Expression and Localization of Phosphoinositide-Specific Phospholipases C in Cultured, Differentiating and Stimulated Human Osteoblasts

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Abstract

The osteoblasts contribute to bone homeostasis maintaining the bone mass, and intervene in bone injuries repair. Insights in the events leading to the proliferation and differentiation of osteoblasts might allow uncover potential molecular targets to control the complex mechanisms underlying bone remodeling. Signal transduction pathways contribute to the differentiation and metabolic activities of osteoblasts, with special regard to calcium-related signaling, including the Phosphoinositide (PI) pathway.

In the present article we aimed to evaluate the role of PI pathway related Phospholipases C (PLCs) enzymes family in human osteoblasts (HOBs). We analyzed the expression of PLC genes and the localization of PLC enzymes in cultured HOBs and in vitro differentiating HOBs after 3, 10, 17 and 23 days, and in HOBs stimulated with Lipopolysaccharide, which affects the differentiation of osteoblasts, after 3, 6, 24 and 48 hours.

Our results confirm the transcription of most PLC genes and the presence of a number of PLC enzymes in HOBs, differently localized in the nucleus, in the cytoplasm or both, as well as in cell protrusions. The localization of PLC enzymes within the cell suggests the activation of both the PI nuclear and of the cytoplasmic cycle in HOBs. Depending on the experimental conditions, transcripts of splicing variants of selected PLC genes were detected and the localization of most PLC enzymes varied, with special regard to enzymes belonging to the PLC β, ε and η sub-families. Further studies addressed to elucidate the complex network involving the signal transduction of PLCs might provide further insights into the complex signal transduction network in bone remodeling, also offering the opportunity to identify promising molecular targets.

Introduction

Bone homeostasis depends on the balance of the activity of osteoblasts and osteoclasts. Osteoblasts are mononucleated cells primarily involved in the formation of bone [1,2]. Osteoblasts are responsible for maintaining the bone mass, and intervene in the repair of bone injuries. The limited number of therapeutic agents able to promote osteogenesis ingenerated great interest addressed to manipulate the activity of osteoblasts. Insights in the events leading to differentiation and proliferation of osteoblasts might allow uncover potential molecular therapy targets in the complex mechanisms underlying skeletal remodeling [3,4].

The activities of osteoblasts are regulated by autocrine, paracrine, and endocrine factors from the external environment to ensure the systemic balance of calcium–phosphate metabolism while maintaining bone homeostasis [5]. External stimuli bind to their receptors on the osteoblasts’ membrane, including G protein-coupled receptors (GPCRs) [6,7]. GPCRs

receiving signals from the microenvironment surrounding the cell, and transuding them to heterotrimeric G proteins, further transducing signals within the cell to downstream effectors [8]. GPCRs comprise of a large seven transmembrane receptor proteins family implicated in a wide range of physiological processes, including bone development and remodeling [9,10]. The activation of GPCRs contributes to modify the bone mass. Signaling pathways related to GPCRs are responsible for bone homeostasis, and disruption or mutation can result in bone diseases [6,7].

The signaling of GPCRs acts by means of several pathways to regulate osteoblast function [11], as Gs and Gi pathways regulate the adenyl cyclase (AC), oppositely modulating intracellular levels of cAMP, and Gqq activates the pathway of Phosphoinositide (PI)-specific Phospholipase C (PLC) to modify intracellular calcium levels [12-16]. The signaling of Secretin GPCRs also acts by means of AC or PLC [17]. Oscillations of calcium act crucially during the remodeling of bone, affecting both the differentiation and proliferation of osteoblasts.

During bone repair or physiological bone regeneration, inflammatory cytokines and inflammatory mediators mobilize osteoblast precursors to the injured part, and guide the proliferation and differentiation of osteoblasts [18]. Lipopolysaccharide (LPS) is one major pro-inflammatory molecule during experimental bone regeneration and induces release of inflammatory cytokines [19-22]. Although LPS can directly induce cell apoptosis/necrosis, low concentrations of LPS may have opposite effects [23,24]. LPS at low concentration promotes the proliferation and vitality of osteoblasts, and inhibits the apoptosis. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is usually activated by LPS-induced Prostaglandin E2 (PGE2) secretion [25]. The enhanced effect of low-concentration LPS upon the proliferation of osteoblasts was associated with the NF-κB signaling pathway and cell autophagy [26]. LPS was demonstrated to inhibit the differentiation in mouse osteoblast-like MC3T3-E1 cells. In fact, addiction of different concentrations of LPS to osteoblast differentiation medium affected the expression of osteoblast differentiation markers, reducing ALP, OCN, OPN, and RUNX2 in MC3T3-E1 cells [27]. Literature data reported LPS to promote fracture healing by affecting the regulation of signal transduction in osteoblasts [26]. LPS was demonstrated to affect the expression of PLC genes in astrocytes and in human endothelial cells [28,29].

The PLC enzymes belong to the PI signal transduction pathway and play an important role in a variety of cell functions [30-34]. PLC enzymes basically cleave the polar head group of Phosphatidylinositol 4,5-bisphosphate (PIP2), producing two further signaling molecules, the inositol trisphosphate (IP3) and diacylglycerol (DAG) [31]. Due to its water-solubility, IP3 diffuses to the cytoplasm, and binds IP3-gated calcium channels within the membrane of the endoplasmic reticulum (ER), promoting calcium release [30,31]. DAG can be cleaved into arachidonic acid [33] or can activate the protein kinase C (PKC) family of enzymes [30,31]. Beside this basic activity, common to each PLC enzyme, PLCs were involved in a wide range of interconnections, including in the bone [34,35].

Mammalian PLC enzymes are multi-domain proteins which differ for the presence of peculiar domains, and subsequently for mechanism of recruitment. Based on specific structural and functional similarities, PLC enzymes were grouped into six sub-families: β (1-4), γ (1-2), δ (1, 3, and 4), ε, ζ, η [34]. Alternative splicing variants were described for most human PLC enzymes [36-40].

Cells own a peculiar panel of PLC enzymes, depending on the cyto-type [29,41-43]. Under abnormal conditions, in a number of cell types the panel of expression of PLCs was described to vary compared to normal counterparts [44,45]. The panel of PLCs was modified by specific stimuli, including inflammation [29,45-49], or by the use of specific inhibitors [50,51]. The subcellular distribution of PLC enzymes influences the activity, suggesting specific roles for each PLC enzyme beside the cleavage of PIP2 [52-54]. PLC enzymes were also recently described in extracellular vesicles (EVs), suggesting a possible role in extracellular signaling [55].

In order to evaluate the role of the PLC signaling during the differentiation of human osteoblasts, we analyzed the expression of PLC genes and the localization of PLC enzymes under different experimental conditions. We analyzed transcription and localization in human osteoblasts (HOBs) cultured through 23 days, in HOBs cultured in differentiating medium through 23 days, and in cultured HOBs stimulated with LPS through 48 hours.

Materials and Methods

Cell culture

HOBs were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and were characterized using antibodies against Osteocalcin, Alkaline Phosphatase, RUNX2, DMP1 and MEPE (data not shown). Frozen cells were thawed, and cultured for 3 days. The initial seeding number of cells was 1x10^5 for each experiment. Cells were grown up in T25 culture flasks for molecular biology experiments or upon 2 cm² coverslips in 24 multiwell plates for fluorescent immunocytchemistry under sub-confluent or confluent conditions. In either culture, cells were maintained at 37°C with 5% CO₂ in Alpha Minimum Essential Medium (MEM) medium (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 µg/ml), and streptomycin (100 U/ml) (Sigma-Aldrich). To maintain cultures, cells were split 1:15 every 3 days through a period lasting 23 days. To culture HOBs in differentiating medium, ascorbic acid (60 mM), and β-glycerophosphate (0.5 mM) were added to MEM medium, and cells were split every 3
days through a period lasting 23 days. In both cultured HOBs and differentiating HOBs, reagent for molecular biology (see below) was added or cells were fixed for immunocytochemistry analyses at the following time points: 0, 3, 10, 17, and 23 days. Finally, LPS 100 ng/ml was added to the culture medium, then Trizol reagent (Invitrogen, see below) was added or cells were fixed after 3 and 48 hours; for some PLC enzymes, such as PLC δ1, PLC ε and PLC n2, two further time points were accounted after 6 and 24 hours from LPS stimulation. In all the indicated experimental conditions, HOBs were counted using a Neubauer haemocytometer (Weber Scientific International Ltd., Middlesex, UK) and morphology was observed using a Nikon optic microscope.

**Molecular biology**

Total RNA was isolated from samples by using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. The obtained RNA was quantitated by the UV absorption ratio 260:280 nm by using Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1 μg of total RNA was reverse transcribed by High-Capacity cDNA (r) Transcription Kit (ThermoFisher Scientific), according to manufacturer’s instructions. GoTaq(R) Colorless Master Mix (Promega, Madison, WI, USA) was used to amplify the obtained cDNA. The following primer pairs (DBA, Segrate, Italy) were used: PLCB1 (OMIM *607120) forward (f) 5'-AGCTTCTGAAACAAGGCTCCCAACA-3'; reverse (r) 5'-ATCTGTCGTGTCACATTCCGT-3'; PLCB2 (OMIM *604114) (f) 5'-AAGGTGAGGCTATCTGAGCCAA-3'; (r) 5'-CGGGCCACGCCAGGCTG-3'; PLCB3 (OMIM *600230) (f) 5'-TATCTTTGACCTTGCTGCG-3'; (r) 5'-GGCCTTCATACCGTCCATC-3'; PLCE1 (OMIM *608414) (f) 5'-GGGGCCACGGTCATCCAC-3'; (r) 5'-CTGAGCGTGAGCTGTTG-3'; PLCD1 (OMIM *602142) (f) 5'-CTGAGCTGATCTTGCCAG-3'; (r) 5'-GACACGTCCCAGTCTGGAACC-3'; PLCD4 (OMIM *605939) (f) 5'-AAGGTGAAGGCCTATCTGAGCCAA-3'; (r) 5'-CTGCTTCCTCTTCCTCATATTC-3'; PLCF (OMIM *600879) (f) 5'-CCAGAAACCATCCCTCAGCT-3'; (r) 5'-GCCA TTGTTGACGACCTGAC-3'; PLDF (OMIM *605939) (f) 5'-AA GACAGCTCCGATCTGGACC-3'; (r) 5'-CTGCTCCCTCCTTCTCATATTC-3'; PLCE (OMIM *608414) (f) 5'-GGGGCCACGGTCATCCAC-3'; (r) 5'-GGGGCCACCTCATCCCTCCAT-3'; PLCH1 (OMIM *612835) (f) 5'-CCAGAACAGCCGCTGTTTCTGGC-3'; (r) 5'-GGATGCTTCTTGTGAG-3'; TCAGCTCTCC-3'; PLCH2 (OMIM *612836) (f) 5'-GGACACTGG CCTCACAACACTGCCCAGG-3'; (r) 5'-GTCCTGGTTTGTGAGATGAC GTCGCCCTCTGC-3'; GAPDH (OMIM *138400) (f) 5'-CGAGATCCCT CCAAATCAA-3'; (r) 5'-GTCTTCTGGGGTCAGTGAT-3'.

The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins. The supplied reagents were mixed in a PCR tube with 0.2 mM primer pairs, and 3–5 μl template cDNA following manufacturer’s instructions to 30 μl final volume. The amplification started with an initial denaturation step at 94°C for 2 min and was followed by 30 cycles consisting of denaturation (30 s) at 95°C, annealing (30 s) at the appropriate temperature for each primer pair, and extension (1 min) at 72°C. The PCR products were analyzed by electrophoresis of 1.5% TAE agarose gel. Gels were previously stained with GelRed® Nucleic Acid Gel Stain (Biotium Inc, Fremont, CA). Chemidoc Imaging System (Bio-Rad, Hercules, CA) was used for gel documentation and image acquisition. Optical densities were normalized to the RNA transcript of glyceraldehyde-3-phosphate dehydrogenase human gene (GAPDH; OMIM *138400), a typical reference constitutive transcript. To exclude possible DNA contamination during the RT-PCR, RNA samples were amplified by PCR without reverse transcription. No band was observed, excluding DNA contamination during the RNA preparation procedure (data not shown).

**Fluorescence immunocytochemistry**

Cells grown upon coverslips were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 10 min at 4°C, and washed three times with PBS. Cells were incubated at 4°C overnight with appropriate primary antibodies diluted (1:100) in PBS. The primary mouse anti-human PLC antibodies against enzymes belonging to the PLC β, PLC γ and PLC δ sub-families (Santa Cruz Biotechnology, Santa Cruz, CA) were detected with the appropriate fluorescent dye conjugated anti-mouse secondary antibodies (Bethyl Laboratories, Montgomery, TX). The primary rabbit anti-human PLC antibodies against enzymes belonging to the PLC ε and η sub-families (Invitrogen, ThermoFisher Scientific) were detected with the appropriate fluorescent dye conjugated secondary antibodies (Thermoscientific, Thermofisher Scientific). Cover-slips were incubated with the specific secondary antibody (1:200) for 1 h at room temperature. Cells were washed twice with PBS for 5 min, then counterstained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining for 20 min. After two washes in PBS for 5 min each, the slides were visualized and images were acquired with a Nikon microscope equipped with NIS Elements software (Nikon Imaging Japan Inc, Tokyo).

**Results**

**Cell culture**

The number of 23 days-cultured HOBs increased 14% after 3 days, 60% after 10 days, and progressively reduced until 23 days about 50%. It was not possible to define the doubling time, as cells did not double (Table 1 and Figure 1). The number of differentiating HOBs increased 331%, slightly decreasing from day 10 to 23, with doubling time of 3 days (Table 1 and Figure 1).

The number of cultured live untreated HOB control cells increased 34% after 3 hours, 70% after 6 hours, 305% after 24 hours and 447% after 48 hours from seeding (Table 1). The number of live HOB cultured cells increased 63% after 3 hours...

Table 1: Number of live cells in quiescent HOBs (HOB) and in LPS stimulated HOBs (HOB-LPS) at the time of seeding (0), and after 3, 6, 24 and 48 hours from seeding (upper). Number of live cells in cultured HOBs (HOB) and in HOBs cultured in differentiating medium (HOB-D) at the time of seeding and after 3, 10, 17 and 23 days (lower).

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Figure 1: (A) Growth curve of long-term cultured HOB (HOB) versus differentiating HOBs (HOB-D). Cells count on y axis and day of culture on x axis. Standard error statistics signs were indicated.

(B) Growth curve of LPS stimulated HOBs (LPS) versus controls (NT). Cells count on y axis and Hours of culture after LPS stimulation on x axis. Standard error statistics signs were indicated.

Figure 1: (A) Growth curve of cultured HOBs (HOB) and HOBs cultured in differentiating medium (HOB-D) at the time of seeding and after 3, 10, 17 and 23 days. (B) Growth curve of quiescent HOBs (NT) and in LPS stimulated HOBs (LPS) at seeding (0), and after 3, 6, 24 and 48 hours. On the x axis the number of live cells.
LPS treatment. After 6 hours from LPS treatment, the number of cells increased 76%. After 24 hours from LPS treatment, HOB cells increased 247% and after 48 hours 627% with respect to live cells at the moment of beginning stimulation (Table 1 and Figure 1). Cells were in well adhering growth and had fusiform shape, after 48 hours from LPS treatment cells were reticulated.

**Statistical analysis**

One-way ANOVA test statistical analysis of the growth data resulted significant with respect to the comparison of 23 days-maintained cultures and differentiating HOBs, with p-value=0.00953 (data in Table 1).

**Molecular biology**

In cultured HOBs cultured 3 days after thawing (day 0), transcripts from PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLC, PLCH1, and PLCH2 genes were detected. PLCB1, PLCB4, and PLCH1 were visualized as a double band (Table 2). PLCD3 and PLCD4 were weakly detected.

The cells were cultured and analyzed after 3, 10, 17 and 23 days and transcripts of all PLC genes were visualized (Table 2).

Transcript of PLCB1 was very weakly detected as two bands in 3, 10, 17, and 23 days cultures (Table 2). The transcript of PLCB2, PLCG1, PLCG2 and PLC were detected after 3 days, more weakly detected after 10 days, and detected after 17 and 23 days (Table 2). The transcript of PLCB4 was visualized as a double band after 3, 10, 17, and 23 days (Table 2). One band transcript was visualized for PLCH1 after 3, 17, and 23 days, while transcription of two bands was detected exclusively after 10 days (Table 2). Transcripts of PLCD3 and PLCD4 were weakly detected (Table 2). The transcript of PLCH2 was visualized as two bands after 3 days and weakly evident after 23 days of culture (Table 2).

In HOBs cultured in differentiating medium through 3, 10, and 17 days, transcripts from all PLC genes were detected (Table 2). Transcription of two bands was visualized for PLCB1 in the differentiation culture, until day 23, when only one band was visualized (Table 2). PLCD3, PLCD4 and inconstantly PLCE were weakly visualized after 3, 10, and 17 differentiation days (Table 2). PLCH1 was strongly visualized at differentiation day 17 (Table 2). In 23 days differentiated HOB cultures, PLCB1, PLCB2, PLCB3, PLCG1, PLCG2, PLCG3, PLCH1 and PLCH2 were detected (Table 2).

In LPS-stimulated HOB cultures, transcripts from PLCB2, PLCB3, PLCG1, PLCG2, PLCG3, PLCH1 and PLCH2 were detected after 3 hours from stimulation (Table 3). Transcripts from PLCB2, PLCB3, PLCG1, PLCG2, PLCD1, PLCD3, PLCE, PLCH1 and PLCH2 were detected after 6 hours after LPS stimulation (Table 3). Transcripts from all PLC genes were detected after 48 hours after LPS stimulation, transcripts from PLCD4 and PLCH1 were visualized as very weak bands (Table 3). PLCB2 in two bands after 24 and 48 hours LPS stimulation (Table 2). PLCH2 was transcribed in two bands after 48 hours LPS stimulation (Table 2).

| Table 2: Gel electrophoresis results of PCR products. HOB= HOB cells cultured 3 days after defrost; LT-C= HOB cells cultured for 23 days; DIFF-C= HOB cells cultured in differentiating medium. (+)= one band transcript; (-)= no transcript; (-/+)= weakly visible transcript; (db)= two bands transcript. |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| PLCB1 | PLCB2 | PLCB3 | PLCB4 | PLCG1 | PLCG2 | PLCD1 | PLCD3 | PLCD4 | PLCE | PLCH1 | PLCH2 | GAPDH |
| HOB | + | - | + | + | + | - | + | + | + | + | + | + | db | + |
| LT-C | 3 | /+ db | - | + | + | -/+ | -/+ | -/+ | + | + | + | db | + |
| Day 0 | 10 | /+ db | -/+ | + | + | + | -/+ | -/+ | -/+ | + | db | + | db | + |
| 17 | /+ db | + | + | + | -/+ | + | + | + | + | + | + | db | + |
| 23 | /+ db | + | + | + | -/+ | -/+ | + | + | -/+ | + | db | + |
| DIFF-C | Day 0 | 0 | + | + | + | + | + | /- | /- | /+ | + | + | + |
| 3 | +db | + | + | + | + | /- | /- | /+ | + | + | + | + |
| 10 | + db | + | + | + | + | + | /- | -/+ | + | + | + | + |
| 17 | /+ db | + | + | + | + | + | + | + | + | + | + | + |
| 23 | /+ | + | + | + | + | + | + | + | + | + | + | + |


Fluorescence immunocytochemistry

In cultured HOBs cultured three days after thawing (day 0), PLC enzymes were all detected (Table 4, Figures 2 and 3). PLC β1 was detected in the nucleus, in the cytoplasm and in cell protrusions. PLC β2 (Figure 2), PLC γ2 (Figure 3), PLC δ4 (Figure 4), and slightly PLCγ1 (Figure 3) were detected in the perinuclear area of the cytoplasm (Table 4). PLC β3 was

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**Table 3:** Gel electrophoresis results of PCR products. HOB= HOB cells cultured 3 days after defrost; LT-C= HOB cells cultured for 23 days; DIFF-C= HOB cells cultured in differentiating medium; LPS-C= cultured HOBs stimulated with LPS. (+)= one band transcript; (-)= no transcript; (/+)= weakly visible transcript; (db)= two bands transcript.

**Table 4:** Fluorescence immunocytochemistry. Localization of PLC enzymes in HOB cells at seeding (HOB), in cultured HOBs (HOBC) and in HOBs cultured in differentiating medium (HOBD) after 3, 10, 17 and 23 days. N: Nuclear; CpN: Cytoplasmic, perinuclear; C: Cytoplasmic; P: Cell protrusions; N (%): Percentage of positive nuclei. Punct: Punctuate distribution; f punct: Finely punctuate distribution; f retic: Finely reticulate distribution; Dep: Localized in deposits or compartmentalized. (-): Undetected; (w): Weakly detected; Pod: Podosome-like structures.
Figure 2: Fluorescence immunocytochemistry of PLCβ enzymes in cultured HOBs (A) and in HOBs cultured in differentiating medium (B) at the time of seeding and after 3, 10, 17 and 23 days. (20X, scale bar in the central figure is intended for all images) (blue: nuclei stained with DAPI; red or green: localization of PLC enzyme).
Figure 3: Fluorescence immunocytochemistry of PLC γ enzymes and of PLC δ1 and PLC δ3 in cultured HOBs (A) and in HOBs cultured in differentiating medium (B) at seeding and after 3, 10, 17 and 23 days. (20X, scale bar in the central figure is intended for all images) (blue: nuclei stained with DAPI; red or green: localization of PLC enzyme).
detected in the cytoplasm and in cell protrusions (Figure 2 and Table 4). PLC β4 was in the cytoplasm, probably accumulated in vesicles (Figure 2 and Table 4). PLC ε (Figure 4) and slightly PLC δ1 and PLC δ3 were detected in the cytoplasm (Figure 3 and Table 4). PLC η1 was detected in the nucleus. PLC η2 was detected both in the nucleus and punctuate in the cytoplasm (Figure 4 and Table 4).

**Figure 4:** Fluorescence immunocytochemistry of PLC δ4 of PLC ε and of PLC η enzymes in cultured HOBs (A) and in HOBs cultured in differentiating medium (B) at seeding and after 3, 10, 17 and 23 days. (20X, scale bar in the central figure is intended for all images) (blue: nuclei stained with DAPI; red or green: localization of PLC enzyme).
In cultured HOBs, after 3 days and 10 days PLC β1 was detected both in the nucleus and in the cytoplasm, where it showed a punctuate distribution exclusively after 3 days, after 17 it was observed in the perinuclear area of the cytoplasm, after 23 days it was observed diffused in the cytoplasm. PLC β2 was detected in the cytoplasm after 3 days accumulated in vesicles, more uniformly distributed after 10 days, weakly detected after 17 and 23 days. PLC β3 was detected in the cytoplasm in a finelly reticulated shape after 3 days, it was not detected after 10 days, but it was detected after 17 days and weakly detected after 23 days in the cytoplasm. PLC β4 was detected finely punctuate in the cytoplasm after 3 days, evenly distributed in the cytoplasm after 10 days, in the cytoplasm and in cell protrusions after 17 days, punctuate both in the cytoplasm and cell protrusions after 23 days (Figure 2A and Table 4). PLC γ1 was located in cytoplasm and protrusions after 3 days, punctuate in the cytoplasm after 10 days, in the perinuclear region of the cytoplasm after 17 days, and in cytoplasm and protrusions after 23 days. PLC γ2 was weakly detected in the perinuclear area after 3 days, punctuate in the cytoplasm after 10 days, weakly detected in the perinuclear area after 17 days, and localized in a side of the cytoplasm after 23 days. PLC δ1 was detected in the cytoplasm after 3 days, both in the nucleus and the cytoplasm after 10 days, very weakly in the cytoplasm after 17 and 23 days. PLC δ3 was detected after 3, 17 and 23 days in the cytoplasm, with a punctuate distribution after 10 days (Figure 3A and Table 4). PLC δ4 was not detected after 3 days, was detected both in the nucleus and cytoplasm after 10 days, in cytoplasm and cell protrusions after 17 days, and both in the nucleus and cytoplasm after 23 days. PLC ε was located in cytoplasm and protrusions after 3 days, in the cytoplasm after 10 days, punctuate in the cytoplasm after 17 days, and filamentous in cytoplasm and protrusions after 23 days. PLC η1 was detected in some nuclei (about 45%) and in the cytoplasm in podosome-like structure after 3 days, in the nucleus, finely punctuate in the cytoplasm and in cell protrusions after 10 days, finely punctuate in the cytoplasm and in cell protrusions after 17 days, and punctuate in the nucleus, cytoplasm and protrusions after 23 days of culture. PLC η2 was detected punctuate in the cytoplasm after 3 days, and both in the nucleus and punctuate in the cytoplasm after 10 days, in the cytoplasm and in cell protrusions after 17 days, and in the cytoplasm, in cell protrusions and in the nucleus of 60% cells after 23 days of culture (Figure 4A and Table 4).

In HOBs cultured in differentiating medium, PLC β1 was detected in both the nucleus and the cytoplasm at day 0, in the nuclei of about 20% cells and in the cytoplasm after 3 days cultures, in the perinuclear area of the cytoplasm after 10 days, punctuate in the cytoplasm in 17 days- and in the perinuclear area in 23 days-differentiating culture. PLC β2 was detected in the perinuclear region at day 0, diffuse in the cytoplasm after 3 and 10 days, punctuate in the cytoplasm after 17 days and punctuate in the perinuclear area after 23 days differentiating culture. PLC β3 was detected in cytoplasm and protrusions at day 0, in the nucleus and in the perinuclear area after 3 days, diffuse in the cytoplasm after 10 days, punctuate in the cytoplasm after 17 days of differentiating culture. PLC β3 was weakly detected in the cytoplasm after 23 days of differentiating culture. PLC β4 was detected in vesicles in the cytoplasm at day 0, punctuate in the cytoplasm after 3, 10, and 17 days differentiating culture. PLC β4 was weakly detected in the cytoplasm after 23 days of differentiating culture (Figure 2B and Table 4). PLC γ1 was weakly detected in the cytoplasm at day 0, in was detected more evidently in the cytoplasm after 3, 10 and 17 days, and punctuate in the cytoplasm after 23 days differentiating culture. PLC γ2 was weakly detected in the cytoplasm at day 0, in the nucleus and in the perinuclear area after 3 and 10 days, and punctuate in the perinuclear area after 17 and 23 days of differentiating culture. PLC δ1 was weakly detected at day 0, it was detected more evidently in the nucleus in 3 days- and 10 days-differentiating cultures, and in the perinuclear area of the cytoplasm after 17 and 23 days in the differentation. PLC δ3 was not detected at day 0, was detected both in the nucleus and the cytoplasm in 3 and 10 days cultures, in the perinuclear area after 17 days and diffuse in the cytoplasm after 23 days (Figure 3B and Table 4). PLC δ4 was weakly detected in the perinuclear area at day 0, punctuate in the cytoplasm after 3 and 10 days, and punctuate in the cytoplasm after 17 and 23 days. PLC ε was detected with a very strong signal in the cytoplasm through the observation period, in cell protrusions in 17 days cultures and with a punctuate distribution after 23 days. PLC η1 was detected punctuate in the cytoplasm through the culture period. PLC η2 was observed both localized in the nucleus and punctuate in the cytoplasm at day 0. It was weakly visible in the nucleus and detected in the perinuclear area with a punctuate distribution after 3 and 10 days. PLC η2 was detected with a very strong signal punctuate in the cytoplasm after 17 days and with a less evident signal after 23 days (Figure 3B and Table 4).

After 3 hours from LPS treatment, PLC β1 and PLC β2 were detected in the perinuclear area (Table 5). PLC δ3 was detected in the cytoplasm, PLC δ4 was detected slightly in the cytoplasm, and PLC ε (Figure 5) was detected both in the nucleus and in the cytoplasm (Table 5). PLC η1 was detected in about 45% nuclei and in the cytoplasm. PLC η2 was detected in about 25% nuclei and punctuate in the cytoplasm (Figure 5 and Table 5). The remaining enzymes were not detected (Table 5). After 48 hours from LPS treatment, PLC β1 was detected in the nucleus, PLC ε (Figure 5) was detected in the cytoplasm, PLC η1 and PLC η2 (Figure 5) were detected punctuate in the cytoplasm (Table 5). Further analyses allowed detect PLC ε both in the nucleus and the cytoplasm after 6 hours. PLC ε was detected in 89% nuclei and in the cytoplasm after 24 hours from LPS stimulation (Figure 5).

The transcript from GAPDH gene was detected in all experiments, as expected (Table 2). Gel electrophoresis of the amplification products revealed one or two DNA bands with nucleotide lengths as expected for the corresponding primers' pair.
Bone is a dynamic tissue destined to continuous remodeling which involves the activity of bone cells, including osteoblasts [56]. Signal transduction pathways contribute to the differentiation and metabolic activities of osteoblasts, with special regard to calcium-related signaling, such as the pathway which involves PLC enzymes.

In our present experiments, we analyzed the variations of PLC enzymes in osteoblasts cultured under different experimental conditions. The results confirm the presence of a number of PLC enzymes in HOBs, differently localized in the nucleus, in the cytoplasm or both. The present experiments also corroborate previous observations indicating that in the same cell type both the expression of PLC genes and the localization of PLC enzymes may vary under different conditions. The behavior of selected PLC enzymes deserves more detailed comments.

The present data confirm the presence of PLC β1 during the proliferation, as well as during the in vitro induced differentiation of HOBs, probably in two splicing isoforms. In HOBs’ cultures maintained 23 days, the transcript of PLCβ1 occasionally comprised of two bands of very slightly different molecular weight, suggesting that two splicing isoforms were transcribed. The presence in the nucleus of PLC β1 during the earlier stage of culture might suggest the activation of the nuclear cycle, accordingly to the presence of both the cytoplasmic and the nuclear splicing isoforms. In quiescent HOBs, the transcript of PLCβ1 gene was variably visualized as one or two bands and PLC β1 was detected in the nucleus, cytoplasm and cell protrusions. In cultured HOBs, the transcript of PLCβ1 was detected as two very weakly visible bands through the observation period, and PLC β1 was...

Initially detected both in the nucleus and the cytoplasm, later exclusively in the cytoplasm. In HOBs cultured in differentiating medium, the transcript of PLCB1 was detected as two visible bands until day 17, but at day 23 one band was visualized. PLCB1 was detected both in the nucleus and in the cytoplasm at day 0, in the nuclei of about 20% cells and in the cytoplasm in 3 days-cultures, and later exclusively in the cytoplasm. In LPS-stimulated HOB cultures, no transcript of PLCB1 was detected after 3, 6, and 24 hours, and by contrast it was visualized after 48 hours. After LPS stimulation, PLCB1 was initially located in the perinuclear area of the cytoplasm, and after 48 hours exclusively in the nucleus, accordingly to molecular biology results. LPS was demonstrated to inhibit osteoblast differentiation [19,27]. The block of PLCB1 transcription shortly after LPS stimulation might confirm literature data indicating that PLCB1 is involved in osteoblast differentiation. In our present experiments, LPS inhibited the transcription of PLCB1, thus reducing the levels of codified PLCB1, considered a positive regulator in the differentiation of osteoblasts [57]. In 23 days-maintained cultures and in differentiating HOBs' cultures, PLCB1 was detected both in the nucleus and the cytoplasm, and in LPS stimulated HOBs it was in the nucleus. That suggests that PLCB1's cytoplasmic membrane cycle or the nuclear cycle or both can be activated under different conditions. Occasionally, PLCB1 in the cytoplasm showed a punctuate distribution that might be related to its presence in vesicles, as already described [55].

The bone can adapt to mechanical load by osteosynthesis and osteoblasts probably own mechanosensitivity [58]. In this perspective, PLC enzymes were described to be involved in the regulation of mechanical loading, with special regard to PLCB2. In the present experiments, no transcript of PLCB2 was visualized in HOB cells shortly cultured after thawing (day 0), according to literature data [51,59], but PLCB2 was observed in the perinuclear area of the cytoplasm. PLCB2 was mainly expressed in hematopoietic cell lines [60,61]. Reports indicated that PLCB2 was not expressed in some quiescent cell lines, as well as in pathological [28,41,42,62] or stimulated counterpart [29,50]. In osteoblasts, PLCB2 was demonstrated to transduce signals from parathyroid hormone (PTH), Prostaglandin E2 (PGE2) and other prostanoids [63,64]. In all the present experimental conditions, PLCB2 was transcribed. Later in the LPS stimulation period, the transcript was comprised of two bands, probably indicating the transcription of two splicing isoforms. Excepting for 48 hours after LPS treatment, PLCB2 was detected and was located in the cytoplasm, occasionally with punctuate distribution. In endothelial cells, LPS silenced the expression of PLCB2 [29]. Interleukin-8 (IL-8), a pro-inflammatory chemokine, activates G protein and subsequently PLCB2 [65]. Several Rac proteins, described in bone tissue, directly bind and activate PLCB2 in vitro, as Rac mediates the membrane migration of PLCB2 and PLCB2 [66]. PLCB2 was involved in the pathogenesis of some autoimmune diseases [67], and it is involved in the inflammation response to viral infection by affecting the activation of growth factor-β-activated kinase 1 (TAK1) [68]. The absence of PLCB2 in late LPS stimulation might be related to its supposed role in inflammation and autoimmunity.

Under all the present experimental conditions, the transcript of PLCB3 was detected. Late in 23 days-cultured and in differentiating cultures and early after LPS stimulation, PLCB3 was not detected. During the 23 days-maintained culture and during differentiation, PLCB3 showed a punctuate distribution. PLCB3 was involved in the development of cerebellar Purkinje neurons [69]. In experimental models, loss of PLCB3 was demonstrated to induce macrophage hypersensitivity to apoptotic induction [70]. Therefore, the reduction of PLCB3 observed in cultured or inflammatory stimulated HOBs might corroborate a relationship with apoptosis.

The transcript of PLCB4 was detected and visualized as two bands slightly differing for molecular weight at each time point in 23 days-cultured and in differentiating cultured HOBs, with the notable exception of 23 days during differentiation, when no mRNA from PLCB4 was detected. During HOB cultures, PLCB4 was early detected in the cytoplasm, both in the cytoplasm and cell protrusions after 17 days and punctuate both in the cytoplasm and in cell protrusions after 23 days. PLCB4 was detected in the cytoplasm occasionally punctuate in 23 days-cultured and differentiating cultured HOBs. LPS stimulation blocked the transcription of PLCB4 exclusively after 3 and 6 hours, accordingly to the observation that PLCB4 was not detected after LPS stimulation. Although PLCB4 was mainly described in the human retina, where it acts crucially in the intracellular transduction of a number of extracellular signals, PLCB4 was detected in further tissues and certainly plays an important general role in intracellular molecular signaling [71,72]. The PLCB4 gene is involved in embryonic development as one of the enzymes of the endothelin pathway [73]. In uveal melanoma a gain-of-function mutation of PLCB4 activates the pathway of GNAQ/GNA11, the genes of which PLCB4 is the downstream target, thus promoting tumorigenesis [74,75]. The Auriculo-condylar syndrome (ARCND2; OMIM #614669), a craniofacial malformation syndrome, was recently recognized as to be due to heterozygous or homozygous missense mutation of the PLCB4 gene, located in chromosome 20p12 [76]. Analyzing cultured osteoblasts of ARCND2 and of controls, PLCB4 was demonstrated to be one of two core signaling components of the endothelin-1-distal-less homeobox 5 and 6 (EDN1-DLX5-DLX6) [77]. Interestingly, one major feature of ARCND2 is the underdevelopment of the mandibular condyle following abnormal development of the pharyngeal arches [76]. PLCB4 is involved in the development of the first and second pharyngeal arches, embryonic structures ultimately developing into the jawbones, facial muscles, middle ear bones, ear canals, outer ears, and related tissues [78]. Further studies are required in order to elucidate the role of PLCB4 in osteoblasts.
In the present experiments, the transcripts of both \textit{PLCG1} and \textit{PLCG2} were detected in 23 days-maintained and in differentiating HOBs' cultures. Although with slight differences, PLC \(\gamma_1\) was detected mainly in the cytoplasm, while PLC \(\gamma_2\) was detected in the cytoplasm and occasionally in the nucleus. PLC \(\gamma_1\) is important in the development, homeostasis, activation and tolerance of T cells as it interacts with the T cell receptor (TCR) signaling [79,80]. PLC \(\gamma_1\) also contributes to the functioning of specific cell types, as immune system cells, and nervous cells [81-88]. In quiescent fibroblasts, the lipase activity of PLC \(\gamma_1\) was demonstrated to be crucial to induce DNA synthesis [89,90], that might responsible of the role of PLC \(\gamma_1\) in the regulation of cell proliferation and survival [91,92]. PLC \(\gamma_2\) is important in the development and maturation of B cells, playing as an effector of pre-B cell receptor (BCR). Loss of PLC \(\gamma_2\) impaired RANKL signaling in hematopoietic cells, leading to defects in osteoclast differentiation [93-95].

After LPS stimulation, we did not detect both PLC \(\gamma_1\) and PLC \(\gamma_2\). Interestingly, previous reports demonstrated that lack of PLC \(\gamma_1\), impairing the activity of regulatory T cells, caused inflammatory/autoimmune symptoms [80]. Moreover, our observations after LPS stimulation accords to literature reports suggesting that PLC \(\gamma_2\) is a critical regulator of cellular and molecular mechanisms during autoimmune inflammation in bone cells, as well as in immune cells [94,95].

Transcript from \textit{PLCD1} was detected in all the experimental conditions, excepting for 3 hours after LPS treatment. The transcripts from \textit{PLCD3} and \textit{PLCD4} were weakly expressed during cultures, and no transcript from \textit{PLCD4} was detected after LPS stimulation. The corresponding enzymes were also weakly detected. PLC \(\delta_1\) and PLC \(\delta_3\) were found in the cytoplasm of 23 days-cultured HOBs. During differentiation both enzymes were detected early in the nucleus and cytoplasm, and later exclusively in the cytoplasm. Late during the 23 days-long culture, PLC \(\delta_4\) was detected both in the nucleus and cytoplasm. After LPS treatment, PLC \(\delta_1\) was not detected, while PLC \(\delta_3\) and PLC \(\delta_4\) were detected after 3 hours and were not detected after 48 hours. Absence of PLC \(\delta_1\) after LPS stimulation might accord to previous observations. Lack of PLC \(\delta_1\) was related to skin inflammation [96]. PLC \(\delta_3\) was described to be down-regulated in endometriosis [45,62], an estrogen-dependent inflammatory disease [97]. PLC \(\delta_4\) is involved in the \textit{in vivo} regulation of transient receptor potential melastatin 8 (TRPM8) ion channel, a major sensor of environmental low temperatures [98]. TRPM8 was described both in human and murine osteoblasts [99]. The overall reduction of the PLC \(\delta\) enzymes might be related to their role in inflammation/autoimmunity.

In the present experiments, transcript from \textit{PLCE} was detected through the 23 days-long culture period, while it was inconstantly detected through the differentiation culture and after LPS stimulation. Fluorescence immuno-cytochemistry allowed detect a surprisingly strong signal for PLC \(\varepsilon\). Throughout the 23 days-maintained culture or differentiation culture periods, PLC \(\varepsilon\) was detected in the cytoplasm and in cell protrusions, occasionally with punctuate or filamentous distribution. During the bone formation, different GPCRs play critically during differentiation and in the activities of osteoblasts [12]. Beside features shared with other PLCs, PLC \(\varepsilon\) is strictly linked to signal transduction pathways involving small GTPases. In fact, PLC \(\varepsilon\) owns a CDC25 domain itself acting as guanine nucleotide exchange factor (GEF) for selected Ras family members. Moreover, the features of PLC \(\varepsilon\) are similar with other Ras effectors that partly underlie the regulation of PLC \(\varepsilon\) by several small GTPases [100]. Previous reports suggested that PLC \(\varepsilon\) might represent a convergence point of regulatory interactions [100]. Thus, PLC \(\varepsilon\) bears unique features enabling it to be activated also through GPCRs. Beside its basic function, PLC \(\varepsilon\) can interact with Ras family GTPases and activate different signaling pathways affecting proliferation, function, and survival [101]. In this perspective, and due to the main role of GPCRs in osteoblasts, the very abundant presence of PLC \(\varepsilon\) within the HOB cells might suggests a preeminent role, which will require further investigations.

The enzymes belonging to the PLC \(\eta\) sub-family were described primarily in the nervous tissue and they are thought to act as calcium sensors during the neural development [102]. In the present experiments, the transcripts of \textit{PLCH1} and \textit{PLCH2} were detected, and splicing variants were observed depending on the experimental conditions, accordingly to literature data. Splicing variants of PLC \(\eta\) enzymes differ in length at the C-terminal end [52], a region rich in serine and proline residues which is thought to facilitate protein–protein interactions [34].

The transcript of \textit{PLCH1} was occasionally represented by two bands in the 23 days-maintained cultures. PLC \(\eta_1\) was early detected in the nucleus, along the 23 days maintained culture the localization differed. It was detected in some nuclei (about 45%), or exclusively in the cytoplasm, occasionally punctuate, and also in podosome-like structure, accordingly to previous literature data reporting PLC \(\eta\) enzymes in podosome-like structures in endothelial cells [48] and in macrophages [49]. In HOBs cultured in differentiating medium, PLC \(\eta_1\) was detected punctuate in the cytoplasm through the whole culture period. Following LPS treatment, PLC \(\eta_1\) was detected in about 45% nuclei and in the cytoplasm after 3 hours and punctuate in the cytoplasm after 48 hours. Recently, mutations of \textit{PLCH1} were associated with holoprosencephaly, a spectrum of developmental disorder of the embryonic forebrain [103]. Moreover, in the human embryo PLC \(\eta_1\) co-localized with Sonig Hedgehog Signaling protein (SHH), involved in a complex network of signaling pathways and the main cause of holoprosencephaly [103].

Transcript of \textit{PLCH2} was comprised of two bands during the 23 days-culture, although the two bands were slightly visible after 23 days. In differentiating cultures, the transcript from \textit{PLCH2} was represented by one band until day 23, when
two bands were visualized. Early in the cultures PLC η2 was detected both in the nucleus and punctuate in the cytoplasm, but the localization slightly differed during the 23 days-long culture. In cultured HOBs, PLC η2 was variably detected in the cytoplasm, occasionally punctuate, in all or in a percentage of the nuclei, and in cell protrusions. In differentiating cultures, PLC η2 was variably observed in the nucleus, in the cytoplasm, occasionally punctuate, with a remarkable strong signal after 17 days. Following LPS treatment, PLC η2 was detected in about 25% nuclei and punctuate in the cytoplasm after 3 hours, and punctuate in the cytoplasm after 6, 24, and 48 hours. The presence of PLC η2 within the nucleus accords to previous reports which demonstrated that it interacts with nuclear and cytoplasmatic LIM domain kinase 1 (LIMK-1) during neurite growth [104]. PLC η enzymes were described to be among the most sensible to low concentration levels of calcium [34]. Enzymes belonging to the PLC η sub-family are activated by very low concentration of calcium, once activated induce increase in calcium levels, subsequently recruiting further PLC enzymes. Studies are required in order to clarify the role of PLC η enzymes in osteoblasts, with special regard to the possible role in the supposed hierarchy control among PLCs.

**Conclusions**

Our data confirm that PLC enzymes play a crucial role in osteoblasts, related to the metabolic activity and calcium signaling. PLC ε was found very abundant in HOBs, regardless of culture conditions, probably related to its role as a convergence point of PI and GTPases signal transduction pathways.

Selected PLC enzymes were observed to move from the nucleus to the cytoplasm. Some PLC enzymes, namely PLC β1, PLC β3, PLC γ2, PLC δ1, PLC δ3, PLC ε, PLC η1 and PLC η2 alternate or move from nucleus to cytoplasm or viceversa depending on different culture conditions, suggesting that nuclear cycles might be activated beside the cytoplasmic membrane cycle.

Many PLC enzymes were detected in the cytoplasm more or less finely punctuate. That might accord with previous descriptions of PLC enzymes in extracellular vesicles [55].

Many items remain to be addressed. Further studies might elucidate why and how selected PLC enzymes shift from nucleus to cytoplasm. Further studies are also required in order to clarify the role of selected PLC enzymes, with special regard to PLC β1 and PLC ε.

Our data overall suggest that PLC signaling might provide further insights into the complex signal transduction network in the study of the bone remodeling, also offering the opportunity to identify new promising molecular targets.

Further studies are required in order to elucidate the role of PLCs in osteoblasts, the meaning of PLC in the nucleus and the mechanisms of activity, as well as the confirmation of those preliminary observations in vivo.

**Conflicts of interest**

All authors disclose any sources of conflict of interest.

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