Phospholipase Cγ2 is required for basal but not oestrogen deficiency–induced bone resorption

Zsuzsanna Kertész*, Dávid Győri†, Szandra Körmenti†, Tünde Fekete‡, Katalin Kis-Tóth‡, Zoltán Jakus*, Georg Schett§, Éva Rajnavölgyi‡, Csaba Dobó-Nagy† and Attila Mócsai*

*Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary, †Independent Section of Radiology, Semmelweis University, Budapest, Hungary, ‡Department of Immunology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary, §Department of Internal Medicine 3, University of Erlangen-Nuremberg, Erlangen, Germany

ABSTRACT

Background Osteoclasts play a critical role in bone resorption under basal conditions, but they also contribute to pathological bone loss during diseases including postmenopausal osteoporosis. Phospholipase Cγ2 (PLCγ2) is an important signalling molecule in diverse haematopoietic lineages. Here, we tested the role of PLCγ2 in basal and ovariectomy-induced bone resorption, as well as in in vitro osteoclast cultures using PLCγ2-deficient (PLCγ2−/−) mice.

Materials and methods The trabecular architecture of long bone metaphyses was tested by micro-CT and histomorphometric analyses. Postmenopausal osteoporosis was modelled by surgical ovariectomy. Osteoclast development and function, gene expression and PLCγ2 phosphorylation were tested on in vitro osteoclast and macrophage cultures.

Results PLCγ2−/− mice had significantly higher trabecular bone mass under basal conditions than wild-type mice. PLCγ2 was required for in vitro development and resorptive function of osteoclasts, but not for upregulation of osteoclast-specific gene expression. PLCγ2 was phosphorylated in a Src-family-dependent manner upon macrophage adhesion but not upon stimulation by M-CSF or RANKL. Surprisingly, ovariectomy-induced bone resorption in PLCγ2−/− mice was similar to, or even more robust than, that in wild-type animals.

Conclusions Our results indicate that PLCγ2 participates in bone resorption under basal conditions, likely because of its role in adhesion receptor signalling during osteoclast development. In contrast, PLCγ2 does not appear to play a major role in ovariectomy-induced bone loss. These results suggest that basal and oestrogen deficiency–induced bone resorption utilizes different signalling pathways and that PLCγ2 may not be a suitable therapeutic target in postmenopausal osteoporosis.

Keywords Gene expression, knockout mice, osteoclasts, osteoporosis, phospholipase Cγ2, signalling.

Eur J Clin Invest 2012; 42 (1): 49–60

Introduction

While osteoclasts are important for normal bone turnover, they also contribute to pathological bone loss during osteoporosis, rheumatoid arthritis or osteolytic bone metastases [1–3]. However, it is incompletely understood how osteoclasts contribute to normal and pathological bone resorption and whether they utilize similar intracellular signalling machineries during the two processes.

Osteoclasts are highly specialized phagocytic cells of haematopoietic origin [4]. They develop by an initial macrophage-like differentiation, followed by reprogramming to the osteoclast lineage and fusion of preosteoclasts to mature multinucleated osteoclasts [1,4]. These processes are directed by the M-CSF and the osteoblast-derived RANK ligand (RANKL) cytokines.

A number of recent studies have indicated similar components of osteoclast biology and immune mechanisms, leading to the emergence of the new field of osteoimmunology [5]. Those similarities include activation by closely related cytokines [5–7], shared use of transcription factors [5,8,9] and the
role of immunoreceptor-like signalling pathways (such as Syk activation by immunoreceptor-associated adapters) in osteoclast development [10–14]. The similarity between bone and immune cells is further supported by the similar components used by neutrophil, macrophage and osteoclast signalling and in vitro inflammatory processes [15–19].

Phospholipase Cγ (PLCγ) proteins link tyrosine kinase-coupled receptors to Ca²⁺ signalling and PKC activation [20]. While the PLCγ1 isoform is ubiquitously expressed and is required for embryonic development [21,22], PLCγ2 is primarily present in haematopoietic lineage cells and its absence triggers defects in haematopoietic lineage cells [23–28]. Most of those phenotypes are also shared with Syk⁻/⁻ mice or mice with genetic defects of immunoreceptor signalling molecules [15,19,29,30].

PLCγ2 is also activated downstream of the immunoreceptor signalling adapters DAP12 and the Fc-receptor γ-chain (FcRγ) in osteoclast precursors while the downstream activation of the NFATc1 transcription factor is mediated by Ca²⁺ signalling through tyrosine phosphorylation pathways [11,31]. The overall similarity between immunoreceptor and PLCγ2-mediated signalling pathways suggests a possible role for PLCγ2 in osteoclast biology.

The above results prompted us to test the role of PLCγ2 in osteoclast development and function, as well as in in vivo bone homeostasis under normal and pathological conditions. Our results indicate that PLCγ2 plays an important role in basal bone resorption, likely due to its role in later phases of osteoclast development. Surprisingly, however, PLCγ2 does not play a major role in ovariectomy-induced bone loss.

Materials and methods

Animals

Heterozygous mice carrying a deleted allele of the PLCγ2-encoding gene (Plcg2tm1jni, referred to as PLCγ2<sup>−/−</sup>) [23] were obtained from James N. Ihle (St. Jude Children’s Research Hospital, Memphis, TN, USA) and has been backcrossed to the C57BL/6 genetic background for more than 10 generations. Because of the limited fertility of homozygous PLCγ2<sup>−/−</sup> mice, the mutation was maintained in heterozygous form as described [26].

For in vivo experiments, PLCγ2<sup>+/−</sup> or PLCγ2<sup>−/−</sup> mice of identical age and sex (mostly litters) from the same colony were used as controls. For in vitro experiments, either PLCγ2-sufficient mice from the PLCγ2 breeding colony or C57BL/6 mice purchased from the Hungarian National Institute of Oncology (Budapest, Hungary) were used as controls. Because of the limited availability of PLCγ2<sup>−/−</sup> animals, some of the in vitro experiments were performed on cells from PLCγ2<sup>−/−</sup> (and appropriate control) bone marrow chimeras generated and tested as described [26]. No difference between the different sources of mice or bone marrow cells has been observed (not shown).

Mice were kept in individually sterile ventilated cages (Techniplast, Buguggiate, Italy) in a conventional facility. All animal experiments were approved by the Semmelweis University Animal Experimentation Review Board.

Ovariectomy

To test oestrogen deficiency–induced bone loss, wild-type and PLCγ2<sup>−/−</sup> females at 8 weeks of age were anesthetized with ketamine and medetomidine and subjected to surgical ovariectomy or sham operation. Six weeks after the operation, the mice were sacrificed and their femurs or tibias were analysed.

Micro-CT and histomorphometry

Bone architecture under basal conditions was tested on age-matched wild-type and PLCγ2<sup>−/−</sup> male mice at 8–10 weeks of age. Ovariectomy-induced bone loss was tested at 14 weeks of age on wild-type and PLCγ2<sup>−/−</sup> females.

Micro-CT studies were performed on the distal metaphysis of the femurs stored in PBS containing 0.1% Na-azide. Samples were scanned on a SkyScan 1172 (SkyScan, Kontich, Belgium) micro-CT apparatus using a 50 kV and 200 μA X-ray source with 0.5-mm aluminium filter, and a rotation step of 0.5° with frame averaging turned on, resulting in an isometric voxel size of 4.5 μm. Three-dimensional images were reconstructed and analysed using the NRecon and CT-Analyser software (both from SkyScan). For quantitative analysis, 400 horizontal sections starting 50 sections above the distal growth plate were selected, and the boundaries of trabecular area were selected manually a few voxels away from the endocortical surface [32]. The density threshold for bone tissue was set manually by an experienced investigator. For graphical presentation, the two-dimensional representation of a horizontal section 250 sections above the distal growth plate, as well as the three-dimensional reconstitution of an axial cylinder of 700 μm diameter, expanding from 150 to 450 sections above the distal growth plate has been prepared.

Histomorphometry studies were performed on the proximal metaphysis of the tibia. After sacrificing the mice, the bones were placed in 70% ethanol, then fixed overnight in 4% formalin and embedded undecalcified in methylmetacrylate (Technovit; Heraeus Kulzer, Wehrheim, Germany). After polymerization, 3- to 4-μm sections were cut with a Jung micrometer (Jung, Heidelberg, Germany) and deplastinated in methoxymethylmetacrylate (Merck, Darmstadt, Germany). Sections were stained with von Kossa and Goldner stains. Bone histomorphometry was performed using a microscope (Nikon, Tokyo, Japan) equipped with a video camera and digital analysis system (OsteoMeasure; OsteoMetrics, Decatur, GA, USA).
Histomorphometry parameters were measured according to international standards [33] as described [34].

**In vitro cultures, resorption assays and flow cytometry**

Suspensions of bone marrow cells were cultured for 48 h in α-MEM (Invitrogen, Carlsbad, CA, USA) in the presence of 10 ng/mL recombinant mouse M-CSF (Peprotech, Rocky Hill, NJ, USA). Nonadherent cells (osteoclast/macrophage precursors) were then plated at 0.5 million per cm² and cultured in the presence of 20 or 50 ng/mL mouse M-CSF or mouse RANKL (Peprotech) with media changes and replacement of the cytokines every 2–3 days. Cellular morphology and tartrate-resistant acid phosphatase (TRAP) expression were determined after 3–5 days of culture in 24-well tissue culture-treated plates using a commercial TRAP staining kit (Sigma, St. Louis, MO, USA). For in vitro resorption assays, osteoclast precursors were plated on BD BioCoat Osteologic slides (BD Biosciences, Bedford, MA, USA), cultured in the presence of M-CSF and RANKL for 10–14 days and processed according to the manufacturer’s instructions. Cultures were observed manually, while the percentage of resorbed area was determined using the **ImageJ** software (NIH, Bethesda, MD, USA). The number of osteoclasts (i.e. TRAP-positive cells with 3 or more nuclei) was counted using a Leica Microsystems (Wetzlar, Germany) DMi6000B inverted microscope. The number of osteoclasts was counted manually, while the percentage of resorbed area was determined using the **ImageJ** software (NIH, Bethesda, MD, USA).

Macrophages were generated by culturing osteoclast/macrophage precursors in the presence of M-CSF but not RANKL. M-CSF was supplied in the form of purified protein (parallel macrophage and osteoclast studies) or as a 10% conditioned medium from CMG14-12 cells [35] (biochemical studies). Expression of the F4/80 macrophage differentiation antigen was tested as described [19].

**Analysis of gene expression**

Osteoclast-specific gene expression was tested using quantitative real-time PCR analysis [36] from wild-type or PLCγ2<sup>−/−</sup> cultures generated in the indicated periods of time using the indicated cytokine concentrations. Total RNA was then isolated from the cells with Trizol reagent (Invitrogen). Reverse transcription was performed at 37 °C for 120 min from 100 ng total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCRs were performed in triplicates with a control reaction containing no reverse transcriptase on an ABI PRISM 7900 (Applied Biosystems) equipment with 40 cycles at 94 °C for 12 s and 60 °C for 60 s using Applied Biosystems Taqman Gene Expression Assay kits. We tested the expression of the mouse Acp5 (TRAP; Taqman Mm00475698_m1), Calcr (Calcitonin receptor; Mm00432271_m1), Cisk (cathepsin K; Mm00484039_m1), Fos (c-Fos; Mm00487425_m1), Nfatc1 (NFATc1; Mm00479445_m1), Oscar (OSCAR; Mm00558665_m1) and Tm7sf4 (DC-STAMP; Mm04209255_m1) genes and normalized it to the expression of the housekeeping gene Gapdh (GAPDH; Mm99999915_g1). The comparative C<sub>T</sub> method was used to quantify transcripts.

**Biochemical and signalling studies**

PLCγ2 expression was determined from Triton X-100-soluble whole osteoclast or macrophage lysates as described [26]. For signalling studies, macrophages were cultured for 5–8 days in bacterial dishes, suspended with 5 mM EDTA and serum starved for 6 h. When indicated, the cells were then incubated with 10 μM PP2 (EMD Biosciences, Darmstadt, Germany) for 8 min. The cells were stimulated with 50 ng/mL M-CSF or 50 ng/mL RANKL in suspension or were plated on 6-cm tissue culture-treated dishes. The reaction was stopped after 30 min at 37 °C, and cell lysates were prepared as described [26]. PLCγ2 was precipitated using the Q-20 PLCγ2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and captured using a 1 : 1 mixture of Protein A Sepharose (Zymed, South San Francisco, CA, USA) and Protein G Agarose (Invitrogen). Whole-cell lysates or PLCγ2 immunoprecipitates were immunoblotted with phosphorylation-specific antibodies (from Cell Signaling Technology, Danvers, MA) against PLCγ2 (pTy7 759; #3874), ERK (p#9101) or the p38 MAP kinase (p#9211); nonphospho-specific antibodies against PLCγ2 (Q-20; Santa Cruz), ERK1/2 (combination of C-16 (ERK1) and C-14 (ERK2) from Santa Cruz), p38 MAP kinase (C-20; Santa Cruz), IκBα (#9242; Cell Signaling) or β-actin (AC-74; Sigma); or antibodies against phosphotyrosine (clone 4G10; Millipore, Billerica, MA, USA). Signal intensity was developed using secondary antibodies and ECL reagents from GE Healthcare (Chalfont St. Giles, UK).

**Statistical analysis**

Experiments were performed at the indicated times with comparable results. Statistical analyses were performed using Student’s unpaired two-population t-test with unequal variance or by two-way ANOVA. Analysis of the interaction between the effects of genotypes and surgical treatments was performed using Tukey’s post hoc test. P values below 0.05 were considered statistically significant.

**Results**

**micro-CT and histomorphometric analysis of wild-type and PLCγ2<sup>−/−</sup> animals**

We first analysed the composition of trabecular bone of wild-type and PLCγ2<sup>−/−</sup> male mice using micro-CT analysis of the distal metaphysis of the femurs. Significantly more trabeculae were visible in PLCγ2<sup>−/−</sup> animals than in the wild-type mice.
both in raw micro-CT slices (Fig. 1a) and in three-dimensional reconstitution images of an axial cylindrical region (Fig. 1b). Quantification of the entire three-dimensional reconstitution image (Fig. 1c) revealed a significant increase in the per cent bone volume (BV/TV) of PLC\(\gamma\)\(_2\)\(^{-/}\) animals (\(P = 0.011; n = 5\)), which was primarily because of increased trabecular number rather than increased thickness of the individual trabeculae (Fig. 1c).

We also performed histomorphometric analysis of the trabecular bone of the proximal tibia of male mice. Those studies confirmed an increased relative bone volume (BV/TV; \(P = 0.0012; n = 4\)) and trabecular number, but not trabecular thickness is PLC\(\gamma\)\(_2\)\(^{-/}\) animals (Fig. 1d). In addition, a significantly lower number of osteoclasts was seen in PLC\(\gamma\)\(_2\)\(^{-/}\) bones while the number of osteoblasts was not affected (Fig. 1e). Taken together, PLC\(\gamma\)\(_2\)\(^{-/}\) animals have increased trabecular bone volume likely due to an osteoclast defect.

**PLC\(\gamma\)\(_2\) is required for in vitro osteoclast development**

To test the role of PLC\(\gamma\)\(_2\) in osteoclasts, we have cultured wild-type and PLC\(\gamma\)\(_2\)\(^{-/}\) bone marrow cells under osteoclastogenic conditions in vitro. As shown in the TRAP-stained images in Fig. 2a and their quantification in Fig. 2b, 20 ng/mL M-CSF and 20 ng/mL RANKL induced significant osteoclast development from wild-type but not from PLC\(\gamma\)\(_2\)\(^{-/}\) bone marrow cells. This defect could not be overcome by increasing the concentration of M-CSF, RANKL or both to 50 ng/mL (Figs 2a,b). However, both wild-type and PLC\(\gamma\)\(_2\)\(^{-/}\) cultures consistently stained positive for TRAP (Fig. 2c), and the percentage of TRAP-positive cells among all mononuclear cells was very similar in the two
genotypes (30-35% at 20 ng/mL M-CSF + 20 ng/mL RANKL and 40-45% at 50 ng/mL M-CSF and 50 ng/mL RANKL, irrespective of the genotype of the cells). Taken together, PLCγ2 is required for the in vitro development of mature multinucleated osteoclasts in the presence of M-CSF and RANKL but likely not for the initial steps of preosteoclast differentiation.

PLCγ2 is not required for macrophage differentiation or expression of osteoclast-specific genes
Our next aim was to address whether PLCγ2 is involved in an earlier or a later phase of osteoclast differentiation. Because we were able to obtain normal numbers of apparently normal macrophages from PLCγ2−/− bone marrow cells (Fig. 2c and data not shown) and those macrophages expressed normal levels of the macrophage differentiation marker F4/80 (Fig. 4a), it is unlikely that PLCγ2 is required for the first steps of general myeloid cell differentiation.

We next tested the time course of osteoclast-specific gene expression in in vitro cultures by quantitative RT-PCR. As concentration. Therefore, PLCγ2 is also required for osteoclast-mediated bone resorption, likely reflecting the previously mentioned osteoclast developmental defect (Fig. 2).

Figure 2  PLCγ2 is required for in vitro osteoclast development. (a) Representative TRAP-stained images of wild-type (WT) and PLCγ2−/− bone marrow cells cultured in the presence of the indicated concentrations of recombinant murine M-CSF and RANKL for 4 days. (b) Number of multinucleated osteoclasts (TRAP-positive cells with 3 or more nuclei) in cultures of WT and PLCγ2−/− bone marrow cells treated with the indicated concentrations of M-CSF and RANKL for 4 days. (c) Enlarged view of TRAP-stained osteoclast and macrophage cultures generated in the presence of 20 ng/mL M-CSF with (osteoclasts) or without (macrophages) 20 ng/mL RANKL for 4 days. Results were obtained from 15 (a,b) or 7 (c) independent experiments per group. Error bars represent SEM. n. d., not detected.
shown in Fig. 4b, the expression of the Acp5 (encoding for TRAP), Calcr (calcitonin receptor), Ctsk (cathepsin K), Fos (c-Fos), Nfatc1 (NFATc1) [11,31] and of DC-STAMP (Tm7sf4), a critical player of the preosteoclast fusion machinery [37,38], were all upregulated normally in PLCγ2−/− cultures (Fig. 4b). These results indicate that PLCγ2 is mostly dispensable for initiation of osteoclast-specific gene expression.

Biochemical characterization of the PLCγ2-mediated osteoclast signalling pathway

Next, we aimed at the biochemical characterization of PLCγ2 activation in osteoclasts. We first tested the presence of PLCγ2 in parallel macrophage and osteoclast cultures and found that PLCγ2 was expressed at comparable levels in wild-type macrophages and osteoclasts but, as expected, not in PLCγ2−/− cells (Fig. 5a).

Osteoclast development is triggered by three major extracellular signals: M-CSF, RANKL and adhesive interactions with the environment (e.g. with tissue culture plastic surface). We next tested which of these three signals trigger PLCγ2 activation, using wild-type macrophages stimulated with M-CSF or RANKL in suspension (which was required to avoid parallel engagement of adhesion receptors), or plated on a tissue culture plastic surface. Both an immunoprecipitation approach followed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 5b) and a direct immunoblotting using phospho-specific PLCγ2 antibodies (Fig. 5c) revealed PLCγ2 phosphorylation upon adhesion of macrophages but not upon M-CSF or RANKL stimulation in suspension. Additional attempts with M-CSF or RANKL stimulation for various periods of time or using various cytokine concentrations ranging from 10 to 100 ng/mL did not reveal a consistent PLCγ2 phosphorylation in suspension either (not shown). On the other hand, M-CSF-induced ERK phosphorylation and RANKL-induced p38 MAP kinase phosphorylation and NFκB activation (degradation of IκBα) could readily be observed under these conditions (Fig. 5c), indicating intact basic M-CSF and RANKL signalling in suspension. Therefore, PLCγ2 appears to be activated by adhesive interactions rather than by stimulation with M-CSF or RANKL cytokines.

We have also tested the role of Src-family kinases in PLCγ2 phosphorylation. As shown in Fig. 5d, pretreatment of macrophages with the Src-family inhibitor PP2 completely abrogated the PLCγ2 phosphorylation response, indicating that the adhesion-induced PLCγ2 activation requires members of the Src kinase family.

PLCγ2−/− mice show normal ovariectomy-induced bone resorption

Because osteoclast-mediated bone resorption contributes to postmenopausal osteoporosis [39], we hypothesized that PLCγ2
may also play a role in oestrogen deficiency–induced bone loss. That possibility was tested by subjecting wild-type and PLCγ2−/− animals to surgical ovariectomy, followed by micro-CT and histomorphometric analysis 6 weeks later. Representative raw micro-CT sections (Fig. 6a), three-dimensional reconstitution images (Fig. 6b) and quantitative micro-CT...

**Figure 4** Normal macrophage development and osteoclast-specific gene expression. (a) Flow cytometric analysis of the expression of the F4/80 macrophage differentiation marker on wild-type (WT) and PLCγ2−/− macrophages generated by culturing bone marrow cells in the presence of recombinant murine M-CSF. Curves of isotype control-stained cells show non-specific labelling. (b) Analysis of the expression of osteoclast-specific genes in WT and PLCγ2−/− osteoclast (OC) and macrophage (Mφ) cultures. WT and PLCγ2−/− bone marrow cells were cultured in the presence of 50 ng/mL with (OC) or without (Mφ) 50 ng/mL RANKL for the indicated period of time, and then the expression of the Acp5 (TRAP), Calcr (Calcitonin receptor), Ctsk (Cathepsin K), Fos (c-Fos), Nfatc1 (NFATc1), Oscar (OSCAR) and Tm7sf4 (DC-STAMP) genes was determined using quantitative RT-PCR. The results shown were obtained from 3 to 6 independent experiments per group. Error bars represent SEM.

**Figure 5** Cellular adhesion triggers PLCγ2 phosphorylation. (a) Expression of PLCγ2 in macrophages (Mφ) and osteoclasts (OC). Wild-type (WT) and PLCγ2−/− bone marrow cells were cultured in the presence of 50 ng/mL M-CSF with (OC) or without (Mφ) 50 ng/mL RANKL for 4 days, followed by preparation of whole-cell lysates (WCL) and immunoblotting for PLCγ2 and β-actin. B-C, Stimulus-induced phosphorylation of PLCγ2. Wild-type macrophages were treated with 50 ng/mL M-CSF, 50 ng/mL RANKL or kept unstimulated in suspension, or they were plated on tissue culture-treated plastic dishes (Adh). After 30 min of incubation, cell was lysed and processed for immunoprecipitation (IP) of PLCγ2 followed by immunoblotting using anti-phosphotyrosine (PY) antibodies, (b) or WCL were immunoblotted using phosphorylation-specific antibodies against ERK, the p38 MAP kinase (p38) and PLCγ2 or nonphospho-specific antibodies against IκBα (c). Immunoblotting for ERK, p38 and PLCγ2 served as loading control. (d) Role of Src-family kinases in PLCγ2 phosphorylation. Wild-type macrophages were pretreated in the presence or absence of 10 μM PP2 and then stimulated and their PLCγ2 phosphorylation tested as in panel b. Results shown represent 3–5 independent experiments with similar results.
analyses (Fig. 6c) indicated that, similar to intact male animals (Fig. 1), sham-operated PLC\(_2\) \(\sim\) females also had increased trabecular bone density, which was reflected in a nearly two-fold increase in relative bone volume (BV/TV; \(P = 0.00031; n = 7\) (wild type) vs. 4 (PLC\(_2\) \(\sim\))). As expected, surgical ovariectomy led to a significant reduction in the per cent bone volume (BV/TV) of wild-type mice (\(P = 0.025; n = 7\)). Contrary to our expectations, however, the per cent bone volume of PLC\(_2\) \(\sim\) animals was also significantly reduced (\(P = 0.00023; n = 4\)) and that reduction was even higher in PLC\(_2\) \(\sim\) mice than in wild-type animals both in terms of absolute reduction in BV/TV values (4.1 vs. 1.6 percentage points, respectively) and in percentage of the BV/TV values of the sham-operated control animals (50% vs. 36%, respectively). The difference of the effect of ovariectomy on wild-type and PLC\(_2\) \(\sim\) animals (interaction of the genotypes and surgical procedures) proved to be statistically significant (\(P = 0.0090\)). Importantly, while the BV/TV values of sham-operated wild-type and PLC\(_2\) \(\sim\) animals were statistically highly significant (\(P = 0.00025\)), there was no significant difference between the two genotypes after the ovariectomy procedure (\(P = 0.25; n = 7\)).

The above-mentioned findings were also confirmed by histomorphometric analysis of ovariectomy-induced bone loss in the proximal tibia. As shown in Fig. 6d, that analysis confirmed the increased per cent bone volume (BV/TV) in sham-operated PLC\(_2\) \(\sim\) animals (\(P = 0.0010\)) and a reduction in per cent bone volume in ovariectomized wild-type mice (\(P = 0.038; n = 3\)). Importantly, the ovariectomy procedure induced a significantly more pronounced reduction in per cent bone volume in PLC\(_2\) \(\sim\) mice than in wild-type animals, both
in terms of absolute reduction in BV/TV values (9.9 vs. 4.3 percentage points, respectively) and in per cent of the BV/TV values of the sham-operated control animals (38% vs. 24%, respectively). The difference of the effect of ovariectomy on wild-type and PLCγ2−/− animals (interaction of the genotypes and surgical procedures) proved again to be statistically significant (P = 0.013). Similar to the micro-CT data (Fig. 6a), while the BV/TV values of sham-operated wild-type and PLCγ2−/− animals were statistically highly significant (P = 0.0010), that difference faded away after the ovariectomy procedure (P = 0.24; n = 3). A similar picture was seen during the analysis of trabecular numbers, whereas the trabecular thickness remained unaffected by the different genotypes and surgical procedures (Fig. 6d).

Additional studies testing the number of osteoclasts and osteoblasts attached to the bone surface indicated that although the number of osteoclasts was significantly lower in the sham-operated PLCγ2−/− mice than in the wild-type ones (3.0 ± 0.1 vs. 1.4 ± 0.5 per mm, respectively; P = 0.0006; n = 3), the number of osteoclasts was strongly increased and reached a comparable, though, still significantly different level (6.3 ± 0.4 vs. 5.0 ± 0.4 per mm, respectively; P = 0.010; n = 3) in the two genotypes after the ovariectomy procedure (Fig. 6e). The difference of the effect of ovariectomy on wild-type and PLCγ2−/− animals (interaction of the genotypes and surgical procedures) did not prove to be statistically significant (P = 0.56; n = 3), indicating that PLCγ2−/− animals were able to upregulate osteoclast numbers upon oestrogen deficiency normally. Analysis of the number of osteoblasts did not find any significant difference between any of the groups tested (Fig. 6e).

Taken together, these results suggest that ovariectomized PLCγ2−/− animals are capable of reducing their bone mass to levels comparable to those seen in similarly treated wild-type animals, likely because of similar oestrogen deficiency–induced increase in osteoclast numbers in the two genotypes.

Discussion

In the first part of this study, we showed that PLCγ2−/− mice have increased basal bone density, likely due to reduced in vivo osteoclast number reflecting the role of PLCγ2 in a later phase of osteoclast development. These results raised the possibility that PLCγ2 may also participate in pathological bone resorption, such as oestrogen deficiency–induced osteoporosis. Much to our surprise, however, PLCγ2−/− mice showed similar, or even more pronounced, ovariectomy-induced bone resorption than their wild-type counterparts. Therefore, PLCγ2 does not appear to be required for oestrogen deficiency–induced bone loss.

The experiments presented in this paper (part of which were published in an abstract form before [40]) were initiated based on our prior experiments showing defective osteoclast develop-
phosphorylation, Mao et al. [42] and Chen et al. [43] suggested that PLC\(\gamma\)2 is activated downstream of RANK. That conclusion, however, is confounded by the ligation of both RANK and adhesion receptors in that assay. In contrast, we did not observe any PLC\(\gamma\)2 phosphorylation upon stimulating wild-type macrophages with RANKL (or M-CSF) in suspension, whereas cellular adhesion consistently triggered robust phosphorylation of the protein in the absence of any cytokines (Fig. 5b,c). These results suggest that PLC\(\gamma\)2 is primarily involved in adhesion receptor rather than in RANK signal transduction, a possibility consistent with the role of PLC\(\gamma\)2 in integrin signalling of neutrophils [24,26] and its proposed modulatory effect on integrin signalling in preosteoclasts [45].

The analysis of ovariectomy-induced bone resorption is likely the most clinically relevant aspect of our study. While the increased basal bone volume (Fig. 1) and the defective in vitro osteoclast development and function (Figs 2 and 3) in PLC\(\gamma\)2\(^{-/-}\) mice suggested a role for PLC\(\gamma\)2 in pathological bone resorption, ovariectomy-induced bone loss in PLC\(\gamma\)2\(^{-/-}\) mice was unexpectedly normal or even more pronounced than in wild-type animals (Fig. 6). Because this was observed both in the distal femur and in the proximal tibia and by two independent approaches (micro-CT and histomorphometry), we believe that PLC\(\gamma\)2 is not a major general component of oestrogen deficiency–induced bone resorption. However, we cannot exclude the possibility that ovariectomy-induced bone resorption at certain sites or under some specific conditions would be defective in PLC\(\gamma\)2\(^{-/-}\) animals.

It is at present unclear to us how exactly PLC\(\gamma\)2\(^{-/-}\) animals are able to reduce their bone mass during oestrogen deficiency. However, the fact that the ovariectomy-induced increase in the number of osteoclasts was similar in the two genotypes despite significantly reduced basal number of osteoclasts in PLC\(\gamma\)2\(^{-/-}\) mice (Fig. 6e) suggests the existence of PLC\(\gamma\)2-independent mechanisms triggering oestrogen deficiency–induced osteoclast development. Whether those are mediated by cell–cell interactions (e.g. with osteoblasts) not present in the in vitro cultures [10,11], by excessive release of cytokines overcoming osteoclast developmental defects [46] or by the amplification of PLC\(\gamma\)2-independent signal transduction pathways, should be the subject of future research.

It has been generally believed that osteoclasts use similar signal transduction pathways during basal and induced (e.g. oestrogen deficiency–induced) bone resorption. Therefore, it has been assumed that the identification of novel osteoclast signalling molecules may provide suitable targets for the therapeutic intervention in pathological bone loss, such as postmenopausal osteoporosis. Our results showing normal ovariectomy-induced bone loss in PLC\(\gamma\)2\(^{-/-}\) animals indicate that this may not be the case. Interestingly, a prior study showed that ovariectomy-induced bone loss in the highly osteopetrotic DAP12\(^{-/-}\)/FeRy\(^{-/-}\) animals is comparable to, or even higher than, that in wild-type mice [47]. All these results suggest differential osteoclast signalling requirements for basal and oestrogen deficiency–induced bone resorption and indicate that care should be taken when extrapolating findings on basal bone resorption to pathological conditions. These results also indicate that limited clinical benefit can be expected from therapeutic targeting of PLC\(\gamma\)2 in postmenopausal osteoporosis.

Acknowledgements
We thank James Ihle for the PLC\(\gamma\)2\(^{-/-}\) animals; Zsuzsa Krasznai for help with initial experiments; Edina Simon and Krisztina Makara for technical assistance; Norbert Gyöngyösi for help with statistical analyses. This work was supported by the Wellcome Trust (International Senior Research Fellowship No. 087782 to A. M.), the European Research Council (Starting Independent Investigator Award No. 206283 (MOLINFLAM) to A. M.), the Hungarian Office for Research and Technology (Anyos Jedlik Award No. NKFP-A1-0069/2006 to A. M.), the Deutsche Forschungsgemeinschaft (SPP1468-IMMUNOBONE to G. S.), the European Union (MASTERSWITCH project to G. S.), the Norway Grants Scheme (0046/NA/2006-2/OP-9 to E. R.) and the University of Debrecen (OEC Mec-10/2008 Award to K. K.-T.).

Conflict of interest
The authors declare that no financial conflict of interest exists.

Address
Department of Physiology, Semmelweis University School of Medicine, 1094 Budapest, Hungary (Z. Kertész, D. Győri, Z. Jakus, A. Mócsai); Independent Section of Radiology, Semmelweis University, 1089 Budapest, Hungary (S. Körmendi, C. Dobó-Nagy); Department of Immunology, Medical and Health Science Center, University of Debrecen, 4032 Debrecen, Hungary (T. Fekete, K. Kis-Tóth, E. Rajnavölgyi); Department of Internal Medicine 3, University of Erlangen-Nuremberg, 91054 Erlangen, Germany (G. Schett).

Correspondence to: Attila Mócsai, Department of Physiology, Semmelweis University School of Medicine, PO Box 259, 1444 Budapest, Hungary. Tel.: +36-1-439-1500 Ext. 60-409; fax: +36-1-266-7480; e-mail: mocsai@eok.sote.hu

Received 10 September 2010; accepted 16 May 2011

References
1 Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289: 1504–8.
2 Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. J Biol Chem 2010;285:25163–8.
3 Schett G, Teitelbaum SL. Osteoclasts and arthritis. J Bone Miner Res 2009;24:1142–6.

4 Teitelbaum SL. Osteoclasts: what do they do and how do they do it? Am J Pathol 2007;170:427–35.

5 Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 2007;7:292–304.

6 McNees IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7:429–42.

7 Cheng X, Kinosaki M, Murali R, Greene MI. The TNF receptor superfamily: role in immune inflammation and bone formation. Immunol Rev 2003;201:287–94.

8 Takayanagi H. The role of NFAT in osteoclast formation. Ann N Y Acad Sci 2007;1116:227–37.

9 Boya JS, Alle N. NF-kB functions in osteoclasts. Biochem Biophys Res Commun 2009;378:1–5.

10 Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC et al. The immunomodulatory adapter proteins DAP12 and Fc receptor γ-chain (FcγR) regulate development of functional osteoclasts through the Syk tyrosine kinase. Proc Natl Acad Sci USA 2004;101:6158–63.

11 Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E et al. Osteopetrosis and thalamic hypomyelination with synaptic degeneration in DAP12-deficient mice. Proc Natl Acad Sci USA 2004;99:1899–910.

12 Shirane M, Sawa H, Kobayashi Y, Nakano T, Kitajima K, Shinkai Y et al. The Syk protein tyrosine kinase is a crucial player in diverse biological functions. Nat Rev Immunol 2010;10:387–402.

13 Jakus Z, Simon E, Frommhold D, Sperandio M, Mocsai A. Critical role of phospholipase C2 in integrin and Fc receptor-mediated neutrophil functions and the effector phase of autoimmune arthritis. J Exp Med 2009;206:577–93.

14 Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin αMβ2 signaling in platelets. J Thromb Haemost 2005;3:1752–62.

15 Watson SP, Herbert JM, Pollitt AY. GPVI and CLEC-2 in hemostasis and vascular integrity. J Thromb Haemost 2010;8:1456–67.

16 Mocsai A, Ruland J, Tyubulewicz VL. The SYK tyrosine kinase: a role of Fc receptor signaling in platelet aggregation. Nature 2004;428:758–63.

17 Kaufl T, Nakahara J, Inui M, Mishima K, Momiyama T, Kaji M et al. Osteopetrosis and thalamic hypomyelination with synaptic degeneration in DAP12-deficient mice. J Clin Invest 2003;111:323–32.

18 Humphrey MB, Ogasawara K, Yao W, Spusta SC, Davs MR, Lane NE et al. The signaling adapter protein DAP12 regulates multination-ation during osteoclast development. J Bone Miner Res 2004;19:224–34.

19 Faccio R, Zou W, Colaianni G, Teitelbaum SL, Ross FP. High dose M-CSF partially rescues the Dap12−/− osteoclast phenotype. J Cell Biochem 2003;90:871–83.

20 Mocsai A, Abram CL, Jakus Z, Hu Y, Lanier LL, Lowell CA. Integrin signaling in neutrophils and macrophages uses adapters containing immunoreceptor tyrosine-based activation motifs. Nat Immunol 2006;7:1326–33.

21 Kiefer F, Brumell J, Al-Alawi N, Latour S, Cheng A, Veillette A et al. The Syk protein tyrosine kinase is essential for Fcγ receptor signaling in macrophages and neutrophils. Mol Cell Biol 1998;18:4209–20.

22 Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA et al. Arthritis critically dependent on innate immune system players. Immunity 2002;16:157–68.

23 Jakus Z, Nemeth T, Verbeek JS, Mocsai A. Critical but overlapping role of FcγRIII and FcγRI in activation of murine neutrophils by immobilized immune complexes. J Immunol 2008;180:618–29.

24 Jakus Z, Simon E, Balázs B, Mocsai A. Genetic deficiency of Syk protects mice from autoantibody-induced arthritis. Arthritis Rheum 2010;62:1899–910.

25 Patterson RL, van Rossum DB, Nikolaidis N, GILL DL, Snyder SH. Phospholipase C-γ; diverse roles in receptor-mediated calcium signaling. Trends Biochem Sci 2005;30:688–97.

26 Ji QS, Winnier GE, Niswender KD, Horstman D, Wisdom R, Magnuson MA et al. Essential role of the tyrosine kinase substrate phospholipase C-γ1 in mammalian growth and development. Proc Natl Acad Sci USA 1997;94:2999–3003.

27 Shirane M, Sawa H, Kobayashi Y, Nakano T, Kitajima K, Shinkai Y et al. Deficiency of phospholipase C-γ1 impairs renal development and hematopoiesis. Development 2001;128:5173–80.

28 Wang D, Feng J, Wen R, Marine JC, Sangster MY, Parganas E et al. Phospholipase C2 is essential in the functions of B cell and several Fc receptors. Immunity 2000;13:25–35.

29 Graham DB, Robertson CM, Bautista J, Mascarenhas F, Diacovo MJ, Montgrain V et al. Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice. J Clin Invest 2007;117:3445–52.

30 Cremasco V, Graham DB, Novack DV, Swat W, Faccio R. Vav- Phospholipase C2-mediated control of a neutrophil-dependent murine model of rheumatoid arthritis. Arthritis Rheum 2008;58:2712–22.
41 Turner M, Mee PJ, Costello PS, Williams O, Price AA, Duddy LP
et al. Perinatal lethality and blocked B-cell development in
mice lacking the tyrosine kinase Syk. Nature 1995;378:298–
302.

42 Mao D, Epple H, Uthgenannt B, Novack DV, Faccio R. PLCγ2
regulates osteoclastogenesis via its interaction with ITAM proteins
and GAB2. J Clin Invest 2006;116:2869–79.

43 Chen Y, Wang X, Di L, Fu G, Bai L, Liu J et al. Phospholipase Cγ2
mediates RANKL-stimulated lymph node organogenesis and
osteoclastogenesis. J Biol Chem 2008;283:29593–601.

44 Miyamoto T, Arai F, Ohneda O, Takagi K, Anderson DM, Suda T.
An adherent condition is required for formation of multinuclear
osteoclasts in the presence of macrophage colony-stimulating factor
and receptor activator of nuclear factor κB ligand. Blood
2000;96:4335–43.

45 Epple H, Cremasco V, Zhang K, Mao D, Longmore GD, Faccio R.
Phospholipase Cγ2 modulates integrin signaling in the osteoclast by
affecting the localization and activation of Src kinase. Mol Cell Biol
2008;28:3610–22.

46 Faccio R, Takeshita S, Zallone A, Ross FP, Teitelbaum SL. c-Fms and
the αvβ3 integrin collaborate during osteoclast differentiation. J Clin
Invest 2003;111:749–58.

47 Wu Y, Torchia J, Yao W, Lane NE, Lanier LL, Nakamura MC et al.
Bone microenvironment specific roles of ITAM adapter signaling
during bone remodeling induced by acute estrogen-deficiency.
PLoS ONE 2007;2:e586.