Osteoarthritis and Cartilage 20 (2012) 440–445

Osteoarthritis and Cartilage

DIO2 modifies inflammatory responses in chondrocytes

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ARTICLE INFO

Article history:
Received 20 July 2011
Accepted 13 February 2012

Keywords:
Chondrocyte
Osteoarthritis
Inflammation
Selenoprotein
Cyclooxygenase
Interleukin-1
Iodothyronine Deiodinase-2

ABSTRACT

Objective: Selenium neutralizes interleukin-1β (IL-1β) induced inflammatory responses in chondrocytes. We investigated potential mechanisms for this through *in vitro* knock down of three major selenoproteins, Iodothyronine Deiodinase-2 (DIO2), Glutathione Peroxidase-1 (GPX1), and Thioredoxin Reductase-1 (TR1) in primary human chondrocytes.

Methods: Primary human chondrocytes were transfected with scrambled small interfering ribonucleic acid (siRNA) or siRNA specific for DIO2, GPX1 and TR1. After 48 h, transfected cells were cultured in serum free media for 48 h, with or without 10 pg/ml IL-1β for the final 24 h. The efficiency of siRNAs was confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis. The gene expression, by qRT-PCR, of cyclooxygenase-2 (COX2), IL-1β, and Liver X receptor (LXR) alpha and beta was evaluated to determine the impact of selenoprotein knockdown on inflammatory responses in chondrocytes.

Results: The messenger RNA (mRNA) expression of DIO2, GPX1, and TR1 was significantly decreased by the specific siRNAs (reduced 56%, $P = 0.0004$; 96%, $P < 0.0001$; and 66%, $P < 0.0001$, respectively). Suppression of DIO2, but not GPX1 or TR1, significantly increased (~2-fold) both basal ($P = 0.0005$) and IL-1β induced ($P < 0.0001$) COX2 gene expression. Similarly, suppression of DIO2 significantly increased (~9-fold) IL-1β induced IL-1β gene expression ($P = 0.0056$) and resulted in a 32% ($P = 0.0044$) decrease in LXRα gene expression but no effect on LXRβ.

Conclusions: Suppression of the selenoprotein DIO2 resulted in strong pro-inflammatory effects with increased expression of inflammatory mediators, IL-1β and COX2, and decreased expression of LXRα suggesting that this may be the upstream target through which the anti-inflammatory effects of DIO2 are mediated.

Introduction

Profound selenium deficiency is associated with the severe osteoarthropathy known as Kashin-Beck Disease (KBD) that affects around seven million individuals in China and neighboring regions[1]. In the US, a low but non-deficiency level of selenium has been shown to be associated with osteoarthritis (OA) presence and severity in a large population study[2,3]. These observations suggest a requirement for selenium for cartilage health and OA prevention. Recently, selenium has been shown to be anti-inflammatory by altering cytokine-inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) gene expression in response to lipopolysaccharide (LPS) stimulation in cultured macrophages[4,5]. Our recent study demonstrated that pretreatment of chondrocytes with selenomethionine (SeMet) attenuated production of interleukin-1β (IL-1β) induced nitric oxide (NO) and prostaglandin E_2 (PGE_2), synthesized *via* the COX and PG synthase pathways[6]. Given the fact that selenium is incorporated as selenocysteine at the active site of a wide range of selenoproteins, we hypothesized that one or more selenoproteins may be responsible for the anti-inflammatory effects of selenium.

To date, 25 mammalian selenoproteins have been identified including three large subfamilies: glutathione peroxidases (GPXs), thioredoxin reductases (TRs), and iodothyronine deiodinases (DIOs)[7]. GPXs and TRs function as antioxidative enzymes to catalyze the reduction of intracellular peroxide and regulate the redox balance in cells[8]. The DIOs regulate the bioactivity of thyroid hormone by controlling levels of thyroxine (T4) and the active hormone, 3,3′,5-triiodo L-thyronine (T3)[9]. DIO2 is responsible for

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doi:10.1016/j.joca.2012.02.006

local (chondrocyte) conversion of T4 to the active form of the hormone, T3. Several studies now implicate specific selenoproteins from all three of these subfamilies in OA. A genetic variant of GPX1 and reduced plasma GPX activity are associated with increased risk of developing KBD, a severe form of OA endemic to China[10]. Gene expression of GPX1 and thioredoxin-interacting protein (TXNIP) are downregulated in cartilage lesions with moderate to severe late-stage OA[11]. A variant of DIO2 is associated with risk for developing OA[12] and has been shown to increase the vulnerability of cartilage to OA in association with non-optimal femoral head bone shapes rather than directly influencing the formation of these shapes[13]. Taken together, this evidence shows modulation of major selenoproteins in OA and suggests that they may be important modifiers of the joint to inflammatory responses and thereby OA susceptibility. Our goal in this study was to elucidate the biological effects of three specific major selenoproteins through analysis of the consequences of DIO2, GPX1 and TR1 knockdown with small interfering RNAs (siRNAs) on inflammatory responses in primary human chondrocytes.

Materials and methods

Chondrocyte isolation and culture

Anonymous surgical waste articular cartilage samples, taken at the time of joint replacement, were used for this project. Tissue was collected under the approval of the Duke Institutional Review Board (IRB) who determined that this protocol met the definition of research not involving human subjects as described in 45CFR46.102(f) and satisfied the Privacy Rule as described in 45CFR164.514. Articular cartilage samples were obtained from six patients undergoing total knee replacement surgery [mean age, 60.3 ± 10.3 years]. Cartilage was harvested from nonlesional areas, further minced, and subjected to pronase and collagenase digestion to isolate primary human chondrocytes, similar to previously published methods[14]. Isolated chondrocytes within the first two passages were used for all experiments.

RNA Interference of DIO2, GPX1 and TR1

siRNA transfection was performed using the program U20 of the Amaxa Nucleofector (Gaithersburg, MD), with either the Amaxa Primary Human Chondrocyte Nucleofector Kit or Mirus Ingenio™ Electroporation Kit (Madison, MD) according to the manufacturers' protocols. Chondrocytes were transfected with the following siRNAs: 3 μg of Silencer Negative Control No.1 siRNA (Ambion, Applied Biosystem) that served as a scrambled transfection control; 3 μg of human DIO2 specific siRNA (s4106); 1 μg of human GPX1 specific siRNA (s804); or 3 μg of human TR1 specific siRNA (s755).

After transfection, cells were cultured for 48 h in Dulbecco's Modified Eagle Medium (DMEM)/F12 media supplemented with 10% fetal bovine serum to allow gene suppression and turnover of the preexisting targeted proteins. Transfected cells were subsequently cultured in the absence of serum in DMEM media for 24 h, then treated for another 24 h with or without 10 pg/ml IL-1β (R & D systems, Minneapolis, MN) in serum free media. The IL-1β concentration of 10 pg/ml was chosen based on evidence for physiological relevance (equivalent to concentrations in human OA synovial fluid) and previously established experiments[6,15,16]. Thus, the total period of knockdown was 96 h (72 h prior to the addition of IL-1β). Cells were treated with RNeasy Lysis buffer (Qiagen Valencia, CA) to isolate RNA for gene expression studies.

RNA isolation and Real Time-Polymerase Chain Reaction (RT-PCR)

Cell lysates, prepared by RNeasy Lysis buffer from each experimental condition were homogenized by passage through a QIAshredder spin column (Qiagen, Valencia, CA). The total deoxyribonucleic acid (DNA) and RNA fractions were further isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The isolated total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for RT-PCR analysis. The ABI Prism 7000 sequence detection system and relative quantification software (Applied Biosystems, Foster City, CA) were used for real-time analyses. The amplification for RT-PCR used the following Applied Biosystems primer and probe sets: 18S rRNA endogenous control; Hs00255341_ml (DIO2); Hs00829989_gH (GPX1); Hs00182418_ml (TR1); Hs01573474_g1 (COX2); Hs01555410_m1 (IL-1β); Hs00172855_ml Liver X receptor (LXRα) and Hs00173195_ml (LXRβ). The real-time reactions were performed in triplicate in a final volume of 25 μl.

mRNA quantification and statistical analysis

Raw mRNA expression values were computed by $2^{-\Delta Ct}$ formula[17] with values normalized to 18S rRNA, where ΔCt represents the difference in Ct (threshold cycle) number of the 18S rRNA gene and target genes. Results were derived from a total of four independent experiments for COX2, IL-1β, LXRα and LXRβ gene expression, performed in triplicate, using a total of four separate primary chondrocyte cell lines. The relative fold changes in mRNA expression levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ formula[17], between cells transfected with selenoprotein siRNA and the cells transfected with scrambled siRNA in different treatments. For the purposes of graphical presentation, the relative mRNA level in scrambled transfected cells without treatment (control group) was set at 100%.

Raw mRNA expression data were evaluated by paired *t*-test of the log transformed $2^{-\Delta Ct}$ values comparing subgroups ($n = 4$ in each group from four separate cell lines): (1) the scrambled transfected group without IL-1β treatment and individual selenoprotein siRNA transfected groups without IL-1β treatment, and (2) the scrambled transfected group with IL-1β treatment and individual selenoprotein siRNA transfected groups with IL-1β treatment. *P* values less than 0.05 were considered significant after correction for multiple comparisons (Bonferroni).

Western blot quantification of protein knockdown

Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Membranes were blocked with 5% BSA in TBS/0.1% Tween 20 (TBS-T). Polyclonal primary antibodies against DIO2, GPX1 and TR1 were obtained from Abcam (Cambridge, MA). A monoclonal antibody against α-tubulin (Sigma) was used as a normalization control at 1:10,000 dilution. Anti-rabbit and anti-mouse IgG-HRP (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were used at a 1:5,000 dilution. The resulting films were scanned using CanoScan LiDE 70 (Canon, Lake Success, NY) and the band intensities were quantified using Adobe Photoshop CS and Image J (National Institutes of Health, Bethesda, MD).

Results

Suppression of selenoproteins by siRNA

To examine the effects of DIO2, GPX1 and TR1 on IL-1β responses in primary human chondrocytes, we suppressed the expression of DIO2, GPX1, and TR1 with sequence specific siRNAs (Fig. 1). Compared with the scrambled siRNA control, the expression of the three selenoproteins was significantly reduced by the specific siRNAs: DIO2 siRNA reduced DIO2 mRNA expression by 56% ($P = 0.0004$) [Fig. 1(A)]; GPX1 siRNA reduced GPX1 mRNA