Oscillatory Fluid Flow Induced Autophagy in Bone Cells

Yanghui Xing, Henry J. Donahue, Jun You.
Division of Musculoskeletal Sciences, Department of Orthopaedics and Rehabilitation, The Pennsylvania State University College of Medicine, Hershey, PA, USA.

Disclosures:
Y. Xing: None. H.J. Donahue: None. J. You: None.

Introduction: Autophagy is a basic catabolic mechanism which involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery. The coordinated action of osteocytes, osteoblasts, and osteoclasts is necessary for the maintenance of bone homeostasis (1). Mechanical loading of bone results in a variety of biophysical signals, such as oscillatory fluid flow that affects bone cellular metabolism. However, whether mechanical loading directly induces bone cell autophagy is unknown. In addition, intracellular calcium mobilization has been demonstrated to play an important role in mechanical loading induced osteoblastic metabolism. The role of calcium in bone cell autophagy is unclear. We hypothesize that mechanically induced oscillatory fluid flow induces bone cell autophagy via a mechanism requiring intracellular mobilization.

Methods: Cell Culture- murine osteocytic MLO-Y4 cells were cultured in minimal essential medium containing 5% fetal bovine serum, 1% penicillin and streptomycin and maintained in a humidified incubator at 37°C with 5% CO2. All cells were sub cultured for 2 days prior to experiments, on glass slides for fluid flow experiments. A 5000 cells/cm² seeding density was used for all experiments. Cells were exposed to oscillatory fluid flow in the same medium for all experiments. A 50nM final concentration of thapsigargin was added to culture to reach autophagy levels. A 5000 cells/cm² seeding density was used for all experiments. Primary Cell Acquisition and Culture- Mice at 6 to 12 weeks were sacrificed and marrow cells from the femurs and tibias were flushed out with a syringe needle system. The bone marrow cells were collected and cultured in cell differentiation medium for experiments. The femurs and tibias were incubated with 0.01 percent collagenase for 70 minutes and chopped into small chips to a size of 1-2 mm in diameter. Then the bone chips were cultured in maintenance medium with an anti-contamination agent for 5 days. The medium was switched into normal maintenance media for another 21 days. Cells were trypsined and sub-cultured in larger culture plates to form primary osteoblastic cells. Fluid Flow Experiments- To expose cells to oscillatory fluid flow, slides were positioned in parallel plate flow chambers and connected to a servopneumatic materials testing device, oscillating at 1Hz, via glass Hamilton syringes and rigid wall tubing. Flow rate was monitored in real time with an ultrasonic flowmeter. Oscillatory fluid flow induces shear stresses associated with the loading and unloading of long bones during normal gait and was implemented at a physiological frequency of 1 Hz and 10 dynes/cm². Western Blot- To examine the effect of fluid flow on LC3 protein levels, MLO-Y4 cells were exposed to oscillatory fluid flow with a shear stress at 10 dynes/cm² for different time periods. Immediately after fluid flow, slides with cells were frozen in -80°C and cells were lysed at a later time with RIPA buffer supplemented with a protease inhibitor cocktail. The total protein concentration in cell lysates was measured with a BCA protein assay kit. 15ug protein from each sample was resolved by SDS-PAGE and transferred to PVDF membranes. The membrane was probed with LC3 antibody. GAPDH was used as control. Visualization of immunoreactive proteins was achieved employing an ECL detection system and membrane exposure to film. Densitometric analysis was carried out with Quality One image analysis software.

Results: Microtubule-associated protein light chain (LC3) is a major regulator of autophagosome formation, which remains associated with the mature autophagosomal membrane. Conversion of a cytosolic truncated form of LC3 (LC3-I) to its autophagosomal membrane-associated, phosphatidylethanolamine-conjugated form (LC3-II) is an indicator of autophagosome formation. We first examined the levels of LC3 in response to oscillatory fluid flow in MLO-Y4 osteocytes. We found that oscillatory fluid flow was able to induce LC3 level changes at 60, 90 120 min at a shear stress level of 10 dynes/cm² (1 Hz) in MLO-Y4 cells. The peak change in response to oscillatory fluid flow was at 90 min in (Fig 1). Next we examined the role of calcium in bone cell autophagy. Thapsigargin raises intracellular calcium concentration by blocking the ability of the cell to pump calcium into intracellular stores which causes these stores to become depleted. We found that thapsigargin treatment resulted in a decrease in autophagy as evidenced by a decrease of 2.1 and 2.4 fold in LC3 levels at 2 and 6 hours, respectively, in MLO-Y4 cells. However, oscillatory fluid flow was still able to increase autophagy in the presence of thapsigargin in MLO-Y4 cells. Finally, we collected primary bone marrow cells and osteoblastic cells from mouse long bones, and examined their LC3 expression levels. Our results indicated that the levels of LC3 in osteoblastic cells were 2.7 fold higher than those in undifferentiated bone marrow cells.

Discussion: Autophagy is an important degradative cellular process, fulfilling a wide range of roles in survival, homeostasis and adaptation. Mechanical loading plays an important role in regulating bone cell metabolism. However, the interaction between
mechanical loading and autophagy in bone cells is unknown. This study first demonstrated that oscillatory fluid flow as an important biophysical signal, increased autophagy in osteocytes, indicating an important role of mechanical loading in regulating osteocyte autophagy. Calcium signaling pathways have been demonstrated to be activated in response to fluid flow. Our thapsigargin results suggest that a calcium signaling pathway was involved in bone cell autophagy. However, mechanically induced autophagy may be independent of intracellular calcium mobilization in osteocytes. Moreover, our results demonstrated that more differentiated osteoblastic cells had higher levels of autophagy, suggesting a more important role of autophagy in osteocytes compared to undifferentiated osteoblastic cells.

**Significance:** To understand the molecular mechanisms of bone cell autophagy in response to mechanical loading will lead to novel targets for pharmacological intervention in bone diseases that have a mechanical component, such as osteoporosis, and improve progression of bone tissue engineering.

**Acknowledgments:** This study was supported by NIH AR054851.


![Image](image_url)

**Fig. 1** LC3 expression level changes in response to fluid flow.


**ORS 2014 Annual Meeting**

*Poster No: 1457*