Journal of Biomechanics 66 (2018) 165–169

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Short communication

Hydrostatic pressure as epigenetic modulator in chondrocyte cultures: A study on miRNA-155, miRNA-181a and miRNA-223 expression levels

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ARTICLE INFO

Article history:
Accepted 28 October 2017

Keywords:
microRNA
Chondrocyte
Osteoarthritis
Mechanical loading
Hydrostatic pressure

ABSTRACT

Mechanical stimuli and hydrostatic pressure (HP) play an important role in the regulation of chondrocytes metabolism. Growing evidence demonstrated the ability of mechanical loading to modulate the expression of microRNA (miRNA) involved in chondrocytes homeostasis and in the pathogenesis of osteoarthritis (OA).

The expression of miR-155, miR-181a and miR-223 in normal and OA chondrocyte cultures, and their potential modifications following exposure to three hours of a cyclic HP (1–5 MPa, frequency 0.25 Hz) were investigated. Also evaluated the expression of Chuk, regulator of the NF-kB pathway activation, which is a target gene of miR-223, was evaluated. Chondrocytes were collected immediately after pressurization (T0), and following 12, 24, and 48 h. Total RNA was extracted, reverse transcribed and used for real-time PCR.

At basal condition, a significant increase of miR-155 and miR-181a was observed in OA in comparison to normal cells; on the contrary, no differences in miR-223 and Chuk expression levels were detected between normal and OA chondrocytes. miR-155 and miR-181a resulted significantly downregulated immediately after pressurization (T0) in OA cells. The pressure effect on miR-155 and miR-181a levels was maintained over time. No modifications of miR-223 were observed in response to HP, while Chuk levels resulted significantly reduced at T0 and after 12 h. Pressurization did not cause any modifications in normal cells.

In conclusion, HP was able to modulate the expression of miRNA associated to OA pathogenesis. The preliminary results about Chuk response to pressure raised interest in its involvement in the possible HP induced NF-kB pathway modulation.

1. Introduction

microRNAs (miRNAs) comprise a class of noncoding RNAs responsible for repression of proteins synthesis, by degradation of target messenger RNAs (mRNAs) or inhibition of their translation. They are involved in the regulation of many cellular processes and in the development of some disorders (De Palma et al., 2017). miRNAs have been associated to the maintenance of healthy cartilage and regulation of chondrocytes homeostasis (Van Meurs, 2017). Furthermore, accumulating evidence reported the role of miRNAs in the pathogenesis of osteoarthritis (OA) (Cong et al., 2017).

Biomechanical force is a well known factor involved in the development and progression of OA (Jørgensen et al., 2017). Several *in vitro* studies demonstrated the role of HP and mechanical loading in regulating chondrocyte morphology and metabolism (Fioravanti et al., 2003, 2010; Furumatsu et al., 2013). Interestingly, mechanical stimuli have been found able to modify miRNAs expression in OA chondrocyte cultures (Jin et al., 2014; Yang et al., 2016).

In a recent study we demonstrated the effect of three hours of a cyclic HP (magnitude 1–5 MPa and frequency 0.25 Hz), in changing expression levels of miR-27a/b, miR-140, miR-146a and miR-365, some of the main miRNAs associated to OA, in affected chondrocytes (Cheleschi et al., 2017).

A recent bioinformatic analysis showed the OA-related roles of miR-155, miR-181 and miR-223 (Cong et al., 2017). So, the aim of

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https://doi.org/10.1016/j.jbiomech.2017.10.044
0021-9290/

this study was to investigate the gene expression of miR-155, miR-181a and miR-223, and their potential modifications following HP exposure, in normal and OA chondrocyte cultures. We also evaluated the expression of the nuclear factor kappa-B kinase subunit alpha (Chuk), target gene of miR-223, with an emerging involvement in OA.

2. Material and methods

2.1. Cell culture

Normal human articular cartilage was aseptically obtained from post-traumatic femoral heads of five subjects (three males and two females; mean age 61) with no known history of joint disease. OA human articular cartilage was obtained from the femoral heads of five patients (two males and three females; mean age 70) with hip OA defined by clinical and radiological ACR criteria (Altman et al., 1991), undergoing total hip replacement surgery. OA grade ranged from moderate to severe (Mankin degree 3–7) (Mankin et al., 1971), and OA chondrocytes derived from the adjacent area to OA lesion. The samples were provided by Orthopedic Surgery of Siena. The Ethics Committee of the Azienda Ospedaliera Universitaria Senese approved the use of human articular specimens (decision No. 726/07) and patients signed an informed consent.

Cartilage was aseptically minced into small pieces; chondrocytes were isolated using sequential enzymatic digestion (Cheleschi et al., 2017). The trypan blue viability test pointed out a 90–95% cell survival for both normal and OA chondrocytes. Cells were incubated for two weeks at 37 °C and 5% CO_2 in DMEM containing 10% fetal calf serum, 200 U/mL penicillin, and 200 μg/mL streptomycin.

In the first passage normal and OA chondrocytes were seeded in Petri dishes (35 × 10 mm) at a starting density of 1×10^5 cells until they became 85–90% confluent. The primary single layer of chondrocyte cultures was evaluated before starting the experiment (basal conditions) and after the HP exposure.

All experiments were performed in triplicate for each donor.

2.2. Pressurization system

The sinusoidal pressure (1–5 MPa, frequency of 0.25 Hz for 3 h) used for this study was generated by our prototype of pressurization system (Nerucci et al., 1998). Chondrocytes were collected at basal conditions and immediately after pressure (T0), as well as after 12 h (T12), 24 h (T24), and 48 h (T48) of retention in culture, following HP. Some dishes, maintained in the same culture conditions for the same time without receiving any pressurization, were used as controls.

2.3. MTT assay

Cell viability was evaluated at the different time points by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium Bromide MTT assay (Cheleschi et al., 2017).

The experiments were carried out on pre-confluent cell cultures to prevent contact inhibition influencing the results.

2.4. RNA extraction and RT-qPCR

Total RNA was extracted using TriPure Isolation Reagent according to the manufacturer's instructions (Roche). The concentration of RNA were evaluated by measuring the OD at 260 nm and the 260/280 and 260/230 ratios by Nanodrop-1000 (Celbio). Reverse transcription for miRNA was performed using the cDNA miScript PCR Reverse Transcription (Qiagen), while that for target gene by using QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions.

Table 1
Primers for real time PCR.

Gene	Cat. No. (Qiagen) oR sequence
miR-155	MS00031486
miR-181a	MS00008827
miR-223	MS00003871
Chuk	QT00040614
SNORD25	MS00014007
HPRT-1	For 5′-TTGCTTTCCTTGGTCAGG-3′ Rev 5′-ATCCAACACTTCGTGGGG-3′

MiRNAs and Chuk expression was analyzed by real-time PCR using miScript SYBR Green kit (Qiagen) and QuantiFast SYBR Green PCR (Qiagen) respectively. The primers are listed in Table 1. All qPCR reactions were performed by Light Cycler 1.0 (Roche) with Software Version 3.5.

Data analysis was performed by the ΔΔCt method using LinReg Software (Pfaffl, 2001).

Normalization was carried out considering SNORD-25 as housekeeping gene for miRNAs and HPRT-1 for Chuk.

2.5. Statistical analysis

The results were expressed as mean ± standard deviation of triplicate values.

Real-time PCR were evaluated by one-way (ANOVA) with a Tukey's post hoc test using $2^{-\Delta\Delta C}_T$ values for each sample. All analyses were performed using GraphPad Prism 6.1. A p-value <.05 was considered significant.

3. Results

3.1. Cell viability

MTT test showed that HP did not affect cell viability, neither immediately after the stimulus nor at the various considered time points, in OA and normal chondrocytes (Fig. 1). These data were confirmed by Trypan Blue test.

3.2. The effect of HP on miRNAs expression levels

The expression of miR-155, miR-181a, miR-223 and of the target gene Chuk in normal and OA chondrocytes were reported in Fig. 2. At basal condition, a significant increase of miR-155 and miR-181a was observed in OA in comparison with normal cells ($p < .05$), while no differences in miR-223 expression levels were observed (Fig. 2A). Also Chuk mRNA levels showed no significant differences between normal and OA chondrocytes (Fig. 2B).

Fig. 3 shows the effect of our HP on the expression of the analyzed genes. Statistically significant down-regulation of miR-155 ($p < .01$) and miR-181a ($p < .001$) immediately after pressurization (T0) were reported in OA chondrocytes compared with the unloaded controls (Fig. 3A and B respectively). Significantly decreased levels of miR-155 ($p < .01$) and miR-181a ($p < .05$) at all analyzed time points after HP compared with T0 were maintained. The reduced expression of miR-155 ($p < .01$) and miR-181a ($p < .05$) was also observed in OA cells exposed to HP at each time point in comparison with the corresponding OA control without receive any pressurization. On the contrary, miR-223 gene expression showed no modifications in response to HP (Fig. 3C). Chuk levels of OA chondrocytes have been found significantly reduced ($p < .01$) at T0 and T12 compared with load-free condition at T0 (Fig. 3D).