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Inorganic polymeric phosphate/polyphosphate as an inducer of alkaline phosphatase and a modulator of intracellular Ca^{2+} level in osteoblasts (SaOS-2 cells) in vitro

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ABSTRACT

Inorganic polymeric phosphate is a physiological polymer that accumulates in bone cells. In the present study osteoblast-like SaOS-2 cells were exposed to this polymer, complexed in a 2:1 stoichiometric ratio with Ca^{2+}, polyP (Ca^{2+} salt). At a concentration of 100 μM, polyP (Ca^{2+} salt) caused a strong increase in the activity of the alkaline phosphatase and also an induction of the steady-state expression of the gene encoding this enzyme. Comparative experiments showed that polyP (Ca^{2+} salt) can efficiently replace β-glycerophosphate in the in vitro hydroxyapatite (HA) biomineralization assay. In the presence of polyP (Ca^{2+} salt) the cells extensively form HA crystallites, which remain intimately associated with or covered by the plasma membrane. Only the tips of the crystallites are directly exposed to the extracellular space. Element mapping by scanning electron microscopy/energy-dispersive X-ray spectroscopy coupled to a silicon drift detector supported the finding that organic material was dispersed within the crystallites. Finally, polyP (Ca^{2+} salt) was found to cause an increase in the intracellular Ca^{2+} level, while polyP, as well as inorganic phosphate (P_i) or Ca^{2+} alone, had no effect at the concentrations used. These findings are compatible with the assumption that polyP (Ca^{2+} salt) is locally, on the surface of the SaOS-2 cells, hydrolyzed to P_i and Ca^{2+}. We conclude that the inorganic polymer polyP (Ca^{2+} salt) in concert with a second inorganic, and physiologically occurring, polymer, biosilica, activates osteoblasts and impairs the maturation of osteoclasts.

1. Introduction

The most abundant and most important inorganic phase in the hard tissue of vertebrates (i.e. bones) is hydroxyapatite (HA). HA represents about 60 wt.% of bone, while water accounts for about 10% and the remainder is organic matter [1]. It is the combination of inorganic and organic phases that provides bone with unique mechanical properties [2], including elastic modulus, hardness and fracture toughness. The process of mineralisation can be subdivided into three mechanisms: (i) matrix vesicle-mediated mineralization; (ii) nucleation of mineral crystals on collagen; and (iii) ectopic mineralization [3]. The detailed processes that lead to the accumulation of early calcium phosphate crystals as well as the transport of the primordial, early mineral to the extracellular matrix space are only incompletely understood. It has been published that initial mineralization of the HA crystallites occurs intracellularly, in matrix vesicles and membrane-bound bodies that are formed from plasma membrane. From there the crystallites migrate into the loose ECM space [4–6]. In a more recent study, Rohde and Mayer [3] provided data suggesting that mineralization by osteoblasts starts with the synthesis of amorphous Ca/P material that is secreted via an exocytotic process from vacuoles of the osteoblasts and is subsequently deposited extracellularly on collagen fibrils under formation of mature HA crystallites.

Extracellular HA deposition on collagen type I constitutes the main framework into which the mineral crystals of bone tissue are incorporated [7,8]. Furthermore, a series of soluble, non-collagenous proteins exist within the collagen scaffold that are synthesized by osteoblasts and subsequently secreted by them, e.g. osteopontin [9,10], osteocalcin [11], osteonectin [12] and bone sialoprotein [13], or by osteoclasts, e.g. the bone morphogenetic proteins [14].

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Those mediators control the maturation and function of the osteoblasts and osteoclasts that form or dissolute the HA crystallites. The substrates for the HA deposits are calcium (Ca^{2+}) and phosphate [15].

The importance of phosphate biochemistry was strengthened in 1923 [16] by the finding that hexosephosphoric esters modulate ossification. Subsequently, the enzyme alkaline phosphatase (ALP) was implicated in phosphate metabolism in bone, due to its high levels and regional accumulation in areas of highest ossification [17]. In humans, four isoenzymes of ALP have been identified; three of them are expressed in a tissue-specific pattern in the intestine, the placenta and germ cells [18], while the fourth is abundant in bone and liver [19]. Hence, this latter isoform was used as a biochemical marker in serum and urine to assess bone formation and bone resorption [20] especially for human metabolic diseases, such as osteoporosis and hyperparathyroidism [21]. Subsequently, this enzyme, the bone-specific alkaline phosphatase (bone ALP) was used as a target to successfully modulate phosphatase activity and, in turn, ossification, e.g. by bisphosphonates [22]. The physiological role of bone ALP is understood to some extent. It has been proposed that bone ALP generates inorganic phosphate (P_i), which is needed for hydroxyapatite crystallization in the bone matrix [23], while in an alternative view it is hypothesized that this enzyme hydrolyzes the mineralization inhibitor inorganic pyrophosphate (PP_i) [24] in order to facilitate mineral precipitation and growth [25]. More recent studies favor the assumption that the major function of bone ALP is to maintain the proper homeostasis of this mineralization inhibitor to ensure balanced bone mineralization [23].

It was proposed in 1925 [26] that β-glycerophosphate (β-GP) is the phosphate donor for HA deposition in vivo and in vitro. β-GP is synthesized intracellularly and then released from the cells to the extracellular space [27]. In turn, β-GP has traditionally been used as one component in the activation cocktail added to HA-mineralizing cells in vitro [28]. Besides being a proposed substrate for HA formation, β-GP has been demonstrated to be a suitable substrate for ALP [29], especially in tissue characterized by physiological and pathological mineralization [30]. It was concluded some time ago that, especially under in vitro conditions, β-GP is completely converted into P_i [31]. The enzyme ALP also hydrolyzes inorganic polyphosphates (polyP) [32], naturally occurring inorganic polymers that are found in pro- and eukaryotes [33,34].

Recently, our group showed that the two naturally occurring inorganic polymers, biosilica and polyP, are potent inducers of biomineralization of HA-forming bone cells in vitro. As a model cell line we used SaOS-2 (sarcoma osteogenic) cells, a nontransformed cell line derived from primary osteosarcoma cells that can undergo differentiation and HA formation [35]. Biosilica, an inorganic polymer synthesized from soluble silicic acid [$Si(OH)_4$] by the enzyme silicatein in vitro [36,37], was found to up-regulate the expression of osteoprotegerin (OPG) while the receptor activator for NF-κB ligand (RANKL) remained unaltered [38]. In line with our earlier finding that polyP has a strong effect on biomineralization in SaOS-2 cells [39], detailed studies with SaOS-2 cells were performed (Schröder et al., submitted for publication). The resulting experiments revealed that polyP causes a dual effect on bone metabolizing cells: first, it promotes HA formation in SaOS-2 cells (osteoblasts) and second, it impairs maturation of the osteoclast-related RAW 264.7 cells.

It was the aim of the present study to investigate the effect of polyP on the level of ALP in SaOS-2 cells and then to demonstrate that the increase in the enzymatic activity measured in the crude extracts might be due to an induction of the tissue-nonspecific type ALP (TNAP). This enzyme has been correlated with the anabolic pathway of bone formation [40]. TNAP plays a key role in bone mineralization, and both initiates and promotes the formation of HA crystals in the matrix vesicles of osteoblasts as well as of hypertrophic chondrocytes, then transports those crystals into the extracellular matrix [5,41,42]. Those matrix vesicles represent extracellular particles that serve as the initial site of mineralization [42]. TNAP also degrades PP_i under formation of free P_i, which forms together with calcium HA. Finally, we studied the effect of polyP on the intracellular Ca^{2+} level of SaOS-2 cells. An increased level of cytosolic Ca^{2+} was measured after exposure of the cells to a stoichiometric complex of polyP to Ca^{2+} (molar ratio of 2 (with respect to phosphate monomer) to 1 Ca^{2+}), an effect that was not seen if polyP was added as an Na^+ salt. From these experiments, we deduced that polyP (Ca^{2+} salt) is a potent inducer of ALP in SaOS-2 cells and contributes with these activities and functions to the synthesis of HA crystallite formation.

2. Materials and methods

2.1. Materials

The following materials were obtained: sodium phosphate glass type 45 (average chain length 45) and Alizarin Red S from Sigma–Aldrich (Taufkirchen; Germany); McCoy's medium from Biochrom (Berlin; Germany); mouse monoclonal antibodies (mAb) against human ALP from R&D Systems (Wiesbaden; Germany); Alexa Fluor 488 goat anti-mouse IgG from Invitrogen (Darmstadt; Germany); Fura-2 AM and Fura-2 Calcium Imaging Calibration Kit from Molecular Probes (Eugene, OR).

2.2. Cells and incubation conditions

SaOS-2 cells (human osteogenic sarcoma cells [43]) were cultured in McCoy's medium (containing 1 mM $CaCl_2$), with 5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and gentamicin (50 μg ml^{-1}) in 6-well plates (surface area 9.46 cm^2; Orange Scientifique, Braine-l'Alleud; Belgium) at a density of 5×10^3 cells cm^{-2} in a humidified incubator at 37 °C and 5% CO_2, as described previously [44,45].

PolyP was mixed together with $CaCl_2$ in a stoichiometric ratio of 2:1 (polyP:$CaCl_2$), designated "polyP (Ca^{2+} salt)". $CaCl_2$ was added to compensate for any effect caused by the chelating activity to Ca^{2+} before and after potential hydrolysis of polyP to monomeric phosphate or pyrophosphate by phosphatases in vitro [46,47]. Unless otherwise mentioned, 100 μM polyP (Ca^{2+} salt) complexed with 50 μM Ca^{2+} was added to the test assays.

Where and when indicated, the cultures were supplemented with the complete activation cocktail required for initiation of HA formation, composed of the standard inducers 5 mM β-GP, 50 mM ascorbic acid (AA) and 10 nM dexamethasone (DEX) [48], or with only the incomplete activation cocktail of 50 mM AA and 10 nM DEX.

2.3. Quantitative alkaline phosphatase assay

Cells were removed from the plates and rinsed with phosphate-buffered saline (PBS) to remove the FCS. The cells were broken in a 12 mM Tris/$NaHCO_3$ buffer (pH 6.8), supplemented with 1 vol.% of Triton X-100. After centrifugation (15,000*g*, 5 min, 4 °C) the supernatant was collected and used for determination of the protein and DNA concentrations and finally for ALP activity. Enzyme determination was performed as described [49]. The assay mixture (200 μl) contained 0.1 M 2-amino-2-methyl-1-propanol (pH 10.5), 2 mM $MgCl_2$ and 2 mM 4-nitrophenylphosphate, to which 20 μl of cell extract was added; the protein concentration was between 1 and 5 μg per assay. After an incubation period of 10 min the reaction was stopped by addition of NaOH and the absorbance