Fluid shear stress changes cell morphology and regulates the expression of ATP6V1A and TCIRG1 mRNA in rat osteoclasts

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Abstract. Fluid shear stress (FSS) is a potent physical cell signal in the regulation of bone remodeling. Although the effects of FSS on bone cells of the osteoblastic lineage have been studied extensively, less is known about the direct effect of FSS on osteoclasts. In this study, we investigated the direct influence of FSS on rat osteoclasts isolated by a classic mechanical anatomical technique. Osteoclasts were exposed to an FSS of 2.9 dynes/cm^2 for a given period, or subjected to various magnitudes of FSS for 30 min. Cell morphology under FSS was observed and the expression of the mRNA of ATP6V1A and TCIRG1, two crucial subunits of the vacuolar H^+-ATPase gene, was analyzed by quantitative real-time PCR. Changes in osteoclast morphology were apparent after the application of FSS, including increased cell volume, strengthened refraction and decreased transmittance. Time-dependent and dose-dependent increases in ATP6V1A and TCIRG1 mRNA expression were seen in response to FSS within 30 min. However, a duration of FSS exceeding 30 min induced a significant decrease in ATP6V1A and TCIRG1 mRNA expression. FSS therefore appears to be a potent stimulus that is sensed by rat osteoclasts and results in significant changes in cell morphology and the gene regulation of ATP6V1A and TCIRG1. These effects might be strictly dependent on the duration of FSS.

Introduction

Maintenance of appropriate bone mass requires the coordination of bone resorption by osteoclasts and bone formation by osteoblasts. It is well known that mechanical loading controls this cell coupling (1), and that mechanical loads applied to bone tissue result in the deformation of skeletal tissue, which induces the pressurization of interstitial fluid and stimulates its movement along pressure gradients (2). Within the confined geometry of lacunar-canalicular and haversian systems, interstitial fluid flow imparts shear stresses upon cell membranes (3). *In vitro* studies have indicated that the predicted levels of shear stress are potent regulators of bone cell behavior (4).

The mechanisms of flow-induced remodeling have been partially elucidated by *in vitro* studies that subjected osteoblasts or osteocytes to fluid shear stress (FSS). It is generally accepted that osteocytes are the primary mechanosensory cells in bone and elicit different biomechanical responses, such as the release of a number of signaling molecules; these in turn modulate the activities of osteoblasts and osteoclasts (5-9). Furthermore, increasing data suggest that not only osteocytes, but also osteoblasts and osteoprogenitors, are responsible for perceiving and responding to fluid flow (10-14). For example, FSS has been shown to modulate the key factors expressed in the osteoblasts and stromal cells that control osteoclast recruitment (10,11). Osteoclasts, which reside in the bone marrow, are also possibly exposed to flow-induced shear stress. However, less is known about the direct effects of FSS on osteoclasts or the potential for osteoclasts to perceive and respond to fluid flow.

Osteoclasts are multinucleated giant cells that originate from hematopoietic precursor cells of the monocytic/macrophage lineage. Under normal conditions, osteoclasts are the only cells that function to resorb the mineralized bone matrix (15). This function is carried out by the active secretion of protons into the resorption lacuna through the activity of a specialized osteoclastic vacuolar H^+-ATPase (V-ATPase) present on the ruffled border membranes (15,16). Suppression of the gene expression of V-ATPase by antisense RNA and DNA molecules inhibits bone resorption (17), and V-ATPase-specific inhibitors reduce bone resorption *in vitro* (18) and *in vivo* (19). Thus, V-ATPase plays an essential role in osteoclastic function.

V-ATPase is composed of a peripheral V_1 domain responsible for ATP hydrolysis and an integral V_0 domain responsible for proton translocation (20,21). Subunit A, a

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protein located in the V_1 domain, is the catalytic nucleotide binding site of V-ATPase, and the knockdown of its gene (ATP6V1A) impairs acid secretion (22). Subunit 'a' is an integral membrane protein of V_0. Presently, four different isoforms of subunit 'a' have been identified, both in mice and humans, designated as a1, a2, a3 and a4. Among the four 'a' isoforms in mammals, a3 is a form that is enriched in osteoclasts and plays an essential role in bone resorption (20,21). Disruption of the mouse or human a3 gene (TCIRG1) results in severe osteopetrosis (23,24). Both subunit A (ATP6V1A) and a3 (TCIRG1) are highly expressed in osteoclasts, indicating their crucial roles in the resorption activity of the osteoclast.

Many *in vitro* studies have reported that FSS may regulate osteoclast differentiation in a co-culture system of osteoblasts and osteoclast precursors. This effect has been attributed to the modulation of key factors expressed in osteoblasts (10,11), while few studies have considered the potential direct effects of FSS on osteoclasts, particularly on V-ATPase activity. In this study, we developed a parallel flow chamber to investigate the effect of FSS on the morphology of cultured rat osteoclasts, and examined the mRNA expression of ATP6V1A and TCIRG1 in response to FSS.

Materials and methods

Cell isolation and culture. Rat osteoclasts were isolated and cultured essentially as previously described (25). All procedures involving animal treatment were approved by the Council on Animal Care of Sichuan University. Briefly, 6-week-old male Sprague-Dawley rats (Western China Animal Center, P.R. China) were sacrificed by cervical dislocation. The tibiae and femura were removed and dissected free from adhering soft tissues under aseptic conditions. Epiphyseal heads were removed and the bones were rapidly cut into pieces with surgical scissors in a small beaker containing 30 ml of α-MEM (Hyclone, USA) culture medium at 4˚C. The beaker was vibrated on a mixer, then the supernatant was centrifuged and the resultant pellets were diluted to 10^9/ml using α-MEM culture medium containing 20% fetal calf serum (Hyclone) and 100 U/ml penicillin and streptomycin (North China Pharmaceutical Corporation, P.R. China). The cell suspension was inoculated onto a glass slide in a petri dish or into 24-well culture plates, with or without dentine slices, and incubated at 37˚C in 5% CO_2 in a humified tissue culture incubator. After 30 min, the culture medium was replaced to remove non-adherent cells, and then changed three times at 3-h intervals.

Tartrate-resistant acid phosphatase staining. The cells cultured for a given period were washed with phosphate buffer solution (PBS) and fixed with glutaraldehyde for 10 min. They were then rinsed with distilled water and air-dried. TRAP staining fluid (Sigma, USA) was added and the plates were incubated at 37˚C for an additional 60 min. After the removal of the Tartrate-resistant acid phosphatase (TRAP) solution, the plates were washed three times with distilled water and air-dried. The slices were sealed and observed. TRAP-positive cells with ≥3 nuclei were considered to be osteoclasts.

Bone resorption assay. The cells were cultured together with dentine slices for three days, then the dentine slices were removed and cleaned ultrasonically for 60 min. The slices were then washed with PBS, fixed in glutaraldehyde for 10 min, dehydrated in a graded alcohol series and incubated with 1% toluidine blue for 3-4 min. The slices were washed with distilled water and the resorption pits were observed by light microscopy (Olympus, Tokyo, Japan).

Fluid shear stress experiments. A parallel plate flow chamber was used to impose an FSS upon osteoclasts as previously described (14,25). The device was essentially a uniform rectangular parallel plate flow chamber and a liquid irrigation system. A glass slide was mounted in the flow chamber by sandwiching a silicone gasket between the glass slide and the chamber base. The liquid irrigation system contained an inlet and outlet so that fluid could perfuse the cells. Initiation of fluid flow generated laminar shear stress due to hydrostatic pressure between two storage pools, where the flow rate could be adjusted to obtain a specific shear stress level based on the Poiseuille law equation detailed in a previous study (25). The entire apparatus was maintained at 37˚C and the medium was aerated with 95% air/5% CO_2 during the experiments. All stress experiments were conducted under sterile conditions.

To investigate the effects of various stress intensities over a fixed time period (30 min), experimental groups were subjected to the following flow stress: 0 (control), 0.9, 2.9, 8.7 and 26.3 dynes/cm^2. In time-course studies, the cells were subjected to an FSS of 2.9 dynes/cm^2 for a given period. Polylysine (Sigma) was used to enhance the adherence of the cells to the glass surface. A flow of α-MEM culture medium was used to impose FSS on the osteoclasts. The exposure time and range of stress intensities were chosen to ensure the maintenance of cell-substrate adhesion while keeping the cytoskeleton intact.

To observe the effects of FSS on the configuration of osteoclasts, the flow chamber was set up on the scanning stage of the microscope. During the process of stress loading, a clear field of view of the cell creeping slice (~10 mm in diameter) was selected at random and captured under a microscope. Image-Pro Plus analytical software (Media Cybernetics Corp., USA) was used to measure the cell area at each level of applied shear stress.

Quantitative real-time PCR (qPCR). Following flow experiments, flow and static control samples were removed from the parallel plate flow chambers and cultured for an additional 2 h. Subsequently, medium was removed and, after washing with PBS, total RNA was extracted with Trizol Reagent (Sigma) following the manufacturer's instructions. The purity and quantity of RNA was determined spectrophotometrically and the integrity of the RNA preparations was examined by gel electrophoresis. Total RNA (2 μg) was reverse transcribed into single-stranded cDNA by AMV reverse transcriptase and oligo(dT) primer (Sigma) following the company's recommended protocol. Amplification reactions were performed in 30 μl PCR buffer containing 10 μM primers and 25 mM dNTPs, as well as 5 U Taq polymerase (Sigma) with 10 μM labeled probes. Aliquots of cDNA were diluted 10 to 10,000-fold to generate relative standard curves, to which sample cDNA was compared. Sequences for the primers and probes are shown in Table I. Temperature cycling was as follows: initial denaturation at 94˚C for 2 min; denaturation at 94˚C for 20 sec,

Table I. Primer and probe sequences for qPCR.

Gene	Primer and probe sequences
ATP6V1A	
Forward	5'-TAGGAGCAGTTTCTCCACCT-3'
Reverse	5'-CTTATCCAAGCCCCAGAACA-3'
Taqman probe	5'-CTGATCCAGTCACTTCTGCAAC-3'
TCIRG1	
Forward	5'-CTCAACTCCTTCAAGATGAAGAT-3'
Reverse	5'-CTTATCCAAGCCCCAGAACA-3'
Taqman probe	5'-CACCCCAAAGGCCATGTGC-3'
GAPDH	
Forward	5'-TGGGTGTGAACCACGAGAA-3'
Reverse	5'-GGCATGGACTTGGTCATGA-3'
Taqman probe	5'-CTGCACCACAACTGCTTAGC-3'

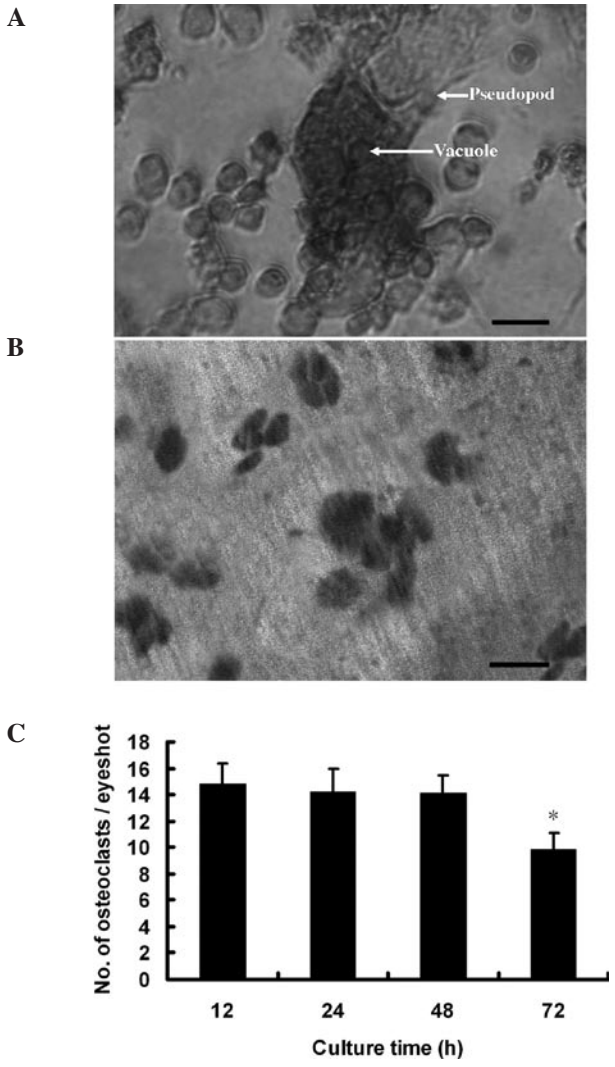

Figure 1. (A) Light microscopic image of a rat osteoclast stained for TRAP. The cell was larger than surrounding cells and had an irregular configuration. Spherical nuclei were dispersed throughout the cytoplasm. Some vesicles and pseudopodia were visible. Magnification x400; scale bar 20 μm. (B) Light microscopic photograph of a dentine slice cultured with marrow cells for 3 days. Irregularly shaped pits were clearly apparent. Magnification x400; scale bar 20 μm. (C) The number of osteoclasts was counted at various culture times. Results are shown as the mean ± SD (n=6). *P<0.05.

primer annealing at 55˚C for 30 sec and extension at 60˚C for 40 sec for 45 cycles. The relative target mRNA expression was computed from target cycle threshold (Ct) values and GAPDH Ct values using the standard curve method.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by the Student's t-test (SPSS 12.0, Chicago, USA) were used for statistical analysis. At least four independent samples were examined for each experiment. A P-value <0.05 was considered statistically significant.

Results

Osteoclast identification. Cells isolated by osteoclast identification yielded a number of TRAP-positive multinucleated cells scattered among the monocytes. These displayed various morphologies (spherical, elliptical, kettle-shaped or irregular). Spherical nuclei were dispersed throughout the cytoplasm, and some vesicles and pseudopodia were occasionally visible (Fig. 1A). The cells were functional, as they produced resorption lacunae typical of osteoclasts when cultured on dentine slices (Fig. 1B). The number of osteoclasts was maintained at a relatively stable level over a culture period of 12-48 h, but osteoclast number clearly decreased after >72 h of culture (Fig. 1C).

Effects of FSS duration and magnitude on osteoclast morphology. As shown in Fig. 2A, significant changes in cell morphology, particularly in cell area, occurred when FSS was imposed on osteoclasts. Although some fluctuation was observed, there was a general increase in osteoclast area as the duration of FSS was extended (Fig. 2B). This area response was also dose-dependent, as the imposition of step-wise increases in FSS above 0.9 dynes/cm^2 resulted in a step-wise change in osteoclast area (Fig. 2C).

Effect of FSS duration on the expression of ATP6V1A and TCIRG1 mRNA. FSS applied for 15 min clearly increased the expression of ATP6V1A and TCIRG1 mRNA compared to the levels seen in control unstressed cells. The levels of ATP6V1A and TCIRG1 mRNA expression increased to a peak at 30 min. However, extending the duration of FSS to 60 min induced a significant decrease in the expression of ATP6V1A and TCIRG1 mRNA compared to the unstressed controls. Further FSS exposure led to further down-regulation of mRNA expression (Fig. 3).

Effect of FSS magnitude on the expression of ATP6V1A and TCIRG1 mRNA. As shown in Fig. 4, application of FSS at 0.9 dynes/cm^2 for 30 min significantly increased the expression of ATP6V1A and TCIRG1 mRNA compared to the unstressed controls. As the magnitude of FSS was increased, the levels of ATP6V1A and TCIRG1 mRNA were up-regulated proportionally.

Discussion

It is difficult to study osteoclasts *in vitro* since they are relatively scarce, terminally differentiated, adherent to

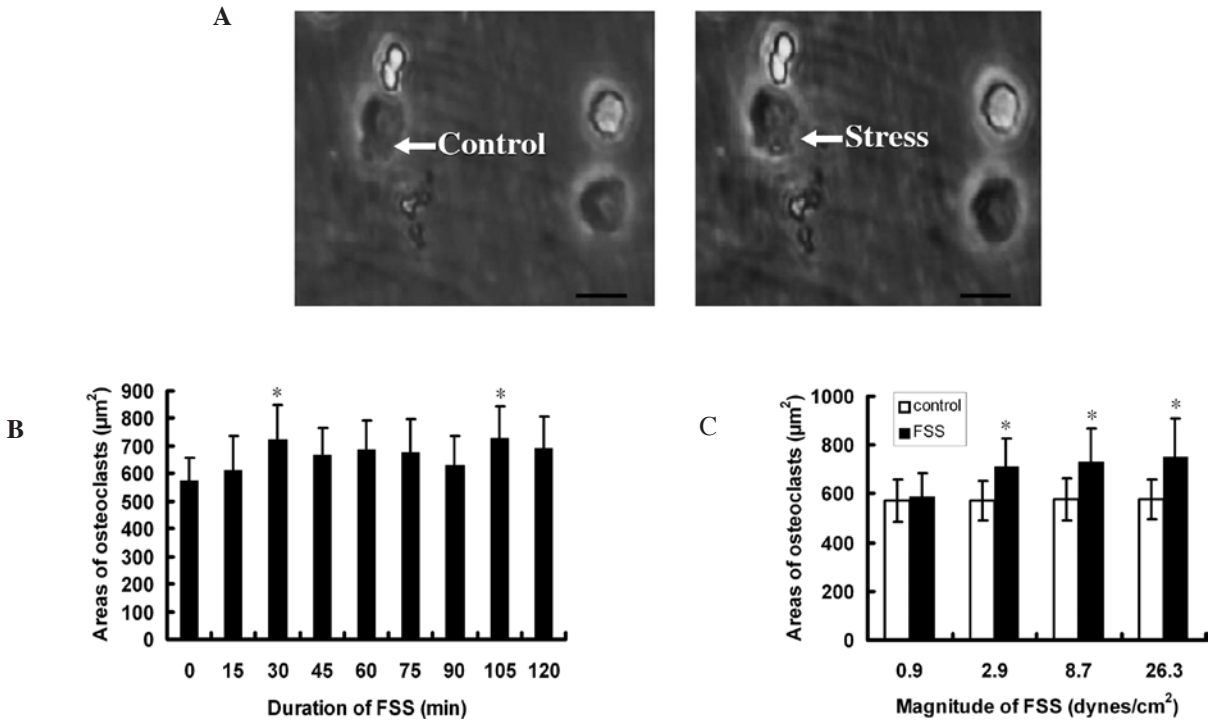

Figure 2. Effects of fluid shear stress (FSS) on cultured rat osteoclast morphology. (A) Light microscopic photographs of osteoclasts before stress (left) and following fluid shear stress for 30 min (right). Magnification x400; scale bar 20 μm. (B) Changes in osteoclast area calculated using Image-Pro Plus analytical software under static conditions or following an FSS of 2.9 dynes/cm^2 for the indicated times. (C) Changes in osteoclast area under static conditions or after being subjected to the indicated FSS stress intensity (dynes/cm^2) for 30 min. Results are shown as the mean ± SD (n=6). *P<0.05.

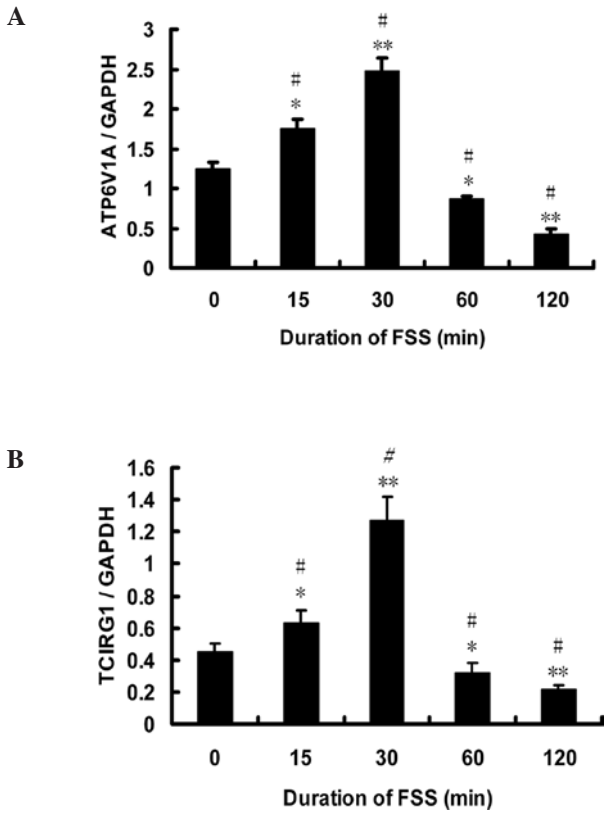

Figure 3. Effect of FSS duration on the expression of (A) ATP6V1A and (B) TCIRG1 mRNA in rat osteoclasts. After a 24-h culture, the osteoclasts were subjected to an FSS of 2.9 dynes/cm^2 for 15, 30, 60 and 120 min. Expression of ATP6V1A and TCIRG1 mRNA was measured by qPCR, and the data were normalized to GAPDH expression. Results are shown as the mean ± SD (n=4). *P<0.05; **P<0.01, compared with the 0-min (control) group. $^\#$P<0.05, compared with the other experimental groups.

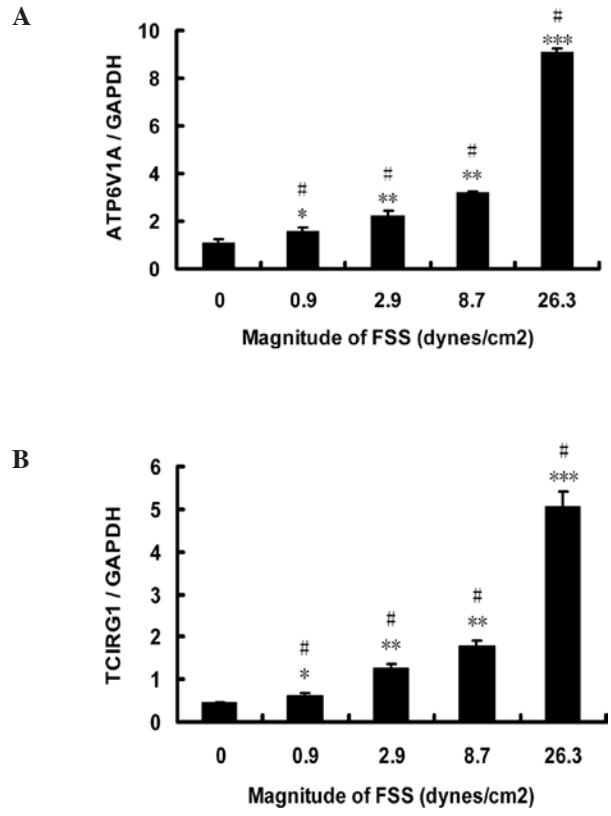

Figure 4. Effect of FSS magnitude on the expression of (A) ATP6V1A and (B) TCIRG1 mRNA in rat osteoclasts. After a 24-h culture, the osteoclasts were subjected to an FSS of 0.9, 2.9, 8.7 and 26.3 dynes/cm^2 for 30 min. Expression of ATP6V1A and TCIRG1 mRNA was measured by qPCR, and the data were normalized to GAPDH expression. Results are shown as the mean ± SD (n=4). *P<0.05; **P<0.01; ***P<0.001, compared with the 0-min (control) group. $^\#$P<0.05, compared with the other experimental groups.

mineralized surfaces and fragile (26). To date, no ideal methods have been established to provide large populations of highly purified and activated osteoclasts. In this study, we used a classic mechanical anatomical technique to obtain osteoclasts derived directly from animal bone marrow tissue. These cells are most likely to have characteristics closely resembling those of osteoclasts in physiological conditions. In addition, the cells obtained by this procedure were sufficient in number for the experiments and, more importantly, the numbers were maintained at a relatively stable level over a period of 12-48 h. All the experiments described in the present study were completed within 24 h.

Changes in osteoclast morphology were apparent after the application of an FSS. This was particularly noticable as increases in osteoclast volume (Fig. 2), which were confirmed by strengthened refraction and decreased transmittance following FSS. These changes in cell morphology may be related to inner alterations of the osteoclast. It has become clear that osteoblasts or osteocytes exposed to fluid flow elicit a number of biomechanical responses, such as the activation of intracellular signaling cascades including mitogen-activated protein kinases (MAPK), PKA and PKC pathways (10,14,27), the release of soluble factors including ATP, nitric oxide (NO) and prostaglandin E_2 (PGE_2) (6,28,29), cytoskeleton reorganization (30), and the regulation of bone-specific marker expression including Cbfa1/Runx2, osteopontin, cyclooxygenase-2 (Cox-2) and several extracellular matrix proteins (14,31,32). We therefore speculated that osteoclasts are also capable of perceiving fluid flow and respond to FSS by the activation of intracellular signaling cascades and subsequent changes in gene regulation, which gave rise to the observed morphological changes. The application of FSS for 30 min resulted in a dose-dependent increase in the mRNA levels of ATP6V1A and TCIRG1, as compared to the unstressed controls (Fig. 3). V-ATPases play a critical role in the resorption activity of osteoclasts, and V-ATPase expression, in particular of ATP6V1A and TCIRG1, increases with the activity of osteoclasts (15,16). Therefore, it is likely that FSS also increased the activity of the osteoclasts in the present study. However, this activation is apparently time-dependent, occurring within the first 30 min of stress; extending the duration of the FSS beyond 30 min resulted in a loss of expression of ATP6V1A and TCIRG1 mRNA. Prolonged FSS may therefore have an inhibitory effect on V-ATPase expression.

These results differ from those reported in other studies using osteoblasts or other bone cells. Many *in vitro* studies in co-cultures of osteoblasts and osteoclasts indicate that mechanical stress induces the activation and differentiation of osteoblasts, while inhibiting osteoclast differentiation (7,8,11,13,29). For example, Kim *et al* reported that physiological levels of loading-induced fluid flow decreased osteoclast formation in a co-culture system of marrow stromal cells and osteoclast precursors by decreasing the RANKL/OPG mRNA ratio (11). Furthermore, Suzuki *et al* suggested that mechanical stress directly suppresses osteoclast differentiation in RAW264.7 cells (33). Taken together, these studies consistently suggest that mechanical load is capable of depressing osteoclastogenesis and inhibiting bone resorption.

On the other hand, there are studies indicating that mechanical stress regulates the key factors expressed in osteoblasts and stromal cells, and induces osteoclast differentiation. Judex *et al* reported that RANKL mRNA was increased in the murine tibia after exposing mice to vibrational stress (34). Zhu *et al* found that the RANKL/OPG ratio was increased during mechanically-induced bone formation by distraction osteogenesis in the mandibula of rats (35). Mehrotra *et al* indicated that fluid flow increased RANKL expression in primary murine calvarial osteoblasts (10). These data suggest that mechanical loading could enhance osteoclastogenesis.

The contradictory results in the literature may partly be due to the use of different mechanical conditions. A study by Kreja *et al* demonstrated that in human primary osteoblastic cells, intermittent stretching increased RANKL expression, whereas continuous loading had no effect, regardless of the strain magnitude or the duration of loading (36). In addition, *in vitro* studies have suggested that altering the flow rate at a fixed fluid shear stress alters the ability of bone cells to respond to fluid flow (13,37). Based on these studies, we deduced that different types and frequencies of stress would have varying effects on bone cells. The present study confirms that different durations of stress induce different osteoclast reactions.

In the current study, a prolonged duration of stress gradually became inhibitory to the expression of ATP6V1A and TCIRG1 mRNA. This may be associated with several cellular events induced by the FSS. Cells isolated from animal bone marrow include not only osteoclasts, but also osteoblasts, macrophages and others (26). In the current experiments, these cells were simultaneously exposed to flow fluid. Many *in vitro* studies have indicated that osteoblasts subjected to FSS release a number of autocrine/paracrine factors, such as ATP, NO and PGE_2, (6,28,29). Furthermore, McAllister *et al* also observed the release of NO and prostaglandin following the exposure of osteoclast-like cells to FSS (38). It is known that NO is antiapoptotic and contributes to osteoblast activation (39,40). PGE_2 has been shown to enhance the anabolic effects of mechanical loading (41). Pharmacological inhibition of PGE_2 or NO production *in vivo* inhibits bone formation after the addition of exogenous loads (42,43). Moreover, the induction of prostaglandin synthesis in response to fluid flow appears to be dependent upon the release of ATP (28). These results indicate that these released factors can decrease the activity of osteoclasts. Taken together, we speculate that the signaling molecules released by osteoblasts or osteoclasts may be transmitted via the cytoskeleton, which is physically linked to ion channels, as well as the intracellular signal transduction pathways, thus regulating the expression of some genes in osteoclasts, such as ATP6V1A and TCIRG1.

In summary, FSS is a potent stimulus that is sensed by osteoclasts, resulting in significant changes in cell morphology and in the expression of ATP6V1A and TCIRG1 mRNA. These effects were strictly dependent on the duration of FSS; however, the mechanisms by which FSS activates osteoclasts and regulates the mRNA expression of ATP6V1A and TCIRG1 is in need of further investigation.

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