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The MEK5/ERK5 mitogen-activated protein kinase cascade is an effector pathway of bone-sustaining bisphosphonates that regulates osteogenic differentiation and mineralization

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ABSTRACT

Bisphosphonates play an important role in the treatment of metabolic bone diseases such as osteoporosis. In addition to their anti-resorptive activity by triggering osteoclast apoptosis, nitrogen-containing bisphosphonates (N-BP) may also influence osteogenic differentiation, which might rely on their capacity to inhibit the mevalonate pathway. In vascular endothelial cells inhibition of this pathway by cholesterol-lowering statins activates the MEK5/ERK5 mitogen-activated protein kinase cascade, which plays an important role in cellular differentiation, apoptosis or inflammatory processes.

Here we evaluated whether N-BP may also target the MEK5/ERK5 pathway and analysed the consequences of ERK5 activation on osteogenic differentiation.

We show that N-BP dose-dependently activate ERK5 in primary human endothelial cells and osteoblasts. The mechanism likely involves farnesyl pyrophosphate synthase inhibition and subsequent functional inhibition of the small GTPase Cdc42 since siRNA-mediated knockdown of both genes could reproduce N-BP-induced ERK5 activation. ERK5 activation resulted in regulation of several bone-relevant genes and was required for calcification and osteogenic differentiation of bone marrow-derived mesenchymal stems cells as evident by the lack of alkaline phosphatase induction and alizarin-red S staining observed upon ERK5 knockdown or upon differentiation initiation in presence of a pharmacological ERK5 inhibitor.

Our data provide evidence that N-BP activate the MEK5/ERK5 cascade and reveal an essential role of ERK5 in osteogenic differentiation and mineralization of skeletal precursors.

1. Introduction

Bone homeostasis is regulated by the interaction of mainly three cell types of mesenchymal origin [1]. Osteoclasts are a specific multinucleated form of the monocyte/macrophage lineage and are responsible for bone resorption. Osteoblasts (OB) are derived from fibroblast-like skeletal precursors and secrete components of the extracellular bone matrix, which is mineralized as a blueprint for the skeleton. Terminally differentiated OBs may become part of the lining cell system that covers sinusoidal surfaces or may be embedded in the matrix to become osteocytes. These cells are specially differentiated OBs with long dendritic processes that form the osteocyte lacunocanalicular network and influence both bone formation and bone resorption [2]. Imbalances of this perfectly coordinated system may result in a variety of diseases. One example is osteoporosis, which is characterised by decreased bone density and increased fragility [3]. Osteoporosis belongs to the most common diseases worldwide and leads to a considerable reduction in quality of life and increased mortality [4].

One option to treat osteoporosis is a therapy based on bisphosphonates (BP) [5]. BPs bind to bone mineral and accumulate in the acidic environment of the resorption space between bone surface and osteoclasts [6,7]. They are taken up into osteoclasts by endocytosis and inhibit their function by different mechanisms. Nitrogen-free bisphosphonates (Non-N-BP) such as clodronate or etidronate are metabolized into cytotoxic ATP-adducts, which cause apoptosis of

Abbreviations: ANOVA, analysis of variance; ANT, adenine-nucleotide-translocase; ALPL, alkaline phosphatase; Apppl, ATP analogue triphosphoric acid-1-adenosine-5′yl ester 3-(3-methylbut-3-enyl) ester; BP, bisphosphonate; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; FDPS, farnesyl pyrophosphate synthase; HMG-Co A, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG-CoA inhibitor, statin; HUVEC, Human umbilical vein endothelial cell; IPP, isopentenyl pyrophosphate; KLF, Krüppel-like factor; N-BP, nitrogen-containing bisphosphonate; Non-N-BP, Nitrogen-free bisphosphonate; MAPK, Mitogen-activated protein kinase; MSC, mesenchymal stem cell; OB, osteoblast; OPN, osteopontin; PTHLH, parathyroid hormone like hormone; Scr, scrambled; shRNA, small hairpin RNA.

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osteoclasts by inhibiting mitochondrial adenine-nucleotide-translocase (ANT) [8,9]. New-generation nitrogen-containing bisphosphonates (N-BP) such as pamidronate, ibandronate or zoledronate cause accumulation of intracellular isopentenyl pyrophosphate (IPP) thereby generating the cytotoxic ATP analogue triphosphoric acid-1-adenosine-5′yl ester 3-(3-methylbut-3-enyl) ester (ApppI) that also interacts with ANT and similarly causes induction of osteoclast apoptosis [10]. In addition, N-BPs interfere with osteoclast function by specifically inhibiting farnesyl pyrophosphate synthase (FDPS), an important enzyme of the mevalonate pathway [11]. By inhibition of FDPS, prenylation of small G-proteins such as Cdc42, Rho, Rac, Ras or Rab is inhibited, leading to a deficient signal transduction that affects the organization of the cytoskeleton and triggers apoptosis [12].

In addition to triggering osteoclast apoptosis, BPs may also influence bone metabolism *via* effects on skeletal precursors, OBs and osteocytes, although according to present knowledge, much higher concentrations of BPs are needed to achieve sufficient intracellular concentrations in mesenchymal, endothelial and epithelial cells, which is achieved *via* endocytosis [13–16]. In very high concentrations N-BPs may also exert apoptosis in these cell types, which is the basis of a long lasting discussion about anti-tumour effects of BPs [17–20]. A possible candidate for mediating the observed effects of BPs in these cells is the mechano-responsive MEK5/ERK5 mitogen-activated protein kinase (MAPK) cascade. It importantly controls differentiation, apoptosis and inflammatory processes in other cells types as well as cardiovascular development and endothelial function [21]. We and others previously demonstrated that inhibition of the mevalonate pathway further upstream at the level of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-Co-A reductase) using HMG-Co-A inhibitors (statins) can potently activate this signalling cascade in primary human endothelial cells (EC) leading to altered gene expression through transcriptional induction of the two Krüppel-like transcription factors KLF2 or KLF4 [22,23]. Experiments, which were independently conducted by that time, also described KLF2 as a direct target of the N-BP zoledronate in tumour cells [19]. While the mode of KLF2 induction by BPs is unknown, a possible mechanism could be decreased GTPase prenylation resulting from interference with the mevalonate metabolism. A likely interaction of GTPase signalling and ERK5 activity is also implicated by transcriptome analysis of genes modulated by constitutive ERK5 activation in ECs, which revealed a statistical overrepresentation of functional gene clusters associated with GTPase function [23]. Remarkably, we also noticed regulation of several bone-relevant genes [23] suggesting a potential influence of ERK5 on bone homeostasis.

In this study, we identify N-BPs as dose-dependent activators of ERK5 and ERK5-dependent gene expression in primary human endothelial cells and osteogenic precursors and provide evidence for a role of FDPS and Cdc42 inhibition therein. We reveal parathyroid hormone like hormone (PTHLH) as novel ERK5-regulated gene induced by zoledronate that may account for the proposed stimulatory effect of N-BPs on OB differentiation and demonstrate an essential role of ERK5 in the regulation of bone mineralization during OB differentiation.

2. Materials and methods

2.1. Cell culture and reagents

Human primary umbilical vein endothelial cells (HUVEC) were purchased from PromoCell and cultured in a 1:2 mixture of endothelial growth medium (EGM, Lonza) and M199 medium (PAA Laboratories) as described [23]. Cells were used until passage 3–4.

Primary human mesenchymal stem cells (MSC) were isolated from bone marrow of different donors and cultivated up to four weeks by a standardized protocol [24]. Bone marrow was isolated after informed consent from the explanted femoral heads of patients undergoing elective hip arthroplasty. The procedure was approved by the local Ethics Committee of the University of Würzburg. Briefly, bone marrow preparations were washed with stem-cell medium (Dulbecco's modified Eagle's medium, (DMEM/F12) supplemented with penicillin/streptomycin (both Thermo Fisher Scientific) and 10% fetal bovine serum (Bio&Sell GmbH) [25], 50 μg/ml ascorbate (Sigma-Aldrich)), and centrifuged at 1200 rpm for 5 min. The pellet was reconstituted in medium and washed four times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and seeded at a density of 3×10^8 cells per 150 cm^2 culture flask. Adherent cells were washed after 2 days and cultured to confluency prior to seeding for the respective experiments.

Amphotropic retrovirus producer cells (φNX ampho) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum. All cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2.

The pharmacological ERK5 inhibitor XMD8-92 (#SC-361408) was purchased from Santa Cruz and used at a concentration of 10 μM. The Non-N-BPs (clodronate and etidronate) and N-BPs (pamidronate, ibandronate and zoledronate) were obtained from Sigma Aldrich and employed at 100 μM unless indicated otherwise.

2.2. Plasmids and retroviral infections

The retroviral constructs for stable expression of constitutively active rat MEK5 S311D/T315D (pBABE puro MEK5D) and small hairpin RNA (shRNA) against ERK5 (pRetroSuper-ERK5, targeting sequence: GAGTCACCTGATGTCAACC) were described previously [23].

Retroviral infections of HUVECs or MSCs were carried out in two consecutive rounds using 5 μg/ml of polybrene as described before [23]. 72 h post infection positively transduced cells were selected for puromycin resistance conferred by co-expression of a puromycin resistance gene from the retroviral backbones by overnight incubation in medium containing 2 μg/ml puromycin (Applichem). Cells were then reseeded into puromycin-free media for the respective experiments.

2.3. Differentiation of OBs from MSCs and detection of matrix mineralization

For osteogenic differentiation, 1×10^5 MSCs were seeded into 6-well plates and cultured in stem cell medium until they reached confluency. Osteogenic differentiation was initiated by switching the regular stem cell medium to osteogenic medium (DMEM high glucose (Thermo Fisher Scientific), 50 μg/ml ascorbate, 10 mM β-glycerophosphate, 100 nM dexamethasone (all purchased from Sigma Aldrich)). Cells were then cultured in osteogenic medium for 14 days with medium replacement every 2–3 days to obtain differentiated OBs. Successful production of mineralized extracellular matrix by the obtained differentiated OBs was routinely monitored by detecting extracellular calcium deposits by Alizarin-red S (#A5533, Sigma Aldrich) staining as described [24]. Where indicated, additionally RNA was isolated at different time points after initiation of osteogenic differentiation and mRNA induction of selected OB differentiation and mineralization markers was determined by qRT-PCR as described below.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted from HUVECs, MSCs or differentiated OBs using the RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. A DNase treatment was performed and first-strand cDNA was generated by reverse transcription of 1 μg RNA using the Quantitect cDNA synthesis kit following the manufacturer's protocol (Qiagen). Relative mRNA expression levels were then determined by SYBR-Green or TaqMan-based quantitative real-time PCR using the comparative threshold cycle (ΔCT) method as described [26]. Briefly, qRT-PCR reactions were run on a StepOne™ Plus qPCR-cycler (Applied Biosystems) using either commercially synthesized gene-specific forward and reverse primers (Sigma) and