Process Biochemistry 44 (2009) 678–684

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

The dose dependent effects of betulin on porcine chondrocytes

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ARTICLE INFO

Article history:
Received 3 September 2008
Received in revised form 19 January 2009
Accepted 17 February 2009

Keywords:
Anti-oxidants
Betulin
Chondrocytes
Extracellular matrix
Osteoarthritis

ABSTRACT

The past two decades triterpenes have attracted attention because of their pharmacological potential, especially its anti-oxidant activity. The present study was aimed to evaluate the possible protective effects of the triterpene betulin on porcine chondrocytes. For this, the cells were treated with different doses of betulin (0.02, 0.32 and 5.12 μg/mL) and without betulin. Biochemical measures of necrosis, mitochondrial activity, DNA content and sulphated glycosaminoglycans (sGAG) were reported. In addition, the gene expression of extracellular matrix molecules (ECM), proteases and soluble factors were examined. The abundance of reactive oxygen species (ROS) was also reported. Among the concentrations tried 0.32 μg/mL of betulin was found to be optimum because it effectively promoted the gene expressions of type II collagen, aggrecan and inhibited the gene expression of matrix metalloproteinase 2 (MMP-2). The chemiluminescence (CL) assay indicated that betulin treated chondrocytes had better free radical scavenging activity than the chondrocytes cultured without betulin. Alcian blue staining revealed that the chondrocytes were functionally active and able to synthesis sGAG. The free radical scavenging activity ensures betulin as protectant of chondrocytes and it further maintains the proliferation and basic activities of chondrocytes.

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1. Introduction

Adult articular cartilage contains no blood vessel, neural network or lymphatic drainage, and has a limited intrinsic capacity to heal once damaged by injury or disease. Once articular cartilage is degenerated or damaged by extensive overuse, injury, inflammation, or ageing, intrinsic repair mechanisms might replace the tissue. Of the many diagnoses characterized as rheumatic disorders (e.g., gout, rheumatic fever, and lyme arthritis), rheumatoid arthritis (RA) and osteoarthritis (OA) account for the very most of the diseases. Conventional surgical treatments for joint lesions are not fully satisfactory, because they often result in a repair tissue that is fibrocartilaginous and exhibit poor mechanical properties [1]. The mechanical performance and physiological function of articular cartilage is dependent on the architecture of extracellular matrix that aids the chondrocytes to maintain the matrix. Cartilage contains a single mesenchyme-derived cell type, the chondrocyte, which secretes the cartilage extracellular matrix (ECM) [2]. The cell death of chondrocytes has been considered to be a prominent factor related to the progression of OA through the promotion of articular cartilage degradation. Several studies have demonstrated that the number of apoptotic chondrocytes is higher in severely affected OA cartilage than in less affected cartilage [3,4].

Reactive oxygen species (ROS) are one of the main factors in cartilage extracellular matrix degradation and death of chondrocytes. The often produced ROS in chondrocytes are superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$) which can further generate derivative radicals such as $ONOO^-$ and H_2O_2 [5,6]. Interaction of NO with $O_2^{\bullet -}$ produces the reactive species peroxynitrite which can lead to energy depletion and modification of proteins, lipids, carbohydrates, and nucleic acids. Thus, NO and related species have been implicated in atherosclerosis, arthritis, endotoxemia, and other pathologies [7]. The oxidative damage products such as peroxidated lipids, nitrotyrosine, and mutagenic 8-oxoguanine gradually accumulate in chondrocytes and disturb normal cellular functions until the chondrocytes cannot endure these structural changes [8].

Betulin (lup-20(29)-ene-3β,28-diol) is an abundant naturally occurring triterpene and it is found predominantly in bushes and trees forming the principal extractive (up to 30% of dry weight) of the bark of birch trees. Betulin and its related derivatives are reported as bioactive, anti-inflammatory, anti-tumor promoting and anti-viral activities [9,10]. The anti-oxidant activity of betulin was greatly explored by several researchers and has been used in Chinese herbal medicines for thousands of years to treat joint pain [11,12]. The anti-oxidant activity of *Ampelopsis brevipedunculata* was used to protect HepG2 cells from H_2O_2-induced oxidative stress stating the presence of triterpene like betulin [13]. However, the biological effects of betulin have not been elucidated in the chondrocytes of cartilage. To examine whether this is doable, we

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doi:10.1016/j.procbio.2009.02.008

investigated the effects of different doses of betulin on porcine chondrocytes *in vitro*. For this purpose, cell proliferation, matrix productivity and anti-oxidant activity of betulin treated and non-treated cells were studied.

2. Materials and methods

2.1. Isolation of chondrocytes

The retrieval and usage of animal tissues and cells (porcine) was approved by the Research Ethical Committee at the author's institute. Porcine chondrocytes were isolated upon 16-h incubation at 37 °C in 0.2% collagenase and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL neomycin [14]. The incubated porcine chondrocytes were resuspended and washed in phosphate buffer saline (PBS) and their viability was determined using trypan blue dye exclusion.

2.2. Growth and maintenance of chondrocytes

The chondrocytes were expanded by monolayer culture in DMEM medium containing 10% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL neomycin. At confluence, the cells were trypsinized and resuspended in DMEM at a concentration of 1×10^6 cells/mL. The chondrocytes were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.3. Betulin treatment

Betulin was purchased from Sigma chemicals, USA and 4× serial dilutions were made from 20.48 mg/mL to 1.95×10^{-5} μg/mL. Among the concentrations tried 0.02, 0.32 and 5.12 μg/mL of betulin was found to be effective in treating chondrocytes based on lactate dehydrogenase (LDH) and cell viability assy. For betulin treatment, the chondrocytes (passage 2; 70–80% confluence) were trypsinized and replated in sterile cell culture microplates for 7 days in DMEM medium. The experimental groups were prepared separately with aforesaid doses of betulin and incubated with chondrocytes for 7 days in a humidified 5% CO_2 incubator at 37 °C. The chondrocytes cultured in medium devoid of betulin served as control. All the experiments were performed in quadruplicate.

2.4. Cytotoxicity assay

The extent of cell damage and death was assessed by measuring LDH levels [15] using a commercial kit, CytoTox 96® Non-radioactive cytotoxicity assay (Promega, USA) and the optical density was measured at 490 nm by a MicroElisa reader (Emax Science Corp., USA).

2.5. Cell viability assay

As a measure of cellular proliferation and cell viability, cells were analyzed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For MTT assay, 1×10^4 cells/well were seeded onto 24-well plates. In brief, the cultured cells were washed thoroughly with PBS and then MTT solution (0.5 mg/mL) was added to the wells containing cells. The cells were incubated for 60 min at 37 °C in 5% CO_2/95% humidified air. Thereafter, the medium was removed and added to blue formazan crystals dissolved in isopropanol and the optical density was measured at 550 nm using a plate reading spectrophotometer.

2.6. Total DNA quantification

The Wizard® Genomic DNA purification kit (Promega, USA) was employed for DNA extraction from both experimental and control groups at the end of 7-day cultivation. The DNA content was measured by UV spectrophotometer (Beckman Instrument Inc., USA) at OD_{260}.

2.7. Quantification of glycosaminoglycans

The 1,9-dimethyl-methylene blue (DMMB) was used to determine the content of sulphated glycosaminoglycans. Briefly, the DMMB solution was prepared by dissolving 21.0 mg of DMMB (Aldrich-Chemie GmbH, Germany) in 5.0 mL of 95% ethanol (Sigma, USA), and the dissolved dye was further added with 2.0 g sodium formate (Sigma, USA) and made up to a final volume of 1000 mL with double distilled water. The solution pH was adjusted to 1.5 with 0.1 M formic acid. At the end of 7-day cultivation, 40 μL of aliquots of media were mixed with 250 μL of DMMB solution in a 96-well microplate at room temperature and the absorbance was measured at 595 nm using a microplate absorbance reader (Emax Science Corp., USA). The concentrations of sGAG in the samples were determined based on a standard curve generated by using chondroitin-6-sulphate (Sigma, USA).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from each sample was isolated by TRI REAGENT™ (T9424, Sigma, USA) according to the instructions of manufacturer and resulted RNA was tested on Beckman spectrophotometer for absorption wavelength at 260 and 280 nm separately. In complementary DNA (cDNA) synthesis, 8 μL of total RNA, 1 μL of 50 μM oligo$(dT)_{20}$ (Invitrogen, CA) and 1 μL of 10 mM dNTP (Clontech) were added and incubated at 65 °C for 5 min. After annealing at 65 °C for 5 min, the mixture was kept in ice for 2 min, followed by the addition of 2 μL 10× RT buffer, 4 μL 25 mM $MgCl_2$, 2 μL 0.1 M DTT, 1 μL RNaseOUT™, and 1 μL SuperScript™ III RT (200 U/μL) into the mixture and the polymerization was allowed to occur for 50 min at 50 °C. The reaction was terminated at 85 °C for 5 min. Finally, the synthesized cDNA was chilled on ice, and 1 μL of RNase H was added and kept at 37 °C for 20 min. RT-PCR was carried out with 10 μL of cDNA (100 ng), 12.5 μL of 2× SYBR® Green Master Mix (Applied Biosystems, USA), 0.75 μL of 1 μM forward primer solution, 0.75 μL of 1 μM reverse primer solution, and 6 μL of nuclease-free water (P1193, Promega Co.). Polymerase chain reaction (PCR) was performed by ABI PRISM 7900 Sequence Detection System with ABI PRISM 7900 Sequence Detection Software 2.2.2 (Applied Biosystems, USA). After the activation of SYBR® Green I dye for 2 min at 50 °C, the PCR cycles were performed with the following conditions: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s, 72 °C for 30 s. Finally, the samples were held at 37 °C for 10 min to inactivate the SYBR® Green I dye. In order to avoid pipetting errors, three replicates of each sample were amplified. The level of mRNA expression of target genes were normalized by dividing with GAPDH as the endogenous housekeeping gene and the fold differences were calculated using the $\Delta\Delta C_T$ ($2^{(-\Delta\Delta C_T)}$) method. The list of target genes and their primer sequences were presented in Table 1:

$$\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{control})$$
$$\Delta C_T(\text{sample}) = C_T(\text{sample; target}) - C_T(\text{sample; GAPDH})$$
$$\Delta C_T(\text{control}) = C_T(\text{control; target}) - C_T(\text{control; GAPDH})$$

2.9. Chemiluminescence assay

Luminol or lucigenin-enhanced chemiluminescence assay was performed to evaluate the ROS scavenging effect of chondrocytes treated with different concentrations of betulin. 0. 2 mM luminol (Fluka) was used to detect the presence of H_2O_2 or ROO and 0.2 mM lucigenin (Sigma, USA) was specifically used to detect the existence of $O_2^{\bullet -}$. The chemiluminescence measurements were carried out on a multi luminescence spectrometer (Tohoku Electronic Industrial Co., Ltd., Japan). Briefly, at the end of 7-day cultivation, the cells were washed twice with PBS and the specimens were placed in TEI CL sample chamber for background measurement for 180 s. After that, 1 mL of lucigenin or luminol was added separately to each specimen, and the CL counts were obtained for further 6 min at 10 s intervals. Finally, the results were given as the area under curve (AUC) for a counting period of 6 min, where the CL count was corrected by subtracting the AUC of the baseline.

2.10. Alcian blue staining

The phenotypic expression of chondrocytes and the evaluation of proteoglycans in ECM was assessed by Alcian blue staining [16].

Table 1
List and description of target gene analysis by RT-PCR.

Gene description	Abbreviation	Forward primer sequences	Reverse primer sequences
Collagen type I	COL1	5′-CAGAACGGCCTCAGGTACCA-3′	5′-CAGATCACGTCATCGCACAAC-3′
Collagen type II	COL2	5′-GAGAGGTCTTCCTGGCAAAG-3′	5′-AAGTCCCTGGAAGCCAGAT-3′
Aggrecan	AGC	5′-CGAAACATCACCGAGGGT-3′	5′-GCAAATGTAAAGGGCTCCTC-3′
Decorin	DCN	5′-GCATTTGCACCTTTGGTGAA-3′	5′-GACACGCAGCTCCTGAAGAG-3′
Membrane type 1 matrix metalloproteinase	MT1-MMP	5′-GCTGTGGTGTTCCAGACAAG-3′	5′-GGATGCAGAAAGTGATCTCG-3′
Matrix metalloproteinase 2	MMP-2	5′-GTTCTGGAGGTACAATGA-3′	5′-ACCACGGCGTCCAGGTTA-3′
Tissue inhibitor of metalloproteinase 1	TIMP-1	5′-AACCAGACCGCCTCGTACA-3′	5′-GGCGTAGATGAACCGGATG-3′
SRY (sex determining region Y)-box 9	SOX-9	5′-ACCTGAAGAAGGAGAGC-3′	5′-CACCGGCATGGGTACCA-3′
Interleukin 1 β	IL-1 β	5′-ACCTCAGCCCTCTGGGAGA-3′	5′-CCTCCTTTGCCACAATCAC-3′
Transforming growth factor β1	TGF-β1	5′-GCACGTGGAGCTATACCAGA-3′	5′-ACAACTCCGGTGACATCAAA-3′

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