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Differential effects of arachidonic acid and docosahexaenoic acid on cell biology and osteoprotegerin synthesis in osteoblast-like cells

by

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SUMMARY

The purpose of the study was to elucidate the mechanisms by which polyunsaturated fatty acids (PUFAs) prevent bone loss. MG-63 human osteoblasts and MC3T3-E1 murine osteoblasts were exposed to the n-6 PUFA arachidonic acid (AA) and the n-3 PUFA docosahexaenoic acid (DHA) as well as oestrogen (E2) and parathyroid hormone (PTH) and the effects thereof tested on a variety of biological parameters characteristic of osteoblasts. These parameters included prostaglandin E_2 (PGE_2) synthesis, proliferation, differentiation to mature mineralising osteoblasts as well as osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) secretion.

Results showed that AA stimulates PGE_2 production significantly in both cell lines. Stimulated PGE_2 production by MC3T3-E1 cells however, was significantly higher, which might be attributed to auto-amplification by PGE_2 itself in this cell line. Pre-incubation of the MG-63 cells with cyclo-oxygenase (COX)-blockers inhibited PGE_2 production significantly, suggesting that both COX enzymes were involved in PGE_2 synthesis.

The number of functional osteoblasts is important for bone formation therefore *in vitro* osteoblastic cell proliferation was investigated. In contrast to the hormones E2 and PTH, both AA and DHA inhibited proliferation significantly. The AA-mediated anti-proliferative effect is possibly independent of PGE_2 production, as PGE_2 *per se* had little effect on proliferation. DHA inhibited proliferation of MG-63 cells more severely, which might be attributed to the osteosarcoma nature of the MG-63 cells. The anti-proliferative effect of these PUFAs might be attributed to modulation of cell cycle progression or anti-mitotic effects of PUFA peroxidation products. Morphological studies showed apoptotic cells after DHA exposure in MG-63 cells.

There is a reciprocal relationship between reduced proliferation and the subsequent induction of cell differentiation *in vitro*. High basal levels of alkaline phosphatase (ALP) activity, a marker of the mature mineralising osteoblastic phenotype, were detected in MC3T3-E1 cells. Long-term exposure to AA inhibited ALP activity in these cells. This process might be PGE_2-mediated. Exposure to PUFAs, however, did not compromise the ability of the MC3T3-E1 cells to differentiate to mature mineralising osteoblasts.

In contrast with MC3T3-E1 cells, MG-63 cells demonstrated low basal ALP activity and were unable to differentiate to mature mineralising osteoblasts. In the absence of osteogenic-inducing supplements, PUFAs induced adipocyte-like features that might be due to the expression of high levels of PPARγ in this cell line. Lipid-filled vacuoles were absent in the MC3T3-E1 cells suggesting that the MC3T3-E1 cell line may not express PPARγ mRNA.

The study furthermore demonstrated that PUFAs are able to modulate OPG and RANKL secretion in osteoblasts. AA inhibited OPG secretion dose-dependently in both cell lines, this could be PGE_2-mediated. AA dose-dependently stimulated soluble RANKL (sRANKL) secretion in MC3T3-E1 cells thereby affecting the OPG/RANKL ratio in a negative way, supporting various reports that AA and PGE_2 do cause bone resorption. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA suggesting that DHA could be protective to bone.

In conclusion, contrary to *in vivo* evidence, this *in vitro* study could not indisputably demonstrate protective effects of PUFAs on the osteoblastic cell lines tested.

KEY WORDS:

Osteoblasts, polyunsaturated fatty acids, arachidonic acid, docosahexaenoic acid, prostaglandin E_2, proliferation, differentiation, alkaline phosphatase activity, mineralisation, transdifferentiation, osteoprotegerin (OPG), receptor activator of nuclear factor κB ligand (RANKL).

OPSOMMING

Die doel van die studie was om die meganismes waardeur poli-onversadigde vetsure (POVS) beenverlies voorkom te verklaar. MG-63 menslike osteoblaste en MC3T3-E1 muis-osteoblaste is blootgestel aan die n-6 POVS aragidoonsuur (AS) en die n-3 POVS dokosaheksaenoësuur (DHS) sowel as estrogeen (E2) en paratiroïedhormoon (PTH) en die effekte daarvan op 'n verskeidenheid biologiese parameters kenmerkend aan osteoblaste getoets. Hierdie parameters sluit in prostaglandien E_2 (PGE_2) sintese, proliferasie, differensiasie na volwasse mineraliserende osteoblaste sowel as osteoprotegerien (OPG) en reseptor aktiveerder van nukluêre faktor κB ligand (RANKL) sekresie.

AS het PGE_2-produksie in beide sellyne betekenisvol gestimuleer. Gestimuleerde PGE_2-produksie was aansienlik hoër by die MC3T3-E1-selle wat moontlik toegeskryf kan word aan outoversterking deur PGE_2 in hierdie sellyn. Voorafblootstelling van die MG-63-selle aan sikloöksegenase (SO)-blokkers het PGE_2-produksie betekenisvol geïnhibeer, wat op die betrokkenheid van beide SO-ensieme by PGE_2-sintese kan dui.

Aangesien die aantal funksionele osteoblaste belangrik vir beenvorming is, is die *in vitro* proliferasie van osteoblaste bestudeer. In kontras met die hormone E2 en PTH, het beide AS en DHS proliferasie betekenisvol geïnhibeer. Die inhiberende effek van AS op selproliferasie is waarskynlik onafhanklik van PGE_2-produksie, aangesien PGE_2 op sigself min effek op selproliferasie gehad het. DHS het proliferasie van MG-63-selle meer geïnhibeer as dié van die MC3T3-E1-selle, wat moontlik aan die tumorigeniese aard van die MG-63-selle toegeskryf kan word. Die anti-proliferatiewe effekte van POVS kan moontlik aan modulering van selsiklusprogressie, of andersins aan antimitotiese effekte van POVS-peroksidasieprodukte toegeskryf word. Morfologiese studies het die teenwoordigheid van apoptotiese selle na DHS-blootstelling by MG-63-selle aangetoon.

Daar bestaan 'n omgekeerde verwantskap tussen 'n afname in proliferasie en die daaropeenvolgende induksie van seldifferensiasie *in vitro*. Hoë basaalvlakke van alkaliese fosfatase (ALF)-aktiwiteit, 'n merker vir die volwasse mineraliserende osteoblastiese fenotipe, is by die MC3T3-E1-selle waargeneem. Langdurige blootstelling aan AS het ALF-aktiwiteit in hierdie selle geïnhibeer, wat moontlik PGE_2-gemedieerd kan wees. Die vermoë van die MC3T3-E1-selle om na volwasse

mineraliserende osteoblaste te differensieer, is egter nie deur blootstelling aan POVS benadeel nie.

In teenstelling met die MC3T3-E1-selle het die MG-63-selle lae basaalvlakke vir ALF-aktiwiteit getoon en hulle was nie in staat om na na volwasse mineraliserende osteoblaste te differensieer nie. In die afwesigheid van osteogenese-induserende supplemente het POVS adiposiet-agtige eienskappe geïnduseer, wat moontlik aan die uitdrukking van hoë PPARγ-vlakke in hierdie selle toegeskryf kan word. Die afwesigheid van lipiedvakuole by die MC3T3-E1-selle dui daarop dat hierdie sellyn moontlik nie PPARγ bRNS uitdruk nie.

Die studie het verder getoon dat POVS daartoe in staat is om OPG en RANKL-sekresie in osteoblaste te moduleer. AS het OPG-sekresie in beide sellyne op ‘n dosisafhanklik wyse geïnhibeer wat moontlik PGE_2-gemedieerd kan wees. AS het verder op ‘n dosisafhanklike wyse die sekresie van oplosbare RANKL (oRANKL) in MC3T3-E1-selle gestimuleer en dus die OPG/RANKL verhouding negatief beïnvloed. Hierdie bevinding ondersteun verslae dat AS en PGE_2 beenresorpsie kan veroorsaak. Geen oRANKL is na DHS-blootstelling aan MC3T3-E1-selle waargeneem nie wat daarop kan dui dat DHS moontlik beskerming aan been kan bied.

Opsommend, in teenstelling met *in vivo* studies, kon hierdie *in vitro* studie nie bo alle twyfel beskermende effekte van POVS op die osteoblastiese sellyne soos getoets, aantoon nie.

SLEUTELWOORDE:

Osteoblaste, poli-onversadigde vetsure, aragidoonsuur, dokosaheksaenoësuur, prostaglandien E_2, proliferasie, differensiasie, alkaliese fosfatase-aktiwiteit, mineralisasie, transdifferensiasie, osteoprotegerien (OPG), reseptor aktiveerder van nukluêre faktor κB ligand (RANKL).

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AA	arachidonic acid (C20,5c,8c,11c,14c-20:4)[n-6]
ALA	α-linolenic acid (C18,9c,12c,15c-18:3)[n-3]
ALP	alkaline phosphatase
α-MEM	alpha modification of Eagle's minimal essential medium
ANOVA	analysis of variance
β-GP	β-glycerophosphate
BMP	bone morphogenetic protein
BMP-2	bone morphogenetic protein-2
BSA	bovine serum albumine
BSS	balanced salt solution
caspases	cysteinyl aspartate-specific proteases
cAMP	cyclic AMP
Cbfa1	core binding factor α-1
cdks	cyclin-dependent kinases
CLA	conjugated linoleic acid
COX	cyclo-oxygenase
COX-1	cyclo-oxygenase-1
COX-2	cyclo-oxygenase-2
Col1a1	type I collagen
cPGES	cytosolic prostaglandin E synthase
cPLA$_2$	cytosolic phospholipase A$_2$
DAG	diacylglycerol
ddH$_2$O	deionised distilled water
DGLA	dihomo-gamma-linolenic acid (C20,8c,11c,14c-20:3) [n-6]
DHA	docosahexaenoic acid (C22,4c,7c,10c,13c,16c,19c-22:6)[n-3]
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
E2	oestrogen (17β-estradiol)
EDTA	disodium ethylene diaminetetraacetate
EFAs	essential fatty acids
EGTA	ethylene glycol-bis[beta-aminoethyl ether]N,N,N$_1$,N$_1$-tetra-acetate
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E$_2$ receptor
EPA	eicosapentaenoic acid (C20,5c,8c,11c,14c,17c-20:5)[n-3]
ER	oestrogen receptor
Erk	extracellular signal-regulated kinase
FCS	fetal calf serum

FGFs	fibroblast growth factors
FITC	fluoroisothiocyanate
GLA	gamma linolenic acid (C18,6c,9c,12c-18:3)[n-6]
GPCRs	G protein-coupled receptors
H^+-ATPase	proton ATPase
H&E	Haematoxylin and eosin
hBMSc	human bone marrow stroma cells
hFOB	human fetal osteoblastic cell line
HOE	Hoechst no 33342
HOE/PI	Hoechst no 33342 and propidium iodide
IGFs	insulin-like growth factors
IGF-1	insulin-like growth factor-1
IGFBPs	insulin-like growth factor binding proteins
IL	interleukin
Indo	indomethacin
JNK	c-jun N-terminal protein kinase
LA	linoleic acid (C18,9c,12c-18:2)[n-6]
LO	lipoxygenase
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MC3T3-E1	mouse calvaria osteoblast-like cell line
MCF-7	human breast carcinoma cell line
M-CSF	macrophage-colony stimulating factor
MEM	minimum essential medium with Earle's salts
MG-63	human osteoblast-like osteosarcoma-derived cells
mPGES	membrane-associated prostaglandin E synthase
mRNA	messenger RNA
MSCs	mesenchymal stem cells
n-3	omega-3. Family of polyenoic fatty acids with 3 or more cis-unsaturated centres separated by methylene groups and having first unsaturated center 3C from the methyl terminal.
n-6	omega-6. Family of polyenoic fatty acids with 2 or more cis-unsaturated centres separated by methylene groups and having first unsaturated center 6C from the methyl terminal.
N.D.	not detected
NF$\kappa\beta$	nuclear factor $\kappa\beta$
NSAIDS	nonsteroidal anti-inflammatory drugs
O.D.	optical density
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand (RANKL)
Osx	osterix

OVX	ovariectomised
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PGs	prostaglandins
PGE_2	prostaglandin E_2
PGG_2	prostaglandin endoperoxide G_2
PGH_2	prostaglandin endoperoxide H_2
PGHS-1	prostaglandin endoperoxide synthase-1
PGHS-2	prostaglandin endoperoxide synthase-2
PGI_2	prostacyclin
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
ρ-NP	para-nitrophenol
ρ-NPP	para-nitrophenylphosphate
PPAR	peroxisome proliferator activated receptor
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
PUFA	polyunsaturated fatty acid
PUFAs	polyunsaturated fatty acids
RANK	receptor activator of nuclear factor-κβ
RANKL	receptor activator of nuclear factor-κβ-ligand
RIA	radioimmunoassay
rpm	revolutions per minute
RXR	retinoid X receptor
SDF-1	stromal cell-derived factor-1
sRANKL	soluble secreted RANKL
SREBP	sterol regulating element binding protein
TBS	tris-buffered saline
TGF-β	transforming growth factor-β
TMB	3'3',5',5' tetramethylbenzidine
TNF	tumor necrosis factor
TNFα	tumor necrosis factor-α
TRAF-6	TNF receptor-associated factor-6
TRAP	tartrate-resistant acid phosphatase
UV	ultra violet
v/v	volume per volume
vit D_3	1,25-Dihydroxy vitamin D_3 (calcitriol)
w/v	weight per volume