Metabolic Activities in Mevalonate Pathway could affect the Intracellular Calcium Signaling of In Situ Chondrocytes

Jie Ma, Yilu Zhou, Liyun Wang, X. Lucas Lu.
University of Delaware, Newark, DE, USA.

Disclosures: J. Ma: None. Y. Zhou: None. L. Wang: None. X. Lu: None.

Introduction: Mevalonate pathway is an important metabolic pathway that converts mevalonate into sterol isoprenoids. Zoledronic acid (ZA) in bisphosphonate family and simvastatin in statin family are widely used to inhibit the mevalonate pathway, although the two drugs are used to treat different diseases. Comprising this pathway could impair farnesyl diphosphate (FPP) synthase of osteoclasts, suppressing bone resorption, which can be used for osteoporosis treatment. Recently, it was found that cartilage degradation could be rescued by blocking the mevalonate pathway of chondrocytes [1]. The resultant chondro-protective effect may suggest a new therapeutic technique for osteoarthritis. However, little is known about the roles of mevalonate pathway in chondrocytes. To elucidate the mechanism responsible for the effects of mevalonate inhibitor and derivative on chondrocytes, we investigated how these chemicals could affect the intracellular calcium ([Ca$^{2+}$]) signaling. [Ca$^{2+}$] signaling, as one of the earliest responses in chondrocytes under mechanical stimulation [2], is involved in the regulation of chondrocyte fate and its metabolic activities [3]. In this study, we hypothesized that the effects of inhibiting mevalonate pathway could be indicated by the spatiotemporal features of [Ca$^{2+}$] signaling of in situ chondrocytes. We aimed to compare the spontaneous [Ca$^{2+}$] responses of in situ chondrocytes that are (i) treated with and without mevalonate pathway inhibitors; (ii) treated with the mevalonate pathway inhibitors and derivatives together.

Methods: **Mevalonate inhibitor Treatment:** Cylindrical cartilage explants (thickness: 2mm, diameter: 3mm) were harvested from femoral condyle head of juvenile bovine (3~6 month-old) knee joints, followed by culturing in chemically defined medium [4] for 3 days. A batch of explants was separated into two groups (n=8 for each) to check the effects of ZA: (1) chemically defined medium as control; (2) medium supplemented with 10 μM ZA. Another batch of samples was separated into two groups (n=8) to check the effects of simvastatin: (3) chemically defined medium with DMSO vehicle; (4) medium supplemented with 1μM simvastatin. After 48 hours treatment, cartilage explants were cut into two identical halves, dyed with 5 μM Fluo-8 AM (AAT Bioquest, CA) for 40 minutes, and mounted on the confocal microscope (Zeiss LSM510), as shown in Fig. 1A. Fluorescent images of the in situ chondrocytes were recorded every 1.5 seconds for 15 minutes. [Ca$^{2+}$] intensity of each cell was analyzed to obtain the percentage of responsive cells and the spatiotemporal parameters of the [Ca$^{2+}$] peaks, as defined in Fig. 1B. Chi-square analysis was performed to detect the difference of responsive percentage between the two groups, and Mann-Whitney U test to detect the differences of spatiotemporal parameters of [Ca$^{2+}$] signaling.

**Mevalonate Derivatives Treatment:** After harvesting, explants were separated into 3 groups: (1) chemically defined medium with 10 μM ZA and DMSO vehicle; (2) culture medium with 10 μM ZA and 5 μM farnesol (FOH); and (3) culture medium with 10 μM ZA and 5 μM geranylgeraniol (GGOH). FOH and GGOH are derivatives of mevalonate, the presence of which may reverse the inhibition of mevalonate
pathway. After 48 hours treatment, spontaneous intracellular calcium signaling of chondrocytes was recorded and analyzed as previously described.

**Results:** *Mevalonate inhibitor Treatment:* Both ZA and simvastatin treatment increased the responsive percentage of \([\text{Ca}^{2+}]_i\) signaling of *in situ* chondrocytes (Fig. 1C & 2A) (p<0.01). After ZA treatment, time to reach a peak (Fig. 1F) and time between two neighboring peaks (Fig. 1H) increased, average number of calcium peaks (Fig. 1D) decreased, suggesting ZA could decrease the frequency of \([\text{Ca}^{2+}]_i\) signaling in chondrocytes although it can stimulate more cells to respond. No significant difference was detected for the magnitude of peaks (Fig. 1E) and peak relaxation time (Fig. 1G). After simvastatin treatment, the magnitude of calcium peaks (Fig. 2C) and the peak relaxation time (Fig. 2E) were significantly decreased, while no significant difference was detected for average number of peaks (Fig. 2B) and time to reach a peak (Fig. 2D).

*Mevalonate derivative Treatment:* The \([\text{Ca}^{2+}]_i\) responsive percentage in the ZA+FOH group (25.0%) dropped back and was lower (p<.001) than that in ZA vehicle group (33.7%), but no significant difference was detected between ZA+GGOH (33.2%) and ZA vehicle group (Fig. 3A). With the existence of GGOH, chondrocytes significantly increased the average number of peaks (p<.05, Fig. 3B), but decreased the magnitude of calcium peaks (p<.05, Fig. 3C). No significant differences were detected for the other spatiotemporal parameters.

**Discussion:** The short-term treatment of the mevalonate inhibitors, both ZA and statin, seem to have biphasic effect on \([\text{Ca}^{2+}]_i\) signaling of chondrocytes, significantly increased the percentage of chondrocytes that exhibit spontaneous \([\text{Ca}^{2+}]_i\) signaling, but reduce the frequency of calcium peaks. It is interesting to find that ZA and statin resulted in different patterns of \([\text{Ca}^{2+}]_i\) peaks. ZA slowed down the rising of \([\text{Ca}^{2+}]_i\) during a peak, which might result from the Ca2+ chelating capability of ZA which dampens the electrochemical gradient across the membrane. Statin accelerated the intracellular Ca2+ being pumped into extracellular space, showing as shorter peak relaxation time, suggesting that statin could attenuate the rise of \([\text{Ca}^{2+}]_i\) concentration of chondrocytes, which is consistent with its effect on cardiomyocytes [6]. FOH offset the positive effect of ZA on the responsive percentage of \([\text{Ca}^{2+}]_i\) signaling, while GGOH did not. This implies that more chondrocytes prone to be active in calcium signaling after the inhibition of the prenylation of small GTPases in the mevalonate pathway, to be specific, protein farnesylation.

**Significance:** This study for the first time demonstrated the effects of mevalonate inhibitor and derivatives on the spontaneous \([\text{Ca}^{2+}]_i\) signaling of *in situ* chondrocytes. It provides new knowledge about the chondro-protective effect of bisphosphonate and statin and may identify a new therapeutic technique for the treatment of osteoarthritis.
Figure 1: (A) Cartilage explant mounted on the confocal microscope for calcium imaging; (B) A typical calcium response intensity curve of a chondrocyte and the definitions of spatiotemporal parameters; (C) Responsive percentage of the in-situ chondrocytes (cells with calcium peaks over the number of total cells); (D) Average number of calcium peaks of responded cells within 15 minutes; (E) Magnitude of all calcium peaks; (F) Time to reach a peak; (G) Time to relax to 50% of the peak; (H) Time between two neighboring peaks. (The error bars are S.E.M. *: p<0.05, **: p<0.01, ***: p<0.001)
Figure 2: (A) Responsive percentage of in-situ chondrocytes treated with Simvastatin; (B) Average number of Peaks of responded cells within 15 minutes; (C) Magnitude of all peaks; (D) Time to reach a peak; (E) Time to relax to 50% of the peak; (F) Time between two neighboring peaks. (The error bars are S.E.M. *: p<0.05, **: p<0.01, ***: p<0.001)
Figure 3: (A) Responsive percentage of the spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} signaling of in-situ chondrocytes between the control and the treated groups; (B) Number of Peaks for each responsive cell in 15 minutes; (C) Magnitude of all peaks; (D) Time to reach a peak; (E) Time to relax to 50% of peaks; (F) Time between peaks. (The error bars are S.E.M. *: p<0.05, **: p<0.01, ***: p<0.001)