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Differential effect of chondroitin-4-sulfate on the immediate and delayed prostaglandin E_2 release from osteoblasts

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ABSTRACT

The present study examines the effect of chondroitin-4-sulfate (C4S) on the immediate (non-inflammatory conditions) and the delayed (inflammatory conditions) prostaglandin E_2 (PGE_2) release from rat calvarial osteoblasts. An immediate low release of PGE_2 was induced by PAF, phorbol ester and arachidonic acid but not by IL1β, TNF-α and LPS whereas a delayed high release of PGE_2 was induced by the inflammatory agents IL1β, TNF-α and LPS but not by PAF, phorbol ester and arachidonic acid. C4S had no effect on the immediate PGE_2 release but inhibited the delayed release of PGE_2. IL1β, TNF-α and LPS enhanced the expression of COX-2 and mPGES1 whereas phorbol ester enhanced COX-2 expression only. PAF and arachidonic acid had no effect on the expression of COX-2 and mPGES1. C4S inhibited the enhanced expression of COX-2 and mPGES1 but had no effect on the IL1β-induced decrease of I-κBα and nuclear translocation of NF-κB. These results indicate that the beneficial effects of C4S in bone inflammatory diseases might be due to a specific inhibition of the delayed high PGE_2 release from osteoblasts.

1. Introduction

Prostaglandins (PG) are considered as important local factors that modulate bone remodeling through their effects both on osteoblasts and osteoclasts and have been linked to many bone-resorptive diseases [1–4]. Among several PG produced, prostaglandin E_2 (PGE_2) is the most prominent eicosanoid in bone tissue. It is discussed on the one hand as a potent stimulator of bone resorption by promoting the formation of active osteoclasts but otherwise also to promote bone-forming osteoblasts [1–3]. The main producers of PGE_2 within bone are osteoblasts which release PGE_2 upon different stimulation but also respond to external PGE_2 [4,5].

The synthesis of PGE_2 involves different enzymatic steps: (a) the release of arachidonic acid from membrane glycerophospholipids, (b) the conversion of free arachidonic acid into PGG/H_2, and (c) the formation of PGE_2 by terminal PGE_2 synthases. The first step can be catalyzed by a cytosolic phospholipase A_2 ($cPLA_2$) [4,6–9]. Activation of $cPLA_2$ requires phosphorylation and an increase in cytosolic free calcium level for its translocation from the cytoplasm to e.g. the perinuclear or endoplasmic reticular membrane [7–10]. Stimuli like platelet-activating factor (PAF), which induce an increase of cytosolic free calcium and an activation of protein kinases, have been reported to activate this pathway [11]. However, there is also evidence that stimuli like phorbol ester, which have no effect on cytosolic free calcium, induce a release of arachidonic acid by activation of phospholipase C (PLC) and/or phospholipase D (PLD) and diacylglycerol lipase [12–15]. Free arachidonic acid is a substrate for the cyclooxygenase(s) (COX) which convert arachidonic acid into PGG/H_2; there are at least two genes encoding two COX isoenzymes, a constitutive COX-1 and an inducible COX-2 isoenzyme [16]. Finally, PGH_2 is converted into PGE_2 by terminal PGE_2 synthases (PGES); there are three distinct terminal PGES, a cytosolic constitutive PGES (cPGES) and two microsomal PGES, the inducible mPGES1 and the constitutive mPGES2 [17]. Dependent on the stimulus, the synthesis and release of PGE_2 can take place within minutes after activation (immediate release) or after a lag phase of hour(s) (delayed release). Stimuli like phorbol ester and PAF induce an immediate release by the activation of constitutively expressed enzymes such as $cPLA_2$, COX-1, cPGES and mPGES2 [6,18–20] whereas stimuli like lipopolysaccharide (LPS), interleukin 1β (IL1β) and tumor necrosis factor-α (TNF-α) induce a delayed release by the expression of inducible enzymes such as COX-2 and mPGES1 [21–23].

Chondroitin sulfate (CS) belongs to the family of glycosaminoglycans (GAG), long unbranched polysaccharides consisting of a repeating disaccharide unit which is often modified by sulfation, acetylation or epimerization [24]. In CS the repeating disaccharide structure is composed of glucuronic acid and N-acetylgalactosamine and is often sulfated, particularly in the 4- or 6-position (C4S, C6S) [25]. In the extracellular matrix all GAG

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subclasses, except hyaluronan (Hya) are covalently linked to certain core protein molecules forming proteoglycans [26].

In clinical trials it has been shown that the application of CS reduces pain and improves the articular function of patients with osteoarthritis (OA), a disease that is characterized by a degeneration of articular cartilage and an altered remodeling of subchondral bone [25,27]. Numerous *in vitro* and *in situ* studies revealed that the beneficial effects of CS in OA are probably mediated by an inhibitory effect of CS on the IL1-induced nuclear translocation of NF-κB (nuclear factor of activated B-cells) [28–32]. In osteoblasts, CS inhibits the osteoblast-mediated activation of osteoclasts [27,33] and in animal studies it has been shown that coating of titanium-collagen implants with CS enhances bone healing and apposition of new bone tissue around the metallic implants [34,35].

In bone, CS and other GAG are actively synthesized by osteoblasts and are localized in and at the membrane of osteoblasts and in the extracellular matrix [36–38]. GAG have been reported to influence the proliferation and differentiation of osteoblasts *per se* [36,39,40] or *via* an interaction with growth factors and cytokines [41,42]. Recently, it has been shown that CS directly regulates the bone morphogenetic protein-mediated differentiation of human mesenchymal stem cells into osteoblasts [43]. Furthermore, Hirata et al. [44] demonstrated that hyaluronan inhibits bone resorption by suppressing PGE_2 synthesis in osteoblasts treated with IL1β. The molecular mechanisms how GAG mediate these effects in osteoblasts are not yet understood. The aim of the present study was to investigate if C4S has an effect on enzymes of the arachidonic acid cascade and PGE_2 release in rat calvarial osteoblasts.

2. Materials and methods

2.1. Chemicals

Collagenase P (from *C. histolyticum*) was from Roche Diagnostics (Mannheim, Germany). Bovine pancreatic trypsin, penicillin/streptomycin antibiotics, Dulbecco's minimum essential medium (DMEM) and phosphate buffered saline (PBS) were purchased from Biochrom (Berlin, Germany) and the fetal calf serum (FCS) was from BioWest (Germany). C4S from bovine trachea, arachidonic acid and LPS from *Escherichia coli* were obtained from Sigma–Aldrich (Steinheim, Germany). Calbiochem (Darmstadt, Germany) provided phorbol 12-myristate 13-acetate (PMA) and PAF. Recombinant rat IL1β and TNF-α were supplied by R&D Systems (Wiesbaden, Germany). Polyclonal antibody against COX-2 was from Cayman Chemical Company (Ann Arbor, USA). Antibodies against NF-κB, I-κBα and $cPLA_2$ were from Cell Signaling (Frankfurt, Germany) and anti-β-tubulin antibody from Sigma. [^{3}H]-arachidonic acid and lipid-free albumin were purchased from Hartmann Analytic (Braunschweig, Germany) and Serva (Heidelberg, Germany), respectively.

2.2. Cell isolation and culture and PGE_2 release

NIH guidelines for the care and use of laboratory animals were applied for the study. Rat calvarial-derived osteoblasts were isolated and cultured according to Geissler et al. [45]. PGE_2 release into the media was determined by an ELISA according to Dieter et al. [46] after treating the osteoblasts with either 10 μM arachidonic acid, 1 μM PMA, 1 μM PAF, 10 ng IL1β/ml, 10 ng TNF-α/ml or 1 μg LPS/ml in the presence or absence of 500 μg C4S/ml.

2.3. [^{3}H]-arachidonic acid release

The amounts of released [^{3}H]-arachidonic acid from [^{3}H]-arachidonic acid-prelabeled cells were determined as described earlier [47].

2.4. Quantitative real-time RT-PCR

Cells were washed with ice-cold PBS and total RNA was isolated using RNeasy Mini Kit (Qiagen, Hildesheim, Germany). Digestion of residual DNA and reverse transcription (1 μg RNA) were carried out with QuantiTect RT-Kit (Qiagen), according to manufacturer's instructions. Real-time PCR reactions were performed using Rotor-Gene PG-3000 PCR machine (Corbett, Wasserburg, Germany). For PCR analysis, 1 μl of total cDNA was amplified in a 20 μl reaction mix containing 20 pmol of forward and reverse primer (MWG Biotech, Ebersberg, Germany; for primer sequences, see Table 1) and 10 μl of QuantiTect SYBR green mixture (Qiagen). After initial activation step at 95 °C for 15 min, PCR cycles were run for denaturation at 94 °C for 20 s, annealing (for temperatures, see Table 1) for 20 s, and for synthesis at 72 °C for 20 s.

2.5. Immunoblotting

Cells were lysed in homogenization buffer containing 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and 0.1 mM aprotinin, scraped off and were centrifuged at 20,800 × *g* for 30 min at 4 °C to remove cellular debris. Protein content was determined using the RotiQuant assay from Carl Roth GmbH (Karlsruhe, Germany). The protein concentration (μg/μl cell lysate) was calculated from a standard curve made with bovine serum albumin and equivalent amounts of protein are subjected to SDS-PAGE.

Extracted proteins (25 μg/lane) were separated on SDS-polyacrylamide gels (10%) under reducing conditions and electrophoretically transferred to nitrocellulose membrane (GE Healthcare, Freiburg, Germany). The membrane was incubated in blocking buffer (5% (w/v) dry milk, 0.05% (v/v) Tween-20 in Tris-buffered saline) for 2 h at 25 °C and incubated overnight at 4 °C with antibodies against β-tubulin (3.7 μg/ml), Phospho-$cPLA_2$ (0.1%), $cPLA_2$ (0.1%), COX-2 (0.5 μg/ml) and I-κBα (0.2%) followed by incubation with 0.05% (v/v) of the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling) diluted in blocking buffer for 1 h at 25 °C. Immunoreactive bands were detected using ECL kit (GE Healthcare) and exposed to a CCD camera (Genegnome from Syngene, Frederick, USA). Prestained and biotinylated SDS-PAGE Standards (Biorad Laboratories, München, Germany) were used as molecular weight standards.

2.6. Immunofluorescence microscopy

Cells were fixed with 4% (v/v) paraformaldehyde for 15 min at 25 °C. Subsequently, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min and incubated in blocking buffer (1% (w/v) BSA, 0.05% (v/v) Tween-20 in PBS) for 10 min. Then, cells were incubated overnight at 4 °C with antibody against NF-κB (2% (v/v) in blocking buffer) followed by incubation in the same buffer with Alexa 568-conjugated secondary antibody (10 μg/ml, Invitrogen) for 1 h at 25 °C, and for 15 min with DAPI (0.2 μg/ml) to stain nuclei. Stained cells on glass slides were then embedded in Mowiol 4-88. The staining was visualized using the AxioPhot fluorescence microscope, and digital images were acquired with an AxioCam MRm (Zeiss). The fluorescence signals were detected with the following optics: Alexa 568: excitation 546 nm, emission 590 nm; DAPI: excitation 365 nm, emission 420 nm.

2.7. Statistical analysis

All presented data were derived from three independent experiments (3 different litter of neonatal rat pups) performed in triplicate. The results are presented as mean ± standard deviation