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Purification and identification of lactoperoxidase in milk basic proteins as an inhibitor of osteoclastogenesis

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ABSTRACT

A milk protein fraction with alkaline isoelectric points (milk basic protein, MBP) inhibits both bone resorption and osteoclastogenesis for in vitro models. We previously identified bovine angiogenin as a component of MBP that inhibits bone resorption. However, purified angiogenin had no effect on osteoclastogenesis, suggesting that MBP contains unidentified component(s) that inhibit osteoclast formation. In this study, we purified lactoperoxidase (LPO) as the predominant inhibitor of osteoclastogenesis in MBP. The LPO treatment downregulated levels of reactive oxygen species in osteoclasts. Signaling by receptor activator of NF-kappa-B ligand/receptor activator of NF-kappa-B (RANKL/RANK) was downregulated in LPO-treated cells, and, in particular, the ubiquitination of tumor necrosis factor receptor associate factor 6 (TRAF6) and activation of downstream signaling cascades (JNK, p38, ERK, and NFκB) were suppressed. Ultimately, LPO treatment led to decreased expression of c-Fos and NFAT2. These results suggest that MBP contains at least 2 components that independently suppress bone resorption through a unique mechanism: angiogenin inhibits bone resorption and LPO inhibits RANKL-induced osteoclast differentiation. These data explain many of the positive aspects of milk consumption on bone health.
Key words: milk basic protein, lactoperoxidase, osteoclast

INTRODUCTION

Among foods, the calcium contained in milk is extremely bioavailable, and milk consumption has pronounced effects on bone metabolism (Kawakami, 2005). We previously showed that treatment of young ovariectomized rats with a milk protein fraction with alkaline isoelectric points (milk basic protein, **MBP**) led to increased femoral bone strength. In aged ovariectomized rats, a well-known model of osteoporosis, MBP treatment prevented bone loss (Toba et al., 2000). Supplementation with MBP also increased bone mineral density and suppressed urine levels of bone resorption biomarkers such as cross-linked *N*-teleopeptides of type-I collagen in healthy women (Aoe et al., 2001; Yamamura et al., 2002; Itabashi, 2006; Uenishi et al., 2007), menopausal women (Aoe et al., 2005), and healthy older women (≥65 yr; Aoyagi et al., 2010). In particular, MBP suppresses osteoclast-mediated bone resorption and leads to reduced osteoclast numbers in animal studies (Uenishi et al., 2007). These results suggest that MBP contains one or more substances that modulate bone metabolism by suppressing bone resorption and osteoclastogenesis.

We have been working to identify the protein fraction(s) responsible for the observed activities of MBP, and we previously reported that bovine angiogenin acts as a bone resorption-inhibitory protein in MBP (Morita et al., 2008). Angiogenin is an angiogenic factor and a member of the ribonuclease family (Komolova and Fedorova, 2002), and its presence in and purification from bovine milk has been reported previously (Maes et al., 1988; Ye et al., 1999; Komolova and Fedorova, 2002). Although we demonstrated that angiogenin inhibits osteoclast-mediated bone resorption, purified angiogenin had no effect on osteoclastogenesis.

In this study, we aimed to identify the component(s) contained within MBP that inhibit osteoclastogenesis, and we demonstrate that lactoperoxidase (**LPO**; EC 1.11.1.7) exhibits this activity when purified from milk. Lactoperoxidase is a heme peroxidase that uses hydrogen peroxide (H_2O_2) as an electron donor for the oxidation of thiocyanate to generate an antimicrobial agent. We examined the biological activity of LPO using an in vitro system of osteoclastogenesis.

MATERIALS AND METHODS

Reagents

Milk basic protein was prepared as described previously (Uenishi et al., 2007). Alpha-modified Eagle's

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medium (**α-MEM**) and fetal bovine serum (**FBS**) were purchased from Invitrogen (San Diego, CA) and Thermo Trace (Melbourne, Australia), respectively. Eagle's MEM (**E-MEM**) was from Nissui Pharmaceutical Co. (Tokyo, Japan), and SP-Sepharose Fast Flow and HiLoad 16/60 Superdex 75 pg were obtained from GE Healthcare (Buckinghamshire, UK). Human macrophage colony-stimulating factor (**M-CSF**; Leukoprol) was purchased from Kyowa Hakko Kirin (Tokyo, Japan). Other chemicals used in the study were purchased from Wako Pure Chemicals (Osaka, Japan). For reverse transcription (RT)-PCR, the following primer sets from Gene Design (Osaka, Japan) were used: *rank*: 5′-GCC CAG TCT CAT CGT TCT GC-3′ and 5′-TAG CTG TCA GCG CTT TCC CT-3′; *c-fms*: 5′-CCG GCC CAC TCT TGG AAT TT-3′ and 5′-AGA CCG TTT TGC GTA AGA CCT-3′; *gapdh*: 5′-CAT GTT CCA GTA TGA CTC CAC TC-3′ and 5′-GGC CTC ACC CCA TTT GAT GT-3′.

In Vitro Osteoclastogenesis and Osteoclast-Mediated Bone Resorption

Bone marrow macrophages (**BMM**) were prepared from 6- to 9-wk-old male C57BL/6 mice as described previously (Ogawa et al., 2006) with some modifications. In brief, mice were killed by cervical dislocation (according to the guidelines of the animal ethical committee of Nara Institute of Science and Technology (NAIST), and bone marrow cells were collected by flushing the femurs and tibias with α-MEM. Red blood cells were removed by treatment with an ammonium chloride solution. After washing, cells were cultured in α-MEM supplemented with 10% FBS and 1% conditioned medium generated from NIH3T3/pCAhMCSF cells (**M-CSF-CM**; Yogo et al., 2006). After 12 to 16 h, nonadherent cells were collected and cultured a further 3 d in α-MEM supplemented with 10% FBS and 3% M-CSF-CM. RAW264 cells were maintained in E-MEM containing 10% FBS without M-CSF. For examining osteoclastogenesis in vitro, RAW264 cells or BMM were seeded at a density of 1 to 2 × 10^4 cells/cm^2 and incubated overnight. Glutathione S-transferase (**GST**) and purified GST-RANKL (receptor activator of NF-kappa-B ligand), hereafter called RANKL, prepared as described (Meiyanto et al., 2001), were added to the culture medium at a final concentration of 250 to 500 ng/mL (RANKL) or 125 to 250 ng/mL (GST) with 30 ng/mL of M-CSF (d 0). On d 3, cells were restimulated with RANKL and M-CSF. On d 4 or 5, cells were fixed and stained for tartrate-resistant acid phosphatase (**TRAP**) as described previously (Collin-Osdoby et al., 2003).

Purification of the Bone Resorption Inhibitory Protein by Column Chromatography

Milk basic protein (2.5 g) was dissolved in 10 m*M* sodium phosphate buffer (pH 7.0) and loaded onto an SP-Sepharose column (25 mm × 155 mm) equilibrated with the same buffer at a flow rate of 5 mL/min. The bound proteins were washed with buffer and eluted in a NaCl gradient in buffer as follows: 0 to 125 min, 0 to 0.6 *M*; and 125 to 200 min, 0.6 to 1.5 *M*. Inhibition of osteoclast formation was assessed by monitoring the formation of TRAP-positive multinucleated (**MN**) cells as described above. Fractions with inhibitory activity were pooled, concentrated with Amicon Ultra 15-Ultracel 5k (Millipore, Billerica, MA), and loaded onto a HiLoad 16/60 Superdex 75 pg column (16 mm × 600 mm) equilibrated with 10 m*M* sodium phosphate buffer (pH 7.0) containing 150 m*M* NaCl at a flow rate of 0.2 mL/min. The fraction containing inhibitory activity was loaded onto a YMC-Pack Protein-RP column (4.6 mm × 250 mm) equilibrated with 10% acetonitrile–0.1% trifluoroacetic acid (**TFA**) buffer (solution A) at a flow rate of 1 mL/min. The bound proteins were eluted with a gradient of 90% acetonitrile-0.1% TFA buffer (solution B) as follows: 0 to 4 min, 0% (vol/vol); 4 to 5 min, 0–25% (vol/vol); 5 to 40 min, 25 to 60% (vol/vol); 40 to 45 min, 60 to 90% (vol/vol); and 45 to 50 min, 90% (vol/vol). The elution profiles were monitored by measuring the absorbance at 280 nm, and inhibition of osteoclastogenesis was monitored.

Cell Viability Assay

Bone marrow macrophages were seeded at 1 × 10^4 cells per well of a 96-well plate. After overnight incubation, medium was replenished with LPO-supplemented medium (10 ng to 1 mg/mL) and incubated for another 48 h. After washing with PBS, a 1:10 dilution of WST-8 (Cell Counting Kit-8, Dojindo, Kamimashiki-gun, Kumamoto, Japan) in PBS was added to each well to determine the cell viability.

Immunoprecipitation and Western Blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (10 m*M* Tris-HCl, 1% Nonidet P40, 0.1% deoxycholic acid, 0.1% SDS, 150 m*M* NaCl, and 1 m*M* EDTA) containing 2 m*M* phenylmethylsulfonyl fluoride, 2 m*M* Na_3VO_4, 20 m*M* NaF, and 100 kIU/mL aprotinin. Immunoprecipitation and Western blotting were carried out as described using protein A-Sepharose (GE Healthcare Bioscience KK, Tokyo, Japan; Yogo et al., 2006). Immobilon Western Chemiluminescent HRP

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