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# Milk basic protein increases ghrelin secretion and bone mineral density in rodents

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ABSTRACT

*Objectives:* Milk basic protein (MBP), a mixture of proteins isolated from bovine milk, is known to increase bone formation. Ghrelin, a stomach-derived peptide hormone, also has been reported to stimulate osteoblast formation. The aim of this study was to determine whether MBP-induced bone formation is mediated via ghrelin.
*Methods:* MBP was chronically administered to mice in their drinking water for 3 wk, and body weight, water intake, and bone mineral density were measured. Additionally, plasma bone-specific alkaline phosphatase, tartrate-resistant acid phosphatase isoform 5b, and ghrelin concentrations were determined by enzyme-linked immunosorbent assay. To examine the direct effect of MBP on ghrelin secretion, gastric tissue culture and primary mucosal cells were stimulated by MBP.
*Results:* The in vivo study of young, growing mice showed that chronic MBP intake for 3 wk increased the plasma ghrelin concentration and bone mineral density of the hind limb tibia. In vitro studies using minced rat gastric mucosa tissues and primary murine isolated gastric mucosal cells revealed that MBP stimulated ghrelin release in a dose-dependent manner. Moreover, MBP-induced ghrelin secretion was partly inhibited by adrenergic blockers.
*Conclusions:* These findings suggest a novel mechanism by which MBP directly acts on ghrelin secretion. Additionally, the elevated ghrelin level induced by MBP may act as a mediator for bone formation.

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## Introduction

Milk basic protein (MBP) is a mixture of positively charged proteins extracted from bovine milk. Strong evidence suggests that MBP promotes bone health, thus MBP is commercially available as a functional food. Several results concerning the effect of MBP on bone have been accumulated in animal models and human trials. For example, MBP promotes the proliferation of osteoblastic MC3 T3-E1 cells, the production of bone matrix protein type I collagen [1], and the inhibition of osteoclastic pit formation in rabbit osteoclasts [2]. Additionally, oral administration of MBP suppressed osteoporosis in an ovariectomized rat model [3]. In human trials, the intake of MBP supplements by different age groups improved bone turnover and increased bone mineral density (BMD) [4–8]. Although MBP shows phenotypic activity on bone metabolism and helps maintain bone health, it is still unknown whether MBP affects bone directly or indirectly.

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Endocrine hormones such as insulin-like growth factor (IGF)-1, thyroid hormone, and sex steroids are involved in bone metabolism [9–11]. Ghrelin, a 28-amino acid peptide mainly produced in the gastric mucosa, has shown involvement in bone formation [12,13]. It has been reported that ghrelin stimulates the proliferation of human osteoblastic TE85 cells via the nitric oxide/cyclic guanosine monophosphate signaling pathway [12]. Moreover, Ma et al. demonstrated that growth hormone secretagogue receptor (GHS-R) null mice have a low bone mass phenotype, and ghrelin regulates bone remodeling through GHS-R in osteoblasts by modulating the cAMP-responsive element-binding and Runx2 pathways [13]. Additionally, ghrelin and its specific receptor, GHS-R, are expressed in rat primary osteoblast-like cells and ghrelin stimulates the proliferation of osteoblastic cells [14,15]. Thus, previous studies have suggested that ghrelin is directly involved in bone formation.

In this study, we hypothesized that MBP affects bone metabolism via ghrelin secretion from gastric ghrelin-producing cells. To verify this hypothesis, we examined the effect of MBP on ghrelin secretion in mice in vivo and also studied the direct effect of MBP on ghrelin secretion using minced gastric mucosal tissues and primary isolated gastric mucosal cells.

## Methods

### *Preparation of milk basic protein*

MBP was prepared as previously described [2]. Briefly, fresh bovine milk was defatted by centrifugation and loaded onto a column packed with sulfonated Chitopearl CS cation exchange resin (Fuji-Bouseki, Tokyo, Japan). The column was washed with deionized water and positively charged proteins bound to the column were eluted with 1 M sodium chloride. The eluate was dialyzed using a cellulose membrane tube (MWCO 14000 Da; Sanko-Junyaku, Tokyo, Japan), freeze-dried, and the lyophilized powder was used as MBP. The protein concentration of MBP was about 98% and contained several minor nonprotein components.

### *Animals and diets*

Six-wk-old male C3 H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan) were divided into two groups ($n = 10$) for experiments to study the effects of chronic administration of MBP. The mice had access to a modified AIN-76 diet [16] (Table 1) and water ad libitum. For the gastric tissue culture study, 8-wk-old Wistar male rats were allowed access to commercial chow for rodents (CE-2; CLEA Japan, Inc.) and water ad libitum. Gastric primary cell cultures were prepared from male C57 BL/6 J mice ages 8 to12 wk provided with CE-2 and water ad libitum. All animals were housed individually in stainless-steel cages in a temperature- and humidity-controlled room (23°C and 40 ± 5% relative humidity) on a 12/12 light/dark cycle under specific pathogen-free conditions. Animals were treated in accordance with the National Institutes of Health guideline for the care and use of laboratory animals and the studies were approved and performed in accordance with the Committee on Animal Research of Saitama University and Milk Science Research Institute, Megmilk Snow Brand Co, Ltd.

**Table 1**
Component of modified AIN-76 diet

| | g/kg diet |
|---|---|
| Casein | 200 |
| DL-Methionine | 3 |
| α-Corn Starch | 150 |
| Sucrose | 500 |
| Cellulose | 50 |
| Corn oil | 50 |
| Mineral mix (AIN-76) | 35 |
| Vitamin mix (AIN-76) | 10 |
| Choline bitartrate | 2 |
| Total | 1000 |

α-corn starch (i.e., pregelatinized cornstarch) was used in this study instead of cornstarch to improve the physical property of the diet and to enable mice to eat it easily

### *In vivo study of the chronic effect of MBP using mice*

Six-week-old male C3 H/HeJ mice were divided into two groups: One group was provided with deionized water (control group) and the other with 1% MBP-containing deionized water (MBP group) as drinking water for 3 wk. Body weight and the amount of food and water consumed were measured for each mouse every other day during the experiment. After 3 wk of administration, micro-computed tomography analysis (μCT) was performed using an R_mCT (Rigaku Corp., Tokyo, Japan). For in vivo scanning, mice were anesthetized with isoflurane, and computed tomography images of the left hind limb were obtained under the following conditions: tube voltage, 90 kV; tube current 100 μA; rotation, 360°; magnification, ×10; exposure time, 17 s; resolution, 20 μm. The data were collected and reconstructed by using the imaging software I-View (J. Morita Mfg. Corp., Kyoto, Japan). BMD at the diaphysis in the tibia ranging 3.63 mm from the joint of the tibia and fibula to the proximal was analyzed by using the three-dimensional bone morphology software TRI/3D-BON (RATOC System Engineering Co. Ltd., Tokyo, Japan). For BMD determination, a hydroxyapatite (HA) calibration curve was prepared from images of phantoms (HA contents: 200–1550 mg/cm$^3$).

After the administration schedule was complete, the mice were deeply anesthetized with isoflurane and blood was withdrawn from the abdominal inferior vena cava into tubes containing 0.5 M EDTA pH 8.0 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1 mM *p*-hydroxymercuribenzoic acid (PHMB; Sigma-Aldrich, St. Louis, MO, USA). The plasma fraction was obtained by centrifuging each blood sample at 3000*g* at 4°C for 30 min and was stored at −80°C until analysis. The plasma concentration of IGF-1 was determined using a mouse/rat IGF-1 immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's guidelines. Bone-specific alkaline phosphatase (BAP; Cusabio Biotech Co. Ltd., Wuhan, China) and tartrate-resistant acid phosphatase isoform 5b (TRACP 5b; Immunodiagnostic Systems Ltd., Boldon, England) were measured by enzyme-linked immunosorbent assay, and acylated ghrelin (AG) was determined using a rat AG enzyme immunoassay kit (Bertin Pharma, Montigny-le-Bretonneux, France) according to the manufacturers' instructions.

### *Ghrelin secretion study using rat gastric tissue culture*

Stomach tissue culture was performed as previously described [17]. Briefly, male rats (CLEA Japan) were sacrificed under deep anesthesia with isoflurane and the stomachs were quickly removed. After washing with saline, the mucosa of the gastric corpus was cut into pieces (~1 mm$^3$) with a sharp razor blade in serum and phenol red-free Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Inc.). The minced tissues were divided equally between each well of a 24-well culture plate, then incubated with medium containing different doses of MBP (final concentrations in the medium of 0.1, 1, 5, or 10 mg/mL), 10 mg/mL whey protein isolate (WPI; Arla Foods AMBA, Aarhus, Denmark), or 10 mg/mL bovine serum albumin (BSA; Iwai Chemicals Co., Ltd., Tokyo, Japan) for 30 min at 37°C in humidified 95% air and 5% carbon dioxide. The incubated media were collected and stored at −80°C until measurement of the ghrelin concentration.

### *Ghrelin secretion experiment using a primary murine ghrelin cell culture*

Primary gastric mucosal cells from adult male C57 BL/6 J mouse stomach were isolated according to the procedure of Sakata et al. [18]. A hemocytometer was used to determine the concentration of the isolated cells. A total concentration of $1 \times 10^5$ cells per well was seeded in 24-well plates and incubated in humidified 95% air and 5% carbon dioxide at 37°C. After 24 h, the medium was slowly aspirated from the cells and serum-free DMEM (Thermo Fisher Scientific, Inc.) containing 50 μM sodium octanoate and 5 mM glucose with MBP (0.1, 1, 5, or 10 mg/mL) or norepinephrine hydrochloride (Sigma-Aldrich) was added. For the β-adrenergic antagonist experiments, cells were pretreated with 10 μM propranolol (Sigma-Aldrich) or 10 μM atenolol (Sigma-Aldrich) for 1 h before MBP treatment. The cells were incubated for 6 h in humidified 95% air and 5% carbon dioxide at 37°C, then the medium was collected from each well and centrifuged at 6000*g* for 5 min at 4°C. An aliquot of each supernatant (100 μL) was transferred to a separate tube, 1 N HCl (10 μL) was immediately added, and then the samples were stored at −80°C until analysis.

### *Statistical analysis*

All data are expressed as the mean ± SEM and statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, CA, USA). Data were standardized as the percentage of the control group and were analyzed by Student's *t* test or one-way analysis of variance with Tukey's post hoc tests. Differences were considered statistically significant at $P < 0.05$.

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