Direct evidence of nitric oxide production induced by lactoferrin and its enhancement by magnesium ions in cultured endothelial cells

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ABSTRACT. Bovine lactoferrin (BLF) reportedly lowers blood pressure and induces vasorelaxation, but its effect on nitric oxide (NO) production has not been established. Accordingly, we aimed to determine whether BLF induces NO production in bovine aortic endothelial cells, and the effects of extracellular free magnesium (Mg) ion concentrations on this NO production. BLF induced NO production time-dependently. NO production was markedly inhibited by the NO synthase inhibitor, L-NAME, in an effect abolished by L-arginine, but not D-arginine. NO production was suppressed at low concentrations, and enhanced at high concentrations, of Mg ions in culture medium. These results suggest that BLF has an important role in hypotensive effects. Mg ions may affect BLF-induced NO production.

KEYWORDS: bovine aorta endothelial cell, cell culture, lactoferrin, magnesium, nitric oxide production

Bovine lactoferrin (BLF) is a natural iron-binding protein found in milk, and in vivo and in vitro experiments have indicated that it may have nitric oxide (NO)-dependent hypotensive and vasorelaxant effects. BLF-induced relaxation is abolished by removal of endothelium in rat aortic rings [4]. BLF increases NO production in macrophages [13] and contributes to opioid-mediated analgesia [5], antinociception [3], and lipopolysaccharide-induced diarrhea [14]. However, there is no direct evidence that BLF induces NO production in vascular endothelial cells. Magnesium (Mg) is the fourth most abundant mineral in the body, represents the second most abundant cation in cells after potassium, and is a cofactor in more than 300 enzymatic reactions involved in energy metabolism and protein and nucleic acid synthesis in the body [2, 8]. Mg also plays an important role in regulating and lowering blood pressure [6]. Accordingly, in the present study, we aimed to determine whether BLF can induce the production of NO in cultured bovine aortic endothelial cells (BAECs) and how extracellular Mg ion concentration affects BLF-induced NO production.

BAECs were isolated from the aortas of slaughtered Japanese black beef cattle and cultured in a growth medium containing 90% Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum, and an antibiotic mixture of 100 units/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B, as described previously [11]. They were isolated using a single gentle scrape with a surgical blade (No 10; Feather Kogyo, Tokyo, Japan) and characterized based on their morphology using phase-contrast microscopy (IX70, Olympus, Tokyo, Japan) and by staining for fluorescent acetylated low-density lipoprotein [15]. Endothelial cells with fewer than six passages were used. Confuent endothelial cells (3–5 × 10⁶) were treated with BLF and four different concentrations of Mg ions.

The following reagents were used: D-arginine, DMEM, Hanks’ balanced salt solution (HBSS), L-arginine, N’-nitro-L-arginine methyl ester (L-NAME) hydrochloride, penicillin, streptomycin, amphotericin B (Sigma-Aldrich Co., St. Louis, MO, USA), heat-

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inactivated fetal calf serum (Invitrogen Corp., Waltham, MA, USA), acetylcholine hydrochloride (Daiichi-Sankyo, Tokyo, Japan), NO₂/NO₃ assay kit (Fujifilm Wako, Osaka, Japan), and fluorescent acetylated low-density lipoprotein (Harbor Bio-Product, Norwood, MA, USA). When nitrite (NO₂⁻) and nitrate (NO₃⁻) levels were measured as indicators of NO production, 10% fetal calf serum was not used, and either 100% DMEM without phenol red, or 100% HBSS without phenol red, was used to avoid disturbance of the fluorometric assay [10]. Sampling times for quantifying NO were set at 1, 3, and 12 hr or 3, 6, and 12 hr after exchanging the new medium and treating with BLF. Results are expressed as mean ± standard error of the mean (SEM). Statistical analyzes were performed using Student’s t-test or Bonferroni test after one-way analysis of variance (Stat View J-4.5, Abacus Concepts Inc., Piscataway, NJ, USA). The significance level was established at P<0.05.

As shown in Fig. 1A, treatment with BLF (10 µM) yielded a time-dependent increase in NO production in cultured BAECs. Although the untreated BAECs showed spontaneous NO production, the NO production was clearly enhanced with BLF treatment. In this experiment, 10 µM of BLF was used as the concentration at which the submaximal response was observed in a previous in vitro study [4]. To investigate the mechanism of BLF-induced NO production, NO synthase (NOS) inhibitor L-NAME, L-isomer of arginine (L-arginine), and D-isomer of arginine (D-arginine) were added to the BAEC cultures. As shown in Fig. 1B, NO production induced by BLF (10 µM) was significantly inhibited by L-NAME (0.1 mM) at 3 hr. The inhibitory effect of L-NAME was completely abolished at 1 hr and largely abolished at 3 hr by L-arginine (1 mM) but it was not abolished by D-arginine (1 mM). A 1 mM concentration was thus set for D-arginine, to confirm that D-arginine did not abolish the inhibitory effect of L-NAME (0.1 mM) at the same concentration that L-arginine did. These results suggested that BLF induces NO production via the NO pathway in endothelial cells, and the results of this cell culture study are consistent with those of previous in vivo and in vitro studies suggesting that BLF produces hypotension via endothelium-dependent vasodilation, which is strongly mediated by NO production [4].

Before investigating the effect of Mg ions on NO production induced by BLF, we compared NO production in two different cell culture media, DMEM and HBSS, because Mg ion concentrations in the HBSS medium may be easily susceptible to alteration. Figure 2A shows a comparison of BLF-induced NO production in DMEM or HBSS at 3, 6 and 12 hr. The two media yielded no significant difference in NO production at 3, 6, or 12 hr. Therefore, HBSS was selected as the medium for use in a comparison of BLF-induced NO production at four different Mg ion concentrations (0.2, 0.4, 0.8, and 1.6 mM). The 0.8 mM Mg ion concentration was regarded as representing a normal level, since serum concentrations of Mg ions range from 0.65–10.5 mM in adults [8]. As shown in Fig. 2B, BLF-induced NO production was increased in a concentration-dependent manner and was found to be significantly higher with a treatment of 1.6 mM Mg ions versus treatment with 0.2 mM Mg ions, at 3, 6 and 12 hr. These results are similar to those reported in studies in which treatment with Mg lithospermate B [9] and Mg tanshinoate B [12] increased NO production in endothelial cells. We consider that an increased Mg ion concentration may induce phosphorylation of endothelial NOS (eNOS) via activating the PI3K/AKT pathway and enhancing NO production, because Mg is a cofactor in more than 300 enzymatic reactions involved in energy metabolism and protein and nucleic acid synthesis [8, 9]. In alveolar macrophages, however, Mg-deficient medium enhanced NO production by activating inducible NOS (iNOS) [16]. Thus, an increase in Mg ions might activate eNOS, but inactivate iNOS. The different mechanisms for the actions of Mg ions on eNOS and iNOS are still unknown, but Mg ions reportedly may increase eNOS expression while decreasing iNOS expression [1, 7]. Elucidation of the mechanism of magnesium-induced enhancement of NO production is a future challenge.

In conclusion, BLF induced NO production in BAECs. This result is consistent with those of previous in vivo and in vitro studies. High concentrations of Mg ions might enhance NO production induced by BLF, whereas a low concentration of Mg ions could reduce it.
CONFLICT OF INTEREST. The authors declare no conflict of interest.

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