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

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Abstract. Previous studies have identified a positive correlation between the intake of n-3 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 (sham) 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 eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Fish oil reversed the increased activity and number of osteoclasts, and decreased calcium (Ca) and hydroxyproline (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 and protein levels of macrophage colony-stimulating factor (M-CSF), PU.1, microphthalmia-associated transcription factor (MITF), receptor for activation of NFκB (RANK) and RANK ligand (RANKL) and serum levels of tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and prostaglandin E2 (PGE2). Fish oil was also found to suppress NFκB activation induced by ovariectomy. These results indicate that increases in plasma n-3 fatty acid levels by fish oil led to the suppression of NFκB activation and subsequent downregulation of TNFα, followed by suppression of M-CSF and RANKL. Dietary fish oil suppressed ovariectomy-stimulated osteoclastogenesis by inhibiting the expression of M-CSF, PU.1, MITF and RANK in the early stages of osteoclastogenesis, upstream of RANKL signaling.

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Abbreviations: AA, arachidonic acid; ALP, alkaline phosphatase; COX2, cyclooxygenase 2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL-6, interleukin-6; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; OPG, osteoprotegerin; OVX, ovariectomy; PGE2, prostagrandin E2; PLA2, phospholipase A2; PUFAs, polyunsaturated fatty acids; RANK, receptor for activation of NFκB; RANKL, receptor for activation of NFκB ligand; TRAP, tartrate-resistant acid phosphatase; TNFα, tumor necrosis factor α

Key words: ovariectomy, n-3 polyunsaturated fatty acids, osteoclastogenesis, M-CSF, PU.1, MITF, RANK

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). Osteoclasts are derived from bone marrow hematopoietic stem cells (3). The number and activity of osteoclasts is determined by cell lineage allocation, proliferation and the differentiation of osteoclast precursors (4). Osteoclastic differentiation requires macrophage colony-stimulating factor (M-CSF) and receptor for activation of NFκB ligand (RANKL) (5). In the early stages of osteoclastogenesis, binding of M-CSF to colony stimulating factor 1 receptor (c-fms) stimulates expression of RANK, the receptor of RANKL, in hematopoietic osteoclast precursor cells (6). At later stages, binding of RANKL to RANK activates c-fos, c-jun and nuclear factor of activated T cells cytoplasmic 1 (NFAT c1) in osteoclast precursors, which then differentiate into mononuclear osteoclasts (5).

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 (7). In animals, dietary supplementation with n-3 PUFA-rich oils, including fish oil, has been linked to improved maintenance of bone mass postovariectomy (8-10). In addition, endogenously produced n-3 PUFAs have been revealed to protect against ovariectomy-induced bone loss in fat-1 transgenic mice (11,12). 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 (13). Previous *in vitro* studies have revealed that n-3 PUFAs are linked to decreased NFκB expression (14,15) and modulation of RANKL signaling in RAW264.7 cells (16). 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 vivo* in ovariectomized rats. By contrast, n-3 PUFAs also affect bone formation in animal models (17,18) and osteoblast functions by increasing Runx2 expression in MC3T3 cells (19). 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. 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 weeks 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 α-tocopherol to match the concentration of corn oil. The fatty acid composition of the oils used in the diets are presented in Table II. After 2 weeks, blood and femoral and tibial bone samples were collected under sodium pentobarbital anesthesia after overnight access to food (non-fasting). Blood samples were used to determine the serum concentrations of estradiol, osteocalcin, TNFα, 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, TNFα, 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 (20,21).

Table I. Diet ingredients.

	Diet	
Ingredient (g/kg)	Corn oil	Fish oil
Casein	200	200
DL-methionine	3	3
Corn oil	50	5
Fish oil (menhaden oil)	0	45
Corn starch	150	150
Sucrose	500	500
Cellulose	50	50
Mineral mix[a]	35	35
Vitamin mix[b]	10	10
Choline bitartrate	2	2
Total (g)	1000	1000

[a]AIN 76 mineral mix (S10001); [b]AIN 76 vitamin mix (V10001). AIN, American Institute of Nutrition.

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, St. Louis, MO, USA) as described previously (20). 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-stained cells with >3 nuclei) were performed at standardized sites (300 x 300 μm) under the growth plate in the metaphysis of the proximal tibia (22).

Fatty acid analysis. Serum total lipids were extracted as described previously (23), with specific modifications (24). 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 methyl ester 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 (Toyobo, Tokyo, Japan). PCR was performed using cDNA or total RNA (negative control) with Thunderbird SYBR qPCR mix (Toyobo) and specific primers, as described previously (21,25). Levels of gene expression were determined relative to an internal standard (actin) and expressed relative to the ShamC values.

Western blot analysis. Bone extracts of the proximal tibia were prepared as described previously (20) for western blot analysis. Protein concentrations were measured using the BCA

Table II. Fatty acid composition of oils.

Fatty acid	(C:D)	Corn oil (g/100 g)	Fish oil[a] (g/100 g)
Myristic acid	14:0	-	6.2
Pentadecanoic acid	15:0	-	0.4
Palmitic acid	16:0	11.0	14.4
Palmitoleic acid	16:1 n-7	-	8.8
Hexadecadienoic acid	16:2 n-6	-	1.4
Hexadecatrienoic acid	16:3 n-3	-	1.4
Hexadecatetraenoic acid	16:4 n-3	-	1.4
Stearic acid	18:0	2.0	2.6
Oleic acid	18:1 n-9	25.0	11.2
Linoleic acid	18:2 n-6	60.2	7.8
α-Linolenic acid	18:3 n-3	1.4	1.4
Octadecatetraenoic acid	18:4 n-3	-	2.8
Arachidic acid	20:0	-	0.2
Eicosanoic acid	20:1 n-9	-	1.4
Eicosadienoic acid	20:2 n-6	-	0.2
Dihomo-γ-linolenic acid	20:3 n-6	-	0.4
Arachidonic acid	20:4 n-6	-	1.8
Eicosapentaenoic acid	20:5 n-3	-	12.8
Henicosapentaenoic acid	21:5 n-3	-	0.6
Docosenoic acid	22:1 n-9	-	0.2
Docosatetraenoic acid	22:4 n-6	-	0.2
Docosapentaenoic acid	22:5 n-3	-	2.6
Docosahexaenoic acid	22:6 n-3	-	9.2
Lignoceric acid	24:0	-	0.6
Tetracosenoic acid	24:1 n-9	-	0.2

[a]Menhaden/corn=9/1.

protein assay kit (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 (26). Antibodies recognizing actin, M-CSF, RANK, RANKL, osteoprotegerin (OPG), microphthalmia-associated transcription factor (MITF), PU.1, 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 fish oil on clinical characteristics and bone biochemical markers. 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 (Table III). Fish oil did not affect these values. The weights of the femur and tibia were significantly lower in OvxC rats than in the Sham groups, and were restored to Sham levels in OvxF rats; however, bone lengths were similar in the four groups (Table III).

ALP activity in the proximal tibia and serum osteocalcin levels were significantly higher in the Ovx groups compared with Sham groups (Table III). The activities of TRAP and CK in OvxC rats increased significantly to 1.6- and 1.3-fold of ShamC levels, respectively. Ca and Hyp levels in 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 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 Sham rats.

Histological analysis. Morphometric measurements revealed that the number of osteoclasts and osteoblasts in the OvxC rats increased to 1.7- and 1.5-fold the ShamC value (Table IV). 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 and proteins involved in osteoclastic differentiation in the proximal tibia. Gene expression levels of the osteoclastogenesis-related factors, M-CSF, RANKL, OPG, c-fms, PU.1, MITF, RANK, c-fos, c-jun and osteoclast-specific proteins, TRAP and cathepsin K, relative to the internal control, actin, are demonstrated in Fig. 1A. mRNA levels of M-CSF and RANKL in 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 OvxC rats did not differ from ShamC values. However, levels of RANK, PU.1, MITF, c-fos and c-jun in the OvxC rats were 2-, 2-, 2-, 3- and 4-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 Sham levels by fish oil, although the expression of TRAP in OvxF rats was significantly higher than the ShamC value but lower than the OvxC level. Fish oil did not affect expression levels in Sham rats.

The results of the western blot analysis are presented in Fig. 1B. Protein levels of M-CSF, RANKL, RANK, MITF and PU.1 in the OvxC group increased to ~2-fold of the ShamC values, while OPG levels were largely unchanged (Fig. 1B and C). Fish oil was observed to suppress these increases to Sham values in OVX rats, but had no effect in Sham animals.

Plasma fatty acid composition and serum concentrations of TNFα, IL-6 and PGE2. OVX significantly increased levels of arachidonic acid (AA, 20:4 n-6), as revealed in Table V. Fish oil reduced this increase. In the fish oil-fed rats, ShamF and

Table III. Effects of fish oil on clinical characteristics and bone biochemical markers.

A, Clinical characteristics.

Parameters	ShamC	ShamF	OvxC	OvxF
Body weight, g				
Start (prior to fasting)	208.4±1.3	206.2±1.7	208.5±1.4	208.0±1.0
Final	222.6±1.9	230.2±2.7	257.1±2.7[a]	253.3±3.9[a]
Serum estradiol, pg/ml	25.0±2.4	27.7±3.4	14.0±0.4[a]	18.3±1.8[a]
Bone length, cm				
Femur	3.38±0.03	3.36±0.01	3.38±0.01	3.37±0.02
Tibia	3.70±0.01	3.71±0.02	3.71±0.02	3.72±0.02
Bone weight, g				
Femur	0.666±0.005	0.664±0.008	0.632±0.011[a]	0.669±0.008[b]
Tibia	0.501±0.003	0.497±0.007	0.481±0.004[a]	0.505±0.006[b]
Proximal tibia	0.223±0.002	0.224±0.003	0.212±0.002[a]	0.224±0.002[b]

B, Bone biochemical markers.

Parameters	ShamC	ShamF	OvxC	OvxF
Proximal tibia				
ALP activity, U/g	20.93±0.81	18.88±0.62	25.58±0.62[a]	25.87±0.89[a]
TRAP activity, U/g	0.764±0.039	0.717±0.035	1.263±0.035[a]	1.049±0.034[a,b]
CK activity, U/g	397.4±34.0	325.7±23.1	518.7±28.6[a]	415.7±34.6[b]
Ca, mg/g	119.8±3.2	118.9±2.8	97.7±1.3[a]	113.6±1.1[b]
Hyp, μmol/g	102.4±1.9	104.0±2.4	91.9±1.3[a]	99.5±1.2[b]
Serum osteocalcin, ng/ml	145.6±5.2	154.1±6.1	191.7±7.4[a]	177.4±4.1[a]

Data are presented as the mean ± SEM. [a]P<0.05 vs. ShamC; [b]P<0.05 vs. OvxC. C, corn oil-containing; F, fish oil-containing; Ovx, ovariectomy; Sham, sham-operation; ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; CK, cathepsin K; Ca^{2+}, calcium; Hyp, hydroxyproline.

Table IV. Bone histomorphometry.

Parameters	ShamC	ShamF	OvxC	OvxF
Trabecular number, no./mm	13.4±0.6	13.7±0.3	13.8±0.6	14.8±1.2
Trabecular bone volume, %	60.0±1.4	61.7±1.8	46.1±1.6[a]	59.2±0.8[b]
Trabecular bone surface, mm/mm^2	26.4±1.1	27.5±0.6	33.7±1.7[a]	27.7±1.9[b]
Trabecular thickness, μm	48.4±3.8	45.2±2.0	27.3±2.1[a]	42.0±2.8[b]
Osteoblast index (no. Ob/mm trabecular bone length)	16.32±0.59	16.45±0.78	23.72±0.96[a]	23.80±1.12[a]
Osteoclast index (no. Oc/mm trabecular bone length)	2.93±0.12	2.75±0.26	5.08±0.26[a]	3.45±0.24[b]

Data are presented as the mean ± SEM. [a]P<0.05 vs. ShamC; [b]P<0.05 vs. OvxC. no., number; Ob, osteoblast; Oc, osteoclast; C, corn oil-containing; F, fish oil-containing; Ovx, ovariectomy; Sham, sham-operation.

OvxF, levels of AA and linoleic acid (18:2 n-6) significantly decreased and those of palmitoleic (16:1 n-7), eicosapentaenoic (EPA, 20:5 n-3), docosapentaeic (DPA; 22:5 n-3) and docosahexaenoic (DHA, 22:6 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 Sham levels in OvxC rats, respectively (Table VI). The increases were suppressed to Sham levels by fish oil. Fish oil did not affect these concentrations in Sham rats.

Table V. Effects of fish oil on plasma fatty acid composition (mol %).

Fatty acid	(C:D)	ShamC	ShamF	OvxC	OvxF
Myristic acid	14:0	0.91±0.18	1.68±0.38[a]	1.09±0.39	1.39±0.37
Palmitic acid	16:0	16.89±5.83	19.00±1.23	17.80±1.62	18.82±7.96
Palmitoleic acid	16:1 n-7	2.25±0.30	4.49±0.78[a]	2.25±0.12	4.26±1.23[b]
Stearic acid	18:0	19.71±1.88	21.49±1.31	20.59±1.81	21.13±3.31
Oleic acid	18:1 n-9	11.14±2.05	8.12±1.49	9.21±1.13	8.56±1.68
Vaccenic acid	18:1 n-7	2.04±0.30	2.10±0.30	1.87±0.25	2.30±0.26
Linoleic acid	18:2 n-6	15.88±0.74	5.38±0.43[a]	14.12±1.11	5.63±0.25[b]
γ–Linolenic acid	18:3 n-6	0.30±0.06	0.20±0.02	0.34±0.06	0.38±0.15
α–Linolenic acid	18:3 n-3	0.16±0.05	0.20±0.01	0.08±0.05	0.25±0.14[b]
Arachidic acid	20:0	0.13±0.01	0.17±0.02	0.17±0.07	0.29±0.13
Eicosanoic acid	20:1	0.38±0.20	0.54±0.14	0.36±0.27	0.58±0.21
Eicosadienoic acid	20:2 n-6	0.20±0.02	0.25±0.18	0.20±0.14	0.21±0.16
Dihomo-γ-linolenic acid	20:3 n-6	0.49±0.07	0.64±0.23	0.57±0.09	0.64±0.08
Arachidonic acid	20:4 n-6	20.95±1.45	11.81±1.75[a]	25.68±1.82[a]	11.53±1.23[b]
Eicosatetraenoic acid	20:4 n-3	ND	0.31±0.04	ND	0.31±0.04
Eicosapentaenoic acid	20:5 n-3	0.12±0.02	13.51±0.72[a]	0.09±0.02	14.89±2.04[b]
Docosapentaenoic acid	22:5 n-3	0.29±0.08	1.30±0.35[a]	0.24±0.08	0.88±0.21[b]
Docosahexaenoic acid	22:6 n-3	2.55±0.25	5.77±0.85[a]	2.26±0.20	4.95±0.74[b]

Data are presented as the mean ± SEM (n=4) . [a]P<0.05 vs. ShamC; [b]P<0.05 vs. OvxC. ND, not detected; C, corn oil-containing; F, fish oil-containing; Ovx, ovariectomy; Sham, sham-operation.

Table VI. Effects of fish oil on plasma concentrations of TNFα, IL-6 and PGE2.

Protein	ShamC	ShamF	OvxC	OvxF
TNFα (pg/ml)	19.8±2.0	20.7±1.9	28.7±1.6[a]	19.6±2.7[b]
IL-6 (pg/ml)	84.7±7.2	87.0±3.0	116.2±4.0[a]	81.6±11.04[b]
PGE2 (ng/ml)	1.30±0.06	1.06±0.1	1.61±0.05[a]	1.11±0.05[b]

Data are presented as the mean ± SEM (n=6). [a]P<0.05 vs. ShamC; [b]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; Ovx, ovariectomy; Sham, sham-operation.

mRNA levels of TNFα, IL-6, cyclooxygenase 2 (COX2) and phospholipase A2 (PLA2) and NFκB activation in the proximal tibia. mRNA levels of TNFα, IL-6, COX2 and PLA2 in the OvxC rats increased to ~2.4-, 1.8-, 1.7- and 2.5-fold the ShamC values, respectively (Fig. 2A). These increases were restored to 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 OvxC rats increased to 7-fold the ShamC level (Fig. 2B and C). Stimulation of phosphorylation was reduced to Sham level by fish oil. Fish oil did not affect the phosphorylation of NFκB p65 in Sham rats.

Discussion

Results of the present study confirm that fish oil suppresses increased bone resorption induced by OVX. OVX 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 (17,27) or osteoblastogenesis in osteoblast-like cells (19).

Osteoclast precursors are derived from hematopoietic stem cells in bone marrow. Differentiation into osteoclasts, however, occurs on the bone surface *in vivo* (1,28). Therefore, in the current study, gene expression of osteoclastogenesis-related factors was examined in the bone. mRNA and protein levels of M-CSF significantly increased in OvxC rats compared with Sham rats, and fish oil suppressed these

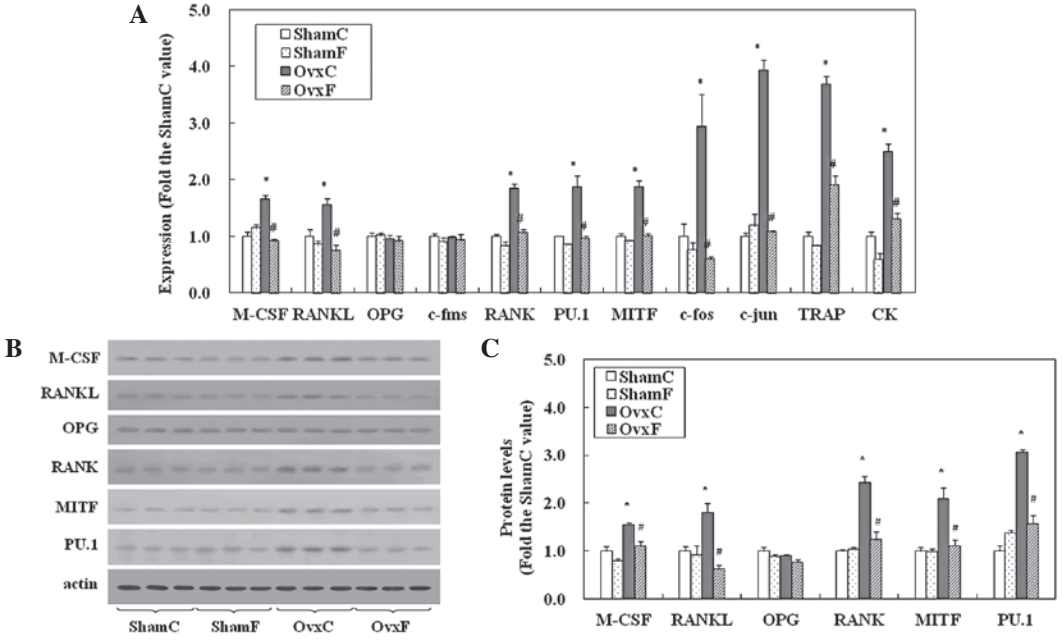

Figure 1. mRNA and protein expression of genes involved in osteoclastic differentiation in the proximal tibia, including M-CSF, RANKL, OPG, c-fms, RANK, PU.1, MITF, c-fos, c-jun, TRAP and CK. (A) RT-PCR and (B) western blot analysis of bone extracts from the proximal tibia. (C) Quantification of protein levels. Data are presented as the mean ± SEM (n=8). *P<0.05 vs. ShamC; #P<0.05 vs. OvxC. M-CSF, macrophage colony-stimulating factor; RANKL, receptor for activation of NFκB ligand; OPG, osteoprotegerin; c-fms, colony stimulating factor 1 receptor; MITF, microphthalmia-associated transcription factor; TRAP, tartrate-resistant acid phosphatase, CK, cathepsin K; C, corn oil-containing; F, fish oil-containing; Ovx, ovariectomy; Sham, sham-operation.

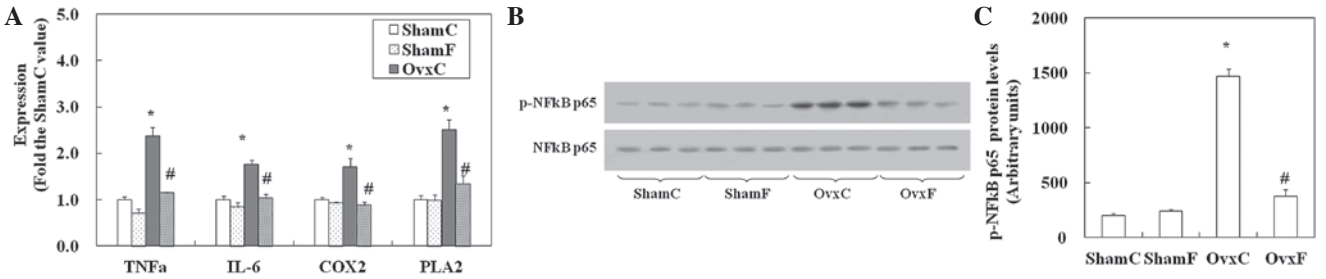

Figure 2. mRNA levels of TNFα, IL-6, COX2 and PLA2 and the activation of NFκB in the proximal tibia. (A) Total RNA was extracted from the proximal tibia and the mRNA levels of TNFα, IL-6, COX2 and PLA2 were assessed by RT-PCR. (B) Western blot analysis of bone extracts from the proximal tibia using p-NFκB 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; PLA2, phospholipase A2; C, corn oil-containing; F, fish oil-containing; Ovx, ovariectomy; Sham, sham-operation.

increases to ShamC and 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 (5). Gene expression of RANK is regulated by the transcription factors PU.1 and MITF (29). In ovariectomized rats, mRNA and protein levels of PU.1 and MITF increased compared with ShamC rats, and fish oil suppressed this 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 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 (30). TNFα expression is induced by NFκB (31). In the present study, increased NFκB activation (p-NFκB p65) was observed in the bones of OvxC rats compared with ShamC rats. Increased NFκB activation was suppressed in the proximal tibia of OvxF rats. Simultaneously, a decrease in the levels of n-6 PUFAs (AA and linoleic acid) and an increase in n-3 PUFAs (EPA, DPA and DHA) was observed in the plasma of 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 (13,32-35). Consistent with these *in vitro* results, the present study suggested that an increase in the n-3/n-6 PUFA ratio induced by dietary fish oil led to the suppression of NFκB activation in the bones of ovariectomized rats in the present study. 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 activa-

tion by increases in serum 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 (36,37), which is known to be upregulated by estrogen deficiency (38,39). 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 OvxC rats compared with ShamC rats. Fish oil restored these levels to Sham values, coinciding with the suppression of M-CSF, in OvxF rats. In addition, increased expression of PLA2, which plays a key role in PGE2 synthesis (40) and is induced by TNFα (41), was suppressed by fish oil. TNFα, IL-6 and PGE2 induced the expression of RANKL (38,42), 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 (16) or activated splenic CD4 cells (13). In the present study, downregulation of RANKL expression by fish oil was confirmed at the mRNA and protein 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.

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