitive potassium channel in mediating BLF-induced relaxation in the aortic rings (Fig. 7B). We found that the selective ATP-sensitive potassium channel blocker glibenclamide did not affect BLF-induced relaxation. This result suggests that the ATP-sensitive potassium channel is not involved in BLF-induced vascular relaxation.

In addition to NO, prostacyclin (PGI₂), which is produced by the EC, is well known to cause vasodilation. There are no
published data on whether LF affects PGI_{2} production from the EC. However, it is reported that BLF inhibits PGE_{2} production from the macrophages in vitro (3). Based on this report, we investigated the role of PG synthesis in BLF-induced hypotension. We found that BLF-induced hypotension did not change in the presence of a cyclooxygenase inhibitor (data not shown), suggesting that no PG-mediated mechanism is involved in BLF-induced hypotension.

The infants of many mammalian species constantly ingest exogenous LF from their mother’s milk (26). As LF is an ubiquitous protein in the bodily fluids (4, 23, 25, 27, 32, 41, 45), endogenous LF is also available to both infants and adult animals and may participate in the physiological blood pressure buffer system. Under inflammatory conditions, LF production is increased in the periphery by neutrophils (6, 23, 45). Thus, when inflammation occurs, BLF-induced vasodilation has biological importance. Especially in local inflammation, BLF-induced vasodilation may increase the blood supply to the injury sites and facilitate their healing. Several studies reported that orally administered BLF shows various functions in adult rats (19, 30, 46). Thus not only endogenous but also exogenous LF can act in adult animals.

We used LF purified from bovine milk in the present study. The LPs of humans, boids, mice, and pigs share 70% overall amino acid sequence and 100% identity in several stretches of 10–15 amino acids at the COOH terminus (42). In addition, we reported that recombinant human LF possesses a level of antiinflammatory activity similar to that of BLF in the rat formalin test (19). Although we used BLF and no LF from other species in this study, it is likely that not only BLF but also LF from different species may possess hypotensive effects.

In summary, the present study demonstrates that BLF produces hypotension via an endothelium-dependent vasodilatation, which is strongly mediated by NO production, and that BLF-induced hypotension is also possibly mediated by the central opioidergic system. In addition to the effect of BLF on the vascular system presented here, LF has many physiological functions, including inducing the primary defense against bacterial and viral infection, anti-inflammation, and antinociception (6, 19, 46). This wide range of LF activity suggests that LF has physiological importance.

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