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Nutritional and biochemical studies on the regulatory mechanisms of osteoclastogenesis

2016

School of Natural Science and Ecological Awareness,
Graduate School of Humanities and Sciences,
Nara Women’s University

Atsuko Nakanishi

Doctoral Thesis
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Background

Bone metabolism

Bone is a dynamic organ that continuously undergoes osteoclast-mediated bone resorption and osteoblast-mediated bone formation. This restructuring process, called ‘bone remodeling’ is needed to remove old or damaged bone in order to insure biomechanical stability and to regulate mineral homeostasis (1, 2). The first step in remodeling is the adhesion of mature osteoclasts to the bone surface, creating a specialized and isolated acid microenvironment (3). Second, the acid dissolve the bone mineral matrix, particularly cathepsin K, that degrade organic matrix. Bone resorption ends when osteoclasts moved away or died by apoptosis. After a certain bone is removed, bone formation takes place in which the cement line is laid down. Osteoblasts then synthesize matrix, which become mineralized (4).

Normally, the equal amount of bone removed will be replaced in the succeeding phase, so no permanent bone loss occurs. Commonly speaking, the nutritional factors, hormones, and diseases could have a marked influence on bone metabolism. Excessive osteoclast activity relative to bone formation leads to pathological bone resorption (5); that is an imbalance between bone resorption and bone formation, resulting in structural deterioration, bone fragility, and osteoporosis. In this phase, resorption is much faster than formation, an area of bone that can be resorbed in 2-3 weeks, however, it takes at least 2-3 months to rebuild it. In fact, bone is remodeled approximately 10 per cent of the skeleton a year in adult vertebrates.
It takes more than 10 years to be replaced entire skeleton (4, 6, 7).

**Bone cells**

Bone has three distinct cell types: osteoblasts, osteocytes, and osteoclasts, which are respectively responsible for the formation, maintenance, and resorption of bone. The osteoblasts are mononucleated, not terminally differentiated ‘bone-forming’ cells deriving from mesenchymal stem cells. They first secrete osteoid, which consists mainly of collagen, and then osteoblasts secrete alkaline phosphate to create sites for calcium and phosphate deposition. Some osteoblasts become trapped in their own bone matrix, giving rise to osteocytes which gradually stop secreting osteoid (8). Osteocytes are thought to control osteoclastogenesis and/or osteoblastogenesis for these decades (9-11). The osteoclasts are the giant multinucleated cells arising from hematopoietic stem cells. They moved to specific sites on the surface of bone and secrete acid phosphatases, hydroxyl chloride, and several proteases to remove bone matrix.

**Osteoclastogenesis**

Osteoclastogenesis is a multi-complex procedure that includes many stages, such as commitment, differentiation, multinucleation, and activation of immature osteoclast. The osteoclasts are derived from hematopoietic stem cells, which give rise to bone marrow cells, and that can further differentiate into monocyte/macrophage precursors, and osteoclasts. The
common and essential factor of osteoclast formation is receptor activator of nuclear factor-κB ligand (RANKL), and macrophage colony-stimulating factor (M-CSF). RANKL and M-CSF act through their receptors c-Fms and receptor for activation of NF-κB (RANK), respectively. (7, 12, 13).

At early stages of osteoclastogenesis, M-CSF stimulates RANK expression in osteoclast precursor cells (c-Fms+RANK−), and induces late-stage precursor cells (c-Fms+RANK+). Hematopoietic transcription factor, PU.1 encoded by the SPI1 gene, and microphthalmia-associated transcription factor (MITF) transactivate RANK expression (14). At later stages, RANKL·RANK interaction activates many transcription factors, including NF-κB, activator protein-1 (AP-1), c-Fos, c-Jun and nuclear factor of activated T cells cytoplasmic 1 (NFATc1) in osteoclast precursors, which then differentiate into mononuclear osteoclasts. NFATc1 induces osteoclast-specific gene expression, such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K. At the end of the osteoclastogenesis, mononuclear osteoclasts fuse to form multinucleated mature osteoclasts (7, 15) and then, activation to bone resorbing osteoclasts.

**Thesis statement**

I have studied nutritional regulation mechanisms of osteoclastogenesis in vitro and in vivo. In this thesis, I describe the effects of ROS and n-3 PUFAs on osteoclastogenesis especially at early stages. Attention must be directed toward the distinction between early-
stage osteoclast precursor cells (c⁻Fms⁺RANK⁻) and late-stage osteoclast precursor cells (c⁻Fms⁺RANK⁺). RANKL-induced intracellular reactive oxygen species (ROS) production is required for late-stage osteoclast differentiation (16, 17). However, the role of ROS in early-stage osteoclast precursor is poorly understood. Estrogen deficiency results in loss of bone mass and can lead to the development of osteoporosis. In addition, dietary n-3 polyunsaturated fatty acids (PUFAs), rich in fish oil have beneficial effect on bone. In chapter 1, I describe the molecular basis for M-CSF-induced ROS generation using early-stage precursor cells: I found the difference the population of these cell types; early-stage osteoclast precursor cells and late-stage osteoclast precursor cells including the murine monocyte cell line, RAW264.7 cells. So in chapter 2 and 3, I investigate the effect of fish oil particularly on early stages of osteoclastogenesis using ovariectomized rats and using various cell culture systems.
References


Chapter 1
The production of reactive oxygen species through a NADPH oxidase in early-stage osteoclast precursor cells

Introduction

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical are generated from endogenous sources such as mitochondria, peroxisomes, and inflammatory cell activation (1), and exogenous sources such as xenobiotic compounds. When their production exceeds the antioxidant capacity in a cellular system, oxidative stress occurs. It has been suggested that oxidative stress is implicated in the pathogenesis of atherosclerosis, cancer, diabetes, and aging (2). However, ROS have been recognized to play a role as a second messenger in various receptor signaling pathways at low levels (3-6).

ROS were reported to regulate the survival or proliferation of monocyte/macrophages induced by macrophage colony-stimulating factor (M-CSF) (7-9). For example, ROS were suggested to play a role in cellular survival in M-CSF-treated human monocyte (7), and in osteoclast differentiation induced by receptor for activation of nuclear factor-κB ligand (RANKL) (10-12). Furthermore, osteoclasts have been shown to produce ROS during resorption (13-15), which leads to fragmentation of collagen and other proteins (16). However, little attention has been given to the role of ROS at the early stages of osteoclastic differentiation and cellular survival.
The plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is recognized as one of the major players in the generation of ROS (17). Aside from the neutrophil Nox2 isoform (18), other Nox isoforms have been discovered and the ROS they generate are suggested to act as messengers in the activation of specific signaling pathways (19). In bone marrow-derived hematopoietic stem cells Nox1, Nox2 and Nox4 were reported to be expressed (20). The expression of Nox2, lower levels expression of Nox1, and Nox1-mediated ROS generation by RANKL were reported in bone marrow-derived monocyte/macrophages (10). It was also suggested that a flexible compensatory mechanism existed between Nox1 and Nox2 for RANKL-stimulated ROS generation to facilitate osteoclast differentiation (21). However, the source of oxidant generated by M-CSF remained to be defined in early-stage osteoclast precursors.

In this study, I investigated the molecular basis for M-CSF-induced ROS generation in the early stages of osteoclastic differentiation. We found that ROS generation by M-CSF stimulation was mediated by Nox2, and was required for the expression of RANK associated with the expression of PU.1 and MITF. In these processes, we revealed that ROS generation was occurred within 5 min after the M-CSF stimulation. Moreover, Nox inhibitor significantly suppressed osteoclastic differentiation of osteoclast precursor cells without affecting cell viability. These data suggested that Nox2-mediated ROS production was required for the RANK expression and osteoclast differentiation in early-stage osteoclast precursor cells.
**Materials and methods**

**Materials**

Recombinant murine M-CSF and recombinant soluble RANKL were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Minimum Essential Medium (MEM), diphenylene iodonium (DPI), and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Penicillin streptomycin, sodium pyruvate, and non-essential amino acid (NEAA) were from Life Technologies (Carlsbad, CA, USA). Fetal Bovine Serum (FBS) was purchased from Biological Industries, Israel Beit-Haemek Ltd. (Kibbutz Beit-Haemek, Israel) and Ficolle paque plus were from GE Healthcare, UK Ltd. (Buckinghamshire, England), respectively.

**Preparation of the bone marrow osteoclast precursor cells**

Bone marrow cells from the femur and tibia of 8-week-old Wistar/ST female rats (Japan SLC, Shizuoka, Japan) were cultured for 16-24 h in MEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml) plus 10% FBS in the presence of M-CSF (5 ng/ml), and nonadherent cells were collected. The monocyte fraction at the interface after Ficoll-Paque gradient centrifugation of the nonadherent cells was used as bone marrow osteoclast precursor cells (22). Animals were treated in accordance with the protocols approved by the Animal Care Research Committee of Nara Women's University.
**Determination of intracellular reactive oxygen species**

Precursor cells were pre-cultured in the absence of M-CSF with or without DPI for 30 min, and then stimulated with M-CSF (20 ng/ml). After the indicated time, cells were washed in MEM and incubated for 10 min with DCFH-DA (10 μM). The fluorescence of DCF was detected by fluorescence microscopy as previously described (23). Fluorescence intensity was measured with WinRoof software (Mitani Co., Tokyo, Japan).

**Determination of the mRNA levels of c-Fms, PU.1, MITF and RANK**

Precursor cells were pre-cultured in the absence of M-CSF with or without DPI for 30 min, and stimulated with M-CSF (20 ng/ml). Cells were harvested after stimulation for 24 h, cells were used to determine the mRNA levels of c-Fms, PU.1, MITF, and RANK by real-time RT-PCR.

**Quantitative real-time RT-PCR**

Total RNA from the cell lysate was prepared using a commercial kit (Sepasol RNA I Super G; Nacalai Tesque, Inc., Kyoto, Japan). The total RNA was reverse-transcribed with a first-strand cDNA synthesis kit (ReverTra Ace qPCR RT Kit; Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed using the cDNA, or total RNA for the negative control, with Thunderbird SYBR qPCR Mix (Toyobo, Co., Ltd., Osaka, Japan) and specific primers.
(Life Technologies Japan Ltd., Tokyo, Japan), (Table I) using a LightCycler real-time PCR
detection system (Toyobo). The amplification program consisted of 1 cycle for 1 min at 95 °C
followed by 45 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Melting curve was
used to verify specific products of the appropriate size. Levels of gene expression were
determined relative to an internal standard (actin).

**Osteoclastic differentiation of bone marrow osteoclast precursor cells**

Precursor cells (1x10^4 cells/well of a 96-well plate or 1.5x10^5 cells/well of a 6-well
plate) were cultured in MEM with 10% FBS containing M-CSF (20 ng/ml) and RANKL (10
ng/ml) with or without DPI. Cultures were maintained with a change of medium every 3 days.
After 5 days, cells were used for counting the tartrate-resistant acid phosphatase (TRAP)−
positive multinucleated cells (MNCs) after TRAP staining and the assessment of cell viability
with a leukocyte acid phosphatase kit 387-A (Sigma) and WST-8 (Cell Counting kit-8;
Dojindo Technologies, Kumamoto, Japan), respectively, as previously described (24).

**RNA interference**

The duplexed Stealth™ siRNA designed against Nox1 and Nox2 (Cybb), and the
negative control were purchased from Invitrogen/Thermo Fisher Scientific Inc. (Rockford, IL,
USA). The sequences were: Nox1 siRNA, 5’-CCAAGG UUGUCAUGCACCACUGUAA-3’
and 5’-UUACAUUGG UGCAUGACACCUCUUGG-3’; Nox2 (Cybb) siRNA,
5′-GAUUCAGGAUGGAGGUGGGACAA UA-3′ and
5′-UAUUGUCCACCUCUCCUCGAAUC-3′. Precursor cells were seeded at a density of 4x10⁵ cells/dish in 35-mm-diameter culture dishes or 2x10⁵ cells/well in 96-well culture plates and transfected with 25 nM of negative control siRNA (Stealth RNAi™ Negative Control; Invitrogen), Nox1 siRNA, or Nox2 siRNA using X-treme GENE HP DNA Transfection Reagent (Roche Applied Science, Penzberg, Germany) for 24 h, according to the manufacturer's instructions. After a change to fresh medium, the expression of Nox1 and Nox2 was determined by RT-PCR. ROS production and RANK expression were determined at 5 min and 24 h after stimulation with M-CSF (20 ng/ml), respectively.

Statistical analysis

All statistical analyses were performed using Welch's method with the Microsoft Excel data analysis program. The differences were considered statistically significant at p<0.05. All data are expressed as the mean ± SEM.

Results

M-CSF generates ROS in osteoclast precursor cells

Stimulation of osteoclast precursor cells with M-CSF resulted in an increase in the intensity of DCF fluorescence (Fig. 1A), indicating that M-CSF induced intracellular ROS
production. The production of ROS rapidly increased to a maximum level at approximately 5 min after the M-CSF treatment and thereafter decreased toward the basal level (Fig. 1).

Effects of NADPH oxidase inhibitor on M-CSF induced ROS production

Treatment of osteoclast precursor cells with DPI, a specific inhibitor for flavoprotein that is a constituent of the Nox complex, eliminated the rise in DCF fluorescence induced by M-CSF (Fig. 2).

Effects of NADPH oxidase inhibitor on mRNA expression, cell viability, and osteoclast formation

M-CSF increased the mRNA levels of RANK, PU.1 and MITF to ~10⁻, 2.4⁻ and 2.8⁻ fold the uninduced (before the stimulation of M-CSF) value, respectively, although the c-Fms mRNA levels were unchanged (Fig. 3A). DPI significantly suppressed these increases. There are no effect on the cell viability by the application of DPI at least 100 μM. The osteoclastic differentiation of precursor cells was significantly inhibited (Fig. 3B).

Expression of Nox isozyme

Precursor cells expressed the mRNA of Nox2, a smaller amount of Nox1, and undetectable levels of Nox3 and Nox4. The mRNA levels of Nox1 were ~0.1% of these of Nox2. M-CSF decreased Nox2 expression to ~30% of the uninduced level, but increased the
Nox1 expression to 4-fold the uninduced level. The levels of Nox3 and Nox4 were undetectable after the induction by M-CSF (Fig. 4).

Effects of Nox1 and Nox2 siRNA on M-CSF-induced ROS generation and RANK expression

To investigate which Nox isozyme is responsible for the responses to M-CSF, precursor cells were treated with negative control, Nox1 or Nox2 siRNA, and ROS production and RANK expression were examined. Nox1 or Nox2 was effectively knocked down by the specific siRNA, as shown by the real-time RT-PCR analysis (Fig. 5). The silencing of Nox2 in precursor cells resulted in a significant decrease in ROS production in response to M-CSF (Fig. 6A). However, Nox1 knockdown had no effect on ROS production. Nox2, but not Nox1, siRNA inhibited the expression of RANK (Fig. 6B). These results suggest that Nox2 is a critical mediator of M-CSF-induced RANK expression in precursor cells (c-Fms+RANK-).

Discussion

M-CSF stimulated RANK expression in early-stage osteoclast precursors (c-Fms+RANK-) and the binding of RANKL to RANK triggers the differentiation of late-stage precursors into osteoclasts (25). The expression of RANK caused by the binding of M-CSF is a key step in the early stages of osteoclastogenesis.

This study clearly demonstrated a critical role for ROS in the differentiation of
early-stage osteoclast precursor cells into late-stage precursors. M-CSF generated ROS in the early-stage of osteoclast precursor cells. The production of ROS was inhibited by a Nox inhibitor, DPI, indicating Nox-mediated ROS generation. Furthermore, the expression of RANK was inhibited by DPI. The mRNA levels of PU.1 and MITF were also reduced by DPI. These data suggested that the inhibition of ROS production resulted in the suppression of RANK expression. This study, for the first time, revealed that Nox-mediated production of ROS was required for the expression of RANK in the early-stage osteoclast precursor cells.

In peripheral blood monocytes, ROS production by M-CSF was suggested to play a role in cellular survival (7). Macrophages from p47phox−/− mice, lacking a key component of the Nox complex required for ROS generation, reduced cell survival compared with wild-type cells (8). Application of DPI was reported to inhibit M-CSF-induced monocyte survival (7). These studies suggested that Nox-mediated ROS generation by M-CSF led to cellular survival in monocyte/macrophages. However, in our experiments using early-stage osteoclast precursor cells, DPI did not reduce the viability of cultured precursor cells, although it decreased osteoclast formation. These results suggested that the effect of ROS generation mediated by Nox in early-stage osteoclast precursor cells was different from that in differentiated monocyte/macrophages. The M-CSF signaling may differ depending on the stage of cell differentiation, and the level of ROS.

Nox is recognized as a major intracellular source of ROS. As observed in monocyte/macrophages (10, 21), Nox2 was found to be the main isotype expressed in
early-stage osteoclast precursor cells. The expression of Nox1 was also detectable at a low level, whereas that of other members such as Nox3 and Nox4 was undetectable. M-CSF decreased the expression of Nox2 and increased that of Nox1. The induced level of Nox1 expression was still 1% of the induced level of Nox2. The downregulation of Nox2 and the upregulation of Nox1 were also observed on RANKL-stimulation (11, 21). Although the upregulation of Nox4 by RANKL-stimulation was also reported (21), the increase in its expression by M-CSF was not observed in this study. The siRNA targeting Nox2, but not Nox1 inhibited the M-CSF-stimulated ROS production and RANK expression. These results clearly indicated the generation of ROS by M-CSF to be mediated through Nox2 in early-stage osteoclast precursors, although a flexible compensatory mechanism between Nox1 and Nox2 for RANKL-stimulated ROS production was suggested in the osteoclast differentiation of bone marrow-derived monocyte/macrophages (21).

This study provides evidence that ROS produced in response to M-CSF via a process mediated by Nox2 act as an intracellular signaling mediator for RANK expression through the expression of PU.1 and MITF in the early-stage osteoclast precursor cells (c-Fms+RANK−).
Summary

In early-stage osteoclast precursor cells (c-Fms+RANK−), Macrophage colony-stimulating factor (M-CSF) transiently increased the intracellular level of reactive oxygen species (ROS) and induced the expression of receptor for activation of nuclear factor-κB (RANK). NADPH oxidase (Nox) is one of the major players in the generation of ROS. The aim of the present study was to determine the molecular mechanisms for M-CSF-induced ROS generation in the early stages of osteoclastic differentiation. Blocking of the activity of Nox with diphenylene iodonium inhibited ROS production, the expression of RANK, PU.1 and MITF, and then inhibited osteoclast differentiation. M-CSF decreased the expression of Nox2 and increased that of Nox1. The induced level of Nox1 expression was still 1% of the induced level of Nox2. The suppression of Nox2, but not Nox1 expression by RNA interference inhibited ROS production and RANK expression. These results suggested that ROS produced in response to M-CSF via a process mediated by Nox2 acted as an intracellular signaling mediator for RANK expression through the expression of PU.1 and MITF in early-stage osteoclast precursor cells.
References


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Primer sets for actin, c-Fms, RANK, MITF, PU.1, Nox1, Nox2, Nox3 and Nox4.
**Fig. 1**  **M-CSF-induced ROS production.** Precursor cells were treated with M-CSF (20 ng/ml) for the period indicated. After the addition of DCFH-DA, the fluorescence of DCF was detected. (A) Representative microscopic fields (x400 magnification). (B) DCF fluorescence. Values are the mean ± SEM of four experiments.
Fig. 2   Effects of DPI on M-CSF-induced ROS production. Precursor cells were pre-cultured in the absence of M-CSF with or without DPI for 30 min and then stimulated with M-CSF (20 ng/ml). After stimulation for 5 min, DCFH-DA was added and the fluorescence of DCF was detected. (A) Representative microscopic fields (x400 magnification). (B) Quantitative calculation data. Values are the mean ± SEM of four experiments. Significantly different from the control value (*p<0.05).
**Fig. 3** Effects of DPI on mRNA expression, cell viability, and osteoclastogenesis. Precursor cells were pre-cultured in the absence of M-CSF with or without DPI for 30 min and then stimulated with M-CSF (20 ng/ml). (A) After stimulation for 24 h, cells were harvested and the expression levels of c-Fms, PU.1, MITF and RANK were determined by real-time RT-PCR. Values are the mean ± SEM of four experiments. Significantly different from the uninduced value (*p<0.05). Significantly different from the induced value (#p<0.05). (B) Precursor cells were cultured with M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 5 days. Cell viability and the number of TRAP-positive MNCs were determined. Values are the mean ± SEM of four experiments. Significantly different from the control value (*p<0.05).
**Fig. 4** Expression of Nox. Precursor cells were pre-cultured in the absence of M-CSF with or without DPI for 30 min and then stimulated with M-CSF (20 ng/ml). (A) After stimulation for 24 h, cells were harvested and the expression levels of Nox1, Nox2, Nox3 and Nox4 were determined by real-time RT-PCR. Levels are expressed relative to the actin level. Values are the mean ± SEM of four experiments. Significantly different from the uninduced value (*p<0.05).

**Fig. 5** Effects of siRNA transfection on the mRNA levels of Nox1 and Nox2. Precursor cells were transfected with or without negative control siRNA (siNC), Nox1 siRNA (siNox1), or Nox2 siRNA (siNox2) using transfection reagent for 24 h. The expression of Nox1 and Nox2 was determined by real-time RT-PCR. Expression levels are expressed relative to the actin level. Values are the mean ± SEM of three experiments. Significantly different from the control value (*p<0.05).
Fig. 6 Effects of negative control, Nox1 and Nox2 siRNA on M-CSF-induced ROS generation and RANK expression. (A) After treatment with M-CSF (20 ng/ml) for 5 min, ROS production was determined. Representative microscopic fields are shown (x400 magnification; upper panel). DCF fluorescence was expressed relative to the control value (lower panel). Values are the mean ± SEM of three experiments. (B) After treatment with M-CSF (20 ng/ml) for 24 h, RANK expression was determined by real-time RT-PCR. Expression levels are expressed relative to the actin level. Values are the mean ± SEM of three experiments. Significantly different from the control value (*p<0.05).
Chapter 2

Fish oil suppresses bone resorption by inhibiting osteoclastogenesis through decreased expression of M-CSF, PU.1, MITF and RANK in ovariectomized rats

Introduction

The pathogenesis of postmenopausal osteoporosis involves increased bone turnover with a relative increase in bone resorption, leading to a marked decline in bone mass with the loss of estrogen following menopause. A number of bone diseases, including osteopenia and osteoporosis, reflect an imbalance in the differentiation and function of two cell types, the osteoblast and osteoclast, which are responsible for bone formation and bone resorption, respectively (1, 2).

Epidemiological and longitudinal studies have revealed a positive correlation between the intake of n-3 long chain polyunsaturated fatty acids (PUFAs) and bone mineral density in postmenopausal women (3). In animals, dietary supplementation with n-3 PUFA-rich oils, including fish oil, has been linked to improved maintenance of bone mass postovariectomy (4-6). In addition, endogenously produced n-3 PUFAs have been revealed to protect against ovariectomy-induced bone loss in fat-1 transgenic mice (7, 8). Administration of n-3 PUFAs for 16 weeks was observed to suppress RANKL expression and NFκB activation in the activated splenic CD4 cells of ovariectomized mice (9). Previous in vitro studies have
revealed that n-3 PUFAs are linked to decreased NFκB expression (10, 11), and modulation of RANKL signaling in RAW264.7 cells (12). These studies indicated that n-3 PUFAs reduced bone resorption by decreasing osteoclastogenesis. However, it remains unknown whether n-3 PUFAs affect the early stages of osteoclastogenesis and which genes or molecules these fatty acids target in ovariectomized rats. By contrast, n-3 PUFAs also affect bone formation in animal models (13, 14) and osteoblast functions by increasing Runx2 expression in MC3T3 cells (15). The effects of n-3 PUFAs on bone formation and osteoblasts remain poorly understood in ovariectomized rats.

In the present study, the effects of fish oil on bone metabolism and the expression of genes involved in osteoclastogenesis were investigated in vivo using ovariectomized rats. This study demonstrated that fish oil reduced the activity and number of osteoclasts without altering the activity and number of osteoblasts. The decrease in the number of osteoclasts was found to be caused by a reduction of osteoclastogenesis, which was associated with the decreased expression of M-CSF in the early stages of osteoclastic differentiation.

**Materials and methods**

**Animals and diets**

Female Wistar/ST rats (9-week old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed individually in a temperature-controlled room with a 12-h light/dark cycle.
Following a 1-week period of adaptation, the animals were subjected to bilateral ovariectomy (Ovx) or sham-operation (Sham). The animals were further divided into two groups and fed American Institute of Nutrition (AIN)-76A-based semipurified diets; corn (C) or fish (F) oil-containing (ShamC, ShamF, OvxC and OvxF; n=10 for each group). C or F diets contained 5% corn or fish oil (4.5% menhaden oil with 0.5% corn oil), respectively (Table I; Research Diets, Inc., New Brunswick, NJ, USA). F diet was supplemented with 6.3 mg/kg \( \alpha \)-tocopherol to match the concentration of corn oil. The fatty acid composition of the oils used in the diets is presented in Table II. After 2 weeks, blood and femoral and tibial bone samples were collected under pentobarbital sodium anesthesia after overnight access to food (non-fasting). Blood samples were used to determine the serum concentrations of estradiol, osteocalcin, TNF\( \alpha \), interleukin (IL)-6 and prostaglandin E2 (PGE2) and the plasma fatty acid composition. Following removal of muscle and tendons, the tibial bone was used for biochemical and histological analyses. Animal experiments were performed in accordance with protocols approved by the Animal Care Research Committee of Nara Women's University.

**Biochemical analysis**

Serum concentrations of estradiol, osteocalcin, TNF\( \alpha \), IL-6 and PGE2 were measured using an Elecsys E2II assay (Roche Diagnostics GmbH, Mannheim, Germany), a Rat Osteocalcin ELISA DS kit (DS Pharma Biomedical Co., Ltd., Osaka, Japan), Quantikine Rat
TNFα and IL-6 Immunoassays (both R&D Systems, Inc., Minneapolis, MN, USA) and a PGE2 Express EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA), respectively.

The activities of alkaline phosphatase (ALP), tartrate resistant acid phosphatase (TRAP) and cathepsin K (CK) and the levels of calcium (Ca) and hydroxyproline (Hyp) in the proximal tibia (the quarter from the aspect of the knee of the tibia) were determined as described previously (16, 17).

**Histomorphometry**

Tibias were fixed in 4% paraformaldehyde, decalcified in 10% EDTA and embedded in paraffin. Sections (4 μm) were stained for TRAP activity using a leukocyte acid phosphatase kit (387-A; Sigma-Aldrich, Co., LLC., St. Louis, MO, USA) as described previously (16). Morphometric measurements of trabecular structure (trabecular bone volume, bone surface, thickness and number) and the number of osteoblasts (cuboidal cells on trabecular surfaces) and osteoclasts (TRAP-positive multinucleated cells) were performed at standardized sites (300 x 300 μm) under the growth plate in the metaphysis of the proximal tibia (18).

**Fatty acid analysis**

Plasma total lipids were extracted as described previously (19), with specific modifications (20). Following methylation, fatty acid methyl esters were separated using a gas chromatograph (GC2014; Shimadzu, Kyoto, Japan) equipped with a 25 m x 0.5 mm capillary
column (HR-SS-10; Shimadzu) and were identified by comparison of retention times with a fatty acid methylester standard (68A; Nu-Chek Prep, Inc., Elysian, MN, USA).

**Quantitative real-time RT-PCR**

Total RNA from the proximal tibia was prepared using a commercial kit (Sepasol RNA I Super G; Nacalai Tesque, Inc., Kyoto, Japan) after bone marrow cells were washed and homogenized in the presence of 0.1 M EDTA. Total RNA was reverse-transcribed using a first-strand cDNA synthesis kit (ReverTra Ace qPCR RT Kit; Toyobo, Co., Ltd., Osaka, Japan). PCR was performed using cDNA or total RNA (negative control) with Thunderbird SYBR qPCR mix (Toyobo) and specific primers (Life Technologies Japan Ltd., Tokyo, Japan), (Table III), using a LightCycler real-time PCR detection system (Toyobo). The amplification program consisted of 1 cycle for 1 min at 95 °C followed by 45 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Melting curve was used to verify specific products of the appropriate size. Levels of gene expression were determined relative to an internal standard (actin).

**Western blot analysis**

Bone extracts of the proximal tibia were prepared as described previously (16) for western blot analysis. Protein concentrations were measured using the BCA protein assay kit (Pierce/Thermo Fisher Scientific Inc., Rockford, IL, USA). Equal amounts of protein were
separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to membranes. Western blotting and reprobing were performed and the chemiluminescent signals were quantified using a densitometer, as described previously (21). Antibodies recognizing NFκB p65 and phosphorylated (p)-NFκB p65 (Ser 276) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Statistical analysis

Data are presented as the mean ± SEM. All statistical analyses were performed by one-way analysis of variance with pairwise comparison by the Bonferroni method using the Microsoft Excel data analysis program. p<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ovariectomy and fish oil on clinical characteristics

Food intake and final body weight were identified to be significantly higher, and serum concentrations of estradiol were significantly lower in the Ovx groups compared with the Sham groups (Fig. 1, Table IV). Fish oil did not affect these values. The weights of the femur and tibia were significantly lower in the OvxC rats than in the the Sham groups, and were
restored to the Sham levels in the OvxF rats; however, bone lengths were similar in the four groups (Table IV).

**Effects of ovariectomy and fish oil on bone biochemical markers**

ALP activity in the proximal tibia and serum osteocalcin levels were significantly higher in the Ovx groups compared with the Sham groups (Table V). The activities of TRAP and CK in the OvxC rats increased significantly to 1.6-fold and 1.3-fold of the ShamC levels, respectively. Ca and Hyp levels in the OvxC rats decreased to ~80 and 90% of the ShamC value, respectively. Fish oil did not affect the increased levels of ALP activity and osteocalcin in the Ovx rats. However, the Ovx-increased activities of TRAP and CK were suppressed and the decreased levels of Ca and Hyp were recovered to the Sham values by fish oil. Fish oil did not affect bone biochemical markers in the Sham rats.

**Histological analysis**

The results of histochemical staining of the tibia for TRAP and ALP, a marker of osteoclast and osteoblast, respectively, are shown in Fig. 2. Morphometric measurements revealed that the number of osteoclasts and osteoblasts in the OvxC rats increased to 1.7-fold and 1.5-fold the ShamC value (Table VI). In OvxC, trabecular bone volume and thickness were decreased and trabecular bone surface was increased, although trabecular numbers were not significantly altered by Ovx. These changes in the number of osteoclasts and trabecular bone
volume, thickness, and surface were reversed by fish oil; however, osteoblast number was not recovered.

Expression of genes involved in osteoclastic differentiation in the proximal tibia

Gene expression levels of the osteoclastogenesis-related factors, c-Fms, M-CSF, PU.1, MITF, RANK, RANKL, OPG, c-Jun, and c-Fos are demonstrated in Fig. 3A. mRNA levels of M-CSF and RANKL in the OvxC rats were ~1.6-fold the ShamC value, although a significant difference was not observed in OPG levels. The expression of c-Fms in the OvxC rats did not differ from the ShamC values. However, levels of PU.1, MITF, RANK, c-Jun and c-Fos in the OvxC rats were 2-, 2-, 2-, 4- and 3-fold the ShamC values, respectively. The expression of TRAP and CK mRNA also increased to 3.7- and 2.5-fold the ShamC value, respectively. These increases induced by Ovx were suppressed to the Sham levels by fish oil, although the expression of TRAP in the OvxF rats was significantly higher than the ShamC value but lower than the OvxC level. Fish oil did not affect these expression levels in the Sham groups.

Plasma fatty acid composition and serum concentrations of TNFα, IL-6 and PGE2

Ovx significantly increased levels of arachidonic acid (AA, C20:4 n-6), as revealed in Table VII. Fish oil reduced this increase. In the fish oil-fed rats, ShamF and OvxF, levels of AA and linoleic acid (LA, C18:2 n-6) significantly decreased and those of palmitoleic (C16:1
n−7), eicosapentaenoic (EPA, C20:5 n−3), docosapentaenic (DPA; C22:5 n−3) and docosahexaenoic (DHA, C22:6 n−3) acid increased compared with the corresponding levels in corn oil−fed rats.

The concentrations of TNFα, IL−6 and PGE2 were significantly increased by 1.4−, 1.4− and 1.2−fold of the Sham levels in the OvxC rats, respectively (Fig. 4). The increases were suppressed to the Sham levels by fish oil. Fish oil did not affect these concentrations in the Sham rats.

mRNA levels of TNFα, IL−6 and cyclooxygenase 2 (COX2), and NFκB activation in the proximal tibia

mRNA levels of TNFα, IL−6 and COX2 in the OvxC rats increased to ~2.4−, 1.8−, and 1.7−fold the ShamC values, respectively (Fig. 5A). These increases were restored to the Sham levels by fish oil, however, significant effects of fish oil were not observed in the Sham rats. The phosphorylation of NFκB p65 (p·NFκB p65) is crucial for NFκB transcriptional activity. The p·NFκB p65 protein in the OvxC rats increased to 7−fold the ShamC level (Fig. 5B and C). Stimulation of phosphorylation was reduced to the Sham level by fish oil. Fish oil did not affect the phosphorylation of NFκB p65 in the Sham rats.
Discussion

Results of the present study confirm that fish oil suppresses increased bone resorption induced by Ovariectomy. Ovariectomy resulted in substantial decreases in Ca and Hyp and increases in osteoclastic and osteoblastic activities. Fish oil suppressed the increase in osteoclastic activity and osteoclast number. However, it did not affect the activity or number of osteoblasts. These results indicate that fish oil suppresses the decrease in Ca and Hyp levels in bone by reducing the increase in bone resorption associated with decreases in osteoclastogenesis. It should be noted that n-3 PUFAs had no effect on bone formation in vivo in ovariectomized rats, although previous studies reported a role in increasing osteoblastic activity in growing rats (13, 22) or osteoblastogenesis in osteoblast-like cells (15).

Osteoclast precursors are derived from hematopoietic stem cells in bone marrow. Differentiation into osteoclasts, however, occurs on the bone surface in vivo (2, 23). Therefore, in the current study, gene expression of osteoclastogenesis-related factors was examined in the bone. mRNA levels of M-CSF significantly increased in the OvxC rats compared with the Sham rats, and fish oil suppressed these increases to the ShamC and the ShamF levels. For the first time, this study revealed the suppression of M-CSF expression by fish oil in the bone of ovariectomized rats. M-CSF induces the proliferation of osteoclast precursor cells, supports their survival and upregulates expression of the receptor of RANKL, RANK, which is a prerequisite for osteoclast precursor cells (24). Gene expression of RANK is regulated by the
transcription factors PU.1 and MITF (25). In the ovariectomized rats, mRNA levels of PU.1 and MITF increased compared with the ShamC rats, and fish oil suppressed these increase. Suppression of M-CSF expression may lead to the reduced expression of PU.1 and MITF and a subsequent decrease in RANK expression in the OvxF rats. This study demonstrated that fish oil suppressed the expression of M-CSF, followed by PU.1, MITF and RANK, in the early stages of osteoclastogenesis, including the differentiation of hematopoietic stem cells into osteoclast precursor cells upstream of RANKL signaling.

M-CSF expression is upregulated by a variety of inflammatory cytokines, including TNFα (26). TNFα expression is induced by NFκB (27). In the present study, increased NFκB activation (p-NFκB p65) was observed in the bones of the OvxC rats compared with the ShamC rats. Increased NFκB activation was suppressed in the proximal tibia of the OvxF rats. Simultaneously, a decrease in the levels of n-6 PUFAs (AA and LA) and an increase in n-3 PUFAs (EPA, DPA and DHA) was observed in the plasma of the OvxF rats. A number of previous in vitro studies have also reported that n-3 PUFAs, which are major fatty acids of fish oil, downregulate NFκB activity (9, 28-31). Consistent with these in vitro results, the present study suggested that a decrease in the n-6/n-3 PUFAs ratio induced by dietary fish oil led to the suppression of NFκB activation in the bones of ovariectomized rats. NFκB suppression was found to be associated with a reduction in serum TNFα levels and the mRNA levels of TNFα in bone. These results indicate that inhibition of NFκB activation by increases in plasma n-3 PUFAs suppresses downstream events, including
TNFα/M-CSF/PU.1/MITF/RANK expression in the proximal tibia. Notably, the effects of increased n-3 PUFAs were observed in ovariectomized rats only and not in normal animals.

M-CSF stimulates the production of IL-6 and PGE2 (32, 33), which is known to be upregulated by estrogen deficiency (34, 35). In the current study, serum concentrations of IL-6 and PGE2 and mRNA levels of IL-6 and a PG synthesis enzyme, COX2, in the proximal tibia increased in the OvxC rats compared with the ShamC rats. Fish oil restored these levels to the Sham values, coinciding with the suppression of M-CSF, in the OvxF rats. TNFα, IL-6 and PGE2 induced the expression of RANKL (34, 36), an additional essential factor for osteoclastogenesis. Downregulation of RANKL, as well as M-CSF expression, by fish oil was observed in bone in the current study. Previous studies have also reported a decrease in RANKL expression in RAW264.7 (12) or activated splenic CD4 cells (9). In the present study, downregulation of RANKL expression by fish oil was confirmed at the mRNA level in vivo in bone. These results indicate that the suppression of RANKL expression resulted from decreased production of IL-6 and PGE2, caused by the suppression of M-CSF, as well as a decrease in TNFα, in the bone of ovariectomized rats.

Results of the present study indicate that fish oil reduces ovariectomy-stimulated osteoclastogenesis by suppressing the expression of M-CSF, PU.1, MITF and RANK in the early stages of osteoclastogenesis and RANKL signaling in later stages.
Summary

Previous studies have identified a positive correlation between the intake of n-3 polyunsaturated fatty acids and bone mineral density in postmenopausal women. The aim of the present study was to determine the effects of fish oil on bone metabolism and to investigate the underlying mechanism using ovariectomized rats. Ovariectomized or sham-operated female rats were fed AIN-76A-based diets containing 5% corn or fish oil for 2 weeks. Fish oil was found to decrease the plasma levels of arachidonic and linoleic acids, but increased the levels of EPA and DHA. Fish oil reversed the increased activity and number of osteoclasts, and decreased Ca and Hyp content of the proximal tibia to sham values without affecting the activity or number of osteoblasts. In addition, fish oil suppressed increases in the mRNA levels of M-CSF, PU.1, MITF, RANK and RANKL and serum levels of TNFα, IL-6 and PGE2. Fish oil was also found to suppress NFκB activation induced by ovariectomy. These results indicate that dietary fish oil suppressed ovariectomy-stimulated osteoclastogenesis by inhibiting NFκB activation and subsequent downregulation of TNFα, followed by suppression of M-CSF and RANKL.
References


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<tr>
<th>Ingredient (g/kg)</th>
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<th>Fish oil</th>
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<td>3</td>
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<sup>a</sup>AIN 76 mineral mix (S10001), <sup>b</sup>AIN 76 vitamin mix (V10001).
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<tr>
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<th>Short illustration</th>
<th>Corn oil (g/100g)</th>
<th>Fish oil* (g/100g)</th>
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*Menhaden/corn = 9/1
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<td>actin</td>
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Primer sets for actin, c-Fms, M-CSF, PU.1, MITF, RANK, RANKL, OPG, c-Jun, c-Fos, TRAP, CK, TNFα, IL-6, and COX2.
Table IV   Effects of ovariectomy and fish oil on clinical characteristics

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<tr>
<th>Parameters</th>
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<th>ShamF</th>
<th>OvxC</th>
<th>OvxF</th>
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<td>Food intake (g/day)</td>
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<td>Bone Length* (cm)</td>
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<tr>
<td>Femur</td>
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<td>3.33 ± 0.02</td>
<td>3.39 ± 0.01</td>
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<td>3.70 ± 0.01</td>
<td>3.66 ± 0.03</td>
<td>3.74 ± 0.02</td>
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<td>Bone Weight *(g)</td>
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<tr>
<td>Femur</td>
<td>0.661 ± 0.007</td>
<td>0.671 ± 0.009</td>
<td>0.632 ± 0.009^a</td>
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<td>Tibia</td>
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<td>0.497 ± 0.007</td>
<td>0.482 ± 0.003^a</td>
<td>0.504 ± 0.006^b</td>
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<td>Serum estradiol (pg/ml)</td>
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<td>27.8 ± 3.5</td>
<td>14.0 ± 0.4^a</td>
<td>18.3 ± 1.8^a</td>
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Values are means ±SEM. ^aSignificantly different from the ShamC (p<0.05). ^bSignificantly different from the OvxC (p<0.05).

C, corn oil-containing; F, fish oil-containing; Sham, sham-operation; Ovx, ovariectomy. *Right bone length, right bone weight.

Fig. 1   Body weight changes in Sham rats and Ovx rats during corn oil feeding or fish oil feeding. Arrow indicated bilateral ovariectomy or sham-operation date after 12 h fasting. Data are presented as the mean ± SEM. *p<0.05 vs. ShamC; ^a p<0.05 vs. OvxC. C, corn oil-containing; F, fish oil-containing; Sham, sham-operation; Ovx, ovariectomy.
<table>
<thead>
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<th>Parameters</th>
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<th>ShamF</th>
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<td>Proximal tibia</td>
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<td>20.1 ± 0.8</td>
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<td>25.9 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>TRAP activity (U/g bone)</td>
<td>0.76 ± 0.04</td>
<td>0.72 ± 0.03</td>
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<td>1.05 ± 0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>CK activity (U/g bone)</td>
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<td>415.7 ± 34.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Ca (mg/g bone)</td>
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<td>118.9 ± 2.80</td>
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Values are means ±SEM. <sup>a</sup>Significantly different from the ShamC (p<0.05). <sup>b</sup>Significantly different from the OvxC (p<0.05).

C, corn oil-containing; F, fish oil-containing; Sham, sham-operation, Ovx, ovariectomy.

ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; CK, cathepsin K; Ca, calcium; Hyp, hydroxyproline.
Fig. 2  TRAP staining of the proximal tibia in (A) ShamC, (B) ShamF, (C) OvxC, and (D) OvxF. Paraformaldehyde-fixed, decalcified and paraffin-embedded tibia obtained from (A) ShamC, (B) ShamF, (C) OvxC, and (D) OvxF, was processed for TRAP staining. TRAP-positive cells appeared red. Hematoxylin counterstaining. Magnification x200. Bar, 100 μm. The results presented here are typical of 10 rats of each group.

### Table VI  Bone histomorphometry

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ShamC</th>
<th>ShamF</th>
<th>OvxC</th>
<th>OvxF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular number (/mm)</td>
<td>13.33 ± 0.64</td>
<td>13.74 ± 0.3</td>
<td>13.80 ± 0.63</td>
<td>14.76 ± 1.23</td>
</tr>
<tr>
<td>Trabecular bone volume (%)</td>
<td>59.72 ± 1.74</td>
<td>61.68 ± 2.17</td>
<td>46.10 ± 1.74a</td>
<td>59.18 ± 1.90b</td>
</tr>
<tr>
<td>Trabecular bone surface (mm/mm²)</td>
<td>26.39 ± 1.13</td>
<td>27.48 ± 0.59</td>
<td>32.84 ± 1.69a</td>
<td>27.74 ± 1.90b</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td>48.43 ± 3.77</td>
<td>45.18 ± 2.04</td>
<td>27.33 ± 2.09a</td>
<td>41.95 ± 2.84b</td>
</tr>
<tr>
<td>Osteoblast index (no.Ob/mm trabecular bone length)</td>
<td>16.32 ± 0.59</td>
<td>16.45 ± 0.78</td>
<td>23.72 ± 0.96a</td>
<td>23.80 ± 1.12a</td>
</tr>
<tr>
<td>Osteoclast index (no.Oc/mm trabecular bone length)</td>
<td>2.93 ± 0.12</td>
<td>2.75 ± 0.26</td>
<td>5.08 ± 0.27a</td>
<td>3.45 ± 0.24b</td>
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</tbody>
</table>

Values are means ±SEM. aSignificantly different from the ShamC (p<0.05). bSignificantly different from the OvxC (p<0.05).

C, corn oil-containing; F, fish oil-containing; Sham, sham-operation; Ovx, ovariectomy; no, number.
Fig. 3  mRNA expression of genes involved in osteoclastogenesis in the proximal tibia, including c-Fms, M-CSF, PU.1, MITF, RANK, RANKL, OPG, c-Jun, c-Fos, TRAP and CK. Total RNA was extracted from the proximal tibia and the mRNA levels of c-Fms, M-CSF, PU.1, MITF, RANK, RANKL, OPG, c-Jun, c-Fos, TRAP and CK were assessed by real-time RT-PCR. Data are presented as the mean ± SEM (n=8). *p<0.05 vs. ShamC; #p<0.05 vs. OvxC. C, corn oil-containing; F, fish oil-containing; Sham, sham-operation; Ovx, ovariectomy.
Table VII  Effect of ovariectomy and fish oil on plasma fatty acid composition (mol %)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>ShamC</th>
<th>ShamF</th>
<th>OvxC</th>
<th>OvxF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>C14:0</td>
<td>0.91 ± 0.18</td>
<td>1.68 ± 0.38</td>
<td>1.09 ± 0.39</td>
</tr>
<tr>
<td>Pentadecanoic acid</td>
<td>C15:0</td>
<td>0.27 ± 0.06</td>
<td>0.61 ± 0.23</td>
<td>0.47 ± 0.18</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>C16:0</td>
<td>16.89 ± 5.83</td>
<td>19.00 ± 1.23</td>
<td>17.80 ± 1.62</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>C16:1 n-7</td>
<td>2.25 ± 0.30</td>
<td>4.49 ± 0.78</td>
<td>2.25 ± 0.12</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>C18:0</td>
<td>19.71 ± 1.88</td>
<td>21.49 ± 1.31</td>
<td>20.59 ± 1.81</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C18:1 n-9</td>
<td>11.14 ± 2.05</td>
<td>8.12 ± 1.49</td>
<td>9.21 ± 1.13</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>C18:1 n-7</td>
<td>2.04 ± 0.30</td>
<td>2.10 ± 0.30</td>
<td>1.87 ± 0.25</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>C18:2 n-6</td>
<td>15.88 ± 0.74</td>
<td>5.38 ± 0.43</td>
<td>14.12 ± 1.11</td>
</tr>
<tr>
<td>γ-Linolenic acid</td>
<td>C18:3 n-6</td>
<td>0.30 ± 0.06</td>
<td>0.20 ± 0.02</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>C18:3 n-3</td>
<td>0.16 ± 0.05</td>
<td>0.20 ± 0.01</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td>Stearidonic acid</td>
<td>C18:4 n-3</td>
<td>0.02 ± 0.02</td>
<td>0.18 ± 0.05</td>
<td>0.07 ± 0.08</td>
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<tr>
<td>Arachidonic acid</td>
<td>C20:0</td>
<td>0.13 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.17 ± 0.07</td>
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<tr>
<td>Eicosenoic acid</td>
<td>C20:1 n-9</td>
<td>0.38 ± 0.20</td>
<td>0.54 ± 0.14</td>
<td>0.36 ± 0.27</td>
</tr>
<tr>
<td>Eicosadienoic acid</td>
<td>C20:2 n-6</td>
<td>0.20 ± 0.02</td>
<td>0.25 ± 0.18</td>
<td>0.20 ± 0.14</td>
</tr>
<tr>
<td>Dihomo-γ-linolenic acid</td>
<td>C20:3 n-6</td>
<td>0.49 ± 0.07</td>
<td>0.64 ± 0.23</td>
<td>0.57 ± 0.09</td>
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<tr>
<td>Arachidonic acid</td>
<td>C20:4 n-6</td>
<td>20.95 ± 1.45</td>
<td>11.81 ± 1.75</td>
<td>25.68 ± 1.82</td>
</tr>
<tr>
<td>ETA</td>
<td>C20:4 n-3</td>
<td>ND</td>
<td>0.31 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>EPA</td>
<td>C20:5 n-3</td>
<td>0.12 ± 0.02</td>
<td>13.51 ± 0.72</td>
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<td>Erucic acid</td>
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<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>Clupandonic acid</td>
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<td>0.41 ± 0.08</td>
<td>0.06 ± 0.02</td>
<td>0.48 ± 0.19</td>
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<tr>
<td>DPA</td>
<td>C22:5 n-3</td>
<td>0.29 ± 0.08</td>
<td>1.30 ± 0.35</td>
<td>0.24 ± 0.08</td>
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<td>DHA</td>
<td>C22:6 n-3</td>
<td>2.55 ± 0.25</td>
<td>5.77 ± 0.85</td>
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<td>Lignoceric acid</td>
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<td>0.40 ± 0.04</td>
<td>1.28 ± 0.20</td>
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<tr>
<td>Tetracosenoic acid</td>
<td>C24:1 n-9</td>
<td>0.31 ± 0.03</td>
<td>0.59 ± 0.27</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
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<td>42.27 ± 2.00</td>
<td>43.98 ± 0.86</td>
<td>42.12 ± 2.58</td>
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<tr>
<td>Monounsaturated fatty acids</td>
<td></td>
<td>16.26 ± 1.93</td>
<td>15.66 ± 2.11</td>
<td>14.19 ± 1.28</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids</td>
<td></td>
<td>41.47 ± 2.43</td>
<td>40.36 ± 2.66</td>
<td>43.69 ± 2.88</td>
</tr>
<tr>
<td>n-6/n-3 PUFA ratio</td>
<td></td>
<td>12.58 ± 1.10</td>
<td>0.85 ± 0.06</td>
<td>14.95 ± 1.10</td>
</tr>
</tbody>
</table>

Values are means ±SEM. *Significantly different from the ShamC (p<0.05). †Significantly different from the OvxC (p<0.05).

ND, not detected; C, corn oil–containing; F, fish oil–containing; Sham, sham–operation; Ovx, ovariectomy.

ETA, Eicosatetraenoic acid; EPA, Eicosapentaenoic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid.
Fig. 4  Effects of ovariectomy and fish oil on the serum concentrations of TNFα, IL-6 and PGE2.

Serum concentrations of TNFα, IL-6, and PGE2 were measured using an ELISA or EIA kit. Data are presented as the mean ± SEM (n=6). *p<0.05 vs. ShamC; #p<0.05 vs. OvxC. TNFα, tumor necrosis factor α; IL-6, interleukin-6; PGE2, prostaglandin E2; C, corn oil-containing; F, fish oil-containing; Sham, sham operation; Ovx, ovariectomy.
Fig. 5  mRNA levels of TNFα, IL-6 and COX2 and the activation of NFκB in the proximal tibia.

(A) Total RNA was extracted from the proximal tibia and the mRNA levels of TNFα, IL-6 and COX2 were assessed by real-time RT-PCR. (B) Western blot analysis of bone extracts from the proximal tibia using p-NFkB p65 (Ser276) or p65 antibodies. (C) Quantification of protein levels. Data are presented as the mean ± SEM (n=8). *p<0.05 vs. ShamC; #p<0.05 vs. OvxC. TNFα, tumor necrosis factor α; IL-6, interleukin-6; COX2, cyclooxygenase 2; C, corn oil-containing; F, fish oil-containing; Sham, sham-operation; Ovx, ovariectomy.
Chapter 3
Effects of n-3 polyunsaturated fatty acids on osteoclastogenesis

Introduction

Osteoclastic differentiation of hematopoietic stem cells requires macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor-κB ligand (RANKL), which are mainly produced by osteoblast/stromal cells. The binding of M-CSF to its receptor c-Fms stimulates RANK expression in hematopoietic stem cells (c-Fms+RANK−) and induces osteoclast precursor cells (c-Fms+RANK+), which are common precursors for osteoclasts and monocytes/macrophages (1) The binding of RANKL to its receptor RANK activates c-Fos and nuclear factor-κB (NFκB), followed by the expression of nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent-1 (NFATc1). NFATc1 induces osteoclast-specific gene expression, such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K. c-Fos expression has also been reported to be mediated by the transcription factor PPARγ (2, 3). The deletion of PPARγ in mouse hematopoietic lineages of osteoclasts but not osteoblasts impaired osteoclast differentiation through suppressing the expression of c-Fos (2). Treatment with synthetic PPARγ agonists, such as rosiglitazone, the thiazolidinedione (TZD) class of antidiabetic agents, increased the expression of c-Fos and TRAP, and
stimulated osteoclastogenesis (2, 4-6). n-3 polyunsaturated fatty acids (PUFAs) are known as natural ligands of PPARγ (7). These suggest that n-3 PUFAs directly act on bone marrow-derived macrophages (BMMs) and increase osteoclastogenesis mediated by PPARγ.

However, n-3 PUFAs, eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), have been reported to decrease osteoclastogenesis in vivo (8-11). I have reported that dietary fish oil, rich in EPA and DHA, suppressed ovariectomy-stimulated osteoclastogenesis by inhibiting the expression of RANKL along with reduction of the expression of COX2, TNFα and IL-6 and the reduced activation of NFκB (11). The transcription factor NFκB is known to regulate the expression of COX2, TNFα and IL-6, which stimulate RANKL expression. Although the precise mechanism of the effect of n-3 PUFAs on osteoclastogenesis is not yet fully understood, it has been suggested that n-3 PUFAs indirectly inhibit osteoclastogenesis of BMMs by a decrease in RANKL expression through the reduction of NFκB activation in the mesenchymal cell lineage, osteoblasts/stromal cells of bone marrow. n-3 PUFAs are known to inhibit the activity of NFκB in several ways, including interference with the initiation of transcription by NFκB via PPARγ (12-14). I hypothesize that n-3 PUFAs inhibit the activation of NFκB by binding to PPARγ in MSCs and indirectly inhibit osteoclastogenesis of BMMs in bone marrow.

PPARγ was originally described as a factor induced during adipocyte differentiation (15-17). Osteoblasts and adipocytes are derived from a common progenitor, MSCs. Lineage commitment of MSCs is determined by the expression of specific transcription factors, such
as Runx2 for osteoblasts, and PPARγ for adipocytes. In mice and rats, the activation of PPARγ by treatment with synthetic PPARγ agonists, TZDs, caused bone loss, which resulted from an increase of marrow adipocytes and a decrease of osteoblasts associated with the decreased expression of Runx2 (18, 19). Clinical studies also reported that TZD treatment decreased bone formation markers in male and female diabetic patients (20), healthy postmenopausal women (21), and postmenopausal women with type 2 diabetes (22). In contrast to my hypothesis mentioned above, these suggest that the binding of n-3 PUFAs to PPARγ does not lead to the inhibition of NFκB activation, but to the diversion of MSCs from osteoblasts to the adipocyte lineage.

In the present study, I investigated the effects of EPA and DHA on osteoclastogenesis and the role of PPARγ using three cell culture systems: the culture of BMMs, that of BMCs comprising whole bone marrow cells (BMCs) except erythrocytes and that of MSCs. I demonstrated that EPA and DHA stimulated osteoclastogenesis through the PPARγ-mediated enhancement of c-Fos expression in the culture of BMMs but suppressed osteoclastogenesis through the PPARγ-dependent inhibition of NFκB activation of MSCs in the culture of BMCs.

### Materials and Methods

#### Materials
Minimum Essential Medium (MEM) was purchased from Sigma-Aldrich, Co., LLC. (St. Louis, MO, USA). Recombinant murine M-CSF and recombinant soluble RANKL were from PeproTech Inc. (Rocky-Hill, NJ, USA). Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LA), arachidonic acid (AA), and the PPARγ antagonists, GW9662 and T0070907, were from Cayman Chemical Co. (Ann Arbor, MI, USA). The PPARγ agonists, rosiglitazone and GW1929, were from Enzo Life Science, Inc. (Farmingdale, NY, USA) and Wako Pure Chemical Industries, Ltd. (Kyoto, Japan), respectively.

**Preparation of BMCs, BMMS, and MSCs**

Bone marrow cells (BMCs) were prepared by removing femurs and tibias from 5-week-old Wistar/ST male rats (Japan SLC, Shizuoka, Japan) and flushing the bone marrow cavities with MEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The BMCs were cultured in MEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml) plus 10% fetal bovine serum (FBS) for 18–24 h in the presence of M-CSF (5 ng/ml) and the non-adherent and adherent cells were collected. After establishing a Ficoll-Paque gradient of the non-adherent cells, the cells at the gradient interface were used as bone marrow-derived monocyte/macrophage precursor cells (BMMs) as previously described (23). Adherent cells were used as mesenchymal stem cells (MSCs) after trypsinization. Animal experiments were performed in accordance with protocols approved by the Animal Care Research Committee of Nara Women’s University.
Cell cultures

BMMs (2x10^4 cells/well of a 96-well plate), BMCs after lysis of red blood cells with 0.83% ammonium chloride (1x10^5 cells/well of a 96-well plate), or MSCs (6x10^5 cells/well of a 6-well plate) were cultured in MEM culture medium containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 1% non-essential amino acids, and 1% sodium pyruvate plus M-CSF (20 ng/ml) and RANKL (10 ng/ml) with or without PUFAs (EPA, DHA, AA, and LA), PPARγ agonists, and PPARγ antagonists. Cultures were maintained with a change of medium every 3 days. After 4 days, cells were used for assessments of cell viability and TRAP staining using WST-8 (Cell counting Kit-8; Dojindo Technologies, Kumamoto, Japan) and leukocyte acid phosphatase kit (Sigma 387-A), respectively, as described previously (24). The TRAP-positive multinucleated cells (not less than 3 nuclei per cell) (MNCs) were manually counted with a light microscope. For the determination of mRNA and protein levels, the cells were harvested at 3 or 4 days and used for quantitative real-time RT-PCR or Western blotting, respectively.

Quantitative real-time PCR analysis

Total RNA from cell lysates of the cultured BMMs, BMCs, and MSCs was prepared using a commercial kit (Sepasol-RNA I Super G; Nacalai Tesque, Inc., Kyoto, Japan). The total RNA was reverse-transcribed with a first-strand cDNA synthesis kit (Rever Tra Ace
qPCR RT; Toyobo, Co., Ltd., Osaka, Japan). Real-time PCR was performed using the cDNA, or total RNA as a negative control, with THUNDERBIRD SYBR qPCR Mix (Toyobo, Co., Ltd., Osaka, Japan) and specific primers (Table I), using a Light Cycler real-time PCR detection system (Toyobo). The amplification program consisted of 1 cycle for 1 min at 95 °C followed by 45 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Melting curve was used to verify specific products of the appropriate size. Gene expression levels were determined relative to an internal standard (actin) and expressed relative to the control values.

**Western blot analysis**

The cell lysates of BMMs were used for western blot analysis. Equal amounts of protein were electrophoresed in SDS-polyacrylamide gels and transferred to membranes. Western blotting and reprobing were performed and the chemiluminescent signals were quantified by a densitometer, as previously described (25). Antibodies recognizing actin (H-300), NFATc1 (H-110), c-Fos (D-1), and PPARγ (H-100) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The protein concentration was measured with the BCA protein assay kit (Pierce/Thermo Fisher Scientific Inc., Rockford, IL, USA).

**Statistical analysis**

All statistical analyses were performed using the Microsoft Excel data analysis program for the Bonferroni method. Values are expressed as the mean ± SEM. Values of p<0.05 were
considered significant.

**Results**

*Effects of n-3 and n-6 PUFAs on osteoclastogenesis and mRNA expressions of osteoclastogenesis-related genes, PPARγ and COX2, of BMMs*

The presence of EPA at 5 or 10 μM increased the number of TRAP-positive MNCs to about 1.6- or 1.8-fold the control, respectively (Fig. 1A). Another n-3 PUFA, DHA, also increased the formation of TRAP-positive MNCs. However, n-6 PUFAs, LA and AA, had no effect on the generation of TRAP-positive MNCs of BMMs. Cell viability was not affected by the presence of these PUFAs.

The mRNA levels of osteoclastogenesis-related genes of BMMs in the presence or absence of PUFAs were examined by real-time RT-PCR. The mRNA levels of c-Fms, PU.1, MITF, RANK, and c-Jun in BMMs were unaltered by the presence of 10 μM of n-3 or n-6 PUFAs. However, the presence of EPA or DHA significantly increased the mRNA levels of c-Fos, TRAP and cathepsin K to about 3.6-, 1.9-, and 2.8-fold the control levels, respectively (Fig. 2A). Furthermore, the expression of PPARγ also increased to 2.2-fold the control level by the addition of EPA or DHA, whereas COX2 expression was unaltered (Fig. 2A). n-6 PUFAs, AA and LA, had no significant effect on the expression of these genes.
**Effects of EPA or DHA on the protein levels of PPARγ, c-Fos, and NFATc1 of BMMs**

The treatment with EPA significantly increased the protein levels of PPARγ, c-Fos, and NFATc1 to about 2.0-, 3.2-, and 1.9-fold the control values, respectively (Fig. 2B). DHA also increased these proteins to levels similar to EPA.

**Effects of PPARγ agonists on osteoclastogenesis and the mRNA expression of osteoclastogenesis-related genes of BMMs**

To examine the effects of PPARγ on osteoclastogenesis of BMMs, a PPARγ agonist, rosiglitazone or GW1929, was added to the culture medium. The presence of rosiglitazone at 1, 5, and 10 μM increased the number of TRAP-positive MNCs to about 1.3-, 1.5-, and 1.6-fold the control values, respectively (Fig. 3A). The highly selective PPARγ agonist GW1929 at lower concentrations of 0.05 and 0.1 μM also increased the formation of TRAP-positive MNCs to about 1.5- and 1.7-fold the control values, respectively. The expressions of c-Fos, TRAP, and cathepsin K significantly increased to about 3.7-, 2.1-, 2.2-fold the control in the presence of these agonists, respectively, while those of c-Fms, PU.1, MITF, RANK, and c-Jun were unchanged (Fig. 3B).

**Effects of PPARγ antagonists on osteoclastogenesis and the c-Fos mRNA expression of BMMs with EPA or DHA**

The presence of PPARγ antagonists, GW9662 and T0070907, decreased the increased
number of TRAP-positive MNCs by EPA or DHA to the control levels without effects on the cell viability (Fig. 4A). The stimulated expressions of c-Fos by EPA or DHA were recovered to the control levels by addition of these antagonists at 1.0 μM (Fig. 4B).

**Effects of PUFAs on osteoclastogenesis and mRNA expressions of osteoclastogenesis-, osteoblastogenesis-, and inflammatory-related factors in BMCs**

In contrast to BMMs, the addition of EPA to BMCs significantly inhibited the formation of TRAP-positive MNCs to 80 and 60% of the control at 5 and 10 μM, respectively, without effects on cell viability (Fig. 5A). DHA also suppressed osteoclast formation to 70 and 30% at 5 and 10 μM, respectively. However, n-6 PUFAs, LA and AA, had no significant effects on the formation of TRAP-positive MNCs in BMCs. The cell viability was not affected by the presence of these PUFAs at 10 μM.

The mRNA levels of M-CSF, c-Fms, PU.1, RANK, and c-Jun of BMCs were unaltered by the presence of EPA, DHA, LA, or AA at 10 μM (Fig. 5B). However, the presence of EPA significantly decreased the mRNA levels of RANKL, MITF, c-Fos, TRAP, and cathepsin K to about 54, 70, 60, 70, and 50% of the control levels, respectively. DHA also decreased these genes to a similar extent. EPA or DHA suppressed the expression of COX2 and the inflammatory cytokines, TNFα and IL-6, to about half level of the control, while no significant effect on PPARγ expression was observed (Fig. 5C). The expression of the transcription factors required for osteoblastogenesis and adipogenesis, Runx2 and aP2,
respectively, were not affected by EPA and DHA. AA and LA had no significant effect on the expressions of these genes of BMCs.

Effects of PPARγ agonists on osteoclastogenesis and the mRNA expressions of osteoclastogenesis-, osteoblastogenesis-, and inflammatory-related genes in BMCs

The PPARγ agonist rosiglitazone at 5 and 10 μM decreased osteoclast formation to 70 and 50% of the control level, respectively (Fig. 6A). GW1929 at 0.05 and 0.1 μM also inhibited osteoclastogenesis to 70 and 55% of the control.

Rosiglitazone or GW1929 suppressed the expressions of RANKL, c-Fos, TRAP, cathepsin K, COX2, TNFα, and IL-6, although no significant effects on the expressions of aP2 and Runx2 were detected, as observed with EPA and DHA (Fig. 6B and C).

Effects of PPARγ antagonists on osteoclastogenesis in BMCs with EPA or DHA

The PPARγ antagonists, GW9662 or T0070907, at 1 μM recovered the decreased number of TRAP-positive MNCs by EPA to the control levels (Fig. 7A). The inhibition of the formation of TRAP-positive MNCs by DHA was also recovered by the antagonists, but even at 2 μM of antagonists, their recovery was about 70% of the control level. The higher concentration (at more than 3 μM) of antagonists decreased cell viability (Fig. 7B).

Effects of PPARγ antagonists on mRNA levels of M-CSF, RANKL, PPARγ, COX2, TNFα, IL-6,
aP2, and Runx2 of MSCs

The addition of EPA and DHA at 10 μM to the culture medium of MSCs decreased the expressions of RANKL, COX2, TNFα, and IL-6 to about 30, 30, 60, and 30% of the control, respectively, although significant changes in the expressions of M-CSF, PPARγ, aP2, and Runx2 were not observed (Fig. 8). The treatment with DHA showed a similar profile of changes in these gene expressions. The presence of GW9662 or T0070907 at 1 μM inhibited the suppression of the expressions of RANKL, COX2, TNFα, and IL-6 by EPA or DHA.

Discussion

In this study, I demonstrated that EPA and DHA stimulated osteoclastogenesis in the culture of BMMs. The stimulation of osteoclastogenesis by EPA and DHA was associated with increased c-Fos expression. The events upstream of the induction of c-Fos in osteoclastogenesis, c-Fms/M-CSF and RANK/RANKL signaling were not affected by EPA or DHA. c-Fos is essential for osteoclastogenesis (26). The binding of RANKL to its receptor RANK induces the expression of c-Fos and then NFATc1, which directly regulates a number of osteoclast-specific genes like TRAP and cathepsin K (27). In addition to RANK/RANKL signaling, c-Fos has been reported to be directly regulated by PPARγ using transgenic gf/f-Tie2Cre mice (2). The increase in c-Fos expression was also associated with the increased expression of PPARγ in the present study. n-3 PUFAs were reported to induce
PPARγ expression more markedly than n-6 PUFAs in the retina or choroid of mice (28).

Indeed, n-6 PUFAs, LA and AA, had no effect on the expression of PPARγ or c-Fos in BMMs in this study. Furthermore, PPARγ agonists, rosiglitazone and GW1929, stimulated the osteoclastogenesis associated with the increased mRNA expression of c-Fos, as observed in the treatment with EPA or DHA. In contrast, the addition of PPARγ antagonists, GW9662 and T0070907, suppressed the n-3 PUFA stimulated c-Fos expression and osteoclastogenesis. These results clearly demonstrate that c-Fos expression stimulated by EPA and DHA was mediated by PPARγ. Inconsistently, the inhibition of osteoclastogenesis by EPA and DHA was reported in the murine monocytic cell line RAW264.7 (29). One of the reasons why the inhibitory effect was observed may be due to higher doses of EPA and DHA (at more than 50 μM) used in RAW264.7 than in this study (at 10 μM). Although the inhibition of osteoclastogenesis by PPARγ agonists was also reported in BMMs (30), the present findings support the suggestion that the activation of PPARγ induces c-Fos and thereby stimulates the osteoclastogenesis of BMMs, in agreement with previous lines of evidence (2, 5, 6, 31), as illustrated in Fig. 9A.

Obviously, the stimulatory effects of EPA and DHA on osteoclastogenesis are inconsistent with the previous in vivo studies, which reported that n-3 PUFAs decreased osteoclastogenesis (8-11). Osteoclastic differentiation of BMMs occurs in bone marrow in vivo, interacting with other types of cells including osteoblasts/stromal cells and immune cells. So, to obtain an environment for osteoclastogenesis similar to that in bone marrow in vivo,
the differentiation of BMMs into osteoclasts was examined using the culture of BMCs, which are whole BMCs depleted of erythrocytes. The osteoclastogenesis in BMCs was inhibited by EPA and DHA associated with a decrease in the expression of RANKL, COX2, TNFα and IL-6, as observed in my previous animal study (11). DHA inhibited osteoclastogenesis more potently than EPA, although no significant difference was observed in the gene expression levels. COX2, TNFα and IL-6, inducers of RANKL, are downstream targets of NFκB. To verify my hypothesis of the PPARγ-dependent inhibition of NFκB activation by EPA and DHA, experiments using PPARγ agonists and antagonists were conducted. As expected, PPARγ agonists, rosiglitazone and GW1929, were found to inhibit the expression of NFκB-regulating genes, COX2, TNFα and IL-6, associated with the inhibition of osteoclast formation, as observed in the treatment with EPA and DHA. The addition of the PPARγ antagonists, GW9662 and T0070907, abrogated the inhibitory effect of EPA and DHA on osteoclastogenesis in the culture of BMCs. This abrogation was complete in EPA but partial in DHA, suggesting the partial involvement of mechanisms other than the PPARγ-dependent pathway in the case of DHA. These results indicate that EPA and DHA inhibit osteoclastogenesis by the PPARγ-dependent inhibition of the transcriptional activation of NFκB in BMCs and strongly suggest that this PPARγ signaling pathway is involved in the inhibitory effect of n-3 PUFAs on osteoclastogenesis in vivo.

Next, to clarify which cells are responsible for the PPARγ-dependent inhibition of NFκB activation in the various types of cells contained in BMCs, the effects of EPA and DHA on
gene expression profiles of MSCs, which are precursors of a high RANKL expressing cell lineage, osteoblasts/stromal cells, were examined. Similarly to the results of the culture of BMCs, mRNA expressions of RANKL, COX2, TNFα and IL-6 in MSCs were inhibited by EPA and DHA. In addition, these inhibitions were completely blocked by PPARγ antagonist. These results suggest that the inhibitory effects of EPA and DHA on osteoclastic differentiation of BMMs in bone marrow were mediated by the PPARγ-dependent inhibition of NFκB activation in MSCs. Although the mechanism by which PPARγ inhibits the NFκB activation is not known, I speculate the presence of the transrepression mechanism by SUMOylation as reported in macrophages (12), or by unknown transcriptional repressors induced by PPARγ, as illustrated in Fig. 9B. The two different PPARγ response networks, the PPARγ-mediated c-Fos expression in BMMs and PPARγ-mediated inhibition of the activation of NFκB in MSCs, are proposed in Fig. 9. PPARγ is known to influence distinct target genes in various types of cells (32). The difference in the PPARγ signaling pathway may be due to the fact that PPARγ function is dependent on the availability of coregulators that differentially regulate the transcriptional activities of PPARγ and its target genes (33). However, the reason why MSCs rather than BMMs respond to n-3 PUFAs in bone marrow remains unknown, although differences in the ability of fatty acid incorporation and/or the population of two cell types might be conceivable.

On the other hand, PPARγ is also known to induce adipogenesis and inhibit osteoblastogenesis (34). The synthetic PPARγ agonists, TZDs, promote adipogenesis and
inhibit osteoblast differentiation in BMCs (18, 35, 36). However, in this study, the effects of EPA and DHA on the expression of aP2 and Runx2 were not observed in BMCs or MSCs. These results are consistent with my previous report that fish oil did not affect the ALP activity nor the number of osteoblasts in the proximal tibia of ovariectomized rats (11). The decrease in the number of osteoblasts of the proximal tibia by rosiglitazone was observed in only adult (6-month-old) mice, and not in young (1-month-old) nor old (24-month-old) animals (37). Bone marrow derived from old mice was reported to produce unknown PPARγ activator(s) that stimulates adipocyte differentiation and suppresses osteoblast differentiation (38). In addition to the PPARγ action, other as yet unknown factors have been suggested to be required to alter the fate of progenitor cells into osteoblasts or adipocytes (39). Regarding n-3 PUFAs, there is abundant evidence that n-3 PUFAs are associated higher bone mass in animals and humans (40-42). Taken together, n-3 PUFAs may not induce the PPARγ-mediated inhibition of osteoblastogenesis, but the PPARγ-mediated inhibition of osteoclastogenesis through inhibiting the activation of NFκB in vivo.

Clinical studies have reported that treatment with TZDs results in bone loss and increased risk of fracture rates in aged and diabetic women (43, 44). Rosiglitazone therapy for 14 weeks to healthy postmenopausal women decreased circulating bone formation markers without affecting bone resorption markers (21). Long-term (12 months) rosiglitazone treatment in patients with type 2 diabetes reduced bone formation markers and elevated bone resorption markers in women, while only a decrease in bone formation markers was observed
in men (20). Taking rosiglitazone for 6 months elevated bone resorption markers and not bone formation markers in women with type 2 diabetes and cardiovascular disease or additional cardiac risk factors (45). Collectively, although TZDs affect both bone formation and bone resorption, longer-term TZD treatment in women, the aged and/or patients with diabetes may elevate bone resorption. It can be speculated that depending on the conditions such as age, sex, health condition and treatment term, PPARγ agonists, probably also EPA and DHA, may act directly on BMMs and stimulate osteoclastogenesis followed by an increase in bone resorption in vivo as in vitro, showing by the culture of BMMs in this study.

In conclusion, this study provides evidence that EPA and DHA stimulated osteoclastogenesis by enhancing the PPARγ-mediated c-Fos expression of BMMs but suppressed osteoclastogenesis via the PPARγ-dependent inhibition of NFκB activation of MSCs in the culture of BMCs. The suppressive effects of EPA and DHA on osteoclastogenesis in the culture of BMCs suggest that n-3 PUFAs are beneficial for bone health in vivo.
Summary

n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been reported to decrease osteoclastogenesis in vivo. In this study, the effect of PUFAs on receptor for activation of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis was examined using bone marrow-derived monocytes/macrophage precursor cells (BMMs) or bone marrow cells (BMCs) in vitro. EPA and DHA stimulated the osteoclastic differentiation of BMMs, but n-6 PUFAs, linoleic and arachidonic acids, had no effect. The stimulation of osteoclastogenesis of BMMs by EPA and DHA was associated with enhancement of the gene expressions of c-Fos, tartrate-resistant acid phosphatase, cathepsin K, and peroxisome proliferator-activated receptor-γ (PPARγ), and the protein levels of c-Fos, PPARγ, and nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent-1 (NFATc1). The PPARγ agonists, rosiglitazone and GW1929, also stimulated the osteoclastic differentiation of BMMs. The PPARγ antagonists, T0070907 and GW9662, inhibited the stimulations of BMM osteoclastogenesis and c-Fos expression by EPA or DHA. However, EPA and DHA inhibited the osteoclastogenesis in BMCs comprised of BMMs and mesenchymal stem cells (MSCs). The inhibition was associated with suppression of the expression of RANKL and nuclear factor-κB (NFκB)-regulating genes, cyclooxygenase 2 (COX2), TNFα, and IL-6 in BMCs and MSCs. The agonists and antagonists of PPARγ showed that the inhibitions of NFκB transcriptional activity and osteoclastogenesis by EPA and DHA were PPARγ-dependent. These results suggest that EPA
and DHA stimulated the osteoclastogenesis of BMMs through enhancing c-Fos expression mediated by PPARγ, but suppressed osteoclastogenesis through the PPARγ-dependent inhibition of NFκB activation of MSCs in BMCs.
References


25. Nakanishi A, Hie M, Itsuka N and Tsukamoto I: A crucial role for reactive oxygen species in


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Primer sets for actin, aP2, c·Fms, c·Fos, c·Jun, cathepsin K, COX2, IL-6, M-CSF, MITF, PU.1, PPARγ, RANK, RANKL, Runx2, TNFα, and TRAP.
**Fig. 1  Effects of PUFAs on osteoclastogenesis of BMMs.** BMMs were cultured in the absence or presence of PUFAs (1, 5, or 10 μM). After the culture of BMMs for 4 days, the cells were fixed and stained for TRAP. Scale bar indicates 100 μm. Representative microscopic fields (x100 magnification; upper panel) and a quantitative graph (lower panel) are shown. Values are means ± SEM. *, Significantly different from the control value (p<0.05).
Fig. 2  Effects of PUFAs on gene expression, and protein levels of BMMs. BMMs were cultured in the absence or presence of PUFAs (1, 5, or 10 μM). (A) After the culture of BMMs in the absence or presence of 10 μM of PUFAs for 72 h, the cells were harvested and used for real-time RT-PCR. The expression levels are shown relative to the control values. (B) After the culture of BMMs in the absence or presence of 10 μM of PUFAs for 4 days, the cells were harvested and used for Western blotting for PPARγ, c-Fos, and NFATc1. The protein levels are shown relative to the control values. Values are means ± SEM.

* Significantly different from the control value (p<0.05).
Fig. 3  Effects of PPARγ agonists on osteoclastogenesis and gene expression of BMMs. BMMs were cultured in the absence or presence of the PPARγ agonists, rosiglitazone (1, 5, or 10 μM) and GW1929 (0.01, 0.05, or 0.1 μM). (A) After the culture of BMMs for 4 days, the cells were fixed and stained for TRAP. Scale bar indicates 100 μm. Representative microscopic fields (x100 magnification; upper panel) and quantitative data (lower panel) are shown. (B) After the culture of BMMs in the absence or presence of rosiglitazone (10 μM) or GW1929 (0.1 μM) for 72 h, the cells were harvested and used for real-time RT-PCR. The expression levels are shown relative to the control values. Values are means ± SEM. *, Significantly different from the control value (p<0.05).
Fig. 4  Effects of PPARγ antagonists on osteoclastogenesis of BMMs. BMMs were cultured in the absence or presence of EPA or DHA (10 μM) plus GW9662 or T0070907 (0, 0.1, 0.5, or 1.0 μM). (A) After the culture of BMMs for 4 days, the cells were fixed and stained for TRAP. Scale bar indicates 100 μm. Representative microscopic fields (x100 magnification; upper panel) and quantitative data (lower panel) are shown. (B) After the culture of BMMs in the absence or presence of EPA or DHA (10 μM) plus GW9662 or T0070907 (None or 1.0 μM) for 72 h, the cells were harvested and used for real-time RT-PCR. The expression levels are shown relative to the control values. Values are means ± SEM. *, Significantly different from the control value (p<0.05). #, Significantly different from the values of EPA or DHA without PPARγ antagonists (p<0.05).
Fig. 5  Effects of PUFAs on osteoclastic differentiation and gene expression in BMCs. BMCs were cultured in the absence or presence of PUFAs (1, 5, or 10 μM). (A) After the culture of BMCs for 4 days, the cells were fixed and stained for TRAP. Scale bar indicates 100 μm. Representative microscopic fields (x100 magnification; upper panel) and a quantitative graph (lower panel) are shown. (B, C) After the culture of BMCs in the presence of 10 μM of PUFA for 72 h, the cells were harvested and used for real-time RT-PCR. The expression levels are shown relative to the control values. Values are means ± SEM. *, Significantly different from the control value (p<0.05).
Fig. 6  Effects of PPARγ agonists on osteoclastogenesis and gene expression in BMCs. BMCs were cultured in the absence or presence of the PPARγ agonists, rosiglitazone (1, 5, or 10 μM) and GW1929 (0.01, 0.05, or 0.1 μM). (A) After the culture of BMCs for 4 days, the cells were fixed and stained for TRAP. Scale bar indicates 100 μm. Representative microscopic fields (x100 magnification; upper panel) and quantitative data (lower panel) are shown. (B,C) After the culture of BMCs in the absence or presence of rosiglitazone (10 μM) and GW1929 (0.1 μM) for 72 h, the cells were harvested and used for real-time RT-PCR. The expression levels are shown relative to the control values. Values are means ± SEM.

*  Significantly different from the control value (p<0.05).
Fig. 7 Effects of PPARγ antagonists on osteoclastogenesis of BMCs. BMCs were cultured for 4 days in the absence or presence of EPA or DHA (10 μM) plus GW9662 or T0070907 (0, 0.1, 0.5, 1.0, or 2.0 μM). (A) After the culture of BMCs, the cells were fixed and stained for TRAP. Scale bar indicates 100 μm. Representative microscopic fields (x100 magnification; upper panel) and quantitative data (lower panel) are shown. (B) Cell viability tests of BMCs, in the presence of DHA (10 μM) plus GW9662 or T0070907 (0–5.0 μM). The cell viability levels are shown relative to the control values. Values are means ± SEM. *, Significantly different from the control value (p<0.05). #, Significantly different from the values of EPA or DHA without PPARγ antagonists (p<0.05).
Fig. 8 Effects of PPARγ antagonists on the expression levels of M-CSF, RANKL, PPARγ, COX2, TNFα, IL-6, aP2, and Runx2 in MSCs. After the culture of MSCs in the absence or presence of EPA or DHA (10 μM) with or without GW9662 or T0070907 (1.0 μM for EPA, or 2.0 μM for DHA) for 72 h, the cells were harvested and used for real-time RT-PCR for M-CSF, RANKL, PPARγ, COX2, TNFα, IL-6, aP2, and Runx2. The expression levels are shown relative to the control values. Values are means ± SEM. *, Significantly different from the control value (p<0.05). #, Significantly different from the values of EPA or DHA without PPARγ antagonists (p<0.05).
Fig. 9  Proposed mechanisms by which EPA and DHA affect osteoclastogenesis. (A) In osteoclast precursor cells (BMMs), ligand (EPA, DHA, their metabolite or PPARγ agonist)–activated PPARγ enhances c-Fos expression, which stimulates osteoclastogenesis (3). (B) In MSCs in the culture of the bone marrow, ligand (EPA, DHA, their metabolite or PPARγ agonist) inhibits the activation of NFκB by the SUMOylation of PPARγ (10) or the induction of transcriptional repressor in MSCs. The inhibition of NFκB transcriptional activity leads to a reduction of the expression of NFκB–regulating genes, TNFα, IL-6 and COX2, followed by a decrease in RANKL. The decreased level of RANKL suppresses the osteoclastic differentiation of BMMs.
Conclusion

Most studies of osteoclastogenesis have used late-stage precursor cells (c-Fms+ RANK+), including the murine monocyte cell line RAW264.7 cells. Previous studies of the role of ROS have also concentrated on the late stages of osteoclastic differentiation. Thus, in this study, I investigated the molecular basis for M-CSF-induced ROS generation using early-stage precursor cells. M-CSF generated ROS at approximately 5 min after the stimulation. The production of ROS was inhibited by a Nox inhibitor, DPI. The mRNA expressions of PU.1, MITF, and RANK, and osteoclastogenesis of precursor cells were also inhibited by DPI. These results suggested that Nox-mediated ROS production was occurred by M-CSF stimulation, and required for the expression of RANK in the early-stage osteoclast precursor cells. In addition, the gene expression of Nox isoforms was examined in precursor cells. The mRNA level of Nox2 was about 1,000-fold higher than that of Nox1, whereas Nox3 and Nox4 were undetectable. M-CSF increased the expression of Nox1 to about 4-fold the uninduced level, but decreased the expression of Nox2 to about 30% the uninduced level. The siRNA targeting Nox2, but not Nox1 inhibited the M-CSF-stimulated ROS production and RANK expression. This study provides evidence that ROS production in response to M-CSF was mediated by Nox2, and was required for the RANK expression in the early-stage osteoclast precursor cells.

The pathogenesis of bone loss in postmenopausal women involves an accelerated bone
turnover with a relative increase in bone resorption to bone formation. In the second chapter of this thesis, I demonstrated that the effect of fish oil on bone resorption and formation, and the underlying molecular mechanisms using ovariectomized rats. As previously reported by various researchers, ovariectomized rats showed bone loss by significant increase in osteoclastic and osteoblastic activities. The enhanced osteoclastogenesis in ovariectomized rats is associated with the up-regulation of M-CSF and RANKL, and the increased serum levels of TNFα, IL-6 and PGE2. These inflammatory related factors regulated by NFκB have been found to induce the expression of RANKL. In addition, significant increase in the plasma levels of AA (20:4, n-6) and NFκB activation were also observed. These results suggest that an increase in plasma AA level or an imbalance in the n-6/n-3 PUFAs ratio induced by ovariectomy activate NFκB, and increase the level of inflammatory related factors, and then stimulate osteoclastogenesis.

In fish oil feeding rats, decreased the plasma levels of n-6 PUFAs (AA and LA) and the increased levels of n-3 PUFAs (EPA, DPA and DHA) were observed. The decreased n-6/n-3 PUFAs ratio led to the suppression of NFκB activation in OvxF, which inhibit downstream events, including TNFα, M-CSF/Pu.1/MITF/RANK and RANKL. The results of this study reveal that fish oil reduces ovariectomy-stimulated osteoclastogenesis by suppressing the expression of M-CSF, Pu.1, MITF and RANK in the early stages of osteoclastogenesis and RANKL signaling in later stages.

As I have reported in the second chapter, n-3 PUFAs, EPA (20:5 n-3) and DHA (22:6
n-3) have been reported to decrease osteoclastogenesis in vivo. I hypothesized that PPARγ is involved in the inhibition of NFκB activation by n-3 PUFAs in osteoclastogenesis. However, the addition of n-3 PUFAs enhanced osteoclastogenesis in the culture of BMMs. This stimulation was associated with the upregulation of c-Fos and PPARγ. The mRNA levels of c-Fms, PU.1, MITF, and RANK were not affected by EPA and DHA. PPARγ agonists, rosiglitazone and GW1929, also stimulated the osteoclastogenesis of BMMs associated with the increased expression of c-Fos. Further, the addition of PPARγ antagonists, GW9662 and T0070907, suppressed the n-3 PUFA-stimulated c-Fos expression and osteoclastogenesis. These results clearly demonstrated that c-Fos expression stimulated by EPA and DHA was mediated by PPARγ.

On the other hand, the osteoclastogenesis of BMCs, a co-culture system containing BMMs and MSCs was inhibited by EPA and DHA associated with a decrease in RANKL expression as reported in vivo. In BMCs, mRNA expression of COX2, TNFα, and IL-6 were also inhibited. These suppressions occurred also in MSCs, were completely blocked by PPARγ antagonists. Further, PPARγ agonists were found to inhibit the expression of NFκB-regulating genes, COX2, TNFα and IL-6, associated with the inhibition of osteoclast formation, as observed in the treatment with EPA and DHA. In this study, mRNA levels of osteoblastogenesis-related genes, and adipogenesis-related genes were not affected by EPA and DHA. In addition, n-6 PUFAs, AA and LA had no effect on the osteoclastogenesis. The result of this study demonstrated that EPA and DHA stimulated the osteoclastogenesis of
BMMs through the PPARγ-mediated enhancement of c-Fos expression, but suppressed osteoclastogenesis through the PPARγ-dependent inhibition of NFκB activation of MSCs in BMCs. However, the reason why MSCs rather than BMMs respond to n-3 PUFAs in bone marrow remains unknown, although differences in the ability of fatty acid incorporation and/or the population of two cell types might be conceivable.

These nutritional and biochemical studies on the regulatory mechanisms of osteoclastogenesis in this thesis will offer insight into understanding the molecular basis for bone metabolism. I hope that this thesis stimulate further research, and contribute to the nutritional prevention and therapy of osteopenia or osteoporosis.
List of Publications

1) Hie M, Iitsuka N, Otsuka T, Nakanishi A and Tsukamoto I.

Zinc deficiency decreases osteoblasts and osteoclasts associated with the reduced expression of Runx2 and RANK.

Bone 49: 1152-9, 2011.

2) Iitsuka N, Hie M, Nakanishi A and Tsukamoto I.

Ethanol increases osteoclastogenesis associated with the increased expression of RANK, PU.1 and MITF in vitro and in vivo.


3) Nakanishi A, Hie M, Iitsuka N and Tsukamoto I.

A crucial role for reactive oxygen species in macrophage colony-stimulating factor-induced RANK expression in osteoclastic differentiation.


4) Nakanishi A, Iitsuka N and Tsukamoto I.

Fish oil suppresses bone resorption by inhibiting osteoclastogenesis through decreased expression of M-CSF, PU.1, MITF and RANK in ovariectomized rats.
5) Nakanishi A and Tsukamoto I.

n-3 polyunsaturated fatty acids stimulate osteoclastogenesis through PPARgamma-mediated enhancement of c-Fos expression, and suppress osteoclastogenesis through PPARgamma-dependent inhibition of NFkB activation.


6) Ootsuka T, Nakanishi A and Tsukamoto I.

Increase in osteoclastogenesis in an obese Otsuka Long-Evans Tokushima fatty rat model.

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