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The PI3K/Akt/FOXO3a/p27^{Kip1} signaling contributes to anti-inflammatory drug-suppressed proliferation of human osteoblasts

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ABSTRACT

Akt has been reported to suppress p27^{Kip1} promoter activity through Forkhead box O (FOXO) in different kinds of cells. Previous studies indicated that anti-inflammatory drugs up-regulated p27^{Kip1}, and this effect might play an important role in anti-inflammatory drug-induced cell cycle arrest of human osteoblasts (hOBs). In this study, we hypothesized that these drugs might increase p27^{Kip1} expression in hOBs by altering the Akt/FOXO signaling. We tested this hypothesis by examining the influences of three anti-inflammatory drugs on the levels and/or activities of Akt, FOXO and p27^{Kip1} as well as the relationship between these factors and proliferation of hOBs. We tested the effects of indomethacin (10^{-5} and 10^{-4} M), celecoxib (10^{-6} and 10^{-5} M), and dexamethasone (10^{-7} and 10^{-6} M) using PI3K inhibitor, LY294002 (10^{-5} M) as the basis of comparison. The three drugs suppressed the canonical level of phosphorylated Akt in hOBs. This was accompanied by elevated FOXO3a level and increased promoter activity, mRNA expression and protein level of p27^{Kip1}. Furthermore, the anti-inflammatory drugs suppressed the EGF-induced increases in proliferation, phosphorylation, and nucleus translocation of Akt. Simultaneously, they suppressed EGF-induced decreases of FOXO3a nucleus accumulation and p27^{Kip1} mRNA expression. On the other hand, FOXO silencing significantly attenuated the drug-induced up-regulation of p27^{Kip1} and suppression of proliferation in hOBs. To the best of our knowledge, this study represents the first to demonstrate that Akt/FOXO3a/p27^{Kip1} pathway contributes to suppression of hOB proliferation by anti-inflammatory drugs. We suggest that anti-inflammatory drugs suppress hOB proliferation, at least partly, through inactivating Akt, activating FOXO3a, and eventually up-regulating p27^{Kip1} expression.

1. Introduction

Anti-inflammatory drugs are widely used to relieve pain and inflammation in orthopaedic patients. However, reports have suggested that these drugs, including glucocorticoids (GCs), non-selective non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors have adverse effects on bone repair [1–8]. Anti-inflammatory drugs have been further reported to suppress proliferation and/or induce apoptosis in different kinds of cells via affecting cell cycle and pro-apoptotic factors [9–16]. Our previous studies also found that NSAIDs inhibited proliferation and arrested cell cycle at G0/G1 phase in both human bone marrow stem cells (hBMSCs) and osteoblasts (hOBs) [17–20]. Moreover, we found that dexamethasone, non-selective NSAIDs and COX-2 selective inhibitors caused the p27^{Kip1}, a cyclin-dependent kinase (cdk) inhibitor, expression increase and accompanied with cell cycle arrest in both hBMSCs and hOBs, and these effects were independent from anti-inflammatory drug-induced PG insufficiency [19,20]. The p27^{Kip1} is an important factor to regulate cell cycle progression and thus suppressed osteoblast proliferation, and enhanced differentiation by controlling proliferation-related events both in osteoblasts and bone marrow stem cells [21,22]. Base on these previous studies, we hypothesized that the up-regulation of p27^{Kip1} may contribute to an important common mechanism of anti-inflammatory drug-induced suppression of proliferation in osteogenic cells.

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The serine/threonine kinase Akt plays an important regulatory role in phosphatidylinositol-3-kinase (PI3K)/Akt signal transduction. Activated Akt regulates the activities of transcription factors such as Forkhead box class O (FOXO), mTOR, NFkB, and MDM2, and subsequently controls cell proliferation, apoptosis, and differentiation [23–25]. Celecoxib, glucocorticoids, and indomethacin have been reported to inhibit PI3K/Akt signaling in several somatic and cancer cell lines [26–36]. Although the effects of dexamethasone on Akt phosphorylation were examined using mouse osteoblastic cells (MC3T3E-1) [37], no studies reported whether GCs, non-selective NSAIDs, and COX-2 selective inhibitors suppress hOB Akt signaling.

PI3K/Akt signaling has been reported to suppress $p27^{Kip1}$ and thus proceed cell cycle [23–25,38,39]. Celecoxib has been reported to arrest cell cycle of human umbilical vein endothelial cells through its inhibition of Akt signaling [40]. In previous studies, we found three classes of anti-inflammatory drugs, GCs, non-selective NSAIDs, and COX-2 selective inhibitors, to increase the expression of $p27^{Kip1}$ mRNA in hOBs (as well as hBMSCs) [19,20]. Based upon these findings, we hypothesized that these drugs might up-regulate the expression of $p27^{Kip1}$ by inhibiting Akt activity in hOBs. FOXOs, are Akt down-regulated transcription factors reported to mediate cell cycle arrest, DNA repair, and apoptosis [41]. These transcription factors, which belong to the 'O' subgroup of winged-helix/forkhead transcription-factor family, consist principally of four members FOXO1, FOXO2, FOXO3a, and FOXO4 [42–44]. FOXO3a has been reported to induce the transcription of $p27^{Kip1}$ in many cell lines [43,45–51], suggesting that it may be a key regulator of anti-inflammatory drug-induced up-regulation of $p27^{Kip1}$. Therefore, we further hypothesized that anti-inflammatory drug-induced $p27^{Kip1}$ up-regulation may occur through the alteration of the Akt/FOXO3a signaling in hOBs. To test these hypotheses, we studied the influences of the anti-inflammatory drugs, celecoxib, indomethacin and dexamethasone, on changes in Akt, FOXOs and $p27^{Kip1}$, and relationship between these changes and the proliferation in hOBs.

2. Materials and methods

2.1. Materials

Dulbeco's Modified Eagle's Medium (DMEM), ascorbic acid, nonessential amino acid, penicillin/streptomycin, fetal bovine serum (FBS), and trypsin/EDTA were purchased from Gibco-BRL (Grand Island, New York, NY, USA). LY294002 (PI3K inhibitor), recombinant human EGF, DMSO, indomethacin and dexamethasone were obtained from Sigma (Saint Louis, MO, USA). Celecoxib was obtained from Pfizer (New York, NY, USA).

2.2. Normal human osteoblasts (hOBs)

Primary hOBs were isolated from bone chips of twelve 40–60-year-old donors (5 men and 7 women) who were generally healthy with no other bone disorders than hip dysplasia for which they received hip arthroplasty at Kaohsiung Medical University Hospital. The protocol for this study was approved by the Institutional Review Board (IRB) at Kaohsiung Medical University and the informed consent was obtained from each donor. The hOBs were cultured in DMEM containing 100 mg/ml of ascorbic acid, non-essential amino acids, penicillin/streptomycin and 10% FBS. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The doubling time of hOBs was 22–24 h under these experimental conditions. To synchronize cell cycle, hOBs were cultured in medium containing 2% FBS for 24 h before being treated with one of the agents according to procedures described previously [19,52–54].

2.3. Drug treatment

The drugs used to treat the hOBs in this study were indomethacin (10^{-5} to 10^{-4} M), celecoxib (10^{-6} to 10^{-5} M), dexamethasone (10^{-7} to 10^{-6} M), LY294002 (10^{-5} M) (Sigma, St. Louis, MO, USA), and recombinant human EGF (20 ng/ml) (Sigma, St. Louis, MO, USA). The therapeutic concentrations of indomethacin, celecoxib and dexamethasone were approximately 10^{-5}, 10^{-6} and 10^{-7} M, respectively [55–57]. Indomethacin, celecoxib, dexamethasone and LY294002 (Sigma, St. Louis, MO, USA) were dissolved in DMSO as stock solutions, and recombinant human EGF (Sigma, St. Louis, MO, USA) was dissolved in 10 mM acetic acid containing 0.1% BSA. All the drugs were diluted with a medium containing 2% FBS immediately before treatment began. DMSO was diluted to 0.1% or less to reduce the possibility of its influence on the process [18,19]. Because we found no significant cytotoxicity in hOBs incubated in a medium containing 0.1% DMSO, control cultures were cultivated in a medium containing neither anti-inflammatory drugs nor DMSO.

2.4. Enzyme-linked immunoassay (ELISA)

The levels of canonical phosphorylated Akt and total Akt were measured in indomethacin-, celecoxib-, dexamethasone-treated cultures and control cultures. The hOBs were seeded in a 6-well plate (2×10^4/well) and cultured to 80% confluence. After 24-h treatment with indomethacin, celecoxib or dexamethasone, the cells were collected for assay. We measured phosphorylated serine residue 473 and total Akt levels using BioSource AKT [pS473] ELISA and BioSource AKT ELISA, respectively (BioSource, Camarillo, CA, USA). We calculated phosphorylated Akt and total Akt level based on standard curves. All assays were performed in triplicate.

2.5. Luciferase assays

Cells were cultured in 10 cm dish to 80% confluence, and then harvested for plasmid transfection. The promoter region of human $p27^{Kip1}$ gene was subcloned into the XhoI site of the pGL2 basic vector (Promega, Madison, WI, USA) to create the p27PF luciferase reporter plasmid. Deletion constructs of p27PF including p27KpnI, p27ApaI, p27MB-435, and p27SacII were generated as described previously [58,59] and were kindly provided by Dr. Sakai. Cells were transfected with 2 μg of control plasmid, p27PF plasmid, or deleted p27 plasmids using a MicroPorator (Digital Bio Technology, Seoul, Korea) [60–65]. Cells were then seeded into 12-well plates and incubated in the absence or presence of indomethacin, celecoxib, or dexamethasone for 24 h. Luciferase activity was measured using TopCount Microplate Scintillation and Luminescence Counters (Packard, Meriden, CT, USA). The luciferase activity was normalized with total protein. Experiments were repeated in triplicate.

2.6. Western blot analysis

Cells were treated with indomethacin, celecoxib or dexamethasone for 24 h and lysed in the PhosphoSafe™ Reagent (Novagen, Darmstadt, Germany). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Cell lysates containing 40 μg of protein were analyzed using 10% SDS-PAGE. Transferred membranes were blocked using 5% skim milk and incubated overnight with antibodies against $p27^{Kip}$ (BD, San Jose, CA, USA), p-Akt (upstate, Charlottesville, VA, USA), FOXO1 (Santa Cruz, Santa Cruz, CA, USA), and FOXO3a (Cell Signaling, Danvers, MA, USA). These membranes were also probed with anti-actin (Sigma, St. Louis, MO, USA) or Akt (Santa Cruz, Santa Cruz, CA, USA) for house-keeping purposes. Membranes were developed