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Effects of arachidonic acid, docosahexaenoic acid, prostaglandin E_2 and parathyroid hormone on osteoprotegerin and RANKL secretion by MC3T3-E1 osteoblast-like cells

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Abstract

Bone is continuously remodeled through resorption by osteoclasts and the subsequent synthesis of the bone matrix by osteoblasts. Cell-to-cell contact between osteoblasts and osteoclast precursors is required for osteoclast formation. RANKL (receptor activator of nuclear factor-κB ligand) expressed on osteoblastic cell membranes stimulates osteoclastogenesis, while osteoprotegerin (OPG) secreted by osteoblasts inhibits osteoclastogenesis. Although polyunsaturated fatty acids (PUFAs) have been implicated in bone homeostasis, the effects thereof on OPG and RANKL secretion have not been investigated. MC3T3-E1 osteoblasts were exposed to the *n*−6 PUFA arachidonic acid (AA) and the *n*−3 PUFA docosahexaenoic acid (DHA); furthermore, the bone-active hormone parathyroid hormone (PTH) and the effects thereof were tested on OPG and RANKL secretion. Prostaglandin E_2 (PGE_2), a product of AA metabolism that was previously implicated in bone homeostasis, was included in the study. AA (5.0–20 μg/ml) inhibited OPG secretion by 25–30%, which was attenuated by pretreatment with the cyclooxygenase blocker indomethacin, suggesting that the inhibitory effect of AA on OPG could possibly be PGE_2-mediated. MC3T3-E1 cells secreted very low basal levels of RANKL, but AA stimulated RANKL secretion, thereby decreasing the OPG/RANKL ratio. DHA suppressed OPG secretion to a smaller extent than AA. This could, however, be due to endogenous PGE_2 production. No RANKL could be detected after exposing the MC3T3-E1 cells to DHA. PTH did not affect OPG secretion, but stimulated RANKL secretion. This study demonstrates that AA and PTH reduce the OPG/RANKL ratio and may increase osteoclastogenesis. DHA, however, had no significant effect on OPG or RANKL in this model.

Keywords: Arachidonic acid; Docosahexaenoic acid; Osteoprotegerin; RANKL; Parathyroid hormone; Osteoblasts

1. Introduction

Bone is continuously remodeled through resorption by osteoclasts and the subsequent synthesis of the bone matrix by osteoblasts [1]. The remodeling cycle is finely regulated by a variety of systemic and local factors [e.g., estrogen, parathyroid hormone (PTH), $1,25(OH)_2D_3$, growth factors and cytokines] [1–3]. Cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and hematopoietic osteoclast precursors (present in the bone marrow, spleen and peripheral blood) is required for osteoclast formation [4]. During the past decade, various research groups have identified some of the proteins involved in the interaction between cells of osteoblastic and osteoclastic lineages. These proteins belong to the families of tumor necrosis factors and receptors [5–9] and comprise RANKL (receptor activator of nuclear factor-κB ligand) and its cognate receptor RANK (receptor activator of nuclear factor-κB), as well as a decoy receptor osteoprotegerin (OPG).

RANKL is a protein expressed on the osteoblast cell membrane that binds to its cognate receptor RANK, which is present on the osteoclast progenitor membrane. The binding of RANKL to RANK activates nuclear factor-κB and c-jun N-terminal protein kinase, which is associated with osteoclastic differentiation and activation [10]. OPG, a secreted glycoprotein, is a member of the tumor necrosis factor receptor family and is produced by cells of osteoblast

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lineage as well as other cells in the bone marrow [7,8]. The binding of RANKL to RANK can be prevented by OPG binding to RANKL. If the binding between RANK and RANKL is interrupted by OPG, the osteoclast precursor cannot differentiate and fuse to form mature resorbing osteoclasts. OPG, therefore, acts as a decoy receptor in the RANKL–RANK signaling system, thereby inhibiting osteoclastogenesis [6,7]. Apart from inhibiting osteoclastogenesis, OPG is also involved in suppressing osteoclast survival [8]. The presence of OPG in the bone microenvironment therefore limits the number of mature osteoclasts and could have a determining effect on resorption rate and bone mass. Various mediators modulate OPG secretion. OPG secretion is stimulated by estrogen [11], phytoestrogens [12], IL-1β [13] and TGF-β [14], but is inhibited by IL-1α [15], PGE_2 [16], PTH [17], glucocorticoids [18] and vitamin A metabolites [19].

Polyunsaturated fatty acids (PUFAs) have been implicated in bone homeostasis both in vitro [20–22] and in vivo [23–27]. There is increasing evidence that lack of certain PUFAs in the diet can induce bone loss [28–30], while dietary supplementation of some PUFAs has been shown to be beneficial for bones [24,25,31,32]. Clinical studies have shown, for instance, that supplementation of calcium, γ-linolenic acid and eicosapentaenoic acid (EPA) in the diets of elderly women decreases bone turnover and increases bone mineral density [31,32]. In vivo studies have shown that supplementation with PUFAs such as γ-linolenic acid, EPA and docosahexaenoic acid (DHA) could decrease bone turnover in ovariectomized animals by decreasing bone resorption [22,24].

Although PUFAs have been implicated in bone homeostasis, their effects on OPG and RANKL secretion by osteoblasts have not been investigated. To determine whether PUFAs and the bone-active hormone PTH affect OPG secretion in vitro, MC3T3-E1 osteoblasts were exposed to these compounds. PGE_2, a product of arachidonic acid (AA) metabolism in osteoblasts that was previously implicated in bone homeostasis [33,34], was included in this study. Since the OPG/RANKL ratio is important for the regulation of the bone microenvironment, RANKL secretion by MC3T3-E1 cells, after exposure to the abovementioned components, was also determined.

2. Materials and methods

2.1. Reagents and materials

Sigma Chemical Co. (St. Louis, MO, USA) supplied L-glutamine, crystal violet, trypan blue, AA, DHA, PTH (1–34), PGE_2, and dextran-coated charcoal. Heat-inactivated fetal calf serum (FCS) was purchased from Highveld Biological (Pty) Ltd. (Sandringham, South Africa). DMEM was obtained from Sterilab Services (Kempton Park, South Africa), and gentamicin was from Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). All other chemicals were of analytical grade and were purchased from Sigma Chemical Co. Sterile cell cluster plates were supplied by LASEC (Johannesburg, South Africa).

2.2. Cell culture and maintenance

Nontransformed MC3T3-E1 mouse calvaria fibroblasts (established from the calvarium of an embryo/fetus C57BL/6 mouse), which were described to differentiate into osteoblasts [35], were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Cell cultures were maintained in DMEM (with 10% heat-inactivated FCS) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All cell cultures were supplemented with 2 mM L-glutamine and gentamicin (25 μg/ml). Fatty acid stock solutions were stored in small aliquots at −70°C, and working solutions were freshly prepared each time prior to their use. Ethanol concentration did not exceed 0.2%. Previous studies in our laboratory showed no toxic effects of the ethanol vehicle at this concentration.

2.3. Determination of OPG concentrations in conditioned media

After trypan blue exclusion, the MC3T3-E1 cells were seeded in sterile 24-well culture plates at a density of 50,000 cells/well in DMEM (without phenol red) containing 5% charcoal-stripped FCS. After cells had attached firmly for 24 h, the culture medium was replaced with fresh medium. Vehicle (0.2% ethanol), PUFAs (AA and DHA; 2.5–20 μg/ml) or PTH (10^{-8} M) and PGE_2 (10^{-8} M) were then added. In some cases, the cyclooxygenase (COX) inhibitor indomethacin (1 μM) was added to the growth medium 45 min prior to the addition of test substances. After 24 h of exposure to the test substances, conditioned media were harvested and stored at −70°C until analyzed.

Following removal of the medium, cell numbers were determined by crystal violet staining, as previously described [36]. In short, cultures were fixed with 1% glutaraldehyde and stained with 1% crystal violet, and the dye was extracted with 0.2% Triton X-100. Absorbance was read on an ELX800 Universal Microplate Reader (Analytical Diagnostic Products; Bio-Tek Instruments, Inc., Weltevreden Park, South Africa) at a wavelength of 570 nm; 0.2% Triton X-100 in water was used as blank. Crystal violet is a basic dye that stains cell nuclei [36]. Spectrophotometer readings of color intensity are an indication of DNA content and, therefore, cell numbers. Results were analyzed using a linear standard curve established from known cell numbers. Three independent experiments were conducted ($n=4$).

2.4. Quantification of OPG concentrations in MC3T3-E1 conditioned media

For the measurement of murine OPG, a sandwich enzyme-linked immunosorbent assay (ELISA) protocol similar to the one used for the detection of human OPG was developed

[16]. A MaxiSorb microtiter plate (NUNC Brand Products, Roskilde, Denmark) was coated with 2 μg/ml monoclonal antimouse OPG antibody (R&D Systems, Inc., Minneapolis, MN, USA) and incubated overnight at 4°C. The plate was then blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 5% sucrose and 0.05% NaN_3 for 1 h at room temperature.[1] Samples and standards (ranging from 31.25 to 5000 pg/ml; recombinant mouse OPG/Fc chimera; R&D Systems, Inc.) diluted in dilution buffer (PBS containing 1% BSA and 0.02% NaN_3) were added and incubated for 2 h at 37°C. Cell culture medium was used as a blank.[1] Thereafter, 0.2 μg/ml biotinylated antimouse OPG-detecting antibody (R&D Systems, Inc.) was added. After 2 h of incubation at 37°C[1], peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark; 1:2000 dilution in PBS containing 0.05% Tween-^{R}20; Merck, Schuchardt, Germany) was added and incubated for 30–40 min at room temperature.[1] Ready-to-use 3′3′,5,5′-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich, Inc., St. Louis, MO, USA) was added, and the plate was incubated at room temperature with shaking (300 rpm) for a period of 10–20 min until color has developed. The reaction was terminated by the addition of 0.9 M H_2SO_4. Optical density was read at 450/620 nm using an ELX800 Universal microplate reader (Analytical Diagnostic Products; Bio-Tek Instruments, Inc.). Results were analyzed using a linear curve that was constructed from standard values. Intra-assay and interassay variability was 6.5% and 16%, respectively.

2.5. Quantification of soluble RANKL (sRANKL) concentrations in MC3T3-E1 conditioned media

A commercial sandwich ELISA kit (Biomedica Medizinprodukte, GmbH and Co. KG, Vienna, Austria) was used for the quantitative determination of free sRANKL in the conditioned media of the MC3T3-E1 cells. In short, recombinant murine OPG was used as "capture antibody" while a polyclonal biotinylated antimouse sRANKL antibody was used as detection antibody. Recombinant mouse sRANKL ranging from 12.5 to 100 pmol/L was prepared in DMEM and used as standard. Cell culture medium was used as a blank. Streptavidin–horseradish peroxidase was used as conjugate, and TMB liquid was used as substrate for color development. The reaction was terminated by the addition of H_2SO_4. Optical density was read at 450/620 nm using an ELX800 Universal microplate reader (Analytical Diagnostic Products, Bio-Tek Instruments, Inc.). Results were analyzed using a linear curve that was constructed from standard values. RANKL concentrations were then expressed as (picograms per milliliter) per 10,000 cells (1 pg/ml=0.05 pmol/L). Intra-assay and interassay variability was 4.2% and 9%, respectively.

2.6. Statistical analysis

Three independent experiments were conducted ($n=4$). Data were expressed as mean±S.D. Statistical analysis was performed using Statistics for Windows software (version 2; Tallahassee, FL, USA). Student's paired t test was use to evaluate differences between the sample of interest and its respective control. For analysis of dose responses, the means of groups were compared by one-way ANOVA, and significance was determined by post hoc testing using Bonferroni method. To test for a significant linear trend between AA concentration and OPG/sRANKL ratio, Pearson correlation coefficient test was performed. $P<.05$ was considered statistically significant.

3. Results

3.1. Effects of AA, PGE_2 and PTH on OPG secretion

Fig. 1 depicts the effects of AA (2.5–20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M) on OPG secretion by the MC3T3-E1 cells. Compared with control values, AA (5.0–20 μg/ml) inhibited OPG secretion by 25–30%. Preincubating the cells with the unselective COX inhibitor indomethacin prior to AA exposure (AA concentrations, 2.5–10 μg/ml) completely abolished the inhibitory effect of AA on OPG secretion and increased OPG secretion to levels higher than that of control values. Indomethacin did not abolish the inhibiting effect of 20 μg/ml AA on OPG secretion. PGE_2 inhibited OPG secretion by 40% while PTH had no effect.

3.2. Effects of DHA on OPG secretion

Fig. 2 depicts the effects of DHA (2.5–20 μg/ml) on OPG secretion by the MC3T3-E1 cells. Compared with control, DHA at lower concentrations (2.5–10 μg/ml) had no significant effect on OPG secretion, but DHA (20 μg/ml) inhibited OPG secretion by 30%. Preincubating the cells with indomethacin prior to DHA exposure (2.5–10 μg/ml) increased OPG secretion to levels slightly higher than that of control values. Indomethacin did not abolish the inhibitory effect of 20 μg/ml DHA on OPG secretion.

3.3. Effects of AA, DHA, PGE_2 and PTH on sRANKL secretion

Fig. 3 depicts the effects of AA (2.5–20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M) on sRANKL secretion by the MC3T3-E1 cells. The concentrations of sRANKL secreted by these cells at control conditions and at the lowest AA concentration (2.5 μg/ml) were too low to be detected by the sRANKL ELISA procedure used. AA at concentrations of 5–20 μg/ml, however, increased sRANKL secretion, with the largest effect observed at

[1] Between steps of ELISA, the plate was washed thrice with PBS containing 0.05% Tween.

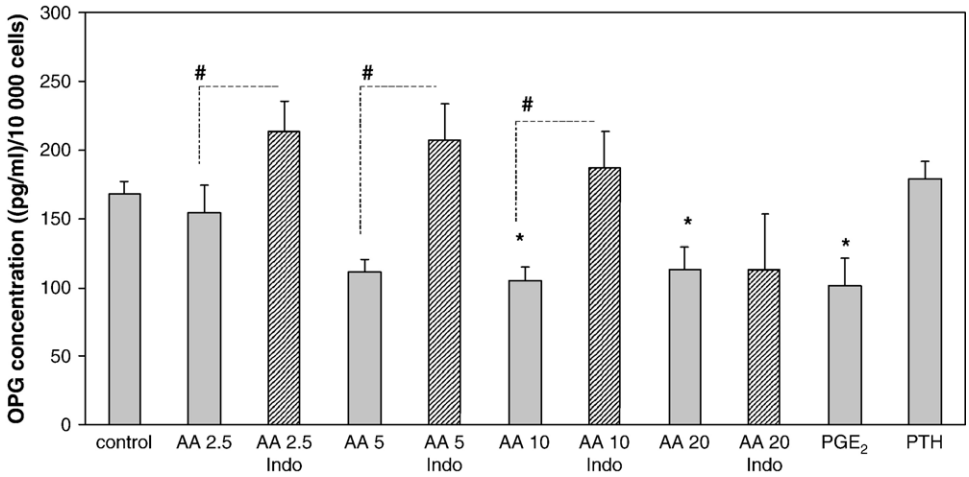

Fig. 1. Effects of AA, PGE_2 and PTH on OPG secretion by the MC3T3-E1 cells. Analysis of OPG levels from the MC3T3-E1 cells that were cultured for 24 h in the presence of vehicle (0.2% ethanol; control), AA (2.5–20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M). Indomethacin (Indo; 1 μM) was added 45 min prior to AA treatment. OPG protein secretion was measured by ELISA from the conditioned medium and then standardized for cell number, as described in Materials and Methods. Three separate experiments were conducted; data are from a representative experiment. $^{*}P<.05$ compared with control cultures ($n=4$). $^{\#}P<.05$ compared with the corresponding indomethacin-treated culture ($n=4$).

20 μg/ml AA. Indomethacin preincubation attenuated the stimulatory effect of AA on sRANKL secretion, especially at 20 μg/ml AA. PGE_2 (10^{-8} M) stimulated sRANKL secretion to levels slightly higher than that of 20 μg/ml AA. Of the agents tested, PTH (10^{-8} M) stimulated sRANKL secretion to the highest degree. Cells exposed to DHA did not secrete sRANKL levels high enough to be detected with the commercial ELISA protocol used and were, therefore, not included in the graph.

3.4. Effects of AA, PGE_2 and PTH on the OPG/sRANKL ratio in MC3T3-E1 conditioned media

The effects of AA, PGE_2 and PTH on the OPG/sRANKL ratio in the MC3T3-E1 cells are shown in Fig. 4. Although

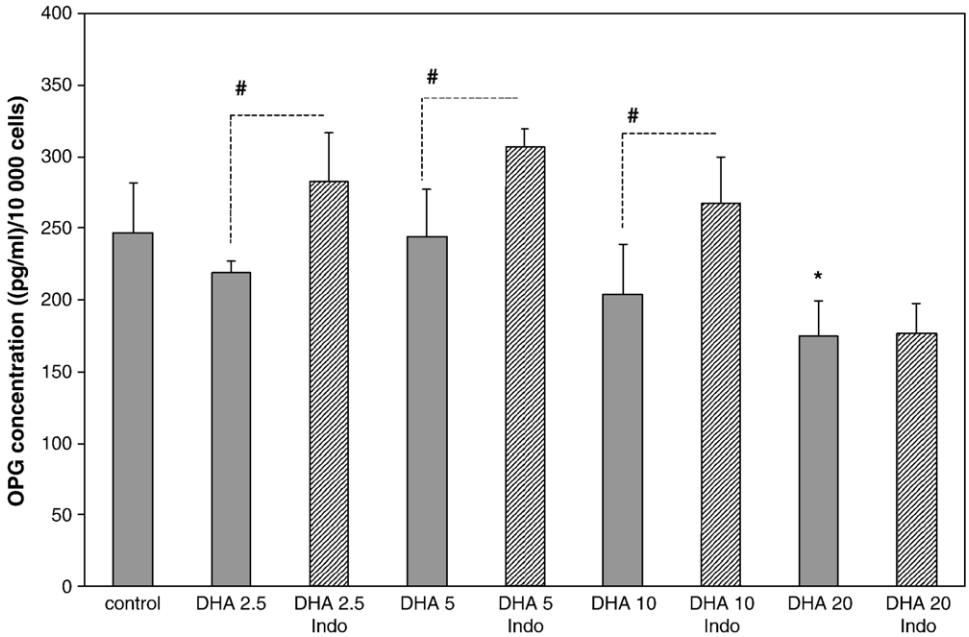

Fig. 2. Effects of DHA on OPG secretion by the MC3T3-E1 cells. Analysis of OPG levels from the MC3T3-E1 cells that were cultured for 24 h in the presence of vehicle (0.2% ethanol; control) and DHA (2.5–20 μg/ml). Indomethacin (Indo; 1 μM) was added 45 min prior to DHA treatment. OPG protein secretion was measured by ELISA from the conditioned medium and then standardized for cell number, as described in Materials and Methods. Three separate experiments were conducted; data are from a representative experiment. $^{*}P<.05$ compared with the corresponding control cultures ($n=4$). $^{\#}P<.05$ compared with the corresponding indomethacin-treated culture ($n=4$).

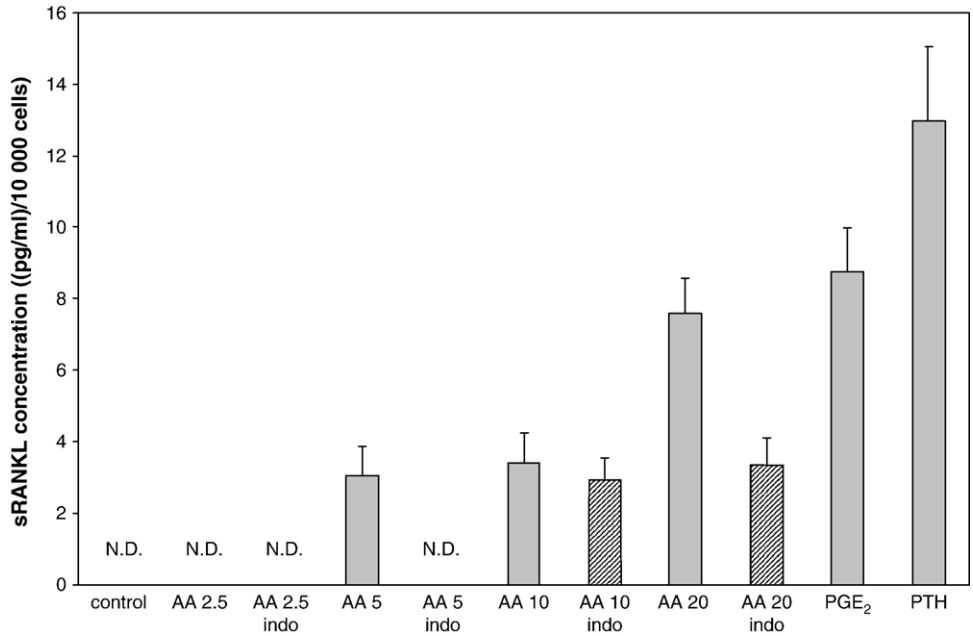

Fig. 3. Effects of AA, PGE_2 and PTH on sRANKL secretion by the MC3T3-E1 cells. Analysis of sRANKL levels from the MC3T3-E1 cells that were cultured for 24 h in the presence of vehicle (0.2% ethanol; control), AA (2.5–20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M). Indomethacin (Indo; 1 μM) was added 45 min prior to AA treatment. sRANKL secretion was measured by ELISA from the conditioned medium and then standardized for cell number, as described in Materials and Methods (ND, not detected). As sRANKL could not be detected in control conditions, it was not possible to perform statistical analysis on the data presented in this graph.

OPG was secreted by cells exposed to vehicle (0.2% ethanol) and 2.5 μg/ml AA, sRANKL could not be detected in these conditions and the OPG/sRANKL ratio could not be determined. A significant linear trend (tested with the Pearson correlation coefficient test, $P=.0009$) was observed between AA concentration and OPG/sRANKL ratio. Exposure to higher AA concentrations (5–20 μg/ml) decreased the OPG/sRANKL ratio, with 20 μg/ml AA causing a decrease of more than 50% compared with 5 μg/ml AA. Compared with 5 μg/ml AA, PGE_2 (10^{-8} M) and PTH (10^{-8} M) decreased the OPG/sRANKL ratio by 75%.

4. Discussion

Apart from having an effect on bone formation, osteoblasts are also coupled with osteoclasts through the

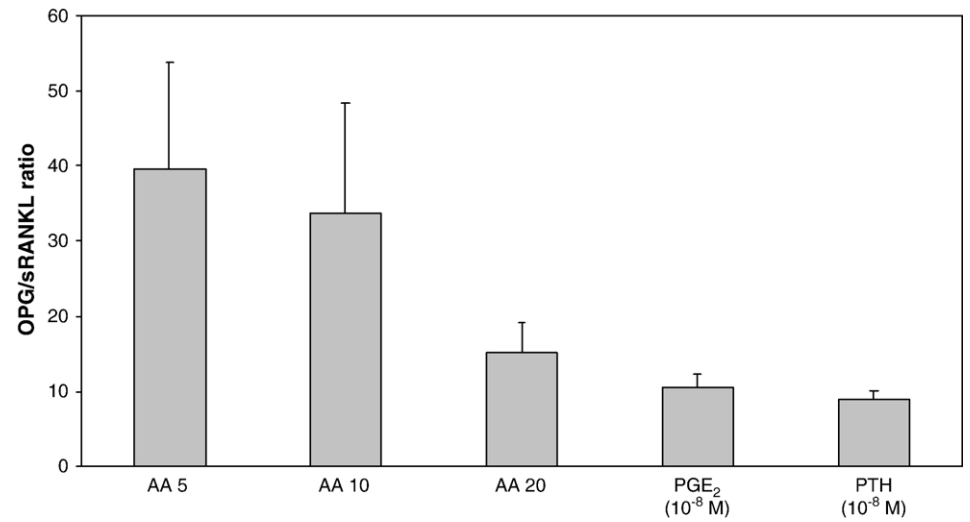

Fig. 4. Effects of AA, PGE_2 and parathyroid on the OPG/sRANKL ratio in the MC3T3-E1 cells. The MC3T3-E1 cells were cultured for 24 h in the presence of vehicle (0.2% ethanol; control), AA (2.5–20 μg/ml), PGE_2 (10^{-8} M) and PTH (10^{-8} M). sRANKL and OPG secretions were measured by ELISA from conditioned media, standardized for cell number (as described in Materials and Methods) and expressed as OPG/sRANKL ratio. To test for a significant linear trend between AA concentration and OPG/sRANKL ratio, the Pearson correlation coefficient test was performed. $P<.05$ was considered statistically significant.

release of various cytokines, including macrophage colony-stimulating factor (M-CSF) and RANKL [6]. Most pro-osteoclastogenic and antiosteoclastogenic cytokines act primarily through osteoblasts to alter levels of RANKL and OPG, the balance of which determines overall osteoclast formation [5,14,37].

We speculated that PUFAs affect bone resorption indirectly through the modulation of the OPG/RANKL ratio via PGE_2 synthesis. To determine whether PUFAs affect OPG secretion, in vitro MC3T3-E1 osteoblasts were exposed to the $n-6$ PUFA AA and the $n-3$ PUFA DHA. To determine whether the effects of PUFAs could be attributed to the modulation of PGE_2 synthesis, cells were preincubated with the unselective COX blocker indomethacin in some experiments [38].

4.1. Effects of AA, PGE_2 and DHA on OPG secretion

Results from our study (Fig. 1) showed that AA (5.0–20 μg/ml) suppressed OPG secretion in the MC3T3-E1 osteoblastic cell line possibly via PGE_2 production, as PGE_2 alone also significantly reduced OPG secretion. Indomethacin pretreatment completely abolished the inhibitory effects of 2.5–10 μg/ml AA on OPG secretion and even enhanced OPG concentrations to levels surpassing that measured in control conditions, thereby suggesting that indomethacin stimulated OPG secretion in these conditions (Fig. 1). However, the culture medium contains FCS-derived AA [39] that could be metabolized to low levels of PGE_2, which could inhibit OPG secretion even in control conditions. Although it is customary to omit FCS from culture media when testing for secreted mediators such as OPG [13,16,19], we chose not to do this as the MC3T3-E1 cells do not tolerate PUFAs in FCS-free conditions. Research in our laboratory reported detectable levels of PGE_2 after 4 h of incubation in control conditions in the presence of FCS [40]. Our results are supported by those of O'Brien et al. [41], who demonstrated higher levels of OPG in the medium from mouse calvaria cultured in the presence of indomethacin than that cultured in the presence of PGE_2. They also reported significant amounts of OPG even in the presence of PGE_2 [41], thereby confirming our results.

Indomethacin pretreatment could not attenuate the inhibitory effect of 20 μg/ml AA on OPG secretion to the same degree as it did at lower AA concentrations. This might be explained by the inability of indomethacin to completely block PGE_2 synthesis at high AA concentrations, as reported by the authors [40] and confirmed by others [42]. Alternatively, high concentrations of AA per se could have an inhibitory effect on OPG secretion independently of PGE_2 synthesis.

PGE_2, produced by osteoblasts from its fatty acid precursor AA, has pronounced effects on the bone. Depending on the concentration and experimental model, both antiresorptive and proresorptive effects of prostaglandins have been reported [33]. Several studies confirmed the importance of PGE_2 in osteoclast formation and bone resorption [43–45]. PGE_2 has been shown to inhibit OPG synthesis in various cell cultures such as primary human bone marrow cells [16] and tissues such as mouse calvaria [41], and to stimulate the mRNA expression of RANKL [46]. PGE_2 thus lowers the OPG/RANKL ratio, thereby favoring osteoclastogenesis and bone resorption. Many bone-active agents that induce bone resorption, such as PTH [47], and cytokines, such as IL-1 and IL-6, are prostaglandin-mediated [48,49], since the COX blocker indomethacin partially inhibits their action.

DHA, one of the $n-3$ PUFAs, is not a substrate for prostaglandin synthesis, but inhibits PGE_2 synthesis [50] possibly by replacing AA in the cell membrane, thereby limiting the amount of AA available for PGE_2 production [51]. MC3T3-E1 osteoblast-like cells were exposed to DHA to determine whether DHA affects OPG secretion. Cells were also preincubated with the unselective COX blocker indomethacin [38] to determine whether the effects of DHA could be attributed to the modulation of PGE_2 synthesis.

In the MC3T3-E1 cell line, DHA suppressed OPG secretion slightly, which was significant only at the highest DHA concentration (20 μg/ml) (Fig. 2). Indomethacin pretreatment, however, affected OPG secretion significantly. It, therefore, seems that AA supplied by the FCS in the culture medium affected OPG secretion, probably via PGE_2 synthesis in this cell line. The mechanism whereby DHA inhibited OPG secretion in this cell line is not known and will have to be investigated. In an in vivo study, Atkinson et al. [52] supplemented the diets of rats with oil rich in DHA. Although the oil did not contain detectable levels of EPA, significantly elevated EPA levels were reported in the membrane phospholipids of a variety of tissues, suggesting considerable retroconversion of DHA back to EPA [52]. EPA is a substrate for COX that catalyzes the formation of PGE_3 [50], and it has been shown that PGE_3 stimulates bone resorption with potency similar to that of PGE_2 in cultured fetal rat bones and neonatal rat calvaria [53]. EPA, however, is only one tenth as effective for PGE_3 synthesis as AA is for PGE_2 synthesis [53], suggesting that replacing AA with EPA in osteoblasts could have a bone-protective effect. It is thus possible that DHA exposure could, via retroconversion of DHA to EPA, result in the formation of low levels of PGE_3, which could inhibit OPG secretion.

4.2. Effects of AA, DHA and PGE_2 on sRANKL secretion and the OPG/sRANKL ratio

Factors that affect bone resorption may affect RANKL and/or OPG secretion, the balance of which determines osteoclastogenesis and ultimately affects bone resorption rate [14]. The biological activity of RANKL in vitro and in vivo has been characterized. When combined with M-CSF, RANKL stimulates osteoclast development and activates mature pre-existing osteoclasts [6]. Apart from cell-bound RANKL present on osteoblast membranes, sRANKL is synthesized as a membrane-anchored precursor, which is

then released from the plasma membrane by a metalloprotease. sRANKL demonstrates potent osteoclastogenic activity [54]. In order to determine whether the compounds we tested impacted on sRANKL as well as on the OPG/sRANKL ratio, this study was extended to include measurements of sRANKL secretion.

The levels of sRANKL in the harvested culture media of the MC3T3-E1 cells were very low and, in some cases, could not be detected by the commercial sRANKL ELISA protocol we used. Others reported low levels of RANKL mRNA expression in the MC3T3-E1 cells [17,37], which could explain the low levels of sRANKL secreted by these cells. No sRANKL could be detected in the media harvested from MC3T3-E1 control cells exposed to vehicle (0.2% ethanol) only. AA, however, stimulated sRANKL secretion in this model (Fig. 3).

Sun et al. [22] demonstrated increased RANKL-positive T cells in corn-oil-fed mice, thereby confirming that oils high in $n-6$ PUFAs, such as AA, could stimulate RANKL expression in lymphocytes. PGE_2 enhanced RANKL secretion in our model. Results from our study suggest that AA-stimulated secretion of sRANKL could be PGE_2-mediated, as indomethacin pretreatment attenuated this effect significantly. Our results confirm those of Nakashima et al. [46], who have shown that PGE_2 increases RANKL mRNA and protein expression in murine osteoblast stromal cells. Making use of EP agonists [55] and antagonists [56,57], it was shown that PGE_2 acts on mouse calvaria cultures mainly via the EP_2 and EP_4 receptors to induce cAMP and the expression of RANKL in osteoblastic cells. PGE_2 could possibly stimulate sRANKL secretion via binding to the EP_4 receptor in the MC3T3-E1 cells, as mRNA for the EP_4 receptor has been detected in these cells [58]. In the MC3T3-E1 cell line, AA affected sRANKL and OPG in opposite directions, which resulted in a markedly decreased OPG/sRANKL ratio (Fig. 4). Whether the change in the OPG/sRANKL ratio is sufficient to increase osteoclastogenesis and eventually enhance bone resorption has to be investigated.

No sRANKL could be detected in the culture media of DHA-exposed MC3T3-E1 cells. It, therefore, seems that DHA did not stimulate sRANKL secretion, in contrast to AA that stimulated sRANKL secretion (Fig. 3). This observation is supported by the findings of Sun et al. [22]. Although not in an osteoblast cell model, it was demonstrated that treatment of mice with fish oil (which contains high levels of $n-3$ PUFAs such as EPA and DHA) had no affect on RANKL expression in stimulated T cells. In contrast, corn oil (which contains high levels of $n-6$ PUFAs) stimulated RANKL expression [22]. As sRANKL secretion could not be detected after DHA exposure in our model, the OPG/sRANKL ratio could not be calculated. However, since OPG was detected and is known for its antiresorptive properties, the absence of sRANKL after DHA exposure suggests a possible bone-protective role for DHA.

4.3. Effects of PTH on OPG and sRANKL secretion, and the OPG/sRANKL ratio

PTH has dual activities in the bone. Continuous PTH administration results in enhanced bone resorption. Intermittent PTH therapy, while having a net anabolic effect on the bone, stimulates both bone formation and bone resorption [2]. Depending on the model used, disparate effects of PTH on OPG and RANKL synthesis, as well as on the OPG/RANKL ratio, have been reported. In murine bone marrow cultures, PTH stimulates RANKL and inhibits OPG expression, thus adversely affecting the OPG/RANKL ratio [59]. In a similar study using murine osteoblasts, no stimulatory effect of PTH on RANKL expression could be demonstrated [17]. As PTH also had no effect on OPG expression, it did not affect the OPG/RANKL ratio in this model [17].

In our model, PTH did not affect OPG secretion in MC3T3-E1 cell lines at the PTH concentration tested (10^{-8} M) (Fig. 1). The inability of PTH to stimulate OPG secretion in the MC3T3-E1 cells has been confirmed by Suda et al. [60] and Lee and Lorenzo [17], who demonstrated that PTH did not affect OPG mRNA expression in these cells. However, in our laboratory, in the MC3T3-E1 cell line, PTH stimulated sRANKL secretion significantly (Fig. 3), which resulted in a decreased OPG/sRANKL ratio (Fig. 4). Our results confirm the findings of others who demonstrated PTH-mediated stimulation of RANKL expression in murine bone marrow cells [61,62], which was mainly dependent on the cAMP/protein kinase A pathway [61,63]. As PTH did not enhance PGE_2 synthesis in the MC3T3-E1 cell line [40], stimulation of sRANKL secretion by PTH is probably not PGE_2-mediated in this model.

5. Conclusions

A large number of stimulators and inhibitors of osteoclast formation converge on the RANKL/RANK/OPG pathway, making this an appropriate target for therapeutic intervention in osteoporosis [14]. A logical therapeutic approach would be to search for ways to either increase OPG formation or decrease RANKL formation by bone cells, causing decreased osteoclast formation and less bone resorption, thus protecting the bone.

Some PUFAs have been shown to protect the bone by demonstrating antiresorptive properties [31,32]. Our study demonstrated that PUFAs could affect OPG secretion in MC3T3-E1 osteoblast-like cells. AA (5.0–20 μg/ml), one of the $n-6$ PUFAs, inhibited OPG secretion. Indomethacin pretreatment prior to AA exposure attenuated the inhibitory effect of AA, suggesting that AA-induced inhibition of OPG could possibly be mediated via PGE_2 synthesis. Although the MC3T3-E1 cells secreted very low levels of sRANKL into the cultured media, AA stimulated sRANKL secretion, possibly leading to bone resorption.

DHA suppressed OPG secretion, but to a smaller extent than AA. This could, however, be due to endogenous PGE_2 production, as DHA itself is not a substrate for PGE_2 synthesis. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA. Since OPG was detected in the media and is known for its antiresorptive properties, the absence of sRANKL could suggest that DHA could be protective of the bone.

Exposure of the MC3T3-E1 cells to PTH did not affect OPG secretion, an observation confirmed by Lee and Lorenzo [17]. However, PTH enhanced sRANKL secretion in the MC3T3-E1 cells, thereby decreasing the OPG/sRANKL ratio. These results confirm that PTH may cause bone resorption.

Our study demonstrates that PUFAs and PTH modulate the OPG/sRANKL ratio and, therefore, could affect bone remodeling at the cellular level. The expression of OPG and RANKL has been shown to be developmentally regulated [64,65], and it has been hypothesized that undifferentiated marrow stromal cells with a high RANKL/OPG ratio can initiate and support osteoclastogenesis, while the mature osteoblastic phenotype, which mostly expresses OPG, acquires an osteogenic phenotype [64,65]. In our MC3T3-E1 model, secreted sRANKL levels were very low and could not be detected in all samples. Others have reported low levels of mRNA RANKL expression in this cell line [17,37], which suggests that the MC3T3-E1 cell line might not be a suitable model for investigating RANKL modulation. It is, therefore, necessary to extend this study to include less differentiated cell lines, such as primary bone marrow stroma cells and primary rat/human osteoblasts.

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