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# Mechanosensitivity of human osteosarcoma cells and phospholipase C β2 expression

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**Abstract**

Bone adapts to mechanical load by osteosynthesis, suggesting that osteoblasts might respond to mechanical stimuli. We therefore investigated cell proliferation and phospholipase C (PLC) expression in osteoblasts. One Hertz uniaxial stretching at 4000 μstrains significantly increased the proliferation rates of human osteoblast-like osteosarcoma cell line MG-63 and primary human osteoblasts. However, U-2/OS, SaOS-2, OST, and MNNG/HOS cells showed no significant changes in proliferation rate. We investigated the expression pattern of different isoforms of PLC in these cell lines. We were able to detect PLC β1, β3, γ1, γ2, and δ1 in all cells, but PLC β2 was only detectable in the mechanosensitive cells. We therefore investigated the possible role of PLC β2 in mechanotransduction. Inducible antisense expression for 24 h inhibited the translation of PLC β1 in U-2/OS cells by 35% and PLC β2 in MG-63 by 29%. Fluid shear flow experiments with MG-63 lacking PLC β2 revealed a significantly higher level of cells losing attachment to coverslips and a significantly lower number of cells increasing intracellular free calcium.


*Keywords:* Phospholipase C β2; Mechanical loading; Bone; Fluid flow; Osteosarcoma; Ecdysone; Antisense

The regulation of reception of mechanical loading in osteoblasts leads to a downstream activation of phospholipase C (PLC) by a less known biophysical/biochemical transducer(s) within 120 s [1]. The activation of PLC might be due to the release of an autocrine cytokine such as IL-4 [2].

Once activated the dynamics of the intracellular free calcium release from intracellular stores are the same as seen upon hormonal PLC stimulation, using, for instance, parathyroid hormone [3]. This is consistent with pharmacological evidence that modulators of the PLC associated GTPase can alter mechanotransduction [4] and indicates that the isoenzyme of PLC in question is of the β-type. Osteoblasts and pre-osteoblasts seem to be the major cellular candidates that regulate and/or undertake bone remodeling after mechanical loading. Downstream regulation of physiological amplitudes and modes (uniaxial) of strain and fluid shear flow seems to be mainly transduced in osteoblasts and other cells by the PLC-PKC pathway [5]. The biophysical nature and site of the mechano-transducer itself is less known. The phosphoinositide-specific phospholipase C (PLC) plays a crucial role in initiating mechano-mediated and receptor-mediated signal transduction by generating two second messenger molecules, inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG), from phosphatidylinositol-4,5-bisphosphate ($PIP_2$). Diacylglycerol is responsible for activating a large family of protein kinase C isoenzymes, which catalyze protein phosphorylation. On the other hand, $IP_3$ binds to a receptor and releases $Ca^{2+}$ from the endoplasmatic reticulum [6,7]. Many other downstream events including prostaglandin

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synthesis, nitric oxide synthesis, and their downstream molecular pathways, such as protein kinase A, protein kinase G, and extracellular receptor kinases (ERKs) can be linked to either PKC activation, PLC activation or both [8–13]. Peake et al. [14,15] showed c-fos and AP-1 upregulation in MG-63 cells and in primary human osteoblasts after mechanical stimulation.

So far, 11 different mammalian PLC isoforms have been described. This family is divided into four subtypes β, γ, δ, and ε, each of which contains different isoenzymes: PLC β1–β4, γ1 and γ2, δ1–δ4, and ε have been identified [10,16]. The isoforms can be divided on the basis of amino acid sequence and their way of activation. G-proteins activate PLC β whereas tyrosine phosphorylation activates the PLC γ-subtype. The regulatory mechanisms of PLC δ are still unknown [9]. For the activation of PLC ε the G-protein $G_{\alpha 12}$ is necessary [10]. The activities of all isoforms of PLC are modified by increases in intracellular free $Ca^{2+}$ ion concentration in vivo. The aim of this study was to identify the isoform of PLC involved in the mechanotransduction in osteosarcoma cells and primary osteoblasts. First, we investigated the mechanosensitivity of the osteosarcoma cell lines MG-63 [17], MMNG/HOS [18], OST [19], U-2/OS [20], and SaOS-2 [21], and primary human osteoblasts for mechanical loading using a 4-point-bending machine [1] and the expression pattern of different isoforms of PLC in these cells. To investigate the reaction of human osteosarcoma cells MG-63 (mechanosensitive) and U-2/OS (not mechanosensitive) to mechanical stimulation, we created a new inducible antisense-expressing system for mechanical loading by expressing specific antisense sequences against PLC β1 and β2. There are only very few studies about the mechanosensitivity of osteosarcoma cells by mechanical loading [4,14,15,22] and none relating the expression of different isoforms of phospholipase C in the cells in this regard in the literature.

In order to examine the feasibility of using the ecdysone system for inducible knockdown of the PLC subtypes β1 and β2, we isolated cDNA fragments of the above-mentioned isoforms and cloned them in a reverse direction into the pIND(SP1)-vector and have attempted to establish steroid hormone inducible isoform-specific PLC suppression using the MG-63 and U-2/OS osteosarcoma cell lines. These cell lines were transfected with the regulatory vector pVgRXR that encodes both the RXR and EcR receptors. Following this first procedure, the cells were further modified by incorporating the pIND(SP1) vector containing the antisense gene of interest, which is under the control of a minimal heat shock promoter, the SP1 promotor, which is activated by the endogenous SP1-transcription factor which is expressed in nearly all mammalian cells (Ecdysone manual of Invitrogen, Netherlands) and five upstream ecdysone-response elements. We report about the proliferation rate of human osteoblasts and sarcoma cell lines after mechanical loading and the participation of PLC β2 in this pathway.

## Materials and methods

*Cell culture*. The human osteoblast-like osteosarcoma cell lines MG-63, HOS/MNNG, OST, U-2/OS, and SaOS-2 were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. All the cells were cultured in RPMI 1640 medium (Life Technologies), supplemented with 2 mM glutamine (Life Technologies) and 10% fetal calf serum (FCS; Seromed, Germany). To select transfected cells, the antibiotics G-418 (Life Technologies) and Zeocin (Invitrogen) were employed. Primary human osteoblasts (PHO) and bovine osteoblasts (PBO) were cultured and characterized as previously described by Jones et al. [3].

*MTT-cell quantification assay*. Human osteoblast-like osteosarcoma cells MG-63, MNNG/HOS, OST, U-2/OS, and SaOS-2, and primary human osteoblasts were seeded at a density of 60,000 cells per $cm^2$ onto sterile polycarbonate plates, each of which has nine wells of 5 mm thick pure silicone rubber (Dow Corning, Germany) with an area of 0.5 $cm^2$ on the surface. Cells were grown for 24 h, reaching a confluence of around 60%. On day two, three, and four, one set of plates was bent in a 4-point-bending machine [3] for 30 cycles at 1 Hz and 4000 μstrain (με). Control groups did not receive mechanical stimulation, but were otherwise subjected to the same conditions. After another 24 h of incubation the cell activity was measured by the colorimetric microassay by Mosmann [23]. Ten microliters of tetrazolium salt (MTT: 3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, 5 mg/ml; Sigma, Germany) was added to each well and then incubated for 4 h. The plates were read with a multiwell scanning Titertek Elisa reader (wavelength 540 nm, reference length 630 nm). The relative absorbance was shown as a percent of the corresponding control. The relative absorbance was calibrated against control cell cultures plated at different densities, which were then counted in a Coulter ZM cell counter equipped with Channelyser to control for dead cells. The accuracy of the MTT method was shown to be ±200 cells as previously described [23].

*Cloning of the antisense vector against phospholipase C β1 and β2*. To obtain an antisense-expression vector against phospholipase C β2 a fragment of the human PLC β2 sequence published by Park et al. [24] was used. A unique DNA fragment at position 160–309 was amplified by PCR as this contains the translation start and has only <10% sequence homology with other members of the PLC β family. For amplification, a set of synthetic oligonucleotide primers were generated: forward primer 5′-CGGAATTCCGACCATGTCTCTGCTCA ACC-3′, reverse primer 5′-CGGGGTACCCAGTATAAGTAGTA GCCCTTA-3′. The primer pair included two restriction sites, *Eco*R1 at the 5′-end of forward primer, *Kpn*I at the 5′-end of reverse primer. The double-stranded cDNA was synthesized directly from the human phospholipase C β2 sequence. PCR conditions were: 94 °C/5 min; 30 cycles of (94 °C/30 s; 55 °C/60 s; 72 °C/60 s). The amplified cDNA was purified and cloned into the ecdysone-expression vector pIND(SP1). Both the PLC fragment and the pIND(SP1) vector were cut with *Kpn*I and *Eco*R1, and the PLC fragment was ligated into the vector. Under the same conditions and methods, the antisense-expression vector against PLC β1 was cloned using the fragment 365–514 of PLC β1 cDNA [25] with the following PCR primers: forward 5′-CGGAATTC CGCACCATGTCTCTGCTCAACC-3′; reverse 5′-CGGGGTACCC AGTATAAGTAGTAGCCCTTA-3′.

*Sequence analysis*. For sequence analysis the T7 DNA-Sequenase-System from USB, Germany, was used. Ten different clones were analyzed with the ecdysone forward primer (Invitrogen):