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Isoquercitrin and polyphosphate co-enhance mineralization of human osteoblast-like SaOS-2 cells *via* separate activation of two RUNX2 cofactors AFT6 and Ets1

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ABSTRACT

Isoquercitrin, a dietary phytoestrogen, is a potential stimulator of bone mineralization used for prophylaxis of osteoporotic disorders. Here we studied the combined effects of isoquercitrin, a cell membrane permeable 3-*O*-glucoside of quercetin, and polyphosphate [polyP], a naturally occurring inorganic polymer inducing bone formation, on mineralization of human osteoblast-like SaOS-2 cells. Both compounds isoquercitrin and polyP induce at non-toxic concentrations the mineralization process of SaOS-2 cells. Co-incubation experiments revealed that isoquercitrin (at 0.1 and 0.3 μM), if given simultaneously with polyP (as Ca^{2+} salt; at 3, 10, 30 and 100 μM) amplifies the mineralization-enhancing effect of the inorganic polymer. The biomineralization process induced by isoquercitrin and polyP is based on two different modes of action. After incubation of the cells with isoquercitrin or polyP the expression of the *Runt-related transcription factor 2* [RUNX2] is significantly upregulated. In addition, isoquercitrin causes a strong increase of the steady-state-levels of the two co-activators of *RUNX2*, the *activating transcription factor 6* [ATF6] and the *Ets oncogene homolog 1* [Ets1]. The activating effect of isoquercitrin occurs *via* a signal transduction pathway involving ATF6, and by that, is independent from the induction cascade initiated by polyP. This conclusion is supported by the finding that isoquercitrin upregulates the expression of the gene encoding for *osteocalcin*, while polyP strongly increases the expression of the *Ets1* gene and of the *alkaline phosphatase*. We show that the two compounds, polyP and isoquercitrin, have a co-enhancing effect on bone mineral formation and in turn might be of potential therapeutic value for prevention/treatment of osteoporosis.

1. Introduction

Osteoporosis is a progressive skeletal disorder characterized by a decrease in bone mass and a compromised bone strength predisposing to an increased risk of fracture (reviewed in [1]). This bone disease has become a major public health problem worldwide. While postmenopausal osteoporosis is most common in women after menopause, osteoporosis after the age of 75 occurs in an almost equal ratio in both females and males. On the cellular level, osteoporosis is caused by an imbalance between bone resorbing (osteoclasts) and bone forming cells (osteoblasts). It is widely proposed that lifestyle changes, including diet, *e.g.*, the intake of calcium or vitamin D, and exercise might decelerate the progression of the disease. Experimentally-based studies propose that phytoestrogens, including flavonols like quercetin, might prevent bone loss, and might be taken for prophylactic use in postmenopausal women [2]. Focusing on quercetin, this flavonol has been described to be a potent inhibitor, at a concentration of ≈1–10 μM, of osteoclasts *in vitro* [3], as well as of osteoblasts, likewise *in vitro* at ≈10 μM [4]. The latter effect is controversial, since it has been reported that quercetin, at 50 μM, is a strong activator of osteoblasts *in vitro* resulting in an increased

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mineralization [5]. This effect has been attributed to the interaction of quercetin with the estrogen receptor [ER], which causes an activation both of the MAPK/ERK pathway and of the alkaline phosphatase [ALP]. Another study reports that quercetin induces the tumor necrosis factor-alpha (TNF-α)-mediated pathway and apoptosis in osteoblasts *in vitro* [6]. Quercetin that penetrates through the plasma cell/membrane lipid bilayer [7] has also been described to reduce the overall ATP release from platelets [8], to interfere with Na^+/K^+-ATPase [9] and to act as a ligand for the aryl hydrocarbon receptor [AhR] [10]. In our studies we demonstrated that polyphosphate [polyP], a natural inorganic polymer, induces bone formation *in vitro* and likely also *in vivo* [11,12], and might be considered, like bio-silica/silica [13] and activators of the carbonic anhydrase [14], for animal test-directed studies for potential osteoporosis-ameliorating activity. These stimulating components have been found to induce the expression of the genes encoding for the osteoclastogenesis inhibitory factor *osteoprotegerin* [OPG], *bone morphogenetic protein 2* [BMP2], and *alkaline phosphatase* [ALP] (reviewed in [12,15]).

It has been proposed that polyP, after hydrolysis to monomeric phosphate [P_i] by phosphatases like ALP [16,17], is involved in regulation of mineralization processes occurring during vertebrate skeleton formation [18]. PolyP is a calcium chelator and might act as a source of Ca^{2+} ions. Both Ca^{2+} and the monomeric P_i, the products released after enzymatic degradation of polyP (Ca^{2+} salt), are the mineralic precursors required for hydroxyapatite deposition [19]. The ALP also hydrolyzes pyrophosphate [PP_i] produced by the ectonucleotide pyrophosphatase/phosphodiesterase-1 [ENPP1]. ENPP1 is an ecto-enzyme that regulates the extracellular levels of PP_i by cleveage of ATP; this enzyme is expressed in matrix vesicles and plasma membranes of osteoblasts [20]. Both PP_i and P_i are regulator molecules of the process of mineral deposition onto bone-forming cells. While PP_i is a well-known inhibitor of mineralization [21,22], P_i is inhibiting the carbonic anhydrase, which is most likely involved in bio-seed formation during mineralization of bone cells [23].

The extracellular level of phosphate can be modulated physiologically, by the action of the hydrolytically-acting enzymes, ALP and ENPP1, or pharmacologically *e.g.*, by quercetin which is also an inducer of *ALP* [5]. Recently, it has been shown that polyP is synthesized in mammalian mitochondria [24]. PolyP is not toxic on cells *in vitro* up to concentrations of 100 μM. PolyP administered in a stoichiometric ratio of 2 M polyP:1 M $CaCl_2$ [polyP (Ca^{2+} complex)], causes in osteoblast-like SaOS-2 cells, on the level of gene transcription, an increased expression of *BMP2*. In addition, in osteoclast-like RAW 264.7 cells polyP (Ca^{2+} salt) inhibits cell proliferation, very likely through a down-regulation of the *tartrate-resistant acid phosphatase* [TRAP] and an inhibition of the phosphorylation of IκBα by the respective kinase [25].

It is the aim of the present study to determine the pharmacological effect of quercetin, acting as a stimulator for the mineralization process *in vitro*, and of polyP (Ca^{2+} salt), functioning as a supplier for P_i. In view of the findings that quercetin-3-*O*-glucoside [isoquercitrin, IQC], the 3-*O*-glucoside of quercetin, provides a superior bioavailability than non-glucosylated quercetin [26] we have used isoquercitrin for our studies. Isoquercitrin [quercetin 3-β-D-glucoside] is also a common secondary metabolite in plants, *e.g.*, in the Mongolian medicinal plant *Dracocephalum ruyschiana* [27].

The published data show that quercetin causes an anabolic effect on SaOS-2 cells within the range of 1 and 5 μM, and hence support previous results obtained with MG-63 human osteoblasts [5]. In contrast, and using MC3T3-E1 osteoblastic cells, Son et al. [6] reported cell toxicity within this concentration range and higher. It might be mentioned here that the quercetin content of edible plants ranges from 10 to 100 mg per 100 g of edible portion [28], while the concentration of isoquercitrin is about 30-fold higher [29]. Administration of quercetin at doses of 40–1900 mg/kg/day to rats revealed treatment-related effects on survival and no treatment-related clinical signs of toxicity, due to its poor absorption [30]. Likewise of low toxicity is isoquercitrin with an acute toxicity (oral LD_{50} [lethal dose]) in Sprague-Dawley rats of >25,000 mg/kg [31].

In the present study, polyP and isoquercitrin individually, but particularly in combination, are shown to increase bone mineral formation in SaOS-2 cells and to have different modes of action *via* signal transduction pathways involving two Runt-related transcription factor 2 [RUNX2] cofactors ATF6 and Ets1. SaOS-2 is a non-transformed cell line that is derived from primary osteosarcoma cells and provided with a (limited) differentiation capacity [32].

2. Materials and methods

2.1. Chemicals

Quercetin 3-β-D-glucoside [isoquercitrin] (#17793) was obtained from Sigma (Taufkirchen, Germany); Na-polyphosphate [Na-polyP] with an average chain length of approximately 40 phosphate units was from Chemische Fabrik Budenheim (Budenheim, Germany). The sources for the enzymes and reagents, used for the molecular biological experiments, were listed recently [14].

2.2. Cultivation of SaOS-2 cells

SaOS-2 cells (human osteogenic sarcoma cells, [33]) were cultured in McCoy's medium (Biochrom-Seromed, Berlin, Germany) containing 2 mM L-glutamine and 1 mM $CaCl_2$ [34]. The medium was supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 units/ml penicillin/100 μg/ml streptomycin. Routinely, the cells were incubated in 25 cm^2 flasks or in six-well plates (surface area 9.46 cm^2; Orange Scientifique, Braine-l'Alleud, Belgium) in a humidified incubator at 37 °C [35,36]. If not mentioned otherwise 3×10^5 cells/well were added to start the culture (total volume, 3 ml). To induce biomineralization the cultures were supplemented with the mineralization activation cocktail (MAC), composed of 5 mM β-glycerophosphate, 50 mM ascorbic acid and 10 nM dexamethasone [37]. The mineralization activation cocktail was added 3 days after starting the experiments. Medium was changed every 3 days and new MAC was added; likewise the test assays were added with the test compounds after each medium change.

If not mentioned otherwise the test compounds [isoquercitrin and polyP (Ca^{2+} salt)] were added after a cultivation period of the cells for 5 days. At this time point also MAC was added to the cultures. The incubation was terminated after a total period of 12 days. PolyP was administered as a Ca^{2+} salt; for this, 2 M polyP (referring to the polymer) was mixed together with 1 M $CaCl_2$ in a stoichiometric ratio 2:1, to compensate for the chelating function of polyP for Ca^{2+} [25]. Isoquercitrin was dissolved in a stock solution of 300 μM in DMSO [dimethyl sulfoxide].

The cell density was determined by the colorimetric method based on the tetrazolium salt XTT (Cell Proliferation Kit II; Roche, Mannheim, Germany), following the recommendations of the supplier and as described [38]. The cultures had been incubated for 72 h; during this period of time the cell density increased from 10×10^3 cells/ml to 355×10^3 cells/ml. Then the cultures were subjected to the XTT assays and the absorbance of the samples was measured against a background control at a wavelength of 450 nm. The non-specific readings, measured at a wavelength of 690 nm, were subtracted.