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

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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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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

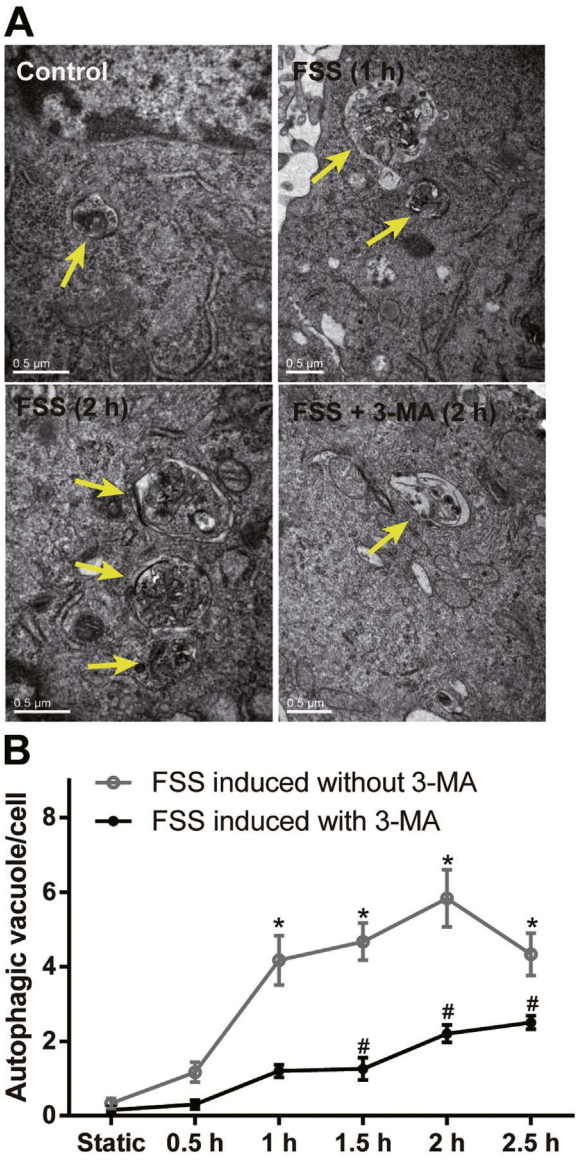

Fig. 1. Effects of FSS on autophagosomes in MLO-Y4 osteocytes. (A) Representative TEM images of MLO-Y4 osteocytes exposed to 12 dyn/cm^2 FSS treated with or without 3-MA. The arrows indicate autophagosomes; (B) The average number of autophagosomes in the FSS-loaded osteocytes increased over time with or without 3-MA, but the peak changes in response to FSS occurred at 2 h for the FSS-loaded without 3-MA group. *: $p < 0.05$ (compared with the Static Controls without 3-MA), #: $p < 0.05$ (compared with the Static Controls-treated with 3-MA). The values are presented as the means ± the SE of three independent experiments.

significance was defined as $p < 0.05$.

3. Results

3.1. Effects of FSS on the accumulation of autophagosomes in MLO-Y4 osteocytes

The double-membrane autophagosomes in the MLO-Y4 cells were observed by TEM (Fig. 1A). Compared with the static control (non-FSS), the average number of the autophagic vacuoles per cell significantly increased due to FSS stimulation. After 1 h of FSS, the cells contained an average of 4.2 vacuoles per cell. The highest average number of autophagosomes in 2-h loaded cells was 5.8 per cell (Fig. 1B). Compared with the experimental groups that underwent FSS loading, the addition of 3-MA in the media resulted in a decrease in autophagosomes over time (Fig. 1B).

3.2. Effects of FSS on LC3 and p62 in the MLO-Y4 osteocytes

The expression levels and the conversion ratio of endogenous LC3-II to LC3-I, important markers of autophagy, were detected by immunoblotting. The FSS induced an increase of the ratio of LC3-II to LC3-I in the MLO-Y4 cells (Fig. 2A and B). The conversion ratio for the 2 h loaded group (2.4 ± 0.3) was greater than that for the 1 h loaded group (1.8 ± 0.2). The treatment of 3-MA resulted in decreased expression levels of LC3-II to LC3-I, and a 1.6-fold reduction in the conversion ratio for the 2 h loaded group compared with the non-treated 2 h loaded group (Fig. 2B). However, FSS was still able to increase autophagy in the MLO-Y4 cells with the 3-MA treatment at the current concentration.

Besides the increased LC3-II isoform level, decreased p62 is another marker for activated autophagic flux. As illustrated in Fig. 2C and D, the p62 level was significantly decreased in the 1- and 2-h- FSS-loaded groups. This decreasing trend was significantly reversed by the addition of 3-MA, and a greater accumulation of p62 occurred relative to the FSS-loaded-alone cells. The 3-MA treated group exhibited an increased p62 level in the static control. Therefore, our data indicated that FSS induced the activation of autophagic flux.

3.3. GFP-LC3 puncta increased in FSS induced MLO-Y4 osteocytes

The GFP-LC3 transformed cells were exposed to FSS for 0.5–2.5 h and then analyzed by fluorescence microscopy for the visualization of green fluorescent puncta. As illustrated in Fig. 3, sparsely punctuated fluorescence was also seen in the static culture with an average of 0.7 puncta per cell, suggesting the presence of basal level of autophagy. Autophagosomes distributed in the cytoplasm were consistently observed in the cells loaded with FSS. The most abundant puncta was observed in the 2 h-loaded cells with an average of 12.5 puncta per cell. The 3-MA treatment partially inhibited the FSS-induced autophagy as shown in the decreased number of fluorescent puncta compared with the non-3MA treated FSS-loaded cells (Fig. 3).

3.4. FSS-induced autophagy affected ATP release and synthesis

The ATP released into the media and that contained in the cells were measured using biochemical assays (Fig. 4A and B). The FSS stimulated an acute surge in ATP release from the MLO-Y4 cells relative to the static control culture cells, and a gradually decreasing trend with time was observed afterward. The total ATP content was raised markedly by the onset of FSS loading; however, no significant differences were observed among 1-h, 1.5-h and 2-h loading groups. The ATP release and synthesis in the 3-MA treated cells under FSS loading were significantly lower than those of the 3-MA non-treated but FSS loaded groups. The 3-MA treatment had no significant effect on ATP release or production in static controls. These results illustrated that FSS-induced autophagy affected the ATP release and production of osteocytes *in vitro*.

3.5. FSS-induced autophagy affected cell viability

To determine whether the cell viability was affected by FSS-induced autophagy activation, the ratios of cell death and Caspase 3/7 activity were measured. Given that significant cell death could only be detected after some time following initial insults, the FSS-loaded cells were cultured for 12 h followed FSS prior to the viability assays. The percentage of dead cells in the FSS-loaded group was higher than that in the static group (Fig. 4C). FSS loading with the 3-MA treatment resulted in a strikingly higher percentage of death than that observed in the static culture and FSS-loaded-alone culture. However, the 3-MA treatment in the static culture only induced a slight increase in dead cells. To further investigate whether the cell death resulted from apoptosis, the Caspase 3/7 activity was measured. The results are displayed in Fig. 4D. After FSS exposure, the Caspase 3/7 activity was increased, but co-

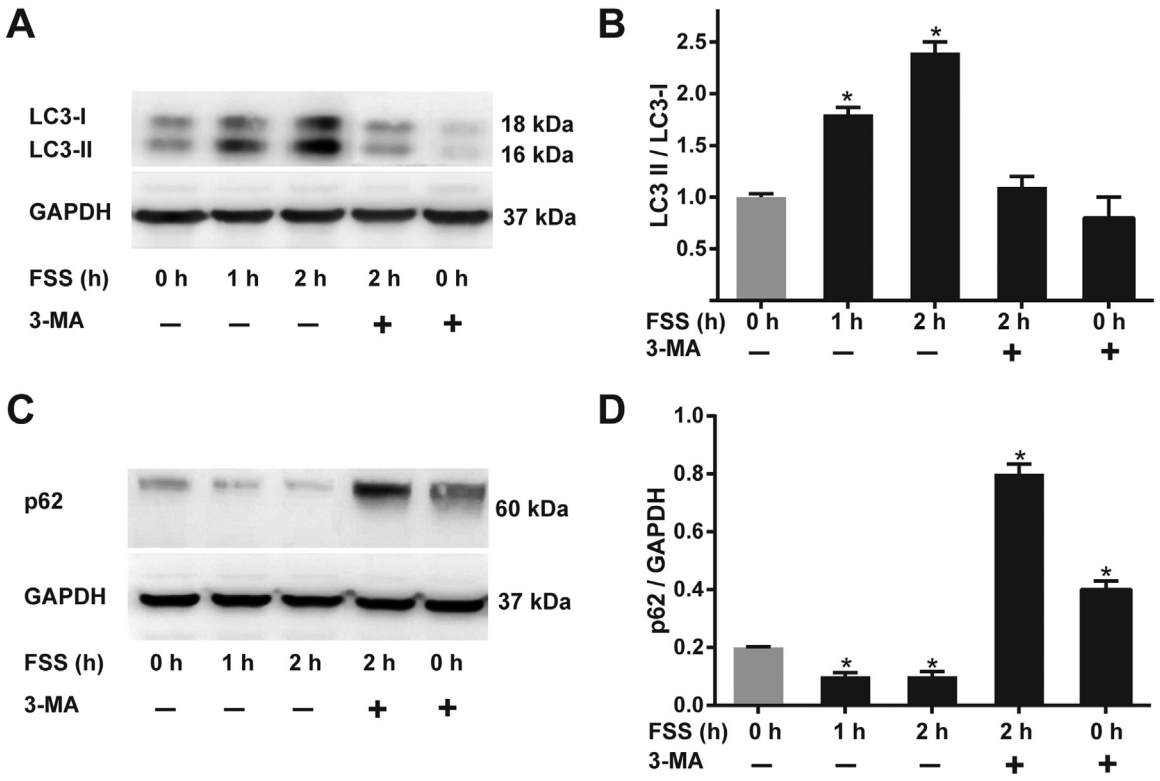

Fig. 2. LC3 conversion and p62 degradation assays. (A) The LC3-II isoform was increased in the FSS-loaded cells except for the 3-MA-treated cells. (B) The ratio of LC3-II to LC3-I based on the quantification of the bands in the immunoblot is shown. (C) The p62 levels were decreased in the FSS-loaded cells and increased when the cells were treated with 3-MA. (D) The intensity of the p62 band normalized to DAPDH is shown. *: $p < 0.05$ (compared with the Static Controls without 3-MA). The values are presented as the means ± the SE of three independent experiments.

treatment with 3-MA did not result in any significant difference from the FSS-loaded cells without 3-MA treatment. These results suggest that autophagy activation is beneficial for the maintenance of the viability of cells that are exposed to fluid shear stress; however, the increased cell death was not due to enhanced apoptosis activity when autophagy was inhibited.

4. Discussion

The characteristics of the osteocytes, such as terminal differentiation, longevity, and their relative hypoxic and nutrient-poor environment, suggest that autophagy may play a crucial role in their survival and function. Because osteocytes are primary sensors to mechanical loading [1,22], the objective of this study was to determine whether autophagy response occurs and plays a role in mechanically loaded osteocytes. During locomotion and other physical activities, bone experiences various mechanical loading, which deforms bone matrix and drives interstitial fluid flow within the porous bone [23]. Shear stress and/or fluid drag forces generated by interstitial fluid flow in the osteocyte LCS system influences are believed to the mechanical signals to which osteocytes respond [2,24], initiating downstream mechanotransduction pathways and bone remodeling processes [1,22]. A numerical model by Weinbaum et al. estimated the FSS induced by physiological mechanical loading within the range of 0.8–3 Pa [24]. Our recent FRAP experiments showed a higher peak FSS (5 Pa) in the canaliculi [25], although the magnitude of FSS experienced by the cell bodies in larger lacunae is expected to be lower.

In the current study, MLO-Y4 cells were exposed to FSS at a physiological magnitude and frequency (12 dyn/cm^2 and 1 Hz). TEM images showed the presence of autophagosomes, confirming our hypothesis that autophagy occurs in osteocytes due to FSS. This conclusion was further supported by the considerable number of fluorescent puncta observed in the GFP-LC3 transfected cells. In addition, western assays provided quantitative evidence of activated autophagy after FSS stimulation as demonstrated by the increased conversion ratio of LC3-II to LC3-I and decreased p62 in fluid sheared osteocytes. Collectively, our study demonstrated that autophagy is one of the mechanobiological effects produced by FSS *in vitro*. Our results are consistent with other *in vitro* studies where the more differentiated osteocyte-like cells MLO-Y4 exhibited elevated autophagy compared to the MSCs, osteoblasts and less differentiated MLO-A5 cells [26]. *In vivo*, autophagic osteocytes were found more often at a distance from the Haversian canal than bone surface osteoblasts [9]. Autophagy thus may be a coping mechanism by which the terminally differentiated osteocytes endure their particular living environment encased in bone matrix.

Osteocytes have been characterized as metabolically inactive cells because of their small size, small volume of cytoplasm, few organelles, and permanently immured environment [27,28]. Growing evidence indicates that osteocytes are metabolically active cells, and play an important role in bone metabolism [27]. Autophagy is thought to generate energy and synthesis materials during starvation or periods of intense metabolism [6]. It is known that cellular energy metabolism can be altered by mechanical stimulus [29,30]. He et al. [31] showed that exercise-induced autophagy in the muscle of mice, while mice deficient with autophagy induction exhibited decreased endurance and altered glucose metabolism during acute exercise. Therefore, we suspected that autophagy may be responsible for energy metabolism in osteocytes, which would allow osteocytes to adapt for stressful environments and particularly nutrient-poor environments.

ATP is a direct cellular energy source and potent signaling molecule and is responsible for a wide variety of cellular activities, including cell proliferation, metabolism, and survival. ATP production and release are altered by mechanical loading, which may subsequently affect cellular activities [29,30]. Herein, we determined whether ATP changes in osteocytes are accompanied by FSS-induced autophagy. Undoubtedly, FSS produced a rapid and significant increase in ATP release. However, the

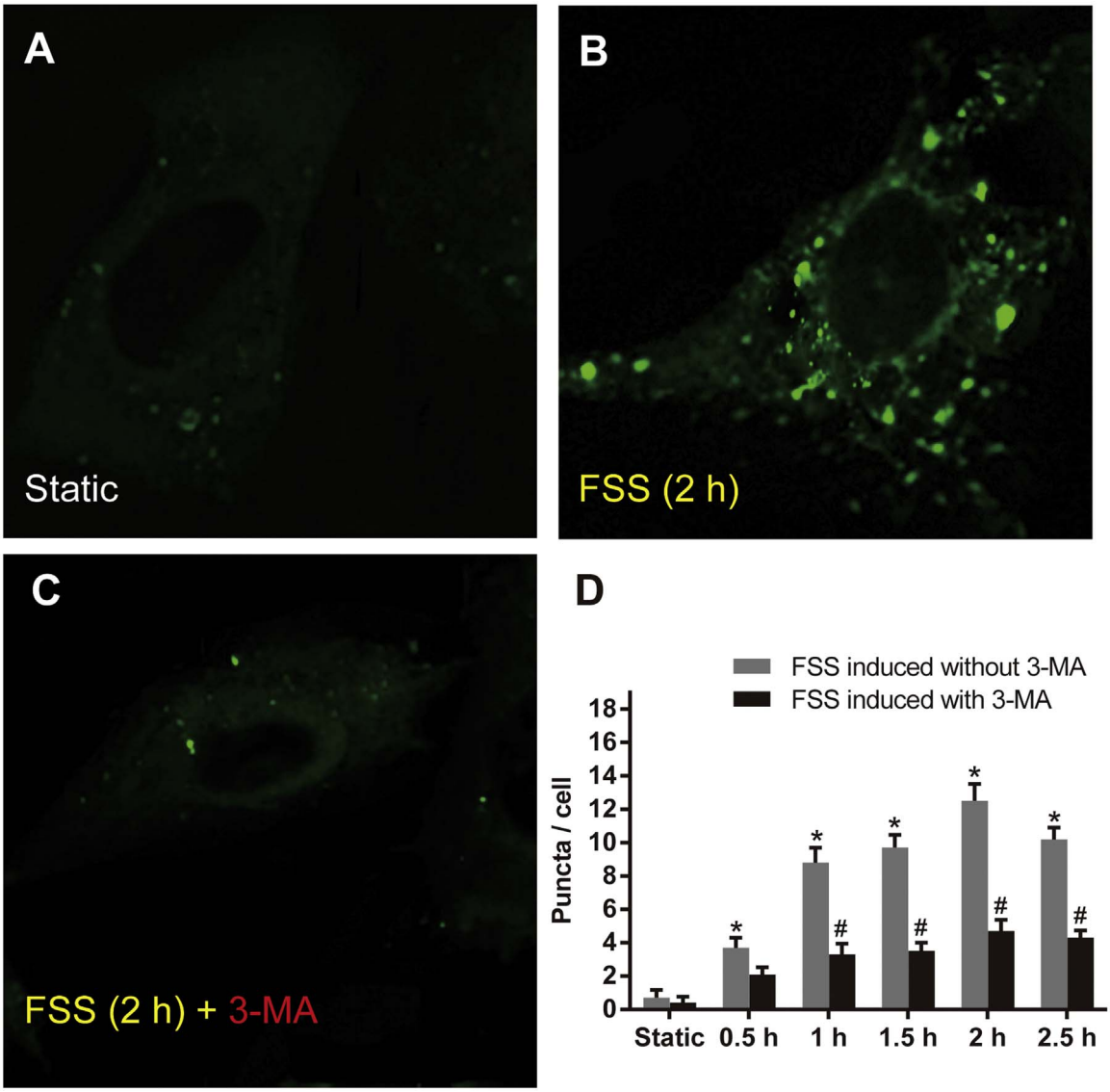

Fig. 3. GFP-LC3 fluorescent puncta formation assays. (A) Static control. (B) Cell FSS-loaded for 2 h. (C) Cells that were FSS-loaded for 2 h in the presence of 5 mM 3-MA. (D) The mean numbers of GFP-LC3 puncta per cell under FSS loading with and without 3-MA over time. *: $p < 0.05$ (compared with the Static Controls without 3-MA), #: $p < 0.05$ (compared with the Static Controls-treated with 3-MA). The values are presented as the means ± the SE of three independent experiments.

inhibition of autophagy with 3-MA attenuated the shear-induced release of ATP but did not affect the basal ATP release in the static cells. Regarding the ATP release pathway, Fader et al. found that ATP can be loaded in autophagic vacuoles and released into the extracellular space by fusion with the plasma membrane in cells in response to starvation [32]. Mechanically induced autophagic vacuoles might be the ATP-loaded carriers, and the decreased autophagy due to 3-MA treatment may partially block the ATP release. A previous study demonstrated that FSS-induced ATP vesicular release is Ca^{2+}-dependent, and ER calcium mobilization induces autophagy [32,33]. The calcium signaling may bridge the FSS-induced ATP release and autophagy, but thus far, there are no affirmative results to support the conjecture.

As a direct cellular energy source, the production of ATP is an indication of cell metabolism, of which the autophagy is a major contributor [6,30]. In cardiomyocytes treated with hypoxia for 30 min, a 6-fold decrease in the ATP level was induced, and the occurrence of autophagy was observed. In our view, mechanically induced autophagy enhances preservation of ATP and promotes osteocyte survival. This study demonstrated that the total ATP production was increased upon the FSS loading, and the inhibition of autophagy resulted in a decrease in ATP production in the FSS-loaded cells but had no significant effect on the static control cultures. Mechanical loading promotes extracellular matrix biosynthesis by osteoblasts, which requires energy in cells [34]. However, osteocytes are not responsible for matrix biosynthesis. Instead, osteocytes are responsible for detecting the mechanical load and the goal for them may just be living longer as the sensors. In such a context, autophagy may provide osteocytes with energy that could allow osteocytes to better respond to mechanical stress, since ATP can also serve as a second messenger to transmit the loading information to other adjacent osteocytes and osteoblasts. Autophagy also promotes cell survival, as demonstrated in our cell viability assays. Although autophagy has been associated with type II programmed cell death [35], the FSS-induced autophagy was not severe enough to induce apoptotic activity in the MLO-Y4 osteocytes. The slightly increased cell death ratio (resulting from 3-MA-alone treatment), might have been due to the effect of 3-MA on other cellular processes [36].

In summary, the occurrence of autophagy induced by fluid shear stress stimulation occurs in osteocytes *in vitro*. The induced autophagy, which promoted cell survival, may be an adaptive cellular response to mechanical stress, which is related to ATP synthesis and release. Literature data show reduced autophagic ability and increased cell

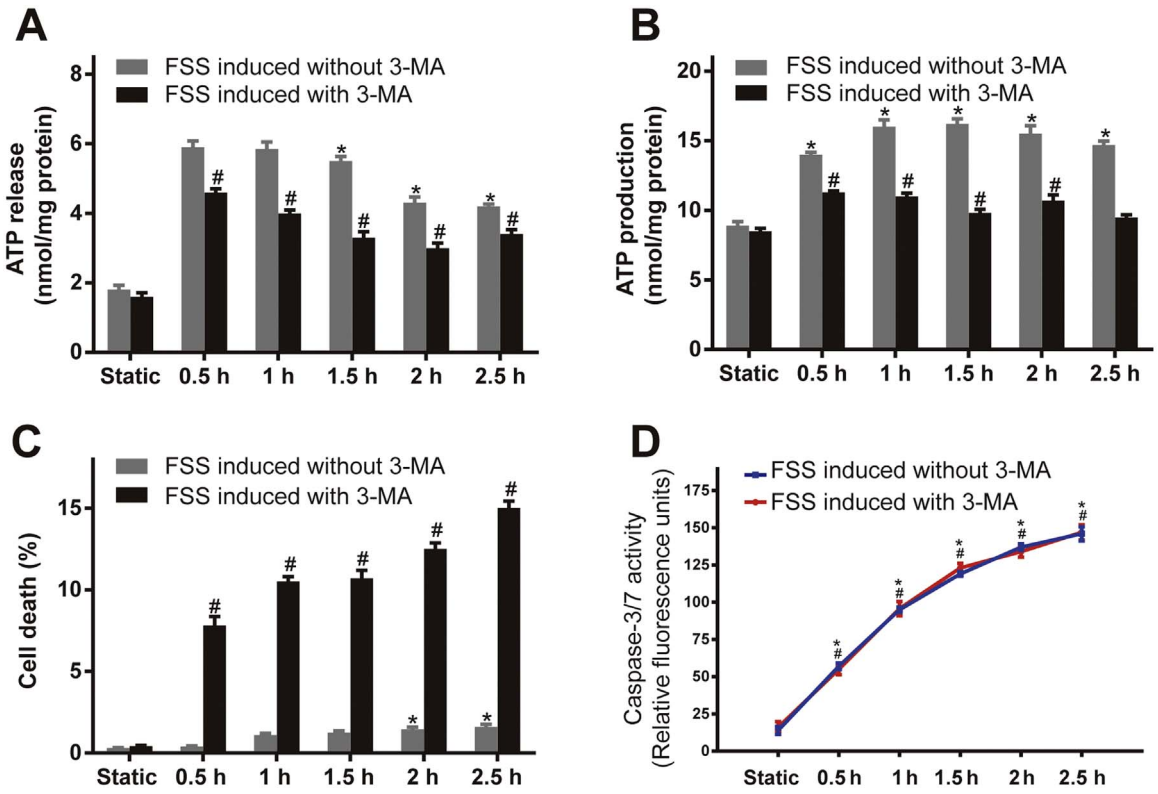

Fig. 4. Effects of mechanically induced autophagy on ATP release, synthesis, and cell viability. (A) FSS-activated ATP release, and the increasing trend was attenuated by combined treatment with 3-MA. (B) Total ATP content was enhanced upon FSS, 3-MA treatment reduced the increasing trend. (C) Inhibited autophagy with 3-MA reduced cell viability in sustained cultures after FSS loading. All the FSS-loaded cell groups were cultured for an additional 12 h before the cell viability assay experiment was performed. (D) Caspase-3/7 activity increased with FSS loading time, but no difference between the presence and absence of 3-MA was noted. The data were read from 20-min of Caspase plate exposure. *: $p < 0.05$ (compared with the Static Controls without 3-MA), #: $p < 0.05$ (compared with the Static Controls-treated with 3-MA). The values are presented as the means ± the SE of three independent experiments.

death in aging osteocytes [37]. Combining our findings, we speculate that enhancing osteocyte survival through drugs that modulate the autophagic state and the autophagy-related pathways may be useful for the prevention of age-related bone disorders.

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Conflict of interest statement

The authors declare no potential conflict of interest.

Author's contributions

Study design: BZ, BW; The experiments and data analysis: BZ, RH, TL, YZ, BW; Manuscript preparation: BZ, ZZ, LW, BW; Manuscript approval: all authors; BW take responsibility for the integrity of the data analysis.

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