Redox Biology 14 (2018) 492–498

Contents lists available at ScienceDirect

Redox Biology

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

Mechanically induced autophagy is associated with ATP metabolism and cellular viability in osteocytes *in vitro*

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ARTICLE INFO

Keywords:
Osteocyte
Fluid shear stress
Autophagy
ATP
Cell survival

ABSTRACT

Both mechanical loading and intracellular autophagy play important roles in bone homeostasis; however, their relationship remains largely unexplored. The objectives of this study were to determine whether osteocytes undergo autophagy upon fluid shear stress (FSS) loading and to determine the correlation between mechanically induced autophagy and ATP metabolism. Autophagic vacuoles were observed by transmission electron microscopy (TEM) in osteocyte-like MLO-Y4 cells subjected to FSS. Increased autophagic flux was further confirmed by the increased amount of the LC3-II isoform and the degradation of p62. Fluorescent puncta distributed in the cytoplasm were observed in the GFP-LC3 transformed cells subjected to FSS. Furthermore, FSS-induced ATP release and synthesis in osteocytes were attenuated by inhibiting autophagy with 3-MA. After FSS exposure, a high ratio of cell death was observed in cultures pretreated with 3-MA, an autophagy inhibitor, with no significantly different Caspase 3/7 activity. Our results indicated that FSS induces protective autophagy in osteocytes and that mechanically induced autophagy is associated with ATP metabolism and osteocyte survival. From the clinical perspective, it may be possible to enhance skeletal cell survival with drugs that modulate the autophagic state, and the autophagy-related pathway could be a potential target for the prevention of ageing-related bone disorders.

1. Introduction

Osteocytes, as the terminally-differentiated cells, are embedded within the mineralized bone matrix and can be away from the vascular supply up to 200–300 μm. Long-term survival and mechanosensitivity are the characteristics of osteocytes [1]. Moreover, the transport system through which osteocytes obtain nutrients for survival consists of the lacunar-canalicular system (LCS), which is very narrow with the annular fluid space in the canaliculi on the order of 100 nm, suggesting high resistance to mass transport in bone [2,3]. This structural constrain may result in a hypoxic and relatively nutrient-poor environment for osteocytes embedded in bone matrix. How osteocytes maintain a prolonged lifespan and continually respond to loading in such a harsh environment deserves careful exploration.

Autophagy is an evolutionarily conserved protein degradation pathway that facilitates to the recycling of damaged organelles, protein aggregates, and unwanted proteins to maintain cellular homeostasis [4,5]. During the period of starvation, cells, through the autophagy pathway, recycle cytosolic components for energy generation to overcome stressful stimuli [6]. Autophagy is thus vital to cells, particularly terminally-differentiated cells, for both quality control and responses to external and internal stressors [7]. There is limited evidence to suggest that the induction of autophagy may be beneficial for osteocyte survival and offer protection against bone loss [8]. Recently, osteocyte autophagy was observed *in vitro* and *in vivo*. The *in vivo* immunolocalization of microtubule-associated protein light chain 3 (LC3) demonstrated that osteocytes at a distance from the Haversian canal in cortical bone are autophagic [9]. This finding was consistent with an *in vitro* study [9] that autophagy is induced following nutrient deprivation and hypoxic culture of the pre-osteocyte-like MLO-A5 cells. Low dose glucocorticoids can induce the development of autophagy and preserve the viability of osteocytes [10]. Onal et al. demonstrated that the suppression of autophagy in osteocytes *via* conditional knock-out of the autophagy-essential gene Atg7 causes a low bone mass in young adult mice that resembles the effect of ageing on the skeleton. The findings of these authors suggest an underlying connection between autophagy and skeletal ageing, and the notion that a decline of autophagy with ageing may contribute to ageing-related low bone mass [11]. Therefore, autophagy could be a critical process for maintaining osteocyte survival in response to environmental stresses.

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http://dx.doi.org/10.1016/j.redox.2017.10.021
Received 15 September 2017; Received in revised form 17 October 2017; Accepted 25 October 2017

Recently, the mechanosensitive autophagy response was observed in several cell types [12–15]. King et al. noted that the autophagosome level increases rapidly in cells upon compression (0.2–1 kPa) and reverts to the basal levels after 90 min [12]. Simulated microgravity conditions induce autophagy in pre-osteoclastic cells and enhance osteoclast differentiation [14]. Hep3B hepatocarcinoma cells exposed to a flow shear stress of 0.5–12 dyn/cm^2 exhibited autophagy and enhanced resistance to shear stress [13]. Tension-induced chaperone-assisted selective autophagy of the cytoskeleton is essential for mechanotransduction in muscle and immune cells [15]. These studies suggest that autophagy is responsible for cellular responses to mechanic stimuli. Since osteocytes experience mechanical stimulations *in vivo* and they are mechano-sensitive, an intriguing question to be answered is whether osteocytes undergo mechanically induced autophagy, and if so, what the role of this process is for osteocyte function.

In the present study, we investigated the osteocytes autophagy response to flow shear stress, with a particular attention on the effects of autophagy on ATP metabolism and cell viability.

2. Materials and methods

2.1. Cell culture

The osteocyte-like MLO-Y4 cell line (kindly provided by Dr. Lynda F. Bonewald) [16], was cultured on type I rat tail collagen-coated glass plates in α-modified Eagle's Medium (α-MEM) supplemented with 5% fetal bovine serum (FBS) and 5% calf serum (CS) in a 5% CO_2 incubator at 37 °C.

2.2. Fluid flow stimulation

The MLO-Y4 cells were seeded onto collagen-coated glass slides (7.5 cm × 2.5 cm × 1.0 cm) at a density of 5000/cm^2 and incubated in an incubator. The cultured cells were allowed to reach up to ~80–85% confluency. The slides were then placed in a parallel plate flow chamber for exposure an oscillatory fluid shear stress (FSS) of 12 dyn/cm^2 at a frequency of 1 Hz, which was predicted to be within the ranges of interstitial fluid flow experienced by osteocytes in the bone microenvironment [17–20]. Osteocytes were exposed to FSS loading for 0.5, 1, 1.5, 2 or 2.5 h at 5% CO_2 and 37 °C. Flow durations longer than 2.5 h were not used in this study due to the concern of cell viability and cell lifting under hypoxic conditions [19]. Following flow stimulation, the autophagy, ATP synthesis and release by the MLO-Y4 cells were analyzed. To measure cell viability, the cells were left in the culture medium for another 12 h following the flow stimulation. Moreover, to compare the effects of FSS with or without autophagy, one group of MLO-Y4 cells were pre-treated with 3-methyladenine (3-MA, 5 mM), a chemical inhibitor of autophagy, for 1 h, followed by FSS for 2 h in the presence of the same reagent. Static osteocytes controls were not subjected to FSS (0 h) and remained cultured in a 5% CO_2 incubator at 37 °C.

2.3. Detection of autophagic vacuoles with TEM

The cells were trypsinized, centrifuged, and fixed in a solution containing 2.5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate (pH7.4), and 8 mM $CaCl_2$. The cells were post-fixed with 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Ultra-thin sections were cut with an ultra-microtome, stained with uranyl acetate and lead citrate, and visualized using the Hitachi-600 transmission electron microscope (Hitachi).

2.4. Monitoring autophagic flux with western blotting analysis

To monitor the autophagic flux in the MLO-Y4 cells, a western blotting assay of endogenous LC3 and p62 proteins was performed as described previously [21]. The cells were collected and then washed with cold PBS and lysed with cold RIPA lysis solution (Santa Cruz Biotechnology, Santa Cruz, CA). Fifty micrograms of protein per lane were loaded on a 12% SDS-PAGE gel and resolved at 200 V. First, the conversion of LC3 from its cleaved cytosolic (LC3-I) form to its lipidated autophagosomal membrane-bound (LC3-II) form, a process indicative of autophagosome formation, was assessed. The anti-LC3 antibody (a rabbit polyclonal antibody, PN ab4894, Abcam, Cambridge, MA) was diluted (1:1000) prior to use. The level of p62, a preferential substrate that is degraded by autophagy, were also used to monitor the autophagic flux using the anti-p62 antibody (a rabbit polyclonal antibody, PN ab4894, Abcam, Cambridge, MA) diluted to 1:1000. The protein levels were expressed as the average level of LC3 or p62 normalized to the level of GAPDH (control).

2.5. GFP-LC3 puncta assay

An autophagy reporter cell line was generated to monitor the autophagosome distributions in the MLO-Y4 cells. The phrGFP expression vector (Invitrogen) was used to produce a hrGFP-tagged microtubule-associated protein 1-light chain 3β (MAP1-LC3β). EcoR1 and BamH1 restriction sites were added to human MAP1-LC3β cDNA (NCBI accession: BC067797) for in-frame fusion of hrGFP to the C-terminus. The MLO-Y4 cell line was transfected with a construct containing the GFP-LC3 sequence using Lipofectamine 2000 in OptiMEM transfection medium according to the manufacturer's protocol (Invitrogen). The transfected cells were maintained in G418 (Invitrogen)-containing medium to screen out the stably transfected cells (GFP-LC3 cells). The GFP-LC3 cells were FSS-loaded and then analyzed for the presence of green fluorescent puncta in the cytoplasm by fluorescence microscopy.

2.6. ATP synthesis and release

The ATP contents in culture media and inside cells were measured using the luciferin-luciferase method (Sigma). The ATP content in the media was used to evaluate the ATP released from the MLO-T4 cells. The combined ATP contents in the media and inside cells were used to assess the total ATP synthesis. The media or cell extracts were mixed with dilution buffer containing luciferase, and the mixture was then transferred into 96-well scintillation microplates. The resultant luminescence, which was detected using a MicroBeta 1450 scintillation and bioluminescence detector (MicroBeta), reflected the ATP concentration. The luminescence data were collected and reported as the ATP contents (nmol). The ATP levels were normalized to the total cellular protein concentrations as determined by BSATM protein assay kit (Pierce) for each plate.

2.7. Cell viability assays

Cell viability and apoptosis were assessed by using the Trypan blue exclusion method and Caspase-3 assay kits (Invitrogen Cat# E13183). The cells underwent FSS loading with or without 3-MA, followed by culture in fresh MEM medium for additional 12 h. The cells were then trypsinized and re-suspended in 1 mL serum-free media. After adding an equal volume of 0.4% trypan blue dye (Sigma), the cells were incubated for 4 min at 37 °C. The numbers of stained (dead cells) and non-stained (survival cells) cells were counted using a hemocytometer. The Caspase-3 assay kit was used to detect the onset of apoptosis by assaying Caspase-3/7 activity per vendor's instruction.

2.8. Statistical analysis

All statistical analyses were performed using the Prism software package (GraphPad Software). All data are shown as means ± SE. Measurements at various time points were analyzed by analysis of variance and two-tailed *t-tests* for pair comparisons. Statistical