Calcium Signaling Of In Situ Chondrocytes During The Stress-relaxation Of Cartilage Explant

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Introduction: Mechanical stimuli can regulate the phenotype and metabolism of chondrocytes in articular cartilage[1]. One of the earliest intracellular responses of chondrocyte under physical stimulation is the fluctuation of intracellular calcium ([Ca^{2+}]_{i}) intensity, i.e., calcium signaling. Previous studies have observed that many mechanical signals, such as fluid flow, AFM indenting, and osmotic stress, can induce [Ca^{2+}]_{i} signaling in chondrocytes[2]. However, most of these studies were performed on monolayer cells, the phenotype of which could have been changed due to the dedifferentiation of chondrocytes on hard surfaces. Little is known about the calcium signaling of in situ chondrocytes residing in their natural extracellular matrix[3]. The viscoelastic nature of cartilage matrix usually incurs significant time-dependent displacement of cells when the tissue is under mechanical loading, which makes it challenging to image cells within deforming matrix. In this study, a new microscope loading device was designed with two opposing actuators to overcome this difficulty. The calcium signaling of in situ chondrocytes was recorded when cartilage was under stress-relaxation stage, and the results were compared with the spontaneous [Ca^{2+}]_{i} signaling of in situ chondrocytes with no stimulation.

Methods: Sample preparation: Five 3 mm diameter cartilage explants were harvested from fresh bovine knee joints within 24 hours of slaughter (Green Village, NJ) and cultured in chemically defined medium[4]. After 3 days culture, samples were cut into 2 halves using a custom-designed cutting tool and stained in 5 µM Fluo-8AM dye medium for 40 minutes. Calcium Imaging: After calcium dye, a half cylindrical sample was mounted on a confocal microscope (Zeiss LSM 510), and the fluorescent images of an interest area in the center of middle zone were taken for 16 minutes (2s per frame) to record the spontaneous [Ca^{2+}]_{i} signaling of in situ chondrocytes. The other half of the sample was placed between two loading platens in an imaging chamber, which was mounted on a custom-designed microscope loading device. Each platen was driven by an independent actuator. When the sample was loaded, two actuators moved in the opposite direction at the same speed so that the center of tissue did not move (Fig 1A, B). During test, 10% strain was applied on the tissue at a speed of 0.8mm/s by two actuators (Fig 1C). Recording of calcium images was started 30s after the loading phase and lasted for 16 minutes (Fig 1D). Data Analysis and Statistics: Calcium images from each sample were first registered using Image J (version 1.47). The [Ca^{2+}]_{i} concentration of each cell was represented by the average image intensity within the cell boundary and analyzed using a custom designed software. The percentage of cells responding with at least one calcium peak (defined as four times of the baseline level) and the spatiotemporal parameters of the [Ca^{2+}]_{i} peaks were obtained as described in our previous study[5]. Chi-square analysis was performed to detect the difference in responsive percentage, and Mann-Whitney U test was performed to detect the differences of spatiotemporal parameters of [Ca^{2+}]_{i} peaks between the two groups.

Results: Due to the slow displacement of cartilage solid matrix in radial direction during the stress relaxation phase under unconfined compression[6], movement of the in situ chondrocytes was observed in the calcium image stack. The shifting of cells can be successfully rescued by image registration.
Mechanical loading induced $[\text{Ca}^{2+}]_i$ responses were captured for the *in situ* chondrocytes (Fig 2A, B). In total, 451 cells in the loaded group and 298 cells in unloaded group were analyzed. The $[\text{Ca}^{2+}]_i$ intensity in cells oscillated vigorously during the stress relaxation phase of cartilage explant (Fig 2B). Many chondrocytes showed more than one $[\text{Ca}^{2+}]_i$ peak in 16 minutes, average 3.8±0.3 peaks per responsive cell for unloaded group and 4.2±0.2 for the loaded group (no significant difference). The responsive percentage of chondrocytes in the loaded group was significantly higher than that of the unloaded group (33.6% vs 23.5%, p<.001) (Fig 2C). For the responsive cells, the magnitude of $[\text{Ca}^{2+}]_i$ peaks (p<.001) was significantly increased in the loaded group, while the time to reach a peak (p<.01) and time between two neighboring peaks (p<.001) were significantly shortened (Fig 3A-E).

**Discussion:** In the present study, a new microscope loading device was built to enable the recording of $[\text{Ca}^{2+}]_i$ responses of loaded *in situ* chondrocytes. Due to the large deformation (10% strain) of stress-relaxation test, recording the cell images during loading phase is still technically challenging. However, the calcium responses of cells were successfully recorded during the stress-relaxation phase. The usage of two opposing actuators minimized the shifting of the region of interest (the center of the sample) and helped to achieve steady focus during the time course of imaging. Furthermore, the new setup allowed us to study how chondrocytes responded to physical stimuli such as interstitial fluid pressure, shear stress from fluid flow, ion flow induced electric current, and the change of electric potential. All these stimuli could be responsible for the strong calcium responses of chondrocytes observed in this study. The higher responsive percentage under loading condition demonstrates the sensitive nature of chondrocytes to these signals experienced *in situ*. Additional differences observed in the spatiotemporal characteristics of $[\text{Ca}^{2+}]_i$ peaks included greater magnitude, shorter time to reach a peak and higher frequency compared to the unloaded cells. The results provide new knowledge about the mechanical stimuli induced calcium signaling of *in situ* chondrocytes.

**Significance:** A new microscope loading device was built to facilitate the record of $[\text{Ca}^{2+}]_i$ responses of *in situ* chondrocytes when cartilage is under mechanical loading. *In situ* chondrocytes showed more vigorous $[\text{Ca}^{2+}]_i$ responses during the stress relaxation phase of cartilage explant than the unloaded cells.
Figure 1: (A) A picture of the microscope loading device with two actuators moving the in the opposing directions. The two loading platens are submerged in the imaging chamber. (B) Schematic illustration of the experiment. A cartilage sample is loaded by the device while the calcium images are taken by the confocal microscope. (C) A typical stress-relaxation curve of cartilage explant under loading and time interval of calcium imaging. (D) A fluorescent image of in situ chondrocytes in cartilage explant.

Figure 2: (A) Representative [Ca$^{2+}$]$_i$ curves of unloaded chondrocytes. (B) Representative [Ca$^{2+}$]$_i$ curves of chondrocytes in loaded cartilage. (C) Percentage of in-situ chondrocytes showed spontaneous [Ca$^{2+}$]$_i$ signaling in 16 minutes.
Figure 3: (A) Average number of peaks of responded cells within 16 minutes; (B) Magnitude of all peaks; (C) Time to reach a peak; (D) Time to relax to 50% of a peak; (E) Time between two neighboring peaks. (The error bars are S.E.M. *: p<0.05, **: p<0.01, ***: p<0.001)