Journal of Steroid Biochemistry & Molecular Biology 123 (2011) 85–89

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Vitamin D metabolites and analogs induce lipoxygenase mRNA expression and activity as well as reactive oxygen species (ROS) production in human bone cell line

D. Somjen[a,*], S. Katzburg[a], M. Grafi-Cohen[a], E. Knoll[a], O. Sharon[a], G.H. Posner[b]

[a] Institute of Endocrinology, Metabolism and Hypertension Tel-Aviv Sourasky Medical Centre and Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, Israel
[b] Department of Chemistry, The Johns Hopkins University, Baltimore, MD, USA

ARTICLE INFO

Article history:
Received 30 August 2010
Received in revised form 8 November 2010
Accepted 17 November 2010

Keywords:
Vitamin D metabolites
Vitamin D less-calcemic analogs
SaOS2
Lipoxygenase
HETE
ROS

ABSTRACT

Vitamin D metabolites and its less-calcemic analogs (vitamin D compounds) are beneficial for bone and modulate cell growth and energy metabolism. We now analyze whether 25(OH)D_3 (25D), 1,25$(OH)_2D_3$ (1,25D), 24,25$(OH)_2D_3$ (24,25D), JKF1624F_2-2 (JKF) or QW1624F_2-2 (QW) regulate lipooxygenase (LO) mRNA expression and its products; hydroxyl-eicosatetraenoic acid (12 and 15HETE) formation, as well as reactive oxygen species (ROS) production in human bone cell line (SaOS2) and their interplay with modulation of cell proliferation and energy metabolism. All compounds except 25D increased 12LO mRNA expression and modulated 12 and 15HETE production whereas ROS production was increased by all compounds, and inhibited by NADPH oxidase inhibitors diphenyleneiodonium (DPI) and N-acetylcysteine (NAc). Baicaleine (baic) the inhibitor of 12 and 15LO activity blocked only slightly the stimulation of DNA synthesis by all compounds, whereas DPI inhibited almost completely the stimulation of DNA and CK by all compounds. Treatments of cells with 12 or 15HETE increased DNA synthesis and CK that were only slightly inhibited by DPI. These results indicate that vitamin D compounds increased oxidative stress in osteoblasts in part via induction of LO expression and activity. The increased ROS production mediates partially elevated cell proliferation and energy metabolism, whereas the LO mediation is not essential. This new feature of vitamin D compounds is mediated by intracellular and/or membranal binding sites and its potential hazard could lead to damage due to increased lipid oxidation, although the transient mediation of ROS in cell proliferation is beneficial to bone growth in a yet unknown mechanism.

1. Introduction

Vitamin D metabolites and its less-calcemic analogs (vitamin D compounds) affect the biology of skeletal cells via genomic [1,2] and non-genomic mechanisms [3]. Adequate availability of vitamin D_3 and its main active metabolite 1,25$(OH)_2D_3$ (1,25D) is essential for skeletal health and modulation of cell growth and differentiation of both osteoblasts and osteoclasts [4]. On the other hand 1,25D causes hypercalcemia [4,5] and therefore optimal bone growth and prevention of osteoporosis which requires its adequate concentrations [6,7], needs the use of the less-calcemic analogs having no adverse calcemic activity [8]. There are also other native metabolites of vitamin D such as the 24,25$(OH)_2D_3$ (24,25D) and 25(OH)D_3 (25D) which are biologically active in the skeletal cells in addition to 1,25D, inducing changes in specific markers of osteoblasts in cultured osteoblasts [9,10] with no effect on calcium metabolism. The metabolite 24,25D binds to specific receptors different from those of 1,25D in skeletal cells such as chondroblasts and "young" less differentiated Obs and regulates different biological functions in a variety of skeletal cells [11,12]. Whether 25D has an independent biological role or it functions only after its conversion to the more hydroxylated metabolites the 1,25D and/or 24,25D is not clear.

We have tested previously the activity of structurally modified less-calcemic analogs of vitamin D and found that JKF1624F_2-2 (JKF) and QW1624F_2-2 (QW) stimulated different parameters in primary cultured human female derived osteoblasts and in human bone cell lines [13,14]. In addition, pre-treatment of the osteoblast-like cells with these analogs up-regulated both their responsiveness and sensitivity to estrogenic compounds and modulated the different estrogen nuclear and membranal receptors [13–15].

In the present report we studied the regulation of LO mRNA expression and activity through the formation of 12- and 15-hydroxyeicosatetraenoic acid (HETE), the arachidonic acid derived metabolites of these enzymes as well as ROS production by the vitamin D compounds. We also studied the involvement of LO, HETE and ROS in the stimulation of cell proliferation (DNA) and energy

* Corresponding author. Tel.: +972 3 697 3812; fax: +972 3 697 3421.
E-mail address: dalias@tasmc.health.gov.il (G.H. Posner).

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doi:10.1016/j.jsbmb.2010.11.010

metabolism (CK). We focused on these enzymes since LO products [16] were shown to induce ROS formation [9], proliferation or survival, playing a role in promoting cell growth [13]. We hypothesized that the growth modulating effects of vitamin D compounds in human osteoblasts might be associated with accelerated production of LO metabolites, whose putative action including effects on ROS production may explain some of the new links between the LO system and other biological activities [17]. This is a complementation of our data presented in the last 14th vitamin D workshop [9], which was on the effects of the vitamin D compounds on primary human female bone cells cultures, and using mainly the less calcemic analogs and not the natural metabolites.

2. Materials and methods

2.1. Materials

12 and 15HETE were obtained from Biomol (Biomol International, Plymouth Meeting, PA). Nitro-blue tetrazolium (NBT), N-acetylcysteine (NAc) and diphenyleneiodonium chloride (DPI) were obtained from Sigma Chemicals Co. (St. Louis, MO). 2′,7′-dichloro-fluorescein diacetate (DCF) was obtained from Molecular Probes (Eugene, OR). Vitamin D metabolites were obtained from Enzo life science (Lausanne, Switzerland) whereas the less-calcemic analogs of vitamin D were synthesized by us [8]. All other chemical used were of analytical grade.

2.2. Cell cultures

SaOS2 human bone cell line was obtained from ATCC (Manassas, VA, USA) and grown according to the instructions.

2.3. Hormonal treatment

Sub-confluent cultured cells were treated with vehicle, 25D at 50 nM; 1,25D at 25 nM; 24,25D at 125 nM; JKF or QW at 1 nM [14];

a. for 1 h with serum-free medium, followed by the addition of vehicle or vitamin D compounds at the concentrations mentioned above for 10 min and HETE were extracted and assayed as previously described [16] or;
b. for 1 h by the addition of vehicle or vitamin D compounds, at the concentrations mentioned above for ROS assay as previously described [9] or;
c. for 24 h with the addition of vehicle or vitamin D compounds, at the concentrations mentioned above for DNA synthesis (DNA) and for creatine kinase (CK) specific activity as previously described [13].

2.4. Determination of mRNA for 12 and 15LO by RT-PCR

RNA was extracted and the expression of mRNA of 12 and 15LO or ERα and ERβ was carried out by RT-PCR as previously described [16].

2.5. Determination of the levels of 12 and 15HETE by HPLC

Cells and medium were extracted for HETE formation and analyzed by HPLC as previously described [16].

2.6. Determination of ROS formation

After hormonal treatment for 1 h, and ROS formation [9] using NBT colorimetric method as previously described [18] or for fluorescent microscopy by using 2′,7′-dichloro-fluorescein diacetate (DCF) [19] was determined.

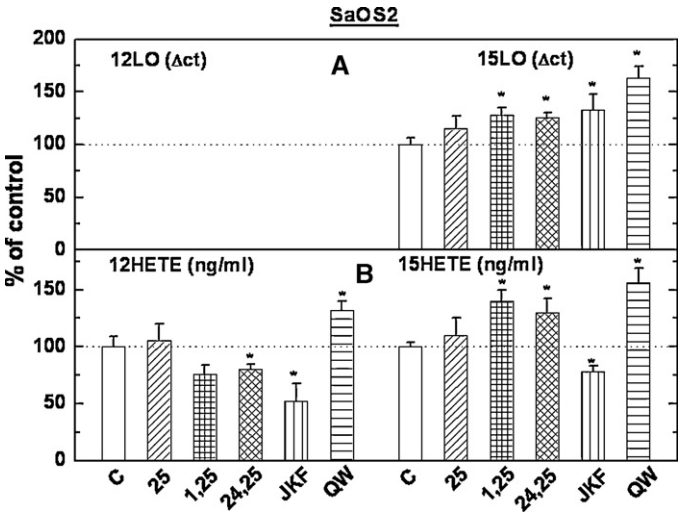

Fig. 1. The effect of vitamin D compounds 25D, 1,25D, 24,25D, JKF and QW on LO mRNAs expression and the production of HETE.
Vitamin D compounds were added daily at their optimal conditions for 3 days to determine LO mRNAs expression (A, Δct) and added for 1 h for the production of HETEs (B, ng/ml) in SaOS2 bone cell line. Details are given in the materials and methods, $^{*}p < 0.05$.

2.7. Assessment of DNA synthesis

After hormonal treatment for 22 h, ^{3}[H] thymidine was added for 2 h and its incorporation into DNA was determined as previously described [14].

2.8. Assessment of creatine kinase specific activity

After hormonal treatment for 24 h, CK was extracted and assayed as previously described [14].

2.9. Statistical analysis

The significance of differences between experimental and control means was evaluated using Student's *t*-test or ANOVA, in which $n = 5–8$ number of cultures.

3. Results

3.1. Modulation of LO mRNA expression and HETE formation in SaOS2 cell line by vitamin D compounds

Three daily additions of 25D (50 nM); 1,25D (25 nM); 24, 25D (125 nM); JKF or QW (1 nM) to SaOS2 cells modulated the expression of mRNA for 15LO but not mRNA for 12LO (Fig. 1, upper panel), which was below detectability in this study. Single treatment for 1 h with the different vitamin D compounds modulated the formation of 12 and 15HETE (Fig. 1, lower panel). All hormones with the exception of 25D stimulated 15LO mRNA expression (Fig. 1, upper panel) and 15HETE formation, whereas JKF inhibited it (Fig. 1, lower panel). On the other hand all hormones with the exception of 25D inhibited 12HETE formation and only QW stimulating it (Fig. 1, lower panel).

3.2. The effect of vitamin D compounds and 12 or 15HETE with/without DPI on ROS formation in SaOS2 cell line

Human bone cell line SaOS2 (Fig. 2) treated for 1 h with 25D (50 nM); 1,25D (25 nM); 24, 25D (125 nM); JKF or QW (1 nM), or 12 and 15HETE at 1 μM showed increased ROS formation as measured

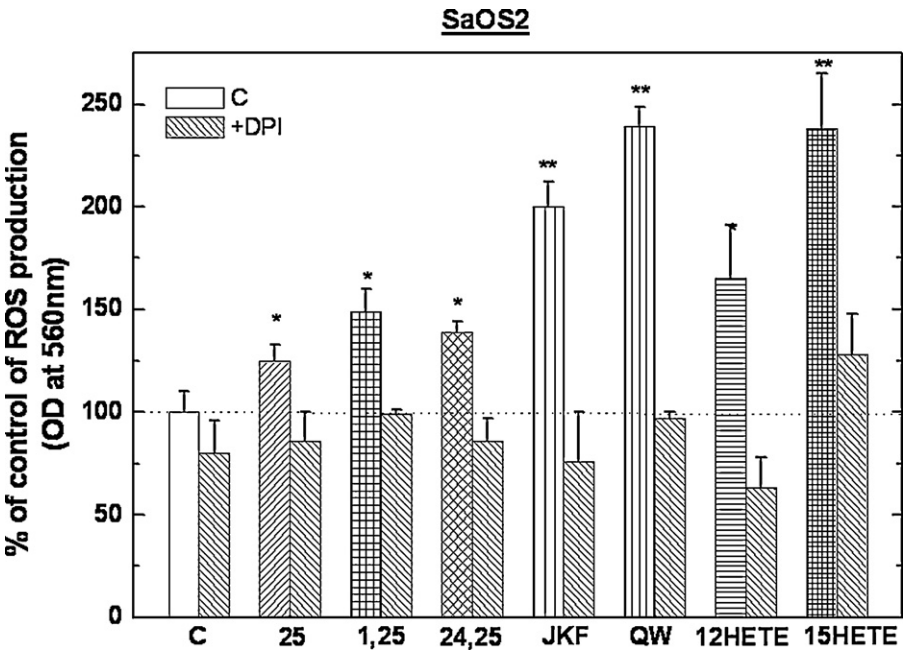

Fig. 2. ROS formation after treatment with vitamin D compounds 25D, 1,25D, 24,25D, JKF and QW and HETEs.
The compounds were added at their optimal conditions for 1 h for the formation of ROS with/without DPI in human bone cell line SaOS2, expressed as % of control of OD at 560 nm. Details are given in the materials and methods, $^{*}p < 0.05$, $^{**}p < 0.01$.

by the colorimetric and quantitative NBT assay induced by all compounds tested. This ROS formation was completely inhibited by the NADPH Oxidase inhibitor DPI (Fig. 2).

3.3. The effect of JKF or QW with/without NAc on ROS formation in SaOS2 cell line

Human bone cell line SaOS2 (Fig. 2, Table 1) treated for 1 h with JKF or QW at 1 nM, showed increased ROS formation as measured by the colorimetric and quantitative NBT assay induced by all compounds tested which was completely inhibited by the NADPH Oxidase inhibitor N-acetylcysteine (Table 1) similar to the inhibition by the another inhibitor DPI (Fig. 2).

3.4. The effect of vitamin D compounds and 12 or 15HETE with/without DPI on DNA synthesis and CK specific activity in SaOS2 cell line

Human bone cell line SaOS2 treated for 24 h with 25D (50 nM); 1,25D (25 nM); 24,25D (125 nM); JKF or QW (1 nM) as well as 12 and 15HETE at 1 μM, resulted in an increased DNA synthesis, blocked completely by the NADPH Oxidase inhibitor DPI except for partial inhibition of the treatment with 12 and 15HETE (Fig. 3, lower panel). CK specific activity was also stimulated by all compounds tested and blocked completely by DPI except slight inhibition of treatment with 12 and 15HETE or JKF and QW (Fig. 3, upper panel).

Table 1
The effect of vitamin D less-calcemic analogs JKF and QW on ROS formation with/without N-acetylcysteine in human bone cell line SaOS2.

Treatments	ROS formation (OD at 560 nm) (% of control)
Vehicle	100 + 9
Vehicle + NAc	63 + 20
JKF	200 + 12**
JKF + NAc	98 + 18
QW	239 + 10**
QW + NAc	100 + 13

The effect of vitamin D less-calcemic analogs JKF and QW on ROS formation with/without N-acetylcysteinein human bone cell line SaOS2. Details are given in the materials and methods.
** $p < 0.01$.

3.5. The effect of vitamin D compounds with/without Baic on DNA synthesis and CK specific activity in SaOS2 cell line

Human bone cell line SaOS2 treated for 24 h with 25D (50 nM); 1,25D (25 nM); 24,25D (125 nM); JKF or QW (1 nM), resulted in an increased DNA synthesis (Fig. 4, lower panel), as well as CK specific activity (Fig. 4, upper panel), but blocked only slightly and partially by the addition of the LO activity inhibitor baicaleine (baic).

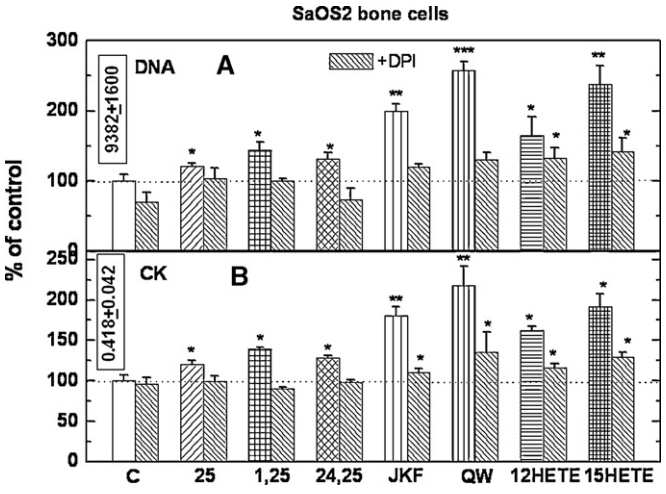

Fig. 3. DNA synthesis and CK specific activity after treatment with vitamin D compounds 25D, 1,25D, 24,25D, JKF and QW and 12 or 15 HETEs.
The compounds were added at their optimal conditions added for 24 h with/without DPI for the changes in DNA synthesis (A, % of control of dpm/well) and CK specific activity (B, % of control of μmol/min/mg protein) in human bone cell line SaOS2. Details are given in the materials and methods, $^{*}p < 0.05$, $^{**}p < 0.01$. Basal levels of cell proliferation and CK specific activity are given in the vertical boxes.

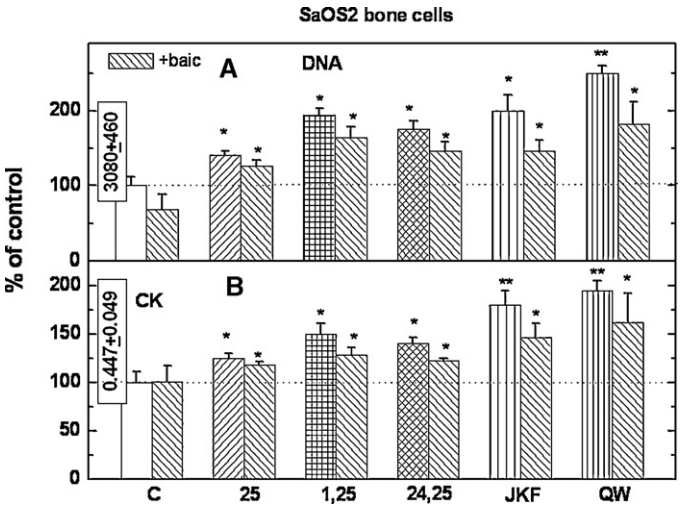

Fig. 4. Modulation by baicaleine of DNA synthesis and CK specific activity after treatment with vitamin D compounds 25D, 1,25D, 24,25D, JKF and QW.
The compounds were added at their optimal conditions added for 24 h with/without baicaleine for the changes in DNA synthesis (A, % of control of dpm/well) and in CK specific activity (B, % of control of μmol/min/mg protein) in human bone cell line SaOS2. Details are given in the materials and methods, $^{*}p < 0.05$, $^{**}p < 0.01$. Basal levels of cell proliferation and CK specific activity are given in the vertical boxes.

3.6. The effect of vitamin D compounds and HETE on ROS formation in SaOS2 cell line analyzed by fluorescent microscopy

Human bone cell line SaOS2 were treated for 1 h with 25D; 25D (50 nM); 1,25D (25 nM); 24,25D (125 nM); JKF or QW (1 nM) or 12 and 15HETE at 1 μM and then for 60 min with the fluorescent probe DCF resulted in fluorescent labelling of the cells. Fluorescent microscopy of the cells showed staining with all compounds (Fig. 5) which was blocked completely by pre-incubation for 30 min with DPI (data not shown), which determined qualitatively intracellular ROS formation. The effect of vehicle, 25D or 1,25D on ROS formation (Fig. 5b,c) shows no staining in vehicle treated cells, only slight staining by 25D, whereas 1,25D was very active. The effects of 24,25D or QW or JKF on ROS formation (Fig. 5d–f) show that the less-calcemic analogs were very effective in the stimulation of the staining. Stimulation with 12 or 15HETE of ROS formation (Fig. 5g,h) demonstrates strong staining with 12HETE and much weaker staining with 15HETE. All stimulated staining was completely inhibited by pre-treatment for 30 min with DPI (data not shown). The fluorescent staining results were similar to those obtained by the colorimetric and quantitative chemically determination of ROS formation by the NBT method shown above (Fig. 2).

4. Discussion

The effects of vitamin D metabolites and its less-calcemic analogs (vitamin D compounds) on ROS formation in human bone cell line SaOS2 complements our findings in studies using primary cultured human bone cells *in vitro*, where the different vitamin D metabolites and the hybrid fluorinated less-calcemic analogs JKF and QW stimulate cell proliferation, energy metabolism as well as membrane-mediated cellular responses [13,14]. Although reports suggest that 12 and 15HETE interact with multiple signals promoting cell replication, their effects are probably not redundant, since inhibition of their production hinders normal cell growth in a variety of cell types [16,17,20]. Recent publications linked also 12/15LO or 15LO and 12LO (platelet type) to bone density [21,22]. In the present study we provide direct evidence for the expression and biological role of LO in bone cell biology. We found that cultured human bone cells and human bone cell lines express mRNA

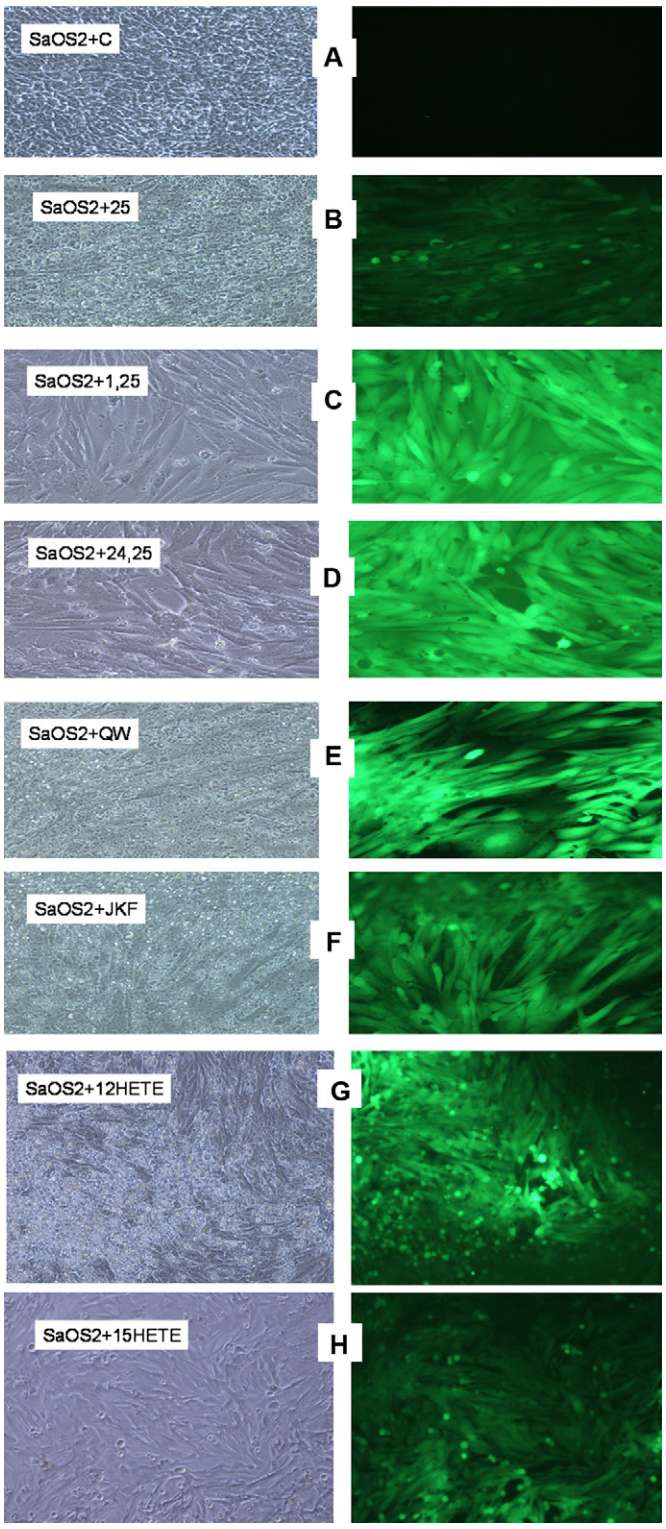

Fig. 5. Fluorescent microscopy analysis of ROS formation after treatment with vitamin D compounds 25D, 1,25D, 24,25D, JKF and QW and HETEs.
The compounds were added at their optimal conditions for 1 h for the analysis of ROS formation in human bone cell line SaOS2 determined by the DCF quantitative fluorescent microscopy (magnification ×20). (a) The effects of vehicle (A) or 25D (B) or 1,25D (C), or; (b) 24,25D (D) or QW 25D (E) or JKF (F), or; (c) 12HETE (G) or 15HETE (H); details are given in the materials and methods.

of three types of LO, i.e., the platelet type 12LO, 15LO type 1 and 15LO type 2 [16]. In the present study we show that the expression of the LO in these cells is modulated by a variety of vitamin D compounds. The expression of these enzymes results in the ability of bone cells to form and secrete 12HETE and 15HETE, the products of LO. Moreover, the generation of 12/15HETE which is driven by vitamin D compounds is linked to vitamin D-dependent osteoblastic growth and to less extent energy metabolism. The 12/15HETE raise local oxidative stress as was shown here by measurements of ROS formation induced by vitamin D compounds as well as by the 12/15HETE themselves. Because oxidative stress, in turn, may lead to inhibition of differentiation of bone osteoblasts-like cells [20] and acceleration of osteoclast differentiation [21], its induction may also result in the release of oxidizing fatty acids which unfavourably affect overall bone osteoblast/osteoclast homeostasis through enhanced oxidative stress. Presently, however, these potential secondary sequels of vitamin D-stimulated LO activities in bone cells remain entirely conjectural and are the subject of future investigation.

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